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13. ABSTRACT ***(Maximum 200 Words)***

The present program of research aims at determining the neuroprotective activity of novel estrogens in both male and female model for brain ischemic damage. We have proposed to achieve 4 technical aims over the course of 3 years. These aims relate to a description of the activity of several estrogens against cerebral damage related to middle cerebral artery occlusion in a rodent model, when the estrogens are administered prior to or following the ischemic event. We also are pursuing our observation that androgen reduction is neuroprotective in male subjects. We will assess (during years 2 and 3 of the grant) the potential mechanism(s) of the neuroprotective activity of estrogens (and of androgen reduction) in these models by determining the extent to which estrogens (or androgen reduction) reduce oxidative damage associated with stroke and then assess the involvement of signal transduction processes and anti-apoptotic proteins in the neuroprotective activity of estrogens. Collectively, these studies will provide the knowledge needed to determine if estrogen therapy and androgen reduction therapy are useful in protection of the brain tissue from damage related to the activities of U.S. Army male and female personnel.

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(4) Introduction: The present program of research aims at determining the neuroprotective activity of novel estrogens in both male and female animal models for brain ischemic damage. We have proposed to achieve 4 technical aims over the course of 3 years. These aims relate to a description of the activity of several estrogens against cerebral damage related to middle cerebral artery occlusion in a rodent model, when the estrogens are administered prior to or following the ischemic event. Also, we will assess (during years 2 and 3 of the grant) the potential mechanism(s) of the neuroprotective activity of estrogens in these models by determining the extent to which estrogens reduce oxidative damage associated with stroke and then assess the involvement of signal transduction processes and anti-apoptotic proteins in the neuroprotective activity of estrogens. Collectively, these studies will provide the knowledge needed to determine if estrogen therapy is useful in protection of the brain tissue from damage related to the activities of U.S. Army male and female personnel.

(5) Body:

Background: This report covers the period of August 1, 1999 to July 30, 2000 for research funded by the grant from the USAMRMC numbered DAMD 17-99-1-9473. During this period, I accepted the position of Chair, Department of Pharmacology and Neuroscience and Director, Institute for Aging and Alzheimer's Disease Research at the University of North Texas Health Science Center at Fort Worth (UNTHSCFW). This change necessitated the transfer of the funds remaining for the second and third years of the grant from the University of Florida to UNTHSCFW. This activity is in progress at the time of preparation of this report.

Also, on August 3, 2000, Major Chessley Atchison, Ph.D., conducted a site-visit at the UNTHSCFW. The progress reported in this document was also reported to Dr. Atchison.

We have made substantial progress in achieving several of the technical aims of the grant and this progress is reported below.

New Compound Synthesis: (Although not specifically funded by this grant, the following program of drug synthesis provided new compounds to our stroke and brain trauma program.)

During Grant year 99-00 we synthesized and conducted evaluations of more than two dozen novel estrogens to identify compound candidates for activity in stroke models.

Our synthetic strategy has been three-fold. First, we employed an enantiomer approach to produce two novel estrogens; 17α-estradiol (17 α-E2) and the complete enantiomer of 17 β-estradiol (17 β-E2), ent-E2. These two compounds have been tested in a variety of *in vitro* assays for neuroprotection (Green et al., 2000) and in an animal model for middle cerebral artery (MCA) occlusion induced cerebral ischemia (Green et al., 2000, See description below). Both compounds are as neuroprotective as 17 β-E2 in both *in vitro* and *in vivo* models for neuronal death.

A second synthetic strategy for production of novel estrogens is based upon the conjugation at the 17-oxygen of 17 β-E2 with alkyl groups. We synthesized 6 new 17-alkylestradiols and assessed their neuroprotective activity in an *in vitro* test (Prokai et al., 2000). One or more of these compounds will be tested in a MCA occlusion model during the next grant period.

A third strategy for production of novel estrogens was to do substitutions at one or more of the other carbons in the estradiol or estrone molecule. This later approach has produced several compounds that are at least as active in *in vitro* assays as is 17 β-E2 (Perez et al., 2000 abstract). During the next grant period, we will continue to synthesize and test additional compounds that may prove to be of interest in neuroprotection.

Pharmacokinetics of Estrogen Preparations: (Although not specifically funded by this grant, the following program of assessment of the iv and sc kinetics of estrogen preparations is important to our preparation of compounds for clinical assessment.)

We tested the kinetics of estrogen preparation intended for rapid distribution to the brain to treat stroke and brain trauma. In an initial study, we administered aqueous soluble preparation of 17 β-E2 and determined the kinetics in serum for 24 hours thereafter. Both preparations (NaSO4-E2 and HPCD-E2) produced a rapid increase and a rapid decline in serum 17 β-E2 concentrations (See Figure 1 attached). The observed rapid increase in 17 β-E2 concentrations is an advantage, as it insures that the active compounds will be distributed to the site of action, the brain, within seconds to minutes after administration. The rapid decline in concentrations, however, is a problem, as 17 β-E2 will be cleared from the brain with about the same time course as it is cleared in serum.

To further optimize a preparation of 17 β-E2 for use in emergency brain protection form stroke and trauma, we assessed various preparation formulated in oil and intended for sc administration (See Figure 2). Administration sc of 17 β-E2 in oil resulted in a prompt increase in serum 17 β-E2 concentrations to levels well above those needed to achieve neuroprotection (20 to 30 pg/ml) within 5 min and these elevated concentrations were maintained for at least 8 hours. This is the distribution profile that we were trying to achieve and represents a means for achieving a rapid and sustained neuroprotection from brain injury.

Studies in Female Rats:

Prophylactic Neuroprotection by Estrogens:

Middle Cerebral Artery (MCA) Occlusion: We have conducted several studies which demonstrate that 17 β-E2 is a potent neuroprotectant when administered prior to the onset of MCA occlusion. Initially, we demonstrated that administration of 17 β-E2 by either a sc or an iv formulation at 1 day prior to MCA occlusion protected animals from much of the cerebral damage related to the infarct (Simpkins et al., 1997). Subsequently, we demonstrated that a slow release preparation of 17 β-E2, administered beginning 1 week prior to the occlusion was potently effective in protecting brain tissue from ischemia damage (Simpkins et al., Unpublished Observations). Similarly, administration of 17 β-E2 at 2 hours prior to the onset of the infarct was protective (Zhang et al., 1998). Finally, using nuclear magnetic resonance (NMR) imaging to perform real time imaging of the development of ischemic damage, we demonstrated that

administration of 17 β-E2 at 2 hours prior to the onset of the occlusion can essentially protect much of the brain tissue from the damaging effects of occlusion (Shi et al., 2000).

Novel estrogens were also assessed for prophylactic neuroprotection. We completed an assessment of the protection against cerebral damage related to MCA occlusion is animals pre-treated with 17 α-estradiol (17 α-E2), through a subcutaneous Silastic® tube containing the steroid at two different doses. We observed that 17 α-E2 provided protection of both cortical and subcortical brain areas from damage induced by the occlusion. The extent of the protection provided by 17 α-E2 was similar to that afforded by 17 β-E2 (Stubley et al., 2000).

Additionally, we tested the complete enantiomer of 17 β-E2, ent-E2, for its neuroprotective activity in an MCA occlusion model of brain damage. We observed that ent-E2 was as effective as 17 β-E2 in protecting both cortical and subcortical tissue from damage (Green et al., 2000). Collectively, these data indicate that non-feminizing estrogens, like 17 α-E2 and ent-E2 are potent neuroprotectant in at least one model for cerebral damage, the MCA occlusion model for stroke.

Subarachnoid Hemorrhage (SAH) Model

We completed an assessment of the prophylactic protective effects of 17 β-E2 in a model for SAH that we developed. Animals were administered 17 β-E2 at a dose of 100 μg/kg at 2 hours prior to the induction of an intraluminal hemorrhage using a thread that punctured the anterior cerebral artery. Pre-treatment with 17 β-E2 preserved blood flow during the bleeding episode, but did not effect the bleeding volume, and markedly reduced the extent of cerebral ischemic damage related to the bleeding episode (Yang et al., 2000a). These data indicate that treatment with 17 β-E2 can provide protection from bleeding as well as occlusion type strokes.

4-Vessel-Occlusion (4-V-O) Model

We have obtained preliminary data to indicate that treatment with estrogens can also protect brain tissue from the damage related to complete reduction in blood flow to the brain, a model for brain damage related to the stoppage of blood flow during a heart attack. Rats were subjected to a 4-vessel occlusion (4-V-O), for 20 minutes, a procedure that essentially stops blood flow to the brain for a brief period of time. This procedure is particularly damaging to hippocampal pyramidal cells of the hippocampus.

We observed that pre-treatment with 17 β-E2 approximately doubles the number of hippocampal pyramidal cells that survived the ischemic event (He et al., 2000).

Post-Trauma Treatment with Estrogens

We assessed the possibility that estrogen treatment after the onset of a cerebral event could provide protection from brain damage. In a cornerstone study which received much public press with its publication, we observed that estrogens were effective for as long as three hours after the onset of a MCA occlusion in protecting brain tissue from ischemic damage (Yang et al., 2000b). This fundamental paper demonstrates that estrogens can be used to treat cerebral damage even after the event has started. These data are consistent with our observation (reported above) that much of the damage related to an ischemic event occurs during re-perfusion, as opposed to during the ischemia itself.

Male Subjects

We assessed the possibility that estrogen could provide cerebral protection in male subjects as well as in females. In an initial study, published prior to the onset of this grant (Hawk et al, 1997) we reported that treatment for 1 week with 17 β-E2 by a sc Silastic® tube containing the steroid, resulted in protection from brain damage induced by MCA occlusion equivalent to that observed in females. Interestingly, however, this protection form brain damage was also observed when males were castrated. This raised the possibility that the effects of estrogens in males were related to their capacity to reduced serum androgens, such as testosterone. To test this possibility, we administered estrogens, but maintained serum testosterone levels relatively constant through sc implants of Silastic® tubes containing the male sex steroid. When testosterone concentrations were maintained, estrogens lost most of their ability to protect brain tissue. These data indicated that serum androgen reduction was a key event in the neuroprotection afforded by estrogens.

In view of this surprising finding, we conducted a series of studies to determine the effects of androgen reduction, through a variety of means, on brain damage subsequent to an infarct. First, we administered the potent LHRH agonist, luprolide, which nearly completely suppresses secretion of LH and hence of testosterone, twice daily for one week. Luprolide reduced testosterone concentration to nearly undetectable levels after a week of administration. These animals were subjected to MCA occlusion and 24 hours later lesion size was evaluated. Luprolide reduced lesion size by 50%, the same extent of protection seen with estrogen pretreatment (Cartright et al., 2000).

Finasteride is a 5α-reductase inhibitor that prevents the conversion of testosterone to 5 α-dihydrotestosterone (HDT). We administered finasteride to male rats, at 6 hours prior to MCA occlusion and evaluated the size of the ischemic infarct 24 hours later. A single dose of finasteride reduced infarct size in male rats by about 40% (Cartright et al., 2000).

To determine if a non-pharmacological means of reducing testosterone would also protect brain from ischemic damage, we subjected male rats to the stress of administration of an anesthetic (or control animals were left undisturbed) at 6 hours prior to the MCA occlusion. Animals which were subjected to stress showed a major reduction in serum testosterone concentrations and a 50% reduction in infarct size (Cartright et al., 2000).

Finally, to fully characterize the duration of testosterone reduction needed to protect animals from ischemic damage, we created a "testosterone clamp" by castrating male rats and implanting them with 2 testosterone filled Silastic® tube. These implants produced serum testosterone concentration which were equivalent to that seen in intact rats (Cartright et al., 2000). Removal of these implants caused an 85% reduction in testosterone concentrations within 1 hour and undetectable levels of the hormone within 2 hours (Cartright et al., 2000). MCA occlusion was initiated at 0 (no removal of the pellets), 1, 2, 4, or 6 hours after removal of the testosterone tubes. We observed a time-dependent reduction in the ischemic damage, with a 50% reduction seen at 6 hours (Cartright et al., 2000). These data indicate that the removal of testosterone for as little as 6 hours can substantially protect the male brain from the damaging effects of ischemia.

(6) Key Research Accomplishments:

- Estradiol and non-feminizing estrogens like 17 α-estradiol and ent-estradiol are potent neuroprotectants against cerebral damage induced by brain ischemia.
- This neuroprotectant activity can be achieved even when the estrogen is administered after the ischemic event.
- The protective effects of estradiol can be observed in male subjects, and appears to have a strong component related to estrogen-induced androgen reduction.
- Acute androgen reduction is potently neuroprotective in male subjects.

(7) Reportable Outcomes:

Published Reports, Manuscripts and Abstracts Primarily Funded by DAMD 17-99-1-9473 (While we cited several sources of funding for the following publications, the primary source of funding for these papers was the grant DAMD 17-99-1-9474. Other funding was coincidental and related to the purchase of reagents used in the studies.)

Cutright, J. S. Yang, Z. He, A. L. Day, and **J. W. Simpkins**, Improvement in cerebral ischemia outcome with non-surgical methods of reducing testosterone in male rats, Society for Neuroscience Abstracts, 2000. **(Appendix N)**

Green, P. S., S.H. Yang, K. R. Nilsson, A. S. Kumar, D. F. Covey and **J. W. Simpkins**, The non-feminizing enantiomer of 17 beta-estradiol exerts protective effects in neuronal cultures and a rat model of cerebral ischemia. Endocrinology, in press, 2000. **(Appendix A)**

Hawk, T., Y-Q Zhang, G. Rajakumar, A.L. Day, and **J.W. Simpkins**. Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats. Brain Research 798: 296-298, 1998. **(Appendix M)**

He. Z, S. H. Yang, Y. J. He, J. Cutright, A. L. Day, and **J. W. Simpkins**, Physiological estradiol delays neuronal death following transient forebrain ischemia in rats, Society for Neuroscience Abstracts, 2000. **(Appendix K)**

Shi, J., J.D. Bui, S.H. Yang, T.H. Lucas, D. L. Buckley. S.P. Blackband, M.A. King, A.L. Day and **J.W. Simpkins**, Estrogens decrease reperfusion-associated cortical ischemic damage: a MRI analysis in a transient focal ischemia model. Stroke, Submitted 2000. **(Appendix H)**

Simpkins, J.W., G. Rajakumar, Y.Q. Zhang, C.E. Simpkins, D. Greenwald, C.J. Yu, N. Bodor, A.L. Day, Estrogens reduce mortality and ischemic damage by middle cerebral artery occlusion in the female rat. J. Neurosurgery 87:724-730, 1997. **(Appendix F)**

Stubley. LA., S.-H. Yang, J. Shi, A. L. Day and **J. W. Simpkins**, The Effects of 17 α-estradiol on cerebral blood flow and cortical protection during middle cerebral artery occlusion in rats, in preparation, 2000. **(Appendix I)**

Yang, S.H., A. L. Day and **J. W. Simpkins,** Estradiol exerts neuroprotective effects when administrated after ischemic insult. Stoke 31: 745-749, 2000. **(Appendix L)**

Yang, S.-H., Z. He, S. S. Wu, Y.-J. He, J. Cutright, W. J. Millard, A. L. Day **and J. W. Simpkins** 17 β-estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrage. J. Cerebral Blood and Metabolism, Under Consideration, 2000. **(Appendix J)**

Zhang, Y.Q., J. Shi, G. Rajakumar, A.L. Day, and **J.W. Simpkins**. Effects of gender and estradiol treatment on focal brain ischemia. Brain Research 784: 321-324, 1998. **(Appendix G)**

He, Z., H. Naritomi, S. H. Yang, T. Yamawaki, A.L. Day, and **J. W. Simpkins,** An experimental model of a small deep infarct involving the hypothalamus in rats: changes in body temperature and postural reflex. Stroke, 30: 2743-2751, 1999.

Shi, J., S.H. Yang, A.L. Day and **J.W. Simpkins**, Hypoperfusion indices over-expression of β-amyloid precursor protein mRNA in a focal ischemia rodent model. Brain Res, 853: 1-4, 2000.

He, Z., T. Yamawaki, S.-H. Yang, Q. Liu, M.A King, A.L. Day, **J.W. Simpkins,** and H. Naritomi, The anterior Choroidal Artery Territory in rats, J Neurological Sciences, submitted, 2000.

Published Reports, Manuscripts and Abstracts Secondarily Funded by DAMD 17-99-1-9473 (While the grant DAMD 17-99-1-9473 was cited as contributing to these papers, their funding from this source was coincidental and related to the purchase of reagents. Also, the research papers listed below contribute heavily to our understanding of the mechanism of the neuroprotective effects of estrogens and estrogen-like compounds, knowledge that will be applied to our cerebral ischemia research during the 2nd and 3rd years of the present grant period.)

Perez, E. J., K. L. Eberst, S. M. Oon, Z. Y. Cai, L. Prokai, D. Covey and **J. W. Simpkins,** Structure-activity relationship of estratrienes against glutamate toxicity in a mouse hippocampal cell line, Society for Neuroscience Abstracts, 2000. **(Appendix C)**

Prokai,L., S.-M Oon, K. Prokai-Tatrai, K. A. Abboud and J. W. Simpkins, Synthesis and biological evaluation of 17 β-alkoxyestra-1,3,5(10)-trienens as potential neuroprotectants against oxidative stress, J. Med. Chem. in press, 2000. **(Appendix B)**

Zaulyanov, L. L., P.S. Green and **J. W. Simpkins**. Glutamate receptor requirement for neuronal death from anoxia-reoxygenation: An in vitro model for assessment of the neuroprotective effects of estrogens. Cell. Mol. Neurobiol. 6:705-718, 1999

G. A. Brazeau and **J. W. Simpkins**, Therapeutic benefits of estrogen replacement therapy, Florida Pharmacy J, December, 1999, pp 14-20.

Simpkins, J. W., The Role of Estrogen replacement therapy in the prevention of neurodegeneration associated with Alzheimer's disease and stroke, Response to an Aging Florida, Spring, 2000.

Persky, A. M., Persky, A.M., P.S. Green, L.A. Stubley, C.S. Howell, L. Zaulyanov, G.A. Brazeau and **J.W. Simpkins**, Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. Proc Soc Expt Biol Med, 223: 59-66, 2000.

Green, P. S. and **J. W. Simpkins**, Neuroprotective effects of estrogens: potential mechanisms of action. Int J Dev Neurosci 18: 347-358, 2000

Simpkins, J. W., P.S. Green, K.E. Gridley, J. Shi, E. M. Monck, Neuroprotective Effects of Estrogens, in "Biology of the Menopause", Ed. F. L. Bellino, Serono Symposium, Springer-Verlag, New York, pp. 103-111, 2000.

In Press

Simpkins, J. W. and Green, P.S., Estrogens: Pleiotropic anti-Alzheimer's disease therapy. In "Promising Directions of Research and New Therapeutic Targets for the treatment of Alzheimer" Disease", Eds. M. Mesulam and Z. S. Khachaturian, 2000, In press.

Green, P.S., S. -H. Yang and **J.W. Simpkins**, Neuroprotective Effects of Phenolic A Ring Estrogens, Novartis Foundation Symposium "Neuronal and Cognitive Effects of Oestrogens" Eds. J. Goode and B. McEwen, in press, 2000.

Green, P.S. and **J. W. Simpkins**, Role of estrogens and estrogen-like non-feminizing compounds in the prevention and treatment of Alzheimer's disease, Annals of the New York Academy of Sciences, in press, 2000.

Green, P. S., E. J. Perez, T. Calloway, and **J. W. Simpkins**, Estradiol attenuation of β-amyloid-induced toxicity: A comparison of MTT and Calcein assays, J. Neurocytology, in press, 2000.

Abstracts In Press

Wen, Y., E. J. Perez, P. Green and **J. W. Simpkins**, Nitric Oxide may medicate estrogen's neuroprotection through a receptor-independent mechanism, Society for Neuroscience Abstracts, 2000.

Wang, J., E. J. Perez, p.S. Green and **J. W. Simpkins**, Estradiol protects against ATP depletion and mitochondrial membrane potential decline induced by 3-nitroproprionic acid in SK-N-SH neuroblastoma cells, Society for Neuroscience Abstracts, 2000.

Watson, D. G., C. Fiola and **J. W. Simpkins**, Inhibition or down-regulation of protein kinase C enhances estrogen-induced neuroprotection in an in vitro model, Society for Neuroscience Abstracts, 2000.

Yang, S. H., Z. He, S. Wu, Y.J. He, J. Cutright, W. J. Millard, A. L. Day, and **J. W. Simpkins,** 17β-estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage, Society for Neuroscience Abstracts, 2000.

Green, P. S. C. T. Fulp and **J. W. Simpkins**, Estrogen modulation of Bcl-2 family protein expression, Society for Neuroscience Abstracts, 2000.

(8) Conclusions: The first grant year has been very successful, resulting in numerous publications, manuscripts and abstracts. These papers demonstrate the following: (1) Estradiol and non-feminizing estrogens like 17 α-estradiol and ent-estradiol are potent neuroprotectants against cerebral damage induced by brain ischemia. (2) This neuroprotectant activity can be achieved even when the estrogen is administered after the ischemic event. (3) The protective effects of estradiol can be observed in male subjects, and appears to have a strong component related to estrogen-induced androgen reduction. (3) Acute androgen reduction is potently neuroprotective in male subjects. As a result of these studies, we are now in a position to conduct detailed analyses of the mechanism of neuroprotective action of the compounds under study. Additionally, we believe that we have sufficient preclinical data with which to initiate a clinical trial (from other sources of funds) to assess the efficacy of estrogens in protection of the brain from damage.

(9) References:
The References in the text are cited by the author's name and are indicated in the Reportable Outcomes Section of this document.

(10) **Appendices:** Appended materials are identified in the text (Reportable Outcomes Section) and are attached.

APPENDIX f. STATEMENT OF WORK (SOW)

Modified 8-30-00 (*modifies materials are in italics*)

The following timelines and specific performance goals are envisioned for the 3 years of the proposed program of research:

Year One: During the first year of funding, we would complete the first technical objective proposed (To assess the prophylactic and post-trauma neuroprotective effects of novel estratrienes in an animal model of cerebral ischemia). This will include the assessment of the efficacy of these compounds when administered either prior to or following the induction of the ischemic event. Research activities involved in achieving this objective include animal procurement and housing, their ovariectomy, estratriene administration, induction of the lesions using middle cerebral artery occlusion, assessment of the lesion volume using staining procedures and perhaps MRI, determination of the dose-dependence of the effects of the estratrienes, statistical evaluation of the data and reporting of the results to the Army and to scientific journals.

Year Two: During the second year of funding, we will achieve technical objective 2 (To determine the effects of novel estratrienes on oxidative damage during and following a cerebral ischemic event) and begin research on technical object 3 (To determine the effects of novel estratrienes on CREB expression and phosphorylation following a cerebral ischemic event). *Also, during year 2 we will continue our assessment of the role of androgen reduction in neuroprotection in male rats. This research resulted from discoveries made during year one and represents a change in direction in the project related to neuroprotection in male subjects. To accommodate this increase in work, we propose the elimination of the testing of intact female rats, a modification in the original SOW made at the suggestion of the Army.* Research activities involved in achieving objective 2 include animal procurement, their ovariectomy, estratriene administration, induction of the lesions using middle cerebral artery occlusion, sampling of tissue and the assay of samples for lipid and protein oxidation, statistical evaluation of the data obtained and reporting of the data. We will also begin the assessment of technical objective 3. The research activities involved in achieving this objective include animal procurement, their ovariectomy, estratriene administration, induction of the lesions using middle cerebral artery occlusion, sampling of tissue and the assay of samples for CREB and the phosphorylated form of CREB using western analysis for protein and northern analysis for CREB mRNA.

Year Three: During year 3 we will complete the work described in technical objective 3 and in technical object 4 (To determine the effects of novel estratrienes on Bcl-2 expression following a cerebral ischemic event). For technical objective 3 we will complete the work begun in year 2 (described above), conduct statistical evaluation of the data obtained and report the data to the Army and compose manuscripts for publication. For technical objective 4, we will conduct assessment of the role of Bcl-2 stimulation by estratrienes in their neuroprotective effects. Research activities involved in achieving this objective include animal procurement, their ovariectomy, estratriene administration, induction of the lesions using middle cerebral artery occlusion, sampling of tissue and the assay of samples for Bcl-2 protein and mRNA by western and northern analysis, respectively.

James Simpkins 8-30-00

DAMA17-99-1-9473
Appendix F
J. W. Simpkins

J Neurosurg 87:724–730, 1997

Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat

JAMES W. SIMPKINS, PH.D., GOPAL RAJAKUMAR, PH.D., YU-QI ZHANG, M.D., PH.D., CHRISTOPHER E. SIMPKINS, B.S., DAVID GREENWALD, M.D., CHUN J. YU, M.D., NICHOLAS BODOR, PH.D., AND ARTHUR L. DAY, M.D.

Department of Pharmacodynamics and the Center for the Neurobiology of Aging, Departments of Neurosurgery and Pharmacology; and Center for Drug Discovery, Colleges of Pharmacy and Medicine, University of Florida, Gainesville, Florida

The present study was undertaken to determine if estrogens protect female rats from the neurodegenerative effects of middle cerebral artery (MCA) occlusion. The rats were ovariectomized and 7 or 8 days later various estrogen preparations were administered before or after MCA occlusion. Pretreatment with 17β-estradiol (17β-E2) or a brain-targeted 17β-E2 chemical delivery system (CDS) decreased mortality from 65% in ovariectomized rats to 22% in 17β-E2–treated and 16% in 17β-E2 CDS–treated rats. This marked reduction in mortality was accompanied by a reduction in the ischemic area of the brain from 25.6 ± 5.7% in the ovariectomized rats to 9.8 ± 4% and 9.1 ± 4.2% in the 17β-E2–implanted and the 17β-E2 CDS–treated rats, respectively. Similarly, pretreatment with the presumed inactive estrogen, 17α-estradiol, reduced mortality from 36 to 0% and reduced the ischemic area by 55 to 81%. When administered 40 or 90 minutes after MCA occlusion, 17β-E2 CDS reduced the area of ischemia by 45 to 90% or 31%, respectively. In summary, the present study provides the first evidence that estrogens exert neuroprotective effects in an animal model of ischemia and suggests that estrogens may be a useful therapy to protect neurons against the neurodegenerative effects of stroke.

KEY WORDS • **17β-estradiol** • **17α-estradiol** • **estradiol chemical delivery system** • **estrogen** • **middle cerebral artery occlusion** • **ischemia** • **rat**

STROKE is the third leading cause of morbidity and mortality in the United States, with over 500,000 cases per year. Poststroke sequelae include mortality and chronic neurological debilitation that result from neuronal damage for which prevention or treatment are not currently available. Inasmuch as postmenopausal women are at increased risk of cardiovascular and neurological diseases[13,14,23,26,28,38] and estrogen replacement therapy (ERT) is associated with a reduction in stroke-related deaths,[13,14,23,26,28,37,38] an assessment of the efficacy of experimental ERT in reducing mortality and ischemic brain damage in animals deprived of endogenous estrogens is warranted.

Recently, we and others demonstrated that estrogens exert neuroprotective effects in vitro against the neurotoxicity that results from serum deprivation,[4,18,33,34] β-amyloid,[2,17,19,33] and *N*-methyl-D-aspartate (NMDA) agonist treatment.[35] In addition, the neuroprotective effects of estrogens appear to be independent of the estrogen potency of the molecule.[18,19,33] The weak or inactive estrogen, 17α-estradiol (17α-E2), is as effective as the naturally occurring potent estrogen 17β-estradiol (17β-E2) in protecting neurons against the toxic effects of serum deprivation,[18,19,33] and β-amyloid toxicity is attenuated by estrogens in a cell type that lacks the estrogen receptor.[2]

Middle cerebral artery (MCA) occlusion has been used to produce focal ischemic lesions in the rat. This procedure causes a unilateral large ischemic area that typically involves the basal ganglion and frontal, parietal, and temporal cortical areas.[15,24] The ischemic lesion begins with a small core at the center of the region perfused by the MCA and enlarges with duration of MCA occlusion.[1] The penumbral area around the core infarct is believed to result from propagation of the lesion from the core outward to tissue that remains perfused by collateral circulation during the occlusion.[24] Death of tissue in this penumbral area may be caused by glutamate released from dying neurons and glia and a subsequent neurotoxic effect mediated through an NMDA receptor mechanism.[3,8,9] As such, the penumbral area around the core ischemic area may be protected by agents that prevent glutamate toxicity or that affect mechanisms downstream from the NMDA receptor and its gating of calcium influx.[17,25]

We undertook a series of studies to determine the efficacy of estrogens in preventing the ischemic lesions caused by MCA occlusion in ovariectomized rats. In addition, we assessed the prophylactic actions of the steroid by pretreating ovariectomized rats, and whether estrogens could prevent ischemic damage when treatment was initiated after the onset of the occlusion.

Materials and Methods

Preparation of Animals

Female Charles Rivers CD rats, each weighing 200 to 225 g, were maintained in our American Association for the Accreditation of Laboratory Animal Care–accredited vivarium for 1 week prior to ovariectomy. All animal procedures were approved by the University of Florida Animal Care and Use Committee. After anesthesia had been induced in the rat by methoxyflurane inhalant, bilateral ovariectomy was performed 7 to 8 days before drug administration and MCA occlusion.

Steroid Treatments

Either 17β-E2 or 17α-E2 was packed into 5-mm-long Silastic tubes that were closed on either end with Silastic adhesive. Sham (empty) pellets were similarly prepared, but without addition of the steroid. All pellets were washed with methanol to remove the steroid adhering to the outside of the tubes. Subsequently, the pellets were washed in physiological saline, a procedure that ensures first-order in vivo release of estradiol to achieve physiologically relevant concentrations.[36] The pellets were implanted subcutaneously into ovariectomized rats 24 hours prior to the MCA occlusion.

The 17β-E2 chemical delivery system (CDS) was synthesized in the manner we previously described in detail.[5,6,12] This system is designed to achieve a rapid and enhanced delivery of active steroid across the blood-brain barrier and for subsequent trapping in the brain.[5,12] We have previously demonstrated that the 17β-E2 CDS achieves brain/blood concentration ratios of 80:1, indicating the effectiveness of the delivery of the steroid.[5,12,29–31] To achieve a formulation that is aqueous soluble and therefore suitable for intravenous injection, we complexed the 17β-E2 CDS with 20% hydroxypropyl-β-cyclodextrin (HPCD).[7] The complex comprised 32 mg 17β-E2 CDS/1 g HPCD.

Middle Cerebral Artery Occlusion

At 7 or 8 days after ovariectomy, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). During surgery, the rat's rectal temperature was monitored and maintained with a heating lamp at between 36.5 and 37°C. For 24 hours following surgery, the rats were housed in a plastic cage that was maintained at 37°C.

With the aid of an operating microscope, a midline skin incision in the neck was made to expose the left carotid artery. The sternohyoid, digastric (posterior belly), and the omohyoid muscles were divided and retracted. The greater horn of the hyoid bone was removed to expose the distal external carotid artery (ECA). The common carotid artery (CCA), the ECA and its branches (occipital and superior thyroid arteries), and the internal carotid artery (ICA) and its extracranial branch (the pterygopalatine artery) were identified and separated from adjacent cranial nerves (vagus and glossopharyngeal). After the dissection was completed, the distal ECA and its branches, the CCA, and the pterygopalatine arteries were cauterized completely. The ECA and the occipital arteries were cut and a microvascular clip was placed on the ICA near the base of the skull.

The tip of a 2.5-cm length of No. 3-0 monofilament nylon suture was heated to create a globule for easy movement and blockade of a vessel lumen. The suture was introduced through a puncture in the ECA lumen and was gently advanced to the distal ICA until it reached the clipped position. The microvascular clip was then removed and the suture was inserted until resistance was encountered. The distance between the CCA bifurcation and the resistant point was 1.8 cm. The resistance indicated that the suture had passed the origin of the MCA and reached the proximal segment of the anterior cerebral artery. This portion of the operative procedure was completed within 10 minutes without bleeding. After the prescribed occlusion time (40 minutes), the suture was withdrawn from the ICA, the distal ICA was immediately cauterized, and the wound was closed.

Quantitation of Mortality and Ischemic Area

The animals that survived until the scheduled time of death (see description of the individual studies that follow) were killed by decapitation. The brains were removed from the cranium and placed in a metallic brain matrix for tissue slicing. The brain tissue slices were then incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium in physiological saline at 37°C. Ischemic lesion size, as estimated by this procedure, correlates very closely with that estimated using hematoxylin and eosin staining.[1] Stained slices were photographed and subsequently imaged for assessment of the cross-sectional area of the ischemic lesion. These images and the calculated area of ischemic damage were stored in a computer for later retrieval and data reduction.

Study 1. This study was conducted to determine the effects of 24-hour pretreatment with two estrogen formulations on ischemic lesion size after MCA occlusion. The animals were treated either with a single intravenous injection of 17β-E2 CDS (1 mg/kg body weight; eight animals) or with a subcutaneous implant of 17β-E2 (eight animals) 24 hours prior to MCA occlusion. A sham-implanted group (six animals) served as the control.

Postischemic times of death were scheduled at 6 hours, 24 hours, and 1 week. For the 6-hour sample, the animals were monitored continuously. For the 24-hour sample, the animals were observed for approximately 4 hours and then returned to their cages in the vivarium. Animals scheduled to be killed 1 week postischemia were similarly monitored for the first 4 hours after surgery and daily thereafter. Only those animals scheduled to be killed 24 hours postischemia were assessed for ischemic area.

Inasmuch as this study was a preliminary assessment of the effectiveness of two estrogen preparations, a limited number of brain slices were evaluated. Three coronal sections were made at 1, 5, and 7 mm posterior to the olfactory bulb.

Study 2. To determine the activity of the presumed weak or inactive estrogen isomer, 17α-E2, we implanted 17α-E2 (13 animals) or an empty Silastic pellet (sham treatment; 10 animals) subcutaneously 24 hours before MCA occlusion. Twenty-four hours after MCA occlusion, five 2-mm-thick coronal sections were made at 5, 7, 9, 11, and 13 mm posterior to the olfactory bulb.

Study 3. To test the extent to which estrogen treatment was effective after the onset of occlusion, ovariectomized rats were treated by intravenous injection of either 17β-E2 CDS or its vehicle, HPCD, 40 minutes after onset of the MCA occlusion. The 17β-E2 CDS was used for these evaluations because it can be administered intravenously and rapidly delivers the active steroid to the brain. The 17β-E2 CDS was administered at a single dose of 1 mg/kg body weight (10 animals) and control animals received 1 ml HPCD/kg body weight (nine animals).

Study 4. To define the post-MCA occlusion time course of the estrogen effect further, we treated rats with 17β-E2 CDS (six animals) or HPCD vehicle (seven animals) 90 minutes after onset of MCA occlusion by using the procedures described in Study 3.

Statistical Analysis

The significance of differences in mortality rates among treatment groups (Study 1) or between groups (Studies 2–4) was determined using chi-square analysis. In Study 1, the significance of differences in ischemic area among groups was determined by analysis of variance, and the Fisher's test was used for the post hoc comparison. In Studies 2 to 4, Student's t-tests were used to determine the significance of differences between sham- and steroid-treated groups, because only two groups were compared for each experiment. To determine the area under the lesion curve for a given treatment, the trapezoidal method was used. Areas calculated for each animal were grouped and the differences between groups (Studies 2–4) were determined using Student's t-test. Area under the curve determinations were not made for Study 1, because only three brain slices were taken—too few to construct a lesion profile for the brain.

Sources of Supplies and Equipment

The rats were obtained from Charles River Breeding Co., Wilmington, MA. The Silastic tubes and Silastic Medical Adhesive were obtained from Dow-Corning, Midland, MI: the 2,3,5-triphenyltetrazolium from Sigma Chemical Corp., St. Louis, MO; and

TABLE 1

*Effects on mortality rates of various estrogen preparations and times of administration after MCA occlusion in ovariectomized rats**

Treatment	Time of Treatment	Planned Time of Death	Percentage Survival
Study 1			
sham	24-hr pre	6 hrs–7 days	35
17β-E2 implant	24-hr pre	6 hrs–7 days	78†
17β-E2 CDS	24-hr pre	6 hrs–7 days	84†
Study 2			
sham	24-hr pre	24 hrs	54
17α-E2	24-hr pre	24 hrs	71
Study 3			
HPCD	40-min post	24 hrs	64
17β-E2 CDS	40-min post	24 hrs	100†
Study 4			
HPCD	90-min post	24 hrs	67
17β-E2 CDS	90-min post	24 hrs	67

* Post = posttreatment; pre = pretreatment.
† $p < 0.05$ compared with sham- or HPCD-treated control group as determined by chi-square analysis.

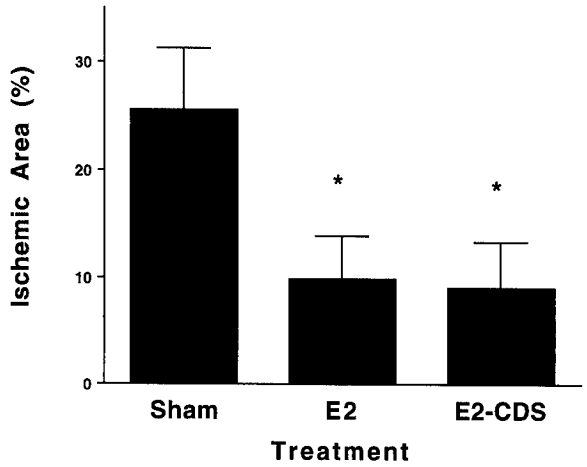


FIG. 1. Bar graph depicting effects of pretreatment for 24 hours with a sham pellet (or injection) (six animals), a 17β-E2 implant (eight animals), or 17β-E2 CDS (eight animals) on the area of ischemic damage induced by MCA occlusion. Values represent the means ± standard error of the means for the percentage of cross-sectional area occupied by the ischemic lesion in three brain slices. *$p < 0.05$ compared with the sham-treated group.

the metallic brain matrix from ASI Instruments, Inc., Warren, MI. A Macintosh Quadra 800 computer (Apple Computer, Inc., Cupertino, CA) with Image 1.47 software program (National Institutes of Health public domain program developed by Wayne Rasband) was used to assess the area of ischemic lesion.

Results

Study 1

Middle cerebral artery occlusion caused a high mortality rate in ovariectomized rats, as only 35% of sham-treated animals survived until the scheduled postischemic time of death (Table 1). In contrast, when treated 24 hours before MCA occlusion with either a subcutaneous implant of 17β-E2 or injection of 17β-E2 CDS, 78% and 84% of animals, respectively, survived until the scheduled postischemic time of death (Table 1). This reduction in mortality rate was evident for rats scheduled to be killed at 24 hours and 1 week, but not at 6 hours postischemia.

Part of the explanation for the reduced mortality rate in the estrogen-treated rats was evident from the assessment of ischemic areas in animals that survived to the scheduled 24-hour or 1-week postischemia time of death (Fig. 1). An examination of slices obtained through the largest extent of ischemic area showed that sham-treated rats had ischemic lesions that occupied 25.6 ± 5.7% of the cross-sectional area of the three brain sections evaluated (Fig. 1). By contrast, rats treated with 17β-E2 or 17β-E2 CDS showed ischemic lesions that occupied only 9.8 ± 4% and 9.1 ± 4.2%, respectively ($p < 0.05$ for both 17β-E2 and 17β-E2 CDS groups compared with the sham-treated group), of the brain area evaluated.

Study 2

To determine the activity of the presumed weak or inactive estrogen isomer, 17α-E2, we subcutaneously implanted the steroid medication 24 hours before MCA occlusion. The survival rate of sham-treated rats in this study was 54%, whereas the survival rate of 17α-E2–treated animals was 71% (Table 1).

Sham-treated rats showed the expected ischemic lesion, with the maximum ischemic area (24.1 ± 2.4%) occurring in the slice obtained 9 mm posterior to the olfactory bulb and a smaller lesion occurring in slices obtained from more rostral and more caudal areas (Figs. 2 and 3). Animals pretreated with 17α-E2 exhibited smaller ischemic areas in all slices evaluated (Fig. 2). Specifically, in the sections obtained 7, 9, and 11 mm posterior to the olfactory bulb, the ischemic area was reduced significantly by 55%, 66%, and 81%, respectively (Figs. 2 and 3). The area under the ischemic lesion curve for the sham-treated and 17α-E2–treated groups was 8.1 ± 0.8% and 3.7 ± 1.3%, respectively ($p < 0.02$; Student's t-test). The mean ischemic area over the five slices was 14 ± 1.5% for the sham-treated group and 6.3 ± 2.3% for the 17α-E2 group ($p < 0.05$).

Study 3

To test the extent to which estrogen treatment was effective after onset of the occlusion, ovariectomized rats were treated intravenously with either 17β-E2 CDS or HPCD at 40 minutes after onset of MCA occlusion. The HPCD-treated animals had a survival rate of 64%, whereas treatment with 17β-E2 CDS increased survival to 100% (Table 1).

In the HPCD-treated rats, large ischemic areas were observed in all slices sampled, with the maximum ischemic area of 25.6 ± 2.7% observed in the slice obtained from 9 mm posterior to the olfactory bulb (Fig. 4). The 17β-E2 CDS treatment reduced the ischemic area in all slices sampled (Figs. 4 and 5). The extent of reduction in ischemic area ranged from 90% in the slice obtained 5 mm posterior to the olfactory bulb to 45% in the slice 9 mm posterior to the olfactory bulb (Figs. 4 and 5). The inte-

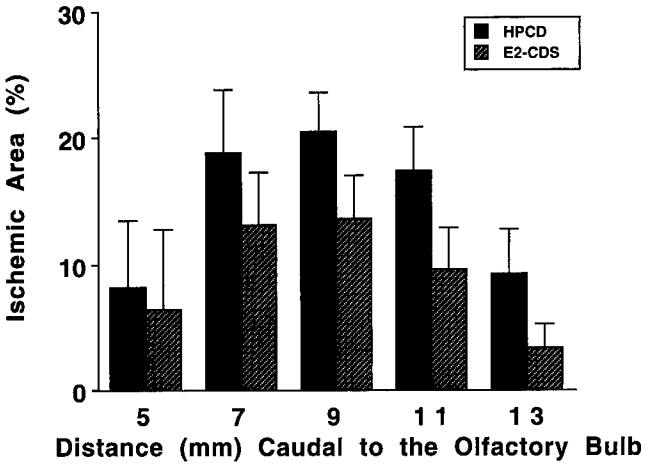


FIG. 6. Bar graph showing effects of 90-minute posttreatment with HPCD (seven animals) or 17β-E2 CDS (six animals) on the area of ischemia damage induced by MCA occlusion. Depicted are the means ± standard error of the means for ischemic areas in five brain slices. Treatment was administered by an intravenous injection 90 minutes after onset of the occlusion.

influx.[8,9,11,26] We observed no interaction of estrogens with the NMDA receptor (JW Simpkins, et al., unpublished observations), but have not ruled out the possibility of an effect of this steroid on Ca^{++} homeostasis of neurons. Alternatively, estrogens could exert the observed neuroprotective effects by reducing the oxidative damage associated with ischemia and reperfusion.[7] We and others have observed that estrogens are potent antioxidants in in vitro systems.[2,16,33,34] Finally, estrogens could reduce ischemic damage by increasing blood flow during the occlusion or by reducing reperfusion hyperemia. In normal human subjects, extreme hyperestrogenemia increases cerebral blood flow velocity.[32] Hurn, et al.,[21] recently reported that continuous treatment of female rabbits with 17β-E2 reduces reperfusion hyperemia following incomplete global ischemia. The same estrogen effect could occur following MCA occlusion ischemia.

In summary, estrogen pretreatment attenuates ischemic damage related to MCA occlusion in the ovariectomized rat in a manner that is independent of the potency of the estrogen, its chemical form, and the route of administration. Similarly, estrogen appears to be effective when administered soon after the ischemic event and this effect does not involve changes in the binding of NMDA to its receptor. In conclusion, estrogens may be effective compounds for the prevention and treatment of ischemic events related to stroke.

Disclosure

James W. Simpkins, Ph.D., holds patents in the area of estrogens and neuroprotection and Nicholas Bodor, Ph.D., holds patents related to the 17β-E2 CDS.

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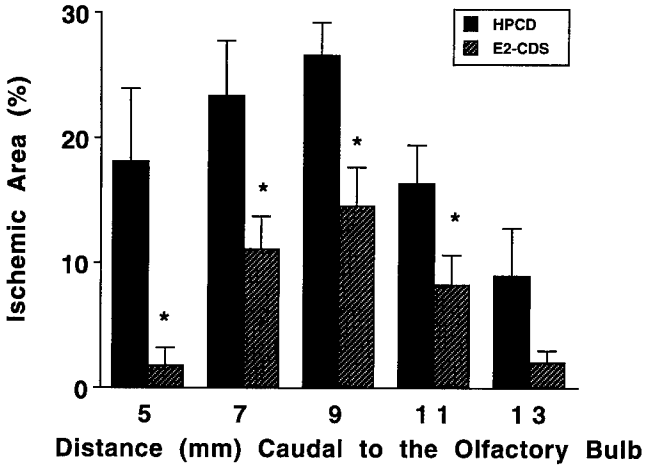


FIG. 4. Bar graph showing effects of 40-minute posttreatment with HPCD (nine animals) or 17β-EC2 CDS (10 animals) on the area of ischemic damage induced by MCA occlusion. Depicted are means ± standard error of the means for the ischemic areas in five brain slices. Treatment was administered by an intravenous injection 40 minutes after onset of the occlusion. *$p < 0.05$ compared with the HPCD group for the same brain slice.

surgery. Neither the surgical techniques nor the anesthesia appeared to affect survival outcome. We assume that the extent and mass effect of the ischemic lesion contributed predominately to survival rates in both estrogen-treated and control animals.

Pretreatment with either 17β-E2 or 17α-E2 by subcutaneous implantation produced the same degree of protection from the neurodegenerative effects of MCA as administration of a large dose of the brain-targeted 17β-E2 CDS. Collectively, these data indicate that it is not the isomer of estradiol or the mode of delivery of the steroid to the brain that affects the protection observed. It appears that the presence of estrogens in the brain before MCA occlusion provided the protection. Such an observation argues against the involvement of the nuclear estrogen receptor–estrogen responsive element in the neuroprotective signal against ischemic damage following occlusion. This is also supported by our earlier observations using in vitro assessments of protection of neuronal cells from a variety of insults, including serum deprivation[4,18,33,34] and β-amyloid treatment,[2,17,19,33,34] in which estrogen antagonists were ineffective in blocking the neuroprotective effects of either 17β-E2 or 17α-E2.[18,33,34] Recent structure–activity relationship evaluations indicate that the phenolic A ring and at least two rings of the steroid structure are critical for neuroprotection in vitro, whereas other modifications in the structure of the estrogen have little influence on neuroprotective activity (PS Green, et al., unpublished observations). Finally, other steroids, including androgens, progestins, glucocorticoids, and aldosterone were inactive in protecting nerve cells in vitro (PS Green, et al., unpublished observations). As such, it appears that among steroids, estrogens have a unique structure that is necessary for neuroprotection.

The 17β-E2 CDS was designed and synthesized by us to achieve a rapid and sustained delivery of 17β-E2 to the brain.[5,12] This chemical means for delivery of 17β-E2 has

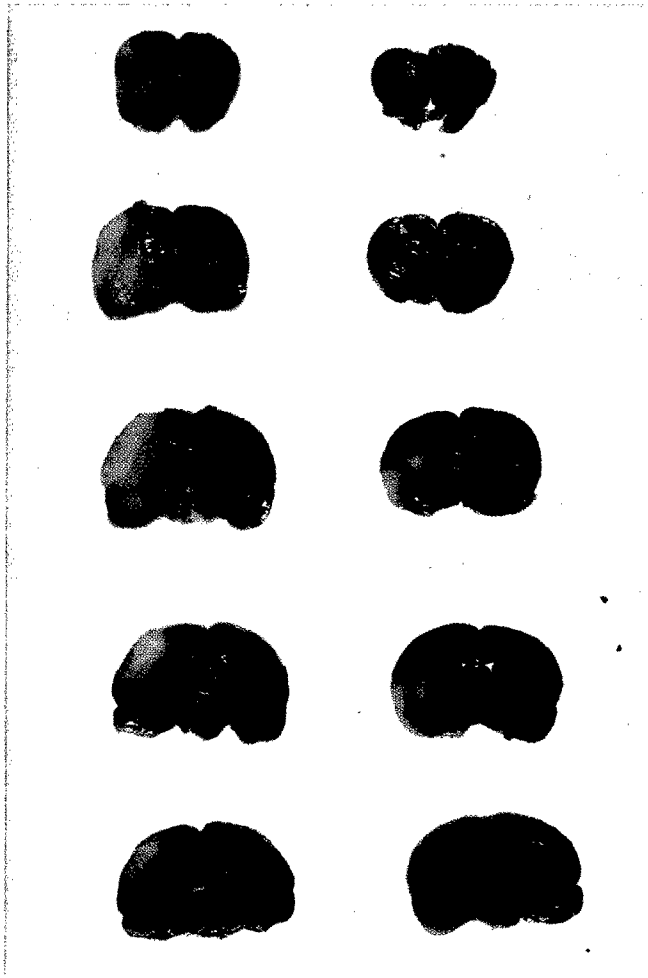


FIG. 5. Photograph displaying representative brain sections obtained from ovariectomized rats treated 40 minutes after onset of MCA occlusion with HPCD *(left)* or 17β-E2 CDS *(right)*.

the advantages of intravenous administration, rapid distribution to the brain, brain trapping of the inactive precursor of 17β-E2, and sustained release of the active steroid.[5,12] We have reported a sustained increase in brain 17β-E2 for at least 21 days[5,12,29–31] and biological effects of the released estradiol for up to 60 days[5,12,29,31] after a single intravenous injection of the 17β-E2 CDS in rats. We administered 17β-E2 CDS before and two times after MCA occlusion. The 17β-E2 CDS was effective when administered 24 hours before or 40 minutes after MCA. This neuroprotective effect persisted through at least 90 minutes postischemia, although the magnitude of the effect was less at this time point. We have not compared 17β-E2 CDS with either 17β-E2 or 17α-E2 when administered after the ischemic event, because soluble preparations of the steroids were not available to us.

The mechanism of neuroprotective effects of estrogens is not currently known, although several possibilities are apparent. Estrogens could interfere with the excitatory amino acid propagation of ischemic damage, by antagonizing the NMDA receptor or by interacting with a mechanism subsequent to the NMDA receptor, such as Ca^{++}

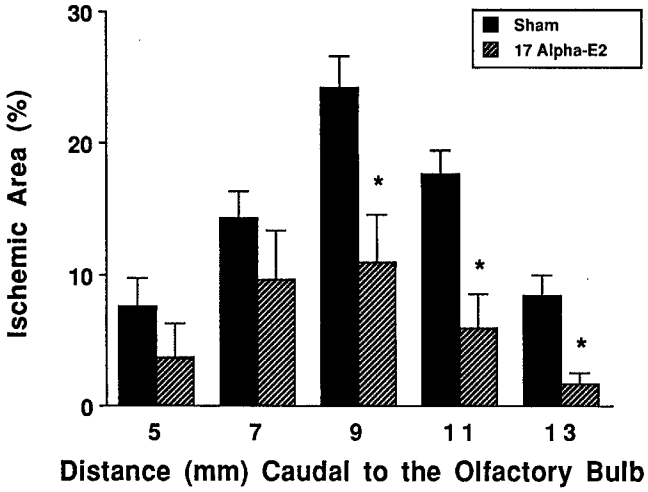


FIG 2. Bar graph showing effects of pretreatment with a sham pellet (10 animals) or 17α-E2 (13 animals) on the area of ischemic damage induced by MCA occlusion. Depicted are the means ± standard error of the means for the percentage of ischemic area in five brain slices. *$p < 0.05$ compared with the sham group for the same brain slice.

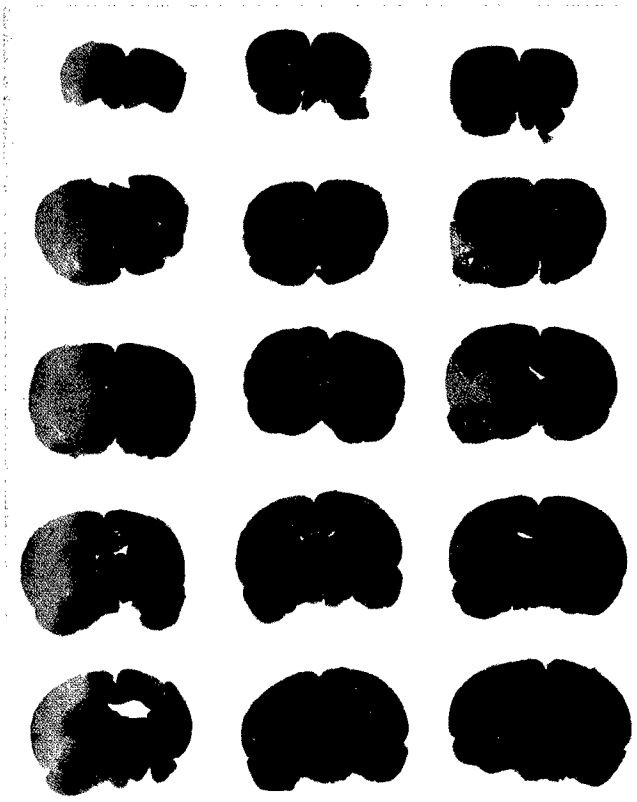


FIG. 3. Photograph displaying representative brain sections from ovariectomized rats pretreated for 24 hours before MCA occlusion with sham implants *(left)* or 17α-E2 (*center* and *right*).

grated area under the ischemic lesion curve was 10.1 ± 1.6% for the HPCD-treated rats and 4.5 ± 0.9% for the 17β-E2 CDS animals ($p < 0.02$; Student's t-test). The mean ischemic area over the five slices was 14.8 ± 3.4% for the HPCD control group and 7.5 ± 1.5% for the 17β-E2 CDS group ($p < 0.05$).

Study 4

To define the post-MCA occlusion time course of the estrogen effect, we treated rats with 17β-E2 CDS or HPCD 90 minutes after onset of the occlusion. At this time point following MCA occlusion, no statistically significant effect of treatment on survival was observed (Table 1).

The HPCD-treated animals showed a large lesion in all slices sampled, with the maximum ischemic area seen in the slice obtained 9 mm posterior to the olfactory bulb (20.5 ± 3.1% of the slice area, Fig 6). Treatment with 17β-E2 CDS reduced the mean ischemic area in all slices examined; however, the differences were not statistically significant (Fig. 6). An evaluation of the area under the ischemia curve for the two groups revealed that treatment with 17β-E2 CDS reduced the ischemic area by 37.1%, from 8.2 ± 1.7% (HPCD-treated animals) to 5.2 ± 1.7% (17β-E2 CDS–treated). The mean ischemic area over the five slices was 14.9 ± 3.4% for the HPCD control group and 9.2 ± 3.4% for the 17β-E2 CDS group.

Discussion

The present study demonstrates for the first time that pretreatment with estrogens or early posttreatment with a brain-targeted delivery system for 17β-E2 following MCA occlusion can dramatically reduce the size of the brain ischemic area. This effect of estrogen pretreatment was independent of the route of administration and of the chemical form of the estrogen. The estrogenic compound 17β-E2 and the presumed inactive isomer, 17α-E2,[10,20,22] as well as the 17β-E2 CDS, which targets the steroid to the brain, were equally effective with administration before MCA occlusion. We infer from these data that exposure of the brain to this important ovarian steroid before the occurrence of an ischemic event provides substantial protection to neurons. These data are consistent with clinical observations that ERT is associated with a reduction in stroke-related deaths.[13,14,23,27,28,38] Interestingly, we also demonstrated a reduced mortality rate in ovariectomized rats treated with estrogen before the onset of MCA occlusion (Table 1).

During optimization of the MCA occlusion method in the male rat model, we typically observed a mortality rate of approximately 20% (JW Simpkins, et al., unpublished observations). In our initial studies of ovariectomized rats, the mortality rate was exceedingly high. Indeed, in the four studies conducted, survival rates for ovariectomized rats ranged from 35 to 67%. That this low survival rate was the result of steroid deprivation rather than an experimental artifact is indicated by the normalization of survival rates with restoration of estradiol levels through subcutaneous implantation of 17β-E2 or its preferential delivery to the brain using the 17β-E2 CDS. Ovariectomized animals fully recovered from surgery and anesthesia and then died, usually within 4 hours after the

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Appended Materials

Appendices A to N

James W. Simpkins

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Appendix A
J. W. Simpkins

The Non-feminizing Enantiomer of 17β-Estradiol Exerts Protective Effects in Neuronal Cultures and a Rat Model of Cerebral Ischemia.

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Running Title: Neuroprotection by the enantiomer of 17β-estradiol

Key Words: Middle Cerebral Artery Occlusion, Neuroprotection, Estradiol, Enantiomer

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Abstract

Estrogens are potent neuroprotective compounds in a variety of animal and cell culture models and data indicate that estrogen receptor (ER)-mediated gene transcription is not required for some of these effects. To further address the requirement for an ER in estrogen enhancement of neuronal survival, we assessed the enantiomer of 17β-estradiol (*ent*-E2), which has identical chemical properties but interacts only weakly with known ERs, for neuroprotective efficacy. *Ent*-E2 was both as potent and efficacious as 17β-estradiol (βE2) in attenuating oxidative stress-induced death in HT-22 cells, a murine hippocampal cell line. Further, *ent*-E2 completely attenuated H_2O_2 toxicity in human SK-N-SH neuroblastoma cells at a 10 nM concentration. In a rodent model of focal ischemia, βE2 (100 μg/kg) or *ent*-E2 (100 μg/kg) injected 2 h prior to middle cerebral artery (MCA) occlusion resulted in a 60 and 61% reduction in lesion volume, respectively. *Ent*-E2 at the doses effective in this study did not stimulate uterine growth or vaginal opening in juvenile female rats when administered daily for 3 days. These data indicate that the neuroprotective effects of estrogens, both *in vitro* and *in vivo*, can be disassociated from the peripheral estrogenic actions.

Introduction

Epidemiological studies associate post-menopausal estrogen replacement therapy with several beneficial neurological outcomes including a reduction in incidence of Alzheimer's disease (1, 2), Parkinson's disease (3, 4), and death from stroke (5, 6). These effects may be mediated, in part, by estrogen-mediated enhancement of neuronal survival. The neuroprotective effects of estrogens, specifically the potent 17β-estradiol (βE2), have been widely described in

neuronal cultures against toxicities including growth factor deprivation, glutamate toxicity, and oxidative stress (for review see 7). Similarly, in rodents, βE2 has been shown to attenuate neuronal loss following cerebral ischemia (8-10), kainic acid administration (11), and physical injury (12).

The role of estrogen receptor (ER)-dependent transcription in estrogen's neuroprotective activity is controversial (for review see 7). The neuroprotective activity of βE2 in culture models is attenuated by the ER antagonists tamoxifen or ICI 182,780 in some studies (13-15); however, others report no effect of these same ER antagonists on βE2-mediated neuroprotection (16-20). Our laboratory (16, 21, 22) and others (17, 23) have reported equipotent (to βE2) neuroprotective efficacy of 17α-estradiol, which only weakly activates ER-dependent gene transcription (24), implicating mechanisms other than ER-mediated transcription in estrogen-mediated protection of neuronal cultures. In mouse models of cerebral ischemia, the data are equally inconclusive. Sampei et al. (25) report no difference in total lesion size between wild-type and ERα-deficient mice. However, ICI 182,780 administration increases striatal lesion volume in the wild-type mouse (26). ICI 182,780 administration did not alter neocortical lesion volume in this study. It is important to note that although protection of neocortical areas are consistently reported with βE2 treatment (8-10), βE2-mediated protection of striatal infarct is not consistently reported (9).

The present study addresses the requirement for ER-dependent transcription in the neuroprotective effects of estrogens both *in vitro* and *in vivo* using a novel enantiomer strategy. *Ent*-17β-estradiol (*ent*-E2), the enantiomer of the naturally occurring βE2 (Fig. 1), has identical physiochemical properties as βE2 except for interactions with other stereospecific molecules such as ERs. *Ent*-E2 is reported to interact only weakly with uterine-derived ERs (27, 28) and

lacks estrogenic effects on reproductive tissues in rodents (29-31). Some reports indicate that *ent*-E2 exerts slight anti-uterotrophic activity and can antagonize the uterotrophic effects of βE2 (32, 33). In contrast, *ent*-E2 has been reported to elicit alterations in lipid profiles identical to βE2 with similar potency (34). Further, *ent*-E2 is not enzymatically converted to βE2 (35) and therefore, is more adequately suited than 17α-estradiol for evaluating the role of ERs in estrogen-mediated neuroprotection. This study evaluates the neuroprotective effects of *ent*-E2 both in culture models of oxidative stress and in a rat transient focal ischemia model, and further, determines if *ent*-E2 can exert neuroprotective effects in the absence of stimulation of peripheral estrogen-responsive tissues.

Materials and Methods

Steroids

βE2 was purchased from Steraloids, Inc. (Wilton, NH). *Ent*-E2 was synthesized from the known starting material, [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxalan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (Chemical Abstracts Registry Number, 139973-49-2) which was prepared by a multistep synthetic pathway as described in the literature (36). This compound was then converted in either of two ways (Method A or Method B) to ent-19-nortestosterone (Chemical Abstracts Registry Number, 4091-86-5). In the first step of Method A, the double bond is reduced using lithium in liquid ammonia and the resulting tricyclic compound is cyclized to *ent*-19-nortestosterone in the second step. In the first step of Method B, the double bond is reduced by catalytic hydrogenation and the resulting tricyclic compound is again cyclized to *ent*-19-nortestosterone in the second step.

Method B has been previously used to prepare 19-nortestosterone (37). The hydroxy group of *ent*-19-nortestosterone is then esterified and the A-ring of the steroid is aromatized using $CuBr_2$ in acetonitrile. This reaction has been reported previously for the conversion of 19-nortestosterone, 17-acetate to 17β-estradiol, 17-acetate (38). Finally, the 17-acetate group is removed by saponification to give *ent*-E2 (Chemical Abstracts Registry Number, 3736-22-9). The structure of *ent*-E2 was proven by experimental data, which showed that the compound had the same melting point (176-177 °C), infrared absorption spectra (3449, 3246, 2936, 2864, 1611, 1587, 1500, 1450, 1283, 1250, 1057, 1012, 930, 874 cm^{-1}), ^1H NMR ((300 MHz, CD_3OD) δ 7.06 (1 H, d, J = 8.7 Hz), 6.54-6.46 (2 H, m), 3.64 (1 H, t, J = 8.4 Hz), 0.75 (3 H)) and ^{13}C NMR ((75 MHZ, CD_3OD) δ 156.07, 138.98, 132.80, 127.32, 116.18, 113.85, 82.57, 51.32, 45.34, 44.36, 40.50, 38.01, 30.67 (2 x C), 28.48, 27.56, 23.99, 11.62) spectra, but opposite optical rotation ($[\alpha]_D^{28}$ = –71.2 (c = 0.99, CH_3OH)) as βE2.

Steroids were initially dissolved in ethanol at a 10 mM concentration and then diluted to the appropriate concentration in culture media or assay buffer for cell culture or *ex vivo* assays, respectively. Steroids were dissolved in corn oil at the concentration necessary to yield the indicated dose in 1 ml/kg injection volume for rodent studies.

Cell Culture

SK-N-SH human neuroblastoma cells were obtained from ATCC (Rockville, MD) and HT-22 cells (immortalized hippocampal neurons of murine origin) were a generous gift of Dr. David Schubert (Salk Institute, San Diego, CA). Cells were maintained in RMPI-1640 and DMEM media (GIBCO, Gaithersburg, MD), respectively, supplemented with 10%

charcoal/dextran-stripped fetal bovine serum (Hyclone, Logan, UT) and 200 μg/ml gentamycin according to standard culture conditions.

Cells were plated 24 h prior to initiation of experiment at a density of 20000 cells/well (SK-N-SH cells) or 5000 cells/well (HT-22 cells) in both clear and white bottomed Nunc® 96-well plates (Fisher Scientific, Orlando, FL). Steroids were added at a concentrations ranging from 0.1 nM to 10 μM either 2 or 24 h prior to exposure to either glutamate (5 mM) or H_2O_2 (3-60 μM). Ethanol was used at a concentrations of 0.001 to 0.1 % v/v as a vehicle control. These concentrations of ethanol had no discernable effect on cell viability. Following 24 h of toxin exposure, cells were rinsed with PBS, pH 7.4 and viability was assessed by the addition of 1 μM calcein AM (Molecular Probes, Eugene, OR) and 1 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) in PBS for 15 min. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm. % Viability was calculated by normalization of all values to the toxin-free control group (= 100 %). % Protection was calculated as the difference between each experimental value and the average of the toxin-only group normalized to the difference between the toxin-free control and toxin-only groups (= 100 % protection). Cells which had been lysed by addition of 1% SDS were used for blank readings. Staining was visualized using a fluorescent Nikon microscope and cells were photographed for qualitative documentation.

Animals

Female Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed in pairs in hanging, stainless steel cages in a temperature controlled room (25 ± 1°C) with a daily light cycle (on from

0700 to 1900 daily). All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before the initiation of the study.

Ovariectomy

Female Sprague-Dawley rats (220-225 g body weight) were given 3-5 days to acclimate then were bilaterally ovariectomized using a dorsal approach. Animals were anesthetized with methoxyflurane (Pitman Moore, Inc., Crossing, NJ) inhalant anesthesia. A small (1 cm) cut was made through the skin, facia, and muscle. The ovaries were externalized, clipped, and removed then the muscle, facia, and skin were sutured closed. Ovariectomy was performed 2 weeks prior to experiments.

MCA Occlusion

Either oil vehicle or 100 μg/kg of βE2 or *ent*-E2 was administered by subcutaneous injection 2 h prior to the onset of MCA occlusion. Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). MCA occlusion was performed as previously described (8). Briefly, the left common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 3-0 monofilament suture was introduced into the ICA lumen and gently advanced until resistance was felt. The suture was kept in place for 60 minutes and then withdrawn to allow MCA reperfusion. The procedure was performed with 20 min with minimal bleeding. Rectal temperature was maintained between 36.5 and 37.0 °C during the entire procedure.

Animals were decapitated and the brain removed 24 h following onset of MCA occlusion. The brain was then dissected coronally into 2 mm sections using a metallic brain matrix (ASI Instruments, Inc., Warren, MI). The sections 3, 5, 7, 9, and 11 mm posterior to the tip of the olfactory bulb were stained by incubation in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TCC) in a 0.9% saline solution at 37°C for 30 min. Slices were then fixed in 10% formalin, photographed, and the ischemic lesion area determined for each slice using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Percent ischemic lesion area was calculated as the sum of the ischemic lesion area for the five slices divided by the total cross sectional area of these five slices.

Plasma levels of βE2

Ovariectomized female Sprague-Dawley rats were injected subcutaneously with either oil vehicle or 100 μg/ml of βE2 or *ent*-E2. Blood samples were obtained by cardiac puncture 5 min prior to injection or 1 h, 4 h, or 24 h post-injection. Plasma was stored at -20°C until assayed using the ultra-sensitive βE2 RIA kit from Diagnostic Systems Laboratories, Inc (Los Angeles, CA) according to the manufacturer's instructions. *Ent*-E2 showed no cross-reactivity with the RIA at concentrations up to 10 μM.

Uterotrophic Assay

Juvenile (25 d old) female Sprague-Dawley rats were injected subcutaneously with oil, βE2 (0.01 to 1 μg/rat), or *ent*-E2 (1 to 100 μg/rat) daily (0830) for 3 d. On the fourth day, the rats were euthanized using methoxyflurane and the uteri excised. Extraneous tissue was gently

removed from the uteri before wet weight was determined. Vaginal opening was assessed prior to uterine removal.

Ligand Competition of Estrogen Receptor Binding

5 nM [2,4,6,7-^{3}H]-17β-estradiol (specific activity 84.1 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) and 400 pM recombinant human ERα or ERβ (Affinity Bioreagents, Inc., Golden, CO) were incubated in ER binding buffer (20 mM Tris, 1 mM EDTA, 400 mM KCl, 1 mM DTT, 10 % glycerol, 0.1 % BSA, pH 7.8) for 1 h at 25°C with no added steroid (total binding), 1.2 μM diethylstilbesterol (non-specific binding), or 0.1 nM to 10 μM βE2 or *ent*-E2. Bound and unbound radioligand were separated using Sephadex G-25 (Amersham Pharmacia Biotech) columns (1.5 ml bed volume) with a 1 ml elution volume. 10 ml scintillation fluid was added and counts determined. This method resulted in greater than 90 % receptor recovery and less than 15 % non-specific binding.

Brain Membrane Oxidation

The brain was removed from ovariectomized female Sprague-Dawley rat and the neocortex was homogenized in ice-cold Tris buffer (100 mM, pH 7.4) with 1 % Triton X-100 using a Teflon/glass tissue homogenizer. The homogenate was centrifuged at 2000 rpm for 10 min. The resulting supernatant was incubated with βE2 or *ent*-E2 at concentrations ranging from 0.1 to 100 μM for 30 min at 37°C. $FeSO_4$ was then added to a final concentration of 200 μM and incubated for an additional 30 min at 37°C. BHT (100 μM) and DPTA (100 μM) were then added. 2-thiobarbituric acid reactive products (TBARs) were immediately determined by

addition of 0.5% 2-thiobarbituric acid, 3.125% trichloroacetic acid and 0.2 N HCl and incubation at 95°C for 1 h. Samples were centrifuged at 10000 rpm for 10 min and the absorbance of the supernatant at 532 nm determined.

Statistical Analysis

All data are presented as mean ± sem. Comparison of ischemic lesion volume was performed using a one-way ANOVA with a Kruskal-Wallis test for planned comparisons between groups. For all other experiments, the significance of differences among groups was determined by one-way ANOVA with a Tukey's Multiple Comparisons Test for planned comparisons between groups when a significant difference was detected. For all tests, $p<0.05$ was considered significant.

Results

Ent-E2 attenuates oxidative stress-induced death in neuronal cultures.

HT-22 cells, transformed hippocampal neurons, are sensitive to glutamate toxicity via a mechanism which involves glutathione depletion and the resulting oxidative stress (39). Exposure of HT-22 cells to 10 mM glutamate caused a 70 - 75 % reduction in neuronal viability by 24 h of exposure (Fig. 2). As previously reported (40), βE2 treatment commencing 2 h prior to glutamate exposure conferred significant protection in this model with a 10,000 nM concentration protecting 35 ± 4% of the cells. *Ent*-E2 performed similarly in this model of neuroprotection with 100 nM and 10,000 nM of *ent*-E2 protecting 16 ± 2 % and 56 ± 4 % of HT-22 cells, respectively.

In another model of oxidative stress, both βE2 and *ent*-E2 significantly attenuated H_2O_2-induced toxicity in HT-22 cells (Fig. 3). H_2O_2 exposure resulted in a concentration-dependent toxicity in HT-22 cells with a 30 μM concentration resulting in 21 ± 5 % reduction in viability (Fig. 3) and a 60 μM concentration resulting in a 97 ± 8 % reduction (data not shown). 10 nM of either βE2 or *ent*-E2 completely attenuated the toxicity of 30 μM H_2O_2 and protected 48 ± 14 % or 63 ± 8 % of the cells from 40 μM H_2O_2 toxicity, respectively (Fig. 3). No protection was seen with the 10 nM concentration of either steroid at H_2O_2 concentrations greater than 40 μM (data not shown). SK-N-SH cells were more sensitive than HT-22 cells to the toxic effects of H_2O_2 exposure with 3 μM H_2O_2 reducing SK-N-SH cell viability by 32 ± 2 % (Fig. 4). This kill was significantly attenuated by *ent*-E2 with a 1 nM concentration conferring 30 ± 9 % protection (Fig. 4). In other studies, 1 nM βE2 prevented 40 ± 5 % of H_2O_2-induced toxicity in SK-N-SH cells (data not shown). Neither steroid exert protective effects in these low nM concentration ranges with higher concentrations of H_2O_2 (data not shown).

Ent-E2 reduces ischemic lesion size following transient MCA occlusion.

Transient (1 h) occlusion of the MCA resulted in an average lesion area of 13 ± 2 % with the lesion localized primarily in the parietal cortex and basal ganglia (Fig. 5). Comparable to our previous reports (8), subcutaneous injection of βE2 2 h prior to onset of ischemia reduced total lesion area by 60 ± 13 %. Administration of *ent*-E2 similarly reduced total ischemic area by 60 ± 12 %. This estrogen-mediated protection was observed in both neocortical and subcortical/allocortical regions of the ischemic infarct. βE2 and *ent*-E2 reduced neocortical lesion size by 77 ± 11% and 59 ± 12 %, respectively. Similarly, subcortical/allocortical lesion

volume was reduced by 48 ± 8 % and 47 ± 7 % by βE2 and *ent*-E2 administration, respectively. The subcortical/allocortical ischemic area includes regions of the caudate/putamen, hypothalmus, and hippocampus.

The protective effects of *ent*-E2 in this model are not due to conversion of *ent*-E2 to the more estrogenically potent βE2. Plasma βE2 levels following *ent*-E2 administration did not change from the pre-injection baseline of 0.05 ± 0.01 nM (Fig. 6). In contrast, subcutaneous injection of βE2 resulted in a rapid rise in plasma βE2 levels with values of 5.16 ± 0.94 nM within 1 h and returned to near baseline (0.24 ± 0.08 nM) by 24 h.

Ent-E2 is a weak ER agonist/antagonist.

Daily administration of βE2 for 3 days caused a dose-dependent increase in uterine wet weight with a 1 μg/rat dose (average dose of 13.8 μg/kg) increasing wet uterine weight by two-fold (Fig. 7). By contrast, *ent*-E2 at doses of 1 to 10 μg/rat had no effect on uterine wet weight. At a dose of 100 μg/rat (average dose of 1400 μg/kg), *ent*-E2 exerted a slight anti-uterotrophic effect, decreasing uterine wet weight by 23 ± 3 %. *Ent*-E2 also slightly antagonized the uterotrophic effects of 1 μg/rat βE2 with a 100 μg/rat dose reducing the uterotrophic effect of βE2 by 27 ± 8 %. These results are comparable to previous reports in immature mice where *ent*-E2 (doses of about 1200 μg/kg) exerted anti-uterotrophic effects (29) and *ent*-E2 antagonized the uterotrophic effects of βE2 when *ent*-E2 was present in a 100-fold excess (32).

Daily injections of βE2 (1 μg/rat) induced vaginal opening in 100 % of the animals examined (Table 1). *Ent*-E2 exerted mixed agonist/antagonist effects on vaginal opening with a 100 μg/rat dose causing vaginal opening in 50 % of the juvenile rats. This dose of *ent*-E2

prevented βE2-induced vaginal opening in 40 % of the rats. No change in body weight was observed with administration βE2, *ent*-E2, or combinations thereof. Body weights of the juvenile rats averaged 72 ± 1g.

In competition binding experiments, *Ent*-E2 showed weak binding to both known ERs with 4.2 % and 6.3 % of the relative binding affinity of βE2 to ERα and ERβ, respectively. *Ent*-E2 has been previously reported to have 0.9 - 6 % of βE2's relative binding affinity to cytosolic uterine ERs (27,28).

Ent-E2 can attenuate brain lipid oxidation ex vivo.

As estrogens have been previously reported to reduced oxidative damage to brain lipids (41-43), we examined the potency of both βE2 and *ent*-E2 in an *ex vivo* assay of brain membrane oxidation. 30 min incubation of the neocortical homogenate resulted in a 16-fold increase in TBAR formation. βE2 and *ent*-E2 were equipotent in the attenuation of FeSO4-induced lipid oxidation as determined by TBAR formation (Fig. 8) with a 50 μM concentration of either steroid significantly attenuating $FeSO_4$-induced TBAR formation.

Discussion

Ent-E2 was both as potent and efficacious as βE2 in culture models of neuroprotection, and further, *ent*-E2 reduced ischemic lesion area following MCA occlusion to the same degree as βE2. In contrast, *ent*-E2 showed only minimal binding affinity for either known ER, was greater than 100-fold less potent than βE2 in exerting effects on uterine growth or vaginal opening, and had weak anti-uterotrophic effects. These data indicate that the neuroprotective effects of

estrogens can occur without stimulation of peripheral estrogen-responsive tissues.

The neuroprotective effects of *ent*-E2 are not likely due to conversion to the more estrogenically potent βE2 as the conversion requires isomerization of five individual chiral carbons. Isomerization of the 17-hydroxy group could be facilitated by 17β-hydroxy steroid dehydrogenase; however, *ent*-E2 is not a substrate for this enzyme (35). Further, there was no detectable increase in plasma βE2 levels during 24 h following subcutaneous injection of *ent*-E2 in female rats indicating that *ent*-E2 is itself neuroprotective.

The minimal neuroprotective concentration of both βE2 and *ent*-E2 varied with the cell culture model utilized. High physiological concentrations (low nM) were sufficient to attenuate H_2O_2-induced toxicity in SK-N-SH cells but significantly higher supraphysiological concentrations (low μM) were required to lessen glutamate toxicity in HT-22 cells. This difference in the neuroprotective potency of estrogens between these models may be due to a number of factors including differences in culture media and differences in toxicity. Further, the concentration of steroid required for protection may depend on the degree of insult as low concentrations of *ent*-E2 or βE2 did not protect SK-N-SH or HT-22 cells from H_2O_2 exposure if viability was reduced by more than 70 % (P.S. Green and J.W. Simpkins, unpublished observations).

The high doses of *ent*-E2 used in some, but not all, of the experiments in the present report could show appreciable ER binding; however, this does not adequately explain the equipotent neuroprotection conferred by the enantiomer. The 16- to 100-fold lower affinity of *ent*-E2 for the known ERs (this report, 27, 28) would be apparent as a similar 16- to 100-fold lower potency in effects mediated by either ERα or ERβ. This potency difference was seen in

uterotrophic and vaginal opening responses but not in assays of neuroprotection. Regardless of the minimum dose required for neuroprotection in each model, *ent*-E2 attenuated neuronal death with a potency equivalent to that of βE2. This result indicates that enantiospecific interactions between estrogens and other cellular molecules are not required for the neuroprotective actions of estrogens.

Several lines of evidence connote that the neuroprotective effects of estrogens do not require ER-dependent gene transcription including potent neuroprotective efficacy of several non-feminizing estrogens including *ent*-E2 (Figs. 2-5) and 17α-estradiol (16, 21-23). Further, functional ERs have not been found in either HT-22 cells (40, 44) or SK-N-SH cells (22) although this study(Fig. 2-4) and others (16, 21-23, 40, 44) demonstrate estrogen-mediated protection of these neuronal cell lines. Similarly, βE2-mediated protection can occur in the presence of ER antagonists (16-20). Together, these findings, while not excluding a role for ERs in neuroprotection, implicate cellular mechanisms other than classical ER activity in the neuroprotective effects of estrogens.

Antioxidant effects have been proposed as one mechanism for the neuroprotective effects of estrogens (19). Interestingly, the structure-activity relationship for the antioxidant effects of estrogens (42) is identical to the structure-activity relationship for the neuroprotective effects (22, 23). Further, it has been reported that the concentrations of βE2 which are capable of exerting *ex vivo* antioxidant effects were required for neuroprotective effects (19). βE2 has been shown to attenuate lipid peroxidation with μM concentrations (19, 41-43). The neuroprotective concentration for βE2 in culture models range from 0.1 nM (21, 45) to 50 μM (18). In this study *ex vivo* antioxidant effects of βE2 and *ent*-E2 required a minimum concentration of 50 μM

whereas neuroprotective effects were seen at much lower concentrations.

Neuronal effects of estrogens with weak ER agonist activity are being increasingly described. The classically inactive estrogen, 17α-estradiol, has been shown to be neuroprotective in both culture (16, 21-23) and MCA occlusion models (8). Similarly, the weak ER agonist dihydroequilin has been shown to exert neurotrophic effects in cultured neurons (46). The cellular mechanisms for these effects of weak ER agonists is not known; however, several cellular effects of 17α-estradiol has been described. Exposure to αE2 can activate the MAP kinase pathway (47) and this pathway is implicated in βE2-mediated neuroprotection (14). In addition, αE2 has also been shown to have several other direct effects on neurons including modulation of the mitochondrial Na+/K+-ATPase activity (48), alteration of membrane fluidity (49), and inhibition of toxin-induced activation of NFκB (P.S. Green and J.W. Simpkins, unpublished observations). It is unknown if *ent*-E2 can also interact with any of these cellular pathways.

A profusion of data indicates that estrogens enhance the survival of neurons both *in vitro* and *in vivo* suggesting that estrogens may be useful in the treatment of neurodegenerative disease or acute neuronal death. Estrogens, such as *ent*-E2, may offer the beneficial neuroprotective effects of estrogens without the complicating peripheral estrogenic actions and could be useful in both men and women for whom estrogen therapy is contraindicated.

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Table 1. Effects of βE2 and *Ent*-E2 on Vaginal Opening in Juvenile Female Rats.

Ent-E2 Dose (μg/rat)	Number with Vaginal Opening	
	Without βE2	With 1 μg/rat βE2
0	0 of 4	5 of 5
10	1 of 4	3 of 4
100	2 of 4	3 of 5

25 d old-female Sprague-Dawley rats were injected subcutaneously with the indicated dose of *Ent*-E2 with or with concurrent administration of 1 μg/kg βE2 daily for 3 d. On day 4, vaginal opening was assessed.

Figure Legends

Figure 1. Structure of the naturally occurring 17β-estradiol (βE2) and the non-naturally occurring *ent*-17β-estradiol (*ent*-E2).

Figure 2. Effects of βE2 and *ent*-E2 on glutamate toxicity in the HT-22 cells. The indicated concentration of steroid was added 2 h prior to the addition of glutamate (5 mM) and viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability and shown is mean ± sem for 4-8 wells and is representative of at least 2 individual experiments. *=$p<0.05$ and **=$p<0.01$ versus toxin only group. Pictured are representative fields stained with calcein AM and propidium iodide.

Figure 3. Effects of βE2 and *ent*-E2 on H_2O_2 toxicity in HT-22 cells. 10 nM of the steroid was added to HT-22 cells 2 h prior to the addition of the indicated concentration of H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 0% reduction in viability and shown is mean ± sem for 4 wells and is representative of at least 2 individual experiments. *=$p<0.05$ versus toxin only group.

Figure 4. Effect of *ent*-E2 on H_2O_2 toxicity in SK-N-SH cells. The indicated concentration of *ent*-E2 was added 24 h prior to the addition of 3 μM H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective

toxin-free group as 100% viability and shown is mean ± sem for 3-4 wells and is representative of at least 2 individual experiments. *=$p<0.05$ and **=$p<0.001$ versus the toxin only group.

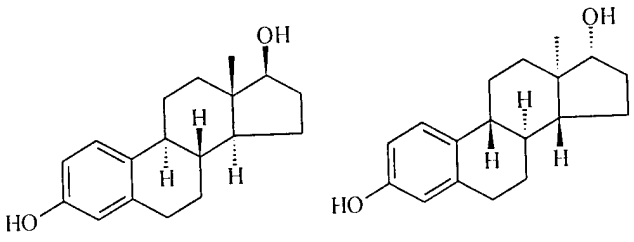
Figure 5. Effects of βE2 and *ent*-E2 on MCA occlusion-induced lesion volume in ovariectomized female rats. Rats were ovariectomized 2 weeks prior to occlusion and steroids were administered by subcutaneous injection 2 h prior to onset of focal ischemia. Following 1 h MCA occlusion and 23 h reperfusion, the brains were removed and 2 mm slices prepared at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Lesion volume was determined by TCC staining. Graphed is mean ± sem for 6 rats per group. *=$p<0.05$ versus vehicle treated rats. Pictured are representative slices for each treatment group.

Figure 6. Plasma βE2 levels following βE2 and *ent*-E2 administration. Ovariectomized female Sprague-Dawley rats were injected subcutaneously with either 100 μg/kg βE2 or *ent*-E2. Blood was drawn by cardiac puncture either 5 min prior to injection, 2 h post injection, 4 h post injection, or 24 h post injection. Plasma was collected and βE2 concentration determined by RIA. Plasma βE2 concentration is given in nM units: 1nM = 272 pg/ml. Shown are mean ± sem for 3 rats per group.

Figure 7. Effects of βE2 and *ent*-E2 on uterine wet weight in juvenile rats. 25 d old-female rats were injected subcutaneously with the indicated dose of βE2 or *ent*-E2, or concurrent administration of the indicated dose of *ent*-E2 with 1 μg/rat βE2 daily for 3 d. On day 4, the uteri were resected and weighed. Shown are mean ± sem for 3 to 9 rats per group. *=$p<0.05$ vs oil

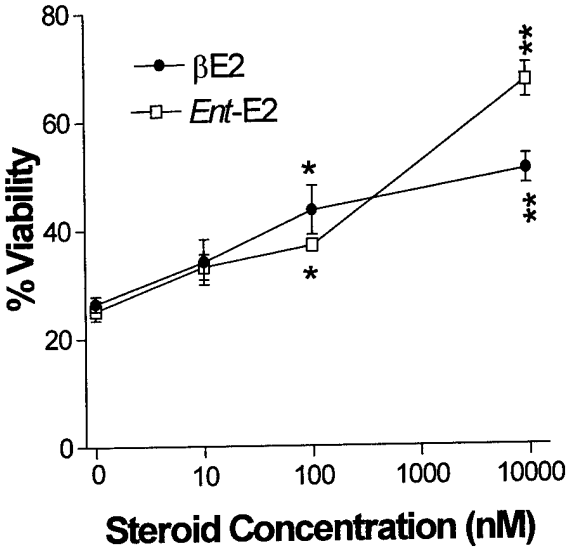
injection.

Figure 8. βE2 and *ent*-E2 inhibit $FeSO_4$-induced lipid oxidation in a rat brain homogenate. Homogenate was prepared from the neocortical tissue of an ovariectomized female Sprague-Dawley rat. Homogenate was incubated with the indicated concentration of steroid for 30 min. and then oxidized by a 30 min incubation with 200 μM $FeSO_4$ at 37°C. The extent of lipid oxidation was determined by TBAR formation. Data were normalized to $FeSO_4$ only group as 100% oxidation. Shown are mean ± sem for 3 samples per group. *=$p<0.05$ versus $FeSO_4$ only group.



17β-estradiol *Ent*-estradiol

Figure 1


80
60
40
20
0
% Viability
βE2
Ent-E2
*
*
**
**
0
10
100
1000
10000
Steroid Concentration (nM)

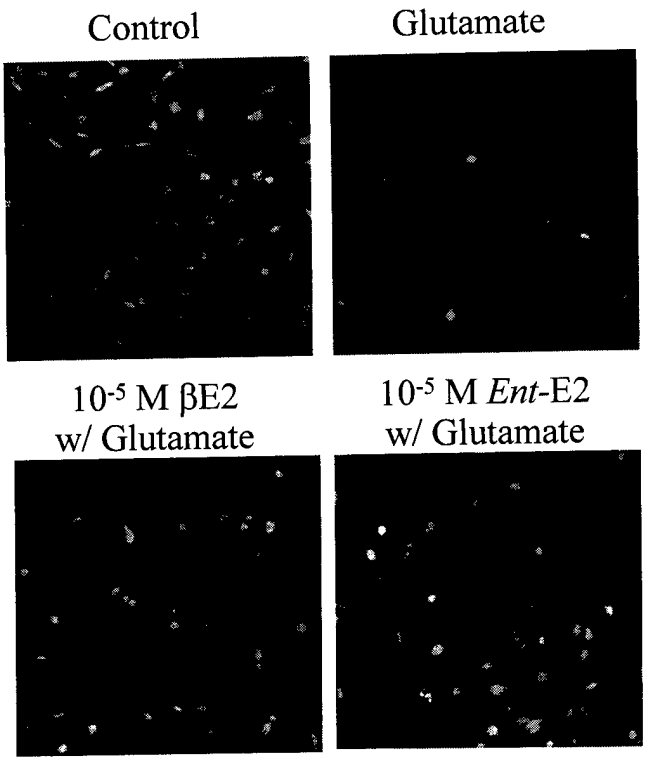

Control
Glutamate
10-5 M βE2
w/ Glutamate
10-5 M Ent-E2
w/ Glutamate

Figure 2

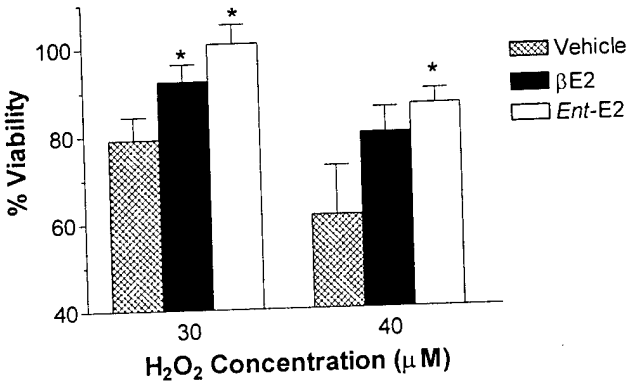

100
80
60
40
% Viability
*
*
*
30
40
H_2O_2 Concentration (μM)
Vehicle
βE2
Ent-E2

Figure 3

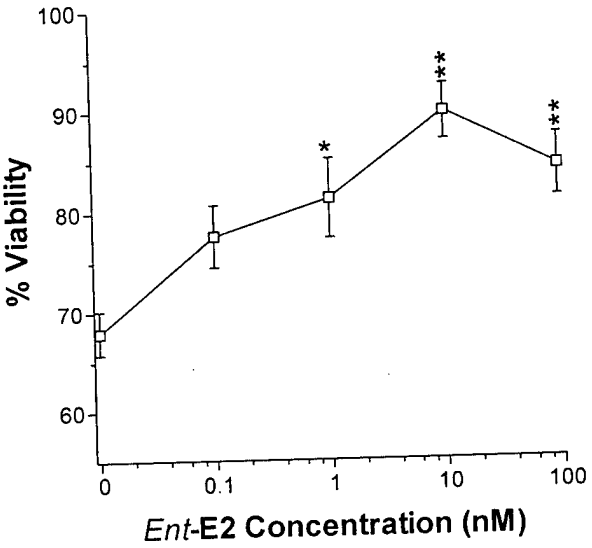


Figure 4

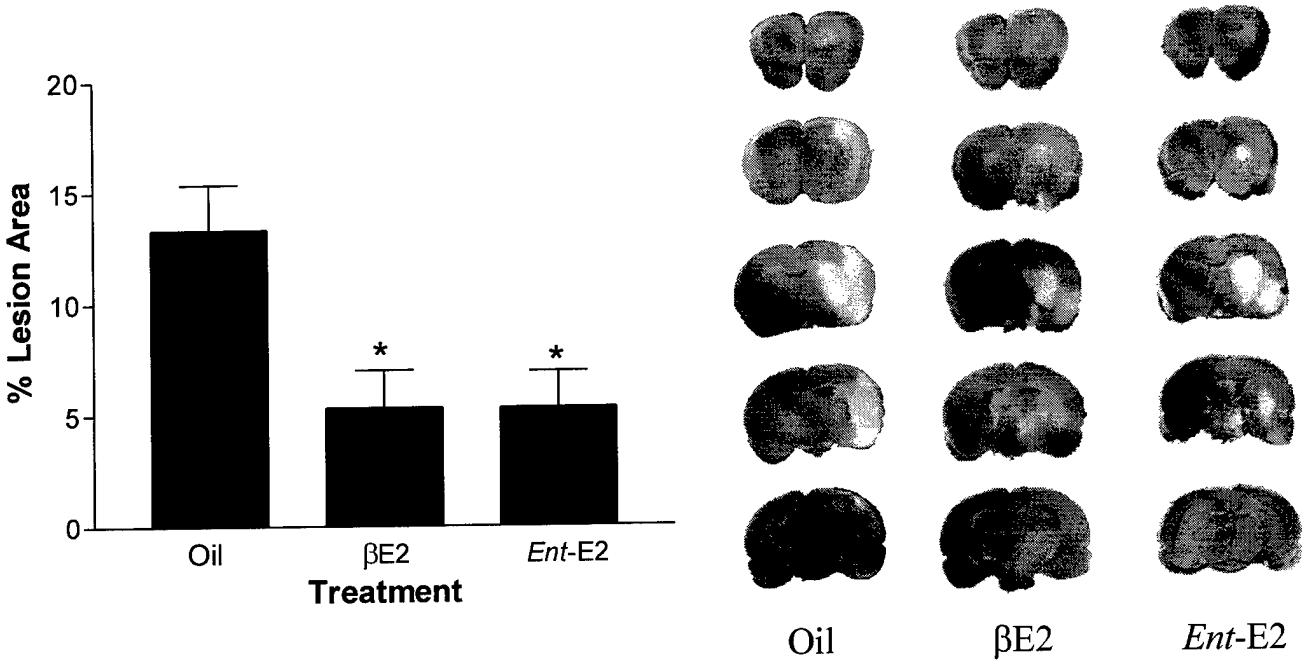


Figure 5

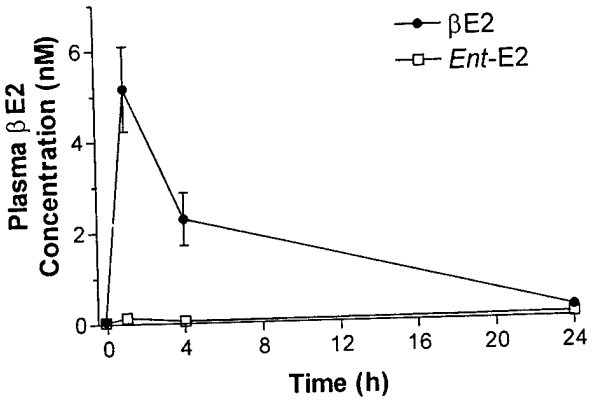

βE2
Ent-E2
Plasma βE2 Concentration (nM)
0
2
4
6
0
4
8
12
16
20
24
Time (h)

Figure 6

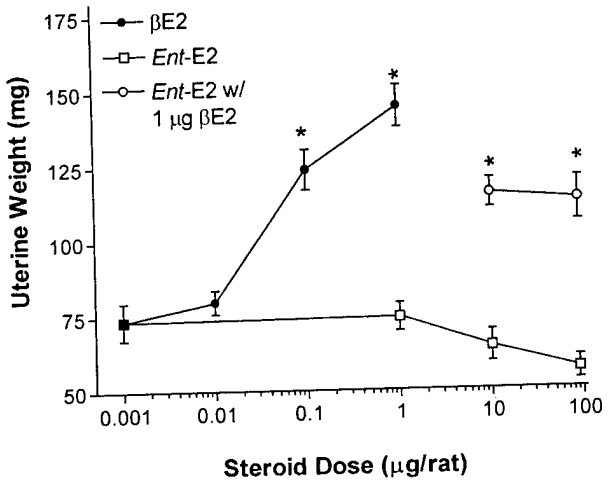

175
150
125
100
75
50
βE2
Ent-E2
Ent-E2 w/
1 μg βE2
Uterine Weight (mg)
*
*
*
*
0.001
0.01
0.1
1
10
100
Steroid Dose (μg/rat)

Figure 7

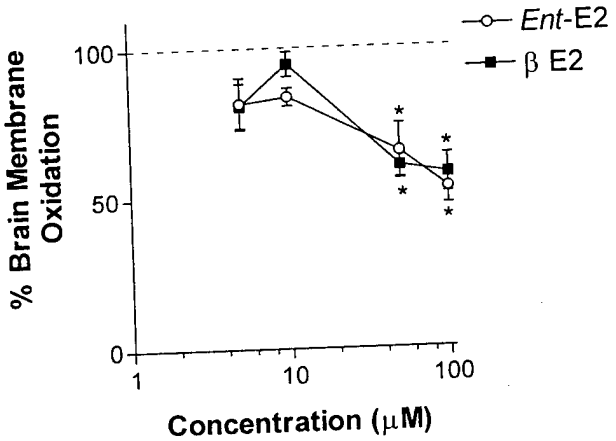

Ent-E2
β E2
% Brain Membrane Oxidation
100
50
0
1
10
100
Concentration (μM)

Figure 8

Synthesis And Biological Evaluation of 17β-Alkoxyestra-1,3,5(10)-Trienes as Potential Neuroprotectants Against Oxidative Stress

DAMA17-99-1-9473
Appendix B
J. W. Simpkins

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Abstract:

17β-O-Alkyl ethers (methyl, ethyl, propyl, butyl, hexyl and octyl) of estradiol were obtained from 3-O-benzyl-17β-estradiol with sodium hydride/alkyl halide, followed by the removal of the protecting group via catalytic transfer hydrogenation. An increase compared to estradiol in the protection of neural (HT22) cells against oxidative stress due to exposure of glutamate was furnished by higher (C-3 to C-8) alkyl ethers, while methyl and ethyl ethers decreased the neuroprotective effect significantly. Lipophilic (butyl and octyl) ethers blocking the phenolic hydroxyl (3-OH) of A-ring were inactive.

Introduction

Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* models. The antioxidant action is believed to be due to their ability to scavenge free radicals that cause neuronal cell death. Oxidative stress has been linked to neuronal cell death resulting from either acute insults due to ischemia, trauma, or chronic neurodegenerative diseases such as Alzheimer's disease (AD)[1] characterized by a progressive loss of memory and cognitive function. Although the histopathogenesis of AD is yet to be fully understood, one theory hypothesizes[2] that the oxidative microenvironment surrounding the accumulated amyloid-β-peptide (Aβ) plaques is responsible for peroxidation of cell membrane lipids leading to cell lysis and death.[3] Another oxidative stressor suggested in AD's pathogenesis is the amino acid glutamate, the major excitatory neurotransmitter in the central nervous system.

Population studies have shown that estrogen replacement therapy in postmenopausal women can decrease the incidence of AD or delay its onset.[4] It has been demonstrated[5] that the most biologically active estrogen, 17β-estradiol (**1**), is a potent antioxidant and has neuroprotective activity; however, the mechanism of action is still unclear. The direct neuroprotective effects of 1 on SK-N-SH human neuroblastoma cells under serum deprivation were first reported in 1994.[6] Numerous recent studies have demonstrated similar effects of **1** against a variety of toxicities, including oxidative stress,[7] in different types of neuronal cells. Interestingly, an enantiomer of estradiol ("ent-estradiol"), its epimer (17α-estradiol), and estratrien-3-ol are equipotent to **1** in protection of neural cells against oxidative damage, although they have significantly reduced estrogenic activities.[6,8] All neuroprotective derivatives or analogs of **1** possess, however, a common structural element which is an intact, unsubstituted phenolic A-

ring with its free 3-hydroxyl group. Since lipophilic phenols also protect neural cells against glutamate and peroxide mediated oxidative damage and cell death,[9] we decided to functionalize the 17-hydroxyl function of **1** by preparing alkyl ethers having a wide range of lipophilicity. We hypothesized that these ethers not only retain, but eventually increase neuroprotection compared to **1** (In the meantime, these 17β-O-ethers also have significantly less "feminizing" effect than **1**). As controls, lipophilic 3-O-alkyl ethers have also been prepared and evaluated to further confirm the structural requirements of a phenolic A-ring for neuroprotection.

Chemistry

The previously reported preparation of 17β-methylestradiol[10] could have offered an attractive and convenient entry to a series of 17β-alkylestradiols. Unfortunately, we were unable to reproduce the procedure. Since alkylation on the phenolic 3-hydroxyl group proceeds under much milder condition than that of the 17 position, we decided to selectively (and reversibly) protect the 3-OH before alkylating on the 17 position under strong basic condition with the relevant alkyl halide. However, the initially prepared 3-*tert*-butyldimethylsilylated estradiol proved to be too unstable in both very mild acidic or basic conditions against alkyl halids, which resulted in a rapid desilylation followed by alkylation of the 3-OH. Similarly, the commercially available 3-benzoyl estradiol was also unsuitable for the preparation of 17-alkylated ethers, since the phenolic ester group also hydrolyzed rapidly under the condition of our attempted alkylations. Therefore, the protection of the 3-OH of **1** as benzyl (Bz) ether[11] (**2**) was employed, followed by elaboration of the 17β-OH to the corresponding 17β-alkoxyl congeners (**3a-f**). The 17-OH group was successfully alkylated with the corresponding alkyl halide in the presence of sodium hydride in

corresponding 17β-alkoxyl congeners (**3a-f**). The 17-OH group was successfully alkylated with the corresponding alkyl halide in the presence of sodium hydride in DMF. However, the subsequent removal of the 3-benzyl protecting group was extremely slow in the usual fashion by using a Parr hydrogenator with Pd/C as the catalyst in glacial acetic acid. On the other hand, the 3-Bz protecting group was removed rapidly under ambient conditions by catalytic transfer hydrogenation using ammonium formate resulting in the desired products (**4a-f**).[12,13] 3-O-Butyl and octyl ethers of **1** (**5b,c**; Scheme 1) as controls were prepared directly from **1** by using alkyl halide in the presence of potassium carbonate.

In addition to NMR, mass spectrometry, chromatographic and combustion analyses to characterize the compounds prepared, crystallography data were obtained for two representative 17β-ethers (**4a** and **4d**). The solid-state conformation (ORTEP-type plot) of **4d** is shown in Figure 1. The crystals were monoclinic and belonged to the P2(1) space group, and confirmed that the 17-methoxy and butoxy groups assumed β-orientation in the D-ring.

Biological Results and Discussion

Compared to **1**, only **4c-f** of the six 17β-O-alkylestradiols tested improved neuroprotection in a dose-dependent manner against the glutamate- induced oxidative damage in murine HT-22 cells at concentrations of 0.1 μM and higher (Fig. 2). These compounds were essentially equipotent at 1 μM (approximately twice as many cells were viable compared to the control), and showed no apparent relationship with a single molecular property such as lipophilicity (based on the calculated log P[14]). The butyl (**4e**) and octyl ether (**4f**) were, for example, no more neuroprotective at a concentration of 10 μM than at 1 μM. The parent

viability compared to **1** at 10 μM, while 17β-ethylestradiol (**4b**) were ineffective even at 10 μM. Similarly to earlier observations regarding the inability of 3-O-methylestradiol to exert neuroprotection,[11] **5b** and **5c** ethers blocking the phenolic hydroxyl in the A-ring also were inactive.

The complex relationship of neuroprotection and 17-alkoxy chain length was surprising. A comparison of the solid-state conformation of **4a** and **4d** revealed no apparent differences in the preferred geometry of the steroid backbone between a representative "active" (**4e**) and an "inactive" (**4a**) ether derivative of **1**. One possible explanation is that the interaction of the alkyl chain of the 17(β)-substituent with the target site or the lipoidal cell membrane plays an important role in the efficacy of the derivative as a neuroprotectant. Thus, **4a** and **4b** having a compact alkyl group may not have the flexibility (i.e., sufficient degrees of freedom for bond rotation) to embed into a cell membrane effectively; however, a longer alkyl chain ($C \geq 3$) may provide this property. Further studies correlating neuroprotection by phenolic A-ring steroids (including **4a-f**) with their effect on membrane fluidity is underway.

In summary, our results indicate that higher 17β-alkyl ethers of estradiol (**4c-f**) show a dose-dependent neuroprotection *in vitro* against oxidative stress in HT-22 cells. Moreover, this effect is manifested at lower concentration (< 1 μM) than that of the parent compound (**1**).

Experimental Section

Instruments and Materials. All solvents and material were obtained from Fisher Scientific (Atlanta, GA) or from Aldrich (Milwaukee, WI) . Estradiol (**1**) and 3-O methyl-17β - estradiol (**5a**) were purchased from Sigma (St. Louis, MO). Sodium hydride was used as a 60%

dispersion in mineral oil. Melting points were determined on a Fisher-Johns melting point apparatus and uncorrected. Thin layer chromatography (TLC) was done on Whatman silica gel plates (on aluminum backing) containing UV fluorescence indicator. All chromatographic purifications were done on gravity columns with 230-435 mesh neutral silica gel using ethyl acetate:hexane 1:4 (v/v) eluent. Elemental analyses were performed by the Atlantic Microlab, Inc. (Norcross, GA). NMR spectral data were recorded for all compounds using a Varian XL-300 spectrometer using TMS as internal standard. Mass spectral data were obtained by using atmospheric-pressure chemical ionization (APCI) on a quadrupole ion trap instrument (LCQ, Finnigan MAT, San Jose, CA). Analytical reversed-phase high-performance liquid chromatography was performed on a ThermoSeparation/SpectraPhysics (Fremont, CA) system consisting of an SP8810 isocratic pump, a Rheodyne (Cotati, CA) Model 7125 injector valve equipped with a 20-µl sample loop, an SP8450 variable wavelength UV/VIS detector operated at 280 nm, and an SP4290 computing integrator. A 15cm x 4.6 mm i.d. octadecylsilica column (Phase Sep S5 ODS2, Queensferry, Clwyd, UK) and a mobile phase of acetonitrile containing 1% acetic acid at a flow rate of 1.0 mL/min were used for the analyses.

X-ray crystallography data were collected at 173 K on a Siemens SMART PLATFORM equipped with A CCD area detector and a graphite monochromator utilizing MoK_α radiation (l = 0.71073 Å). Cell parameters for each structure were refined using up to 8192 reflections and a hemisphere of data (1381 frames) was collected using the w-scan method (0.3° frame width). The first 50 frames were remeasured at the end of data collection to monitor instrument and crystal stability (maximum correction on I was < 1 %). Absorption corrections by integration were applied based on measured indexed crystal faces. Both structures were solved by the Direct

Methods in *SHELXTL5*,[15] and refined using full-matrix least squares. The non-H atoms were treated anisotropically, whereas the hydrogen atoms were calculated in ideal positions and were riding on their respective carbon atoms, except the hydroxyl protons H_{18} in **4a** and H_{18} and H_{26} in **4d**. These protons were obtained from a Difference Fourier map and refined without any constraints. While no solvent crystallized with **4a**, a methanol molecule was found in general position in the lattice of **4d**. A total of 196 parameters of **4a** were refined in the final cycle of refinement using 2961 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 5.03% and 12.66%, respectively. For **4d**, a total of 247 parameters were refined in the final cycle of refinement using 3294 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 3.71% and 8.90%, respectively. Refinement was done using F^2. Tables of geometric data, indicating H-bonding interactions, are available as Supporting Information.

3-Benzyloxyestra-1,3,5(10)-trien-17β-ol (2)[11]**.** To 5 g (18 mmol) of **1** and 10 g (72 mmol) potassium carbonate in 100 ml of acetone 5.7g (4.0mL, 34 mmol) benzyl bromide was added. The mixture was refluxed overnight. Upon cooling the solid was removed by filtration. The filtrate was collected and acetone was removed *in vacuo* leaving behind clear yellowish oil, which solidified on standing. Recrystallization from ethyl acetate/hexane gave 6.1g (93% yield) of a white fluffy solid, m.p. 119-121°C; TLC R_f 0.23; ^{1}H-NMR ($CDCl_3$) δ: 7.44-7.19 (m, 5H); 6.78(dd, J=8.7 Hz and J=2.7 Hz, 1H); 6.72 (d, J=2.4 Hz, 1H); 5.05 (s, 3H); 3.37 (tr, J=8.4 HZ, 1H); 2.87-2.82 (m, 2H); 2.34-1.18 (m, H); 0.78 (s, 3H). MS: *m/z* 363 $[M+H]^+$.

General Procedure for the Preparation of 3-Benzyloxy-17β-alkoxyestra-1,3,5(10)-triene (3a-f). Compound **2** (0.8 g, 2.2 mmol) was dissolved in 5 ml anhydrous DMF and, then, sodium

hydride (0.3 g) was added. The mixture was stirred at room temperature for 30 min before the addition of 20 mmol alkyl-halide. The stirring was continued overnight. The reaction mixture was quenched by pouring it into 20 mL of dilute hydrochloric acid and extracted with methylene chloride. The organic phase was dried over Na_2SO_4 and the solvent removed in vacuo leaving behind a clear, yellowish oil which solidified on standing. The crude products were purified by either recrystallization or column chromatography.

3-Benzyloxy-17β-methoxyestra-1,3,5(10)-triene. (3a) Recrystallization from methanol, 63% yield. Yellowish solid, m.p. 92-94°C; TLC R_f 0.83; ^{1}H-NMR ($CDCl_3$) δ: 7.32-7.48 (m, 5H), 7.22 (dd, J=8.7 and J=2.10 Hz, 1H), 6.80 (d, J=2.4, 1H), 5.05 (s, 2H), 3.39 (s, 3H), 3.33 (t; 1H, J=8.7), 2.83 (m, 2H), 1.22-2.34 (m, 13H), 0.80 (s, 3H). MS: *m/z* 377 $[M+H]^+$.

3-Benzyloxy-17β-ethoxyyestra-1,3,5(10)-triene (3b). Column chromatography, 49 % yield. TLC R_f 0.71; ^{1}H-NMR ($CDCl_3$) δ: 7.45-7.30 (m, 5H), 6.79 (dd, J=8.7 and J=2.10 Hz, 1H), 6.71 (d, J=2.5, 1H), 5.02 (s, 2H), 3.55 (dq, J= 6.9 Hz and 2.1 Hz, 1H), 3.48 (dq, J= 7.0 Hz and 2.1 Hz, 1H), 3.39 (t, J=8.1 Hz, 1H), 2.84-2.81 (m, 2H), 2.31-1.37 (m, 13H), 1.18 (t, J=6.9 Hz, 3H), 0.79 (s, 3H). MS: *m/z* 391 $[M+H]^+$.

3-Benzyloxy-17β-propoxyestra-1,3,5(10)-triene (3c) . Column chromatography. Yield. 54%. White solid. TLC R_f 0.68, ^{1}H-NMR ($CDCl_3$) δ: 7.44-7.37 (m, 5H), 6.75 (dd, J=8.6 and J=2.1 Hz, 1H), 6.70 (d, J=2.7, 1H), 5.02 (s, 2H), 3.41 (dt, J= 6.9 Hz and 2.4 Hz, 2H), 3.37 (t, J=8.4 Hz, 1H), 2.84-2.81 (m, 2H), 2.31-1.37 (m, 15), 0.92 (t, J=6.6 Hz, 3H), 0.79 (s, 3H). MS: m/z 405 $[M+H]^+$.

3-Benzyloxy-17β-butoxyestra-1,3,5(10)-triene (3d) . Column chromatography, yield 52%. White solid. TLC R_f 0.65, ^{1}H-NMR ($CDCl_3$) δ: 7.45-7.30 (m, 5H), 6.79 (dd, J=8.7 and J=2.10 Hz, 1H), 6.71 (d, J=2.5, 1H), 5.02 (s, 2H), 3.55 (dq, J= 6.9 Hz and 2.1 Hz, 1H), 3.48 (dq, J= 7.0 Hz and 2.1 Hz, 1H), 3.39 (t, J=8.1 Hz, 1H), 2.84-2.81 (m, 2H), 2.31-1.37 (m, 13H), 1.18 (t, J=6.9 Hz, 3H), 0.79 (s, 3H). MS: *m/z* 419 $[M+H]^+$.

3-Benzyloxy-17β-hexyloxyestra-1,3,5(10)-triene (3e) . Column chromatography, yield 63%. White solid. TLC R_f 0.75, ^{1}H-NMR ($CDCl_3$) δ: 7.49-7.34 (m, 5H), 6.74 (dd, J=8.7 and J=2.7 Hz, 1H), 6.71 (d, J=2.7, 1H), 4.98 (s, 2H), 3.44 (dt, J=7.6Hz and 2.7 Hz, 2H) 3.36 (t, J=8.1 Hz, 1H), 3.55 (dq, J= 6.9 Hz and 2.1 Hz, 1H), 3.48 (dq, J= 7.0 Hz and 2.1 Hz, 1H), 3.39 (t, J=8.1 Hz, 1H), 2.84-2.81 (m, 2H), 2.31-1.37 (m, 13H), 1.18 (t, J=6.9 Hz, 3H), 0.79 (s, 3H). MS: *m/z* 447 $[M+H]^+$.

3-Benzyloxy-17β-octyloxyestra-1,3,5(10)-triene (3f) . Column chromatography, 55% yield, yellow oil. TLC R_f 0.85, ^{1}H-NMR ($CDCl_3$) δ: 7.45-7.30 (m, 5H), 6.79 (dd, J=8.7 and J=2.10 Hz, 1H), 6.71 (1H, J=7.7), 5.02 (s, 2H), 3.55 (dq, J= 6.9 Hz and 2.1 Hz, 1H), 3.48 (dq, J= 7.0 Hz and 2.1 Hz, 1H), 3.39 (t, J=8.1 Hz, 1H), 2.84-2.81 (m, 2H), 2.31-1.37 (m, 13H), 1.18 (t, J=6.9 Hz, 3H), 0.79 (s, 3H). MS: *m/z* 475 $[M+H]^+$.

General Procedure for the Preparation of 17β-alkoxyestra-1,3,5(10)-triene (4a-f). To a solution of 2.0 mmol **3a-f** in 10mL of methanol was added 0.2 g of Pd/C (10%) and ammonium formate (1.00 g, 16mmol). The reaction mixture was stirred at room temperature for 1 hr. Then the Pd/C was then removed by filtration and solvent was removed in vacuo. To the oily residue

water was added and the resulting solid was collected by filtration. Either recrystallization or column chromatography was used for purification.

17β-Methoxyestra-1,3,5(10)-trien-3-ol (4a). Recrystallization from methanol, 50% yield. White solid, m.p. 242-244°C; TLC: R_f 0.48; ^{1}H-NMR (DMSO) δ: 7.05 (d, J=8.40 Hz, 1H), 6.51 (dd, J=8.40 Hz and 2.10 Hz, 1H), 6.45 (d, J= 2.40 Hz, 1H), 3.30 (s, 3H), 3.28 (t, j=8.25 Hz, 1H); 2.73-2.72 (m, 3H); 2.56-2.50 (m, 1H); 2.30-1.22 (m, 13H); 0.74 (s, 3H). ^{13}C-NMR (DMSO) δ: 156.7, 139.3, 132.7, 128.0, 116.8, 114.5, 92.2, 58.7, 51.7, 45.6, 44.6, 40.2, 39.8, 31.1, 29.2, 28.8, 28.1, 24.4, 13.6; MS: *m/z* 287 $[M+H]^+$, 255 $[M-OCH_3]^+$. Anal. C, H.

17β-Ethoxyestra-1,3,5(10)-trien-3-ol (4b). Recrystallization from methanol, 50% yield, white solid; TLC: R_f 0.57; ^{1}H-NMR ($CDCl_3$) δ: 7.08 (d, J=8.7 Hz, 1H), 6.55 (dd, J=8.4 Hz, 2.1 Hz, 1H), 6.48 (d, J=2.4 Hz, 1H), 3.65 (qd, J= 7.02 Hz and 2.48 Hz, 1H), 3.56 (qd, J= 7.05 Hz and 2.48 Hz, 1H), 3.44 (t, J=8.4 Hz, 1H), 2.76-2.72 (m, 2H), 2.20-1.10 (m, 13H), 1.20 (t, J=7.2 Hz, 3H), 0.80 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ: 155.72, 138.83, 132.64, 127.19, 116.1, 113.7, 89.8, 66.1, 50.8, 44.5, 43.8, 39.3, 38.6, 30.1, 28.6, 27.8, 27.01, 23.5, 15.8 11.9; MS: *m/z* 301 $[M+H]^+$, 255 $[M-OC_2H_5]^+$. Anal. C, H.

17β-Propoxyestra-1,3,5(10)-trien-3-ol (4c). Recrystallization from methanol, 50% yield, white solid; TLC: R_f 0.54; ^{1}H-NMR ($CDCl_3$) δ: 7.08 (d, J=8.7 Hz, 1H), 6.55 (dd, J=8.4 Hz, 2.1 Hz, 1H), 6.48 (d, J=2.4 Hz, 1H), 3.45 (dt, J=6.77 Hz and 1.67 Hz, 2H), 3.31 (m, 3H), 2.76-2.72 (m, 2H), 2.20-1.10 (m, 13H), 0.94 (td, J=7.2 Hz and 1.92 Hz, 3H), 0.72 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 154.0, 137.9, 131.7, 126.2, 115.0, 112.5, 89.0, 71.9, 50.1, 43.8, 43.2, 38.5, 38.0, 29.5, 27.9, 27.1, 26.3, 23.1, 22.9, 11.4, 10.4; MS: *m/z* 315 $[M+H]^+$, 255 $[M-OC_3H_7]^+$. Anal. C, H.

17β-Butoxyestra-1,3,5(10)-trien-3-ol (4d). Recrystallization from methanol, 50% yield, white solid, m.p. 77-81°C; TLC: R_f 0.47; ^{1}H-NMR ($CDCl_3$) δ: 7.08 (d, J=8.7 Hz, 1H), 6.55 (dd, J=8.4 Hz, 2.1 Hz, 1H), 6.48 (d, J=2.4 Hz, 1H), 3.50 (dqn, J=7.00 Hz and 2.01 Hz, 1H), 3.45 (dqn, J=7.11 Hz and 1.85 Hz, 1H), 3.31 (t, J=8.4 Hz, 1H), 2.76-2.72 (m, 2H), 2.20-1.10 (m, 17H), 0.85 (t, J=7.2 Hz, 3H), 0.72 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 153.3, 138.3, 132.7, 126.5, 115.2, 112.5, 89.1, 70.0, 50.3, 43.9, 43.3, 38.6, 38.1, 32.3, 29.6, 28.2, 27.1, 26.5, 23.0, 19.4, 14.0, 11.6; MS: *m/z* 329 $[M+H]^+$, 255 $[M-OC_4H_9]^+$.

17β-Hexyloxyestra-1,3,5(10)-trien-3-ol (4e). Column chromatography, 70% yield, white semi-solid. TLC: R_f 0.47; ^{1}H-NMR ($CDCl_3$) δ: 7.12 (d, J=8.4 Hz, 1H), 6.62 (dd, J=8.3 Hz, 2.7 Hz, 1H), 6.54 (d, J=2.5 Hz, 1H), 3.43 (dt, J=7.6Hz and 2.7 Hz, 2H) 3.36 (t, J=8.1 Hz, 1H), 2.80-2.77 (m, 2H), 2.25-1.25 (m, 18H), 0.89-0.85 (m, 6H), 0.78 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 153.2, 138.2, 132.6, 126.4, 115.1, 112.5, 89.0, 70.3, 50.2, 43.8, 43.3, 38.5, 38.0, 31.6, 30.1, 29.5, 28.1, 26.5, 25.8, 23.0, 22.6, 14.0, 11.6; MS: *m/z* 357 $[M+H]^+$, 255 $[M-OC_6H_{13}]^+$. Anal. C, H.

17β-Octyloxyestra-1,3,5(10)-trien-3-ol (4f). Column chromatography, 75% yield, pale yellow semi-solid. TLC: R_f 0.50; ^{1}H-NMR ($CDCl_3$) δ: 7.12 (d, J=8.7 Hz, 1H), 6.62 (dd, J=8.4 Hz, 2.2 Hz, 1H), 6.53 (d, J=2.3 Hz, 1H), 3.49 (qd, J=6.79 Hz and 2.52 Hz, 1H), 4.31 (qd, J=6.72 Hz and 2.55 Hz, 1H), 3.37 (t, J=8.5 Hz, 1H), 2.81-2.76 (m, 2H), 2.22-1.18 (m, 22H), 0.87-0.83 (m, 6H), 0.79 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 153.3, 138.2, 132.6, 126.5, 115.2, 112.6, 89.1, 70.3, 50.2, 43.9, 43.3, 38.6, 38.0, 31.8, 30.1, 29.7, 29.4, 29.3, 28.1, 27.1, 26.4, 26.2, 23.0, 22.6, 14.0, 11.6; MS: *m/z* 385 $[M+H]^+$, 255 $[M-OC_8H_{17}]^+$. Anal. C, H.

General Procedure for the Preparation of 3-Alkoxyestra-1,3,5(10)-triene (5b,c). To compound **1** (0.5g, 1.8 mmol) and potassium carbonate (1.00g, 7.2 mmol) in 5 ml of acetone 10 mmol of 1-bromobutane or 1-bromooctane was added. The mixture was refluxed overnight then allowed to cool down and it was filtered. The acetone was removed and the oily residue was purified.

3-Butoxyestra-1,3,5(10)-trien-17β-ol (5b). Recrystallization from methanol:water 1:1 (v/v), 68% yield. White solid; m.p. 86-88°C; TLC R_f 0.62; ^{1}H-NMR ($CDCl_3$) δ: 7.17 (d, J=8.7 Hz, 1H), 6.70 (dd, J=8.4 Hz and 2.40 Hz, 1H), 6.62 (d, J=2.4 Hz, 1H), 3.93 (t, J=6.30 Hz, 2H), 3.71 (t, J=8.1 Hz, 1H), 2.86-2.80 (m, 2H), 2.20-1.10 (m, 17H), 0.96 (t, J=7.2 Hz, 3H), 0.77 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 156.9, 137.7, 132.3, 126.1, 114.4, 111.9, 81.7, 67.5, 49.9, 43.8, 43.1, 38.7, 36.6, 31.3, 30.4, 29.7, 27.2, 26.3, 23.0, 19.2, 13.7, 10.9. MS: *m/z* 311 $[M\text{-}OH]^+$.

3-Octyloxyestra-1,3,5(10)-trien-17β-ol (5c) Column chromatography, 72 % yield. White solid, m.p. 64-66°C; TLC R_f 0.70; ^{1}H-NMR ($CDCl_3$) δ: 7.18 (d, J=8.7 Hz, 1H), 6.71 (dd, J=8.7 Hz and 2.7 Hz, 1H), 6.62 (d, J=2.8 Hz, 1H), 3.91 (t, J=6.6 Hz, 2H), 3.73 (t, J=8.4 Hz, 1H), 2.85-2.82 (m, 2H), 2.20-1.10 (m, 25 H), 0.88 (t, J=6.6 Hz, 3H), 0.77 (s, 3H); ^{13}C-NMR ($CHCl_3$) δ: 156.9, 137.8, 132.4, 126.2, 114.5, 112.0, 81.9, 70.3, 67.9, 50.0, 43.9, 43.2, 38.8, 38.1, 36.6, 30.1, 29.7, 29.4, 29.2, 27.2, 26.4, 26.2, 23.1, 22.6, 14.0, 11.0. MS: *m/z* 368 $[M\text{-}OH]^+$. Anal. C, H.

Cytotoxicity Studies. All studies were done on mouse clonal hippocampal HT-22 cells. These cells were gift from Dr. David Schulbert (Salk Institute, La Jolla, CA), and cultured in DMEM media supplemented with 10% fetal bovine serum under the usual conditions. All wells in the 96 well culture plate contained approximately 5,000 cells as determined by a Neubauer

hemacytometer and the cells were incubated for 24 hrs before the compounds were added. The estradiol derivatives were purified by recrystallization or column chromatography and were free from **1** as determined by HPLC. All agents were dissolved in absolute ethanol and diluted, with the culture media, to a final concentration of 0.01 μM, 0.1 μM, 1.0 μM, and 10 μM in their respective wells. The cells were further incubated for 24 hrs before sodium glutamate in a solution of phosphate buffer was added. Cell viability was quantified 2 hrs later by the calcein AM assay in a phosphate buffer solution.

Statistical Analysis. ANOVA was used to determine the significance of differences among groups. Comparison between groups were done using the Tukey test. A $p < 0.05$ was considered significant.

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oxidative stress-induced cell death *in vitro*. *Biochem. Biophys. Res. Commun.* **1995**, 216, 473-482. (b) Sawada, H.; Ibi, M.; Kihara, T.; Urushitani, M.; Akaike, A.; Shimohama, S. Estradiol protects mesencephalic dopaminergic neurons from oxidative stress-induced neuronal death. *J. Neurosci. Res.* **1998**, 54, 707-719.

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14. The logarithm of the 1-octanol/water partition coefficient (log P) was calculated by an an atom fragment method implemented in the molecular modeling package HyperChem version

6.0 (Hypercube, Gainesville, FL): Ghose, A.K.; Pritchett, A.; Crippen, G.M. Atomic physicochemical parameters for 3-dimensional structure directed quantitative structure-activity-relationships. 3. Modeling hydrophobic interactions, *J. Comput. Chem.* **1988**, *9*, 80-90. The obtained log P values were as follows: 4.01 (**1**), 4.29 (**4a**), 4.63 (**4b**), 5.10 (**4c**), 5.49 (**4d**), 6.29 (**4e**), and 7.08 (**4f**). The calculated log P for the 3-alkylestradiols were 4.09 (**5a**), 5.25 (**5b**), and 6.83 (**5c**).

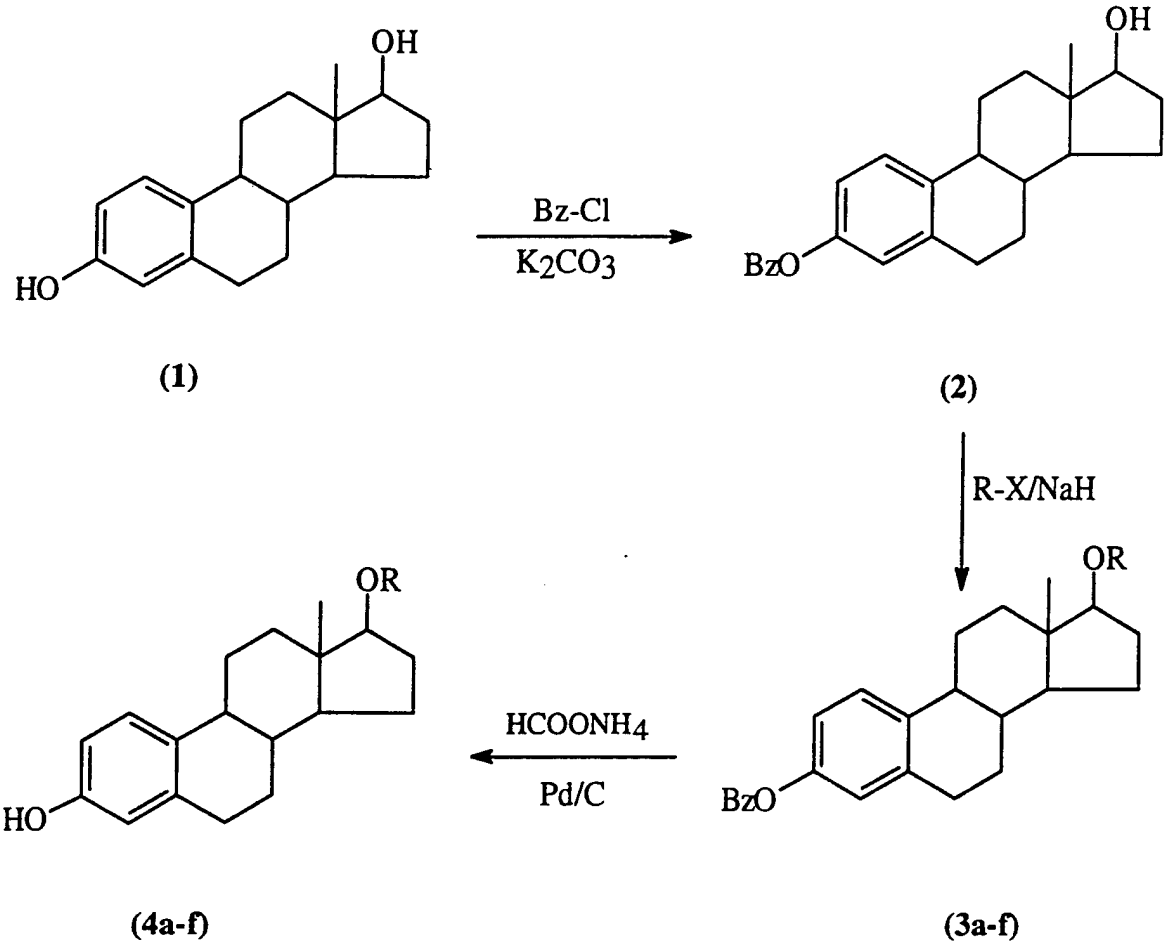
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Legend to the Figures:

Scheme 1. Synthesis of 17- and 3-alkyl ethers of estradiol.

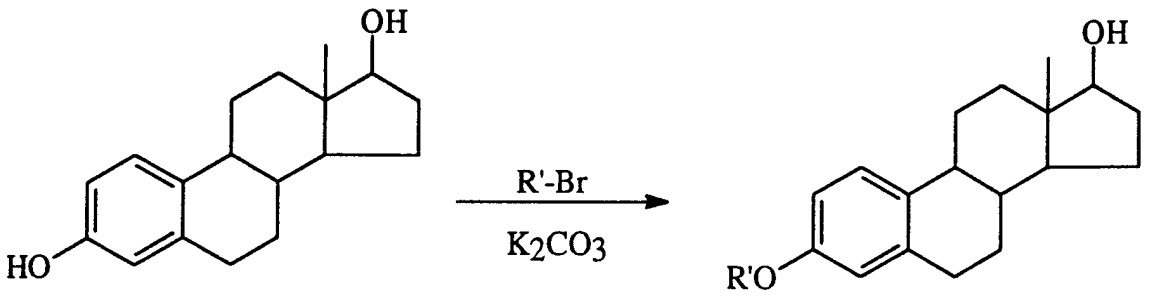
Figure 1. ORTEP plot of the X-ray crystal structure of 17-O-butylated 17β-estradiol (**4d**). Thermal ellipsoids are shown at the 30% probability level.

Figure 2. HT-22 cell viability *in vitro* after glutamate exposure (20 mM) following treatment with estradiol (**1**), its 17β-alkyl ethers (**4a-f**) and 3-butyl estradiol (**5b**, as a typical representative of the 3-alkyl ethers). Statistically significant differences between groups were tested by analysis of variance (ANOVA) followed by *post hoc* Tukey test: * significant increase ($p < 0.05$) vs vehicle control, ** significant increase ($p < 0.05$) vs vehicle control, but decrease compared to 10 μM estradiol (**1**), *** increase ($p < 0.05$) vs vehicle control, and statistically significant increase compared to 10 μM estradiol (**1**).



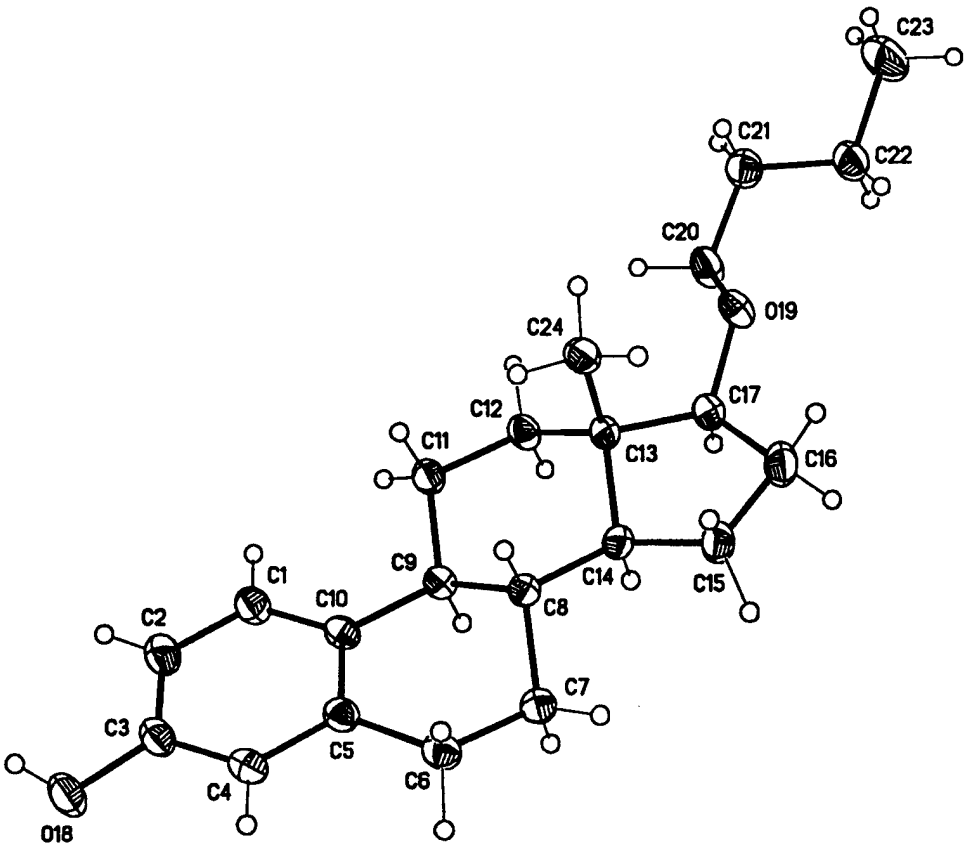
(1) **(2)** **(3a-f)** **(4a-f)**

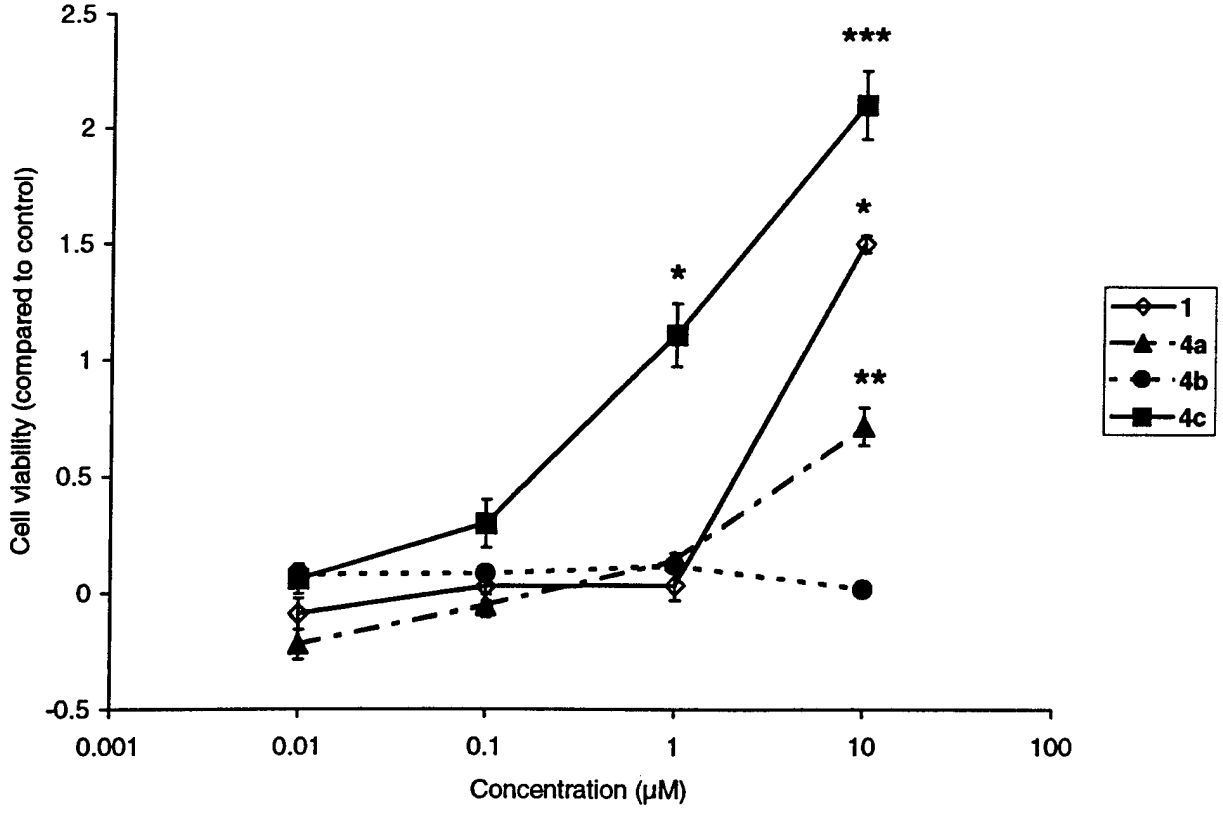
a) R=CH_3, **b)** R=C_2H_5, **c)** R=C_3H_7,
d) R=C_4H_9, **e)** R=C_6H_{13}, **f)** R=C_8H_{17}

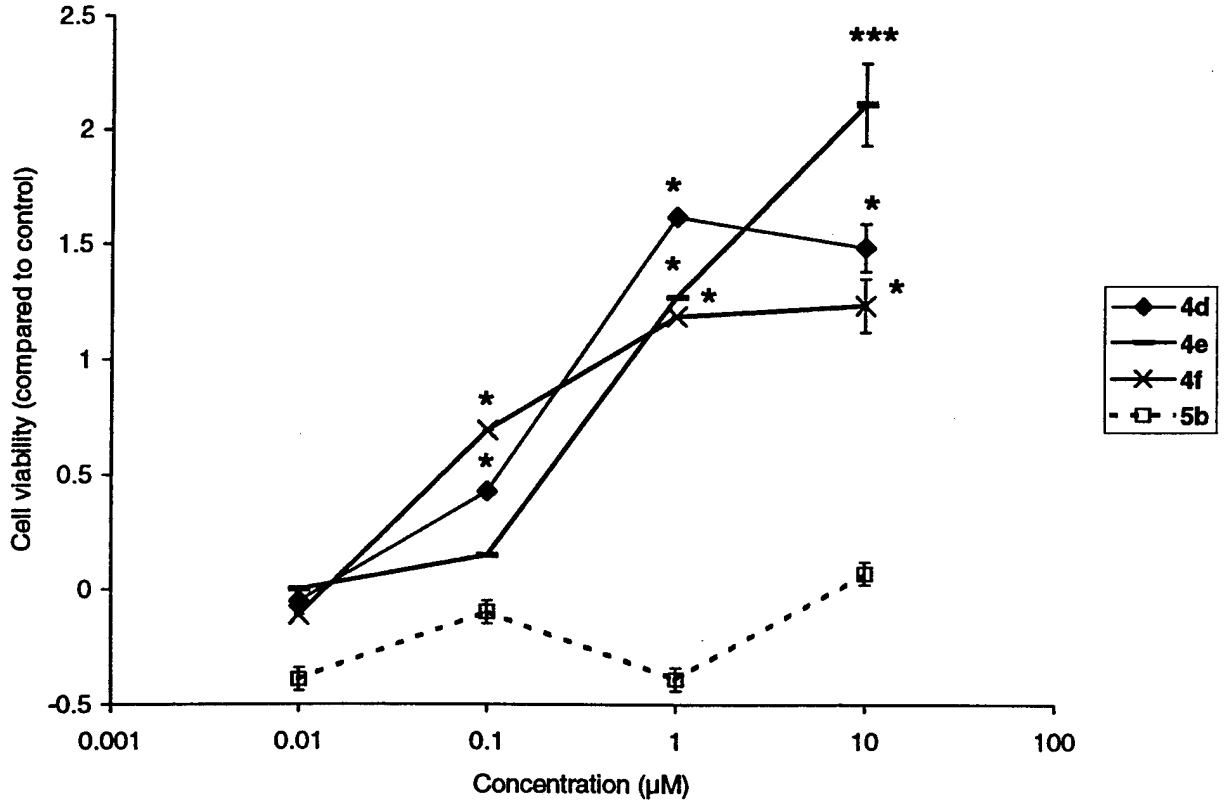


(1) **(5b,c)**

b) R'= C_4H_9, **c)** R'=C_8H_{17}


C23
C21
C22
C20
O19
C24
C17
C12
C11
C13
C16
C1
C9
C14
C15
C10
C8
C2
C7
C3
C5
C6
C4
O18


2.5
2
1.5
1
0.5
0
-0.5
Cell viability (compared to control)
0.001
0.01
0.1
1
10
100
Concentration (μM)
***
*
*
**
1
4a
4b
4c


2.5
2
1.5
1
0.5
0
-0.5
Cell viability (compared to control)
0.001
0.01
0.1
1
10
100
Concentration (μM)
***
*
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5b

Supporting Information

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Title: Synthesis And Biological Evaluation of 17β-Alkoxyestra-1,3,5(10)-Trienes as Potential Neuroprotectants Against Oxidative Stress

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Table 1. Crystal data and structure refinement for **4a**.

Identification code	**4a**	
Empirical formula	C19 H26 O2	
Formula weight	286.40	
Temperature	173(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)	
Unit cell dimensions	a = 6.3746(6) Å	α= 90°.
	b = 17.521(1) Å	β= 103.630(2)°.
	c = 7.0611(6) Å	γ= 90°.
Volume	766.45(1) $Å^3$	
Z	2	
Density (calculated)	1.241 Mg/m^3	
Absorption coefficient	0.078 mm^{-1}	
F(000)	312	
Crystal size	0.51 x 0.33 x .04 mm^3	
Theta range for data collection	2.32 to 27.49°.	
Index ranges	-8• h• 3, -22• k• 22, -9• l• 9	
Reflections collected	5235	
Independent reflections	3364 [R(int) = 0.0436]	
Completeness to theta = 27.49°	99.4 %	
Absorption correction	Integration	
Max. and min. transmission	0.9969 and 0.9700	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3364 / 1 / 196	
Goodness-of-fit on F^2	1.044	
Final R indices [I>2sigma(I)]	R1 = 0.0503, wR2 = 0.1266 [2961]	
R indices (all data)	R1 = 0.0596, wR2 = 0.1362	
Absolute structure parameter	-1.3(13)	
Largest diff. peak and hole	0.249 and -0.190 $e.Å^{-3}$	

$R1 = \Sigma(||F_o| - |F_c||) / \Sigma|F_o|$

$wR2 = [\Sigma[w(F_o^2 - F_c^2)^2] / \Sigma[w(F_o^2)^2]]^{1/2}$

$S = [\Sigma[w(F_o^2 - F_c^2)^2] / (n-p)]^{1/2}$ $w = 1/[\sigma^2(F_o^2)+(0.0370*p)^2+0.31*p]$, $p = [\max(F_o^2,0)+ 2* F_c^2]/3$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for **4a**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
C1	5160(3)	4032(1)	-1139(3)	24(1)
C2	5382(3)	4530(1)	-2601(3)	26(1)
C3	7323(3)	4919(1)	-2448(3)	25(1)
C4	9019(3)	4788(1)	-835(3)	25(1)
C5	8792(3)	4288(1)	644(3)	23(1)
C6	10690(3)	4193(1)	2387(3)	29(1)
C7	10071(3)	3841(1)	4155(3)	27(1)
C8	8619(3)	3149(1)	3552(3)	20(1)
C9	6473(3)	3404(1)	2183(3)	20(1)
C10	6836(3)	3900(1)	512(3)	20(1)
C11	4969(4)	2719(1)	1543(3)	25(1)
C12	4558(3)	2269(1)	3286(3)	24(1)
C13	6671(3)	2019(1)	4661(3)	20(1)
C14	8095(3)	2731(1)	5268(3)	22(1)
C15	9916(4)	2438(2)	6940(3)	37(1)
C16	8795(4)	1840(2)	7974(3)	32(1)
C17	6485(3)	1750(1)	6675(3)	22(1)
O18	7675(3)	5428(1)	-3805(2)	32(1)
O19	5654(2)	989(1)	6636(2)	27(1)
C20	5007(4)	795(1)	8376(3)	34(1)
C21	7790(4)	1399(1)	3715(3)	31(1)

Table 3. Bond lengths [Å] and angles [°] for **4a**.

C1-C2	1.384(3)
C1-C10	1.403(3)
C1-H1A	0.9500
C2-C3	1.394(3)
C2-H2A	0.9500
C3-O18	1.366(2)
C3-C4	1.393(3)
C4-C5	1.395(3)
C4-H4A	0.9500
C5-C10	1.404(3)
C5-C6	1.518(3)
C6-C7	1.525(3)
C6-H6A	0.9900
C6-H6B	0.9900
C7-C8	1.524(3)
C7-H7A	0.9900
C7-H7B	0.9900
C8-C14	1.519(3)
C8-C9	1.544(2)
C8-H8A	1.0000
C9-C10	1.526(3)
C9-C11	1.536(3)
C9-H9A	1.0000
C11-C12	1.536(3)
C11-H11A	0.9900
C11-H11B	0.9900
C12-C13	1.527(3)
C12-H12A	0.9900
C12-H12B	0.9900
C13-C17	1.529(2)
C13-C21	1.537(3)
C13-C14	1.543(3)
C14-C15	1.536(3)
C14-H14A	1.0000

C15-C16	1.545(3)
C15-H15A	0.9900
C15-H15B	0.9900
C16-C17	1.549(3)
C16-H16A	0.9900
C16-H16B	0.9900
C17-O19	1.432(2)
C17-H17A	1.0000
O18-H18	0.85(3)
O19-C20	1.426(3)
C20-H20A	0.9800
C20-H20B	0.9800
C20-H20C	0.9800
C21-H21A	0.9800
C21-H21B	0.9800
C21-H21C	0.9800
C2-C1-C10	122.16(19)
C2-C1-H1A	118.9
C10-C1-H1A	118.9
C1-C2-C3	119.69(18)
C1-C2-H2A	120.2
C3-C2-H2A	120.2
O18-C3-C4	117.22(19)
O18-C3-C2	123.68(18)
C4-C3-C2	119.10(18)
C3-C4-C5	121.21(18)
C3-C4-H4A	119.4
C5-C4-H4A	119.4
C4-C5-C10	120.09(17)
C4-C5-C6	118.18(18)
C10-C5-C6	121.72(17)
C5-C6-C7	113.42(17)
C5-C6-H6A	108.9
C7-C6-H6A	108.9
C5-C6-H6B	108.9
C7-C6-H6B	108.9

H6A-C6-H6B	107.7
C8-C7-C6	110.52(16)
C8-C7-H7A	109.5
C6-C7-H7A	109.5
C8-C7-H7B	109.5
C6-C7-H7B	109.5
H7A-C7-H7B	108.1
C14-C8-C7	113.21(15)
C14-C8-C9	108.04(16)
C7-C8-C9	109.58(16)
C14-C8-H8A	108.6
C7-C8-H8A	108.6
C9-C8-H8A	108.6
C10-C9-C11	114.52(15)
C10-C9-C8	112.02(16)
C11-C9-C8	110.98(15)
C10-C9-H9A	106.2
C11-C9-H9A	106.2
C8-C9-H9A	106.2
C1-C10-C5	117.74(17)
C1-C10-C9	120.78(17)
C5-C10-C9	121.26(16)
C12-C11-C9	112.20(16)
C12-C11-H11A	109.2
C9-C11-H11A	109.2
C12-C11-H11B	109.2
C9-C11-H11B	109.2
H11A-C11-H11B	107.9
C13-C12-C11	111.43(17)
C13-C12-H12A	109.3
C11-C12-H12A	109.3
C13-C12-H12B	109.3
C11-C12-H12B	109.3
H12A-C12-H12B	108.0
C12-C13-C17	115.44(16)
C12-C13-C21	110.90(17)

C17-C13-C21	109.37(16)
C12-C13-C14	108.53(16)
C17-C13-C14	99.26(15)
C21-C13-C14	112.92(18)
C8-C14-C15	120.38(18)
C8-C14-C13	113.20(15)
C15-C14-C13	103.36(17)
C8-C14-H14A	106.3
C15-C14-H14A	106.3
C13-C14-H14A	106.3
C14-C15-C16	103.89(18)
C14-C15-H15A	111.0
C16-C15-H15A	111.0
C14-C15-H15B	111.0
C16-C15-H15B	111.0
H15A-C15-H15B	109.0
C15-C16-C17	105.70(16)
C15-C16-H16A	110.6
C17-C16-H16A	110.6
C15-C16-H16B	110.6
C17-C16-H16B	110.6
H16A-C16-H16B	108.7
O19-C17-C13	112.27(15)
O19-C17-C16	113.72(17)
C13-C17-C16	104.34(16)
O19-C17-H17A	108.8
C13-C17-H17A	108.8
C16-C17-H17A	108.8
C3-O18-H18	109.8(18)
C20-O19-C17	112.73(16)
O19-C20-H20A	109.5
O19-C20-H20B	109.5
H20A-C20-H20B	109.5
O19-C20-H20C	109.5
H20A-C20-H20C	109.5
H20B-C20-H20C	109.5

C13-C21-H21A	109.5
C13-C21-H21B	109.5
H21A-C21-H21B	109.5
C13-C21-H21C	109.5
H21A-C21-H21C	109.5
H21B-C21-H21C	109.5

Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for **4a**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
C1	22(1)	24(1)	22(1)	0(1)	-2(1)	-2(1)
C2	27(1)	25(1)	21(1)	2(1)	-3(1)	-1(1)
C3	28(1)	23(1)	23(1)	4(1)	5(1)	1(1)
C4	23(1)	26(1)	25(1)	3(1)	4(1)	-3(1)
C5	20(1)	24(1)	22(1)	0(1)	1(1)	4(1)
C6	21(1)	35(1)	27(1)	7(1)	-3(1)	-6(1)
C7	23(1)	33(1)	21(1)	1(1)	-4(1)	-6(1)
C8	19(1)	23(1)	17(1)	1(1)	-1(1)	0(1)
C9	18(1)	21(1)	18(1)	2(1)	-1(1)	1(1)
C10	22(1)	18(1)	18(1)	1(1)	0(1)	0(1)
C11	24(1)	28(1)	17(1)	4(1)	-4(1)	-6(1)
C12	21(1)	27(1)	22(1)	4(1)	-1(1)	-4(1)
C13	23(1)	22(1)	14(1)	0(1)	2(1)	-1(1)
C14	21(1)	28(1)	15(1)	-1(1)	-2(1)	-2(1)
C15	28(1)	50(1)	26(1)	13(1)	-8(1)	-10(1)
C16	29(1)	44(1)	19(1)	9(1)	-3(1)	-6(1)
C17	24(1)	25(1)	15(1)	1(1)	2(1)	-1(1)
O18	31(1)	34(1)	28(1)	13(1)	2(1)	-2(1)
O19	34(1)	25(1)	19(1)	2(1)	3(1)	-5(1)
C20	42(1)	36(1)	25(1)	4(1)	7(1)	-7(1)
C21	43(1)	29(1)	24(1)	1(1)	13(1)	6(1)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for **4a**.

	x	y	z	U(eq)
H1A	3829	3771	-1258	29
H2A	4218	4606	-3703	31
H4A	10356	5042	-740	30
H6A	11352	4699	2755	35
H6B	11789	3866	2007	35
H7A	11392	3685	5122	33
H7B	9307	4225	4772	33
H8A	9360	2786	2830	25
H9A	5739	3737	2980	24
H11A	3574	2903	737	30
H11B	5621	2375	727	30
H12A	3735	2591	4008	29
H12B	3675	1813	2807	29
H14A	7224	3093	5868	27
H15A	10532	2857	7839	44
H15B	11083	2201	6431	44
H16A	8745	2018	9292	38
H16B	9578	1348	8095	38
H17A	5496	2103	7162	26
H18	6470(50)	5560(15)	-4560(40)	29(7)
H20A	6259	821	9489	52
H20B	4417	276	8262	52
H20C	3901	1155	8574	52
H21A	6783	977	3278	47
H21B	9057	1209	4669	47
H21C	8245	1615	2596	47

Table 6. Torsion angles [°] for **4a**.

C10-C1-C2-C3	-0.2(3)
C1-C2-C3-O18	-179.1(2)
C1-C2-C3-C4	1.1(3)
O18-C3-C4-C5	178.76(19)
C2-C3-C4-C5	-1.5(3)
C3-C4-C5-C10	0.9(3)
C3-C4-C5-C6	-178.1(2)
C4-C5-C6-C7	162.30(19)
C10-C5-C6-C7	-16.6(3)
C5-C6-C7-C8	46.3(3)
C6-C7-C8-C14	175.18(17)
C6-C7-C8-C9	-64.2(2)
C14-C8-C9-C10	174.27(15)
C7-C8-C9-C10	50.5(2)
C14-C8-C9-C11	-56.3(2)
C7-C8-C9-C11	179.89(17)
C2-C1-C10-C5	-0.5(3)
C2-C1-C10-C9	174.35(19)
C4-C5-C10-C1	0.1(3)
C6-C5-C10-C1	179.0(2)
C4-C5-C10-C9	-174.67(18)
C6-C5-C10-C9	4.3(3)
C11-C9-C10-C1	36.5(3)
C8-C9-C10-C1	164.01(17)
C11-C9-C10-C5	-148.88(18)
C8-C9-C10-C5	-21.4(2)
C10-C9-C11-C12	-176.66(17)
C8-C9-C11-C12	55.3(2)
C9-C11-C12-C13	-54.8(2)
C11-C12-C13-C17	165.10(16)
C11-C12-C13-C21	-69.8(2)
C11-C12-C13-C14	54.8(2)
C7-C8-C14-C15	-55.7(3)
C9-C8-C14-C15	-177.25(19)

C7-C8-C14-C13	-178.58(17)
C9-C8-C14-C13	59.9(2)
C12-C13-C14-C8	-59.4(2)
C17-C13-C14-C8	179.68(16)
C21-C13-C14-C8	64.0(2)
C12-C13-C14-C15	168.73(17)
C17-C13-C14-C15	47.8(2)
C21-C13-C14-C15	-67.9(2)
C8-C14-C15-C16	-161.82(19)
C13-C14-C15-C16	-34.3(2)
C14-C15-C16-C17	7.3(3)
C12-C13-C17-O19	77.9(2)
C21-C13-C17-O19	-48.0(2)
C14-C13-C17-O19	-166.37(16)
C12-C13-C17-C16	-158.51(18)
C21-C13-C17-C16	75.6(2)
C14-C13-C17-C16	-42.78(19)
C15-C16-C17-O19	145.16(19)
C15-C16-C17-C13	22.5(2)
C13-C17-O19-C20	-166.47(17)
C16-C17-O19-C20	75.4(2)

Symmetry transformations used to generate equivalent atoms:

Table 7. Hydrogen bonds for **4a** [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O18-H18...O19#1	0.85(3)	1.90(3)	2.733(2)	163(3)

Symmetry transformations used to generate equivalent atoms:

#1 -x+1,y+1/2,-z

Table 8. Crystal data and structure refinement for **4d**.

Identification code	**4d**	
Empirical formula	C23 H36 O3	
Formula weight	360.52	
Temperature	173(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)	
Unit cell dimensions	a = 8.6418(4) Å	α= 90°.
	b = 9.5698(5) Å	β= 102.021(1)°.
	c = 12.8534(7) Å	γ= 90°.
Volume	1039.67(9) $Å^3$	
Z	2	
Density (calculated)	1.152 Mg/m^3	
Absorption coefficient	0.074 mm^{-1}	
F(000)	396	
Crystal size	0.21 x 0.21 x 0.13 mm^3	
Theta range for data collection	1.62 to 27.50°.	
Index ranges	-11• h• 11, -12• k• 8, -16• l• 16	
Reflections collected	7032	
Independent reflections	3784 [R(int) = 0.0233]	
Completeness to theta = 27.50°	99.8 %	
Absorption correction	Integration	
Maximum and minimum transmission	0.996 and 0.987	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3784 / 1 / 247	
Goodness-of-fit on F^2	0.976	
Final R indices [I>2sigma(I)]	R1 = 0.0371, wR2 = 0.0890 [3294]	
R indices (all data)	R1 = 0.0434, wR2 = 0.0917	
Absolute structure parameter	-0.6(10)	
Extinction coefficient	0.007(2)	
Largest diff. peak and hole	0.205 and -0.172 e.$Å^{-3}$	

$R1 = \Sigma(||F_o| - |F_c||) / \Sigma|F_o|$

$wR2 = [\Sigma[w(F_o^2 - F_c^2)^2] / \Sigma[w(F_o^2)^2]]^{1/2}$

$S = [\Sigma[w(F_o^2 - F_c^2)^2] / (n-p)]^{1/2}$ $w = 1/[\sigma^2(F_o^2)+(0.0370*p)^2+0.31*p]$, $p = [\max(F_o^2,0)+ 2* F_c^2]/3$

Table 9. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for **4d**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
C1	11008(2)	1772(2)	2646(1)	33(1)
C2	9638(2)	2008(2)	1885(1)	36(1)
C3	8198(2)	1518(2)	2055(1)	32(1)
C4	8164(2)	830(2)	2999(1)	31(1)
C5	9533(2)	583(2)	3763(1)	27(1)
C6	9380(2)	-165(2)	4773(1)	34(1)
C7	10944(2)	-633(2)	5461(1)	30(1)
C8	12190(2)	511(2)	5518(1)	24(1)
C9	12523(2)	730(2)	4394(1)	26(1)
C10	11003(2)	1054(2)	3592(1)	27(1)
C11	13868(2)	1783(2)	4401(1)	30(1)
C12	15368(2)	1440(2)	5240(1)	30(1)
C13	15012(2)	1284(2)	6352(1)	25(1)
C14	13726(2)	160(2)	6291(1)	26(1)
C15	13681(2)	-141(2)	7451(1)	39(1)
C16	15431(2)	-37(2)	8022(1)	44(1)
C17	16322(2)	618(2)	7220(1)	31(1)
O18	6797(1)	1672(2)	1344(1)	41(1)
O19	17503(1)	1553(1)	7780(1)	33(1)
C20	18801(2)	1799(2)	7279(1)	38(1)
C21	19992(2)	2723(2)	7972(1)	42(1)
C22	20759(2)	2061(2)	9032(1)	40(1)
C23	21971(3)	3025(3)	9697(2)	72(1)
C24	14553(2)	2697(2)	6760(1)	32(1)
C25	6496(3)	4418(3)	9335(2)	63(1)
O26	6978(2)	3036(2)	9538(1)	61(1)

Table 10. Bond lengths [Å] and angles [°] for **4d**.

C1-C2	1.388(2)
C1-C10	1.398(2)
C1-H1A	0.9500
C2-C3	1.389(2)
C2-H2A	0.9500
C3-O18	1.3650(18)
C3-C4	1.386(2)
C4-C5	1.391(2)
C4-H4A	0.9500
C5-C10	1.408(2)
C5-C6	1.513(2)
C6-C7	1.521(2)
C6-H6A	0.9900
C6-H6B	0.9900
C7-C8	1.527(2)
C7-H7A	0.9900
C7-H7B	0.9900
C8-C14	1.5208(19)
C8-C9	1.5443(19)
C8-H8A	1.0000
C9-C10	1.523(2)
C9-C11	1.537(2)
C9-H9A	1.0000
C11-C12	1.5395(19)
C11-H11A	0.9900
C11-H11B	0.9900
C12-C13	1.5308(19)
C12-H12A	0.9900
C12-H12B	0.9900
C13-C24	1.532(2)
C13-C14	1.537(2)
C13-C17	1.552(2)
C14-C15	1.527(2)
C14-H14A	1.0000

C15-C16	1.540(2)
C15-H15A	0.9900
C15-H15B	0.9900
C16-C17	1.542(2)
C16-H16A	0.9900
C16-H16B	0.9900
C17-O19	1.4328(19)
C17-H17A	1.0000
O18-H18	0.93(2)
O19-C20	1.4256(19)
C20-C21	1.501(2)
C20-H20A	0.9900
C20-H20B	0.9900
C21-C22	1.524(2)
C21-H21A	0.9900
C21-H21B	0.9900
C22-C23	1.519(3)
C22-H22A	0.9900
C22-H22B	0.9900
C23-H23A	0.9800
C23-H23B	0.9800
C23-H23C	0.9800
C24-H24A	0.9800
C24-H24B	0.9800
C24-H24C	0.9800
C25-O26	1.395(3)
C25-H25A	0.9800
C25-H25B	0.9800
C25-H25C	0.9800
O26-H26	0.88(2)
C2-C1-C10	122.41(15)
C2-C1-H1A	118.8
C10-C1-H1A	118.8
C1-C2-C3	119.63(15)
C1-C2-H2A	120.2
C3-C2-H2A	120.2

O18-C3-C4	117.41(14)
O18-C3-C2	123.79(15)
C4-C3-C2	118.80(13)
C3-C4-C5	121.91(14)
C3-C4-H4A	119.0
C5-C4-H4A	119.0
C4-C5-C10	119.87(14)
C4-C5-C6	118.24(13)
C10-C5-C6	121.88(13)
C5-C6-C7	114.37(13)
C5-C6-H6A	108.7
C7-C6-H6A	108.7
C5-C6-H6B	108.7
C7-C6-H6B	108.7
H6A-C6-H6B	107.6
C6-C7-C8	110.44(14)
C6-C7-H7A	109.6
C8-C7-H7A	109.6
C6-C7-H7B	109.6
C8-C7-H7B	109.6
H7A-C7-H7B	108.1
C14-C8-C7	112.56(13)
C14-C8-C9	109.54(11)
C7-C8-C9	108.64(12)
C14-C8-H8A	108.7
C7-C8-H8A	108.7
C9-C8-H8A	108.7
C10-C9-C11	114.53(13)
C10-C9-C8	111.02(11)
C11-C9-C8	111.83(12)
C10-C9-H9A	106.3
C11-C9-H9A	106.3
C8-C9-H9A	106.3
C1-C10-C5	117.34(14)
C1-C10-C9	122.04(13)
C5-C10-C9	120.57(13)

C9-C11-C12	112.95(13)
C9-C11-H11A	109.0
C12-C11-H11A	109.0
C9-C11-H11B	109.0
C12-C11-H11B	109.0
H11A-C11-H11B	107.8
C13-C12-C11	111.78(11)
C13-C12-H12A	109.3
C11-C12-H12A	109.3
C13-C12-H12B	109.3
C11-C12-H12B	109.3
H12A-C12-H12B	107.9
C12-C13-C24	110.53(14)
C12-C13-C14	107.75(12)
C24-C13-C14	113.50(12)
C12-C13-C17	116.96(12)
C24-C13-C17	108.54(12)
C14-C13-C17	99.24(12)
C8-C14-C15	118.94(12)
C8-C14-C13	113.70(12)
C15-C14-C13	104.34(12)
C8-C14-H14A	106.3
C15-C14-H14A	106.3
C13-C14-H14A	106.3
C14-C15-C16	103.35(13)
C14-C15-H15A	111.1
C16-C15-H15A	111.1
C14-C15-H15B	111.1
C16-C15-H15B	111.1
H15A-C15-H15B	109.1
C15-C16-C17	106.59(13)
C15-C16-H16A	110.4
C17-C16-H16A	110.4
C15-C16-H16B	110.4
C17-C16-H16B	110.4
H16A-C16-H16B	108.6

O19-C17-C16	108.71(12)
O19-C17-C13	116.21(14)
C16-C17-C13	104.85(12)
O19-C17-H17A	108.9
C16-C17-H17A	108.9
C13-C17-H17A	108.9
C3-O18-H18	107.1(14)
C20-O19-C17	114.91(12)
O19-C20-C21	109.63(14)
O19-C20-H20A	109.7
C21-C20-H20A	109.7
O19-C20-H20B	109.7
C21-C20-H20B	109.7
H20A-C20-H20B	108.2
C20-C21-C22	113.75(17)
C20-C21-H21A	108.8
C22-C21-H21A	108.8
C20-C21-H21B	108.8
C22-C21-H21B	108.8
H21A-C21-H21B	107.7
C23-C22-C21	111.79(19)
C23-C22-H22A	109.3
C21-C22-H22A	109.3
C23-C22-H22B	109.3
C21-C22-H22B	109.3
H22A-C22-H22B	107.9
C22-C23-H23A	109.5
C22-C23-H23B	109.5
H23A-C23-H23B	109.5
C22-C23-H23C	109.5
H23A-C23-H23C	109.5
H23B-C23-H23C	109.5
C13-C24-H24A	109.5
C13-C24-H24B	109.5
H24A-C24-H24B	109.5
C13-C24-H24C	109.5

H24A-C24-H24C	109.5
H24B-C24-H24C	109.5
O26-C25-H25A	109.5
O26-C25-H25B	109.5
H25A-C25-H25B	109.5
O26-C25-H25C	109.5
H25A-C25-H25C	109.5
H25B-C25-H25C	109.5
C25-O26-H26	112.4(16)

Symmetry transformations used to generate equivalent atoms:

Table 11. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for **4d**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
C1	30(1)	40(1)	27(1)	0(1)	4(1)	-3(1)
C2	38(1)	42(1)	26(1)	1(1)	1(1)	-1(1)
C3	31(1)	32(1)	29(1)	-5(1)	-3(1)	4(1)
C4	25(1)	32(1)	34(1)	-7(1)	3(1)	-1(1)
C5	27(1)	26(1)	27(1)	-3(1)	3(1)	-1(1)
C6	27(1)	41(1)	33(1)	3(1)	3(1)	-7(1)
C7	28(1)	32(1)	30(1)	3(1)	6(1)	-4(1)
C8	22(1)	25(1)	26(1)	1(1)	4(1)	-1(1)
C9	24(1)	28(1)	24(1)	-1(1)	4(1)	-1(1)
C10	27(1)	28(1)	24(1)	-6(1)	4(1)	-1(1)
C11	27(1)	37(1)	26(1)	4(1)	3(1)	-5(1)
C12	22(1)	38(1)	28(1)	1(1)	3(1)	-2(1)
C13	22(1)	27(1)	25(1)	2(1)	1(1)	0(1)
C14	25(1)	24(1)	28(1)	4(1)	2(1)	1(1)
C15	34(1)	48(1)	32(1)	14(1)	1(1)	-8(1)
C16	37(1)	54(1)	35(1)	16(1)	-3(1)	-6(1)
C17	27(1)	33(1)	30(1)	2(1)	0(1)	1(1)
O18	33(1)	47(1)	35(1)	-2(1)	-8(1)	1(1)
O19	23(1)	43(1)	28(1)	-4(1)	-1(1)	-1(1)
C20	29(1)	56(1)	28(1)	4(1)	1(1)	-4(1)
C21	35(1)	48(1)	39(1)	6(1)	3(1)	-8(1)
C22	34(1)	48(1)	36(1)	2(1)	-1(1)	-3(1)
C23	60(1)	87(2)	56(1)	0(1)	-16(1)	-29(1)
C24	29(1)	29(1)	34(1)	-3(1)	0(1)	0(1)
C25	84(2)	50(1)	53(1)	-12(1)	9(1)	1(1)
O26	83(1)	66(1)	34(1)	2(1)	12(1)	27(1)

Table 12. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for **4d**.

	x	y	z	U(eq)
H1A	11985	2111	2518	39
H2A	9684	2503	1251	44
H4A	7178	517	3128	37
H6A	8697	-995	4582	41
H6B	8845	465	5198	41
H7A	11311	-1493	5158	36
H7B	10795	-850	6187	36
H8A	11758	1399	5752	29
H9A	12910	-187	4176	31
H11A	13495	2729	4542	36
H11B	14136	1796	3688	36
H12A	15836	560	5041	36
H12B	16155	2195	5250	36
H14A	14149	-702	6011	32
H15A	13254	-1086	7531	47
H15B	13029	559	7731	47
H16A	15860	-976	8240	52
H16B	15541	557	8664	52
H17A	16854	-141	6891	37
H18	7010(30)	2190(30)	775(19)	64(7)
H20A	18420	2253	6580	46
H20B	19301	900	7157	46
H21A	19465	3601	8110	50
H21B	20831	2967	7583	50
H22A	19929	1834	9433	48
H22B	21281	1178	8899	48
H23A	22780	3271	9296	108
H23B	22470	2551	10357	108
H23C	21445	3877	9867	108
H24A	15435	3353	6808	48

H24B	14309	2575	7466	48
H24C	13621	3066	6269	48
H25A	6344	4857	9996	95
H25B	5498	4438	8808	95
H25C	7307	4930	9060	95
H26	7120(30)	2600(30)	8965(19)	58(7)

Table 13. Torsion angles [°] for **4d**.

C10-C1-C2-C3	-0.1(3)
C1-C2-C3-O18	-178.45(17)
C1-C2-C3-C4	1.5(3)
O18-C3-C4-C5	178.10(16)
C2-C3-C4-C5	-1.9(3)
C3-C4-C5-C10	0.8(2)
C3-C4-C5-C6	179.43(16)
C4-C5-C6-C7	168.87(16)
C10-C5-C6-C7	-12.5(2)
C5-C6-C7-C8	42.5(2)
C6-C7-C8-C14	173.79(12)
C6-C7-C8-C9	-64.71(16)
C14-C8-C9-C10	178.79(14)
C7-C8-C9-C10	55.46(16)
C14-C8-C9-C11	-51.94(17)
C7-C8-C9-C11	-175.27(14)
C2-C1-C10-C5	-0.9(3)
C2-C1-C10-C9	176.60(16)
C4-C5-C10-C1	0.6(2)
C6-C5-C10-C1	-178.00(16)
C4-C5-C10-C9	-176.97(15)
C6-C5-C10-C9	4.5(2)
C11-C9-C10-C1	28.6(2)
C8-C9-C10-C1	156.40(15)
C11-C9-C10-C5	-153.99(15)
C8-C9-C10-C5	-26.2(2)
C10-C9-C11-C12	178.61(12)
C8-C9-C11-C12	51.21(17)
C9-C11-C12-C13	-54.01(19)
C11-C12-C13-C24	-68.52(18)
C11-C12-C13-C14	56.03(18)
C11-C12-C13-C17	166.64(14)
C7-C8-C14-C15	-57.39(19)
C9-C8-C14-C15	-178.37(15)

C7-C8-C14-C13	179.11(13)
C9-C8-C14-C13	58.13(16)
C12-C13-C14-C8	-59.95(16)
C24-C13-C14-C8	62.79(16)
C17-C13-C14-C8	177.76(12)
C12-C13-C14-C15	168.92(14)
C24-C13-C14-C15	-68.34(16)
C17-C13-C14-C15	46.63(15)
C8-C14-C15-C16	-164.84(16)
C13-C14-C15-C16	-36.85(18)
C14-C15-C16-C17	11.8(2)
C15-C16-C17-O19	141.84(16)
C15-C16-C17-C13	16.9(2)
C12-C13-C17-O19	86.22(17)
C24-C13-C17-O19	-39.62(16)
C14-C13-C17-O19	-158.36(12)
C12-C13-C17-C16	-153.75(15)
C24-C13-C17-C16	80.40(16)
C14-C13-C17-C16	-38.33(16)
C16-C17-O19-C20	155.90(15)
C13-C17-O19-C20	-86.18(16)
C17-O19-C20-C21	-176.74(14)
O19-C20-C21-C22	64.4(2)
C20-C21-C22-C23	179.12(18)

Symmetry transformations used to generate equivalent atoms:

Table 14. Hydrogen bonds for **4d** [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
O18-H18...O26#1	0.93(2)	1.78(3)	2.695(2)	167(2)
O26-H26...O19#2	0.88(2)	1.91(3)	2.7839(19)	176(2)

Symmetry transformations used to generate equivalent atoms:

#1 x,y,z-1 #2 x-1,y,z

Elemental Analyses

Compound		Calc.		Found	
		C	H	C	H
3a	$C_{26}H_{32}O_2$	82.83	8.34	83.01	8.35
3b	$C_{27}H_{34}O_2$	83.03	8.77	83.33	8.52
3c	$C_{28}H_{36}O_2$	83.12	8.97	83.40	8.75
3d	$C_{29}H_{38}O_2$	83.21	9.15	83.10	9.35
3e	$C_{31}H_{42}O_2$	83.36	9.48	83.15	9.55
3f	$C_{33}H_{46}O_2$	84.49	9.77	84.19	9.85
4a	$C_{19}H_{26}O_2$	79.68	9.15	79.63	9.25
4b	$C_{20}H_{28}O_2$	79.96	9.39	80.11	9.42
4c	$C_{21}H_{30}O_2$ x H_2O x 0.5 CH_3OH	76.06	9.80	76.04	9.85
4d	$C_{22}H_{32}O_2$ x 0.5 CH_3OH	78.44	9.55	78.77	9.67
4e	$C_{24}H_{36}O_2$ x 0.5 H_2O	80.62	10.43	80.45	10.34
4f	$C_{26}H_{40}O_2$ x H_2O	77.56	10.51	77.84	10.73
5b	$C_{22}H_{32}O_2$	80.44	9.82	80.54	10.05
5c	$C_{26}H_{40}O_2$	81.20	10.48	81.09	10.44

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Appendix C
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See list of themes and topics, pp. 17–18. Indicate below a first and second choice appropriate for programming and publishing your paper.

1st theme title: Disorders of NS & Aging theme letter: J
1st topic title: Degenerative dis: Alzheimer's topic number: 129

2nd theme title: Disorders of NS & Aging theme letter: J
2nd topic title: Ischemia:neuropr & Tolerance topic number: 134

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Include nonrefundable **abstract handling fee** of $50. Fill out payment information form below. Purchase orders will not be accepted. Submission of abstract handling fee does not include registration for the Annual Meeting.

STRUCTURE-ACTIVITY RELATIONSHIP OF ESTRATRIENES AGAINST GLUTAMATE TOXICITY IN A MOUSE HIPPOCAMPAL CELL LINE

E.J. Perez*, K.L. Eberst, S.M. Oon, Z.Y.Cai, L. Prokai, D. Covey, and J. W. Simpkins. Dept. of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, FL, College of Medicine, University of Washington, St. Louis, MO.

Estrogens are neuroprotective against a variety of in vitro and in vivo insults that include peroxide, serum deprivation, Aβ peptides, anoxia, and glutamate toxicity. In several of these cases, estrogen protection is mediated by its intrinsic antioxidant properties. The present study was performed to determine the structure-activity relationship (SAR) for the neuroprotective activities of estratrienes. HT-22 (murine hippocampal) cells that lack estrogen receptors and are sensitive to glutamate were used as our model for this study. Glutamate (10 and 20 mM) for ~16 hours caused a 50-80% decrease in cell viability, as assessed by calcein AM. Each estratriene-derivatized compound was tested for neuroprotection at concentrations that ranged from 0.01 to 10μM. 17β estradiol was used to compare potency and efficacy of each novel compound. Hydroxyl additions to the B- and C-rings of estratrienes eliminated protection of HT-22 cells against glutamate, and opening the B-ring reduced neuroprotection. The addition of alkyl chains to the 17-oxygen or pentanyl groups to the 16 carbon of the D-ring enhanced the neuroprotection potency of the estratrienes. In constrast, addition of alkyl chains to the 3-oxygen of the A-ring completely eliminated neuroprotective activity. These data indicate that modification of estratrienes that are expected to substantially reduce estrogenicity can be achieved with equivalent or enhanced neuroprotection activity, even in a cell type which lacks estrogen receptors. These data support an estrogen receptor-independent mechanism for the neuroprotective effects of estratrienes. The compounds may be useful drug candidates for the treatment of neurodegenerative diseases.

(This work was supported by AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant, DAMD 17-99-1-9473).

Key Words: (see instructions p. 4)

1. Estrogen
2. Neuroprotection
3. Structure-Activity Relationships
4. Neurotoxicity

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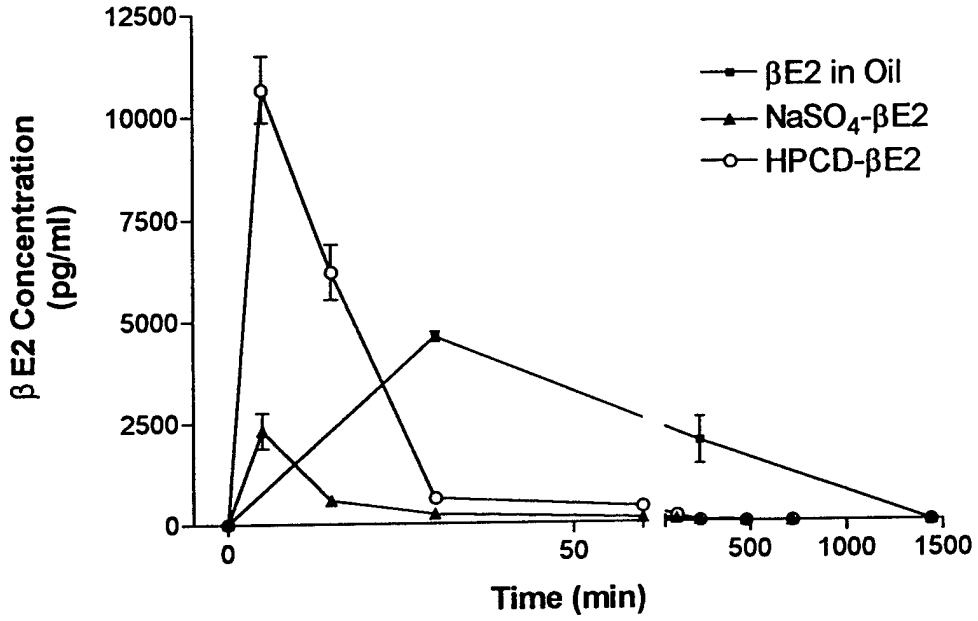
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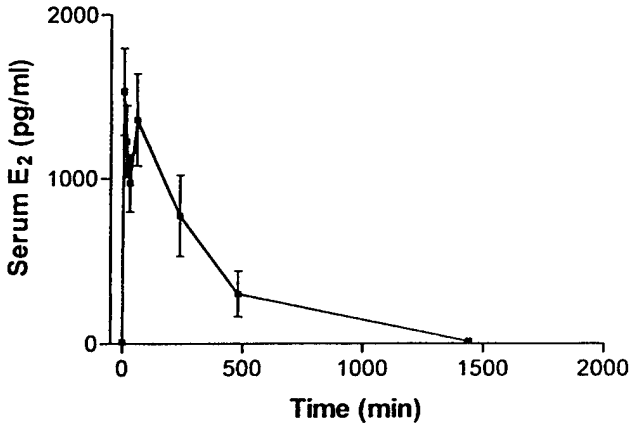
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Appendix D
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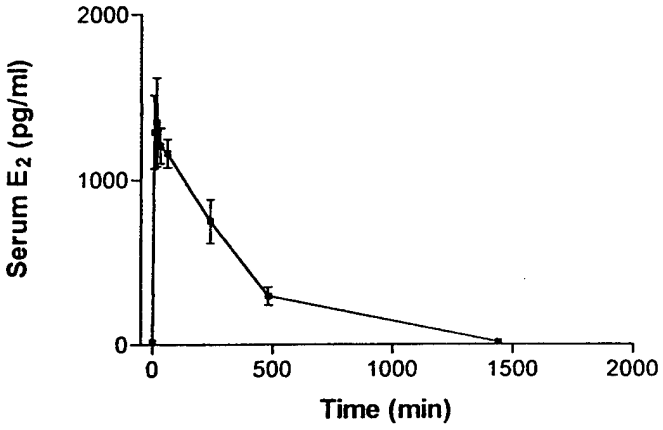


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Appendix E
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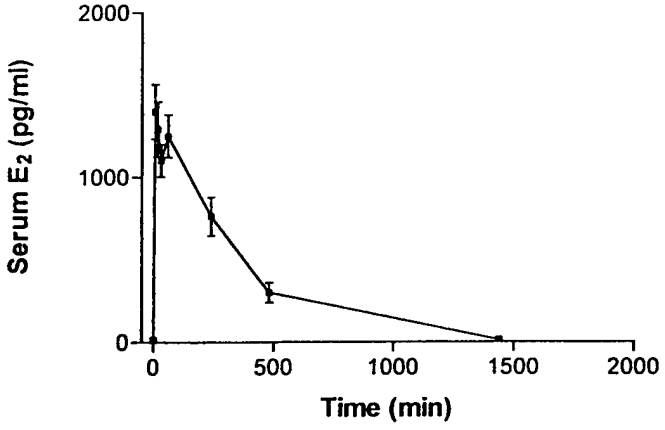
Estradiol
100 μg/kg in oil, sc



Estradiol-2% benzyl alcohol
100 μg/kg in oil, sc



Composite



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Appendix G
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Short communication

Effects of gender and estradiol treatment on focal brain ischemia

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Abstract

The present studies were undertaken to investigate the effects of gender and estrogen treatment on focal cerebral ischemia in male and female rats. Focal ischemia was created by inserting a 3-0 surgical suture through the left cervical internal carotid artery to obstruct the blood flow into the middle cerebral artery (MCA). The MCA was reperfused by removing the suture in 40 min. All rats were sacrificed for measurement of infarct area after 24 h. In the first study, mortalities from MAC occlusion were 12.5% (2/16) each for intact male rats and intact female rats, and 23.5% (4/17) for ovariectomized (OVX) female rats. The coronal infarct area (mean ± S.E.M.) was 9.5 ± 1.0% for intact female rats, 16.6 ± 1.6% for intact male rats ($p = 0.0001$ vs. intact female rats), and 16.0 ± 1.4% for OVX female rats ($p = 0.0002$ vs. intact female rats). In a second experiment, OVX-female rats were administrated either 17β-estradiol (E_2) or its vehicle, hydroxypropyl-β-cyclodextrin (HPCD), at 40 min after the onset of MCA occlusion. Mortalities were 40% (4/10) for vehicle treated OVX rats and 0% for E_2 treated OVX rats. The coronal infarct area (mean ± S.E.M.) was 19.3 ± 1.8% for vehicle treated rats vs. 8.0 ± 1.2% for E_2 treated rats ($p < 0.01$). Serum estrogen levels for vehicle treated OVX rats were 14.5 ± 1.2% pg/ml vs. 142.7 ± 23.6 pg/ml for E_2 treated OVX rats ($p < 0.01$). These results strongly suggest that the level of circulating estrogens play an important role in protecting brain tissues against ischemia induced by MCA occlusion. © 1998 Elsevier Science B.V.

Keywords: Middle cerebral artery occlusion; Estrogen; 17β-Estradiol; Ovariectomy; Ischemia; Sex-dependent mortality

Cerebral stroke is one of the leading causes of death in the United States. Numerous clinical studies have demonstrated beneficial effects of estrogens against heart disease [11,14,24] and cerebral stroke [8,18,20] in postmenopausal women.

We and others have demonstrated that estrogens exert protective effects against events associated with ischemia. Estrogens may act indirectly on the vascular system to reduce platelet aggregation, to reduce the thrombotic and vasoconstrictive effects of thromboxane, to modulate serum lipid levels [1,15,26] and to reduce post-ischemic hyperemia [13]. Estrogens may also promote neuronal survival by activating neurotrophins and their receptors [12,17,18,25], by protecting against neurotoxicity associated with serum-deprivation, β-amyloid and glutamate [3,4,9,10,22,23]. We have undertaken a series of studies to investigate the effects of gender and the efficacy of estrogens in preventing and/or rescuing brain tissue from focal ischemic damages induced by unilateral MCA occlusion.

The purpose of this study was two-fold. First, we investigated the gender-dependent susceptibility to focal ischemia. Second, we explored the protective effects of circulating estrogens against cerebral damages after the onset of focal ischemia induced by unilateral MCA occlusion in female rats.

Adult male and female Sprague–Dawley rats (200–225 g) were purchased from Charles Rivers Laboratories (Wilmington, MA) and were maintained for 1 week before female rats were subjected to ovariectomy (OVX). All rats were allowed free access to food and water throughout the procedure.

After the female rats were anesthetized with Metofane™ (methoxyglurane, Pitman Moore, Crossings, NJ) inhalant, both ovaries were removed through bilateral abdominal incisions using the dorsal approach. For rats receiving sham surgery, skin incisions were made, but the ovaries were not disturbed. After recovering from anesthesia, the rats were returned to their cages.

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MCA occlusion was achieved according to the methods described by Longa et al. [16] and Nagasawa and Kyuya [19] with the following modifications. One week after ovariectomy, anesthesia was induced with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The left carotid artery was exposed through a midline cervical incision. The left sternohyoid, sternomastoid, digastric and omohyoid muscles were divided and retracted. Part of the greater horn of hyoid bone was cut to facilitate exposure of the distal external carotid artery (ECA). The common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were then gently dissected away from adjacent nerves. The distal ECA and its branches, the CCA and pterygopalatine arteries were coagulated completely. A microvascular clip was placed on the ICA near skull base and a 2.5-cm length of 3-0 monofilament nylon suture was introduced into the ECA lumen. The suture was then gently advanced to the distal ICA until it reached the clipped position. After removing the microvascular clip, the suture was inserted until resistance was felt. The distance between the CCA bifurcation and the resistive point was about 1.8 cm. The operating procedure was performed within 10 min with little bleeding. Rectal temperature was monitored and maintained between 36.5–37.0°C with a heating lamp during the entire stroke procedure. The suture was withdrawn from the ICA after 40 min to allow MCA reperfusion. The distal ICA was coagulated immediately.

The 17β-estradiol (E_2) used for estrogen replacement treatment was complexed with hydroxypropyl-β-cyclodextrin (HPCD) [6,7]. The formulation achieved contained 32 mg E_2/g HPCD is aqueous soluble; a preparation suitable for intravenous (i.v.) injection [6,7]. For drug (E_2 or HPCD vehicle) treated rats, when the suture was withdrawn and the ICA was coagulated, a single dose of HPCD only or HPCD complexed with 17β-estradiol (1 mg/kg) was administrated i.v. As such, HPCD or E_2 was administrated at 40 min following the onset of the MCA occlusion.

All survived rats were sacrificed by decapitation 24 h after the MCA occlusion. The brains were removed immediately after sacrifice. Five coronal brain slices were made by a metallic brain matrix at 5, 7, 9, 11 and 13 mm posterior to the olfactory bulb. The slices were placed in a 2% solution of 2,3,5-triphenyltetrazolium hydrochloride (TTC) in a normal saline at 37.0°C for 30 min [2] and then were fixed in 10% formalin. The ischemic lesion area for each section was analyzed using the Image 1.47 software program. Trunk blood was collected and serum 17β-estradiol was measured by radioimmunoassay.

The chi-squared test was applied to assess mortality related to the MCA occlusion. The analysis of variance and Scheffe F-tests were applied to determine the significance of the difference among the experimental groups for infarct area, $p < 0.05$ was considered significant.

Our MCA occlusion procedure resulted in mortality of 12.5% each in the intact male and female rats, 23.5% and 40% in the OVX and OVX + HPCD female rats, respectively, and no mortality in OVX female rats with E_2 replacement (Table 1). As such, it appears that the absence of ovarian steroids increases mortality and the estrogen replacement reduces mortality associated with MCA occlusion in the female rats.

We first evaluated the gender-dependent vulnerability to cerebral ischemia induced by MCA occlusion. The coronal infarct area was 9.5 ± 1.0% for intact female rats, 16.6 ± 1.6% for intact male rats ($p < 0.01$ vs. intact female rats), and 16.0 ± 1.4 for OVX female rats ($p < 0.01$ vs. intact female rats) (Fig. 1).

Furthermore, we evaluated the effect of estrogen-replacement on infarct area following MCA occlusion. OVX rats were treated with either HPCD vehicle or 17β-estradiol complexed with the HPCD at 40 min after the onset of the MCA occlusion. Vehicle-treated rats showed an average coronal ischemic area of 19.3 ± 1.8%, while E_2 treatment reduced the ischemic area to 8.0 ± 1.2% ($p < 0.01$) (Fig. 2).

The present study demonstrates gender- and estrogen-dependent effects on mortality and the focal cerebral ischemic damages induced by unilateral MCA occlusion. These data are consistent with clinical observation that death associated with stroke is reduced in women on estrogen-replacement therapy at the time of the ischemic

Table 1
Effects of gender and estrogen replacement on mortality and ischemic brain lesion area in rats

Groups	Number (#) of rats	Death due to MCA occlusion (# rats)	Mortality (# death/# rats)	Average lesion area (% of coronal area)[a]
Study 1				
Intact males	16	2	12.5	16.6 ± 1.6%
Intact females	16	2	12.5	9.5 ± 1.0%**
OVX females	17	4	23.5	16.0 ± 1.4%
Study 2				
OVX + HPCD	10	4	40	19.3 ± 1.8%
OVX + E_2	10	0	0*	8.0 ± 1.2%**

* $p < 0.05$ vs. OVX + HPCD.
** $p < 0.01$ vs. intact males and OVX females.
[a]Average lesion area was determined for the five brain sections from each rat.

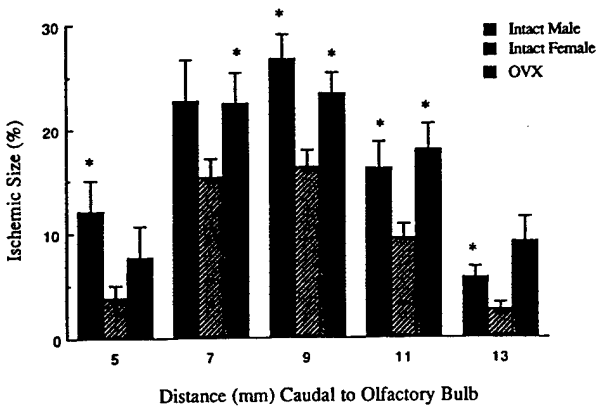


Fig. 1. Effects of gender on ischemic lesion area. Intact male rats, intact female rats and OVX female rats underwent MCA occlusion surgery. Twenty-four hours later, rat brains were dissected coronally producing section corresponding to 5, 7, 9, 11 and 13 mm caudal to the olfactory bulb. Brain slices were stained by TTC. $^{*}p < 0.05$ vs. intact female rats. The average infarct area (mean ± S.E.M.) was 9.5 ± 1.0% for intact female rats, 16.6 ± 1.6% for intact male rats ($p < 0.001$ vs. intact female rats) and 16.0 ± 1.4% for OVX rats ($p < 0.01$ vs. intact female rats).

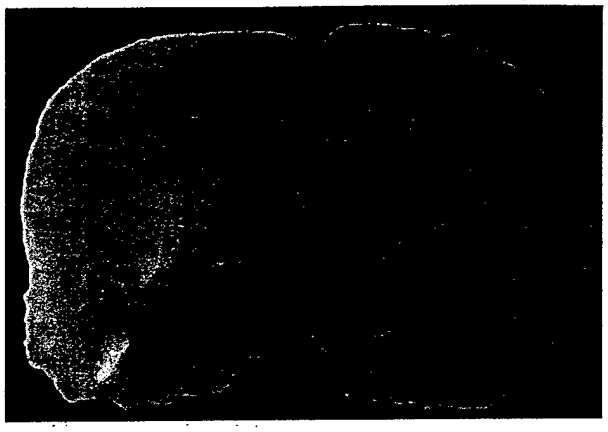


Fig. 3. Rat brain ischemic lesion caused by MCA occlusion. The left MCA was occluded for 40 min. TTC staining was done at 24 h after MCA occlusion. The ischemic lesion was not stained and remains white, while the normal brain area was stained red. This photo represents a section from an intact female rat brain taken at 9 mm caudal to the olfactory bulb. The infarct area occupied 28.6% of the coronal brain area.

event [8,11,20]. It appears that circulating estrogens reduce mortality and ischemic damage associated with MCA occlusion, the most common type of stroke in human beings [8,18] (Fig. 3).

The OVX rats had the highest mortality induced by MCA occlusion while the OVX rats with estrogen replacement had the lowest mortality. The mortalities of the intact male and female rats were intermediate between these two extremes. Similarly, cerebral infarct area caused by focal ischemia was the largest in the OVX rats, the smallest in the OVX-estrogen replaced rats and intermediate in the intact male and female rats. These data suggest that increased mortality is associated with increased lesion area. If this is the case, the ischemic area of OVX rats, albeit large, may be an underestimate, as 23 to 40% of OVX rats were lost to ischemic area assessment by their death prior to the scheduled sacrifice time.

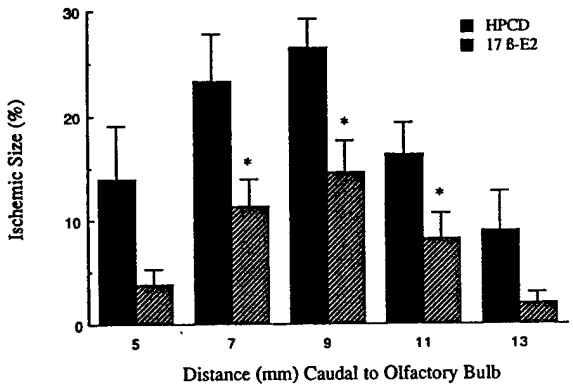


Fig. 2. Effects of estradiol treatment of OVX rats on ischemic lesion area. OVX rats were treated with HPCD vehicle or 17 β-estradiol 40 min after the onset of MCA occlusion. Twenty-four hours later, rat brains were dissected coronally producing section corresponding to 5, 7, 9, 11 and 13 mm caudal to the olfactory bulb. Brain slices were stained by TTC. $^{*}p < 0.05$ vs. the HPCD only group. The average infarct area (mean ± S.E.M.) was 19.3 ± 1.8% for vehicle treated OVX rats vs. 8.0 ± 1.2% for 17 β-estradiol treated OVX rats ($p < 0.01$).

The importance of circulating estrogen in attenuating ischemic damage is documented by the observation that in female rats used in the present study, estrogen concentrations were inversely related to ischemic area caused by MCA occlusion. Circulating estradiol concentration of 15 pg/ml in OVX was associated with the largest lesion area; 20 to 150 pg/ml in intact female rats [23] with intermediate lesion area; and 143 pg/ml for E_2 replacement treated rats with the smallest lesion area. Additionally, male rats are reported to have low circulating E_2 concentrations [21] and exhibited large lesion in response to MCA occlusion. As such, plasma E_2 concentration may be a prediction of the extent of brain damage associated with MCA occlusion.

The protective effects of estrogen observed with surgical MCA occlusion could result from several actions of the steroid. Estrogen could protect neurons from the toxicity associated with the release of glutamate during and following MCA occlusion by affecting excitatory amino acid receptors or the influx of calcium associated with NMDA or AMPA receptor stimulation [3,9,22]. Estrogens have been shown to reduce glutamate/NMDA toxicity [3,9,22] and to affect neuronal calcium homeostasis [9]. Alternatively, estrogen could protect brain tissue by antagonizing the damage initiated by oxygen free radicals during reperfusion [27]. In tissue culture, estrogens have been shown to exert potent antioxidant activity in response to a variety of oxidative stimuli [Refs. [4,9], Gridley et al., unpublished observation]. Finally, estrogen could reduce ischemic damage by enhancing glucose uptake across the blood–brain barrier during MCA occlusion. We have previously demonstrated that estradiol enhances by about 40% extrac-

tion of blood glucose by the brain [5,28] and increases the expression of the type 1 glucose transporter in the endothelial cells in both normal [28] and ischemic conditions [29].

In summary, there is gender-dependent vulnerability to focal ischemia and estrogen post-treatment rescues cerebral tissue from damage induced by focal ischemia. This study demonstrates the importance of estrogen in reducing brain damage associated with an ischemic event.

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Estrogens decrease reperfusion-associated cortical ischemic damage: a MRI analysis in a transient focal ischemia model

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Estrogens decrease reperfusion-associated cortical ischemic damage: an MRI analysis in a transient focal ischemia model

1 table

3 figures

Key words: Focal ischemia, MRI, Estrogen

Background and Purpose Early identification of irreversible cerebral ischemia is critical in defining strategies that influence neuronal survival following stroke. We used magnetic resonance imaging (MRI) to investigate the effects of 17 β-estradiol (E_2) on the temporal evolution of focal ischemia.

Methods Female rats were ovariectomized and divided into one of two groups, ovariectomy alone (OVX, n=4) or ovariectomy with estrogen replacement (OVX + E_2, n=3) groups. Both groups were then subjected to a 1-hour middle cerebral artery occlusion (MCAO), using a standardized transvascular monofilament model, followed by reperfusion. Serial diffusion-weighted (DWI) and T_2-weighted (T_2WI) MRI were obtained during and after the MCAO.

Results DWI detected similar lesion characteristics during MCAO in both groups. In the OVX group, lesion size did not change during reperfusion, but the signal intensity

ratio (SIR) increased early and stabilized during the latter stages. In contrast, DWI lesion size decreased during reperfusion in OVX + E_2 rats by 50-60% ($p<0.05$), a size reduction almost exclusively limited to cortical regions. During MCAO, the SIR in OVX + E_2 rats was reduced compared to OVX rats. Reperfusion further attenuated the SIR in cortical but not subcortical regions (vs. OVX, $p<0.05$). T_2WI revealed no lesions in either group during MCAO, but it detected similar lesion sizes to that of DWI during reperfusion. Further, similar patterns and magnitudes of estrogen treatment-related decrease in lesion size were noted following reperfusion. T_2WI demonstrated less intense SIR changes in both groups compared to DWI.

Conclusion This study strongly suggests that estrogens selectively protect cortical tissue from ischemic damage during MCAO, and that this protection is primarily exerted during the reperfusion phase of ischemia.

Stroke ranks as the third leading cause of death and the leading cause of disability in the United States [1]. Stroke patients must not only survive the acute stages of infarction, but must then cope with significant mental, physical, and economic stresses associated with neurologic impairment. Considering the cost both in loss of life and subsequent self-esteem and productivity, the need for effective therapeutic interventions is obvious. Most stroke occur when perfusion to the middle cerebral artery is reduced by a clot within the major cerebral arteries, producing a region of focal cerebral ischemia and a subsequent cascade of neuronal and microvascular changes ultimately leading to infarction [2]. Experimental ultrastructural evidence suggests that some of the damage occurs in the interval of reduced or absent perfusion (the occlusion phase), but most arises during the reperfusion stage, after flow has been restored by clot lysis or opening of collateral channels [3]. Since new thrombolytic therapy can now dissolve clots and restore arterial patency, the search for neuroprotective agents that can blunt the reperfusion-associated injury and elongate the tissue interval for safe intervention assumes critical strategic importance [4].

Observations from our and other laboratories indicate that estrogens are potent neuroprotective agents and decrease focal ischemia-induced lesion size by about 50% [5-9]. An understanding of the events affected by estrogen during occlusion and reperfusion will allow us to define the therapeutic window for application of estrogens in stroke. The histologic methods used in these previous studies limited our ability to dynamically assess estrogens' protective effects during occlusion and reperfusion. In the present

study, we applied MRI techniques to analyze non-invasively, for the first time, estrogen's temporal and spatial effects in focal cerebral ischemic events.

Materials and Methods

Animals:

Sprague-Dawley female rats (200-225 grams body weight) purchased from Charles Rivers Laboratories, Inc. (Wilmington, MA) were housed in pairs in hanging, stainless steel cages in a temperature controlled room (25+/-1 °C) with daily light cycle (light on 0700 to 1900 h daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Two weeks before the focal ischemia was induced, all rats were ovariectomized to eliminate endogenous estrogens. Rats in the OVX + E_2 group were administrated a single dose of E_2 (100 μg /kg) two hours before focal ischemia surgery, while those in the OVX group received no estrogen replacement. The sample size needed for MRI study was calculated based on our and other's observation that E_2 treatment caused a 50% reduction in MCAO-induced lesion size [7-9]. A small amount of animals sufficient to show statistical significance were assigned to each group.

Focal Ischemic model:

MCAO was achieved according to the methods described previously [7]. Briefly, following administration of anesthetics, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) on the left side were exposed and dissected through a midline cervical incision. A 3-0 monofilament suture was introduced into the left ICA lumen and gently advanced until resistance was felt, indicating MCAO

and compromised blood flow. The suture was kept in place for 60 minutes and then withdrawn to allow blood flow reperfuse to MCA. The operating procedure was performed within 20 minutes with little bleeding. Rectal temperature was monitored and maintained between 36.5 and 37.0 ^{0}C during the entire stroke procedure.

Magnetic resonance imaging

Imaging was performed at 4.7 T 33 cm magnet with a Brucket Console (Billerica, MA, USA), using an actively shielded gradient set capable of 220 mT/m. The animals were supported on a cradle and their heads placed in a home-built birdcage coil with a 5 cm outer diameter (operating in quadrature transmit/receive mode). Following the acquisition of scout images, 6 coronal plane images were prescribed beginning 3 mm behind the olfactory bulb. The slices were each 1.5-mm thick and were separated by 2 mm. All images were acquired over a 5 cm field of view using a 128 x 128 matrix (0.39 x 0.39 mm in-plane resolution) with a repetition time (TR) of 1.75 s and 2 signals averaged. Each set of 6 images was acquired in 7.5 min. DWI were acquired using a standard pulsed gradient spin echo technique with an echo time (TE) of 33 ms. The gradient pulses were each applied for 9 ms and were separated by 13 ms around the 180° refocusing pulse. The gradient amplitude used was 152 mT/m resulting in a b-value of 1400 s/mm^2. T_2WI were acquired using a standard spin echo technique with a TE of 75 ms. Both DWI and T_2WI were captured sequentially for each animal 30 minutes into the MCAO (the occlusion interval), and 2, 4, 6 hours after withdrawal of the monofilament (the reperfusion period).

Histological Staining

The experimental animals were decapitated, and the whole brains were dissected coronally from the olfactory bulb to the cerebellum in a metallic brain matrix (model RBM 4000C, ASI Instruments, Inc., Warren, MI). Sections were then made at 3, 5, 7, 9 and 11 mm posterior to the tip of olfactory bulb and were correlated to the MRI dissection. Sections were immediately stained by immersion in 2% TTC at 37 ^{0}C for 30 minutes and preserved in 10% formalin solution.

Quantitation of ischemic lesion sizes and intensity

The ischemic lesion sizes, as well as lesion intensity, of MRI and TTC staining images were anatomically matched and measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The lesion area was sub-divided to cortical and sub-cortical areas according to neuroanatomic landmarks. The percentage of the lesion size over the whole brain coronal section was calculated. The lesion intensity ratio was calculated with the intensity of the non-lesioned hemisphere assigned a value of 1.

Statistics

The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX + E_2 groups. The linear regression statistics was used to correlate measurements of lesion sizes between TTC staining and MRI. R square was calculated to analyze the coherence of the two measurements. A value of $p < 0.05$ was considered significant.

Results

DWI detected early changes in lesion sizes at 30 minutes into the MCAO. This MCAO-induced total lesion was similar in both groups (33.7% and 33.5% of the whole hemisphere in the OVX and OVX + E_2 groups, respectively), but was larger in cortical regions in the OVX groups (26.5% vs. 17.1% of OVX + E_2) (Fig.1, 2). During reperfusion, the lesion size remained constant in the OVX group but decreased in the OVX + E_2 groups by 50-60% ($p<0.05$). The size reduction was primarily located in cortical regions (Fig.1, 2). The intensity of the ischemic lesion, as measured by the signal intensity ratio (SIR), increased at 30 minutes during MCAO in the OVX group (lesion side vs. nonlesion side, 2.35 and 2.20 in cortical and subcortical regions, respectively) and reached a plateau during the latter stages of reperfusion until at 6 hours of reperfusion, when SIR dropped to 1.80 and 1.87 in cortical and subcortical regions, respectively (Table I). By comparison, the SIR during MCAO in OVX + E_2 animals was reduced (1.63 and 1.51 in cortical and subcortical regions respectively, $p<0.05$). Reperfusion further attenuated the SIR in cortical but not subcortical regions to 1.20 (vs. OVX, $p<0.05$) in the OVX + E_2 group (Table I).

T_2WI failed to detect the early vasogenic edema induced by MCAO in either group. During reperfusion, the OVX group demonstrated a continuous increase in lesion sizes in cortical (19.7%, 22.1% and 24.7% at 2, 4, 6 hours during reperfusion, respectively) and subcortical (8.3%, 10.1% and 12.5% at 2, 4, 6 hours during reperfusion, respectively) regions (Fig.3, 4). In contrast, the OVX + E_2 group showed a 70-80% decrease in lesion size in cortical ($p<0.05$) and a 10-25% decrease in subcortical regions (Fig.3, 4). T_2WI showed less attenuation of SIR by E_2 treatment when compared to DWI.

E_2 treatment did not significantly decrease SIR in subcortical regions when compared to OVX groups (Table I), but did cause a 20% decrease ($p<0.05$) in SIR in cortical regions during later reperfusion.

The changes shown by DWI and T_2WI were compared with those seen on histological 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining. The pattern and volume of TTC staining was consistent with changes seen on T_2WI during MCAO, *i.e.*, it failed to show any early changes in lesion sizes at 30 minutes during MCAO. During the reperfusion interval, however, TTC staining detected changes in lesion size of both groups that comparable to both DWI (r^2 =0.571, p =0.0018) and T_2WI (r^2 =0.631, $p<0.001$) (data not shown).

Discussion

Early detection and localization of potentially reversible ischemic damage is crucial for designing and investigating clinical therapeutic interventions against stroke. The present study demonstrates that MRI can provide a wealth of critical information about the initiation, progression and localization of cerebral ischemic events, and herein is used to define the location and the component of the developing ischemic lesion as affected by estrogens. DWI that is sensitive to the random movement of water molecules is thought to reveal the early changes associated with stroke induced-cytotoxic edema. On the other hand, conventional T_2WI is sensitive to vasogenic edema which occurs later in the pathophysiology of stroke [10]. It can detect subacute ischemic damages, although it fails to show acute ischemic changes. Histological TTC staining of ischemic lesions requires disruption of normal mitochondria function to detect the ischemic area. TTC staining lesions are indicative of neuronal dysfunction. It is noteworthy that TTC staining during MCAO does not reveal lesion, while DWI does, but by 6 hours of reperfusion, TTC staining correlates well with MRI measures of reperfusion-associated neuronal damages.

The early detection of ischemic lesion volumes by DWI is predictive of clinical severity and outcome of stroke patients [11;12]. In our study, DWI was the earliest detected evidence of cerebral ischemia and was the parameter most affected by E_2-treatment. This reduction in DWI changes by E_2-treatment can account for most of the observed beneficial effects of estrogen pre-treatment [5-9].

The preferential protection provided by estrogen to cortical versus subcortical tissue could reflect the differential blood supply to these two brain regions. The

penumbra of cortical ischemic region receives collaterals from leptomeningeal anastamosis, as well as from the watersheds between the anterior cerebral artery and the MCA, and between the posterior cerebral artery and the MCA. While the core of cortical ischemic region and the subcortical region are only supplied by terminal arteries of the MCA [13]. During MCAO, the penumbra may continue to receive limited blood flow (20% of basal level) from the ACA, while the core and basal ganglion are believed to be more severely occluded. Our MRI demonstration of E_2 exerting its protective effects at the penumbra region during MCAO may be related to this residual blood supply to the penumbra. Consistently, we have previously shown that estrogens increase brain glucose utilization [14;15] and enhance the expression of glucose transporter 1 in the blood brain barrier at the penumbra, but not the core ischemic region, during focal ischemia [6]. This modulation of cerebral energy metabolism may explain at least in part, an estrogen role in protection from ischemia-induced energy deficits.

E_2-treatment appears to exert its protective effects by preventing permanent damage associated with reperfusion. Reperfusion causes structure alteration of the Golgi apparatus and compromises the energy supply to brain cells. Hoehn-Berlage et al. [16] applied bioluminescence and fluorescence techniques to correlate DWI and energy disturbance during MCAO, and found a depletion of ATP in the ischemic core while the area of tissue acidosis spreads beyond the ATP-depleted core region. These findings are consistent with our TTC staining observation that the white core infarct region is surrounded by the pink ischemic penumbra [5-7]. The close correlation between DWI and TTC staining suggests that DWI detection is predictive of energy deficits during early stroke.

Reperfusion of ischemic tissue can produce an influx of oxygen followed by an accumulation of oxygen-derived free radicals [2]. The oxidative stress may damage unsaturated fatty acids in the plasma membrane, which in turn could increase calcium influx into the cell and worsen ischemia-initiated neuronal injuries. We and others have shown that estrogens can attenuate free radical-induced peroxidative damage [17;18], modulate calcium homeostasis in the neurons [19], and interact with neurotrophins, their receptors and signaling pathways [20]. All these effects of estrogen may contribute to its protective effects during reperfusion.

The suggestion that estrogens may have significant protective properties during reperfusion could have profound impact in stroke therapies. Many centers in the United States are now treating stroke acutely, using thrombolytic agents to dissolve the offending clot. Clinical trials have demonstrated a significant clinical improvement in such patients, especially when the treatment is delivered within 3 hours of stroke onset. Reopening an occluded intracranial vessel, however, is not without serious risks, which may include acute or delayed intracerebral hemorrhage, and reperfusion hyperemia and progressive to infarction despite a patent lumen. The identification of an agent that can protect against such mechanisms, if delivered before or early after the vessel is re-opened, could minimize an otherwise pre-programmed infarction, and perhaps also positively influence hemorrhagic risks by stabilizing energy metabolism in vascular endothelium.

The present study suggests that estrogens are good candidates for producing such effects. During reperfusion, E_2 treatment dramatically decreases ischemic lesion sizes

and intensities, as demonstrated by both DWI and T_2WI, and these decreases are almost exclusively located in cortical regions.

In summary, we applied MRI techniques to demonstrate the temporal and spatial ischemic changes in a focal ischemic animal model. We have demonstrated that estrogens selectively protect cortical tissue from ischemic damage and that this protection is primarily exerted during the reperfusion phase of damage. This study strongly suggests that estrogen could have direct clinical applications and potentially could widen the therapeutic window by protecting against thrombolytic-induced reperfusion injury.

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Table I: The Effects of Estrogen in the Single Intensity Ratio of Ischemic Lesions Assessed by MRI.

Single Intensity Ratio DWI						Single Intensity Ratio T_2WI			
Time (hours)		Occl	2	4	6	Occl	2	4	6
Cortex	OVX	2.35±0.15	2.43±0.25	2.34±0.27	1.81±0.09	0	1.45±0.05	1.67±0.06	1.84±0.05
	E_2	1.63±0.12*	1.15±0.38*	1.23±0.40*	1.28±0.26	0	1.26±0.08	1.36±0.08*	1.40±0.11*
Sub-Cortex	OVX	2.20±0.21	2.38±0.15	2.27±0.22	1.87±0.06	0	1.40±0.04	1.59±0.06	1.76±0.06
	E_2	1.51±0.32	1.86±0.05*	2.11±0.11	1.94±0.09	0	1.28±0.01	1.35±0.04	1.52±0.06

Changes in ischemic lesion intensity were measured by single intensity ratio. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX + E_2 groups. Mean ± SEM are depicted. * $p < 0.05$ vs. the OVX group.

Figure 1: Sequential DWI from Representative OVX and OVX + E_2 Rats During MCAO and Following Reperfusion. Two weeks after ovariectomy, female rats were divided into ovariectomy alone (OVX, n=4) and ovariectomy with estrogen replacement (OVX + E_2, n=3) groups. Both groups were then subjected to a 1-hour MCA occlusion. Serial DWI were then obtained beginning at 30 minutes of the MCA occlusion and at 2, 4 and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

Figure 2: The Effects of Estrogen on MCAO-Induced Lesion Sizes in Female Rats Assessed by DWI. Serial DWI was applied to assess the total (2-A), cortical (2-B) and sub-cortical (2-C) lesion sizes in both OVX and OVX + E_2 groups. An average measurement of lesion sizes at 7 mm and 9 mm were used for statistical analysis, because they represented the widest extent of the MCAO lesions. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX + E_2 groups. Mean $\pm$ SEM are depicted. When SEM is not shown, it is too small to be depicted. * $p < 0.05$ vs. the OVX group.

Figure 3: Sequential T_2WI from Representative OVX and OVX + E_2 Rats During MCAO and Following Reperfusion. Two weeks after ovariectomy, female rats were divided into ovariectomy alone (OVX, n=4) and ovariectomy with estrogen replacement (OVX + E_2, n=3) groups. Both groups were then subjected to a 1-hour MCA occlusion. Serial T_2WI were then obtained halfway through the MCA occlusion and at 2, 4 and 6 hours after

monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

Figure 4: The Effects of Estrogen on MCAO-Induced Lesion Sizes in Female Rats Assessed by T_2WI. Serial T_2WI was applied to assess the total (4-A), cortical (4-B) and sub-cortical (4-C) lesion sizes in both OVX and OVX + E_2 groups. An average measurement of lesion sizes at 7 mm and 9 mm were used for statistical analysis, because they represented the widest extent of the MCAO lesions. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX + E_2 groups. Mean $\pm$ SEM are depicted. When SEM is not shown, it is too small to be depicted. * $p < 0.05$ vs. the OVX group.

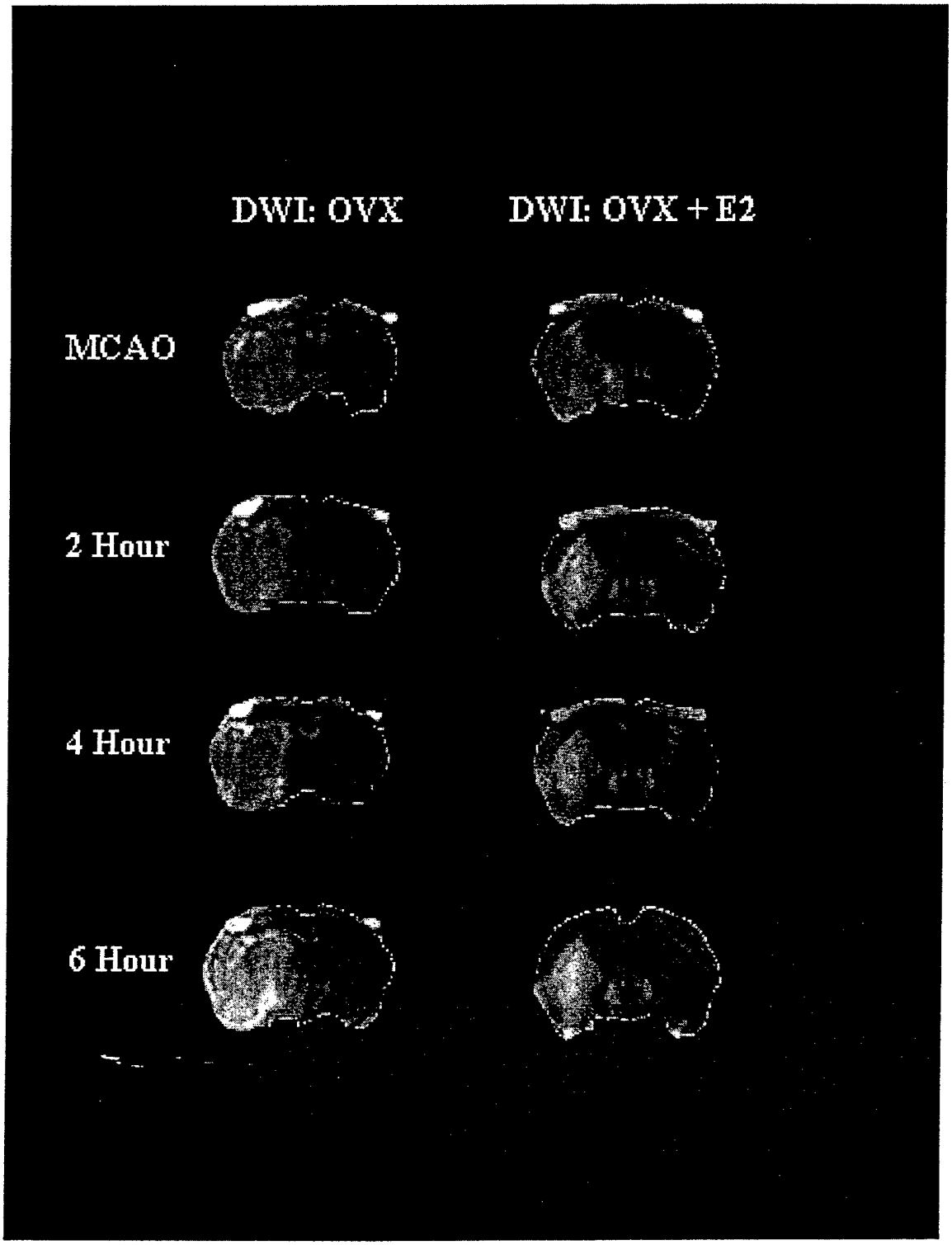


Fig. 1 (JS, JDB, SHY and et al.)

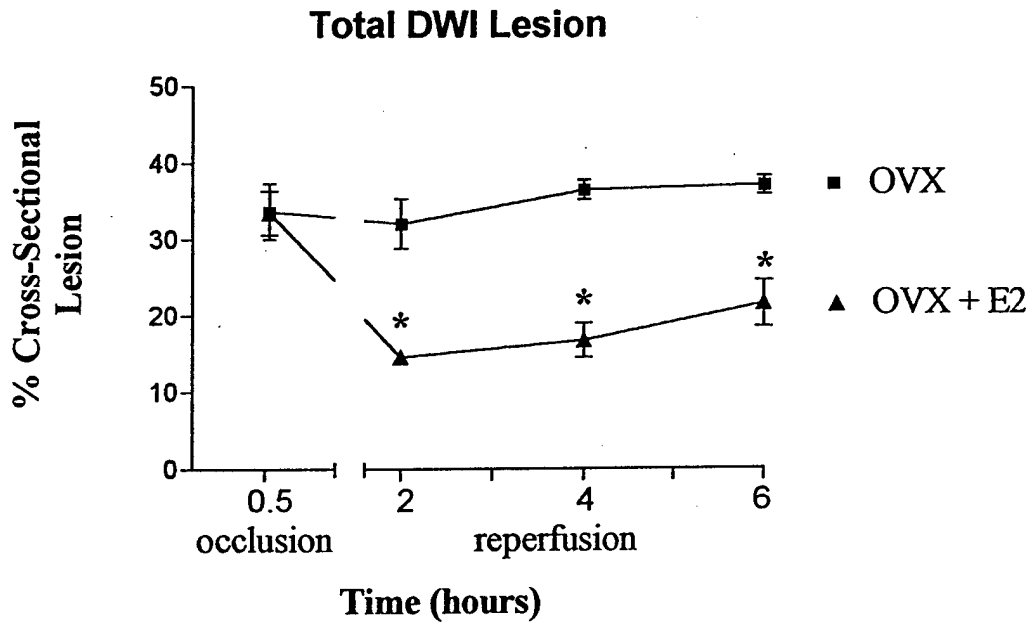


Fig. 2-A (JS, JDB, SHY and et al.)

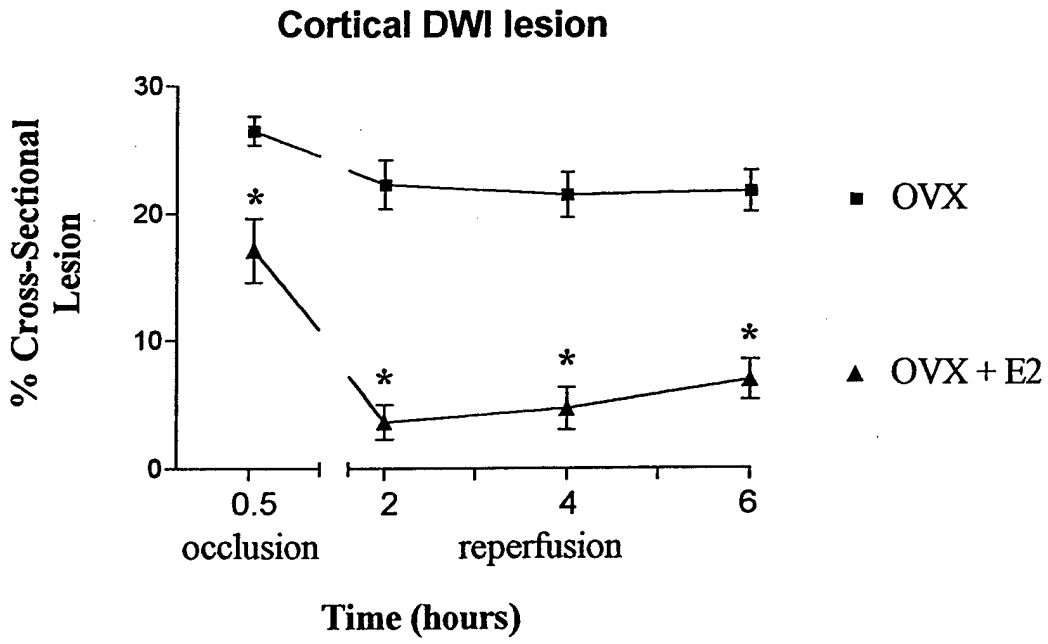


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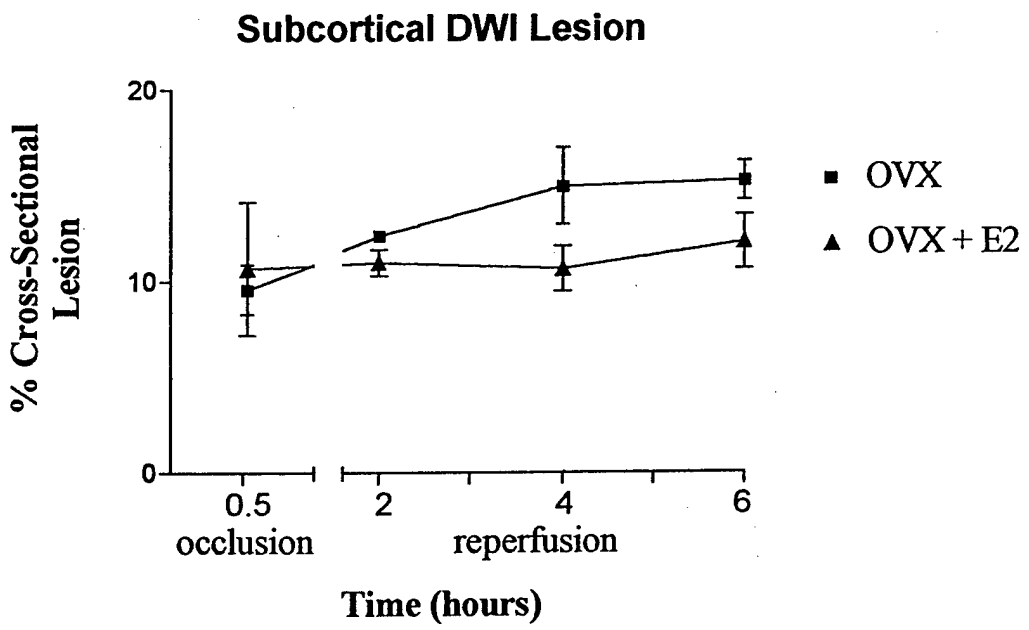


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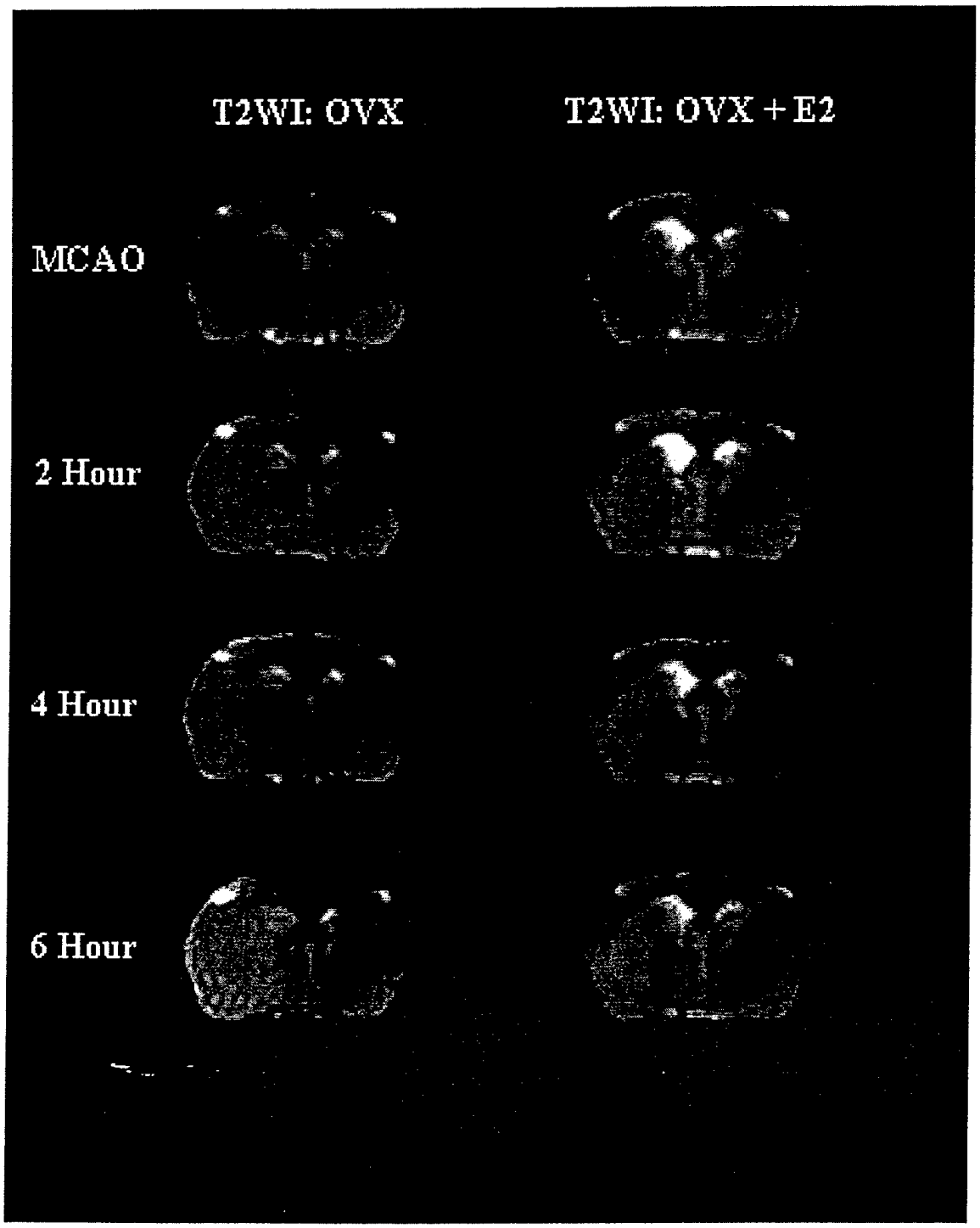


Fig. 3 (JS, JDB, SHY and et al.)

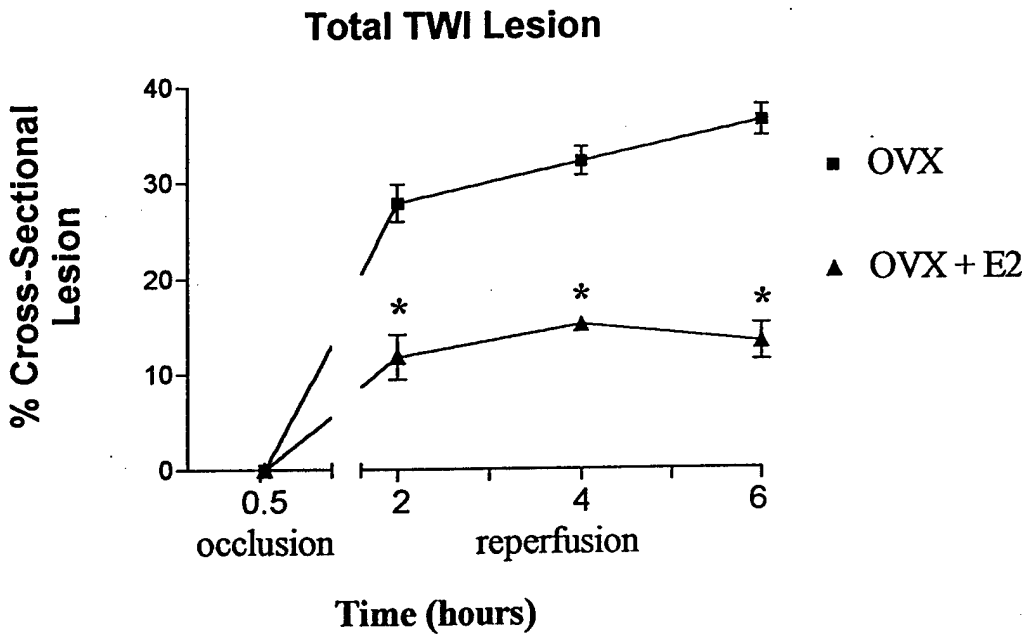


Fig. 4-A (JS, JDB, SHY and et al.)

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Appendix I
J. W. Simpkins

The Effects of 17 α-Estradiol on Cerebral Blood Flow and Cortical Protection During Middle Cerebral Artery Occlusion in Rats

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The Effects of 17 α-Estradiol on Cerebral Blood Flow and Cortical Protection During Middle Cerebral Artery Occlusion in Rats

Cover title: Effects of 17α-Estradiol on MCA Occlusion in Rats

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Abstract

Background and Purpose- Stroke is the third leading cause of mortality in the U.S. and there are currently no neuroprotective therapies available. Furthermore, research concerning estrogen's role in cerebral circulation remains limited. Therefore, the present study examined effects of 17α-estradiol (17α-E2) on cerebral blood flow (CBF) and neural degeneration during and after acute middle cerebral arterial occlusion (MCAO).

Methods- Female rats were ovariectomized and six days later were implanted with Silastic pellets according to treatment: 17α-E2, 17α-E2:Cholesterol [17α-E2 (1:2)], or Sham. 24 hrs later, rats underwent unilateral 1 hr MCAO. CBF Laser doppler measurements were taken prior to and during MCAO, and immediately and 24 hrs following the ischemic episode.

Results- Baseline CBF readings did not differ among groups, however significantly reduced left hemisphere CBF was demonstrated during MCAO for all animals. During reperfusion, sham-treated and 17α-E2 (1:2) rats showed increases in CBF to 77% and 72%, respectively, of pre-MCAO levels. 17α-E2-treated rats exhibited increases in CBF to 83% of baseline levels. All groups showed slight reductions in CBF at the 24-hr readings. No significant right hemisphere changes were found. Although only slight group differences were shown across CBF measurements, a highly significant effect of 17α-E2 on ischemic lesion volume was observed.

Conclusions- Treatment with 17α-E2, as well as 17α-E2 (1:2), was associated with protection from the large infarcts seen in sham-treated animals. Therefore, 17α-E2 may act as a neuroprotective agent and does not appear to be dose-dependent, since both doses demonstrated similar neural ischemic protection.

Cerebral stroke is a leading cause of disability and is the third leading cause of mortality in the United States. When not fatal, the debilitating effects of ischemic episodes can range from mild (e.g., transient loss of motor control) to severe (e.g., neurological damage leading to paralysis and/or dementia). Furthermore, many morphological changes can typically occur in susceptible neuronal populations for which no neuroprotective therapies are currently available. Thus, the current research emphasis focuses on the treatment and prevention of stroke.

Although there has been an effort to provide effective treatment for stroke via vascular and neuroprotective drugs[1,2], there are currently no successful therapies available which prevent the extensive poststroke neuronal damage which typically leads to neurological debilitation. While neuroprotective strategies such as glutamate receptor antagonists, calcium channel blockers, free-radical scavengers, and even mild hypothermia have been employed[3], numerous clinical studies have demonstrated beneficial effects of estrogens against heart disease[4,5,6] and cerebral stroke[7,8,9] in postmenopausal women. This is an especially relevant area of study since postmenopausal women demonstrate an increased risk of cardiovascular and neurological disease estrogen replacement therapy is associated with reductions in stroke-related mortality[6,7,10,11,12,13,14].

Through the work of our laboratory and others, it has recently been demonstrated that estrogens exert significant neuroprotective effects both *in vitro*[15,16,17,18,19,20,21,22,23], as well as in *in vivo* via animal models for cerebral ischemia[24,25,26,27,28,29,30,31,32]. While many mechanisms of neuroprotection via estrogens have been suggested[25], these protective effects do not appear to be solely attributable to the naturally occurring potent estrogen, 17β-Estradiol (17β-E2). In fact, our laboratory[17,18] has recently demonstrated that the weak estrogen 17α-Estradiol (17α-E2) was as effective as 17β-E2 for protecting SK-N-SH neurons in deprived conditions. Thus, the ability

of 17α-E2 to protect neurons against insult is quite encouraging as a clinical treatment perspective since it lacks "classic" genomic mediated estrogenic effects.

Therefore, the present study examined the effects of 17α-E2 on cerebral blood flow (CBF) and neural protection in ovariectomized (OVX) rats. Specifically, we were interested in determining the efficacy of 17α-E2 in protecting against focal ischemia induced via unilateral middle cerebral arterial occlusion (MCAO). Furthermore, we wanted to assess the possible moderating effects of 17α-E2 on cerebral circulation. We employed the MCAO technique, which was first introduced by Tamura et al.[33] and later modified by Longa et al.[34], since it has become the one of the most widely used models for assessing the pathophysiology and therapeutic conditions in both permanent[33] and transient[34] focal cerebral ischemia. We utilized transient MCAO, which allows for reperfusion, since recirculation following cerebral ischemia is a common clinical event[35]. Furthermore, we employed two different doses of 17α-E2, one straight (17α-E2) and one mixed with cholesterol at a ratio of 1(17α-E2):2(cholesterol) to examine the possible dose-range of neuroprotection. With this study we were interested in addressing the following hypotheses: 1) 17α-E2 would normalize reperfusion of CBF following MCAO and would result in baseline levels 24 hours following the MCAO procedure; 2) 17α-E2 (1:2) would also moderate changes in CBF, however these effects would be significantly less than those seen with straight 17α-E2; 3) 17α-E2 and 17α-E2 (1:2) would provide significant protection, in a dose-dependent manner, against ischemic cellular damage; 4) sham-treated animals would demonstrate significantly reduced CBF following MCAO and would show the greatest lesion infarct volume size; and 5) there would be no differences among the treatment groups in plasma pH, ions, or gases.

Materials and Methods

Subjects and Surgery

Twenty-eight female Sprague-Dawley rats (Harlan Sprague—Dawley, 230-260 g) served as subjects. Animals were housed in an AAALAC-approved vivarium, with temperature regulation and a light/dark cycle initiated at 0630 h, and all procedures performed were in accordance with institutional guidelines. All animals were provided free access to laboratory rat chow and water, however food was removed the night before surgery. Prior to surgery, each animal was randomly assigned to one of three experimental groups, designating their respective treatments: 17α-E2 (n=10), 17α-E2 (1:2) (n=10), or Sham (n=8). On day 1, all animals were anesthetized via Metofane™ (methoxyglurane, Pitman Moore, Crossings, NJ) and a bilateral ovariectomy was performed via a dorsal approach utilizing abdominal incisions. Wound clips were used to close the incision and once the animal recovered from surgery, it was returned to its home cage. Following six days of post-operative recovery, all animals were subcutaneously implanted with their respective steroid treatments.

Steroid Treatment

Either straight 17α-E2 or a combination of 17α-E2:Cholesterol (1:2) was packaged into 5 mm-long Silastic pellets (#602-285; Dow Corning Corp., Midland, MI) which were then sealed on both ends with Silastic Medical Adhesive (Silicone Type A). Sham pellets were prepared similarly except the pellets were empty. All pellets were washed with methanol to remove any steroid adhering to the outside of the pellet following packaging. Furthermore, all pellets were soaked in saline 36 hours prior to implantation to ensure *in vivo* release of estradiol[36]. Pellets were then subcutaneously implanted within each respective animal 24 hours prior to MCAO.

Middle Cerebral Arterial Occlusion

Rats were anesthetized via ketamine (60 mg/kg, *ip*) and xylazine (10 mg/kg,*ip*), during which time rectal temperatures were monitored and maintained with a heating pad within a range of 36.5° and 37.0° C. This MCAO procedure has been previously described by our laboratory[24,25,26,31,32]. Briefly, a midline cervical incision was made to expose the left common, external, and internal carotid arteries (CCA, ECA, ICA, respectively). All were identified and separated from adjacent cranial nerves, and the distal ECA and its branches, the CCA, and the pterygopalatine arteries on the left were completely cauterized. A vascular clip was placed at the ICA base and a small ECA lumen incision was made. A 3-0 nylon suture (Ethicon) was inserted into the ECA and advanced to the clip. The clip was removed and the suture inserted until the point of resistance, which indicated that the suture had passed the MCA and lodged into the ACA. This procedure was performed within 20 minutes with minimal bleeding. The suture was withdrawn after 1 hour to allow MCA reperfusion. The ICA was then immediately cauterized and the skin incision closed.

Laser Doppler Flowmetry (LDF)

To assess CBF, recordings were made via LDF (laser doppler flowmetry) (Oxford Optronix Ltd., MicroFlo DSP, Oxford, England) equipped with two channel probes (48 kHz sampling rate) to record right and left parietal cortices. To obtain readings, animals were placed in a Kopf stereotaxic apparatus (Model #900) and bilateral burr holes (1mm diameter) were placed 1.5 mm posterior and 3.5 mm lateral from bregma over the left and right hemispheres. The dura mater was exposed and the probes were placed directly above the tissue via micromanipulators. Values obtained from LDF estimate number of red blood cells passing through 1 mm^3 tissue per second,

and are referred to as Blood Perfusion Units (BPU). Recordings were taken during baseline, MCAO, Recovery, and 24 hr. post-MCAO Next Day readings.

Hemodynamic Data

Venous draws were taken from the jugular vein immediately prior to and immediately following the MCAO procedure to assess hemodynamic content. Jugular draws were utilized to avoid excessively altering arterial conditions. These measurements were collected to assess possible changes in blood gases and electrolyte balance both between and within groups. Values measured via a blood gas and electrolyte analyzer (CIBA Corning) were pH, pCO2, pO2, Na^+, K^+, and Ca^{2+}.

Infarct Volume Analysis

24 hours following MCAO, all animals were anesthetized and CBF recordings were taken. Following LDF, animals were sacrificed via decapitation and the brains immediately removed. The brains were then placed into a brain matrix for standardized tissue sectioning and were cut into 2-mm thick coronal sections at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. The slices were placed into a 2% 2,3,5-triphenyltetrazolium hydrochloride (TTC) solution in saline for 30 min. at 37° C. TTC staining has been shown to effectively and accurately detect areas of infarction 24 hr following ischemic episodes[37]. Sections were then fixed in 10% formalin and analyzed using an Image Scan 1.47 software program for detection of infarct size. This software program allows for the detection and analysis of total area of tissue, total area of "healthy cells" (as detected by a "dark" field on the computer) and total area of infarct tissue (as detected by a "light" field on the computer). All areas of ischemic damage were calculated to provide total percentage of lesion as well as percentages of cortical and subcortical damage.

Statistical Analyses

Statistical analyses were performed via SigmaStat 2.0 Software (Jandel Scientific,San Rafael, CA). Hemodynamic differences among groups were analyzed via paired t-tests to determine changes in blood content from pre-MCAO to post-MCAO readings. Baseline and Next Day CBF readings were analyzed across groups with a one-way analysis of variance (ANOVA). Readings taken during the MCAO procedure, and for the one hour recovery following MCAO, were examined across groups and divided into right and left hemisphere CBF readings. Data from each hemisphere were then examined according to time blocks: 0-5, 5-15, 15-30, 30-45, and 45-60 minute sections. These data were analyzed via two-way ANOVA (group X time) with repeated measures on the second factor. In an attempt to normalize the CBF data, blood flow readings were further analyzed as a total percentage change from baseline for all LDF measurements. These data were then analyzed via one-way ANOVA's for each hemisphere. Finally, lesion percentages were examined according to lesion area (total, cortical, and subcortical regions) and significance was determined across all groups via one-way ANOVA. For all above analyses, Tukey post-hoc tests were used to further evaluate significant main effects, with a level of significance set at 0.01.

Results

Hemodynamic Data

Hemodynamic measurements for venous blood content demonstrated no significant differences across groups for either the pre-MCAO or post-MCAO values (Table 1). When examining each individual group across the pre and post time points, differences were found for all three groups. The paired t-tests for the 17α-E2 animals showed pre to post changes for K^+ ($t=3.87$, $p < 0.01$) and Ca^{2+} ($t=-3.31$, $p < 0.025$). The significant changes for the 17α-E2 (1:2) groups included

pCO_2 ($t=-4.49$, $p < 0.01$), K^+ ($t=2.81$, $p < 0.05$), and Ca^{2+} levels ($t=-2.98$, $p < 0.025$). Finally, similar differences were also noted for the Sham group from pre to post measurements for pCO_2 ($t=-4.72$, $p < 0.01$), K^+ ($t=5.53$, $p < 0.01$), and Ca^{2+} levels ($t=-2.95$, $p < 0.025$).

Insert Table 1 about here

CBF Data

Changes in CBF, as recorded by LDF, were analyzed across the different time points and were divided into Baseline, MCAO, Recovery, and Next Day readings for both left (ischemic) and right (non-ischemic) hemispheres. The one-way ANOVA for the baseline readings demonstrated no significant differences across groups for either hemisphere, thus indicating that 24 hour subcutaneous steroid treatment did not alter cerebral blood flow as compared to Sham-treated control animals, which allowed for accurate comparisons across groups for all subsequent measurements. Following placement of the monofilament suture, dramatic changes in the left hemisphere could be seen for all animals across the one hour MCAO. While there were no differences between groups, there was a significant effect across time points ($F_{4,100} = 3.50$, $p < 0.01$). Post-hoc analyses demonstrated differences between the 17α-E2 and 17α-E2 (1:2) groups at the 15-30 minute reading (Fig.1). Changes also occurred in the right (non-ischemic) hemisphere as well. Again, no group differences were found, however there were significant effects of time ($F_{4,100} = 2.57$, $p < 0.05$) as well as a group X time interaction ($F_{8,100} = 2.05$, $p < 0.05$) (Fig. 2). Post-hoc comparisons demonstrated that all time points differed, with the Sham group showing lower CBF readings across each time point than the other two groups. Furthermore, the 17α-E2 and 17α-E2 (1:2) groups also differed from each other at the 0-5

minute reading. Therefore, significant changes in CBF levels across time could be found for both the left and right hemispheres in all groups during the occlusion procedure. When analyzing the data obtained during the recovery readings, no statistically significant differences could be found for either hemisphere across groups or time for any of the recordings taken during recovery. Although there appeared to be an effect of group, with the 17α-E2 animals demonstrating higher mean blood flows for both the left and right hemispheres, the variation within groups obscured these differences (see Figs. 1 and 2).

Insert Figures 1 and 2 about here

The one-way ANOVA conducted on data obtained during the 24 hour Next Day readings also demonstrated no differences among the three groups for either the left (ischemic) or right (non-ischemic) hemisphere readings. This and the above analyses suggest that steroid treatment did not significantly alter CBF as compared to Sham, non-treated control animals for any of the time points before, during, or after MCA occlusion. In further support of this, one-way ANOVA's conducted on the normalized data, presented as a percentage of CBF reduction from baseline (see Figs. 3 and 4), showed that there were no differences among groups at each of the time points measured (MCAO, Recovery, and Next Day). There were, however, differences found across time points for the left hemisphere ($F_{8,75} = 8.64$, $p < 0.01$), specifically each group's Recovery and Next Day readings differed from those obtained during the MCAO procedure. This effect across time points was not seen on the right hemisphere.

Insert Figures 3 and 4 about here

Infarct Volume Data

While the previous data showed that steroid treatment did not alter the effect of CBF across groups, treatment did significantly affect the resulting lesion size following MCAO (Fig. 5). Statistically significant effects were found for all three ischemic lesion measurements: total lesion ($F_{2,25} = 7.44$, $p < 0.01$), cortical lesion ($F_{2,25} = 4.88$, $p < 0.025$), and subcortical lesion size ($F_{2,25} = 7.34$, $p < 0.01$) (Table 2). Post-hoc analyses revealed that the Sham group had significantly greater lesion size for all three measurements than did the other two groups (Fig. 5). The 17α-E2 and 17α-E2 (1:2) groups did not differ from each other however. Thus suggesting that steroid treatment, regardless of the doses utilized in this study, significantly protects against the resulting neuronal ischemic damage induced by unilateral occlusion of the MCA.

Insert Table 2 and Figure 5 about here

Discussion

The present study was conducted to determine the effects of 17α-E2 on both cerebral circulation and neuroprotection against ischemic infarct. We determined that subcutaneous pellet administration of 17α-E2 24 hours prior to MCAO did not significantly alter CBF as compared to control animals. All animals in the three groups exhibited similar baseline, MCAO, Recovery, and Next Day laser doppler BPU readings. These findings did not support our

original hypothesis that 17α-E2 would be able to normalize the reperfusion of cerebral blood flow and stabilize cerebral circulation 24 hours following the MCAO procedure. In fact, no differences in CBF could be detected for either the left (ischemic) or right (non-ischemic) hemispheres. In fact, during reperfusion (i.e., Recovery phase on all graphs) the left CBF levels only increased to 83.1%, 72.7%, and 77.5% of baseline for 17α-E2, 17β-E2, and Sham groups, respectively. Furthermore, during the Next Day readings 24 hours following MCAO, CBF levels remained at 68.9%, 68.4%, and 57.3% of baseline readings, respectively (see Fig. 3). While estrogens have effects on the peripheral system such as increasing cardiac output and uterine blood flow as well as decreasing systemic vascular resistance[38], 17 α-E2 appeared to be without major effects on cerebral blood flow before or during occlusion. The recovery of blood flow following re-perfusion may be related to the marked neuroprotective effects observed. We have reported that 17 β-E2 protects cerebral endothelial cells from ischemia *in vivo* and from glucose and/or oxygen deprivation *in vitro*[32]. The recovery of blood flow during re-perfusion may be a result of the maintenance of the integrity of the cerebral vasculature during and following the ischemic event. Thus, the beneficial effects of estrogen replacement therapy for cardiovascular health[6,7,10,11,12,13,14,39] as well as for cognitive function[19,20,40,41,42,43,44,45] may have a predominate cytoprotective component.

It could be hypothesized that significant differences were not found among groups due to the fact that subcutaneous administration of 17α-E2 was not as effective as direct delivery into the central nervous system. However, this is unlikely considering that we have shown in the past that subcutaneous implantation of both 17α-E2 and 17β-E2 produces the same degree of neuroprotection from the effects of MCAO than does a large dose 17β-E2 delivered directly across the blood brain barrier via a chemical delivery system[24].

Although changes in cerebral microcirculation as related to 17 α-E2 treatment were not seen in the present study, the neuroprotective effects of 17α-E2 were highly significant. In fact, 17α-E2 and 17α-E2 (1:2) both significantly reduced MCAO-induced lesion size as compared with Sham-treated control animals. The effects of steroid treatment were profound and can be seen in the total lesion size for each group: 9.4% (17α-E2), 9.2% [17α-E2(1:2)], and 20.3% (Sham). Interestingly, 17α-E2 and 17α-E2 (1:2) did not differ with respect to their neuroprotective abilities, thus suggesting that the reductions in lesion size were not dose-dependent as was originally hypothesized. As can be seen in Fig. 5, the most beneficial effects of subcutaneous pretreatment with 17α-E2 was in reducing ischemic damage to the cortex. Although neuroprotective effects were also seen within the subcortical structures, the cortex region was virtually undamaged in animals treated with the steroid. Table 2 shows the differences between the three groups with regard to their total, cortical, and subcortical infarct percentages. While the mechanism for this effect is unknown, we have previously hypothesized on the possibilities[15,16,17,18,19,20,24,45], which include estrogen's interactions with mechanisms influencing Ca^{2+} influx or its effects as related to reductions in oxidative stress following ischemia and reperfusion. The present finding that 17α-E2 demonstrates neuroprotective effects further supports similar results from our laboratory[17,18,18,20,45] and adds to a body of literature suggesting the beneficial effects of this "weak" estrogen.

In conclusion, we have found that pretreatment with 17α-E2 attenuates the focal ischemic damage seen in OVX rats following transient MCAO. The neuroprotective effects of this steroid do not appear to be dependent on the potency of the estrogen since doses of straight 17α-E2 and the 1:2 17α-E2:cholesterol mixture both significantly reduced the neuronal damage associated with the ischemic episode. Although 24 hour pretreatment with 17α-E2 did not appear to have a

modulating effect on cerebral blood flow, the possibility that estrogens affect central blood flow dynamics in a manner undetected by these procedures can not be ruled out. Thus, we have shown that 17α-E2 is a neuroprotective agent and may be a clinically relevant compound in the prevention and/or treatment of stroke.

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Figure Legends

Figure 1. Changes in Cerebral Blood Flow in the Left (Ischemic) Hemisphere. Laser Doppler measurements of CBF prior to, during, and following MCAO for all groups, as measured in BPU's (Mean ± SEM). Laser probe was positioned over the left parietal cortex. "S" indicates measurements taken during MCAO stroke and "R" represents Recovery time points. * $p < 0.01$ versus 17 α-E2 (1:2).

Figure 2. Changes in Cerebral Blood Flow in the Right (Non-Ischemic) Hemisphere. Laser Doppler assessment of CBF, across treatment groups, prior to, during, and following MCAO, as measured in BPU's (Mean ± SEM). Laser probe was positioned over the right parietal cortex. "S" indicates measurements taken during MCAO stroke and "R" represents Recovery time points. * $p < 0.05$ versus 17 α-E2.

Figure 3. Changes in Left (Ischemic) Hemisphere Cerebral Blood Flow as a Percentage from Baseline. Mean (± SEM) group percentage during MCAO, Recovery, and Next Day readings. Laser Doppler recordings were taken from left parietal cortex. * denote that each group's Recovery and Next Day readings significantly differed from MCAO (stroke) readings, but the groups themselves did not differ.

Figure 4. Changes in Contralateral Cerebral Blood Flow as a Percentage from Baseline. Mean (± SEM) group percentage during MCAO, Recovery, and Next Day. Laser Doppler readings were taken from the right (non-ischemic) parietal cortex. No significant effects were found among groups or CBF recording times.

Figure 5. Effects of 17α-Estradiol Treatment on Lesion Size Following MCAO. Percentage of ischemia in total, cortical, and subcortical regions. Each group's mean (± SEM) is presented. * $p < 0.025$, * * $p < 0.01$ versus Sham controls.

Table 1. Venous Plasma pH, Ions, and Gases for All Groups Prior to and Immediately Following MCAO.

PRE-MCAO:

Group	pH	pCO_2	pO_2	Na^+	K^+	Ca^{++}
Sham	7.34 (0.01)	59.03 (1.10)	43.19 (1.36)	147.54 (1.93)	7.78 (0.38)	3.38 (0.12)
17α-E2 (1:2)	7.33 (0.01)	60.99 (3.28)	42.51 (1.30)	146.98 (1.36)	7.10 (0.45)	3.15 (0.17)
17α-E2	7.33 (0.02)	59.35 (3.70)	43.76 (2.19)	145.70 (2.11)	7.48 (0.38)	3.21 (0.23)

POST-MCAO:

Group	pH	pCO_2	pO_2	Na^+	K^+	Ca^{++}
Sham	7.20 (0.01)	68.40* (1.79)	38.71 (1.64)	146.71 (1.12)	6.88* (0.43)	3.58* (0.14)
17α-E2 (1:2)	7.29 (0.01)	71.25† (2.76)	40.68 (3.25)	148.51 (0.75)	5.62† (0.61)	3.93† (0.20)
17α-E2	7.29 (0.01)	66.96 (3.42)	40.01 (2.95)	148.06 (0.73)	5.91‡ (0.56)	4.01‡ (0.35)

All data presented as group means (± SEM)

*, †, ‡ denote paired t-test significance within a group for pre- versus post-MCAO

Table 2. Total, Cortical, and Subcortical Lesion Percentages (mean ± SEM) for All Groups 24 hours Following MCAO.

Group	Total %	Cortical %	Subcortical %
Sham	20.28 (9.51)	9.57 (6.35)	10.67 (3.42)
17α-E2 (1:2)	9.18* (4.79)	3.10† (3.21)	6.08* (2.27)
17α-E2	9.37* (6.0)	3.69† (4.57)	5.68* (3.28)

* $p < 0.01$ and † $p < 0.025$ and denote significant differences from Sham animals

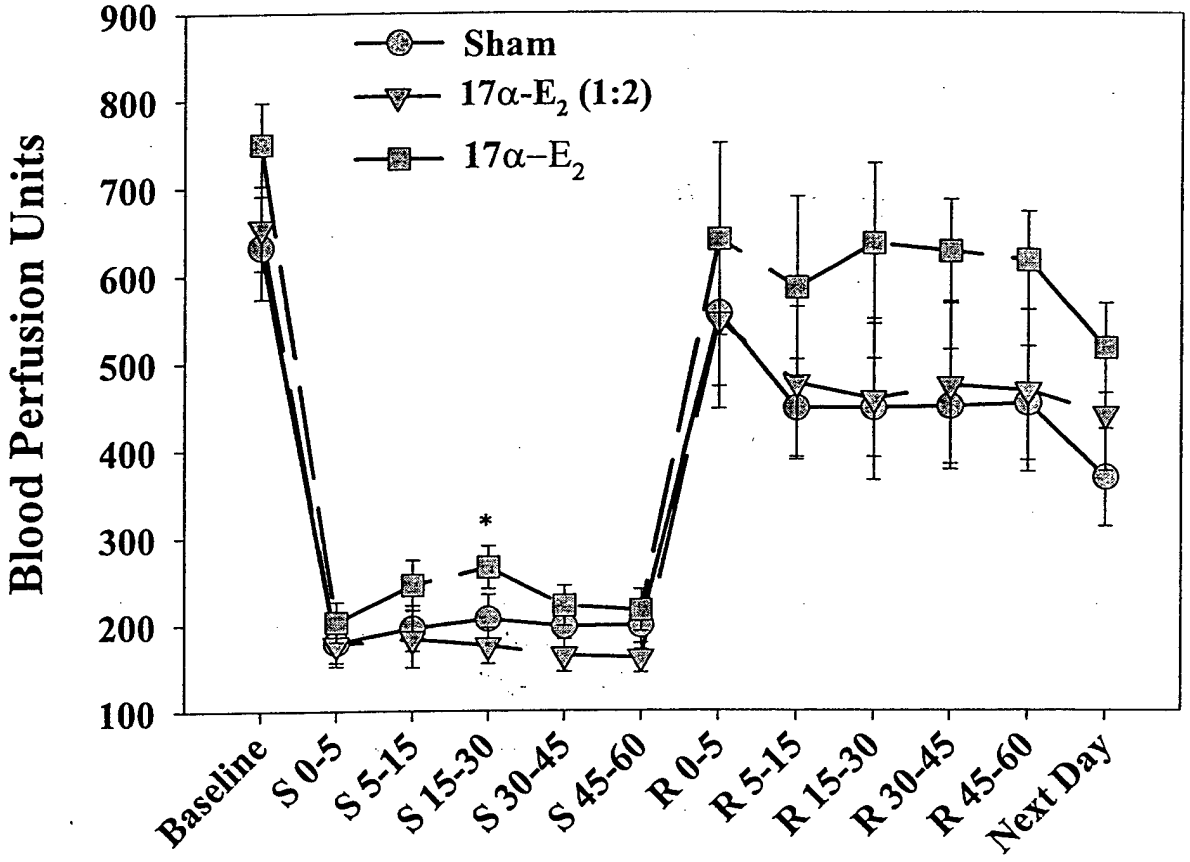


Figure 1: Stubley et al

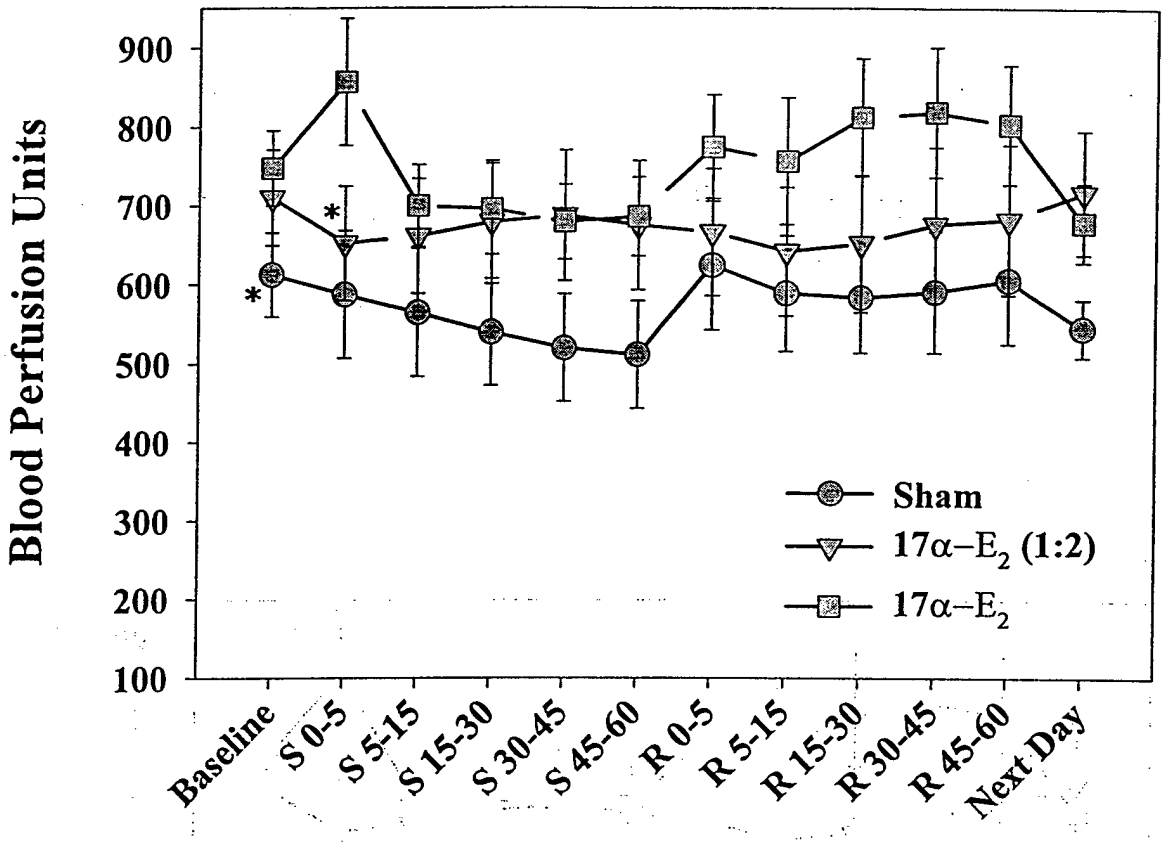


Figure 2: Stubley et al

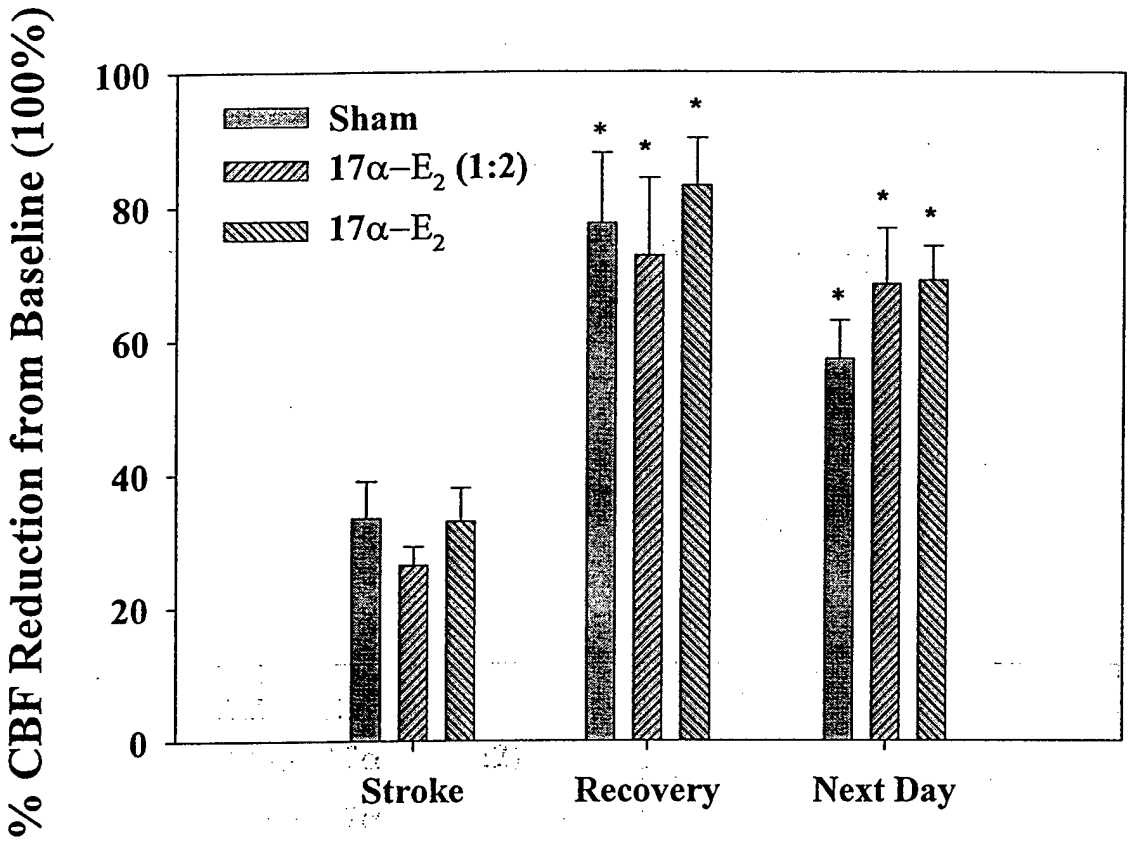


Figure 3: Stubley et al

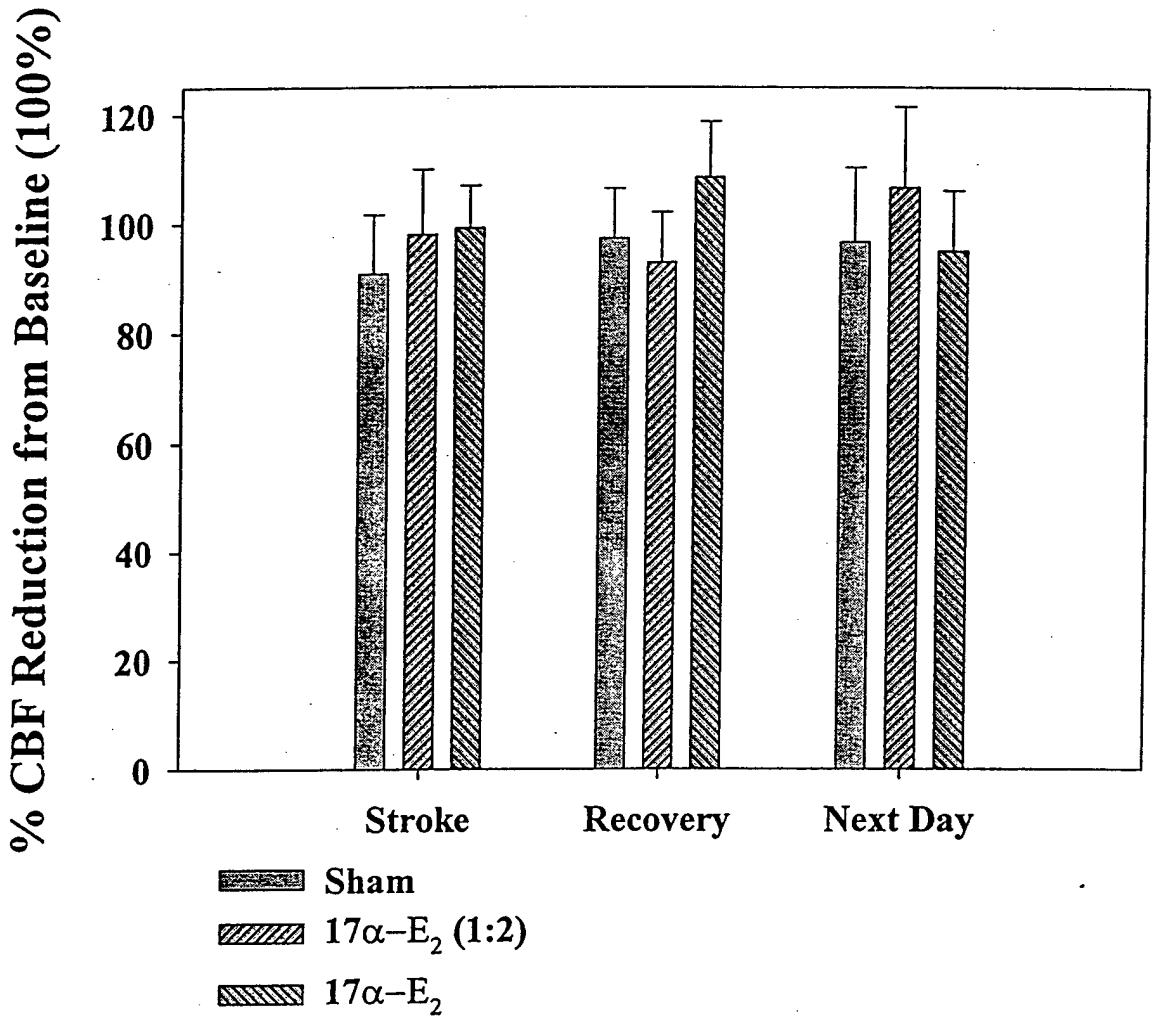


Figure 4: Stubley et al

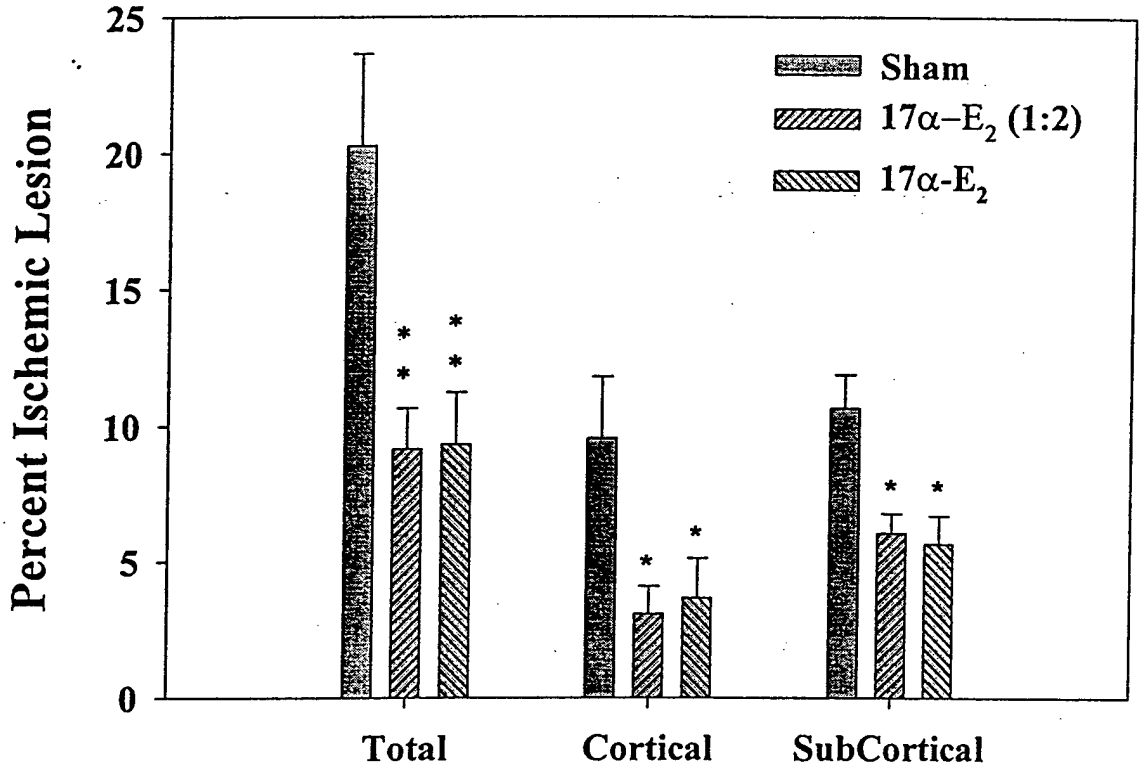


Figure 5: Stubley et al

17-β Estradiol Can Reduce Secondary Ischemic Damage and Mortality of Subarachnoid Hemorrhage

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Abstract

Subarachnoid hemorrhage (SAH) is a unique disorder commonly occurring when an aneurysm ruptures, leading to bleeding and clot formation, with higher incidence in female. To evaluate the influence of 17-β estradiol (E2) in the outcome of SAH, SAH was induced by endovascular puncture of the intracranial segment of internal carotid artery in 15 intact female (INT), 19 ovariecomized female (OVX) and 13 ovariecomized female rats with E2 replacement (OVX+E2). Cerebral blood flow was recorded before and after SAH. All the animals were decapitated immediately after death or 24 hours after SAH for clot volume analysis. The brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for secondary ischemic lesion analysis. The CBF decreased to 29.6±17.7%, 22.8±8.3% and 43.5±22.9% on the ipsilateral side ($p=0.01$) and to 63.4±14.1%, 57.4±11.0% and 66.6±17.9% on the contralateral side ($p=0.26$) in INT, OVX and OVX+E2, respectively. The mortality was 53.3%, 68.4% and 15.4% in INT, OVX and OVX+E2, respectively ($p=0.01$), while no significant difference in clot volume was noted among groups. The secondary ischemic lesion volume was 9.3±8.4%, 24.3±16.3% and 7.0±6.4% in INT, OVX and OVX+E2, respectively ($p<0.01$). This study demonstrated that E2 can reduce the mortality and secondary ischemic damage in a SAH model without affecting the clot volume.

Key words: estrogens, subarachnoid hemorrhage, ischemia, neuroprotection.

Running title: Estrogen' s Neuroprotection in SAH.

Introduction:

Stroke is the third most common cause of death in the adult population in the United States, following ischemic heart disease and all forms of cancer (Camarara et al., 1994). Subarachnoid hemorrhage (SAH) accounts for about 10% of all strokes (Selman et al., 1999). However, SAH affects a younger population and results in death in more than 50% of subjects, most of whom died within the first 24 hours. SAH accounts for more premature mortality than ischemic stroke (Broderick et al., 1994; Zhang et al., 1998). SAH can result in vascular changes such as acute vasospasm and intracranial hypertension, which lead to decrease of cerebral perfusion pressure (CPP) and cerebral blood flow (CBF). All of these can contribute to secondary ischemic damage after SAH. Histological studies of brains of patients who died shortly after SAH show extensive ischemic damage, and secondary ischemia has been report to be one of the major cause of death shortly after SAH (Adams et al., 1981).

Unlike other kind of strokes, aneurysmal SAH occurs more frequently in women than in men (Davis 1994). Sex differences in outcome of SAH are controversial, and the influence of female sex hormone is unclear (Kongable et al., 1996; Simpson et al., 1991; Johnston et al., 1998). On the other hand, estrogens have been found to exert neuroprotective effect in models of ischemic stroke both *in vitro* an *in vivo* (Gridley et al., 1997; Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998;). Whether estrogens exert similar protective effects in SAH as is observed in ischemia is currently unknown. The purpose of this study was to determine whether 17β-estradiol (E2) influence the outcome of SAH and if so whether the influence relates to the ischemia associated with SAH.

Materials and methods

Preparation of Animals

Female Charles Rivers Sprague-Dawley rats (250g, Wilmington, MA) were maintained in laboratory acclimatization for three days prior to ovariectomy. Bilateral ovariectomy was performed two weeks before SAH under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

E2 administration and serum concentration

To obtain a sustained stable elevation in serum E2 concentration, implantation of a Silastic® pellet containing the steroid were utilized. To assess serum concentrations of E2 following this treatment regimen, OVX animals were anesthetized with methoxyflurane inhalant and a control blood sample was taken via the jugular vein. Then a 30mm long Silastic® tube (1.57 mm ID; 3.18 mm OD) containing E2 (4mg/ml in corn oil) was implanted subcutaneously in 5 OVX animals. The animals were returned to their cages and blood samples were then taken via the jugular vein at 24 and 48 hours following steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood cells by centrifugation and stored frozen (-20°C). Serum E2 concentrations were determined using duplicate serum aliquots in a radioimmunoassay (Diagnostic Lab, ultrasensitive estradiol kit).

Endovascular subarachnoid hemorrhage model:

Animals were anesthetized by intraperitoneal injection of ketamine (60mg/kg) and xylazine (10mg/kg). Rectal temperature was monitored and maintained between 36.5

and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline cervical skin incision. A 3-0 monofilament suture with a blunt tip was introduced into the ICA via the ECA lumen, and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was about 1.8 cm. The suture was advanced further for 5 mm, and then withdrawn immediately. The CCA and ICA were coagulated and the skin incision was closed.

Measurement of regional cerebral blood flow

A Laser Doppler Flowmeter (LDF) was used for CBF measurements. The scalp as incised on the midline, and bilateral 2-mm burr holes were drilled 1.5mm posterior and 4.0mm lateral to the bregma. The dura was left intact to prevent cerebral spinal fluid leakage. LDF probes were held in place by a micromanipulator were stereotactically advanced to gently touch the intact dura mater. The lower stable readings were obtained and recorded for at least 10 minutes from both sides (baselines measurement) (Dubal et al., 1998; Cholet et al., 1997). For each animal, the lower CBF reading was recorded at the same sites within ½ hour after SAH. The CBF values were calculated and expressed as a percentage of the baseline values. CBF values reported represent the mean ± SD for the average of the CBF recordings obtained.

Measurement of clot and lesion volume

Each group of animals was decapitated immediately after death or 24 hours after SAH, the brain was removed and the base of the brain was photographed for the measurement of the clot volume. Then the brain was placed in a metallic brain matrix for tissue slicing (Harvard). 2-mm sections were made beginning at 3, 5, 7, 9, 11 and 13mm

posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37 ° C, and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). The clot volume was calculated as the percentage if the base of the brain covered by clot. Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the six slices divided by the total cross sectional area of these six brain slices.

Experimental protocol:

47 animals were placed into three groups: 15 intact females (INT), 19 ovariectomized females (OVX) and 13 ovariectomized females with estrogen replacement (OVX+E2), respectively. In OVX+E2 animal, a 30mm long Silastic® tube containing E2 (4 mg/ml oil) was implanted subcutaneously 24 hours before SAH under methoxyflurane inhalant anesthesia. INT and OVX animals received a Silastic® tube containing oil as a control.

24 hours later, after a baseline CBF reading was obtained, SAH was induced in each animal. CBF was recorded bilaterally at the same sites within ½ hour after SAH. Then the animals were returned to her home cage under careful observation. Each animal was decapitated for clot and ischemic volume analysis immediately after death or 24 hours after SAH.

Statistic study

Statistical analyses were performed using SAS Software (SAS Institute, Cary, NC). Paired t-tests were performed to check the serum concentration of E2 treatment. We

compared the clot volumes, lesion volumes and CBF from the three groups. For each comparison p-values from one-way ANOVA were provided. Chi-square test was used to compare the mortality rate among the three groups.

Results

E2 administration and serum E2 concentration:

Implantation of a 30mm E2 (4mg/ml corn oil) pellet maintained serum E2 concentrations stable for at lest 48 hours after administration. E2 concentration was 31.3±11.5 pg/ml and 24.9±6.6 pg/ml at 24 and 48 hours after administration, respectively, compared with 3.5±1.2 pg/ml in OVX animals ($p<0.01$) (Fig. 1).

Effect of E2 on SAH mortality:

Most of the animals began to recover from the anesthetic at about 3 hours after SAH. The mortality was 53.3%, 68.4% and 15.4% in INT, OVX and OVX+E2 group, respectively ($p=0.01$). In INT group, 3 died within 6 hours after SAH and 5 died between 6 to 24 hours after SAH. In OVX group, 4 died within 6 hours and 9 died between 6 to 24 hours after SAH. In OVX+E2 group, 1 each died within 6 hours and between 6 to 24 hours after SAH, respectively (Table 1).

Effect of E2 on clot volume and secondary ischemic lesion volume:

Clotted and unclotted blood was found around the circle of Willis, distributed to both sides of the brain with the majority on the ipsilateral side. Blood was also found as a thin layer overlying the both sides of the cortex (Fig. 2). No significant difference of clot volume was noted among INT, OVX and OVX+E2 group, which was 6.6±5.0%, 5.9±4.5% and 7.6±3.2%, respectively, of the brain base surface ($p=0.56$) (Fig. 3).

The secondary ischemic lesion was confined in the ipsilateral somatosensory cortex and basal ganglia in most of the animals. Contralateral somatosensory cortex was also involved in 8 of the 19 OVX (42.1%), 2 of the 15 INT (13.3%) and 3 of the 13 OVX+E2 animals (23.1%), respectively. The secondary ischemic lesion volume was 9.3±8.4%, 24.3±16.3% and 7.0±6.4% in INT, OVX and OVX+E2 group, respectively ($p<0.01$) (Fig. 4).

Effect of E2 on CBF after SAH:

The ipsilateral cortex CBF was reduced to 29.6±17.7%, 22.8±8.3% and 43.5±22.9% of the baseline in INT, OVX and OVX+E2, respectively ($p=0.01$) (Fig. 5). The contralateral cortex CBF was reduced to 63.4±14.1%, 57.4±11.0% and 66.6±17.9% of baseline in INT, OVX and OVX+E2, respectively ($p=0.26$) (Fig. 5).

Discussion

Subarachnoid hemorrhage (SAH) is a unique disorder and a major clinical problem that commonly occurs when an aneurysm in a cerebral artery ruptures, leading to bleeding and clot formation, and early morbidity and mortality. The incidence of SAH in females is higher than in males. But the influence of female sex steroid on SAH outcome remained controversial. This study evaluated the influence of female sex steroid on SAH outcome and for the first time demonstrated that 17β-estradiol (E2), which is the most active natural estrogen, can reduce the mortality and secondary ischemic damage in a SAH model.

Several studies have demonstrated that E2 exerts neuroprotective effects in ischemic stroke (Simpkins et al., 1997; Dubal et al., 1998; Toung et al., 1998). But the effect of E2 on secondary ischemic damage of SAH, which has been demonstrated both

in clinic and experimental studies and is one of the major reasons of death shortly after SAH, is unknown. The secondary ischemic damage can be caused by severe CBF reduction immediately after SAH, and / or processes involving vasospasm and edema leading to the reduction of CBF (Warnell, 1996).

The vasospasm caused by hemorrhage is directly responsible for the decrease in CBF, which occurred acutely after SAH in humans and experimental animals (Bederson et al., 1998; Fukuda et al., 1998). Studies have shown through angiographic methods biphasic response of vasospasm after SAH, with the first vasospasm taking place immediately after the bleeding and the second occurring days later (Taneda et al., 1990; Brawley et al., 1968). The reduction of CBF is closely related to the present of blood and blood breakdown products within the perivascular spaces, acting either directly upon the cerebral vessel wall or perhaps more indirectly via perivascular nerves and central brain stem afferent connections to produce an acute vasospasm (Jackowski et al., 1990). Clinically, the primary contributing cause of death and disability is the initial hemorrhage, while delayed arterial vasospasm plays a very minor role in mortality caused by SAH (Broderick et al., 1994, Proust et al., 1995). On the other hand, profound elevations of intracranial pressure (ICP) immediately after SAH, with attendant reduction in CPP, could be another important factor for the reduction of CBF.

Our study showed that CBF was reduced to around 20% and 50% in the ipsilateral and contralateral side respectively immediately after SAH. An immediately 50% CBF reduction was found after autologous blood was injected into subarachnoid space through the cistern magna, which last for several hours (Jackowski et al., 1990). The profound reduction of CBF in our study could attribute to the injury to the artery in our model,

which is very importance in the pathophysiology of SAH. Our model which uses focal puncture of the native internal carotid artery, results in extensive distribution of blood throughout the subarachnoid space. This blood distribution is more similar to that observed with SAH in human subject and make the studies of acute pathophysiological changes of SAH such as secondary ischemic damage more clinically relevant (Veeklken et al., 1995; Bederson et al., 1995; Matz et al., 1996). Diffusion MR imaging study in this SAH model has showed that the acute secondary ischemic damage is confined primarily to the ipsilateral somatosensory cortex and basal ganglia, and involved the contralateral somatosensory cortex in some of the cases, which were consistence to our secondary ischemia result (Busch et al., 1998). The asymmetric ischemic damage could have resulted from the asymmetric clot distribution in this model. It is clear that there is a direct relationship between the location of the thickest blood clots and location of the most severe vasospasm (Camarara et al., 1994).

Deprivation of ovarian steroids, which result from ovariectomy, increased secondary ischemic damage in the present study. In contrast, replacement of E2 in OVX animals decreased the secondary ischemic damage to the level below that of normal females. This study demonstrated that E2 exerted the similar neuroprotective effects in secondary ischemic damage of SAH as we and others have reported for ischemic stroke (Simpkins et al., 1997; Dubal et al., 1998; Toung et al., 1998). The neuroprotective effects of endogenous ovarian steroids or exogenous estradiol on secondary ischemic damage are of the same magnitude as is reported for primary ischemia. Thus, it appears that both endogenous and exogenous E2 exert neuroprotective effects in secondary

ischemic damage of SAH in a manner that is not associated with changes of clot volume in SAH.

The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia (Gridley et al., 1997; Hall et al., 1991), blockade of excitatory amino acid toxicity (Weaver et al., 1997; Singer et al., 1996), modulation of calcium homeostasis (Chen et al., 1998; Collins et al., 1993; Mermelstein et al., 1996; Collins et al., 1996), induction anti-apoptotic protein (Singer, et al., 1998; Pike, 1999), and / or enhancement of brain glucose uptake (Shi et al., 1997; Alvarez et al., 1997). E2 could protect from secondary ischemic damage through the similar mechanisms as in ischemic stroke. However other mechanisms could be operative. Estrogen has been reported to exert both neuroprotective and flow-preserving effects (Toung et al., 1998), but the ischemic protective effects of estrogens seem independent of flow-preserving effect (Dubal et al., 1998; Wang et al., 1999). Our result suggest that estrogens protective effect on ischemia is flow independent, because the secondary ischemia lesion volumes were also significant reduced in INT group even the residue CBF was at same level as that in OVX group.

In our study, residual CBF on the ipsilateral side of SAH in E2 replacement group remained higher than that in OVX and INT group immediately after SAH, while no significant difference was noted on the contralateral side among groups. The flow preserving effect could relate to estrogens' effect on vasospasm. Nitric oxide (NO) has been demonstrated to be related to the vasospasm after SAH (Sayama et al., 1999; Sobey

et al., 1998), and estrogen appears to alter mygenic tone by increasing cerebrovascular NO production and / or action (Geary et al., 1998). Estrogen can also causes the rapid dilation of blood vessels by stimulating endothelial nitric oxide syntheses (eNOS) (Sahul, 1999). Our result demonstrated that the anti-vasospasm effect could only induce by exogenous estrogen. One explanation for the lack of anti-vasospasm effect in endogenous steroids is the present of progestins in intact rats. However, the observation that secondary ischemia is similarly reduced in both INT and OVX+E2 rats suggest that progestins are not influencing the anti-vasospasm effect through a blockade of the estrogen effect on ischemia.

Although both endogenous female steroid and exogenous E2 reduce secondary ischemic damage in SAH, endogenous female steroids (INT group) was not associate with a significant reduction of mortality, while E2 replacement markedly reduced mortality. The observation of effect of endogenous ovarian steroids on morality is consistent to the clinic studies of the outcome of SAH. Kongable' s study showed that the SAH outcome of women and men is the same even women were older and harbor more aneurysms (Kongable et al. 1996). Simpson found that man have a high risk of unfavorable outcome following SAH (Simpson et al. 1991). In contrast to above studies, Johnston et al. (1998) found the mortality of SAH was 62% greater in females than in males. Overall, sex differences in the clinical outcome of SAH are unresolved. The different effect of exogenous estrogen and endogenous female steroids on CBF, which result from vasospasm after SAH, could attribute to the different mortality between INT and OVX+E2 group. Not only secondary ischemia, but also hydrocephalus, which occurs in 20% of patients with SAH and associated with additional morbidity and mortality, has

been reported to associate to vasospasm after SAH (Black 1986; Suarez-Rivera 1998). Further studies of ovarian steroids and their interaction in acute vasospasm and vasospasm related hydrocephalus could help to explain the difference of endogenous female steroid and exogenous E2 replacement in mortality after SAH.

In summary, our study demonstrated that E2 could reduce the secondary ischemic damage and mortality of SAH by exerting neuroprotective effects. These effects are not associated with the change of the clot volume in SAH.

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Table 1. Effects of ovarian steroids environment on mortality and time of death in rats subjected to SAH.

Group	Mortality	Died within 6 hours	Died between 6-24 hours
INT	53.3%	3 (20.0%)	5 (33.3%)
OVX	68.4%	4 (21.0%)	9 (47.4%)
OVX+E2	15.4%	1 (7.7%)	1 (7.7%)

INT: intact female n=15; OVX: ovariectomized female n=19;

OVX+E2: ovariectomized female with E2 replacement n=13.

OVX vs OVX+E2 p=0.01; INT vs OVX+E2 p=0.09; INT vs OVX p=0.59

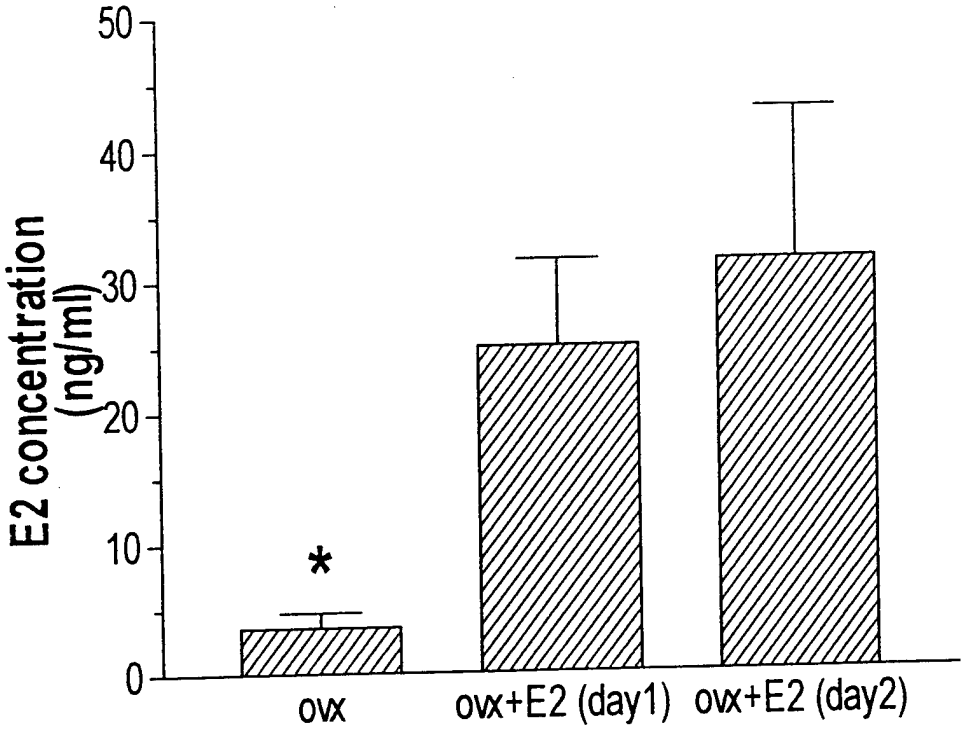


Figure1. Serum E2 concentrations after treatment (n=5). E2 administration: subcutaneous implantation of a 30 mm long Silastic Pellet filled with E2 oil (4mg/ml). * OVX vs OVX+E2 (Day 1) p=0.0002; OVX vs OVX+E2 (Day 2) p=0.0028.

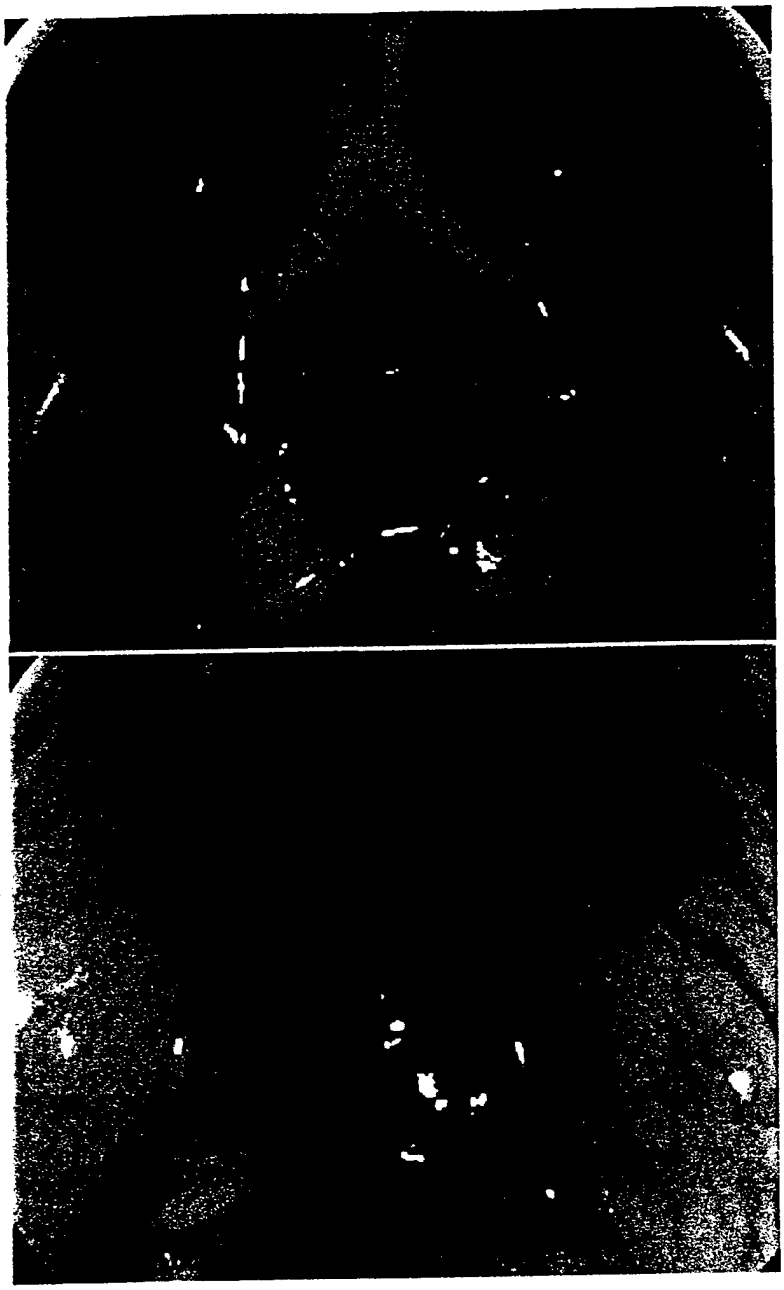


Figure 2. Picture depicted sham (upper) and the blood distribution in SAH (lower). Cloted and uncloted blood distributes to both sides of the brain with the majority on the ipsilateral side. Blood was also found as a thin layer overlaying the both sides of the cortex

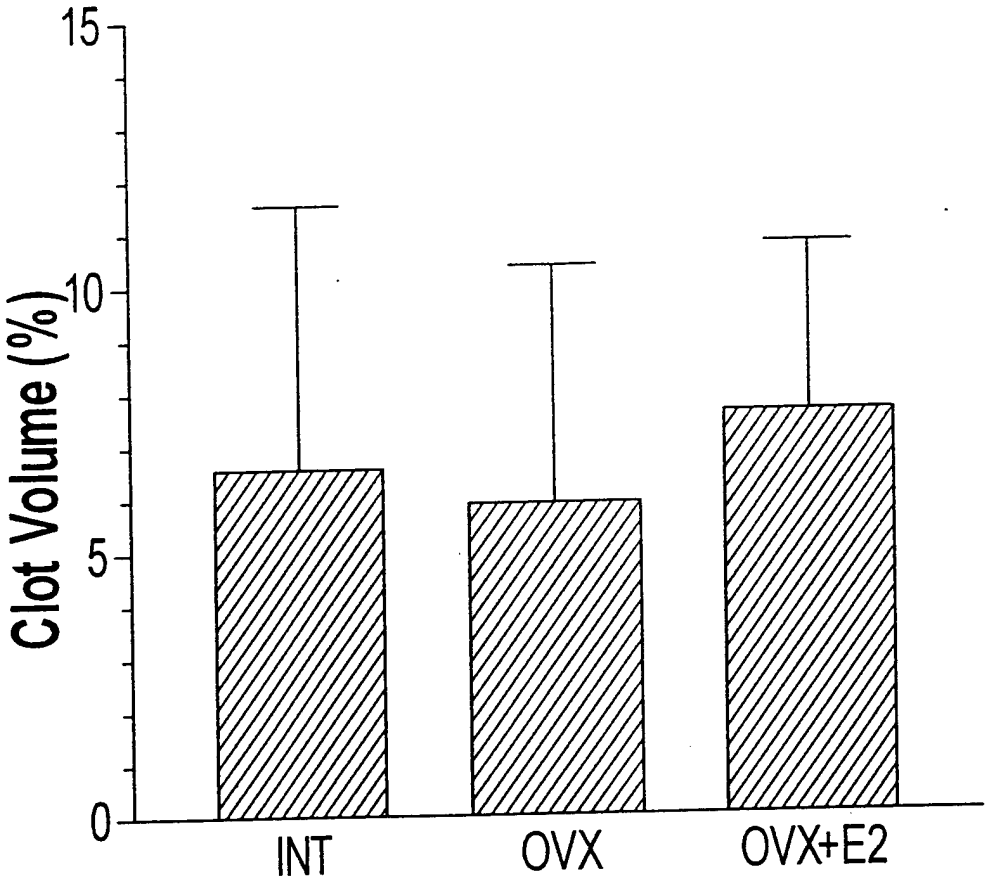


Figure 3. Clot volumes in INT (n=15), OVX (n=19) and OVX+E2 (n=13). No significant difference was noted among each group (p=0.5617).

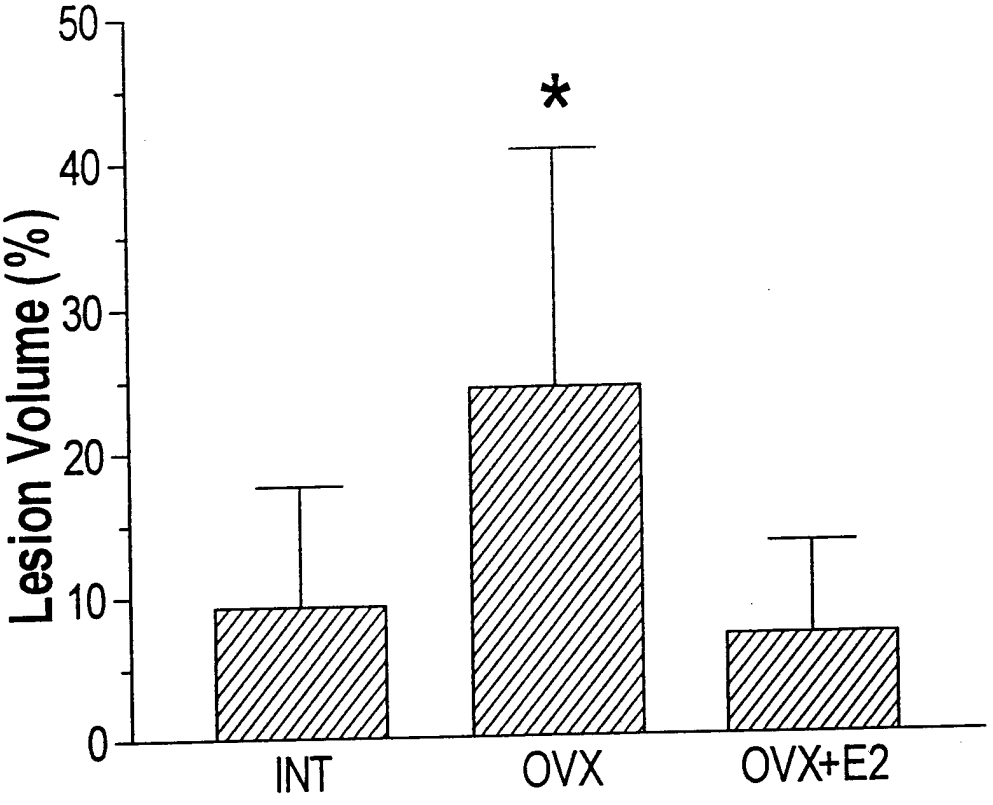


Figure 4. Secondary ischemic volumes in INT (n=15), OVX (n=19) and OVX+E2 (n=13). Lesion volume of OVX was significant larger than that of INT and OVX+E2 (p=0.0003).

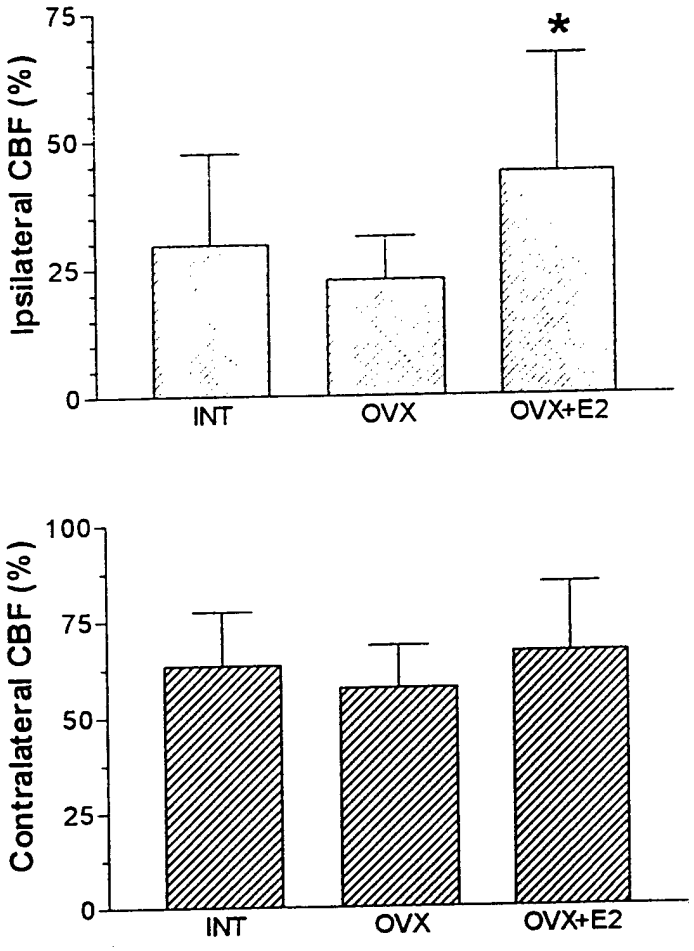


Figure 5. CBF in both side of INT (n=15), OVX (n=19) and OVX+E2 (n=13). In the left side (ipsilateral), CBF of OVX+E2 was significant higher than that of OVX and INT (p=0.0119). In the right side (contralateral), no significant difference was noted among each group (p=0.2624).

DAMA17-99-1-9473
Appendix K
J. W. Simpkins

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Read all instructions before typing abstract.
See *Call for Abstracts* and reverse of this sheet.
Complete abstract and **all boxes at left and below.**
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MANDATORY: The present scientific work may involve a real or perceived financial conflict of interest. (See page 4, item 11.)
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Check one: ❑ poster only ☒ slide/poster

Themes and Topics

See list of themes and topics, pp. 17–18. Indicate below a first and second choice appropriate for programming and publishing your paper.

1st theme title: Disorder of the Nervous System and Aging theme letter: J
1st topic title: Ischemia: Neuroprotection and Tolerance topic number: 134

2nd theme title: Disorder of the Nervous System and Aging theme letter: J
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Special Requests (for example, projection, video, or computer requirements)

Include nonrefundable **abstract handling fee** of **$50.** Fill out payment information form below. Purchase orders will not be accepted. Submission of abstract handling fee does not include registration for the Annual Meeting.

PHYSIOLOGICAL ESTRADIOL DELAYS NEURON DEATH FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA IN RATS. Z. He*, S.H. Yang, Y.J. He, J. Cutright, A.L. Day, J.W. Simpkins. Dept. Pharmacodynamics and Neurosurgery, Center of the Neurobiology of Aging, Univ. of Florida, Gainesville, FL 32610

Hippocampal neurons undergo a delayed death over days following transient global ischemia; and understanding the time-course of this phenomenon is necessary to determine whether a therapeutic intervention provides any effective protection. We conducted a study to see whether physiological levels of estradiol would permanently salvage hippocampal neurons in rats subjected to a global ischemic insult. Ovariectomized female rats were subjected to 20 minutes of ischemia using a four-vessel occlusion technique, and then allowed to survive for 48 or 168 hours. Estradiol was administered subcutaneously by implanting a Silastic® pellet containing the steroid (4mg/ml) 24 hours before the ischemia onset. In both groups, hippocampal blood flow was decreased 54-62% from resting levels (41-48 ml/min/100g) at 10 minutes after ischemia, and recovered to 77-91% and 95-126% of resting levels at 10 and 30 minutes after reperfusion, respectively. Serum 17β-estradiol levels in estradiol-treated rats were 92±12 pg/ml at 48 hours and 37±7 pg/ml at 168 hours ($p<0.05$ vs. 48-hour group) after ischemia, and were significantly higher than their corresponding controls. Live cell counts in the hippocampal CA1 subregion were significantly different between the estradiol- and the vehicle-treated groups at 48 hours (25±1% vs. 44±3% cell number reduction, $p = 0.0019$) but not at 168 hours (47±5% vs. 58±4% cell number reduction) following ischemia. Sustained physiological levels of estradiol appear to delay but not prevent hippocampal damage following transient global ischemia. The significant difference between the 48-hour and 168-hour live cell counts may be dependent on serum estradiol concentration, and supra-physiologic levels may be required to produce any sustained neuronal protective effects.

(Supported by NIH grant AG 10485, Apollo BioPharmaceutics, Inc, and U.S. army grant DAMD 17-99-1-9473)

Key Words: (see instructions p. 4)

1. Ischemia
2. CA1
3. Estradiol
4. Neuron Death

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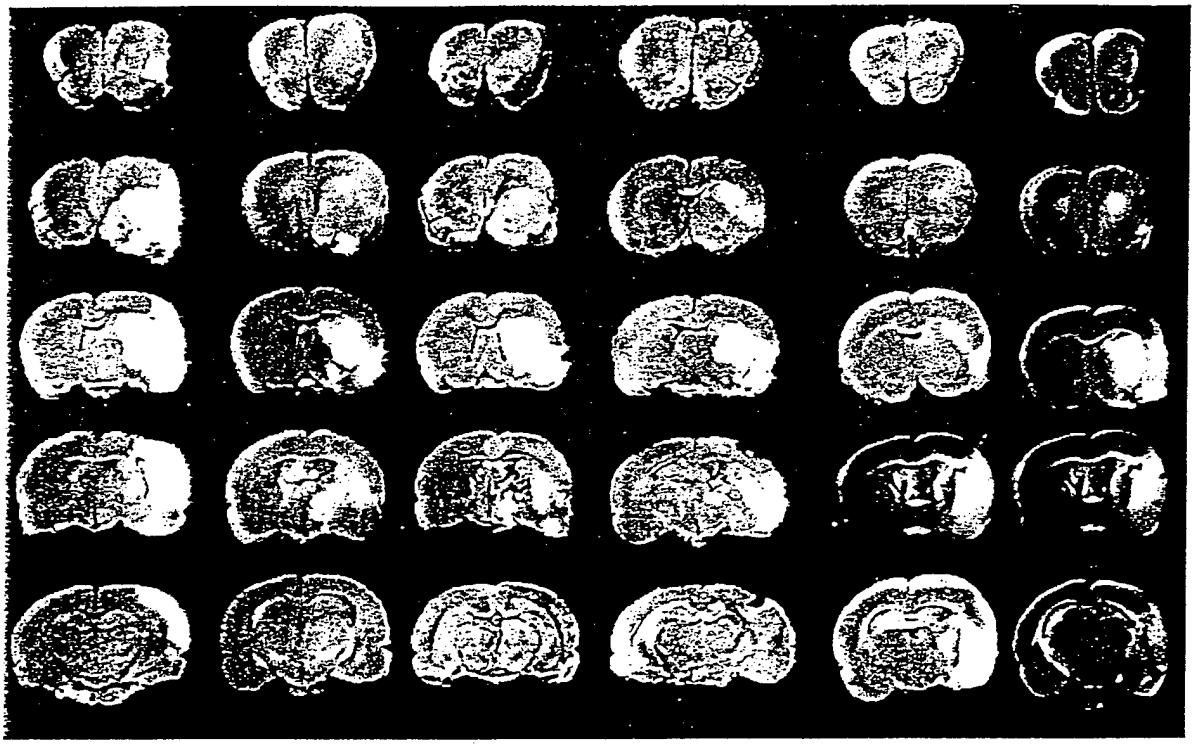
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DAMA17-99-1-9473
Appendix L
J. W. Simpkins

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Stroke

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Estrogen Neuroprotective Effects Time Limited

■ **Original Contributions**
Racial Variation in Initial Stroke Severity
Stress Reduction and Carotid Atherosclerosis in Blacks
Sex Differences in Predictors of Carotid Atherosclerosis
Stroke Management Patterns and Costs
Increased Platelet Sensitivity in Aspirin Nonresponders
Edema in Thrombolysis-Related Hemorrhage
Abciximab in Acute Ischemic Stroke
tPA and TCD for Ischemic Stroke
Carotid Plaque Surface Morphology and Stroke Risk
Low Stroke Risk With Poststenotic Narrowing of the ICA
Internal Borderzone Infarction and Carotid Stenosis
Functional Neuroanatomic Correlations in Poststroke Depression
Neurobiochemical Markers and Neuropsychological Outcome After Cardiac Surgery
Severity of Infarcts and Case Fatality of Stroke
Evolution of fMRI Activation During Stroke Recovery
Functional Extremity Movement Measured by Accelerometry
Somatotopic Mapping After Cortical Infarct
Relating MRI Changes to Motor Deficit After Stroke
MR Perfusion Imaging in Acute Stroke
Effect of Multiple Infarction on Diffusion MRI
MRI Pontine Hyperintensity and Stroke Outcome
TCD and Types of Carotid-Cavernous Fistula
Cerebral Microemboli and Duration of Cardiac Surgery
Intracranial Artery TCD Abnormalities and Clinical Classification
Endothelin-1 in Ischemic Stroke
Intracerebral Hemorrhage and MRI
Renal Artery Lesions in Moyamoya Disease
Stroke and Estrogen Receptor
Estradiol Administered After Ischemia
Electromechanical Changes in the Cerebrovasculature of SHRsp
Endothelial Function in Transgenic Mice
Neuroprotection and Glutamate Release

■ **Comments, Opinions, and Reviews**
The Symptomatic Carotid Plaque
Carotid Wall Material Stiffness and Blood Pressure

■ **Letters to the Editor**

■ **Abstracts of Literature**

Estradiol Exerts Neuroprotective Effects When Administered After Ischemic Insult

Shao-Hua Yang, MD; Jiong Shi, PhD; Arthur L. Day, MD; James W. Simpkins, PhD

Background and Purpose—17β-Estradiol (E2) has been reported to exert neuroprotective effects when administered before an ischemic insult. This study was designed to determine whether E2 treatment after ischemia exerts the same effects and, if so, how long this therapeutic window remains open, and whether the effects are related to changes in cerebral blood flow (CBF).

Methods—Female Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). In protocol 1, E2 was administered (100 μg/kg IV followed immediately by subcutaneous implantation of crystalline E2 in a silicone elastomer tube) to ovariectomized females (OVX+E2) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Intact (INT; n=6) and ovariectomized females (OVX; n=12) were subjected to MCAO and received vehicle instead of E2. Two days after MCAO the animals were killed, and ischemic lesion volume was determined by 2,3,5-triphenyltetrazolium chloride staining. In protocol 2, CBF was monitored before and at 1, 24, and 48 hours in a group of animals receiving E2 or vehicle 0.5 hour after ischemia induction (INT, n=6; OVX, n=8; OVX+E2, n=6).

Results—Lesion volume was 20.9±2.2% and 21.8±1.2% in the INT and OVX groups, respectively. E2 was found to decrease lesion volume significantly when administered within 3 hours after MCAO. The lesion volumes were 6.3±0.5%, 10.3±2.1%, 11.8±1.8%, 13.5±1.6%, and 17.9±2.8% when E2 was administered at 0.5, 1, 2, 3, or 4 hours after MCAO, respectively. CBF decreased to 43.1±2.2% and 25.4±1.0% in the INT and OVX animals, respectively, at 5 minutes after MCAO. In comparison to OVX rats, CBF was not different at 1 hour after E2 administration but was increased significantly in the OVX+E2 group 1 and 2 days after E2 administration.

Conclusions—E2 exerts neuroprotective effects when administered after ischemia, with a therapeutic window in a permanent focal cerebral ischemia model of approximately 3 hours. This effect of estradiol was associated with no immediate change in blood flow but with a delayed increase in CBF. (*Stroke*. 2000;31:745-750.)

Key Words: cerebral blood flow ■ estrogens ■ ischemia ■ neuroprotection

Both retrospective and prospective epidemiological studies have demonstrated beneficial effects of estrogen replacement therapy in reducing stroke-related mortality that is associated with stroke in postmenopausal women.[1,2] Recently, several laboratory studies have also emphasized the neuroprotective effects of estrogens.[3-6] Both chronic and acute pretreatment can reduce ischemic damage in focal cerebral ischemia, indicating that estrogens may be a new therapeutic class of drugs to prevent neuronal damage associated with cerebral ischemia.

Presently, it is not known whether postischemic treatment with estrogen is beneficial. The purpose of this study was to determine (1) whether 17β-estradiol (E2) can protect against brain injury when administered after cerebral ischemia; (2) the duration of any therapeutic window offered by E2, and (3) whether any E2 neuroprotective effects are associated with changes in cerebral blood flow (CBF).

See Editorial Comment, page 749

Materials and Methods

Female Charles River Sprague-Dawley rats (225 to 250 g; Wilmington, Mass) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO) under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

Middle Cerebral Artery Occlusion

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. MCAO was achieved according to the methods described by others, with the following modifications.[7,8] With the aid of an operating microscope, the left common carotid artery, external carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 4-0 monofilament suture with its tip

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rounded by heating was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistive point was approximately 1.9 cm. A 6-0 silk ligature was placed around the external carotid artery and tightened around the intraluminal monofilament suture to prevent bleeding and change of the suture position. The common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

Measurement of Regional CBF

A laser-Doppler flowmeter was used for CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebrospinal fluid leakage. Laser-Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. CBF was measured before and within 1.5 hours after MCAO. The incision was stapled, and the animals were then returned to their home cages. At 1 and 2 days after MCAO, the animals were reanesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP), and stable CBF recordings were obtained bilaterally at the same sites for at least 10 minutes. The CBF values were calculated and expressed as a percentage of the baseline values. CBF values reported represent the mean±SEM for the average of the CBF recordings obtained.

Measurement of Lesion Volume

Each group of animals was decapitated 2 days after MCAO, and the brain was removed and placed in a metallic brain matrix for tissue slicing (Harvard) immediately after decapitation. Five slices were made at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride in physiological saline at 37°C and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the 5 slices divided by the total cross-sectional area of these 5 brain slices.

E2 Administration and Serum Concentration

To obtain a prompt and sustained elevation in serum E2 concentration, intravenous injection of an aqueous soluble E2 preparation combined with simultaneous implantation of a silicone elastomer pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, 6 OVX animals were anesthetized with methoxyflurane inhalant, and a control blood sample was taken via the jugular vein. Then E2 (100 μg E2/kg body wt) complexed with hydroxypropyl-β-cyclodextrin (E2-HPCD, Sigma), which was dissolved in 0.9% normal saline, was administered via tail vein injection, and a 5-mm-long silicone elastomer tube (1.57 mm ID; 3.18 mm OD) containing crystalline E2 was immediately implanted subcutaneously. The animals were put back into their cages, and blood samples were then taken via the jugular vein at 5 minutes and 0.5, 1, 2, 4, 6, 12, 24, and 48 hours after steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined with the use of duplicate serum aliquots in a radioimmunoassay (ultrasensitive estradiol kit, Diagnostic Laboratory).

Protocol 1

To determine whether E2 exerts any beneficial neuroprotective effect when administered after the ischemic insult and the duration of any therapeutic window, E2 was administered (100 μg/kg, by tail vein injection combined immediate with subcutaneous implantation of an E2-containing silicone elastomer tube) in ovariectomized female rats (OVX+E2 group) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. As controls, ovariectomized females (OVX group: n=12) and intact females (INT group: n=6) were treated with equivalent volumes of saline and empty pellets at 0.5, 1, 2, 3, or 4 hours after MCAO.

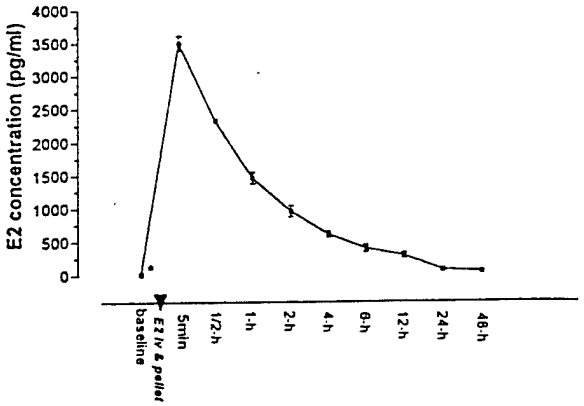


Figure 1. Effect of simultaneous intravenous injection of E2 (100 μg/kg complexed in hydroxypropyl-β-cyclodextrin) and subcutaneous implantation of an estradiol-filled silicone elastomer pellet on serum estradiol concentration in OVX rats. At the time indicated, 0.5-mL blood samples were obtained for subsequent E2 concentration analysis. n=6 rats per time point. Mean±SEM values are depicted. *$P<0.05$ vs all other sample times.

Protocol 2

To determine whether any neuroprotective effects of E2 were associated with blood flow changes, a laser-Doppler flowmeter was used to monitor CBF. After a baseline CBF reading was obtained, CBF was continuously recorded for 1.5 hours after MCAO induction. E2 was administered (100 μg/kg tail vein injection and subcutaneous implantation of an E2 pellet) 0.5 hour after MCAO induction (OVX+E2 group; n=6), and CBF was obtained for 1 hour thereafter and at 24 and 48 hours after MCAO. Intact females (INT group: n=6) and ovariectomized females (OVX group: n=8) received equivalent volumes of saline and empty pellet as controls.

Statistical Analysis

Statistical analyses were performed with SigmaStat 2.0 Software (Jandel Scientific). All data were expressed as mean±SEM. The lesion volumes in each group comparison were analyzed with 1-way ANOVA. The CBF values in each group were analyzed among groups at each sampling time with 1-way ANOVA and multiple comparisons. The difference for each comparison was considered significant at the $P<0.05$ level.

Results

Effects of E2 Administration on Serum E2 Concentration

In young cycling female rats, serum levels of E2 vary between 11±1 pg/mL at diestrus and 41±5 pg/mL at proestrus. Serum E2 concentrations increased and peaked at 3487±110 pg/mL 5 minutes after E2 administration, then decreased to 76±16 pg/mL 24 hours after administration (Figure 1). With the slow release from the E2 pellet, serum E2 concentration remained high at 45±5 pg/mL 48 hours after administration, compared with 13±4 pg/mL in OVX animals.

Therapeutic Window of E2

E2 treatment after the ischemic insult exerted neuroprotective effects (Figures 2 and 3). The ischemic lesion volume was significantly reduced in the OVX+E2 group when E2 was administered at 0.5, 1, 2, or 3 hours after the ischemic insult, with lesion volumes of 6.3±0.5%, 10.3±2.1%, 11.8±1.8%,

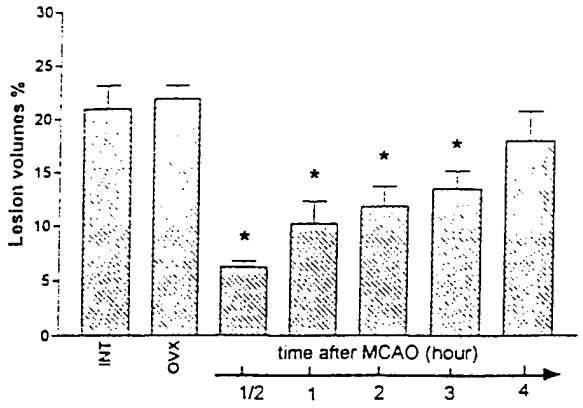


Figure 2. Effects of E2 treatment on lesion volume after MCAO in OVX and OVX+E2 rats. E2 was administered by simultaneous intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and INT.

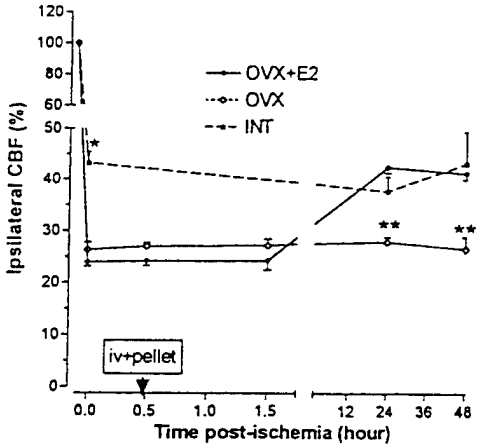


Figure 4. Effect of ovarian status and E2 replacement on ipsilateral CBF after MCAO. E2 was administrated by an intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 hour after MCAO. CBF was measured before and within 1.5 hours after MCAO (OVX, n=8; OVX+E2, n=6; and INT, n=6) and at 24 and 48 hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and OVX+E2; **$P<0.05$ vs INT and OVX+E2.

and 13.5±1.6%, respectively ($P<0.05$), indicating a therapeutic window of up to 3 hours in permanent focal cerebral ischemia. No significant difference of lesion volume was noted between OVX and INT groups (21.8±1.2% and 20.9±2.2%, respectively).

Effect of E2 on CBF

The ipsilateral CBF was higher immediately after MCAO in the INT group compared with the OVX and OVX+E2 groups: values for INT, OVX, and OVX+E2 groups were 43.1±2.2%, 26.2±1.5%, and 23.9±0.9%, respectively ($P<0.01$). After E2 administration, ipsilateral CBF increased at 1 and 2 days after E2 administration but not at 1 hour (Figure 4). The effects of MCAO on the contralateral CBF were similar in all groups and were independent of the estrogen status of the animal.

Discussion

This study demonstrates 3 potentially important clinical effects of E2. First, E2 exerts neuroprotective effects even when administered after the onset of an ischemic insult, with a therapeutic window up to 3 hours. Second, the neuroprotective effects of E2 are not associated with an immediate blood flow augmentation effect but with a later improvement in CBF. Third, at the dose used, neuroprotective effects of E2 are flow independent and in this permanent focal cerebral ischemia model are only observed with exogenous E2.

Several studies have demonstrated that E2 is a potent neuroprotective agent that decreases focal ischemia–induced lesion size by approximately 50% with E2 chronic pretreatment.[3–6] E2 also exerts neuroprotective effects when administered immediately before occlusion.[9] The present study, for the first time, systematically defines the therapeutic window of E2 in a model of permanent focal ischemia when the drug is administered after the ischemia has been induced.

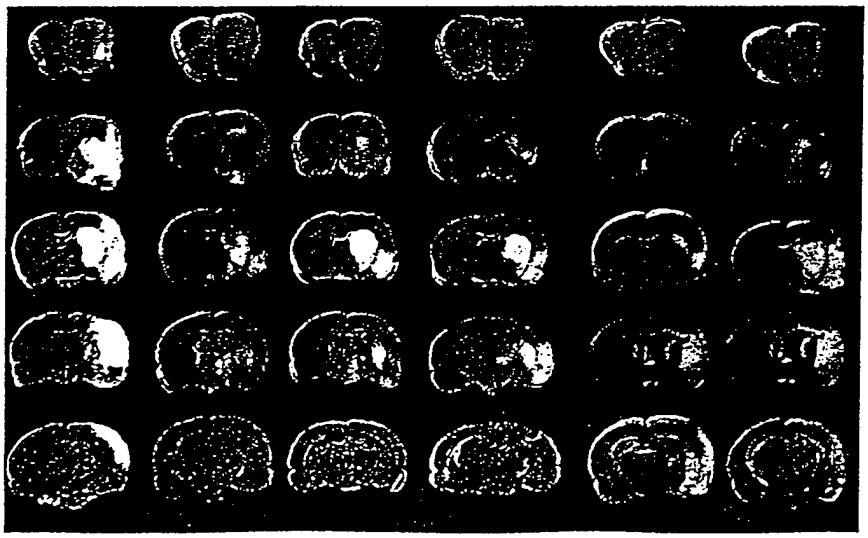


Figure 3. Photographic display of representative brain sections from OVX and OVX+E2 rats at different time points (0.5 hour, n=8; 1 hour, n=6; 2 hours, n=7; 3 hours, n=6; and 4 hours, n=9). Note the increasing infarct size with greater delays in drug administration.

The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia,[10] blockade of excitatory amino acid toxicity,[11,12] modulation of calcium homeostasis,[13–15] induction of neurotrophins and their receptor and intracellular signaling pathway,[16,17] induction of antiapoptotic protein,[18,19] and/or enhancement of brain glucose uptake.[20] E2 could also improve the outcome of cerebral ischemia through a protective effect on brain vascular endothelial cells,[20] resulting in the presently observed delayed improvement in CBF in E2-treated rats.

E2 has been shown to act on both of the peripheral and intracranial vascular systems.[21–24] In young cycling female rats, serum levels of E2 varied between 11±1 pg/mL on diestrus and 41±5 pg/mL on proestrus.[25] In our study, deprivation of endogenous ovarian steroids resulted in low residual CBF ipsilateral to the MCAO.[5,26] Acute administration of exogenous E2 (in which serum levels of E2 vary from 3487±110 to 45±5 pg/mL) increased ipsilateral CBF after stroke, but this effect was delayed until 1 to 2 days after occlusion. It appears that low levels of endogenous ovarian steroids resist the ipsilateral CBF effects after permanent MCAO. Acute treatment with high doses of E2 caused a delayed preserving effect on CBF, an effect that only occurred in the side ipsilateral to the MCAO.

The mechanism of any blood flow–preserving effects of E2 is still not well known, but 3 possibilities have been proposed. First, we have found that exposure of endothelial cells to E2 helps to maintain their viability during an ischemic episode.[20] Findings in this experiment suggest that the delayed effect of E2 on CBF maybe be secondary to a vascular cytoprotective action of the hormone. Alternatively, estrogen could induce vasodilation in cerebral arteries.

Second, E2 has been found to modulate serum lipid levels, reducing aggregation of platelets and the thrombotic and vasoconstrictive effects of thromboxane.[27,28] E2 withdrawal after ovariectomy increases the sensitivity of the rabbit basilar artery to serotonin.[29] Using a mouse carotid model, Sullivan et al[23] found that physiological levels of E2 replacement could significantly suppress the response of the carotid artery to injury. The endothelium produces a variety of vasoactive mediators such as prostacyclin and endothelium-derived nitric oxide, both of which have roles in regulating not only vascular tone but also smooth muscle cell proliferation.[21] Goldman et al[30] have also reported that within 10 minutes of injection of a supraphysiological dose of E2, CBF increases to most regions of the brain. In contrast, our study showed that the blood flow–preserving effects of E2 are not immediate but occur from 1 to 24 hours after E2 administration. These blood flow–preserving effects could be likely due to a slower genomic effect, since the cellular effects of E2 on gene expression occur hours to days after any insult.[31]

Finally, E2 could cause a delayed improvement in CBF through angiogenic mechanisms. Recently, Morales et al[32] found that E2 exerted angiogenic effects in peripheral vessels. While angiogenic effects of E2 may play a potential role in protecting against cerebral ischemia, we are not aware of studies demonstrating that estrogens can induce angiogenesis within 2 days of steroid replacement. However, by promoting neovascularization and collateral formation, E2 could restore cerebral perfusion in ischemic areas and hence lessen the impact of occlusion.

Both low and high circulating concentrations of E2 have been reported to exert neuroprotective effects in the temporary cerebral ischemia model in E2 pretreatment studies.[5,33] Both low and high physiological levels of E2 have exerted similar effects in a 1-day permanent cerebral ischemia study when administered before ischemia.[6] The present study showed that E2 neuroprotective effects could be induced by high-level exogenous E2 in 2-day permanent cerebral ischemia when administered after ischemia. Subsequent assessment of the dose dependence of this neuroprotection is clearly needed.

Assessments of efficacy also need to be conducted in both male and female rats. E2 has been found to exert neuroprotective effects in males, although in males the effects are dependent in part on the suppression of testosterone secretion.[34] Additionally, the neuroprotective effects of estrogens do not appear to be mediated by an estrogen receptor mechanism. 17α-Estradiol, a very weak estrogen, exerts neuroprotective effects equivalent to E2 both in vitro and in vivo.[3,35] Additionally, we have recently reported that ent-estradiol, the enantiomer of E2 that lacks estrogenic activity, is as potent as E2 in protecting cerebral tissue from MCAO.[36] These data indicate that several nonfeminizing estrogens that lack classic genomic-mediated estrogenic effects are potential clinical candidates for stroke neuroprotection.

In summary, our study demonstrates that E2 exerts neuroprotective effects when administered after an ischemic insult, with a therapeutic window of approximately 3 hours. The neuroprotective effect has a delayed CBF-preserving component and a blood flow–independent component. This study raises the possibility that estrogen compounds could be a useful therapy in preserving brain tissue, even if administered after the ischemic insult.

Acknowledgments

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Editorial Comment

Clinical studies have demonstrated that chronic estrogen use reduces stroke-related mortality.[1,2] Along similar lines, animal models of cerebral ischemia have demonstrated that the presence of estrogen in physiological amounts is protective.[3–8] Although much evidence exists that estrogen reduces stroke-related morbidity and mortality when present at the time of injury, it has been unclear whether estrogen is of therapeutic utility when administered after an ischemic event has occurred. To be of utility in the treatment, as opposed to the prevention, of stroke, estrogen must exert a protective effect when given within a reasonable time window after the ischemic event. The article by Yang et al demonstrates that postischemic administration of estrogen affords protection against ischemic damage similar to preischemic administration and that it acts within a clinically useful therapeutic window. However, this postischemic protection only occurs at supraphysiologic doses of estrogen. Another study[5] has suggested that preischemic administration of supraphysiologic doses of estrogen lacks the neuroprotective activity exhibited by physiological doses of estrogen. Differences between the mechanisms of action of physiological and pharmacological amounts of estrogen must be determined to account for the differing actions when estrogen is administered before or after the ischemic event. It remains to be seen whether the mechanism by which supraphysiologic doses of estrogen exert a protective effect is a novel one or represents a nonspecific action of estrogen at a previously described neuroprotective site.

Susan E. Robinson, PhD, *Guest Editor*
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Additional Appended Materials

James W. Simpkins

Other articles included as a general appendix, supported in part by this grant and related to our brain injury program

DAMA17-99-1-9473
Appendix M
J. W. Simpkins

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Brain Research 796 (1998) 296–298

Short communication

Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats

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BRAIN RESEARCH

Short communication

Testosterone increases and estradiol decreases middle cerebral artery occlusion lesion size in male rats

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Abstract

This study was undertaken to determine the effects of estrogen and testosterone on cerebral ischemic lesion size induced by middle cerebral artery (MCA) occlusion in male rats. Rats were gonadectomized and treated with testosterone, estrogen, or testosterone plus estrogen filled Silastic® pellets. The animals were divided into 6 groups: intact, intact + estrogen (E2), castrate, castrate + testosterone (T), castrate + E2, and castrate + T + E2. One week after treatment, cerebral ischemia was induced by MCA occlusion for 40 min, followed by reperfusion. After 24 h, rats were sacrificed and slices were then stained to assess lesion size. The presence of testosterone increased and the removal of testosterone decreased lesion size. A strong positive correlation ($r^2 = 0.922$) between plasma testosterone concentrations and ischemic lesion size was observed. Estradiol treatment reduced ischemic area. In summary, the present study provides evidence that testosterone exacerbates and estrogens ameliorate ischemic brain damage in an animal model of cerebral ischemia. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Testosterone; Estradiol; Stroke; Brain ischemia; Male rat; Middle cerebral artery occlusion

Men are at a relatively increased risk of cardiovascular and neurological disorders when compared premenopausal women [1,9,10]. Further, estrogen replacement therapy in post-menopausal women is associated with improved survival in subjects who suffer from stroke [4,5]. Recently, we and others have demonstrated that estrogens exert neuroprotective effects in vitro against the neurotoxicity subsequent to serum deprivation [3,13,15], β-amyloid [2,7,13], and glutamate agonist treatment [16] in a manner that appears to be estrogen receptor-independent [8,13,14]. Therefore, the next logical step in evaluating the neuroprotectivity of estrogen is to determine its actions in males.

Middle cerebral artery (MCA) occlusion has been used to produce focal ischemic lesions in the rat, because this type of occlusion mimics the mechanism of thrombotic cerebral stroke that occurs in approximately 70% of the victims of stroke [9]. This procedure causes a unilateral large cerebral ischemic area that typically involves the basal ganglion and frontal, parietal, and temporal cortical areas [6,11].

We present here the results of the studies to determine the effect estrogen and testosterone on the ischemic lesion caused by MCA occlusion in gonadectomized male rats.

Male Charles Rivers rats weighing approximately 250 g were purchased from the Willington, MA colony and were maintained in our AAALAC accredited vivarium for 1 week prior to gonadectomy. Bilateral gonadectomy was performed under methoxyflurane (Metophane®, Pitman Moore, Crossings, NJ) inhalant anesthesia 7 days prior to MCA occlusion.

17 β-Estradiol was packed into 5-mm-long Silastic® tubes (Dow-Corning, Midland, MI) and testosterone was packed into 10-mm-long Silastic® tubes that were closed on either end with Silastic Medical Adhesive® (Dow-Corning). Sham (empty) pellets were similarly prepared. All pellets were washed with methanol to remove the steroid adhering to the outside of the tubes. Subsequently, pellets were washed in physiological saline, a procedure that assures first order in vivo release of estradiol to achieve physiologically relevant concentrations [17]. The pellets were implanted subcutaneously (sc) at the time of castration (1 week prior to the MCA occlusion).

At 7 days after gonadectomy and steroid implantation, animals were anesthetized with ketamine (60 mg/kg, ip) and xylazine (10 mg/kg, ip). During surgery, rectal tem-

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perature was maintained between 36.5 and 37.0°C by a heating lamp. MCA occlusions were performed using procedures that we have described in detail elsewhere [12,14,18]. The operative procedure was completed within 10 min with minimal blood loss. After 40 min of occlusion time, reperfusion was allowed to occur.

Animals that survived until the scheduled sacrifice time (24 h after MCA occlusion) were killed by decapitation. Prior to sacrifice, cardiac puncture was used to obtain blood samples for subsequent assessment of plasma testosterone concentration using a Coat-A-Count RIA kit purchased from Diagnostics Products, Los Angeles, CA. Brains were removed 24 h after MCA occlusion and ischemic area was quantified using methods previously described [12,14,18].

The significance of differences in lesion size among the 6-treatment groups was determined by ANOVA and the Fischer's test was used for the post-hoc analysis. $p < 0.05$ was considered significant. The correlation between testosterone and the lesion size was analyzed by regression analysis. The significance of the mortality of the rats prior to sacrifice was analyzed by Chi-square analysis.

The effects of castration and testosterone replacement on ischemic damage following MCA occlusion is shown in Fig. 1. Intact rats showed the expected rostral to caudal extent of ischemic damage with peak ischemic lesions observed at 7 and 9 mm caudal to the olfactory bulb. Lesion size was small at more rostral and caudal brain sections. As we have previously reported [14], the MCA occlusion lesion occupied the expected brain regions, i.e., the frontal and parietal cortex and basal ganglia, supplied by the MCA. Castration of adult male rats reduced ischemic lesion size in each section evaluated (Fig. 1) and reduced the overall mean ischemic area from 17 ± 3% in intact rats to 8 ± 2% in castrate rats (Fig. 2). Testosterone replacement of castrate rats increased lesion size in all sections evaluated (Fig. 1) and increased overall mean ischemic area to 14 ± 2% (Fig. 2).

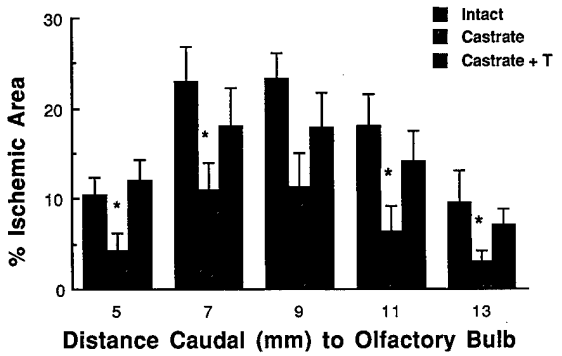


Fig. 1. Effects of androgen environment on ischemic lesion size in male rats. Depicted are the percentage of the cross-sectional areas of the brain (mean ± S.E.M.) in various slices taken from gonadally intact, castrated and castrated animals with testosterone replacement (Castrate + T). * Indicates $p < 0.05$ vs. intact rats for individual brain sections.

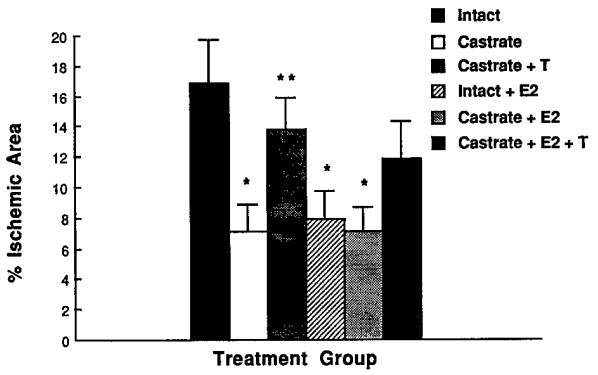


Fig. 2. Effects of endocrine manipulation on mean percent ischemic area (mean ± S.E.M.) in male rats. * Indicates $p < 0.05$ vs. intact rats. * * Indicates $p < 0.05$ vs. castrate and castrate + E2 groups.

Since we have previously reported that estrogens are neuroprotective against MCA occlusion-induced ischemic brain damage in female rats [12,14,18], we evaluated the effects of estrogen treatment in males. Treatment of intact male rats with 17 β-estradiol reduced overall mean ischemic area from 17 ± 3% to 8 ± 2% (Fig. 2). Treatment of castrated males with estradiol did not change the already reduced size of the MCA occlusion-induced lesion (Fig. 2). Simultaneous treatment with both testosterone and estradiol in castrated rats resulted in an ischemic lesion of 12 ± 2%, intermediate between that of castrate + T (14 ± 2%) and castrate + E2 (7 ± 2%) (Fig. 2).

Castrate + E2 treatment profoundly reduced plasma testosterone concentration from 1.556 ± 0.409 ng/ml in intact male rats to 0.069 ± 0.029 ng/ml and 0.054 ± 0.010 ng/ml in castrate and intact + E2-treated animals, respectively. Testosterone replacement in castrate or castrate + E2 rats increased plasma testosterone concentration to 0.668 ± 0.067 ng/ml and 0.590 ± 0.055 ng/ml, respectively.

We conducted an analysis of covariance to assess the relationship between plasma testosterone and overall mean percent ischemic area. An r^2 of 0.922 was observed when ischemic area was analyzed on the basis of plasma testosterone concentrations.

Mortality (deaths prior to the scheduled 24-h sacrifice time) was high in the intact group (46%) and the intact + E2 group (30%), but was low (9–15%) in all castrate groups, regardless of their hormone replacement.

The present study demonstrates that in male rats, plasma testosterone concentrations are highly correlated with increased ischemic brain damage from middle cerebral artery occlusion. As such, reduction in plasma testosterone, either through castration or treatment with estradiol, is associated with approximately a 50% reduction in ischemic lesion size. To our knowledge, this is the first direct demonstration of an adverse influence of testosterone during brain ischemia.

Castration reduced the ischemic lesion by 59%. Testosterone replacement only partially restored lesion size to that seen in intact animals. However, when compared to

intact animals, our testosterone implants only restored plasma testosterone concentration to 42.9 and 37.9% in castrate + T and castrate + E2 + T animals, respectively. As such, we relied on an analysis of covariance to establish the relationship between lesion size and plasma testosterone concentration. A strong positive relationship was observed with an r^2 value of 0.922. These data suggest that regardless of other endocrine factors, plasma testosterone is a primary determinant of the size of ischemic lesions following MCA occlusion in the male rat.

Estrogens have been shown to be neuroprotective in a variety of in vitro and in vivo models for neurodegeneration [2,3,7,8,12–16,18]. In female rats, ovariectomy enhances and estrogen treatment reduces by about 50% ischemic lesion following MCA occlusion [12,14,18]. In the present study, 17 β-estradiol exerted a profound protective effect in intact male rats that was associated with a marked reduction in plasma testosterone concentrations. In the presence of testosterone from a Silastic® implant, estradiol was only partially effective in reducing lesion size. These data indicate that a primary effect of estrogens in male rats may be mediated through a reduction in circulating testosterone levels.

In summary, the extent of ischemic damage following MCA occlusion is correlated with plasma testosterone concentration in the male rats. To our knowledge, this study is the first to demonstrate that testosterone is a negative risk factor in the outcome of cerebrovascular ischemia in an animal model for stroke. These data indicate that testosterone has a detrimental effect on the extent of ischemic damage associated with stroke. This effect could contribute to mortality and morbidity associated with cerebrovascular ischemia in men.

Acknowledgements

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Improvement In Cerebral Ischemia Outcome With Non-Surgical Methods of Reducing Testosterone In Male Rats. J. Cutright, S.Yang, Z. He, A. L. Day, J. W. Simpkins*. Center for The Neurobiology of Aging, Department of Pharmacodynamics, and Neurological Surgery, University of Florida, Gainesville, FL 32610

Cerebral ischemia is less damaging in females than in males. While estrogen's neuroprotective effects have been established, the role of androgens in this sex difference is less clear. We have reported that surgical castration reduces cerebral damage following ischemia in male rats (Brain Research 796: 296-298, 1998). The present study further assesses the role of testosterone in cerebral ischemia using a variety of non-surgical methods to reduce serum testosterone. Male SD rats were treated with leuprolide (0.1 mg/kg every 12 h for 7 d) or saline vehicle, which produced serum testosterone of 0.4 ng/ml and 7 ng/ml, respectively. Anesthetic stress (ketamine 60 mg/kg and xylazine 10 mg/kg) 6 h prior to cerebral infarct reduced serum testosterone to 0.5 ng/ml from 2.2 ng/ml in unstressed controls. All animals were then exposed to cerebral ischemia using 1 h middle cerebral artery occlusion followed by 23 h of reperfusion. Chronic treatment with leuprolide reduced cerebral infarct volume from 16±1% to 8±1%. Additionally, the stress-induced reduction in testosterone was associated with a reduction in infarct volume from 18±3% to 8.5±1.5%. Collectively these data indicate that non-surgical methods of reducing serum testosterone limit cerebral damage following an infarct and suggest that testosterone reduction could be useful in limiting cerebral damage associated with planned ischemic events.

(Supported by NIH AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant DAMD 17-19-1-9473.)

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Estradiol Protects Against ATP Depletion, Mitochondrial Membrane Potential Decline and the Generation of Reactive Oxygen Species Induced by 3-Nitroproprionic Acid in SK-N-SH Human Neuroblastoma Cells.

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ABSTRACT

Mitochondria are increasingly recognized as the important targets of toxicity during ischemia, hypoxia and toxic chemical exposure, wherein mitochondria dysfunction leading to ATP depletion may be a common pathway of cell death. Estrogens have been reported to be neuroprotective against excitotoxicity, oxidative injury and chemical toxicity in experiment models and been proposed to play a role in the modulation of cerebral energy/glucose metabolism. To address whether estrogens are involved in the regulation of mitochondrial function, we chose 3-nitroproprionic acid (3-NPA, a succinate dehydrogenase inhibitor) to uncouple oxidative phosphorylation. ATP levels in SK-N-SH cells were measured after exposed to 3-NPA. 3-NPA (10 mM) initially increased ATP levels with peak maxima occuring at 2 hr then caused a 40% and a 50%-80% decrease in ATP levels when treated for 12 hrs and 24 hrs, respectively. The ATP depletion induced by 3-NPA was attenuated by pretreating cells for 4-5 hours with 17β-estradiol (17β-E_2). At 12 hr and 24 hr of 10 mM 3-NPA exposure, 17β- E_2 pretreatment caused a dose-dependent increase in ATP level back to 80% and 50%, respectively, of that in control cells. The function of mitochondria following exposures to 3-NPA were also assessed by evaluating the mitochondrial transmembrane potential ($\Delta\psi_m$) using the probe rhodamine 123. 3-NPA induced a 60% decrease in fluorescence intensity at 12 hr exposure, an effect that persisted for 24 hr. 17β-E_2 pretreatment eliminate this effect of 3-NPA on mitochondria transmembrane potential. 3-NPA also induced significant increases in levels of cellular hydrogen peroxide and peroxynitrite at 2 hr and estradiol pretreatment blunted these effects.

The present study provides evidence that exposure of SK-N-SH cells to 17β- E_2 can attenuates 3-NPA induced ATP depletion, mitochondrial membrane potential declines and reactive oxygen species generation, actions which may contribute to the neuroprotective effects of estrogens.

INTRODUCTION

Mitochondria are unique organelles in their involvement in the consumption of oxygen, production of ATP, oxygen radicals, and mobilization of calcium (Gunter and Pfeiffer, 1990; Traystman et al., 1991; Coyle and Puttfarcken, 1993; Imberti et al., 1992; Mills et al., 1995; Pang and Geddes, 1995; Seo et al., 1999; Schinder et al., 1996). Mitochondria play pivotal roles in the determining if cells undergo apoptotic versus necrotic cell death (Ankarcrona et al., 1995; Bonfoco et al., 1995) and increasing evidence shows that mitochondrial dysfunction is involved in neuronal damage associated with ischemia, hypoxia, toxicant exposure, and neurodegenerative diseases (Lemasters et al., 1997; Beal, 1998).

Estrogens have long been recognized as antioxidants and recent studies have showed that estrogens are also potent neuroprotective agents. In vitro studies reveal that estrogens reduce the cellular toxicity of beta amyloid peptide (Aβ) and other oxidative insults (Behl et al., 1995; Goodman et al., 1996; Green et al., 1996,1998; Nishino et al., 1998; Leal et al., 1998) and this correlates with a decrease in production of lipid peroxides (Lacort M et al., 1995; Miura et al., 1996; Keller et al., 1997; Gridley et al., 1997). 17 β-Estradiol (17 β-E2) protects neurons from anoxia-reoxygenation, glutamate or AMPA toxicity (Singer et al., 1996; Keller et al., 1997; Bonnefont et al., 1998; Culmsee et al., 1999; Singer et al., 1999; Zaulyanov et al., 1999; Honda et al., 2000). In vivo, treatment with 17β-E2 or 17α-E2 markedly reduces ischemic brain damage induced by middle cerebral artery occlusion in ovariectomized rats (Simpkins et al., 1997; Yang

et al., 2000) and estrogens protect against vulnerability of the lateral striatal artery to 3-NPA (Nishino et al., 1998).

To address the role of mitochondria in the neuroprotection effects of estrogens, we chose 3-nitroproprionic acid (3-NPA), a succinate dehydrogenase inhibitor, to model condition in which interrupted energy metabolism is observed, such as cerebral ischemia (Du et al., 1996; Fink et al., 1996; Hansford, 1985) and chronic neurodegenerative disease (Beal, 1995). We assessed the ability of 17β-E2 to affect the 3-NPA modulation of ATP production, mitochondrial transmembrane potential, reactive oxygen species and cell viability.

MATERIALS AND METHODS

SK-N-SH neuroblastoma cell culture SK-N-SH cells were obtained from American Type Tissue Collection (Rockville, MD). Cell cultures were maintained in RPMI-1640 media (Fisher Scientific, Pittsburgh, PA) supplemented with 10% charcoal- stripped FBS in monolayers in plastic Corning 150-cm^2 flasks at 37°C and under 5% CO_2, 95% air. Media was changed every two days. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300, Nikon, Tokyo, Japan). SK-N-SH cells used in the following experiments were in passes 35 to 40.

Cell viability Cell viability was assessed using observations of nuclear changes occurring during apoptosis with the chromatin-specific dye, Hoechst 33258 (Molecular Probes, Eugene, OR). Cells were fixed in 4% paraformaldehyde for 1 hr, membranes were permeabilized with 0.2% Triton X-100 for 30 min and cells were stained with

1μg/ml fluorescent DNA-binding Hoechst 33258 dye for 30 min. Hoechst-stained cells were visualized under a Zeiss epifluorescence microscope with 340 nm excitation and 510 nm barrier filter using a 40X objective. 200 cells per culture were counted and 5 separate culture were assayed per treatment group. The percentage of cells with condensed and fragmented DNA in each culture was determined.

Succinate dehydrogenase activity The effects of 3-NPA on mitochondrial succinate dehydrogenase (SDH) activity were assessed by measuring the conversion of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) to formazen crystals. Cell cultures were exposed to various doses of 3-NPA for 10 to 48 hr and then were rinsed with medium and incubate with MTT. After solubilization of the fromazen crystals in dimethylsulfoxide, the optical density was determined at 575/690 nm.

Measurements of ATP levels Experiments were initiated by plating 1 x 10^6 cells per well in 24-well plates, allowing growth in regular media for 48 hr. Cells were exposed to 10 mM 3NPA with or without different doses of 17 β-E_2 6 hr pretreatment for 24 hr. Cellular ATP levels were quantified using luciferin and luciferase-based assay. Cells were rinsed with PBS and lysed with ATP-releasing buffer containing 0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 2 mM EDTA, 1 mM DTT; 10 μl of the lysate was taken for protein determination. Another 10 μl of the lysate was taken and added to 96-well plate. ATP concentrations in lysates were quantified using MLX Microtiter Plate Luminometer (Dynex Technologies Inc., Chantilly, VA). A standard

curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nM of ATP per μg of protein and normalized to levels in untreated control cultures.

Assessments of mitochondrial function Mitochondrial transmembrane potential was assessed by the extent of retention of rhodamine-123 (Rh123), a fluorescent cationic dye that is sequestered by mitochondria in relationship to the negativity of the transmembrane potential. Cell cultures were incubated for 12 hr in the presence or absence of 3-NPA with or without 5-6 hr pretreatment with 17β-E_2 in an atmosphere of 95% air/5% CO_2. The mitochondria were stained by adding to cell cultures a stock solution of Rh123 (10 mM in distill water), to a final concentration of 1 μM and incubating for 15 min in the dark at room temperature. Cell cultures were then rinsed three times with the appropriate treatment medium followed by a 5 min wash with a HEPES-buffered control salt solution containing (in mM): NaCl 145, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and glucose 10, pH-adjusted to 7.4 with NaOH. Cells were suspended and centrifuged at 1000 rpm for 2 min and resuspended in the standard saline buffer at a concentration of 1 x 10^6 cells/ml. The cells suspensions were then filtered through a nylon mesh screen of 40 μm into borosilicate glass tubes at a cell density of 1ml of the cell suspension (10^6 cells) per tube. Changes in Rh123 fluorescence intensity were measured by flow cytometry (Becton Dickinson, CA) with excitation at 488 nm using an air-cooled argon-ion laser and emission at 525 nm using an optical band-pass filter.

Measurement of Reactive Oxygen Species Levels of cellular oxidative stress was estimates by monitoring levels of hydrogen peroxide and peroxynitrites. Following the indicated treatment with 3-NPA, 17 β-E2 or their combination, cells were exposed to either 2,7-dichlorofluorescin diacetate (DCF-DA) or dihydrorhodamine 123 (DHR123). Cells were exposed for 30 min with 40 μM DHR 123, followed by a 5 min wash with HEPES-buffered control salt solution (HCSS) containing (in mM): NaCl 145, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and glucose 10, pH-adjusted to 7.4 with NaOH. Fluorescence intensities were measured using flow cytometry. DCF-DA is mainly oxidized hydrogen peroxide in the cytoplasm (Page et al., 1993). Nonfluorescent DHR123 is also oxidized by ROS, in particular, peroxynitrite, to the positively charged, fluorescent rhodamine 123 derivative (Kooy et al., 1994).

Data analysis Fluorescence data were obtained using the flow cytometry (Becton Dickinson, CA). The Rh123 or DCF fluorescence from 10,000 cells was analyzed and the mean fluorescence intensity from these cells were calculated using CELLQuest3_3 software (Becton Dickinson). All data are expressed as mean ± SEM of Rh123 or DCF fluorescence ratio of these cells from 4 to 6 separate cultures, and calculated as the percentage of fluorescence over basal values. The significant treatment effects on cell viability, MTT reduction, mitochondrial membrane potential, reactive oxygen species were determined using ANOVA followed by Tukey's *post hoc test*, with significance determined at $p < 0.05$.

RESULTS

Effects of 3-NPA on Conversion of MTT to Formazen

3-NPA caused a dose-dependent decline in formazen production when measured at 24 hr, with the 10 mM concentration causing an approximate 50% reduction in succinate dehydrogenase activity, as measured by the inhibition of reduction of MTT to formazen crystals (Fig 1A). When tested at this 10 mM concentration, 3-NPA caused a time-dependent reduction in formazen formation which peaked at 24 to 36 hours of exposure (Fig 1B).

Effect of 17 β-E2 on 3-NPA-Induced Apoptosis

The basal rates of apoptosis in SK-N-SH cells under the conditions used was less than 10% (Fig 2). This rate was not effected by exposure to 17β-E_2. 3-NPA caused and increase in apoptosis to 80% of cells after 24 hr and pretreatment with 2 micromolar 17β-E_2 attenuated this response to 3-NPA (Fig 2). Prolonged incubation (2-7 days) with lower dose (200 nM) had similar effect (data not shown).

Effects of 3-NPA, 17 β-E2 and their combination on ATP concentrations

In control cultures, cellular ATP concentrations were 27.8 ± 7.0 nmol/mg protein (n=6). 3-NPA caused time- and dose- dependent changes in ATP levels (Fig 3). Exposure to 3-NPA at concentrations ranging from 1 mM to 20 mM resulted in an initial increase in ATP levels with peak maxima occurring at 2 hr followed by a time-related decrease thereafter (Fig 3). ATP levels of cells treated with 1 mM 3-NP decreased back to control levels by 3 hr and remained constant thereafter (Fig 3). At 12 hr, the 10 mM,

15 mM and 20 mM concentrations of 3-NPA-induced declines in ATP of 40%, 60%, and 80%, respectively (Fig 3). ATP level had further decrease at 24 hr to 50%, 70%, and 95% of that of control cells (Fig 3).

We then evaluated the effect of 17β-E_2 on the ATP changes induced by 2 and 12 hr of exposure to 3-NPA. Treatment with 17β-E_2 alone for 12 hr exerted no significant effects on cellular ATP levels (Fig 4). Pretreatment with 17β-E_2 caused a dose-dependent blockade of the increase in cellular ATP induced by 3-NPA (Fig 5). Similarly, 17β-E_2 pretreatment blunted in a dose-dependent manner, the decline in ATP concentrations seen at 12 hr of 3-NPA exposure (Fig 6).

Effects of 17 β-E2 on 3-NPA-induced mitochondrial depolarization

A decrease in mitochondrial transmembrane potential has been shown to be associated with an increase in reactive oxygen species and subsequent cell death in a variety of experimental models (Mancini et al., 1997; Hortelano et al., 1997; Esteve et al., 1999; Wullner et al., 1999; Castilho et al., 1999; Tatton and Olanow, 1999; Arai et al., 1999). We used the cationic and lipophilic dye Rh123 that permeates into the negatively charge mitochondria and therefore reflects the mitochondrial transmembrane potential (Johnson et al., 1980; Emaus et al., 1986) to examine the effects of 3-NPA on mitochondrial potentials. Levels of Rh123 fluorescence was decreased to 40% of the control level in cultures exposed to 3-NPA for 12 hr (Fig. 7, 8). Two doses of 17 β-E2, 0.2 μM and 2 μM, were chosen to pretreat cells for 4-5 hr. E2 alone did not change the fluorescence levels of cells (Fig. 7, 8). However, 17 β-E2 treatment caused a dose-dependent blockade

of the reduction in mitochondrial transmembrane potential induced by 3-NPA treatment (Fig 7, 8).

Effects of 17 β-E2 on 3-NPA-Induced Production of Reactive Oxygen Species

Inhibition of the production of ATP and the resulting depolarization of mitochondria is associated with enhanced production of reactive oxygen species (ROSs). We assessed the effects of 3-NPA, 17β-E2 and their combination on cellular levels of two ROSs, hydrogen peroxide (Fig 9) and peroxynitrite (Fig 10). Estradiol alone reduced levels of DCF fluorescence (a marker for hydrogen peroxide) by 25 % below levels seen in vehicle treated cultures (Fig 9). 3-NPA treated more than doubles the apparent hydrogen peroxide concentrations, while treatment with 17β-E2 prevented this 3-NPA-induced increase in hydrogen peroxide (Fig 9).

The same patterns of responses to treatment were seen with peroxinitrite. 17β-E_2 alone caused a modest decline in peroxynitrite concentrations. 3-NPA induced a 50% increase in DHR fluorescence, as effect that was blocked by 17β-estradiol treatment. Lower dose (200 nM) of E_2 for the same incubation time had no effect on the cellular levels of both hydrogen peroxide and peroxynitrite.

DISCUSSION

The present study demonstrates that in cultured SK-N-SH human neuroblastoma cells, 3-NPA toxicity is associated with an early increase in ATP production and the generation of ROSs, followed by a reduction in ATP, a decline in mitochondrial transmembrane potential and subsequent apoptosis. Our observation that 17 β-E2 can attenuate each of

these effects of 3-NPA suggest that the neuroprotective effects of this steroid may be related to its direct or indirect effects on mitochondrial function. Since SK-N-SH human neuroblastoma cell line lacks a functional estrogen receptor as determined by nuclear exchange assay (Green et al., 1998) and failed attempts to express the message or protein for ER-α or ER-β (Green et al., Unpublished Observations), alternative mechanisms of neuroprotection are indicated. The present observation of an action of 17 β-E2 on mitochondrial function may provide a key to these non-genomic neuroprotective effects of estrogens.

3-NPA is used as *in vitro* and *in vivo* to model acute insults such as ischemia (Sandberg et al., 1999), as well as neurodegenerative disorders, including Huntington's disease (Beal, 1994) and Alzheimer's disease (Beal, 1995). 3-NPA-induced cell death have been proposed to result from the synergistic effects of inhibition of SDH and activation of glutamate receptors which included rapid necrotic and delayed apoptotic cell death (Pang and Geddes, 1997). The temporal pattern of cellular response to 3-NPA indicates that an elevation in ATP is associated with an increase in ROSs within 2-4 hr of treatment. These changes are followed by a decline in ATP and a reduction in mitochondrial transmembrane potential by 12-13 hr, and progression through apoptosis by 24hr. This pattern suggests that activation of mitochondrial respiration and production of ROSs are among the earliest effects of 3-NPA. Since increased calcium level in the mitochondria can initially stimulate mitochondrial respiration (Hansford, 1985; McCormack et al., 1990; Li et al., 1996), a primary neurotoxic action of 3-NPA may be Ca^{++} dyshomeostasis in close association with increased production of ROSs, rather than an inhibition of SDH, which occurs later. This early increase in ATP production,

however, is not necessarily associated with the later decline in ATP concentration and cell death, since a robust increase in ATP is observed at low doses of 3-NPA that do not result in the subsequent ATP loss or cell death. The late loss of ATP is likely associated with a cell death program as it has been reported that a substantial loss of ATP is a very late event in apoptosis in PC12 cells deprived of NGF (Mills et al., 1995).

3-NPA provoked depletion of intracellular ATP causes a decline in transmembrane potentials and precede cell death. In cells with mitochondrial dysfunction, the glycolytic maintenance of ATP is important for generating ATP. However, high doses of 3-NPA may also inhibit glycolysis. Exposure to hydroperoxides can result in a rapid depletion of GSH, which can in turn inhibit the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (G3PD) by oxidation of critical thiol groups located at the active site of the enzyme (Vaidyanathan, 1993; Brodie et al., 1987,1990). The observed early 3-NPA-induced increase in the generation of ROSs can result in the loss of G3PD activity. Human glutathione peroxidase overexpressing transgenic mice are less susceptible to SDH inhibitor (Zeevalk et al., 1997). Further, reduction in cellular levels of total glutathione render GABAergic and dopaminergic neurons were more sensitive to the neurotoxic effects of 3-NPA (Ludolph et al., 1992; Zeevalk et al., 1995a, b). Collectively, this indicates that 3-NPA produces multiple stresses on the cell which directly or indirectly reduce available ATP.

We observed that estradiol pretreatment blunted or ablated each of the observed effects of 3-NPA, although the concentration of the steroid need to achieve the effects differed between parameter. The parameters most sensitive to estrogens were the time-dependent changes in ATP and the reduction in mitochondrial transmembrane potential

induced by 3-NPA. Blockade of these effects of 3-NPA occurred at 17β-E_2 concentrations of 2 to 200 nM. Estradiol prevented the production of ROSs and apoptosis only at low μM concentrations. This marked difference in concentration of the steroid needed to antagonize the observed effects of 3-NPA could reflect the activation of different mechanisms at different concentrations of 17β-E_2 or may be related to the fact that the length of exposure to the steroid, magnitude of the stress and the timing of the observation can dramatically influence the dose-dependency of the effects of estrogens (Green et al., 2000).

Pretreatment of 17β-estradiol prevented the mitochondrial depolarization in response to 3-NPA. This observation is consistent with the report that estrogens stabilize mitochondrial potential against actions of mutant presenilin-1 (Mattson et al., 1998). Whether the effect was due to the inhibition of the opening of permeability transition pores, the inhibition of the mitochondrial calcium uniport, or recovery of ATP levels, remain unknown. The maintenance by 17β-E_2 of mitochondrial membrane depolarization may be attributed to its antioxidant and/or energy increasing effects.

Uncoupling oxidative phosphorylation could stimulate the F0F1-ATPase, causing hydrolysis of ATP generated by glycolysis and loss of glycolytic protection (Lemaster et al., 1997). Recent studies show that estrogens can bind to one of the subunits of F1F0-ATPase (Ramirez et al., 1996; Zheng and Ramirez, 1999), and 17β-E_2 inhibited the F0F1-ATPase activity at micromolar concentration with 1-2 min of estrogen exposure (Zheng et al., 1999). Inhibition of F1F0-ATPase could serve to blunt ATP loss by 17β-estradiol.

The well characterized antioxidant activity of estrogens (Jellinck PH et al., 1991; Romer et al., 1997; Belh and Holsboer, 1999; Culmsee et al., 1999; Moosmann and Belh, 1999) could account for both the maintenance of cellular ATP concentrations and mitochondrial transmembrane potential in the face of 3-NPA exposure. The antioxidant effects of 17β-E_2 could prevent lipid peroxidation in mitochondrial membranes (Lacort M et al., 1995; Miura et al., 1996; Keller et al., 1997) as well as oxidation of mitochondrial ion channel proteins (Keller et al., 1997). Additionally, an antioxidant effect of 17β-E_2 could preserve the capacity of the cell to produce ATP. Estrogens have been shown to increase GSH levels (Diaz-Flores M et al., 1999), an effect that can increase ATP synthesis via glycolysis (Astiazaram et al., 1989). Estrogens increase the cellular NADH/NAD+ ratio, probably as a result of its cytosolic metabolism by dehydrogenases, and also induces NADH-generating system (Astiazaram et al., 1988, 1989). 17β-E_2 increased synaptic glucose uptake and protects against Aβ and FeSO4 insults (Keller et al., 1997), and enhances GLUT-1 transporter in endothelial cells of the brain vasculature (Shi and Simpkins, 1997a; Shi et al., 1997b), which increases the availability of glycolytic substrate glucose. High glucose, and presumably the resulting increase in ATP generation, protected cells against NMDA, free radical, and oxygen-glucose deprivation (Seo et al., 1999). Finally, neurons maintained in high glucose media show enhanced mitochondrial transmembrane potential that likely counteracts toxic insults (Seo et al., 1999).

In conclusion, by preserving ATP level, reducing reactive oxygen species and preventing mitochondrial depolarization, 17β-estradiol exert a protective effect on cells

exposed to 3-NPA. These results may help explain the beneficial effects of estrogen in neurodegenerative diseases.

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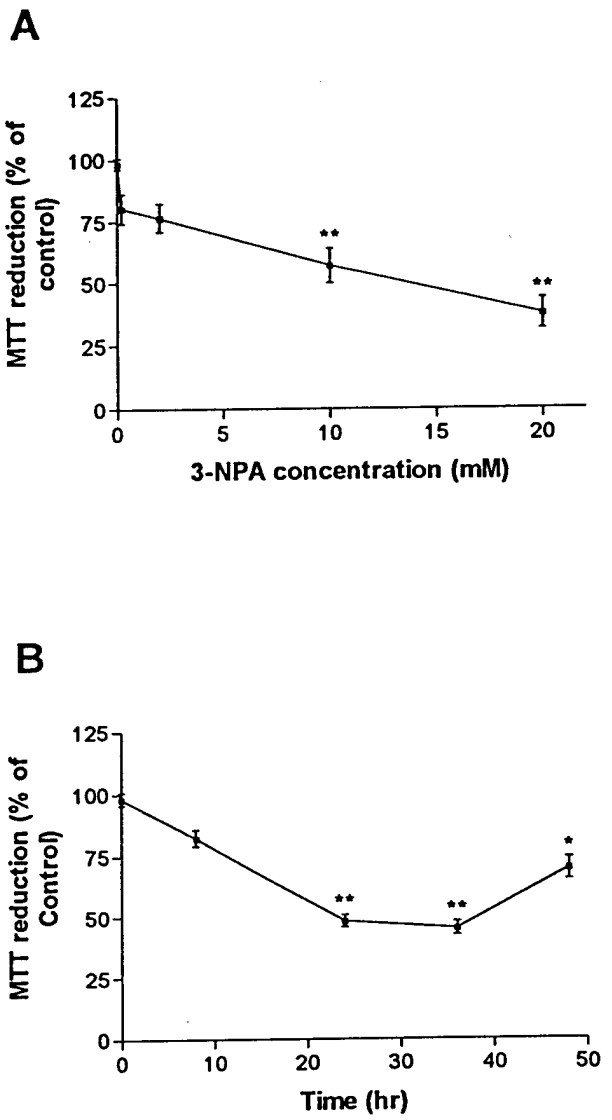


Figure 1. Effect of 3-nitroproprionic acid on MTT reduction. A. Dose-response: SK-N-SH cells were exposed to different doses of 3-NPA for 24 hr. B. Time-course: SK-N-SH cells were exposed to 10 mM 3-NPA for the indicated times. Values are the mean ± SEM of determinations made in 16-20 cultures. *$p<0.01$, **$p<0.001$ vs. 0 mM or 0 hr.

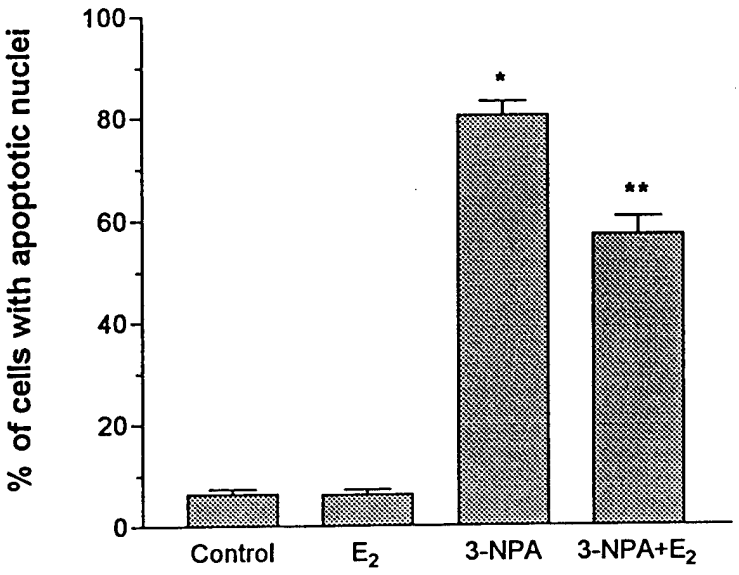


Figure 2. Effect of 5.5 hr 17β-E_2 preatment on 3-NPA induced apoptosis in SK-N-SH cells. Cultures were exposed to the indicated treatments. At 24 hr after exposure to 3-NPA, cells were stained with Hoechst dye and the percentages of cells with condensed fragmented nuclei were quantified. Values are the mean ± SEM for five cultures. *$p<0.001$ compared with control value; **$p<0.001$ compared with cultures not pretreated with 17β-E_2.

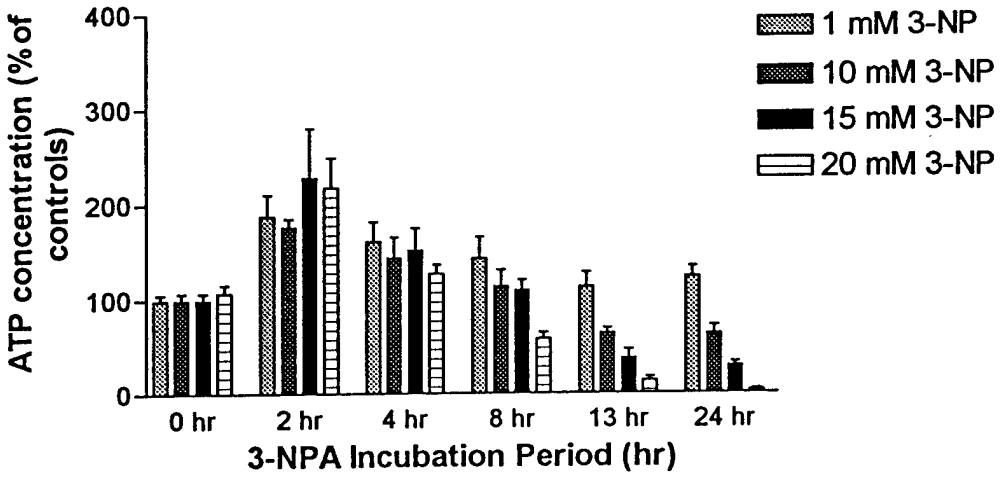


Figure 3. Time course of the effects of various doses of 3-NP on ATP concentrations in SK-N-SH cells. Depicted are mean ± SEM for 3-6 cultures/groups.

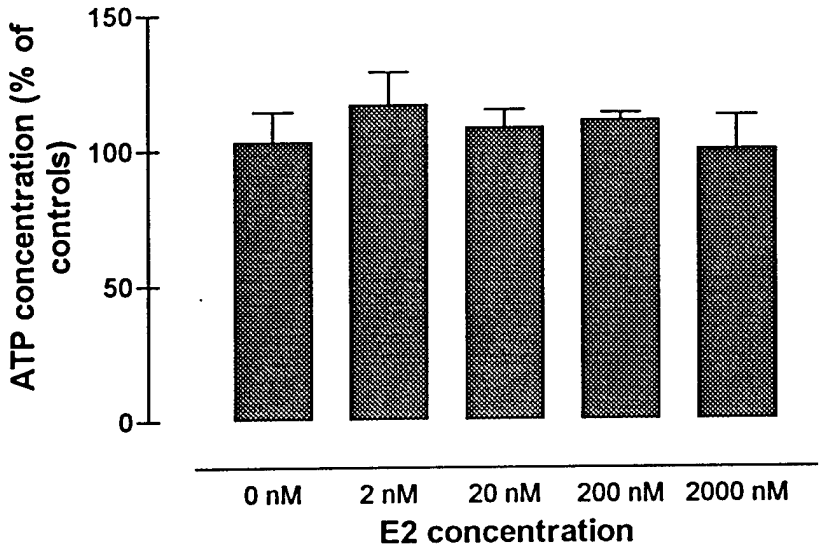


Figure 4. Effect of 12 hr treatment with 17β-E_2 alone on ATP concentrations in SK-N-SH neuroblastoma cells. The ATP concentrations were normalized as the percentages of controls. Results are the means ± SEM for 3 - 4 cultures/groups. ATP levels in control cells were 34.3 ± 6 nmole/mg protein.

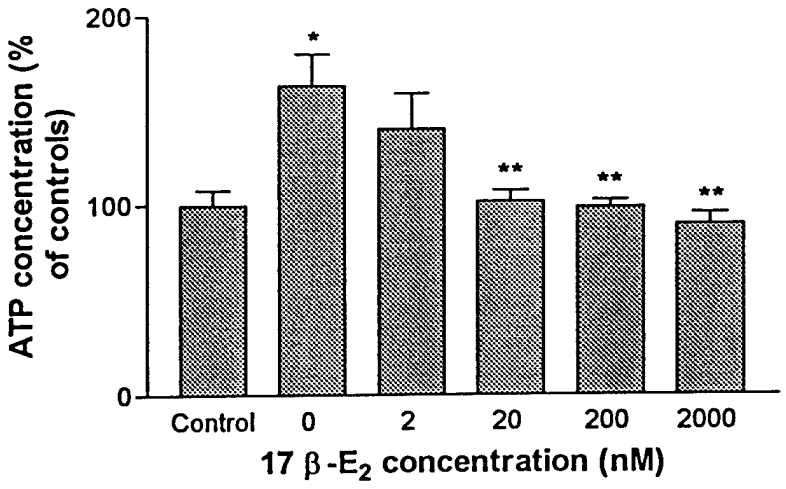


Figure 5. Effects of 4-5 hr pretreatment with 17β-E_2 on ATP concentrations in SK-N-SH cells at 2 hr following treatment with 10 mM 3-NPA. Values are expressed as a percentage of basal levels and represent the mean ± SEM for 4-6 cultures/groups. Basal ATP levels in control cultures were 29.8 ± 8 nmol/mg protein. *$p<0.05$ compared to the control group, **$p<0.05$ compared to the group of 0 nM E_2.

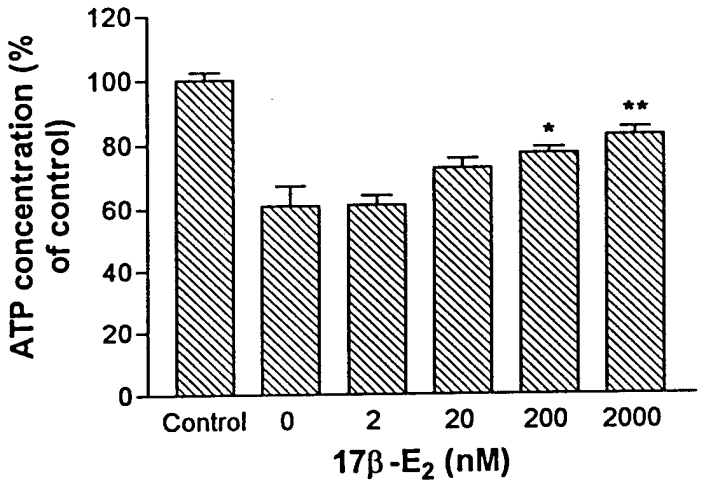


Figure 6. Effects of 5-6 hr 17β-E_2 pretreatment on ATP levels in SK-N-SH cells at 12 hr of 3-NP treatment. Values are expressed as a percentage of basal levels and represent the mean ± SEM of determinations made for 4-8 cultures. Basal ATP levels in control cultures were 46.8 ± 7 nmol/mg protein. *$p<0.05$, **$p<0.01$ compared to the 0 nM E2 group.

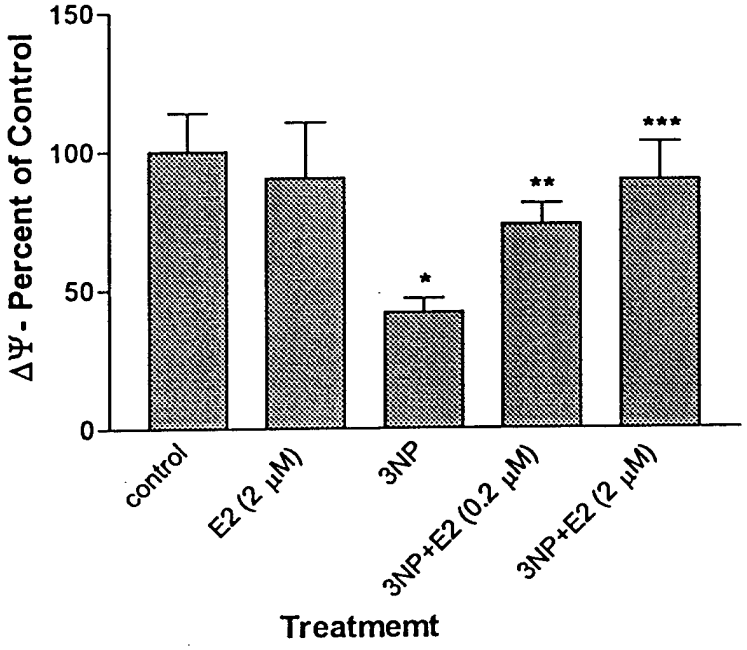
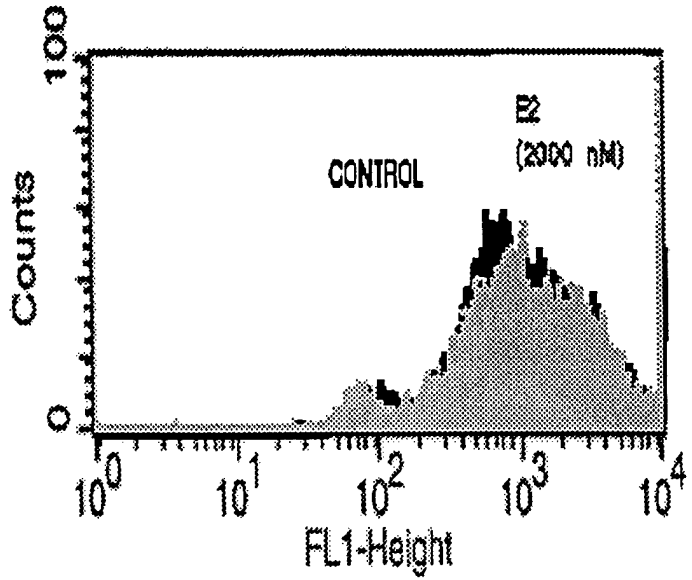
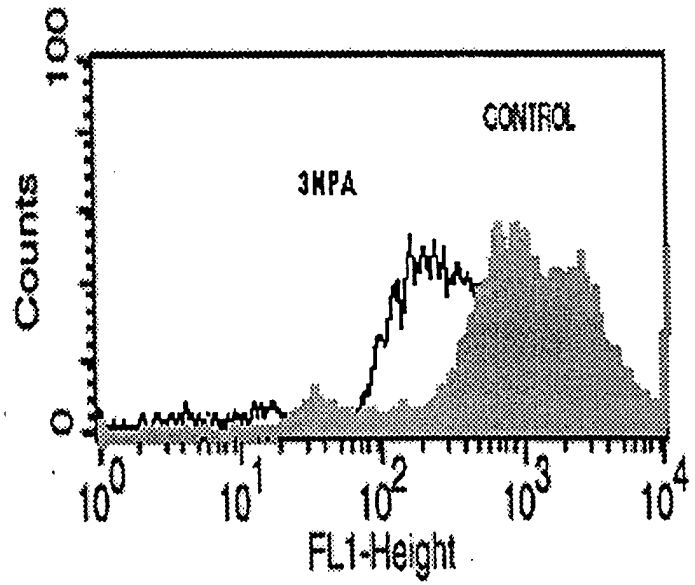
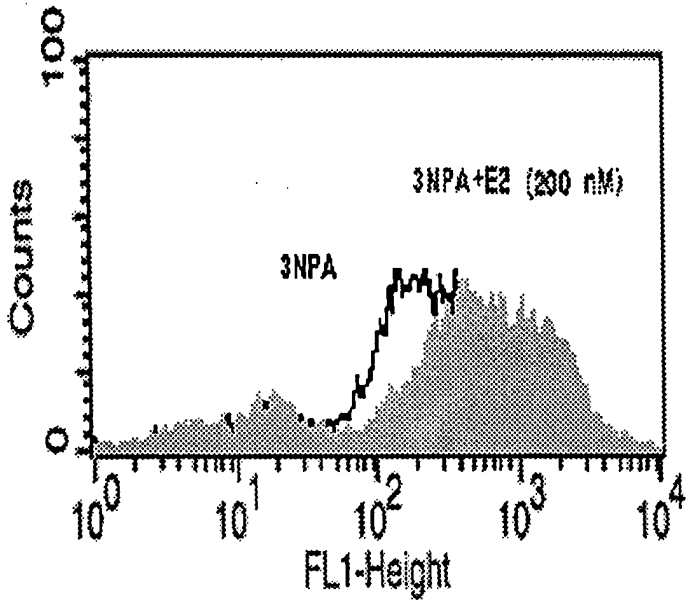


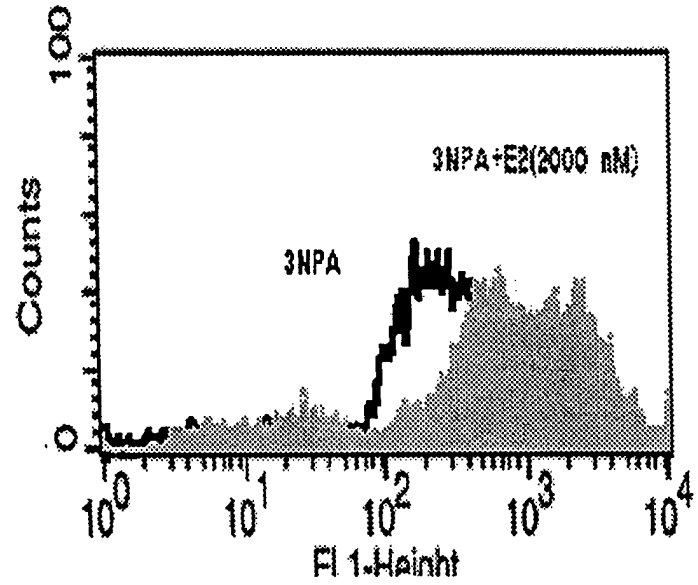
Figure 7. Effect of 5-6 hr pretreatment with 17β-E_2 on 3-NPA induced changes in mitochondrial transmembrane potential. Values are expressed as a percentage of basal levels and represent the mean ± SEM for 4-8 cultures. *$p<0.001$ compared to control and E_2 groups. **$p<0.05$ compared to 3NPA group. ***$p<0.001$ compared to 3NPA group.

Figure 8. Representative histograms of Rhodamine 123 fluorescence in cells with indicated treatments. The ordinate is the number of cells at each fluorescence intensity (abscissa).


100
0
Counts
CONTROL
E2
(2000 nM)
10^0
10^1
10^2
10^3
10^4
FL1-Height


100
0
Counts
CONTROL
3NPA
10^0
10^1
10^2
10^3
10^4
FL1-Height


100
Counts
0
3NPA+E2 (200 nM)
3NPA
10^0
10^1
10^2
10^3
10^4
FL1-Height


100
Counts
0
3NPA+E2(2000 nM)
3NPA
10^0
10^1
10^2
10^3
10^4
Fl 1-Hainht

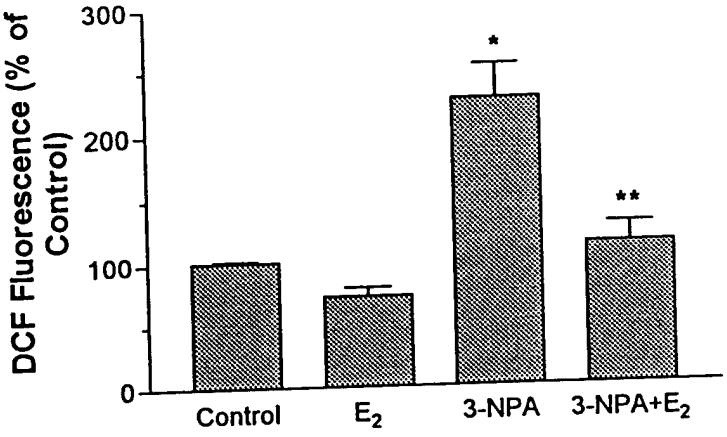


Figure 9. Effect of pretreatment with 17β-E_2 for 5.5 hr on hydrogen peroxide levels after exposure to 3-NPA. Cultures were exposed for 2 hr to the indicated treatments. Values are the mean ± SEM for 4-6 group cultures. *$p<0.001$ compared with control and E_2 alone values; **$p<0.001$ compared with cultures not pretreated with E_2.

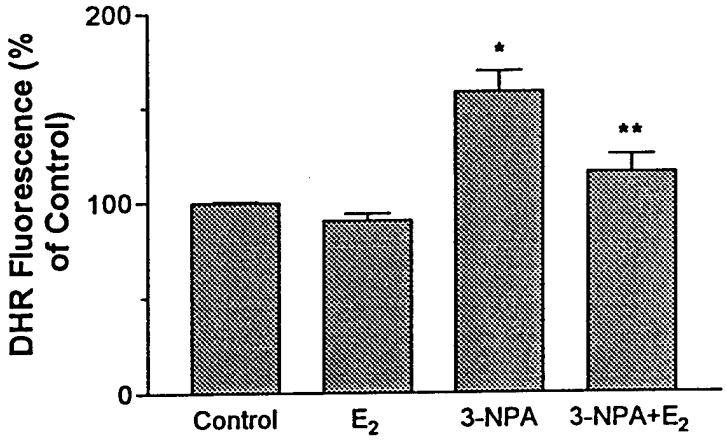


Figure 10. Effects of pretreatment of 17β-E_2 for 5.5 hr on peroxynitrite levels after exposure to 3-NPA. Cultures were exposed for 2-4 hr to the indicated treatments. Values are the mean ± SEM of determinations made in 4-6 group cultures. *$p<0.001$ compared with control and E_2 alone values; **$p<0.01$ compared with cultures not pretreated with E_2.

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Glutamate Receptor Requirement for Neuronal Death from Anoxia–Reoxygenation: An *in Vitro* Model for Assessment of the Neuroprotective Effects of Estrogens

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SUMMARY

1. Previous studies demonstrated that estrogens, specifically 17β-estradiol, the potent, naturally occuring estrogen, are neuroprotective in a variety of models including glutamate toxicity. The aim of the present study is twofold: (1) to assess the requirement for glutamate receptors in neuronal cell death associated with anoxia–reoxygenation in three cell types, SK-N-SH and HT-22 neuronal cell lines and primary rat cortical neuronal cultures, and (2) to evaluate the neuroprotective activity of both 17β-estradiol and its weaker isomer, 17α-estradiol, in both anoxia-reoxygenation and glutamate toxicity.

2. SK-N-SH and HT-22 cell lines, both of which lack NMDA receptors as assessed by MK-801 binding assays, were resistant to both anoxia–reoxygenation and glutamate-induced cell death. In contrast, primary rat cortical neurons, which exhibit both NMDA and AMPA receptors, were sensitive to brief periods of exposure to anoxia–reoxygenation or glutamate. As such, there appears to be an obligatory requirement for NMDA and/or AMPA receptors in neuronal cell death resulting from brief periods of anoxia followed by reoxygenation.

3. Using primary rat cortical neuronal cultures, we evaluated the neuroprotective activity of 17β-estradiol (1.3 or 133 n*M*) and 17α-estradiol (133 n*M*) in both anoxia–reoxygenation and excitotoxicity models of cell death. We found that the 133 n*M* but not the 1.3 n*M* dose of the potent estrogen, 17β-estradiol, protected 58.0, 57.5, and 85.3% of the primary rat cortical neurons from anoxia–reoxygenation, glutamate, or AMPA toxicity, respectively, and the 133 n*M* dose of the weak estrogen, 17α-estradiol, protected 74.6, 81.7, and 85.8% of cells from anoxia–reoxygenation, glutamate, or AMPA toxicity, respectively. These data demonstrate that pretreatment with estrogens can attenuate glutamate excitotoxicity and that this protection is independent of the ability of the steroid to bind the estrogen receptor.

KEY WORDS: SK-N-SH neuroblastoma cells; HT-22 cells; rat primary cortical neurons; anoxia–reoxygenation; glutamate; 17β-estradiol; 17α-estradiol.

INTRODUCTION

L-Glutamate, the major excitatory amino acid neurotransmitter in the brain, is involved in many neurologic functions such as cognition, memory, sensation, and

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movement (Gasic and Hollmann, 1992). However, glutamate neurotoxicity, also referred to as excitotoxicity (Olney, 1986), may mediate neuronal injury and cell death in several pathologic conditions including stroke, epilepsy, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and AIDS-related dementia (for review article see Lipton and Rosenberg, 1994). Excitotoxicity appears to be a multifaceted process that includes excessive influx of sodium and calcium ions into neurons through cationic channels which have been triggered by glutamate receptor activation (Choi, 1992). When released from presynaptic endings, glutamate activates several receptors including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate and the *N*-methyl-D-aspartate (NMDA) ionotropic receptors. Sodium influx through the AMPA/kainate channels causes membrane depolarization and initiates calcium influx through the NMDA channel. The resulting high concentrations of intracellular calcium leads to a cascade of events including the production of toxic free radicals (Braughler and Hall, 1989).

Extracellular glutamate levels rise in proportion to the duration of experimental ischemia (Benveniste *et al.,* 1984; Hagberg *et al.,* 1985; Choi, 1987), and further, extracellular glutamate is directly involved in the accumulation of intracellular calcium and the generation of free radicals resulting from ischemic episodes (Valazquez *et al.,* 1997). In focal ischemia and cerebral hypoxia, waves of spreading depression have been shown to cause glutamate release which may contribute to the ischemic damage seen in penumbral tissue (for review article see Siesjo and Bengtsson, 1989). Glutamate receptor antagonists have been shown to ameliorate the damage seen with excess glutamate. Both NMDA receptor antagonists, such as MK-801, and selective AMPA receptor antagonists, such as NBQX, are effective in reducing damage following focal ischemia caused by middle cerebral artery occlusion in animal models (Park *et al.,* 1988; Buchan *et al.,* 1991). Although glutamate antagonists are neuroprotective, their evaluation in stroke and head injury studies reveal that these compounds produce psychomimetic effects (Bullock, 1995).

Recent studies have revealed that estrogens are potent neuroprotective agents. *In vitro* studies have shown that 17β-estradiol (17β-E2), the naturally occurring potent feminizing estrogen, reduces neuronal damage caused by serum deprivation (Bishop and Simpkins, 1994; Green *et al.,* 1997a, b), β-amyloid treatment (Behl *et al.,* 1995; Goodman *et al.,* 1996; Green *et al.,* 1996, 1998), and exposure to glutamate receptor agonists (Behl *et al.,* 1995; Goodman *et al.,* 1996; Singer *et al.,* 1996). Also, 17β-E2 enhances outgrowth of cortical neurons (Brinton, 1993; Brinton *et al.,* 1997a, b) and neuronal survival through an NMDA-dependent mechanism (Brinton *et al.,* 1997a). 17α-Estradiol (17α-E2), the presumed inactive isomer of 17β-E2 and the naturally occurring weaker nonfeminizing estrogen, has also been shown to be neuroprotective in several studies (Green *et al.,* 1996, 1997a, b, 1998). Recently, we demonstrated that treatment with either 17β-E2 or 17α-E2 markedly reduces ischemic brain damage induced by middle cerebral artery occlusion in ovariectomized rats (Simpkins *et al.,* 1997). In addition, clinical studies suggest that estrogens may have beneficial effects against several pathological conditions including stroke (Wren, 1992; Finucane *et al.,* 1993; Paganini-Hill, 1995) and dementia of the Alzheimer's type (Fillit *et al.,* 1986; Ohkura *et al.,* 1994, 1995; Henderson *et al.,* 1994).

In view of the aforementioned evidence that glutamate may mediate part of

the neuronal damage during ischemia and that estrogens are potent neuroprotectants, we undertook the present study with two aims: first, to determine if functional glutamate receptors are required for anoxia/reoxygenation-induced neuronal cell death *in vitro* and, second, to assess the efficacy of 17β-E2 and 17α-E2 in protecting neurons from the effects of anoxia-reoxygenation and glutamate excitotoxicity.

MATERIALS AND METHODS

SK-N-SH and HT-22 Cell Cultures

SK-N-SH human neuroblastoma cells were obtained from the American Type Tissue Collection (Rockville, MD). HT-22, an immortalized mouse hippocampal cell line, was a gift from Dr. D. Schubert (The Salk Institute, San Diego, CA). Both cell lines were grown to confluency in RPMI-1640 medium (Fisher Scientific, Inc., Orlando, FL) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin G, and 100 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) in monolayers in 150-cm^2 Nunc flasks (Fisher Scientific, Inc.) at 37°C under 95% air, 5% CO_2. Twenty-four hours prior to experimentation, cells were plated at a density of 50,000 cells per well in 16-well chamber plates (Fisher Scientific, Inc.).

Primary Rat Cortical Cultures

Primary neuronal cultures were prepared according to methods described elsewhere (Chandler *et al.,* 1993). Briefly, cortical tissue from 1-day-old Sprague–Dawley pups (Charles River Farms, Wilmington, MA) was separated from blood vessels and pia mater and sectioned into approximately 2-mm chunks. Tissue was then trypsinized in an isotonic salt solution (pH 7.4) at 37°C. Cells were treated with DNAse 1 in the presence of Dulbecco's modified eagle's medium (DMEM; GIBCO, Gaithersburg, MD) containing 10% plasma-derived horse serum (PDHS; Central Biomedia, Irwin, MO) and triturated. The cell suspensions were centrifuged and the resulting cell pellet was suspended in DMEM with 10% PDHS, plated on precoated poly-L-lysine 35-mm culture dishes at a density of 4×10^6 cells per dish, and incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°C. Cells were exposed to β-cytosine arabinoside on days 3 and 4 and neurons in the following experiments were 9–12 days *in vitro.* At this time, cultures consisted of approximately 90% neurons and 10% astroglia.

Cell Viability

Viability for all three cell types was determined using 1 μM calcein AM (Molecular Probes, Eugene, OR) and either 1 μM ethidium homodimer-1 (Molecular Probes) or 10 μM propidium iodide (Sigma Chemical Co.) in phosphate-buffered saline, pH 7.4 (PBS; GIBCO). Live cells were distinguished by the presence of intracellular esterase activity, which cleaves the calcein AM dye, producing a bright

green fluorescence when excited. Both ethidium homodimer and propidium iodide enter cells with damaged membranes and, upon binding to nucleic acids, produce a red fluorescence. Culture plates were viewed with a fluorescent microscope (Nikon Diaphot-300). Two microscopic fields were randomly selected and photographed, and the average number of live cells per field was determined by counting the number of bright green cells.

Anoxia–Reoxygenation, AMPA, and Glutamate Exposure

Anoxic insults were conducted using a modular incubator chamber (Billups–Rothenberg, Del Mar, CA). Cells were placed in the sealed chamber, flushed with 100% N_2 for 15 min, and incubated in the chamber under anoxic conditions for 15 min to 24 hr at 37°C. Cells were returned to a normal atmosphere (95% air and 5% CO_2) and viability was determined 24 hr from completion of anoxia. SK-N-SH and HT-22 cells were exposed to 10 m*M* glutamate (Sigma Chemical Co.), and primary rat neuronal cultures were exposed to 20 μM AMPA (Sigma Chemical Co.) or 50 μM glutamate for 24 hr prior to viability assessment.

^{3}H-MK-801 Receptor Binding Assay

Cerebral cortex, obtained immediately after sacrifice of adult female Sprague–Dawley rats, was homogenized in 50 vol (wt/v) of ice-cold Tris buffer (50 m*M*, pH 7.4) containing 1 m*M* Na_2EDTA and 0.1 m*M* phenylmethanesulfonyl fluoride. SK-N-SH and HT-22 cells were lifted with 0.02% EDTA and the primary rat cortical neurons were removed from 35-mm dishes using a manual cell-lifter (Fisher Scientific, Inc.). The homogenates were centrifuged at 39,000*g* for 10 min at 4°C. The pellets were resuspended in buffer and centrifuged three times, and aliquots were stored in separate tubes at −80°C for receptor binding assays.

MK-801 (Research Biomedicals International, Natick, MA) binding was measured in 5 m*M* Tris buffer that contained 100 n*M* ^{3}H-MK-801 (Amersham Life Sciences, Inc., Arlington Heights, IL), 300 μM glycine, and 1 m*M* glutamate. Nonspecific binding was defined by the addition of 1 m*M* unlabled MK-801. After 30 min of incubation at room temperature, the samples were filtered using GF/C filters in a Brandel tissue harvester and washed with ice-cold Tris buffer. Bound radioactivity was determined using scintillation spectrophotometry. Protein in the homogenates was assayed using the Bradford (1976) method.

Steroid Treatment

Cultures were treated with the following: DMEM as volume controls or 2.78 μM hydoxypropyl-β-cyclodextrin (HPCD, Pharmos, Alachua, FL) and 3.25 μM glutathione (GSH, reduced; Sigma Chemical Co.) as vehicle controls. Steroids were assessed in the aforementioned media by the addition of 1.33 or 133 n*M* 17β-E2 (17β-E2, cyclodextrin-encapsulated; Sigma Chemical Co.) or 133 n*M* 17α-E2 (Steraloids Inc., Wilton, NH) complexed in HPCD. All groups were pretreated 24 hr before anoxia or glutamate challenge.

The 17α-E2 and HPCD was complexed by adding 68 mg of the steroid dissolved in 1 ml of ethanol (95%; Aaper Alcohol and Chemical Co., Shelbyville, KY) to the HPCD dissolved in ddH_2O, mixed, and filtered (0.02 μm; Nalgene, Rochester, NY) under vacuum, and the filtrate was freeze-dried and stored frozen (−80°C). High-performance liquid chromatographic (HPLC) assay was used to determine the degree of incorporation of the steroid into the hydroxypropyl-β-cyclodextrin complex. The system consisted of a Thermo Separation/Spectra Physics (Fremont, CA) SP 8810 isocratic pump, a Rheodyne (Cotati, CA) Model 7125 injector valve equipped with a 20-μl sample loop, a SP 8450 variable wavelength UV/VIS detector operated at 254 nm, and a SP 4290 computing integrator (Spectra Physics, Fremont, CA). A 15-cm × 4.6-mm-I.D. Alltima octadecylsilica (d_p = 5 μm) reversed-phase column (Alltech, Deerfield, IL) was used for separation. The mobile phase was a water/acetonitrile mixture (60/40; v/v) and the flow rate was maintained at 1.0 ml/min. The inclusion complex was dissolved in the mobile phase at 10 mg/ml concentration, and the amount of steroid per gram of complex was determined based on an external calibration curve obtained by analyzing a series of steroid solutions of known concentration.

Statistical Analysis

All statistical evaluations used two-tailed Student's *t*-tests followed by Scheffe's test. $P < 0.05$ was considered significant. Each group consisted of 3–10 samples. All statistical analyses were performed on raw data.

RESULTS

The Effects of Anoxia–Reoxygenation and Glutamate on SK-N-SH, HT-22, and Primary Rat Neuronal Cell Cultures

Four hours of anoxia followed by 24 hr of reoxygenation was extremely toxic to primary rat neurons but not to SK-N-SH or HT-22 cells (Fig. 1). This protocol caused a 90.4% decrease in the number of live primary rat cortical neurons but only a 0.7% decrease in live SK-N-SH cell number and a 5.9% decrease in live HT-22 cell number. When SK-N-SH cells were challenged with 24 hr of anoxia and 24 hr of reoxygenation, there was a 98.1% decrease in the number of live cells (data not shown), indicating that prolonged anoxia can kill SK-N-SH cells. Glutamate exposure was toxic to primary rat neurons but not to SK-N-SH or HT-22 cells (Fig. 1). A 24 hr exposure of primary rat neuronal cultures to 50 μM glutamate resulted in a 74.3% decrease in the number of live cells per field examined. Treatment of either SK-N-SH or HT-22 cells with 10 mM glutamate for 24 hr caused no significant cell loss, therefore no protective studies could be performed on the latter two cell types.

^{3}H-MK-801 Binding in SK-N-SH, HT-22, and Primary Rat Neuronal Cells

The number of MK-801 binding sites was significantly lower in SK-N-SH and HT-22 cells than in rat cerebral cortex (Table I). Rat cerebral cortex demonstrated

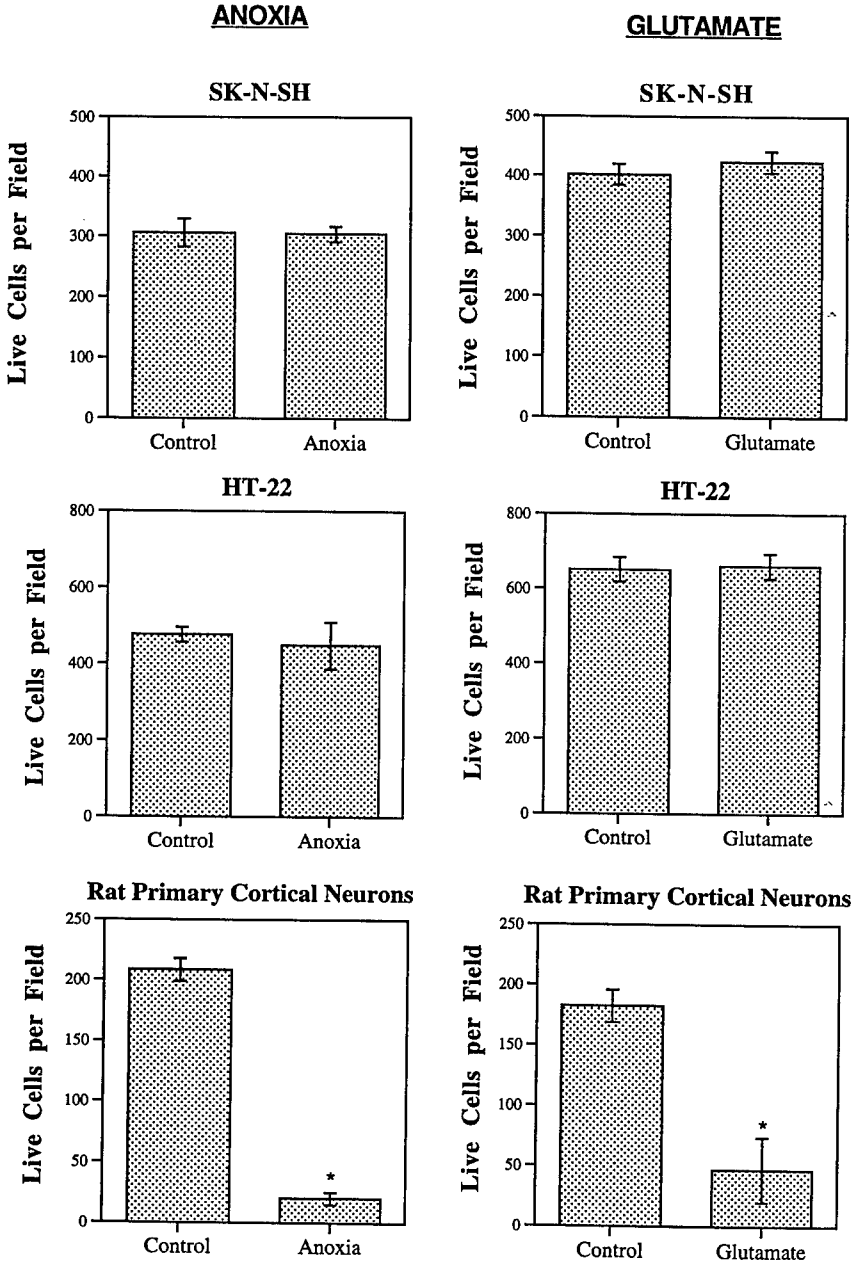


Fig. 1. Effects of anoxia–reoxygenation or glutamate exposure on SK-N-SH, HT-22, and primary rat cortical neuronal cultures. All three cell types were exposed to anoxia for 4 hr and reoxygenated for 24 hr prior to the determination of cell viability. SK-N-SH and HT-22 cells were exposed to 10 m*M* glutamate and primary rat neuronal cultures to 50 μM glutamate for 24 hr prior to viability assessment. $^*P < 0.05$ versus control cultures. The mean ± SE for $n = 3$–8 wells per group is shown.

Table I. MK-801 Binding to Rat Cortex and Various Cell Types

Cell type	*n*	MK-801 binding (fmol/μg protein ± SE)
Cortex	4	2.11 ± 0.26
HT-22	7	0.44 ± 0.21*
SK-N-SH	7	0.71 ± 0.22*
Rat primary neurons	3	1.12 ± 0.05

* $P < 0.05$ versus cortex.

specific ^{3}H-MK-801 binding of 2.11 ± 0.26 fmol/μg protein and primary rat cortical neurons exhibited a specific binding of 1.12 ± 0.05 fmol/μg protein, whereas HT-22 and SK-N-SH cells specifically bound only 0.44 ± 0.21 and 0.71 ± 0.22 fmol/μg protein, respectively.

Time-Dependent Response of Primary Rat Cortical Neurons to Anoxia–Reoxygenation

The time-dependent effect of anoxia–reoxygenation on primary rat cortical neurons is depicted in Fig. 2. After 15 and 30 min of anoxia and 24 hr of reoxygenation, primary rat cortical neurons exhibited a 25.2 and a 34.6% decline in live cells, respectively. Following 1 hr of anoxia and 24 hr of reoxygenation, the live cell number decreased by 65.0%. Therefore, we chose 1 hr of anoxia to test the efficacy of 17β-E2 as a neuroprotective agent.

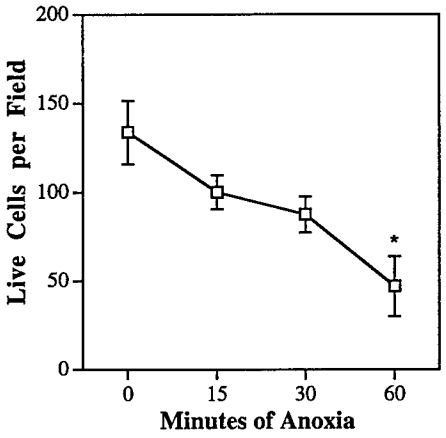
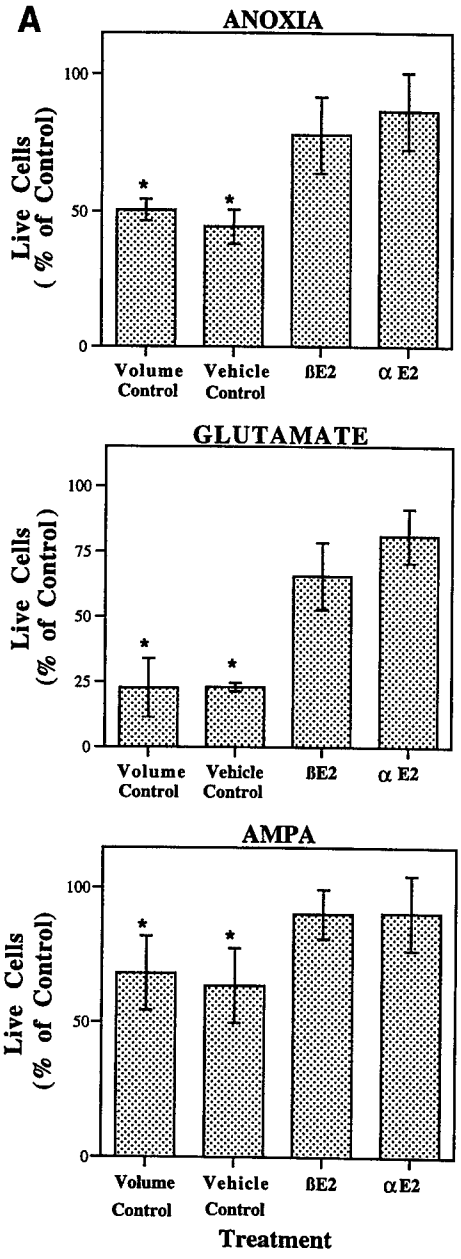
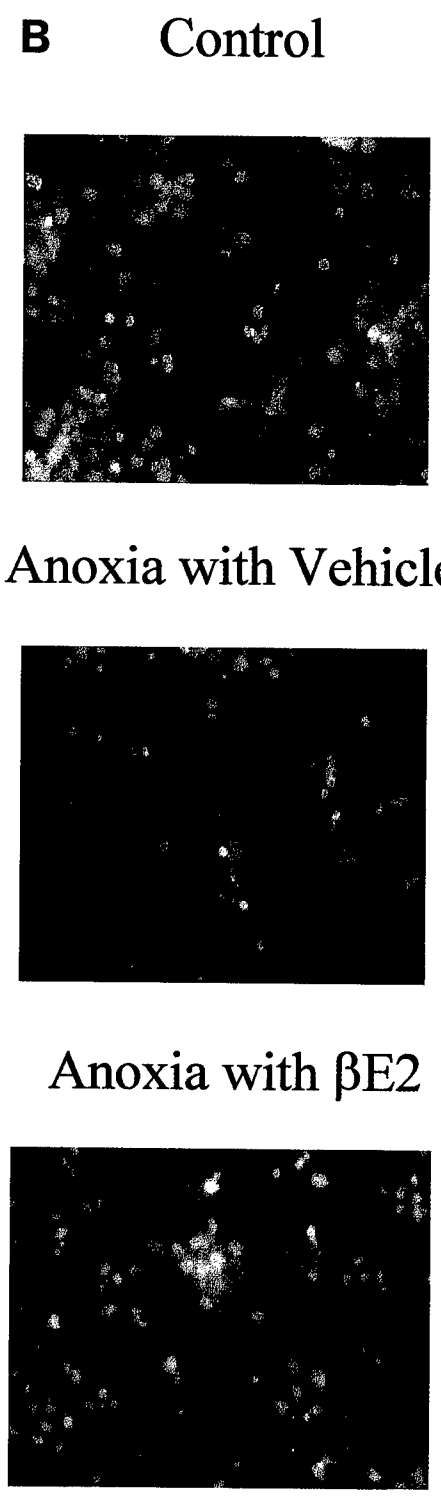


Fig. 2. Time-dependent response of primary rat cortical neurons to anoxia–reoxygenation. Cells were exposed to 15, 30, or 60 min of anoxia and reoxygenated for 24 hr at 37°C. *$P < 0.05$ versus control. The mean ± SE for $n = 4$ is shown.


A
ANOXIA
Live Cells (% of Control)
100
50
0
Volume Control
Vehicle Control
ßE2
α E2
GLUTAMATE
Live Cells (% of Control)
100
75
50
25
0
Volume Control
Vehicle Control
ßE2
α E2
AMPA
Live Cells (% of Control)
100
50
0
Volume Control
Vehicle Control
ßE2
αE2
Treatment


B
Control
Anoxia with Vehicle
Anoxia with βE2

Protection of Primary Rat Neuronal Cultures with 17β-Estradiol or 17α-Estradiol Against Anoxia–Reoxygenation, Glutamate, and AMPA Toxicity

17β-E2 and 17α-E2 exhibited neuroprotective activity against anoxia–reoxygenation (Fig. 3), glutamate (Fig. 3A), and AMPA (Fig. 3A) toxicity in primary rat neuronal cultures. Primary neurons exposed to 1 hr of anoxia and 24 hr of reoxygenation as volume controls or as vehicle controls showed a 49.6 and 55.7% reduction in live cell number, respectively; neurons pretreated with 17β-E2 (133 n*M*) or 17α-E2 (133 n*M*) protected 58.0 and 74.6% of cells, respectively. Similar observations were made with the groups challenged with 50 μ*M* glutamate (Fig. 3A). Primary neurons exposed to glutamate as volume controls or as vehicle controls demonstrated a 74.3 and 77.4% reduction in live cell number, respectively, while neurons pretreated with 17β-E2 (133 n*M*) or 17α-E2 (133 n*M*) protected 57.5 and 81.7% of cells, respectively. We did not observe a neuroprotective effect at a lower concentration of 17β-E2 (1.3 n*M*) during 1 hr of anoxia and 24 hr of reoxygenation (data not shown). Primary neurons exposed to AMPA as volume controls or vehicle controls demonstrated a 31.7 and 36.3% reduction in live cell number, respectively, while neurons pretreated with 17β-E2 (133 n*M*) or 17α-E2 (133 n*M*) protected 85.3 and 85.8% of cells, respectively.

DISCUSSION

The present study demonstrates that glutamate receptors are obligatory for the cell death caused by short-term anoxia followed by reoxygenation. Anoxia–reoxygenation and glutamate exposure was toxic to primary rat cortical neurons but not HT-22 or SK-N-SH cells. In comparison to the rat cerebral cortex, the number of MK-801 binding sites was significantly lower in SK-N-SH and HT-22 cells but not in primary rat cortical neurons. This suggests that the number of NMDA receptors correlates with the response of cells to anoxia–reoxygenation and glutamate excitotoxicity.

17β-E2 provided significant protection of primary rat cortical neurons against anoxia–reoxygenation, glutamate, and AMPA toxicity. This finding is consistent with previous observations where blockade of AMPA receptors (Frandsen *et al.*, 1989; Buchan *et al.*, 1991) or NMDA receptors (Goldberg *et al.*, 1987; Pauwels, 1989; Michaels and Rothman, 1990) was found to be protective against hypoxic, ischemic, or excitotoxic insults *in vitro* and *in vivo*. The findings are also consistent with the observations of Singer *et al.* (1996) and of Goodman *et al.* (1996), who

Fig. 3. (Opposite) (A) Protection of primary rat cortical neuronal cultures with 17β-estradiol and 17α-estradiol. Cells were exposed to 1 hr of anoxia and 24 hr of reoxygenation, 50 μ*M* glutamate for 24 hr, or 20 μ*M* AMPA for 24 hr prior to cell viability assessment. Groups received either DMEM as volume controls, 2.78 μ*M* HPCD and 3.25 μ*M* GSH as vehicle controls, or either 133 n*M* 17β-E2 and 3.25 μ*M* GSH or 133 n*M* 17α-E2 and 3.25 μ*M* GSH as treatment groups. Data are presented as the percentage of live cells with reference to control cultures not exposed to the toxic insult. $*P < 0.05$ versus DMEM control cultures. The mean ± SE for $n = 5$–10 is shown. (B) Photomicrographs of representative primary rat cortical neurons exposed to anoxia–reoxygenation in the presence or absence of 17β-E2. Cells were photographed at 200× total magnification.

demonstrated that estrogen protects primary rat cortical neurons and hippocampal neurons, respectively, from glutamate excitotoxicity. Further, Behl *et al.* (1995) demonstrated that 17β-E2 protects HT-22 cells from oxidative stress induced by glutamate. Finally, estrogen-induced neuronal growth is blocked by NMDA receptor antagonists (Brinton *et al.,* 1997a). The concentration of 17β-E2 at which we observed neuroprotection (133 n*M*) is within the range of previous *in vitro* studies examining glutamate toxicity [15 n*M* (Singer *et al.,* 1996) to 10 μ*M* (Behl *et al.,* 1995)].

It is interesting to note that the effective concentration of 17β-E2 needed for neuroprotection against glutamate and anoxia-reoxygenation far exceeded the K_i of 17β-E2 binding, which is approximately 0.13 n*M* for both known estrogen receptors (Kuiper *et al.,* 1997). An estrogen receptor-mediated neuroprotective effect should have been, but was not, observed at concentrations that approach the K_i for 17β-E2 binding. The high concentration of estradiol required for neuroprotection suggests that this effect is not mediated by an estrogen receptor.

We also demonstrated that the weak or inactive nonfeminizing estrogen, 17α-E2, is as effective as the naturally occurring, potent feminizing estrogen, 17β-E2, in protecting primary rat cortical neurons against anoxia–reoxygenation, glutamate, or AMPA toxicity. Behl *et al.* (1997) observed that 17α-E2 (10 μ*M*) protects HT-22 cells against glutamate (1 m*M*). 17α-E2 has long been characterized as a weak estrogen receptor agonist (Huggins *et al.,* 1954; Korenman, 1969; Clark *et al.,* 1982; Kneifel *et al.,* 1982; Clark and Markaverich, 1983; Lubahn *et al.,* 1985). With this in mind, the findings argue against the obligatory involvement of the nuclear estrogen receptor–estrogen responsive element in the neuroprotective signal that follows anoxia–reoxygenation or glutamate toxicity.

HT-22 cells have been shown to exhibit glutamate toxicity in other studies (Davis and Maher, 1994; Behl *et al.,* 1995). The two major differences that exist between these previous studies and the present study are the plating density and the type of culture medium used. The plating density in our HT-22 studies was 10-fold higher than those in the previous studies. It has been demonstrated several times that high plating densities increase the survival of cells *in vitro* (Speicher *et al.,* 1981; O'Malley *et al.,* 1991), which is probably due to greater concentrations of growth factors at higher plating densities (Hartikka and Hefti, 1988). We have recently observed that HT-22 cells are more sensitive to β-amyloid at lower plating densities (data not shown). Moreover, our studies used HT-22 cells cultured in RPMI-1640 medium, which includes the antioxidant glutathione (3.25 μ*M*), while the other studies used DMEM, which lacks glutathione. Murphy *et al.* (1989) demonstrated that glutamate toxicity can result from inhibition of the cysteine transport, which leads to a subsequent decrease in intracellular glutathione levels and results in oxidative stress. We found that HT-22 cells subjected to 4 hr of anoxia and 24 hr of reoxygenation plated in DMEM supplemented with glutathione (3.25 μ*M*) exhibited only a 5.9% decrease in live cells, while HT-22 cells plated in DMEM without glutathione revealed a significant 34.6% reduction in live cell number (data not shown). This finding supports the findings of Murhpy *et al.* (1989) which suggest that glutathione may be protecting cells from oxidative stress and subsequent cell death.

Cultured primary rat cortical neurons had lower MK-801 specific binding sites

than observed in adult rat cortex, an observation consistent with that reported using thienylcyclohexylpiperidine to identify NMDA receptors (Brinton *et al.,* 1997a). It is likely that the culturing process is selective for those cells that possess the lowest number of NMDA receptors by killing cells with high NMDA receptor numbers. We suspected that glutamate-induced toxicity may account for the low binding of MK-801 to SK-N-SH and HT-22 cells because the RPMI-1640 medium, used for culturing both cell types, contains L-glutamate (180 μM) and that the high concentration of glutamate in the medium may have selected for cells which lack significant numbers of glutamate receptors. However, we found that SK-N-SH and HT-22 cells cultured only in DMEM prior to glutamate exposure (10 mM for 24 hr) were still nonresponsive (data not shown). This finding supports that both SK-N-SH and HT-22 cells lack functional glutamate receptors and therefore are resistant to anoxia–reoxygenation and glutamate toxicity.

The mechanism of neuroprotection by estrogens during anoxia–reoxygenation is not currently known, although several possibilities are apparent. Estrogens could interfere with glutamate neurotoxicity by antagonizing the AMPA or the NMDA receptor, as our findings suggest, or estrogens could interact with a mechanism that occurs subsequent to the glutamate receptor activation, such as sodium or calcium influx (for review see Choi, 1988; Dugan and Choi, 1994; Morley *et al.,* 1994). Wong and Moss (1992) demonstrated that 17β-estradiol, but not 17α-estradiol, has short-term electrophysiological potentiation effects on AMPA, kainate, and quisqualate, but not on NMDA receptor responses, and that depolarizing responses to exogenous glutamate were also potentiated by the steroid. Recently, Weaver *et al.* (1997) reported that 17β-E2 (50 μM) antagonized NMDA-induced calcium currents. However, based on previous MK-801 binding studies, we observed no interaction of 17β-E2 with the MK-801 site on NMDA receptors derived from rat cerebral cortical plasma membranes (C. E. Simpkins *et al.,* unpublished observations). This does not exclude the possibility of other estradiol interactions that are independent of antagonist binding.

Another possibility is that estrogens exert the observed neuroprotective effects through an antioxidant mechanism. Sugioka *et al.* (1987) demonstrated antioxidant activities of estrogens against lipid peroxidation. The proton donating capacity of the phenolic A ring of estrogen may account for this activity. Our laboratory and others have used structure–activity relationship studies to reveal that a phenolic A ring (Green *et al.,* 1997b; Behl *et al.,* 1997) and at least three rings of the steroid structure (Green *et al.,* 1997b) are required for the molecule to demonstrate neuroprotection, suggesting an antioxidant component in neuroprotection. Glutamate toxicity involves reactive oxygen species (Reynolds and Hastings, 1995) and lipid peroxidation is a component of excitotoxic death (Azbill *et al.,* 1997). Further, several studies have demonstrated that estrogens decrease lipid peroxidation (Nakano *et al.,* 1987; Sugioka *et al.,* 1987; Lacort *et al.,* 1995; Takanashi *et al.,* 1995; Goodman *et al.,* 1996; Tang *et al.,* 1996; Behl *et al.,* 1997; Gridley *et al.,* 1998).

In summary, the present study has demonstrated that glutamate receptors are obligatory for neuronal death due to brief exposures of anoxia–reoxygenation or glutamate excitotoxicity and that both 17β-E2 and 17α-E2 are effective agents in the protection of cultured primary rat cortical neurons from either insult. Our

findings are consistent with animal studies showing a neuroprotective effect of estrogen in stroke (Shi *et al.,* 1997; Simpkins *et al.,* 1997; Zhang *et al.,* 1998; Alkayed *et al.,* 1998; Dubal *et al.,* 1998, 1998) and with clinical observations that estrogen replacement therapy is associated with a reduction in stroke-related deaths (Wren, 1992; Finucane *et al.,* 1993; Paganini-Hill, 1995). The therapeutic implications of these results suggest that the use of feminizing as well as nonfeminizing estrogens may offer protection during ischemia as well as during other neuropathological conditions.

ACKNOWLEDGMENTS

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INHIBITION OR DOWN-REGULATION OF PROTEIN KINASE C ENHANCES ESTROGEN-INDUCED NEUROPROTECTION IN AN *IN VITRO* MODEL.

D.G. Watson*, C. Fiola, and J.W. Simpkins. Department of Pharmacodynamics and Center for the Neurobiology of Aging. University of Florida College of Pharmacy. Gainesville, FL 32610.

The neuroprotective effects of estrogen compounds have been reported in a wide variety of animal and cell culture models. However, the mechanism(s) responsible for estrogen's neuroprotective effects are currently not well understood. In the present studies, the role of protein kinase C (PKC) in mediating estradiol-induced neuroprotection was examined in an immortalized hippocampal cell line. HT-22 cells lack classical nuclear estrogen receptors (ER), eliminating ER-mediated effects as a potential site of neuroprotective action. The neuroprotection model utilized calcein fluorescence to quantitate cell viability following an 18-24 h exposure to glutamate (5-10 mM). In this model, estradiol-induced neuroprotection was observed at concentrations above 1 μM. Inhibition of PKC by bis-indolylmaleimide (BIM, 1 μM) or LY333531 (1 μM) significantly enhanced estradiol-induced neuroprotection when administered prior to the glutamate insult. Similarly, down-regulation of PKC by exposure of HT-22 cells to the phorbol ester PMA also enhanced estrogen-induced neuroprotection. Furthermore, we demonstrated that PKC inhibition and down-regulation were also neuroprotective in a dose-dependent manner in the absence of estradiol. In this model, neuroprotection was also observed following inhibition of MAPK signaling pathway by the MEK inhibitor PD98059. Since we observed that exposure of HT-22 cells to estradiol alters the expression and intracellular distribution of at least one PKC isozyme (epsilon), these data suggest a potential role for PKC and the MAPK signaling pathway in mediating the neuroprotective effects of estrogens. These data support the hypothesis that PKC inhibition or down-regulation mediates a neuroprotective signal from estrogen, which is then propagated through the MAPK pathway and ultimately results in altered gene expression beneficial to cell survival.

(Supported by NIH AG10485, U.S. Army grant DAMD 17-99-1-9473, and Apollo BioPharmaceutics, Inc.)

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Nitric Oxide May Mediate Estrogen's Neuroprotection Through a Receptor-Independent Mechanism.

Y. Wen*, E.J. Perez, P. Green and J.W. Simpkins, Department of Pharmacodynamics and Center for Neurobiology of Aging, University of, Florida Gainesville, FL 32610

Estrogens are known to have neuroprotective activity in both *in vivo* and *in vitro* models. Our observation of the lack of correlation between structure-activity relationships for neuroprotection and estrogenicity and potent neuroprotection by estrogens in cell types which lack known estrogen receptors indicate that this activity of estrogens is not mediated by classical estrogen receptor transcriptional activity. In the present study we use H_2O_2 as an insult to measure cytotoxicity using assays for both cell viability and Caspase3-like activity. We observed that both 17β-estradiol (E2) and NO are protective against H2O2 toxicity in SK-N-SH cells. We use DAF2-DA, a cell permeable NO specific dye to examine the effects of E2 on NO production. We observed that E2 induces NO within 10 min, with an EC50 of 8 nM. The protection of E2 can be blocked by a partial nNOS inhibitor L-NNA. We also examined cGMP which is a major mediator of NO's biological functions. We observed that cGMP also protects against H_2O_2 toxicity and NO's protection can be attenuated by ODQ, a cGMP inhibitor. The above data indicate NO may mediate estrogen's potent neuroprotection in a manner that is independent of classical estrogen receptors. (Supported by AG 10485, Apollo BioPharmaceutics, Inc. and U.S. Army Grant, DAMS 17-99-1-9473).

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ESTRADIOL PROTECTS AGAINST ATP DEPLETION AND MITOCHONDRIAL MEMBRANE POTENTIAL DECLINE INDUCED BY 3-NITROPROPRIONIC ACID IN SK-N-SH HUMAN NEUROBLASTOMA CELLS.

J. WANG*, E. J. PEREZ, P. S. GREEN, J. W. SIMPKINS. Dept. of Pharmacodynamics, Center for the Neurobiology of Aging, University of Florida, Gainesville, FL 32610

Mitochondria are increasingly recognized as the important target of toxicity during ischemia, hypoxia and toxic chemical exposures. Mitochondria dysfunction leading to ATP depletion may be a common pathway of cell death in these cases. Estrogens have been reported to be neuroprotective against excitotoxicity, oxidative injury and chemical toxicity in experiment models and been proposed to play a role in the modulation of cerebral energy/glucose metabolism. To address whether estrogens are involved in the regulation of mitochondrial function, we chose 3-nitroproprionic acid (3-NPA, a succinate dehydrogenase inhibitor) to uncouple oxidative phosphorylation. ATP levels in SK-N-SH cells were measured after exposed to 3-NPA. The result showed that 3-NPA (10 mM) initially increased ATP levels with peak maxima occuring at 2 h then caused a 40% and a 50%-80% decrease in ATP levels when treated for 12 h and 24 h, respectively. The ATP depletion induced by 3-NPA was attenuated by pretreating cells for 4-5 h with 17β-estradiol. For 12 h and 24 h 10 mM 3-NPA exposures, 17β-estradiol pretreatment caused a dose-dependent increase in ATP level back to 80% and 50%, respectively, of that in control cells. The function of mitochondria following exposures to 3-NPA were also assessed by evaluating the mitochondrial transmembrane potential ($\Delta\psi_m$) using the probe rhodamine 123. 3-NPA induced a 60% decrease in fluorescence intensity at 12 h exposure, an effect that presisted for 24 h. 17β-estradiol pretreatment eliminated this effect of 3-NPA on mitochondria transmembrane potential. This study provides evidence that these mitochondrial actions of 17β-estradiol may contribute to the neuroprotective effect of estrogens. (Supported by AG 10485, U.S. Army Grant DAMD 17-99-1-9473 and Apollo BioPharmaceutics, Inc.)

Key Words: (see instructions p. 4)

1. 17-beta-Estradiol
2. 3-nitroproprionic acid
3. ATP
4. mitochondrial transmembrane potential

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17-β ESTRADIOL CAN REDUCE SECONDARY ISCHEMIC DAMAGE AND MORTALITY OF SUBARACHNOID HEMORRHAGE

S-H. Yang*, Z. He, S. S. Wu, Y-J. He, J. Cutright, W. J. Millard, A. L. Day, J. W. Simpkins.
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Subarachnoid hemorrhage (SAH) is a unique disorder commonly occurring when an aneurysm ruptures, leading to bleeding and clot formation, with higher incidence in female. To evaluate the influence of 17-β estradiol (E2) in the outcome of SAH, SAH was induced by endovascular puncture of the intracranial segment of internal carotid artery in 15 intact female (INT), 19 ovariectomized female (OVX) and 13 ovariecomized female rats with E2 replacement (OVX+E2). Cerebral blood flow was recorded before and after SAH. All the animals were decapitated immediately after death or 24 hours after SAH for clot volume analysis. The brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for secondary ischemic lesion analysis. The CBF decreased to 29.6±17.7%, 22.8±8.3% and 43.5±22.9% on the ipsilateral side ($p=0.01$) and to 63.4±14.1%, 57.4±11.0% and 66.6±17.9% on the contralateral side ($p=0.26$) in INT, OVX and OVX+E2, respectively. The mortality was 53.3%, 68.4% and 15.4% in INT, OVX and OVX+E2, respectively ($p=0.01$), while no significant difference in clot volume was noted among groups. The secondary ischemic lesion volume was 9.3±8.4%, 24.3±16.3% and 7.0±6.4% in INT, OVX and OVX+E2, respectively ($p<0.01$). This study demonstrated that E2 can reduce the mortality and secondary ischemic damage in a SAH model without affecting the clot volume. (Supported by NIH grant AG 10485, Apollo BioPharmaceutics, Inc, and U.S. Army grant DAMD 17-99-1-9473)

Key Words: (see instructions p. 4)

1. Estradiol
2. Stroke
3. Neuroprotection
4. Cerebral Blood Flow

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ESTROGEN MODULATION OF BCL-2 FAMILY PROTEIN EXPRESSION.

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Estrogens are potent neuroprotective agents in a variety of animal and cell culture models. The purpose of this study was to explore the possible role for modulation of bcl-2 family protein expression in βE2-mediated neuroprotection. We examined βE2-induced changes in expression of bcl-2, bcl-x_L, and bax by Western blot analysis in three different neuronal types. SK-N-SH neuroblastoma cells, HT-22 transformed neuronal cells, and primary neocortical neurons were chosen as βE2 attenuated Aβ-induced toxicity at a 2 nM concentration in all three neuronal types. All three neuronal types showed significant bax immunoreactivity. Bcl-x_L immunoreactivity was readily detectible in both SK-N-SH cells and primary neuronal cells but not HT-22 cells. Bcl-2 was detected by our Western analysis only in the SK-N-SH cells. Exposure of SK-N-SH and primary neuronal cultures to βE2 for 24 h caused a dose-dependent increase in bcl-x_L immunoreactivity. The maximal effect was seen at a 10 nM βE2 concentration and resulted in about a 2.5-fold increase in bcl-x_L band density. No significant effect of βE2 on either bcl-2 or bax immunoreactivity was seen. These data suggest that modulation of bcl-2 family proteins, specifically increased bcl-x_L expression, could contribute to βE2-mediated neuroprotection in SK-N-SH cells and rat primary neocortical neurons.

(Supported by NIH AG 10485, US Army DAMD 17-99-1-9473, and Apollo BioPharmaceutics, Inc.)

Key Words: (see instructions p. 4)

1. Estradiol
2. Neuroprotection
3. BCL-XL
4. A Beta

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RESEARCH ARTICLE

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Effects of 17β-estradiol on glucose transporter 1 expression and endothelial cell survival following focal ischemia in the rats

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Abstract Estrogen replacement therapy in postmenopausal women is associated with a decreased mortality and morbidity from stroke. The present study was undertaken to investigate the effects of estrogen on endothelial cell glucose transporter 1 (GLUT 1) and on the cell viability during focal ischemia in a rat model. Female rats were ovariectomized (OVX) and 2 weeks later 17β-estradiol (E_2) was injected subcutaneously at a dose of 100 μg/kg 2 h before unilateral middle cerebral artery (MCA) occlusion. Ischemic lesion size was quantified using 2,3,5-triphenyl tetrazolium chloride (TTC) staining and GLUT 1 protein was analyzed by Western blotting. E_2 treatment decreased ischemic lesion size in slices taken at 9 and 11 mm posterior from the olfactory bulb by 46.3% and 44.1%, respectively ($P < 0.05$). GLUT 1 protein decreased in both OVX and E_2 groups by 24.6% and 22.7% respectively ($P < 0.05$) compared with the non-lesioned side in the core ischemic region, including the basal ganglia. GLUT 1 protein was increased in the E_2-treated group compared with the control group (23.3%, $P < 0.05$) in the penumbral ischemic region of the cortex. Primary rat brain capillary endothelial cell (BCEC) cultures were established as an in vitro model for ischemic effects on endothelial cells. Estrogen reduced BCEC loss by 35.9%, 28.4% and 23.5% ($P < 0.05$) when glucose in the culture medium was reduced to 50%, 20% and 10%, respectively; and by 28.4% and 18.4% ($P < 0.05$) following 1 or 4 h of anoxia, respectively. This study demonstrates that estrogen treatment increases GLUT 1 transporters and protects BCEC loss which may in turn reduce focal ischemic brain damage.

Key words Estrogen · Middle cerebral artery occlusion · Glucose transporter · Cerebral endothelium · Glucose metabolism

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Introduction

Stroke is the second major cause of death for women in the United States (Barnett 1990). Retrospective clinical trials reveal the beneficial effects of postmenopausal hormone replacement therapies (HRT) with estrogens alone or estrogens plus progestins. Both therapies reduce the morbidity and mortality of stroke and ischemic heart diseases (Duprez 1993; Berga 1994; Ravn et al. 1994). Using an animal model for focal ischemia, we have demonstrated that pretreatment with estradiol reduces by more than 50% the ischemic damage following middle cerebral artery (MCA) occlusion (Simpkins et al., unpublished observation). The underlying mechanism of this effect remains to be determined.

Glucose tranmsporter 1 (GLUT 1) protein and its mRNA, which are exclusively localized in the blood-brain barrier (BBB), account for almost 100% of the glucose uptake by brain capillary endothelial cells (BCEC) (Boado and Pardridge 1990; Pardridge et al. 1990), and increases in GLUT 1 may fulfill a compensatory neuroprotective role during and following ischemia (Gerhart et al. 1994; Lawrence et al. 1996). Ischemic brain insult in gerbil induces a 175% increase in the expression of GLUT 1 mRNA 1 day after ischemia (Gerhart et al. 1994). Additionally, rats transfected with the GLUT 1 transporter gene prior to MCA occlusion showed a greater neuronal survival in the ischemic hemisphere than rats treated with the control vectors only (Lawrence et al. 1996). This suggests that BBB GLUT 1 expression is a compensatory response to the ischemic episode and can reduce neuronal loss following ischemia.

We have demonstrated a modest but significant increase of 40% in GLUT 1 protein and mRNA expression

and in glucose transport in BCEC in response to estrogen replacement in ovariectomized (OVX) rats (Shi and Simpkins 1996). The present study was undertaken to determine the effects of 17β-estradiol (E_2) on GLUT 1 expression in the BBB of focal ischemic rats and to determine whether E_2 has protective effects on BCEC in primary cell culture.

Materials and methods

Animals

Female Charles Rivers SD rats (200–225 g) were purchased from Charles Rivers (Wilmington, Mass). They were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25 ± 1°C) with a daily light cycle (light on 0700–1900 hours daily) for a minimum of 3 days for acclimation. All rats had free access to Purina Rat Chow and tap water. All surgical procedures in this study were reviewed and approved by the institutional animal care and use committee at the University of Florida before initiation of the study.

Two weeks before MCA occlusion surgery, all rats were bilaterally ovariectomized under Metofane (methoxyglurane, Pitman Moore, Crossings, N.J.) inhalant anesthesia to eliminate endogenous estrogens. Aseptic surgeries were performed between 1000 and 1400 hours. Rats were injected intramuscularly with 20 000 units Combiotic (Butler, Columbus, Ohio) after ovariectomy to prevent infection.

Focal ischemia model using MCA occlusion

MCA occlusion was performed using the modified methods of Longa and Nagasawa (Longa et al. 1989; Nagasawa and Kyvya 1989) 2 h after a single injection of E_2 (100 μg/kg, s.c.). Anesthesia was induced by ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A cervical incision was made at the midline. The left sternohyoid, sternomastoid, digastric and omohyoid muscles were retracted to expose the common carotid artery (CCA), the internal carotid artery (ICA) and the distal external carotid artery (ECA). The distal ECA, the CCA and the pterygopalatine artery were coagulated completely. A microvascular clip was placed on the ICA near the skull base, and a 2.5-cm long 3–0 monofilament nylon suture with a global tip was introduced into the ECA and advanced until it reached the clipped site of the ICA. The clip was removed to allow the further advancement of the suture until resistance was felt at the bifurcation of the ICA and MCA. The distance from the CCA bifurcation to the site of resistance was about 1.8 cm. The whole procedure was performed within 15 min with little bleeding. The suture was remove after 30 min of MCA occlusion and the distal ICA was coagulated. Rectal temperature was maintained at 37°C by placing animals on a warming plate during surgery and during recovery from anesthesia. The rat brains were subjected to lesion size evaluation and GLUT 1 protein assay 24 h later.

Ischemic lesion size assessment

Rats were decapitated and the whole brains dissected coronally from the olfactory bulb to the cerebellum by making 2 mm thick sections in a metallic brain matrix (model RBM 4000C, ASI Instruments, Warren, Mich.). Regions A, B, C, D and E began at 3, 5, 7, 9 and 11 mm posterior to the tip of olfactory bulb. Sections were immediately stained by immersion in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 30 min (Bederson et al. 1986) and then in 10% formalin solution for preservation. The area of ischemic lesion for each section was analyzed using the Image 1.47 software program.

Preparation of brain microvessels

Microvessels were isolated from rat brains by the modified method of Goldstein (1975). After decapitation, regions C and D together were further dissected into penumbral and core regions. We chose coronal sections of cortical brain from longitudinal cerebral fissure to lateral cerebral fissure. The superior third of this cortical area was defined as the penumbral region, which is supplied predominantly by the anterior cerebral artery (ACA) and, to small extent, by the MCA. The inferior two thirds was defined as core region, which is perfused exclusively by MCA. This definition of core and penumbra was made on the basis of rat neurological anatomy.

The cortices were homogenized in 1 : 10 (w/v) ice-cold modified Ringer's solution (137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 15 mM HEPES, 3 mM $CaCl_2$, 12 mM $NaHCO_3$, 5 mM dextrose, pH 7.4) with 1% bovine serum albumin (BSA) using a Teflon glass homogenizer (0.25 mm clearance; Wheaton, Millville, N.J.). Homogenate was filtered through two nylon meshes (210 μm, 105 μm) and centrifuged at 1000 *g* at 4°C for 10 min. The pellet was resuspended in 10 ml modified Ringer's solution with 30% BSA and centrifuged at 3000 *g* at 4°C for 20 min. The pellet was then resuspended in modified Ringer's solution with 1% BSA and passed through a 12-cm^3 syringe, fitted with a 21.5 G needle and containing 5 cm^3 of 0.20 mm diameter glass beads (USA/Scientific Plastics, Ocala, Fla.). Capillaries were removed from the glass beads by gentle agitation and were recovered from the supernatant. The microvessel suspension was centrifuged at 1000 *g* at 4°C for 10 min and the pellet was washed twice in buffer A (154 mM NaCl, 20 mM TRIS-HCl, pH 7.4). The entire procedure was conducted in ice-cold conditions to help prevent protein degradation. With the exception of the homogenization step, only plastic tubes were used to avoid capillaries sticking to glass vessel walls.

Western blot protein analysis of GLUT 1 protein

Microvessel protein was solubilized with the addition of an equal volume of TRIS-SDS buffer [0.05 M TRIS, pH 7.4, 2% sodium dodecyl sulfate (SDS), 0.1 mM EDTA] and was measured with bicinchonic acid (BCA) protein assay reagent (Pierce, Rockford, Ill.) by spectrophotometry (Beckman DU7400, Beckman Instruments, Norcross, Ga.). The microvessel protein concentration achieved was about 1 mg protein/ml buffer.

Aliquots of 2 μg endothelial cell proteins were prepared in loading buffer (10% glycerol, 0.1% bromophenol blue, 2% SDS, 50 mM TRIS-HCl, pH 6.8, 0.1 M dithiothreitol) and spotted onto SDS-polyacrylamide gels (12% T, 2% C of resolving gel and 6.3% T, 4% C of stacking gel) in a Mini-protein II cell (BioRad, Hercules, Calif.) using the method of Burnette (1981), except that the samples were not boiled to avoid aggregation of the glucose transporter protein. Loading buffer and GLUT 1 protein from fresh human red blood cells were applied as negative and positive control, respectively. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), gels were equilibrated in the transfer buffer [0.025 M TRIS-base, pH 8.8, 0.25 M glycine, 20% (v/v) methanol] for 15 min and transferred electrically to nitrocellulose membrane (BioRad) using a Mini trans-blot cell (BioRad) at 100 V for 45 min. Blots were incubated first with blocking buffer (0.1% Tween 20, 10 mM TRIS-HCl, pH 7.5, 100 mM NaCl, 5% nonfat dry milk) to minimize nonspecific binding and then with primary antibody (rabbit anti-glucose transporter, code RaGLUTRANS, lot 903028; Eastacres Biologicals, Southbridge, Mass.) which was diluted 1 : 5000 in blocking buffer. Finally blots were exposed to the secondary antibody (donkey anti-rabbit Ig, horseradish peroxidase linked whole antibody, batch 100; Amersham, Arlington Heights, Ill.) which was diluted 1 : 10 000 in blocking buffer. Each incubation was done for 60 min at room temperature. After each incubation, blots were washed three times with washing buffer (blocking buffer without 5% nonfat dry milk) for 5 min each. After washing, air-dried blots were treated with chemiluminescent reagents (ECL Western blotting analysis system, Amersham) for 1 min and exposed to X-ray film (Hyper film-MP, Amersham) for 5–20 min. The film was analyzed by an imaging densitometer (Model GS-670, BioRad).

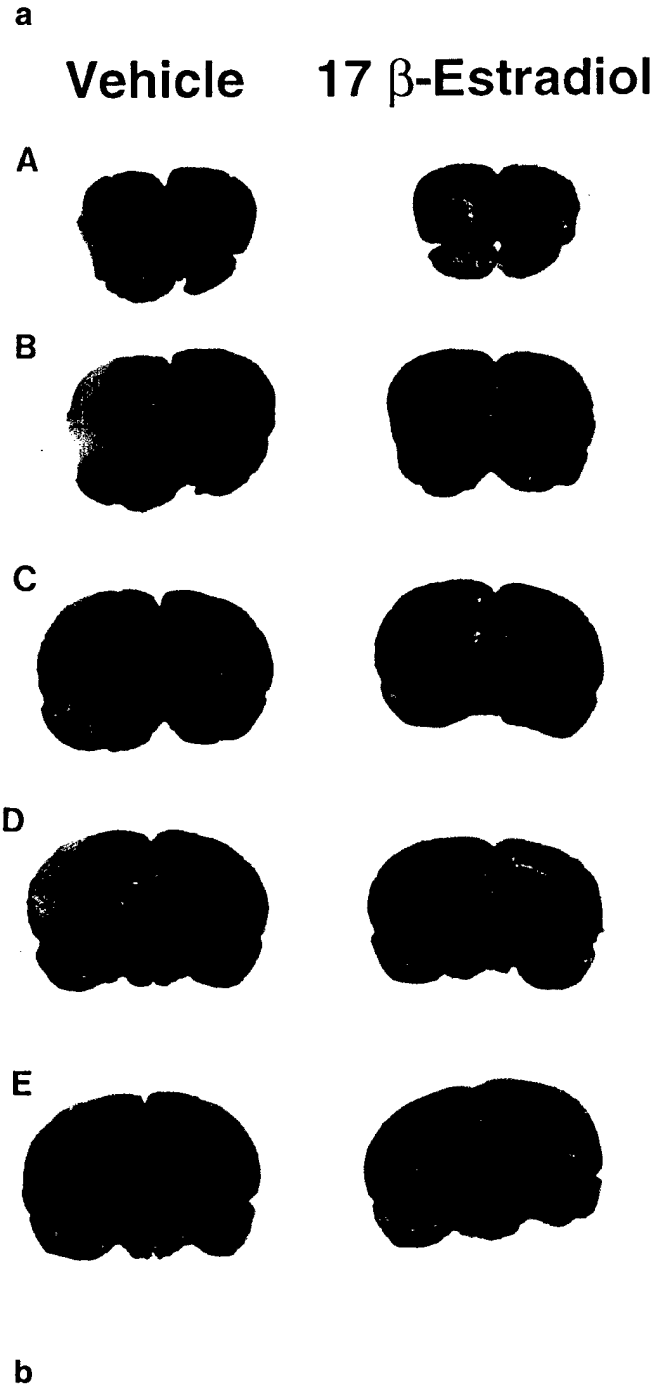


Fig. 1a, b Effects of in vivo 17β-estradiol (E_2) administration on the whole brain focal ischemic lesion area. Ovariectomized (*OVX*) rats were injected with 100 μg/kg E_2 or oil vehicle 2 h before middle cerebral artery (MCA) occlusion surgery. Rat brains were dissected coronally as sections *A* to *E* 24 h after MCA occlusion. Coronal sections A, B, C, D and E corresponded to 5, 7, 9, 11 and 13 mm caudal to the olfactory bulb. Brain sections were stained by 2,3,5-triphenyl tetrazolium chloride (TTC). **a** Slices of rat brains after MCA occlusion. The ischemic area was excluded from TTC staining and remained *white*, while the intact area was stained by TTC and became *red*. **b** The percentage of ischemic lesion area was calculated as lesion area/whole brain section area ×100%. Mean ± SEM are depicted ($n = 8$ in OVX + E_2 group and $n = 6$ in OVX group). * $P < 0.05$ versus corresponding vehicle control groups

Primary rat BCEC culture

New born (1–3 days old) Charles Rivers SD rats were used for primary BCEC cultures (Dropulic and Masters 1987). Rats were anaesthetized by intraperitoneal injection of 0.1 ml 10% (v/v) phenobarbital (Bulter Company), washed with 70% ethanol, and brain tissues isolated in a laminar flow hood. Cerebral cortices free from visible small vessels were placed in sterile petri dishes with modified Ringer's solution with 1% BSA, cut into 1–2 mm^2 pieces and incubated with 0.5% crude collagenase (type II-S, pH 7.4) at 37°C for 30 min. Tissues were homogenized using a Teflon glass homogenizer and centrifuged at 1000 *g* at room temperature for 10 min. Pellets were washed twice, resuspended in modified Ringer's solution with 25% BSA and centrifuged at 3000 *g* at room temperature for 20 min. The floating neural segments were centrifuged again to maximize the recovery. Vascular sediments were collected and added to a colloidal silica gradient solution containing 45% Percoll (Pharmacia Biotech, Uppsala, Sweden) in Dulbecco's phosphate-buffered saline and centrifuged at 20 000 *g* at room temperature for 20 min. The uppermost band containing BCECs was collected and washed. BCECs were cultured in an incubator at 37°C on gelatin (1%) coated plastic culture dishes containing the culture medium RPMI 1640 (glucose free, Gibco BRL, Grand Island, N.Y.), D-(+)-glucose [200 mg/dL (mg%)], 20% heat-inactivated fetal bovine albumin, 50 μg/ml endothelial cell growth supplement (from bovine pituitary gland), 1% (v/v) antibiotic-antimycotic. Culture dishes were washed 1 h later to remove cellular debris and nonadhesive cells. Selective weeding by glass pipette flame was carried out to help homogeneous cells become confluent. Medium was changed every 3 days. When confluency was reached, cells were dissociated from culture dishes by addition of 0.25% trypsin and 1% EDTA for 3 min. Cells were rinsed with fresh medium and diluted at a ratio of 1 : 4 with medium. All reagents were purchased from Sigma (St. Louis, Mo.) unless otherwise specified. We observed that the BCECs slowed growth at approximately the 11th passage, so we used cells at sixth to eighth passage in the present studies.

To determine the purity of the BCEC preparation, we stained cells for the presence of von Willebrand factor, which is specific to vessel endothelial cells using the peroxide anti-peroxidase (PAP) technique (Dropulic and Masters 1987). The slice containing vessel endothelial cells was photographed and counted.

BCEC hypoglycemia and anoxia

To assess the effects of E_2 treatment on BCEC viability during hypoglycemia, BCEC cultures were plated with glucose concentrations in the medium of 200, 100, 40 and 20 mg% by adding appropriate amount of D-(+)-glucose to the glucose-free medium. Glucose concentration in the medium was monitored by Glucose and L-Lactate Analyzer (YSI model 2300 STAT plus, YSI, Yellow Springs, Ohio). Cell viability was analyzed at 24 h after the treatment.

Anoxia was achieved by placing culture dishes containing BCECs in a Modular Incubator Chamber (Billups-Rothenberg, Delmar, Calif.). Nitrogen gas was influxed for 10 min to replaced the oxygen inside the chamber. The chamber was sealed and placed

in the incubator for 1 or 4 h. The combination of anoxia and hypoglycemia was achieved by growing cells in medium containing 100 mg% glucose for 20 h and then placing cultures in the anoxic chamber as described above for 4 h.

Cell mortality was counted using trypan blue staining method (Spence and Peyman 1976). Cell death percentage was calculated as dead cells/total cells ×100%.

Statistics

Two-way analysis of variance was applied to determine the significance of the difference among the experimental groups. Kruskal-Wallis nonparametric analysis was used for data presented as percentage. The Mann-Whitney *U*-test was used when Kruskal-Wallis analysis showed significance among groups. $P < 0.05$ was considered significant.

Results

The administration of 100 μg E_2/kg 2 h before MCA occlusion reduced focal ischemic damage in four of five brain sections analyzed (Fig. 1a, b). E_2 replacement in OVX rats reduced by 46.3% and 44.1% ($P < 0.05$) the ischemic lesion size of the whole coronal brain sections at regions C and D, respectively (Fig. 1b). These regions corresponded to sections taken at 9 and 11 mm caudal to the olfactory bulb. Since we chose only cortices of regions C and D to assess GLUT 1 protein expression in BBB, we separately analyzed the cortical ischemic lesion sizes of regions C and D. Regions C and D showed a decrease of 29.4% and 23.7% ($P < 0.05$) in lesion sizes, respectively (Fig. 2).

In the core ischemic region where cortices are supplied exclusively by the MCA, MCA occlusion reduced GLUT 1 protein expression in BBB of both OVX and OVX + E_2 rats by 24.6% and 22.7% ($P < 0.05$), respectively, compared with corresponding regions at contralateral non-lesion sides (Fig. 3a, b). In the penumbral ischemic region, where cortical tissues are supplied primarily by the ACA but also, to small extent, by the MCA, E_2 replacement caused a 23.3% ($P < 0.05$) increase in GLUT 1 protein, compared with GLUT 1 at the lesion side of OVX rats, and a 9.3% increase over the basal level of GLUT 1 on the contralateral non-lesion side of OVX + E_2 rats (Fig. 3a, b).

The exclusive expression of GLUT 1 in the BBB implies that GLUT 1 may reflect the integrity of the BBB and the capability of its endothelial cells to transport glucose. The decrease in GLUT 1 following ischemia could indicate the breakdown of the BBB and/or a reduced capability in glucose uptake. We established primary BCEC cultures to assess the effects of E_2 on endothelial cell viability. The purity of the BCEC preparation was monitored by immunohistochemistry staining for von Willebrand factor, which is widely used as an endothelial cell marker (Lynch et al. 1983). Approximately 81% of cultured cells were stained dark brown, indicative of the expression of von Willebrand factor.

The normal glucose concentration in RPMI medium is 200 mg%. Reduction in medium glucose by 50%, 80% and 90% increased cell death by 46.7%, 69.2% and

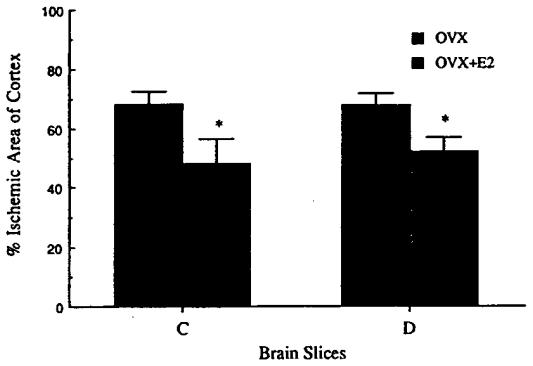


Fig. 2 Effects of in vivo E_2 administration on the cortical ischemic lesion area. Rat treatment and brain lesion assessment were as described in Fig. 1. The percentage of ischemic lesion area was calculated as cortical lesion area/cortical area on the lesioned side ×100%. Mean ± SEM are depicted ($n = 8$ in OVX + E_2 group and $n = 6$ in OVX group). * $P<0.05$ versus corresponding vehicle control groups

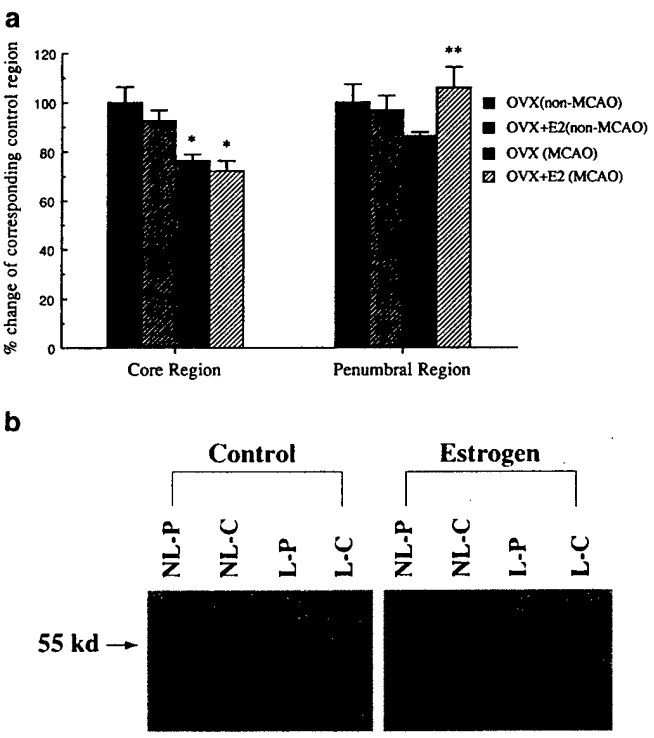


Fig. 3a, b Effects of in vivo E_2 administration on endothelial cell glucose transporter (GLUT 1) protein in the core and penumbral areas of focal ischemic rats. **a** Ovariectomized rats were injected with 100 μg/kg E_2 or ethanol vehicle 2 h before MCA occlusion surgery (*MCAO*). Sections C and D together were dissected into core and penumbral regions 24 h after MCA occlusion. Proteins from the whole endothelial cells were separated by SDS-PAGE, electrically transferred to nitrocellulose membrane and immunoblotted with rabbit anti-rat glucose transporter 1 antiserum. The GLUT 1 protein optical densities of OVX (non-MCAO) were normalized to 100%. Mean ± SEM are depicted ($n = 7$ in OVX + E_2 and OVX groups). * $P < 0.05$ versus corresponding non-MCAO sides at the core region; ** $P < 0.05$ versus OVX (MCAO) at the penumbral region. **b** Typical Western blotting gel that depicts the changes of GLUT 1 at core and penumbral areas in sections C and D

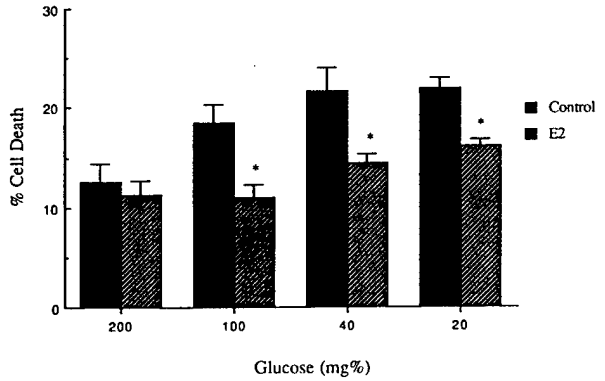


Fig. 4 Effects of in vitro E_2 administration on rat brain capillary endothelial cell (BCEC) mortality following hypoglycemia. BCECs were treated with either 2 nM E_2 or ethanol vehicle. The glucose concentrations in the cell media were adjusted from 20 mg% to 200 mg% by adding an appropriate amount of d-(+)-glucose to the glucose-free medium. BCECs were incubated for 24 h. Trypan blue staining then was used to distinguish live cells from dead cells. Two cell counts at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n = 8–12). * $P < 0.05$ versus corresponding vehicle control

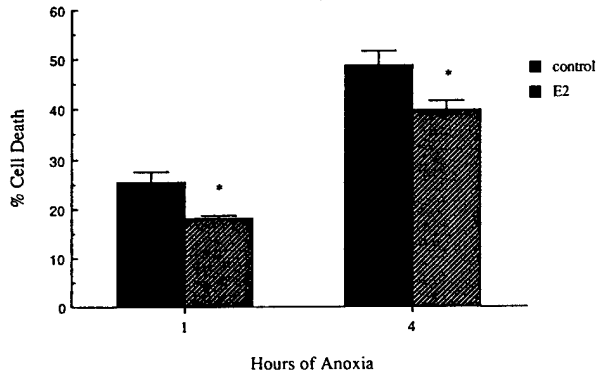


Fig. 5 Effects of in vitro E_2 administration on BCEC mortality following anoxia. BCECs were treated with either 2 nM E_2 or ethanol vehicle. Cell media contained 200 mg% glucose. Culture dishes containing BCECs were placed in a nitrogen-filled chamber for 1 and 4 h. Trypan blue staining was then used to distinguish live cells from dead cells. Two cell counts at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n = 8–12). * $P < 0.05$ versus corresponding vehicle control

73.1%, respectively. Addition of E_2 to the culture medium reduced cell loss by 35.9%, 28.4% and 23.5% ($P < 0.05$, Fig. 4), respectively, compared with corresponding control groups. It is noteworthy that there were more cells floating in the medium in the control groups than in the E_2-treated groups. Since these cells were destined to die, but were excluded when counting cells, the protective effects of E_2 may be underestimated.

Anoxia had a more dramatic effect than hypoglycemia on cell viability. One and four hours of anoxia induced 25.1% and 48.8% cell death, respectively, in the control cultures (Fig. 5). E_2 reduced cell death by 28.4% ($P < 0.05$) at 1 h and 18.4% ($P < 0.05$) at 4 h of anoxia (Fig. 5).

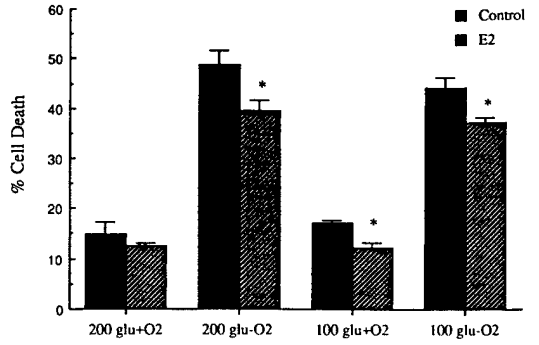


Fig. 6 Effects of in vitro E_2 administration on BCEC mortality following a combination of anoxia and hypoglycemia. BCECs were treated by either 2 nM E_2 or ethanol vehicle. Cell medium contained 100 mg% or 200 mg% glucose. Culture dishes containing BCECs were placed in either an incubator or a nitrogen-filled chamber for 4 h. Trypan blue staining was then used to distinguish live cells from dead cells. Two cell counts at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n = 8–12). * $P < 0.05$ versus corresponding vehicle control

Inasmuch as MCA occlusion reduces both oxygen and glucose supplies to BBB endothelial cells, we subjected BCECs to a combination of hypoglycemia (50% glucose reduction for 24 h) and anoxia (for the last 4 h) to determine whether the two insults exerted synergistic effects on BCECs and to assess the effects of E_2. The combined insults of hypoglycemia and anoxia did not cause more cell loss than anoxia alone and E_2 protected BCECs from cell loss to the same extent as it did with anoxia alone (Fig. 6).

Discussion

Estrogen replacement therapy in women has been reported to have beneficial effects on the outcome of stroke (Grady et al. 1992; Meade and Berra 1992) and our previous studies demonstrated that estrogen replacement decreased ischemia-induced mortality and ischemic lesion area in OVX rats subjected to MCA occlusion (Simpkins et al., unpublished observation). The present study demonstrates that estrogen replacement at 2 h prior to MCA occlusion reduced focal ischemic lesion sizes by nearly 50% and preserved GLUT 1 transporters in the penumbral region of the ischemic lesion. Inasmuch as MCA occlusion is the most frequently encountered stroke event (Kandel and Schwartz 1985), the present observation indicates that E_2-induced preservation of GLUT 1 transporter and the viability of endothelial cells may contribute to the observed improved outcome from stroke.

The MCA supplies blood to the cortical surfaces of frontal, parietal and temporal lobes (regions B, C and D in our sample paradigm), as well as basal ganglia and internal capsule. In regions B, C and D, the MCA is the terminal artery. The lack of collateral arteries supplying in this MCA-distributed area makes ischemia induced by MCA occlusion uncompensatable. On the other hand,

anastomoses between the MCA and ACA in region A and between the MCA and posterior cerebral artery in region E, may compensate for the loss of blood flow during MCA occlusion. In these latter two regions, MCA occlusion causes smaller ischemic lesions.

During stroke, depletion of cellular glucose and oxygen causes a rapid drop in membrane potential to almost 0 mV. Following membrane depolarization, the levels of extracellular excitatory amino acids increase to toxic concentration, causing calcium influx, free radical formation and lactacidosis, leading to cellular damage (Krause and Tiffany 1993; Kempski 1994; Martin et al. 1994). Estrogens have been documented to protect neurons from a variety of oxidative insults, such as β-amyloid fragments (Behl et al. 1995; Goodman et al. 1996; Green et al. 1996), hydrogen peroxide (Behl et al. 1995), $FeSO_4$ (Goodman et al. 1996) and glutamate (Behl et al. 1996; Goodman et al. 1996; Singer et al. 1996). Since excitotoxic amino acids, glutamate, and the consequential intracellular calcium accumulation are involved in oxidation-induced neuronal death, it is suggested that the mechanism of estrogen's cytoprotective effects may be blockade of *N*-methyl-d-aspartate (NMDA) receptors, attenuation of intracellular calcium accumulation (Goodman et al. 1996; Singer et al. 1996), and/or blockade of glutamate-induced cellular DNA degradation (Behl et al. 1995).

GLUT 1 facilitation of glucose uptake across the BBB is the first step in brain glucose utilization. The phosphorylation and oxidation of glucose in the mitochondria is the rate-limiting step in the glucose-turnover-to-energy process as long as there is sufficient glucose and oxygen supply. In the face of ischemic insult, the normal function of mitochondria is compromised and the demand for glucose is increased. Under these conditions, GLUT 1 transport of glucose across the BBB becomes the rate-limiting step in glucose utilization. In this situation, the observed estrogen-induced blunting of the loss of GLUT 1 transporters resulting from MCA occlusion could provide the penumbral area with glucose and thereby serve to increase neuronal survival under the ischemic insult.

Upregulation of GLUT 1 protein and its mRNA has been reported following either global or focal ischemic in animal models (McCall et al. 1996; Vannucci et al. 1996). However, GLUT 1 protein distribution in regions with different degrees of ischemic damage was not reported in those studies. Suzuki et al. (1994) reported a decrease in V_{max} and an increase in K_m of 2-deoxyglucose uptake in the BBB in the ischemic group in a regional perfusion study, which suggested decreases in the affinity and number of functioning glucose transporters. Consistently, our present study showed a reduction in GLUT 1 protein in the BBB in the core ischemic region. In this core ischemic area, estrogen had no effect on GLUT 1 transporter protein.

The depletion of glucose and oxygen caused extensive death of BCECs. This suggests that the observed decrease in GLUT 1 protein in vivo may originate from the breakage of BBB capillaries following ischemia. Unfortunately, the extent to which the loss of endothelial cells contributes to the ischemic damage is unknown. However, in view of our observation that estrogen protects BCEC from hypoglycemia, anoxia and their combination, we believe that the in vivo protective effects of estrogen against ischemia are due, in part, to the preservation of endothelial cells. This rescue of BCEC by estrogen may also contribute to the increase in GLUT 1 transporter observed in vivo. Taken together, these results suggest that estrogen rescues BCEC and increases GLUT 1 transporters in the BBB in penumbral ischemic areas, which leads to a decrease in the sizes of focal ischemic lesion. To our knowledge this is the first report demonstrating estrogen protection of brain capillary endothelial cells against ischemic insults. Such an effect could contribute to cerebral protection by estrogen during cerebral ischemic attacks.

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Neuroprotective Effects of Estrogens

JAMES W. SIMPKINS, PATTIE S. GREEN, KELLY E. GRIDLEY, JIONG SHI, AND EILEEN K. MONCK

Postmenopausal estrogen replacement therapy (ERT) is associated with numerous overall health benefits, including reduced risk of osteoporosis and a decrease in mortality (1). Of particular interest to neurodegenerative disease, ERT correlates with a decreased incidence of Alzheimer's disease (AD) (2,3), reducing the onset of the disease by as much as 10 years in one study (3). Further, several small clinical studies support a role for estrogen therapy in improving cognitive function in AD patients (4–6). Epidemiological studies have also demonstrated a beneficial effect of ERT in reducing the mortality and morbidity associated with myocardial infarction and stroke in postmenopausal women (7–10).

Estrogen, specifically the naturally occurring 17β-estradiol (βE2), is a potent neuroprotective agent in multiple experimental models of neurotoxicity and neurodegeneration. In this chapter, we will first describe neuroprotective effects of estrogens in both cell culture and animal models, and then discuss possible cellular mechanisms of this protection.

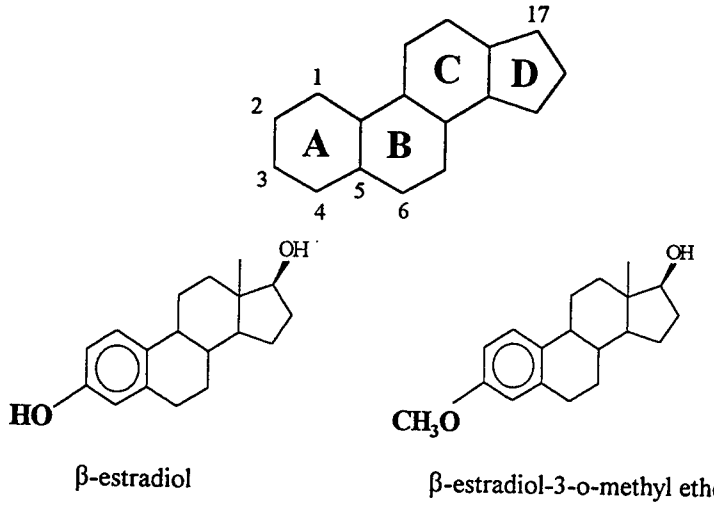
Estrogen and Neuroprotection

In Vitro Models

Our laboratory first demonstrated that physiological doses of the potent estrogen, βE2, could exert direct cytoprotective effects on a neuronal cell line using a human neuroblastoma cell line, SK-N-SH, under the conditions of serum-deprivation (11). Treatment with βE2 did not increase ^{3}H-thymidine uptake in these cells (11), verifying that this is a cytoprotective rather than a mitogenic effect of βE2. In addition, βE2 attenuates oxidative stress-induced toxicity, such as exposure of neurons to the Alzheimer plaque associated β-amyloid peptide (Aβ) (12–14) or exposure to H_2O_2 (12). βE2 treatment also attenuates cell death in rat primary hippocampal and cortical neurons due to

excitotoxic insults, such as glutamate exposure (13,15,16) and anoxia/reoxygenation (Zaulynov et al., unpublished observations).

Several lines of evidence suggest that these neuroprotective effects of βE2 are not mediated by a classical estrogen-receptor (ER) mediated mechanism. First, the structure–activity relationship for the neuroprotective effects of estrogens differs markedly from the structure–activity relationship for binding to the ER. We have demonstrated the 17α-estradiol (αE2) has similar neuroprotective efficacy and potency to the potent estrogen, βE2 (17), although αE2 binds only weakly to the ER and the αE2–ER complex binds only transiently to the estrogen responsive element (18,19). Both we (20) and Behl et al. (15) have demonstrated that estrogens with a hydroxyl group in the C3 position of the A ring (estratrienes) are neuroprotective (Fig. 10.1). If the phenolic nature of the A ring is removed by a 3-O-conjugation, the neuroprotective effects are abolished. The necessity of the phenolic A ring is demonstrated by the diphenolic estrogen mimic, diethylstilbesterol (DES). DES is neuroprotective, and retention of a single hydroxyl function on an aromatic ring is sufficient to retain neuroprotective activity (20). The di-O-methyl ether of DES does not demonstrate protective activity. Furthermore, steroids that lack a phenolic A ring, such as testosterone, progesterone, or cholesterol, do not demonstrate protective effects (20). This suggests that it is the possession of a phenolic A ring rather than binding to the ER that confers neuroprotective potential to estratrienes.



β-estradiol β-estradiol-3-o-methyl ether

FIGURE 10.1. A structural representation of the cyclopentaphenanthrene ring (top), the neuroprotective estrogen, 17β-estradiol, and the nonprotective 17β-estradiol-3-methyl ether. The bold letters indicate ring designation, and the numbers are the carbon positions in the molecule. A hydroxyl group at the 3 position and an aromatic A ring are necessary for neuroprotective activity.

Second, ER antagonists fail to block the neuroprotective effects in SK-N-SH cells (17,21). Exposure to 2 nM 17β-estradiol (βE2) during 48 hours of serum deprivation increases live cell number by an average of two- to three-fold over vehicle controls and concurrent treatment with a 100-fold excess of tamoxifen, which is a mixed ER agonist/antagonist, does not significantly alter the degree of protection conferred by βE2 (17). Tamoxifen alone has no effect on cell viability in this assay system. Further, ICI 187,780, which is a pure ER antagonist that contains a phenolic A ring, is itself protective against Aβ toxicity (21). These results indicate that antagonism of the ER does not antagonize the protection conferred by estratrienes.

Finally, estrogens have been shown to protect a neuronal cell line that lacks an estrogen receptor. The HT-22 cell, which is a mouse hippocampal cell line, does not demonstrate specific ^{3}H-βE2 binding in crude nuclear extracts or whole cell preparations (22). Further, when HT-22 cells are transfected with an ERE- reporter plasmid construct, no increase in reporter plasmid expression was seen with estrogen exposure (12). Estrogens have been shown to protect these neuronal cells from the toxic effects of Aβ, glutamate, buthionine sulfoximine, and H_2O_2 (12,15,22). Further, we have shown that this protection can be achieved with physiologically relevant doses of estrogens (2 nM) (22).

Animal Models

In animal models, we and others have demonstrated that estrogens protect against events associated with ischemia (23–27). We have reported that the treatment with various forms of estrogens at a variety of time points exerts neuroprotective effects against the damages associated with middle cerebral artery (MCA) occlusion-induced focal ischemia in female rats. Twenty-four-hour pretreatment of ovariectomized rats with βE2-CDS, the brain-targeted chemical delivery system, or βE2 reduced post-MCA occlusion mortality by more than 50% compared with vehicle treated ovariectomized rats (23). In a separate study, ovariectomy similarly decreased 24-hour postocclusion survival from 87.5% in intact female rats to 76.5% (24). Pretreatment with the presumed inactive estrogen, αE2, consistently reduced mortality from 36 to 0%. The reduction in the ischemic lesion size may underlie this remarkable reduction in mortality. Twenty-four-hour pretreatment with βE2-CDS or βE2 in ovariectomized rats caused a reduction in ischemic lesion sizes from 25.6 ± 5.7% in ovariectomized rats to 9.1 ± 4.2% and 9.8 ± 4%, respectively.

Pretreatment with aE2 similarly reduced the ischemic lesion sizes by 55–81%. Of greater importance, treatment of ovariectomized rats with bE2 after onset of either temporary or permanent occlusion continued to reduce ischemic lesion sizes by about 50% (23; Shi et al., unpublished observations). βE2 treatment also reduced ischemic lesion size from 17 to 8% in intact male rats (25).

Potential Mechanisms of Action

Antioxidant Effects

Phenolic A ring estrogens may exert their neuroprotective actions through an antioxidant mechanism because lipophilic phenols are well known to be antioxidants (28). Mukai et al. have demonstrated that estrogens, specifically phenolic A ring estrogens, are potent antioxidants (29). 17β-estradiol also not only protects neurons from oxidative insults, such as hydrogen peroxide (12) and β-amyloid peptide (12–14) toxicity, but prevents the increase in lipid peroxidation that accompanies these toxicities (13,30). This is significant as increased lipid peroxidation is associated with a variety of neurodegenerative diseases, including ischemic/anoxic insults (31–32) and Alzheimer's disease (33–34).

Synergism with Glutathione

In attempts to reconcile our previous work, in which low concentrations of estradiol (2 nM) were protective against βAP 25-35-induced toxicity (14), with others where protection was obtained using estrogens at much higher concentrations (12,13), we identified that our work was done with reduced glutathione (GSH) present in the cell culture milieu, whereas others used culture media where GSH was absent. Further experimentation showed that when GSH was absent in the extracellular milieu, the EC_{50} for βE2 protection against Ab toxicity was 126 ± 89 nM for SK-N-SH cells (21) and 3.2 ± 408 μM for HT-22 cells (22). The presence of GSH (3.25μM) shifted the EC_{50} for βE2 neuroprotection to 0.03 ± 0.031 nM in SK-N-SH cells (21) and 5 ± 2 nM in HT-22 cells (22). We also evaluated the effect of GSH on the protection conferred by aE2 and estratriene-3-ol, which are two classically weak estrogens that we have formerly demonstrated to be as potent as βE2 in neuroprotection assays. GSH increased the neuroprotective potency of these estrogens by approximately 400-fold (22).

The low concentrations of GSH used in the aforementioned studies did not have any effect on cell viability in the absence of estrogen; however, GSH also has neuroprotective properties and protected SK-N-SH cells from Aβ toxicity with an ED_{50} of 82.6 ± 60 μM in the absence of estrogens. A physiologically relevant dose of βE2 (2 nM) significantly potentiated the neuroprotective potency of GSH to an ED_{50} 0.04 ± 0.02 μM (21). This implies a synergistic interaction as the neuroprotective potency of both molecules are markedly shifted by the presence of the other. We performed similar experiments in rat primary cortical neurons and obtained similar results (21). Although differences exist in the magnitude of the GSH-induced shift in the neuroprotective potency of E2 in these cell types, intracellular concentrations of GSH may play a role because we have determined that primary rat cortical neurons have higher intracellular GSH concentrations (172 ± 12 μM)

than do SK-N-SH cells (15 ± 2) (21). In addition, this effect appears to be independent of the type of cytotoxic insult used because results obtained using serum deprivation and zinc toxicity (unpublished observations) were comparable to the data obtained from βAP 25-35 (21,22) and βAP 1-40–induced (22) toxicities.

The specificity of estrogens and glutathione for synergism in neuroprotection is supported by several lines of evidence. First, there are no apparent interactions noted between estrogen and the other thiols tested, lipoic acid or taurine, or any other antioxidants tested, ascorbic acid or α-tocopherol (21). Second, oxidized glutathione (GSSH) works in this model (21), and lends credence to the idea that the glutathione-estrogen interaction may involve the glutathione peroxidase/reductase system.

Estrogens are lipophilic and likely to partition to membrane constituents. They should associate their phenolic A rings with the charged hydrophilic head groups of the membrane phospholipids (Fig. 10.2). As a result, estratrienes are well placed to attenuate lipid peroxidation, and we predict the hydroxyl hydrogen of estradiol is donated to prevent the peroxidative cascade. This is further substantiated by the aforementioned structure-activity relationship with neuroprotection (15,17,20). Further, high potency of estratrienes may result from their ability to donate hydrogen ions from several positions on the A ring (35). An oxidized form of estrogen could result from this hydrogen ion donation that would be relatively stable, and glu-

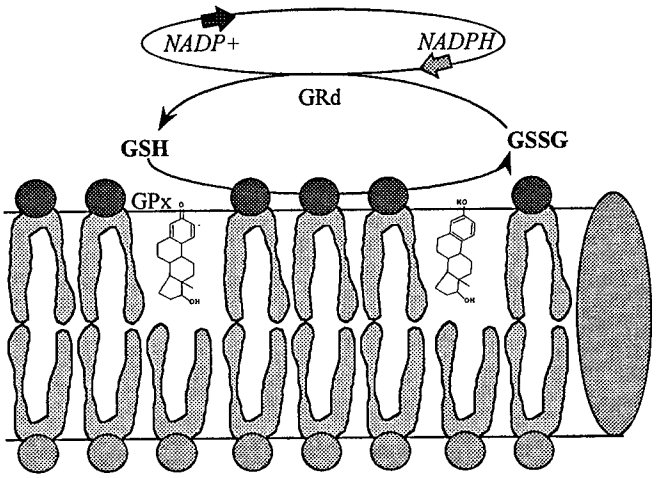


FIGURE 10.2. A schematic representation of the proposed relationship between glutathione and estradiol. The reduced and oxidized forms of estradiol and the reduced and oxidized forms of glutathione (GSH and GSSG, respectively) are depicted. Oxidative stress drives estradiol to the oxidized form, thereby protecting membrane lipids. The proposed role of glutathione is to reduce estradiol. GSSH is then reduced by glutathione reductase (GRd) using NADPH as a substrate.

tathione peroxidase would likely regenerate the reduced form of estrogen by using GSH as a substrate, thus explaining the synergy between the two molecules.

Effects on CREB

Estrogen's neuroprotective effects may also be mediated via interaction with cyclicAMP (cAMP) response element-binding protein (CREB), a constitutively expressed transcription factor that is activated by phosphorylation. Two independent studies have suggested that CREB may play an important role in neuroprotection. Walton et al. (36), using a hypoxic–ischemic injury model, demonstrated a decline in phosphorylated CREB (PO_4–CREB) immunoreactivity in CA1 pyrimidal cells, which do not survive mild hypoxia–ischemia injury. In contrast, the more resistant dentate granule cells and cortical cells showed an increase in PO_4–CREB immunoreactivity. We have previously reported that hypoglycemic seizure results in a selective reduction of CREB immunoreactivity (37). This decline in CREB immunoreactivity does not appear to be due to cell loss because the rats were sacrificed 90 minutes postseizure. It is interesting to note, however, that the regions of CREB decline correlated with regions that have previously been shown to have massive cell loss at 1 week after hypoglycemic seizure (38).

Estrogen replacement has been shown to reduce the seizure-induced decline in CREB immunoreactivity (38). We have examined the effect of serum deprivation on both CREB and PO_4–CREB levels in SK-N-SH cells (Green et al., unpublished observations). These cells showed a decline in both PO_4-immunoreactivity and CRE binding capacity at 4–6 hours of serum deprivation that was normalized by 12 hours. No loss of cell viability as determined by propidium iodide exclusion was detected at these time points. It is interesting that 10 nM βE2 prevented this decline in PO_4–CREB immunoreactivity and CRE binding capacity.

Zhou et al. (39) demonstrated that estrogen treatment of ovariectomized rats increases CREB phosphorylation within 15 minutes of injection in the preoptic area (POA) and bed nucleus of the stria terminalis. This rapid response suggests a non–ER-mediated effect of estrogen. We have demonstrated similarly that βE2 rapidly increases phosphorylation of CREB in SK-N-SH cells (unpublished observations). We observed a 50% increase in PO_4–CREB immunoreactivity within 1 hour using a physiological dose of βE2 (2 nM) (Fig. 10.3). We did not see a change in CREB immunoreactivity at this time point with βE2 doses of up to 10 μM. This rapid βE2-induced phosphorylation of CREB may be due to increased cAMP production because βE2 has been shown to activate adenylate cyclase activity in MCF7 breast tumor cells (40).

These data collectively suggest the possibility that estrogen regulates the phosphorylation state of CREB by interactions with signal transduction pathways other than the traditional estrogen receptor.

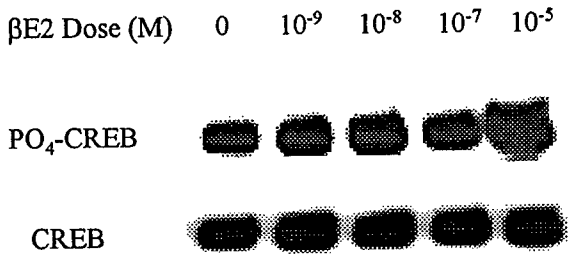


FIGURE 10.3. Western blot analysis of CREB phosphorylation by 17β-estradiol. SK-N-SH cells were treated with the indicated dose of steroid for 1 hour. Nuclear protein was extracted and analyzed by Western blot using an antibody specific to PO_4–CREB (upper blot) and an antibody for CREB. 17β-estradiol increases immunoreactivity of PO_4–CREB while having no effect on overall CREB immunoreactivity.

Conclusion

Neuronal death due to normal aging, neurodegenerative diseases, and acute conditions (e.g., ischemia) is not currently treatable. There is mounting evidence that estrogens are potent cytoprotective compounds against a variety of neurotoxic insults. These actions of estrogens do not appear to require an ER and may involve antioxidant properties of the estratriene molecule as well as interaction with signal transduction pathways. Further, these neuroprotective actions may contribute to the beneficial effects of estrogens seen in both clinical trials and epidemiological studies of AD and/or stroke. As such, estrogen use should be further evaluated for treatment of various neurodegenerative conditions.

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Estradiol Attenuation of β-Amyloid-Induced Toxicity: A Comparison of MTT and Calcein AM Assays.

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Summary

17β-estradiol (βE2) has been shown to attenuate the toxicity of β-amyloid peptides (Aβ) in neuronal cultures with the effective concentration of βE2 ranging from low nM to high μM. This study compares the effective neuroprotective concentration of βE2 against both Aβ-mediated toxicity in a human neuroblastoma cell line, SK-N-SH using cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an endpoint to the effective βE2 concentration obtained using a calcein acetoxymethyl ester (calcein AM) viability assay. The minimum βE2 concentration required for protection varied 1000-fold between the two viability assays with 1 nM βE2 conferring significant protection in the calcein AM assay but 1 μM βE2 required for significant protection in the MTT assay. Interestingly, the maximal inhibition of MTT reduction occured at sub-toxic Aβ concentrations and did not correlate with other markers of cellular viability including calcein fluorescence, dye exclusion (propidium iodide or trypan blue), cellular ATP levels, or reduction of another tetrazolium dye, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium (MTS). By contrast, there was no difference between the MTT and calcein AM assays with respect to H_2O_2 toxicity or the neuroprotective effectiveness of 10 nM βE2 against H_2O_2 toxicity. These results indicate that low concentrations of βE2 can attenuate Aβ and H_2O_2 toxicity in a human neuroblastoma cell line. Further, these results suggest that the MTT assay is not an appropriate assay for the determination of βE2-mediated attenuation of Aβ toxicity.

Introduction

Epidemiological data suggest a role for estrogens in preventing the neurodegeneration

associated with Alzheimer's disease (AD) (Tang et al., 1996; Kawas et al. 1997). Estrogens are potent neuroprotective agents in several models of neuronal death (for review see Green and Simpkins, 2000). Of specific interest to AD, 17β-estradiol (βE2) has been shown to attenuate the neurotoxicity associated with aggregated β-amyloid peptides (Aβ). While the role of Aβ in the etiology of AD remains unclear, these 40 to 43 amino acid peptides comprise a key component of senile plaques in AD and can exert toxic effects on neuronal cultures (Yanker et al. 1990; Pike et al. 1991).

Colorimetric tests based on the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple, formazan product have been widely used to assess both Aβ-induced toxicity (for review see Shearman, 1999) and βE2 mediated neuroprotection (Behl et al., 1995; Bonnefont et al., 1998; Roth et al., 1999). As MTT can be reduced by viable mitochondria (Slater et al., 1963), the Aβ-induced decrease in MTT conversion has been interpreted as evidence for metabolic compromise and an early event in Aβ-induced neuronal death (Shearman et al., 1994; Kaneko et al., 1995). However, the Aβ attenuation of MTT reduction does not correlate with neurotoxic effects in as much as Aβ concentrations in the nM range lead to rapid inhibition of MTT conversion but prolonged exposure to μM concentrations are required for neuronal death as determined by other endpoints (Behl et al., 1994; Shearman et al., 1994).

Liu and Schubert (1997) have shown that Aβ can decrease MTT reduction by enhancing exocytosis of the resulting formazan crystals. Interestingly, they also found that high concentrations of βE2 (>10 μM) diminished exocytosis of the formazan product of MTT reduction (Liu and Schubert, 1998). This suggests the possibility that the effects of βE2 seen

against Aβ toxicity in the MTT assay may not represent estrogen mitigation of neuronal death. However, βE2-mediated protection against Aβ toxicity has also been demonstrated using other assays including dye exclusion techniques (trypan blue) (Green et al 1996; Green et al. 1998), morphological criteria/cell counting (Mook-Jung et al. 1997) and vital dyes (calcein AM) (Pike 1999). Interestingly, these studies reported neuroprotective effects of βE2 in the nM range whereas the studies using MTT reduction as an endpoint required μM concentrations of βE2 to achieve neuroprotection (Behl et al., 1995; Bonnefont et al., 1998; Roth et al., 1999). No conclusion can be drawn from these studies concerning the effect of the viability endpoint chosen on the concentration of βE2 required as different neuronal types, culturing conditions, βE2 treatment paradigms and times of Aβ exposure were used. The present study directly compares βE2-mediated neuroprotection against Aβ and H2O2 toxicity in the MTT assay and calcein AM assay.

Materials and Methods

Cell Culture

SK-N-SH cells (ATCC, Rockville, MD) are a human neuroblastoma cell line. These cells were maintained in RPMI-1640 media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) and 20 μg/ml gentamycin under standard culture conditions (5% CO_2, 95% air, 37°C). Cells used in these experiments were from passages 39 - 45. Cells were plated at a density of 12,000 to 20,000 cells per well in Nunc 96-well plates 24 h prior to initiation of experiments.

Cell Treatments

1 mg of lyophylized Aβ 25-35 (Bachem, Torrance, CA) was suspended in 1 ml of sterile PBS and incubated for 1 h at 37°C immediately before use. Aβ was diluted to the final concentration (0.05 to 20 μM) in culture media and cells were exposed to Aβ for 24 h.

βE2 (Steraloids, Wilton, NH) was initially dissolved in absolute ethanol at a concentration of 10 mM and diluted to the appropriate concentration (1 nM to 10 μM) in culture media. Exposure to βE2 was initiated 3 h prior to addition of Aβ. Ethanol was used at a final concentration of 0.1% as a vehicle control. This concentration of ethanol had no discernable effects on cell viability or Aβ 25-35 toxicity.

Viability Assays

MTT reduction was determined by incubation with 0.25 mg/ml MTT (Sigma Chemical Co., St. Louis, MO) for 3-4 h at 37°C. Following overnight solubilization of the formazan product in 50% N,N-dimethyl formamide, 20% sodium dodecyl sulfate, pH 4.8, the optical density was determined at 575/690 nm. For the calcein AM assay, cells were rinsed once with PBS and incubated with 25 μM calcein AM dye (Molecular Probes, Eugene, OR) at room temperature for 20 minutes. Fluorescence was determined using an excitation/emission filter set of 485/538 nm. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium (MTS; Promega Corp., Madison, WI) reduction was determined by incubation with 0.25 mg/ml MTS and 5 μg/ml phenazine methosulfate (PMS) for 3 h at 37°C followed by determination of optical density at 490/690 nm. Cellular ATP levels were determined using the ATP determination kit (Molecular Probes) according to the manufacturer's instructions. Wells containing media without

cells or wells containing cells which had been lysed prior to dye addition were used for background determination in both assays.

Statistical Analysis

The significance of differences among groups was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p<0.05$ was considered significant and each group consisted of 4 to 12 wells. All values are expressed as mean ± sem.

Results

The MTT and calcein AM viability assays are differentially affected by Aβ 25-35.

Exposure of SK-N-SH neuroblastoma cells to Aβ 25-35 for 24 h results in a potent inhibition of MTT reduction with an IC_{50} of 115 nM (Fig. 1). Maximal inhibition of MTT conversion averaged 56 ± 3 % and was achieved with concentrations of Aβ as low as 0.5 μM. Incubation times up to 96 h did not further increase the Aβ-induced attenuation in MTT reduction (data not shown).

The Aβ-induced reduction in MTT conversion did not correlate with a reduction in viability as determined by the calcein AM fluorescence assay (Fig. 1). Calcein fluorescence requires active cellular esterases (deesterfication of calcien AM) and plasma membrane intergrity (retention of the fluorescent calcein product). No decrease in calcein fluorescence was observed with Aβ 25-35 concentrations less than 10 μM even with incubation times up to 96 h (data not shown). 24 h exposure to 20 μM Aβ resulted in a statistically significant reduction in calcein fluorescence in 7 out of 12 experiments with the average reduction in fluorescence of 26 ± 6 % in these

experiments. Similar results were obtained using trypan blue or propidium iodide dye exclusion (markers of plasma membrane integrity) to assess Aβ-toxicity (data not shown).

Of particular interest, Aβ-inhibition of cellular reduction of the tetrazolium dye MTS did not correlate with potent inhibition seen in the MTT assay. MTS is a tetrazolium dye which is similar in function to MTT with the exceptions that the formazan product of MTS is aqueous soluble and MTS conversion requires the addition of an electron coupling reagent (PMS). A 24 h exposure to 20 μM Aβ 25-35 produced a 12 ± 2 % reduction in MTS conversion as compared with 56 ± 3 % reduction in MTT conversion. Aβ-induced reductions in cellular ATP levels correlated with the reductions in cellular viability rather than reductions in MTT conversion. No reduction in cellular ATP levels was observed with 5 μM or less of Aβ. A reduction in cellular ATP levels of 42 ± 8 % and 65 ± 4 % was observed following 24 h exposure to 10 and 20 μM Aβ 25-35, respectively.

βE2 attenuates Aβ-induced toxicity in SK-N-SH cells.

βE2 attenuates Aβ 25-35-induced reduction in both MTT conversion and calcein fluorescence in SK-N-SH neuroblastoma cells; however, the potency of βE2 differs in the two assays (Fig. 2). A 1 μM concentration of βE2 was required to significantly abate the reduction in MTT conversion induced by 5 μM Aβ 25-35. Lower concentrations of βE2 did not diminish the Aβ-induced effect on MTT conversion even at Aβ 25-35 concentrations of 0.1 μM (data not shown). By contrast, 1 nM βE2 attenuated 72 ± 11 % of the Aβ-induced reduction in calcein fluorescence and 10 nM βE2 completed abolished the Aβ effect (Fig. 2). Differentiation of SK-N-SH cells with 10 μM retinoic acid for 4 days prior to βE2 addition yielded similar results with a 23 ± 3 % reduction in viability with 20 μM Aβ 25-35 exposure and complete protection with 100 nM βE2

(data not shown).

βE2 attenuates H_2O_2-induced toxicity in SK-N-SH cells.

In contrast to Aβ-induced toxicity, H_2O_2-induced toxicity showed similar results between the MTT and calcein AM assays (Fig. 3). H_2O_2 exposure of SK-N-SH cells resulted in significant reductions in MTT conversion and calcein fluorescence with IC_{50} values averaging 4.1 μM and 5.2 μM, respectively. Low concentrations of βE2 significantly attenuated the toxicity associated with 5 μM H_2O_2 in both assays with 10 nM reducing H_2O_2-induced toxicity by 24 ± 3 % and 18 ± 2 % in the MTT and calcein AM assays, respectively. No protective effect of 10 nM βE2 was observed with concentrations of H_2O_2 which resulted in greater than a 70 % reduction in cell viability (data not shown).

Discussion

The present study finds that the apparent neuroprotective potency of βE2 against Aβ toxicity differs by more than 1000-fold when assessed by MTT reduction and calcein AM fluorescence. βE2 abated Aβ-toxicity as assayed by the calcein AM assay at low nM concentrations whereas μM concentrations were required to attenuate Aβ inhibition of MTT reduction. Further, in this model, Aβ-induced inhibition of MTT reduction does not correlate with the cytotoxic effects of the peptide as assessed by calcein AM fluorescence, dye-exclusion methods (propidium iodide and trypan blue), other indicators of cellular reducing potential, or cellular ATP levels. No difference was seen in the two assays between either the degree of toxicity or βE2-mediated protection with H_2O_2 exposure.

A lack of correlation between concentrations of Aβ required to induce cytotoxicity and to inhibit MTT reduction has been widely reported (Shearman et al., 1994, 1995; Kaneko et al., 1995; Hertel et al., 1996; Liu and Schubert, 1997). As MTT can be reduced by components of the electron transport chain (Slater et al., 1963), the differences in Aβ concentrations required for inhibition of MTT reduction and cytotoxicity has been interpreted as evidence that inhibition of MTT reduction is an early indicator of Aβ-induced metabolic compromise (Shearman et al., 1994, 1995; Kaneko et al., 1995). However, at the low concentrations at which Aβ peptides lead to inhibition of MTT reduction, we and others (Shearman et al., 1995; Hertel et al., 1996) report no change in cellular redox potential as measured by other tetrazolium dyes. Similarly, no change in cellular ATP levels was observed at the nM concentrations which exerted maximal inhibition of MTT reduction although cytotoxic concentrations (10 μM) Aβ result in a decrease in ATP levels which has been reported to precede Aβ-induced neuronal death (Zhang et al., 1996; Mark et al., 1997).

Other mechanisms for Aβ inhibition of cellular MTT conversion have been proposed including exacerbation of MTT formazan-induced cell lysis (Hertel et al., 1996) and enhancement of MTT formazan exocytosis (Liu and Schubert, 1997). Liu and Schubert (1998) have also demonstrated that βE2 at concentrations greater than 10 μM attenuate MTT formazan exocytosis (resulting in enhanced MTT reduction) and that this activity may be responsible for the βE2 attenuation of Aβ-induced inhibition of MTT conversion. Our results indicate that the effect of high concentrations of βE2 on Aβ-induced alterations in MTT reduction do not represent the neuroprotective activity of the steroid. Rather, low concentrations of βE2 are capable of attenuating Aβ-induced toxicity in SK-N-SH cells, and this neuroprotection is not detected in assays of cellular MTT reduction.

Lobner (2000) has recently reported that although the MTT reduction and lactate dehydrogenase (LDH) release are similarly affected by nifedipine or C2-ceramide toxicity, the MTT assay did not detect the neuroprotective efficacies of cyclohexamide and the caspase inhibitor ZVAD. These results indicate caution is warranted when interpreting neuroprotection data obtained in MTT reduction as a viability endpoint.

Reports of attenuation of Aβ toxicity with low concentrations of βE2 (1 to 30 nM) used viability endpoints other than cellular MTT reduction including trypan blue exclusion (Green et al., 1996; Green et al., 1998), calcein fluorescence (Pike, 1999), and morphological criteria/cell counting (Mook-Jung et al., 1997). In contrast, studies which used MTT reduction as a viability endpoint required 0.1 to 10 μM βE2 to significantly attenuate the Aβ effect (Behl et al., 1995; Bonnefont et al., 1998; Roth et al., 1999). Together, these studies suggest that high, non-physiological βE2 concentrations are required to alter MTT reduction/exocytosis but low concentrations of βE2 are capable of blocking the neurotoxicity associated with Aβ exposure.

The mechanism by which estrogens exert neuroprotective effects is multi-faceted and remains to be elucidated (for review see Green and Simpkins, 2000). Further study of the neuroprotective effects of estrogens requires accurate methods of assessing cell viability. The present study demonstrates that MTT reduction is not an appropriate endpoint for assessment of Aβ-induced toxicity and the corresponding estrogen-mediated neuroprotection.

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Figure Legends

Figure 1. Comparison of MTT and calcein AM assays for assessment of Aβ 25-35 toxicity in SK-N-SH cells. The indicated concentration of Aβ 25-35 was added 24 h prior to viability assessment. Depicted are mean ± sem for 6-12 wells per group. The data are representative of 7-9 individual experiments. In the MTT assay, $p<0.01$ versus untreated control cells for all concentrations above 0.1 μM. In the calcein AM assay, $p<0.05$ versus untreated control cells only for the 20 μM concentration.

Figure 2. Comparison of MTT and calcein AM assays for assessment of βE2 attenuation of Aβ 25-35-induced toxicity in SK-N-SH cells. The indicated concentration of βE2 was added 3 h prior to addition of Aβ 25-35. Cells were exposed to Aβ 25-35 (5 μM in the MTT assay and 20 μM in the calcein AM assay) for 24 h prior to viability assessment. Depicted are mean ± sem for 12 wells per group. The data are representative of 4 individual experiments. *=$p<0.05$ versus the representative control wells.

Figure 3. Comparison of MTT and calcein AM assays for assessment of H_2O_2-induced toxicity and βE2 neuroprotection. Cells were incubated with the indicated concentration (A) or 5 μM (B) H_2O_2 for 24 h prior to viability assessment. In (B), 10 nM βE2 was added 3 h prior to H2O2. Depicted are mean ± sem for 6-8 wells per group. The data are representative of 2-4 individual experiments. *=$p<0.05$ versus the respective untreated control wells.

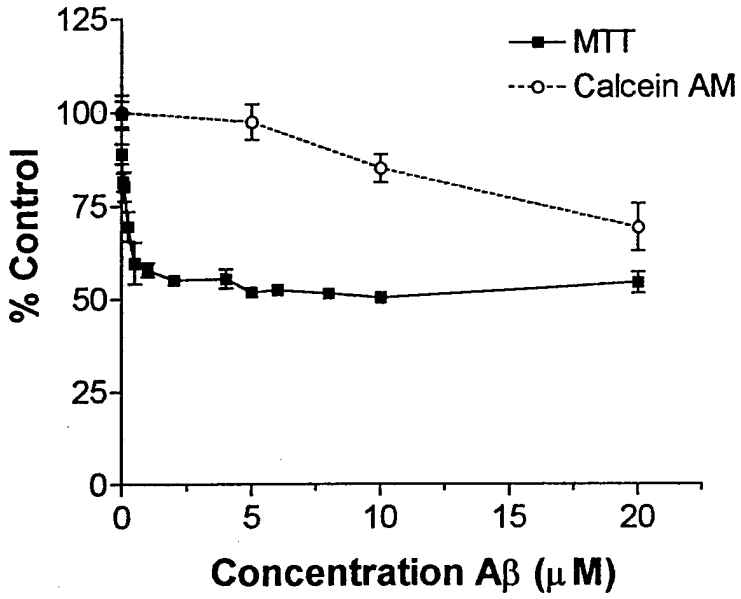

125
100
75
50
25
0
% Control
0
5
10
15
20
Concentration Aβ (μM)
MTT
Calcein AM

Figure 1

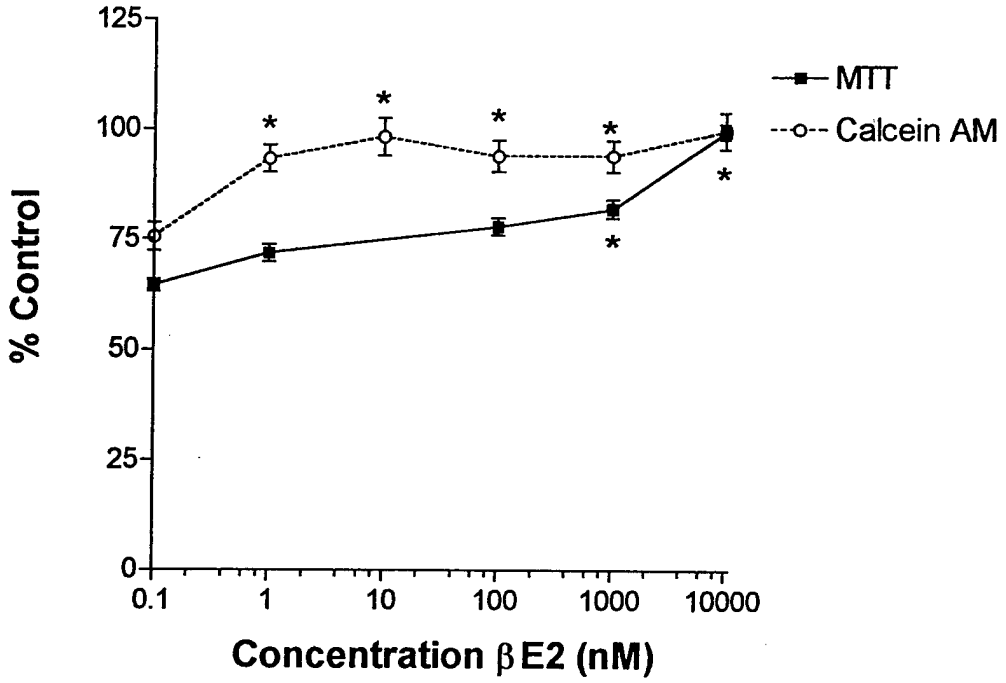

125
100
75
50
25
0
% Control
0.1
1
10
100
1000
10000
Concentration βE2 (nM)
MTT
Calcein AM
*

Figure 2

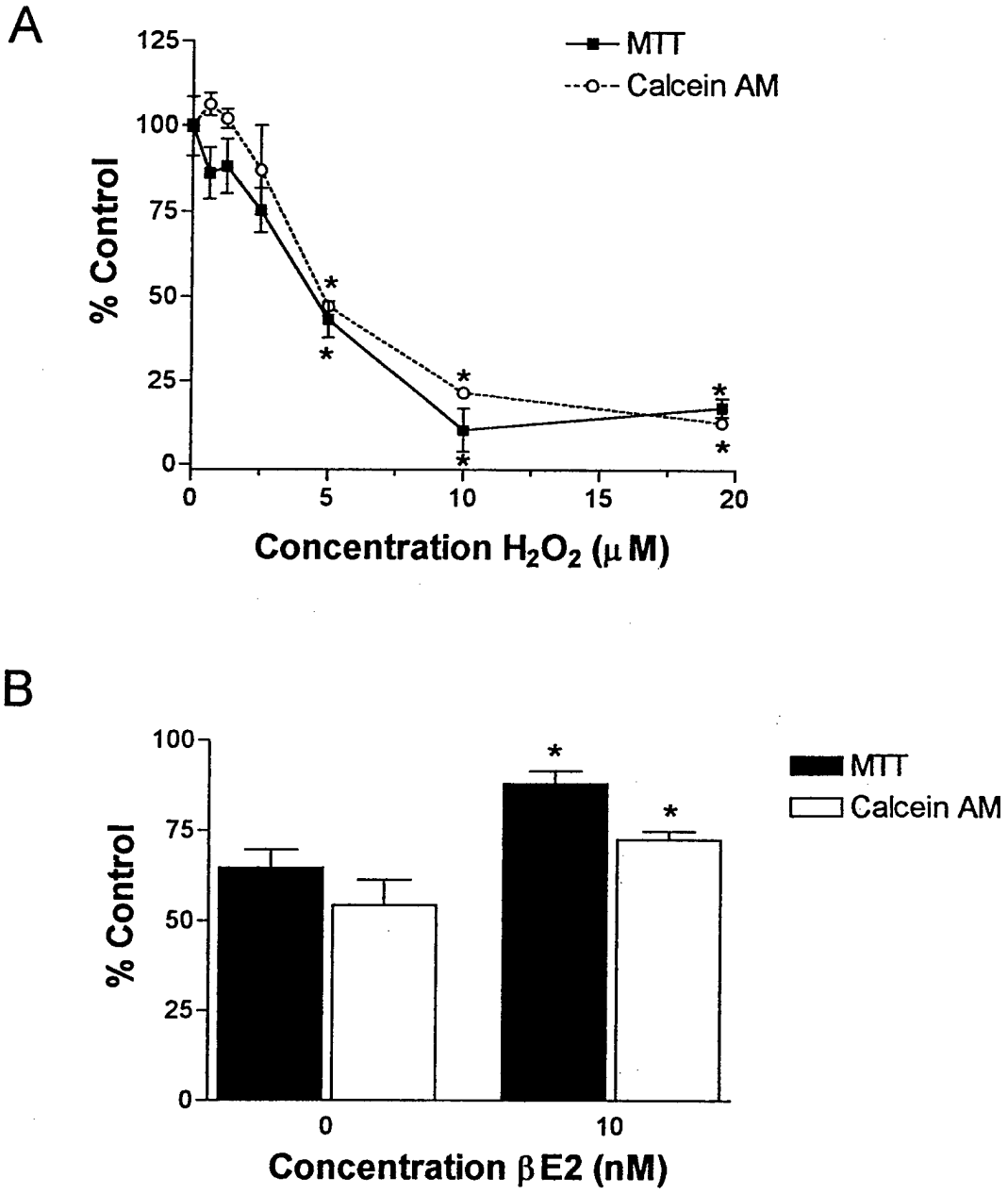

A
MTT
Calcein AM
% Control
125
100
75
50
25
0
0
5
10
15
20
Concentration H_2O_2 (μM)
B
MTT
Calcein AM
% Control
100
75
50
25
0
0
10
Concentration βE2 (nM)

Figure 3

Spontaneous Reperfusion and Associated Reduction of Lesion Volumes in a Stroke Model

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21 pages and 4 figures

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Abstract

The influence of spontaneous reperfusion on stroke severity after acute intracranial vascular occlusion has not been well established in laboratory or clinical models. In this report, twenty-four rats were divided into five groups and subjected to permanent middle cerebral artery occlusion (MCAO) for 1 to 7 days. MCAO was achieved by inserting a 4-0 suture into internal carotid artery via external carotid artery and advancing it intracranially. Cerebral blood flow (CBF) was measured by a Laser Doppler Flowmetry before and after MCAO and before sacrifice. The brain was sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for assessment of ischemic lesion volumes. CBF decreased to 43±11% of the pre-occlusion baseline after MCAO, and remained stable at this level for 2 days. CBF thereafter gradually increased to 64±13%, 92±24% and 102±13% of baseline at 3, 4 and 7-days, respectively. The ischemic lesion volume peaked at 2 days at 21±4%, then decreased significantly to 11±2%, 10±5% and 7±3% at 3, 4 and 7 days, respectively ($p<0.05$). This study demonstrates spontaneous reperfusion in a "permanent" occlusion model for focal cerebral ischemia, and suggests a potential association with reduction in ischemic volume.

Theme: Disorders of the nervous system

Topic: Ischemia

Key words: spontaneous reperfusion, cerebral ischemia, model, cerebral blood flow.

Introduction

Reduced cerebral blood flow is a major contributor to the damage sustained during acute cerebral ischemia, and its restoration is crucial in preserving neural tissue. Shortly after the initial insult adjacent collateral channels begin to vasodilate in an effort to reperfuse the oligemic region. Although the exact timing and benefits of these physiologic attempts at reperfusion after acute stroke is variable, it is a frequently observed phenomenon in human subjects [1,2,3].

Experimental reperfusion is usually investigated using an ischemia-reperfusion model, during which the occlusion time of a cerebral artery (usually the middle cerebral artery) is transient and controlled, and reperfusion is achieved at the end of that time by re-opening the artery to allow recirculation of its prior territory. In spontaneous reperfusion, however, the method of establishing occlusion of the cerebral artery is not reversed but is left in place, and reperfusion (and/or recanalization) allowed to take place via whatever physiologic resources are available. This scenario most closely matches that encountered in human clinical situations, and has not been studied well in an animal model.

The present study outlines our findings on the time course of spontaneous reperfusion in an intraluminal suture rat model for stroke and associated lesion reduction, as measured by changes in lesion size on TTC staining, which was correlated with changes in cerebral blood flow measurements over the same time interval.

Materials and Methods

Preparation of Animals

Female Charles Rivers SD rats (225-250g) purchased from Charles Rivers (Wilmington, MA) were maintained for 1-week prior to surgery in a centralized vivarium on a daily 12-hours light-12-hours dark cycle. All rats were allowed free access to food and water throughout the procedure. All animal procedure was performed aseptically and approved by the University of Florida Animal Care and Use Committee.

Middle Cerebral Artery Occlusion

Rats subjected to permanent middle cerebral artery occlusion (MCAO) were divided into 5 groups according to the duration of MCAO: 1 day (n=5), 2 days (n=5), 3 days (n=4), 4 days (n=5) and 7 days (n=5).

Animals were anesthetized by intraperitoneal injection of ketamine (60mg/kg) and xylazine (10mg/kg). Rectal temperature was monitored and maintained at between 36.5 and 37.5°C. MCA occlusion was achieved using the methods described by others [4,5], with the following modifications. With the aid of an operating microscope, a midline cervical skin incision was made to expose the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). The distal ECA and its occipital and superior thyroid branches were cauterized. The CCA was temporarily occluded by a microvascular clip placed proximal to the bifurcation. A 6-0 silk suture was tied loosely around the proximal ECA. The ICA and its branch, the pterygopalatine artery, were then occluded temporarily by two microvascular clips. A 4-0 monofilament suture with its tip rounded by heating was introduced into the ICA via the ECA lumen. After removal of the clip on the ICA, the suture was advanced until resistance was

encountered. The distance between the CCA bifurcation and the resistive point was about 1.9 cm. The silk suture previously placed around the ECA was tightened around the intraluminal monofilament suture to prevent bleeding and to maintain the occlusive position of the suture. The temporary clips on the CCA and pterygopalatine artery were then removed, and the skin incision was closed.

Measurement of regional cerebral blood flow

A Laser Doppler Flowmeter (LDF) was used for CBF measurements. The scalp as incised on the midline, and bilateral 2-mm burr holes were drilled 1.5mm posterior and 4.0mm lateral to the bregma. The dura was left intact to prevent cerebral spinal fluid leakage. LDF probes held in place by a micromanipulator were stereotactically advanced to gently touch the intact dura mater, and vessels were avoided under microscopic guidance. The lowest stable readings were obtained and recorded for at least 10 minutes from both sides (baselines measurement). For each group, the CBF was measured at least 10 minutes before and after occlusion. Then the rats were allowed to back to their home cages. Each animal was re-anesthetized with ketamine and xylazine, the lowest CBF readings were recorded at the same site before sacrifice according to the schedule. The CBF values were calculated and expressed as percentage of the baseline values. Values reported for CBF are the mean ± SD for the average of the CBF recordings obtained.

Measurement of Ischemic Volume

Each group of animals was decapitated according to the aforementioned schedule, and the brain was immediately removed and the suture position was checked. The brain

was placed in a metallic brain matrix for tissue slicing. Five slices were made at 3, 5, 7, 9 and 11mm posterior to the olfactory bulb. The slices were incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37 °C, and were then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). The analysis was done by an investigator blinded to the treatment group. Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the five slices divided by the total cross sectional area of these five brain slices.

All data was expressed as mean ± SD. The CBF and lesion volumes in each group comparisons were analyzed with one-way ANOVA. All differences were considered significant at $p<0.05$.

Results

Regional CBF

Introduction of a 4-0 suture to block blood supply to the MCA produced a 50 to 65% decline in ipsilateral CBF in each group, beginning 5 minutes following MCAO (Fig. 1). Contralateral hemisphere CBF remained near baseline and was comparable among all groups. Ipsilateral hemispheric CBF decreased to 43±11% of baseline after insertion of the suture, and remained stable at 1 and 2 days. CBF increased to 64±13% of the baseline at 3 days, and returned to baseline at 4 and 7 days, despite permanent placement of the 4-0 suture (Fig. 2).

Lesion volume

As shown in Fig. 3, a time-depend increase in ischemic lesion volume was observed through the first two days of occlusion, with lesion volume peaking at 21±4% on day 2. Ischemic lesion volume then decreased significantly to 11±2%, 10±5% and 7±3% at 3, 4 and 7 days of occlusion, respectively ($p<0.05$).

Correlation between spontaneous reperfusion and lesion volume

Fig. 4 showed that the reduction of ischemic lesion volume was coincident with the observed spontaneous reperfusion. The lesion volume decreased significantly from 21±4% on day 2 to 10±5% on day 4, coincident with a progressive increase in CBF over that same period of occlusion.

Discussion

Spontaneous reperfusion after acute stroke is a common clinical phenomenon that may occur many hours or days after stroke onset. Hakim et al. [1] found that the ischemic cortex was reperfused in one third of studied patients within 48 hours of clinical symptoms. In a study by Jorgensen et al. [2], the incidence of spontaneous reperfusion was 77% within first 2 weeks after stroke onset, and spontaneous reperfusion was reported to improve clinical outcome. Bowler et al. [3] found that spontaneous reperfusion after stroke occurred in 42% of subjects within the first week, but was associated with clinical improvement in only 3% of cases. Unfortunately, spontaneous reperfusion and its effects on stroke sequelae has been little studied in animal focal ischemia model.

The intraluminal suture focal stroke model was first used by Koizumi et al. and subsequently by others [4,5,6]. Different sizes of suture have been used as the occluder in this model, including 4-0 and 3-0 monofilament suture with the tip blunted by heating or

coated with silicone [7,8]. In the study, using 4-0 suture as occluder with the reopening of the CCA and pterygopalatine artery, spontaneous reperfusion was noted in the series observation of 7 days.

There are several explanations for this spontaneous reperfusion of the ischemic area. First, the pulsation of the CCA can cause vibration of the suture in the ICA, which can result in gradual dilation of the artery. Second, branches of the pterygopalatine artery anastomose with the vessels that supply the nasal cavity and this can establish collateral flow via the nasal-olfactory artery and the ophthalmic artery to the ACA and MCA [9]. Third, during dissection of the pterygopalatine artery in rats, we found that there was an anastomosis between pterygopalatine artery and the intracranial segment of the ICA (Yang et al. unpublished observation). Reopening of the pterygopalatine artery may also contribute to the observed spontaneous reperfusion. Finally, the reperfusion could be attributed to a shift of the suture even though it has been secured at the ECA by tight ligature [10]. In this study, 4-0 monofilament was used as occluder. And the suture was inserted over 1.9 cm distal from the CCA bifurcation compared to 1.8 cm for 3-0 monofilament [11]. The tip of the suture was found in the proximal segment of the ACA and measured about 3 mm distal of the MCA bifurcation after sacrifice in this model. So a shift in the suture can be ruled out.

Bolander et al. and Persson et al. have also found gradual increase of CBF in a permanent craniectomy focal cerebral stroke model [12,13]. In their studies, the CBF in the lesion area dropped to below 20% of baseline after coagulation of the MCA and gradually increased to about 40% at 7 days after MCAO. By 28 days, there was no significant different between the lesion side and the contralateral side. As the MCA was

coagulated completely in their model, the reperfusion can only come from the leptomeningeal collateral flow from the anterior cerebral (ACA) and posterior cerebral (PCA) arteries [14]. In our study, spontaneous reperfusion began after the second day of MCAO, and CBF gradually increased to normal by the fourth day. In this model, besides the collateral flow from ACA and PCA, the spontaneous reperfusion could also come from ipsilateral CCA, which was reopened after insertion of the suture.

The peak lesion volume time appears to depend on the degree of CBF reduction during MCAO. In one study using 3-0 suture as occluder without reopening the CCA, CBF was decreased to about 15% of baseline during MCAO [15]. Using a similar model, Memezawa et al. found that 15 minutes of MCAO was sufficient to initiate neuronal necrosis, and the lesion volume peaked at 2 to 3 hours after occlusion [16]. Kaplan et al. decreased the CBF to about 21% of baseline in a craniectomy stroke model, and they found that the lesion volumes peaked at 3 to 4 hours of occlusion time at a volume no different from that observed with 24 hours of occlusion [17]. In our study, CBF was decreased to about 43% of baseline, and it takes two days for the lesion volume to peak. Then as the spontaneous reperfusion, the lesion volume decreased.

Several reasons maybe attribute to the lesion reduction. First of all, spontaneous reperfusion in the model could be the major reason for the lesion reduction. The restoration of blood flow after stroke is crucial for the recovery of impaired neural tissue. Whether the reperfusion is beneficial or harmful to brain tissue not only depends on the duration of the ischemic insult and the degree of cellular alteration that occur during the period of ischemia [18,19,20,21], but also depends on the pattern of the reperfusion. Acute reperfusion can be achieved by withdrawal of the suture or by removal of the

surgical clamp or ligature around a major cerebral artery [22,23]. Rapid re-establishment of normal blood flow is not ideal for tissue subjected to prior ischemia. Reactive hyperemia and delayed hyporemia that have been reported during acute reperfusion and both are thought to be harmful to the ischemic tissue [24,25,26,27]. Further, ischemic edema and blood-brain barrier (BBB) disruption have been found to be exacerbated after the acute reperfusion [15,28], particularly related to the sudden surge of reperfusion in the present of hyperemia [29]. In contrast, gradual blood flow restoration significantly reduces ischemic edema and BBB opening [18,29]. The spontaneous reperfusion observed in our study is gradual, both the reactive hyperemia and delayed hyporemia were avoided, could benefit the recovery of the impaired neural tissue. On the other hand, when residual CBF between 20-40% of the baseline was maintained during MCAO, no significant lesion reduction was noted for 7 days occlusion [12,13].

The potential for post-ischemia recovery of impaired cells is determined by both the level and duration of the flow disturbance [30]. Using a model similar to ours, Garcia et al. found irreversible cell injury involving all cell types in the preoptic area, striatum, and portions of the cortex at 3 to 4 days after permanent MCA occlusion. Even at that time, however, some normal neuron remained [31]. It is possible that spontaneous reperfusion rescue brain cells that originally detected by TTC staining as being sick or dead such as that which we observed at 2 days after MCAO [32,33,34].

It is also possible that the apparent reduction in ischemic lesion volume was a reflection of inflammatory cell infiltration into the lesion area or resorption of fluids as brain edema decreased with time. Inflammatory cell invasion of the lesion could reduce TTC staining, creating the histological perception of a reduction in lesion volume. In

Garcia's study, the infiltration of inflammatory cells was detected in the microvessels of the ischemic hemisphere as early as 30 minutes after occlusion. Intravascular neutrophil numbers peaked at 12 hours, and intraparenchymal granulocytes were most numerous at 24 hours [35]. The timing of these immune events is too early to account for the apparent reduction in lesion volume that began in our study after 48 hours. Ischemic brain edema usually peaked at 3 to 4 days after occlusion [36]. Thus, a reduction in fluids in the area of the lesion could attribute to the apparent lesion reduction in the present study.

In summary, we have observed spontaneous reperfusion following permanent insertion of a 4-0 suture in rats. The temporal pattern of spontaneous reperfusion correlates with an apparent reduction in the volume of the resulting ischemic lesion.

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Fig. 1. CBF ipsilateral to the MCAO side immediately after occlusion in each group. Depicted are means ± SD expressed as a percentage of the baseline (pre-occlusion) CBF. There were no significant differences among groups and all groups exhibited a 50-65% decline in CBF in response to MCAO.

Fig. 2. Upper panel: Temporal pattern of regional CBF contralateral to the MCAO side. No significant differences among groups were observed.

Low panel: Temporal pattern of regional CBF ipsilateral to the MCAO side. *$p<0.05$ compared with the baseline.

Fig. 3. Upper panel: Temporal pattern of ischemic lesion volume following MCAO with a 4-0 suture. *$p<0.05$ compared with 1, 3, 4 and 7-day. Depicted are the mean ± SD for the lesion volume as calculated as the percentage of cross-sectional area occupied by the ischemic lesion of the five brain slices assessed.

Low panel: Photographic display of representative brain sections from each group. *$p<0.05$ vs. others.

Fig. 4. Correlation between spontaneous reperfusion and ischemic lesion volume.

*$p<0.05$ compared with baseline, 3-day, 4-day and 7-day

+$p<0.05$ compared with all other groups.

Fig. 1

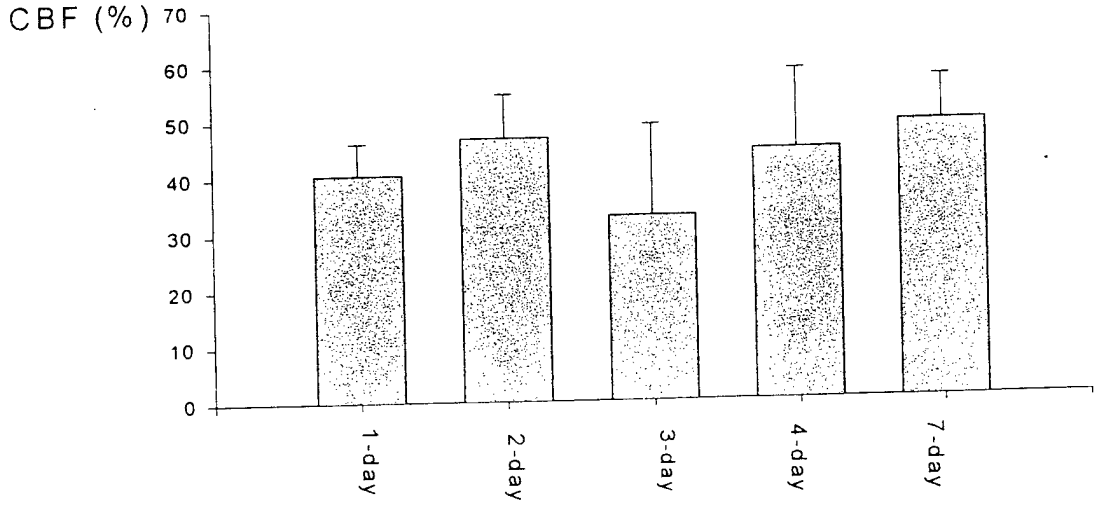


Fig. 2

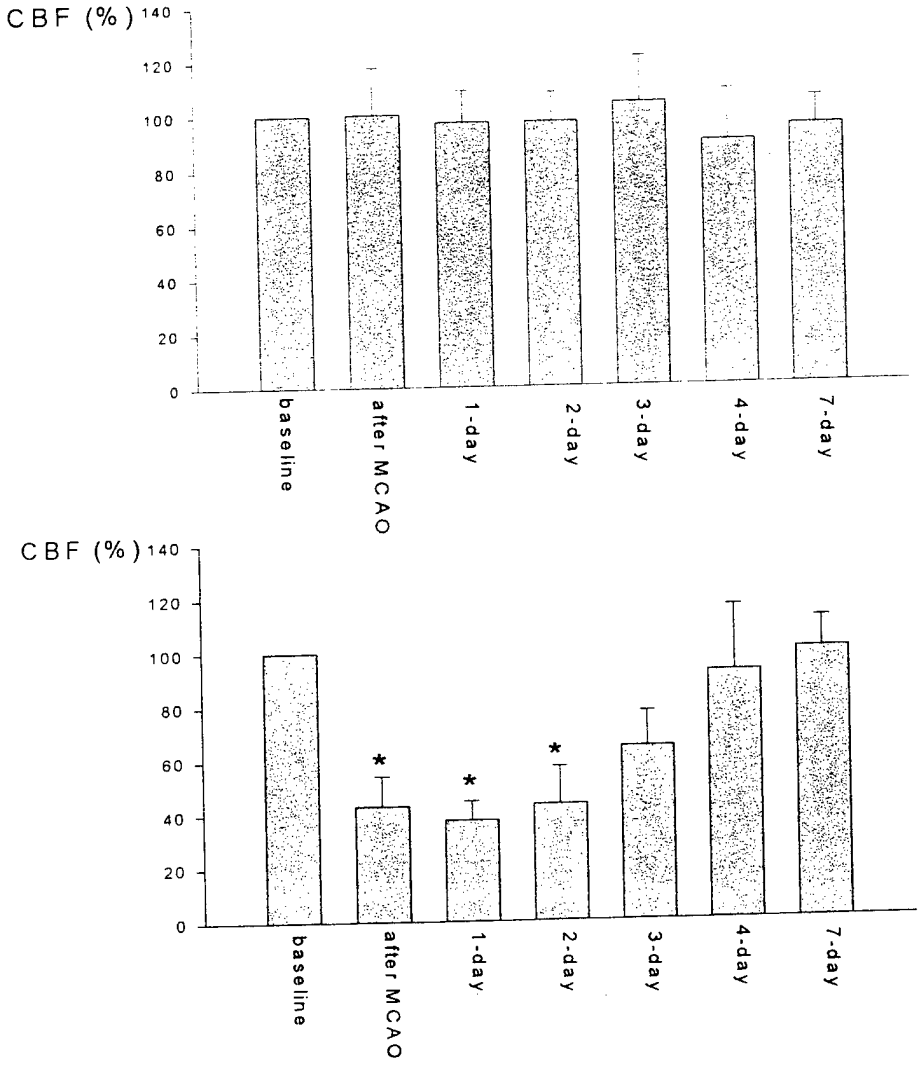
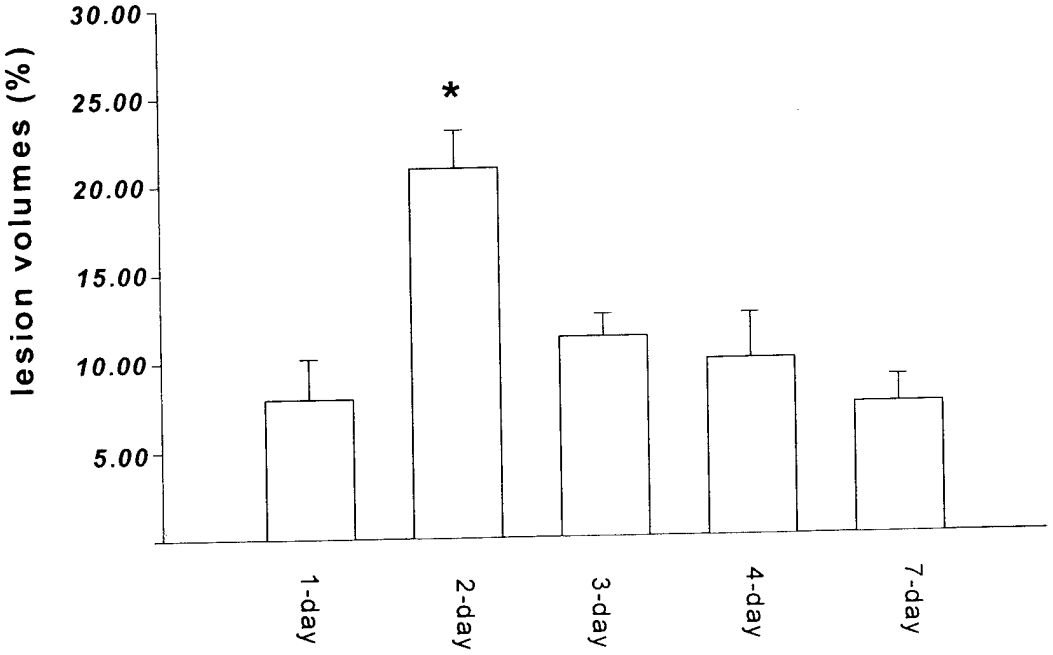
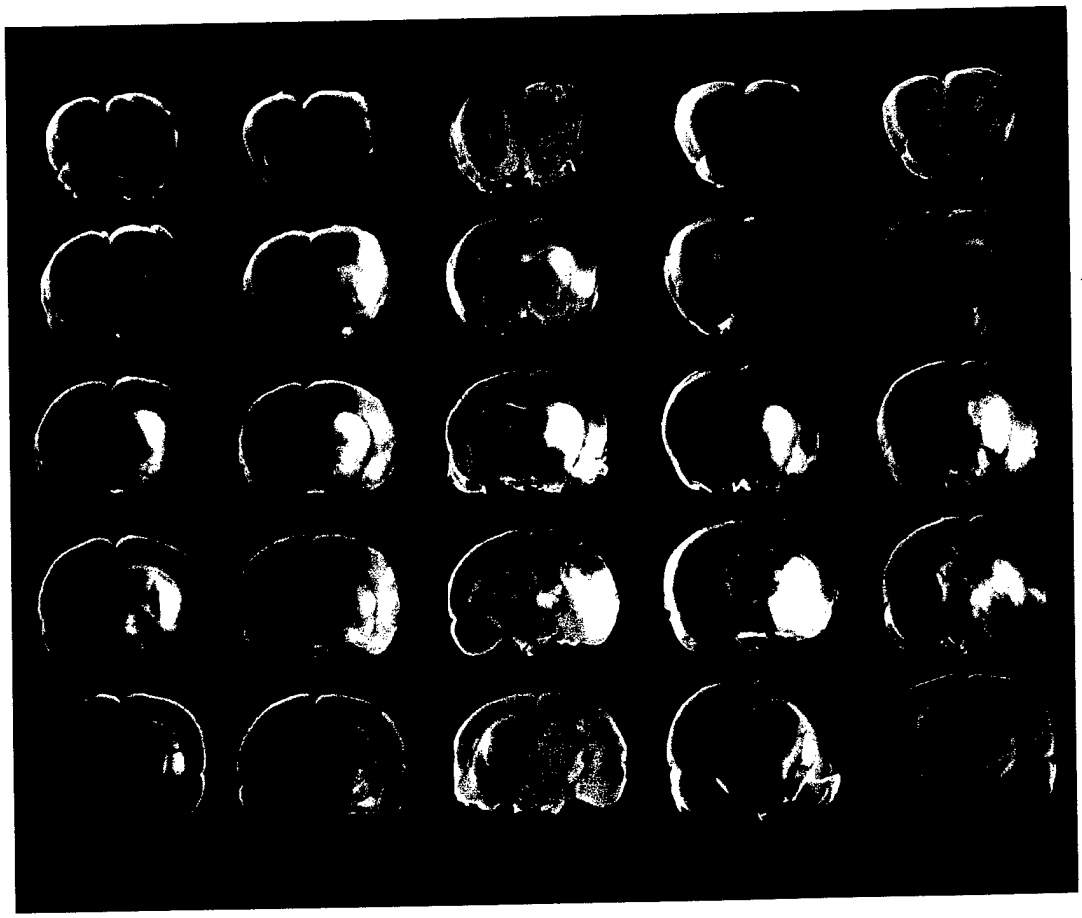

CBF (%)
140
120
100
80
60
40
20
0
baseline
after MCAO
1-day
2-day
3-day
4-day
7-day
CBF (%)
140
120
100
80
60
40
20
0
*
*
*
baseline
after MCAO
1-day
2-day
3-day
4-day
7-day

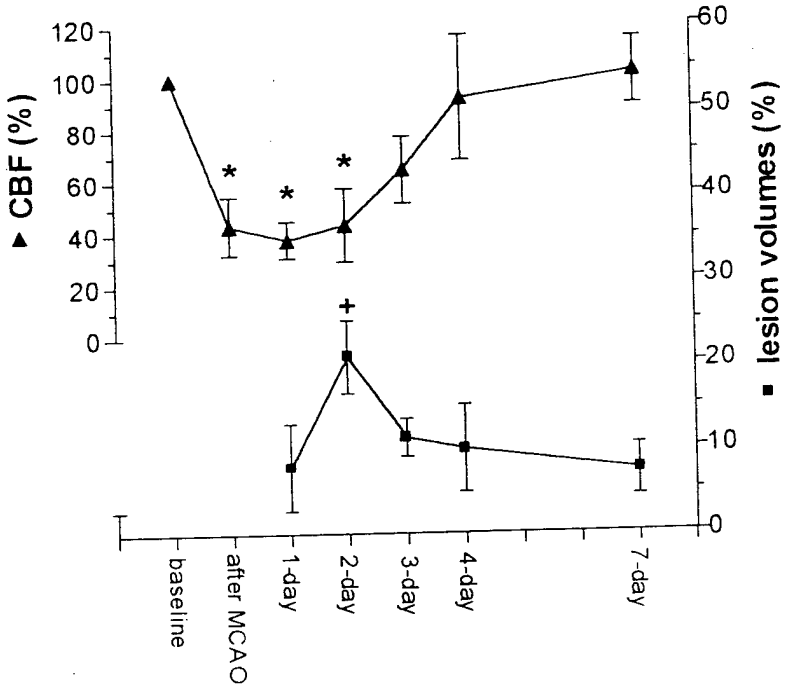
Fig 3





1-day 2-day * 3-day 4-day 7-day

Fig. 4


▲ CBF (%)
120
100
80
60
40
20
0
■ lesion volumes (%)
60
50
40
30
20
10
0
*
*
*
+
baseline
after MCAO
1-day
2-day
3-day
4-day
7-day

Definition of the Anterior Choroidal Artery Territory in Rats Using Intraluminal Occluding Technique

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Summary

This manuscript delineates the territory of the anterior choroidal artery (AChA) in rats, as defined by the induction of an AChA infarction. By advancing a 0.24 mm surgical suture up the internal carotid artery (ICA) to a point 0.5-2 mm proximal to the middle cerebral artery (MCA) origin, the AChA could be occluded and a reliable AChA distribution infarction was produced in 62% (23/37) of animals. The infarct volume, as defined by TTC staining, was 55±7 mm^3. Maps of the infarction, generated by measuring the entire area of overlapping coronal slices, demonstrated that the internal capsule was always damaged. Other areas that might be affected included the hippocampus, thalamus, amygdaloid complex, piriform cortex, dorsal caudatoputamen, and lateral ventricular wall. Positioning the coated suture proximal to the AChA produced a much smaller infarct involving the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus exempt from AChA territory damage. A causative relationship between AChA occlusion and a deep cerebral infarct centered on the internal capsule was further established by: (1) identifying the AChA on the non-ischemic side with colored silicone perfusion, and subsequent similar delineation on the ischemic side, and (2) delineating infarction in the silicone perfused AChA region using hematoxylin and eosin staining and the TUNEL method. The AChA usually originated from the ICA (91% of cases), 1.75±0.12 mm proximal to the MCA bifurcation. Approximately 27% of the AChAs had periamygdaloid branch(es) on its initial segment.

Introduction

Intraluminal middle cerebral artery (MCA) occlusion has gained increasing acceptance as an experimental rat model of focal cerebral ischemia [1]. Advantages of this technique over other methods include avoidance of a craniectomy and the easy ability to establish reperfusion. Disadvantages, however, include variation in infarct sizes. When 4-0 nylon surgical sutures are obtained from two separate companies, infarct size may vary because of difference in suture diameter and thread quality [2]. More severe ischemia results from a thread coated with silicone, which alters the diameter and the quality of the thread, compared to those without silicone coating [3].

Intraluminal MCA occlusion is generally performed by inserting a nylon thread into the internal carotid artery (ICA) and advancing the tip to the bifurcation at the anterior communicating artery [4]. The final thread position at least partially obstructs the entire course of the ipsilateral anterior cerebral A-1 segment and the ICA, raising the possibility that the thread introduced via the ICA can produce ischemia in the anterior hypothalamus and preoptic area of male Wistar rats [5]. Based on the vascular anatomy of the rat hypothalamus, this ischemia localization has been attributed to occlusion of the anterior cerebral and anterior choroidal arteries (AChA) [6,7]. We have observed similar results in male Sprague-Dawley rats [8].

Differentiation of infarction subtypes is essential to the treatment and prevention of recurrences [9]. Recent advanced imaging techniques have renewed interest on AChA infarct territory [10,11,12]. The AChA may supply portions of many anatomical structures [13], leading to discrepancies on the definition of its territory. The pathogenesis of AChA territory stroke is also uncertain [14]. Proposed causes of spontaneous AChA-territory infarction include hypertensive or diabetic-related intrinsic vascular disease of the artery, thromboembolism, and vasospasm after subarachnoid hemorrhage, or surgery for ICA aneurysms [15]. Several subtypes of lacunar stroke, as classified by Fisher [16], involve the AChA territory. The pathogenesis of lacunar stroke is not fully understood, and establishing an experimental model of selective AChA occlusion may provide important clinical and pathological information needed for clarification.

Surgical AChA occlusion has been attempted in dogs, but the operative trauma confused the experimental results [17]. Coyle [6] and Seremin [7] have provided principal information about the AChA territory in rats using vascular cast, cerebral section, and ink injection techniques. Their studies, however, have not led to the conclusions whether selective occlusion of the AChA will absolutely cause AChA territory infarction and which structure/s in the territory will be affected consistently in case of occlusion of the AChA, because there exists rich anastomosis between the AChA and the posterior choroidal artery, the longitudinal hippocampal artery, and the dorsal thalamic artery, respectively [6,7]. Our recent report demonstrated that advancing an occluder up to 0.7-1.9 mm proximal to the MCA in the intracranial segment of the internal carotid artery (ICA) produced a small infarct in the AChA territory [18]. However, the successful rate of the model is less striking (33%). In the present study, we tested whether a variation in the placement of the intraluminal thread could cause AChA territory infarction without affecting the MCA territory with rather higher successful rate. We used infarct overlap maps to define the AChA territory infarction using 2-mm coronal brain slices stained by 2,3,5-triphenyltetrazolium chloride (TTC). Furthermore, We used perfusion of colored silicone followed by microsurgical dissection to define the AChA on the non-ischemic side, and to guide the identification of AChA occlusion on the ischemic side. Perfusion-fixed samples were then examined using 5μ coronal slices stained by hematoxylin and eosin (H&E) and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method to delineate the ischemic lesion.

Materials and Methods

The experimental protocols have been approved by the Institutional Animal Care and Use Committee of the National Cardiovascular Center Japan and the University of Florida, USA. Female Wistar rats, 10-week old and 223-265 grams, were purchased from Oriental Yeast Co., Japan and Charles Rivers Laboratories, (Wilmington, MA), USA and a total of 74 rats were used. Five rats each were kept in a cage and given laboratory chow and tap water ad libitum under a fixed light-dark cycle for two to three weeks prior to

experimentation to allow acclimation to the laboratory condition.

Experimental design and surgical procedures

Rats were anesthetized with 2% isoflurane together with a mixture of 30% oxygen and 70% nitrous oxide. Body temperature during the surgical procedure was monitored with a rectal probe and controlled by a heating pad set at 37.0 °C. The time necessary for introduction of cerebral ischemia was 25 minutes or less. Post-operatively, each rat was kept in a separate cage and allowed to recover from anesthesia at room temperature (21±1°C).

Five of 37 rats subjected to attempted AChA occlusion (experiment 1), together with 5 rats each from the sham-operated and positive control groups, were randomly selected for determination of arterial physiological parameters and blood pressure. The femoral artery in these rats was cannulated for continuous recording of arterial blood pressure until 10 minutes after the onset of cerebral ischemia. Blood sampling was performed twice, at rest before surgery and at 10 minutes after the onset of ischemia, for pH, $PaCO_2$, and PaO_2 measurements.

Body temperature was measured by a probe temporarily inserted 2 cm into the rectum. Measurements were recorded before, 0, 1, and 2 h following surgery in hand-handled, non-anesthetic rats. The resting level temperature before surgery was measured at 3:00±0:30 p.m. in all animals. To evaluate sensorimotor function, the postural reflex test was carried out at the same time as body temperature determination as described previously [18].

Experiment 1. This experiment was designed to induce an AChA territory infarct without affecting the MCA territory, using a variation of the intraluminal technique [8,18]. The protocol was designed to advance an occluder to the position about 1 mm proximal to the MCA bifurcation (Fig. 1). Briefly, under an operating microscope, a 3-0 nylon surgical suture (diameter 0.24 mm) coated with 0.1% poly-L-lysine, was introduced through the right common carotid artery (CCA) and advanced intracranially in the ICA until resistance was encountered. When the advanced distance from the CCA bifurcation was shorter than 18 mm, the occluder was retreated by 1-1.5 mm. If the advanced distance from the CCA bifurcation was longer than 18 mm without feeling

resistance, the occluder was retreated to a position 15.5, 16 or 16.5 mm from the CCA bifurcation. Measured another way, the distance varies between the CCA bifurcation and the base of the front teeth ($\leq$ 32, 32-34 or $\geq$ 34 mm, respectively), since there was subtle variation in the location of the CCA bifurcation. A total of 37 animals were subjected to attempted AChA infarction. Another 16 were used as positive controls (n=11) by advancing the coated suture more than 18 mm from the CCA bifurcation or until resistance was felt. Sham-operated controls (n=5) were prepared by advancing the suture 13 mm from the CCA bifurcation. In all groups, the occluder was fixated intraluminally, and rats were allowed to live for 72 hours.

Since the induction of isolated deep cerebral ischemia was done blindly, at least 4 results could be predicted: no infarction or infarction in the hypothalamic artery (HTA), AChA, and MCA territories, as pointed out in our previous study [18]. If abnormal postural reflex did not appear within 1-2 hours after surgery (supposing that the manifestation indicates AChA territory ischemia), the rat was subjected to a second operation, advancing the occluder 0.5-1 mm further.

Experiment 2. Eleven rats were used to define the AChA and to establish a causative relationship between AChA occlusion and a small deep infarct involving the internal capsule. These rats were perfused with colored silicone after perfusion of 100 ml of saline and 100 ml of 10% of phosphate-buffered formalin, respectively, 72 hours after the onset of ischemia. The circle of Willis was then exposed using microsurgical dissection. Photography was used to validate that the contralateral AChA was filled well with colored silicone, and that the vessel on the ischemic side was obstructed by the occluder. Brain samples were harvested and subjected to H&E staining and the TUNEL method to identify any AChA territory infarction.

Assessment of the infarct size and volume

At 72 hours after ischemia onset, each animal was re-anesthetized with pentobarbital (50 mg/kg body weight) and sacrificed by decapitation. In Experiment 1, the occluder tip position relative to the MCA bifurcation was verified by naked eye visualization before each brain was removed from the cranial base. Photographs were taken in some cases to verify the occluder tip position.

Each brain was then coronally sectioned into seven 2-mm-thick slices, starting from the frontal pole, and stained by TTC. Areas not stained red with TTC in the ipsilateral cerebral structures were considered infarcted. Rats in Experiment 1 were divided into subgroups according to whether the tip of the occluder obstructed the MCA, and whether the infarct was limited to the subcortex involving the internal capsule. We defined a lesion involving the caudatoputamen and adjacent neocortex as an infarct in the MCA territory, even though the lesion might extend to the structures in the non-MCA territory such as the thalamus, dorsal hippocampus, and those supplied by the AChA. We also defined a lesion encompassing the hypothalamus and internal capsule without affecting the MCA territory as AChA territory infarct.

Infarct size of each slice was determined using computer programs as described previously [19]. The total infarct volume was calculated by numerical integration of the infarct volume from all brain slices (Σ(the slice thickness X (the infarcted area on the rostral surface + the infarcted area on the caudal surface)/2)). Similarly, the calculation of the infarct volume in the non-neocortical structures was determined by subtraction of the value of the neocortex from that of the total area. For convenience of analysis, the cerebral cortex above the rhinal fissure was considered as neocortex, while the piriform cortex was defined as non-neocortical structure. Overlap maps of the infarct area were constructed to find common infarct areas and regions at risk. Common infarct area was identified by demarcating the infarct area spatially overlapped in all rats, while the risk infarct area was outlined by demarcating the area encompassing all infarct areas on the overlap maps in all rats. Infarct frequency in respective cerebral regions was calculated by counting each cerebral region with ischemic damage on the overlap maps.

H&E staining and in situ detection of TUNEL-positive cells.

The formalin perfusion-fixed brain samples, cut into seven 2-mm-thick coronal sections, were paraffin-embedded, then cut further into 3-6 slices of 5 μm-thick from the caudal surface of each section. Adjacent sections were used to evaluate histologic changes with H&E staining and TUNEL method.

The presence of TUNEL-positive cells was assessed in situ by direct immunoperoxidase detection of digoxigenin-labeled 3'-OH DNA strand breaks using the

TUNEL method with the Apop Tag IN Situ Apoptosis Detection Kit- Peroxidase (Oncor Technical Assistance Inc. MD 20877 U.S.A.). Sections confirmed by color silicone perfusion with an infarct limited to the AChA territory (n=5) were stained with H&E to clarify the extent of ischemic injury. Lesions were further defined in closely adjacent slices by the TUNEL method [18]. Anatomically matching areas localized contralateral to areas within the occluded vascular territory were used as internal controls for each brain slice.

Statistical analysis

The Mann-Whitney and Chi-square tests were used to compare the infarct area and volume between experimental groups. The statistical software used was Statview-J 4.1 (Abacus Concepts, Inc., Berkeley, CA). A p value <0.05 was considered significant.

Results

Experiment 1. Twenty-three of 37 rats had an AChA infarct. The fate of the other 14 rats included a MCA infarct (4), an incomplete MCA infarct involving the caudatoputamen but with little effects on the adjacent neocortex (5), a lacunar-like infarct limited to the hypothalamus (3), and death before histological assessment (2). All eleven positive controls developed MCA infarcts but 4 of them died before evaluation of histological changes. All negative controls showed normal TTC brain staining.

Body temperature was 37.0-38.0 °C before surgery. During surgery, it decreased to 32.5-35.5 °C due to anesthesia. Body temperature spontaneously increased to 37.6-39.3 °C 1-2 hours after induction of cerebral ischemia. Thirty-two of rats had abnormal postural reflex after two hour of cerebral ischemia (8 of 37 rats received the second operation).

Slight increases in blood pressure and subtle changes in pH and $PaCO_2$ were noted after CCA occlusion and insertion of surgical suture (Table 1). The tip of the suture was advanced to a position 0.5-2.0 mm proximal to the MCA bifurcation in all rats with an infarct in the HTA or AChA territories, and about 0.5-1mm across the MCA bifurcation in four rats with an MCA territory infarct. Incomplete MCA blockage by the

occluder tip (5 rats) produced an infarct involving the caudatoputamen but had little effect on the adjacent neocortex. Withdrawing the occluder tip to a position more than 3 mm proximal to the MCA bifurcation caused no infarct (sham controls).

Fig.2 shows the infarct area (Fig.2a) and volume (Fig.2b) on 2-mm-thick coronal slices in the AChA and MCA infarct groups. Since no significant differences in infarct area or volume between those animals in this group with inadvertent MCA infarct (n=4) and MCA infarction in positive controls (n=7), the data from these two groups were pooled as a single group. The total infarct area on each slice and the infarct area in non-neocortical structures on all slices in the MCA infarct group were significantly larger than those in the AChA infarct group. The AChA infarct group volume was 55±7 mm^2, which constituted about 15% of the total infarct volume and 31% of the non-neocortical infarct volume, respectively, compared to that produced by conventional MCA intraluminal occlusion.

Positioning the coated suture proximal to the AChA produced a much smaller infarct involving the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus exempt from AChA territory damage (data not shown).

Intraluminal MCA occlusion produced infarction not only in the ipsilateral caudatoputamen and adjacent neocortex but also in the ipsilateral hippocampus, hypothalamus, thalamus, amygdaloid complex, piriform cortex, dorsal caudatoputamen, and lateral ventricular wall and internal capsule, even though the latter structures are generally not supplied by the MCA (Fig.3, the lower two lines). Withdrawal of the suture to a position 0.5-2 mm proximal to the MCA bifurcation produced an infarct only encompassing non-neocortical structures, namely an AChA infarct (Fig.3, the upper two lines). The lesion was limited to the two slices 8 and 10 mm from the frontal pole in 17 of 23 rat brains. The infarction might involve the amygdaloid complex, internal capsule, or part of the caudal caudoputamen located to the margin of the MCA territory. The AChA territory lesion was almost always associated with hypothalamic infarction, although generally limited to the lateral hypothalamic area. In the remaining 6 rats, lesions expanded to slices 2 - 4 mm rostral and 2 mm caudal to the 8 and 10 mm slices. Additional structures involved in these infarcts included the rostral caudoputamen,

hippocampus, and part of the thalamus.

Overlap maps for infarct areas were constructed on coronal slices 8 and 10, because all brain samples with an infarct in the AChA territory showed lesions on these two surfaces regardless of staining methods (TTC or H&E). Since MCA infarcts were only evaluated by TTC staining, AChA infarct images stained by H&E or the TUNEL method were not used for making overlap maps. The common (100%) infarct area was located only on the 8 mm slice (about 6 mm^2), while the risk infarct area on overlap maps made from the 8 and 10 mm slices measured about 42-48 mm^2. Structures constantly affected by intraluminal AChA occlusion were the internal capsule and endopeduncular nucleus. Part of the caudal caudatoputamen and the lateral hypothalamus were affected in 83% of rats having an infarct in the AChA territory, while the frequency of involvement in the entire caudal caudatoputamen was 13% (Fig.4a). In contrast, all aforementioned structures and the cortex were infarcted on the 8 mm slice in the MCA infarct group (Fig.4b).

On the 10 mm slice, the risk area following AChA infarction included part of the thalamus (70%), dorsal hippocampus (17%), amygdala and piriform cortex (17%) (Fig.4c). Intraluminal MCA occusion increased infarct frequency to 80-90% for the thalamus, 91% for the amygdaloid and piriform cortex, and 36% for the hippocampus (Fig.4d).

Experiment 2. Perfusion with colored silicone on the non-ischemic side demonstrated the AChA from its ICA origin to the point where the vessel entered parenchyma (Fig. 5a,b). The distance between the AChA and MCA origins from the ICA was 1.74±0.12 mm, with a range from 0.85 to 2.64 mm. Several variations were identified: 1) the AChA might stem from the posterior cerebral artery (1/11); 2) branch(es) to the periamygdaloid cortex might originate from the ICA (8/11), and 3) and/or the AChA had two branches (3/11), one of which passed to the periamygdaloid cortex, the other entered the brain parenchyma. Obstruction of the AChA on the ischemic side was defined by 1) a distance between the occluder tip and the MCA between 0.5 and 2 mm, 2) the AChA was not filled with colored silicone and 3) blood residue remained in the occluded AChA (not always evident). AChA occlusion specimens showed ischemic lesions in the internal capsule both on H&E staining and TUNEL method (Fig.6a,b). Withdrawing the occluder

proximal to the AChA caused a much smaller infarct limited to the hypothalamus and sparing the AChA territory (Fig.6c,d). Damage in marginal regions rarely supplied by the AChA such as the hippocampus could be readily identified by H&E staining and TUNEL method (Fig.6e,f)

Discussion

Ischemic infarction accounts for about 75% of all strokes [20], and those involving the AChA territory comprise about 3% of all hospitalized infarct patients [21]. The use of physiologically regulated and reproducible ischemic brain injury animal models is crucial in the understanding of both the mechanisms governing occurrence and potential therapeutic strategies [22]. We herein report that a suture advanced to a position 0.5-2 mm proximal to the MCA bifurcation obstructs the initial segment of the AChA and produces a small deep infarction in the AChA territory of rats. The brain regions affected by this maneuver were limited to non-neocortical structures. Confirmed by mapping of the infarct area, the internal capsule was constantly affected, suggesting that the ischemic core in AChA occlusion involve the structure.

The role of the AChA in the variation of infarct size produced by intraluminal MCA occlusion: The AChA is the largest branch of the intracranial ICA between the MCA and posterior cerebral artery (PCA), and originates from the ICA at a position 1.75±0.12 mm proximal to the MCA bifurcation as demonstrated in the present study. This gap makes it possible to advance an occluder up to a position between the AChA and the MCA origins. If the occluder diameter is large enough to effectively close the entire intracranial ICA lumen at that position, then flow into adjacent ICA branches could cease, while MCA flow could be maintained through retrograde anterior cerebral artery collaterals. The 3-0 surgical suture, coated with poly-L-lysine, was ideal in achieving this goal. The coating process did not increase the diameter of the suture, but appears to increase adhesive forces around the suture, leading to effective occlusion of the target site by adhesion of the occluder to the surrounding vascular endothelium [23].

Furthermore, a mild abnormality of the postural reflex (grade ≤ 2) with or without increase of body temperature during the first 30-60 minutes following surgery was highly

predictive of an AChA infarct [18]. Although the passage of the suture is done "blindly," the present protocol produced AChA territory infarction in 62% of rats (the AChA originates from the PCA in 9% of cases herein). The infarct caused by the AChA occlusion constituted 15% of the total infarct volume induced by conventional intraluminal MCA occlusion.

Our model of AChA occlusion eliminates some of the shortcomings of intraluminal occlusion of the MCA, in which other branches of the ICA are involved in addition to the MCA. By advancing an occluder proximal to the AChA, we excluded the possibility that the medial and lateral hypothalamus, preoptic region, optic chiasm, and marginal region of the internal capsule near to the lateral hypothalamus area are supplied by the AChA. Using color silicone perfusion followed by microsurgery technique, the medial, lateral and abdominal aspects of the ICA segment between the MCA and the PCA were well shown, and in general, the AChA, branch to optic nerve, branch to periamygdaloid cortex, and HTA originate from the segment. Since the branch to periamygdaloid cortex originates from the AChA in about 30% frequency, its domain could be defined to the AChA territory. The territory of the branch to optic nerve is readily excluded according to its location and definition. Although we can not role out the other branches originating from the dorsal segment of the ICA between the HTA and the MCA, the results of the present study are consistent with the works of Coyles [6] and Seremin [7], as well as the results conducted in dog [17], indicating that our model provides, at least, a mimic of selective occlusion of the AChA alone.

AChA Territory: In humans, as established by autopsy injection techniques in postmortem specimens and modern imaging techniques, the AChA gives off branches to the medial segment of the globus pallidus, the piriform cortex, amygdala, anterior hippocampus and dentate gyrus. In addition, the AChA supplies the middle third of the cerebral peduncle, subthalamus, part of the thalamus, and part of the internal capsule before it terminates in the choroid plexus [11,15]. AChA occlusion in dogs produces gross infarction in a similar pattern [17]. To our knowledge, an isolated AChA occlusion model in rats has not been reported.

The arterial patterns in the rat brain are remarkably similar to those in humans

[6,24,25], and the similar distribution of infarction in our study strongly suggests that AChA occlusion is responsible for much of the deep ischemia seen in our model. That a suture withdrawn to the position 3 or more mm proximal to the MCA bifurcation failed to produce any infarct also argues that AChA occlusion contributed to ischemia in those structures. Although the AChA has many variations in branching in humans, the most constant branches are those to the optic tract, cerebral peduncle, posterior limb of the internal capsule, and choroid plexus [26,27]. The common infarct structures produced by AChA occlusion in the present study, verified by the infarct area maps, encompassed the internal capsule and endopeduncular nucleus, suggesting that the AChA give off constant branches to these structures in rats.

The AChA territory in humans and dogs does not include the hypothalamus. Although 57%-83% of AChA infarcts in the present study showed ischemic damage in the medial and/or lateral hypothalamus, our ability to produce a hypothalamic lesion independent of AChA territory infarction argues that at least one artery branch to the hypothalamus originates from the ICA proximal to the AChA.

Collateral circulation of the AChA: The AChA may not supply the hippocampus or caudoputamen in rats [6,24,28], and any damage in the caudal caudatoputamen may be due to occlusion of branches from the caudal part of the circle of Willis [28]. The AChA anastomoses with the MCA, posterior communicating artery and PCA. An intraluminal thread tip in the position just proximal to the MCA bifurcation may interfere with other ICA branches to the PCA or small direct perforators [29]. An arterial loop is generally present between the posterior lateral choroidal artery (a branch of the PCA) and the AChA (Fig. 1) [6]. Anastomoses are also present between the MCA and the longitudinal hippocampal artery (a branch of the PCA, Fig. 1) and between the MCA and AChA in the amygdaloid region. As demonstrated on infarct area overlapping maps, intraluminal MCA occlusion markedly increases infarct frequency of the amygdaloid complex. Therefore, whether infarction occurs in the hippocampus and amygdalar region may depend on the extent of collaterals. Furthermore, because the branch to the periamygdaloid cortex directly originates from the ICA in most cases, advancing the tip of occluder up to a position between the branch to periamygdaloid cortex and the AChA

might produce an infarct sparing damage of the amygdaloid complex. Another possibility is the presence of "steal phenomenon", since the choroidal artery loop may divert blood supply from the PCA to the AChA when the AChA origin is occluded.

The advantages and disadvantages of the present model: It is thought that the brain vasculature in rats is different between not only rat strains but also bleeders, from the fact of the probability of successful forebrain ischemia in 4-vessel-occlusion model. In experiment 1, we used rats purchased from a Japanese company and in experiment 2, we used an American bleeder. Furthermore, we have tested both sexes of another stain, Sprague-Dawley rat, and demonstrated that advancing the occluder proximal to the MCA to obstruct the AChA caused an infarct involving the internal capsule.

The advantage of the present model has been already used in our recent study, demonstrating that a correlation between abnormal postural reflex and AChA territory infarction involving the internal capsule [18,30]. The findings are consistent with the report conducted in human beings, in which axonal injury in the internal capsule correlates with motor impairment after stroke [31]. Human stroke usually affects cerebral white matter. To our lower case knowledge, this is the first focal cerebral ischemic model in rodent that selectively affects the white matter. Since white matter consists of axons and glia but not synapses, ischemic injury of white matter presumably is mediated by nonsynaptic cellular mechanisms, which are different from those of neuron death. Our model provides a tool suitable for investigating the mechanisms of ischemic damage in the white matter.

The disadvantage of the present model is the large variability in infarct size. For example, this model may not be suitable for investigating interventions aimed at reducing infarct size. On the other hand, the model addresses that the variation is the nature of vasculature. For example, using vascular perfusion followed by micro-dissection to investigate the supply of the hypothalamus, it was reported that "the optic area is supplied by vessels that originate in the anterior cerebral arteries and run upwards and backwards at both sides of the third ventricle"[32]. Our recent study demonstrated that advancing the occluder proximal to the AChA in the ICA caused optic area infarction in approximately 15% of rats [18], indicating a variation that this region may be supplied by the vessels

that originates from the ICA proximal to the AChA. High frequency of ischemic damage in the thalamus in the present study (Fig. 4b) suggests that a part of the thalamus be supplied by the AChA in rats. The results are consistent with the work of Scremin [7]. However, an endocast and scanning electron microscopic study argues against that the thalamus is supplied by the AChA [29]. The vasculature variation may explain for the discrepancy.

The territorial variation of the AChA may also include the hippocampus. In the present study, 17% of rats with an AChA infarct had damage in the hippocampus using TTC staining to delineate ischemic lesion. The frequency of damage in the hippocampus should increase when H&E staining and the TUNEL method are employed (Fig. 6e,f) [18]. This finding is consisted with those reported both in dog and humans. Several hypotheses have been proposed to explain hippocampal damage following intraluminal MCA occlusion in rats [33]. They include edema-related elevation of intracranial pressure [33], loss of trophic support [34], excessive excitation of hippocampal neurons resulting from loss of the inhibitory input, overactivation of the excitatory input from the ischemic core or penumbra projecting to the hippocampus [35], and opening of the blood-brain barrier following MCA occlusion, which may expose hippocampal neurons to blood-born molecules such as iron-containing heme protein [36]. Our findings argue a much simple explanation for the damage: shortage of blood flow from branches of the AChA that can supply the hippocampus.

In conclusion, intraluminal occlusion of the MCA in rats may produce an infarct beyond the MCA territory by obstructing other ICA branches, including the AChA. An occluder tip placed 1-2 mm proximal to the MCA origin is capable of producing a small deep infarct in AChA territory in rats. The internal capsule is absolutely damaged in such cases, while wider territories of AChA infarction depends on variation of AChA branching and its collaterals.

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Table 1. Arterial Physiological Parameters and Blood Pressure (BP) Before and After the Onset of Focal Cerebral Ischemia

	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	pH	BP (mmHg)	
				Systolic	Diastolic
Pre-occlusion					
Control	40 ± 4	198 ± 16	7.39 ± 0.02	122 ± 3	88 ± 3
AChAI	36 ± 1	214 ± 7	7.41 ± 0.01	122 ± 3	85 ± 5
MCAI	40 ± 2	193 ± 15	7.40 ± 0.02	129 ± 5	81 ± 3
10 min after occlusion					
Control	42 ± 3	189 ± 18	7.36 ± 0.03	140 ± 4	92 ± 6
AChAI	39 ± 1	211 ± 7	7.40 ± 0.02	140 ± 7	97 ± 5
MCAI	45 ± 3	195 ± 15	7.36 ± 0.02	145 ± 6	92 ± 6

Values are expressed as mean±SEM based on 4-5 rats in each group.

A 3-0 nylon surgical suture coated with 0.1% poly-L-lysine was used for induction of cerebral ischemia by intraluminal occlusion. In the Control group, the occluder was advanced to 13 mm from the carotid artery bifurcation (CAB) via the internal carotid artery (ICA). In the AChAI group, the occluder was similarly advanced until resistance was felt, and then retreated by 1.5 or 3 mm, if the advanced distance from the CAB was less than 18 mm, or equal to or more than 18 mm, respectively. (see text for details). In the MCAI group, the occluder was advanced to the cranium via the ICA until resistance was felt.

Legends for Figures

Fig. 1. A schematic illustration of intracranial arteries showing the position of the occluder for intraluminal occlusion of the anterior choroidal artery (AChA). The surgical suture was advanced up to about 1 mm proximal to the middle cerebral artery (MCA). ACA, anterior cerebral artery; AcomA, anterior communicating artery (It is also used to represent anterior common artery in rats); BA, basilar artery; ICA, internal carotid artery; PCA, posterior cerebral artery; PcomA, posterior communicating artery. The large arrow with the thick solid line represents the position of an occluder for occlusion of the ICA segment proximal to the MCA bifurcation, while the arrow with the dotted line indicates the usual position of the occluder for MCA occlusion by the conventional intraluminal technique. The choroidal artery (ChA) loop with the dotted line represents an arterial loop connecting the AChA to the posterior lateral choroidal artery. The LHipA-MCA with the dotted line indicates the anastomosis between the longitudinal hippocampal artery (LHipA) and the MCA.

Fig. 2. The infarct area (a) on 2-mm-thick coronal slices, starting from the frontal pole, and the infarct volume (b) after 72 h of focal cerebral ischemia induced by insertion of a 3-0 nylon surgical suture coated with 0.1% of poly-L-lysine via the internal carotid artery (ICA). Anterior choroidal artery infarct (AChAI, see text for details) was successfully produced in 23 of 37 rats. Middle cerebral artery infarct (MCAI) was erroneously produced in four rats. The infarct data in these rats were pooled together with the positive control group (n=7), for which the MCA was occluded by advancing the occluder across the MCA. T-MCAI represents the total infarct area or total infarct volume including both neocortex and non-neocortical structures. N-MCAI indicates the infarct area or infarct volume of the non-neocortical structure. Bars represent S.E.M. *$p<0.05$, **$p<0.01$ vs. the AChAI group.

Fig.3. Four representative rat brains with 4 slices (2 mm-thick) each between 6-12 mm from the frontal pole were stained by 2,3,5-triphenyltetrazolium chloride immersion method. The areas without staining (white) represent infarction on right side of the slices.

The upper two lines show a small deep infarct produced by intraluminal occlusion of the anterior choroidal artery (AChA). The internal capsule (IC) was always affected. Intraluminal occusion of the middle cerebral artery (MCA) caused a huge infarction (shown in the lower two lines) encompassing the ipsilateral dorsal hippocampus (Hip), thalamus (Th), hypothalamus (HT) and the AChA territory which are outside the area supplied by the MCA. CPU: caudatoputamen.

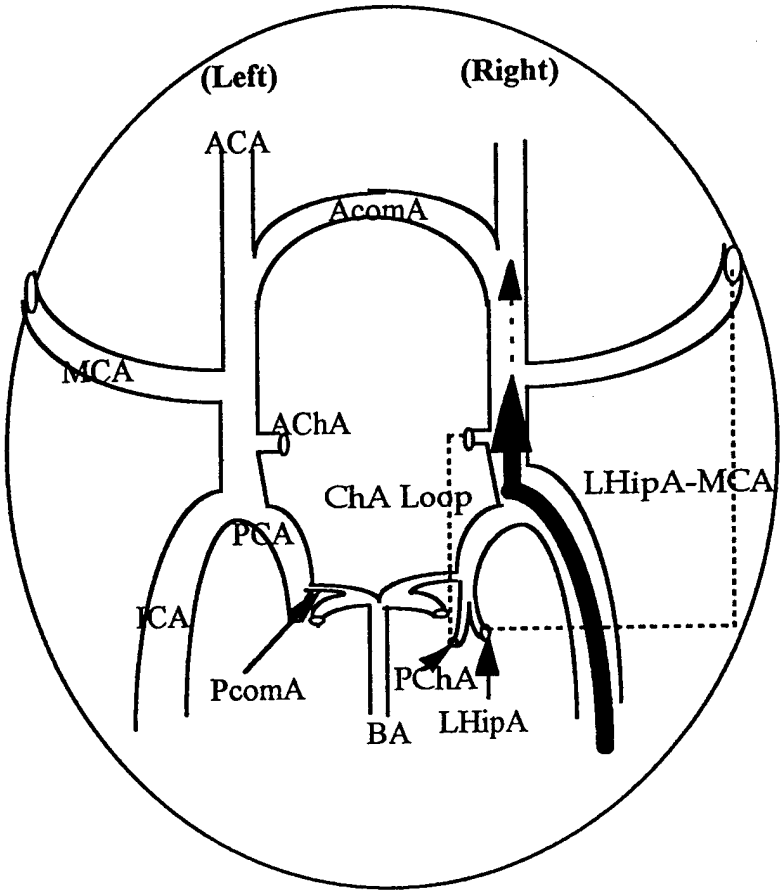
Fig. 4. Infarct area overlap maps of the coronal slices were made from 23 brains with anterior choroidal artery (AChA) infarct (a,b) and from 11 brains with middle cerebral (MCA) infarct. The slices are 8 (a,b) and 10 mm (c,d) from the frontal pole, respectively. The common infarct area in AChA infarct was confined to the slice shown in (a) and involved the internal capsule and endopeduncular nucleus according to Pellegrino et al. [37]. Infarct frequency in the marginal regions such as cortical amygdaloid nucleus was very low in the AChA infarct group, while intraluminal occlusion both the AChA and MCA significantly increased the infarct frequency. CPU, caudoputamen, IC, internal capsule, EP, endopeduncular nucleus, LHA, lateral hypothalamic area, VMH, ventromedial nucleus of the hypothalamus, Hip, hippocampus, TH, thalamus, PC, cerebral peduncle, V, lateral ventricle, ACO, cortical amygdaloid nucleus.

Fig. 5. The arterial circle and its branches at the brain base and occlusion of the anterior choroidal artery (AChA) in rats. To define the AChA and to establish causative relationship between occlusion of the AChA and a small deep, rats were subjected to a surgery advancing an occluder via the internal carotid artery (ICA) up to about 1 mm proximal to the middle cerebral artery. Seventy-two hours later, the rats were perfused with colored silicone following perfusion of 100 ml of saline and 100 ml of 10% of phosphate-buffered formalin, respectively. The circle of Willis was exposed by microsurgical dissection. The normal arterial circle with its branches at the brain base was shown on panel (a). Panel (b) shows that the AChA is divided into three parts, I, II and III from the same sample of panel (a). The common trunk is named as segment 'I', which can be seen from a ventral view because the AChA courses laterally on the surface of the brain before entering the brain parenchyma. The superficial branches are named as

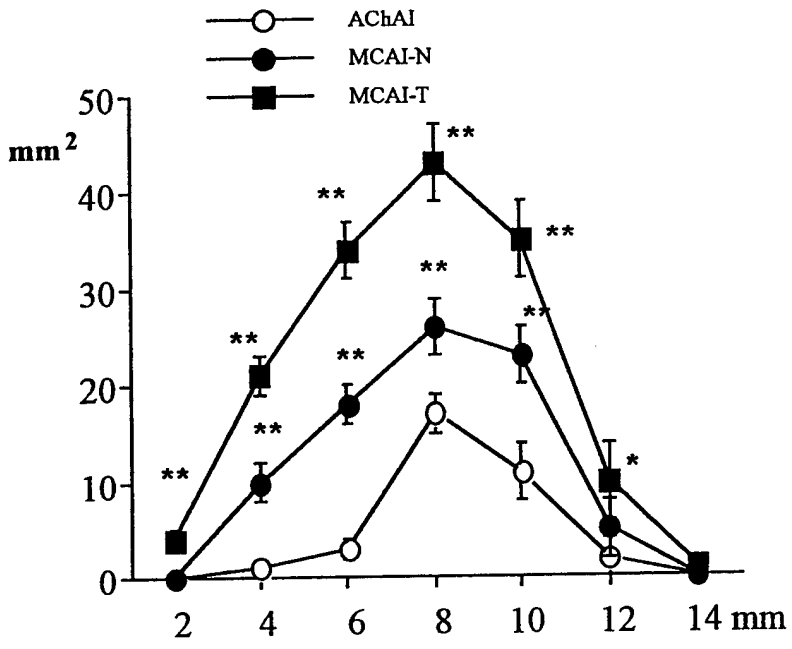
the segment 'II'. The segment of the AChA entering the parenchyma is named 'III'. On the panel (b), the overlying brain tissue of segment 'III' was dissected. Advancing an occluder about 1 mm proximal to the MCA led to obstruct the AChA (c,d), which originated from the ICA 1.74±0.12 mm (n=11) proximal to the MCA. ACA, anterior cerebral artery; HTA, hypothalamic artery; OC, optic chiasm; PCA, posterior cerebral artery; PcomA, posterior communicating artery; PS, pituitary stalk.

Fig. 6. Representative histologic findings showing small infarcts in anterior choroidal artery (AChA) territory. Ischemic damage was visualized by hematoxylin and eosin (a,c,e) staining and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method (b,d,f). Intraluminal occlusion of the AChA caused a small deep infarct centered on the internal capsule (a). Advancing the occluder proximal to the AChA produced a much smaller infarct limited to the hypothalamus exempt from AChA territory (c). The square notes the regions where staining with the TUNEL method on the closely adjacent slices showed TUNEL-positive cells (b,d). Large arrow heads indicates the wall of the third ventricle (a,c). Large arrows indicates infarction in (a,c). The damaged cells in the hippocampal CA1 region ~~were~~ are shown at high magnification (630x, Panel: e,f). Small white triangles indicate the damaged cells stained by hematoxylin and eosin (e). Small black triangles indicate TUNEL-positive cells (b,d,f).

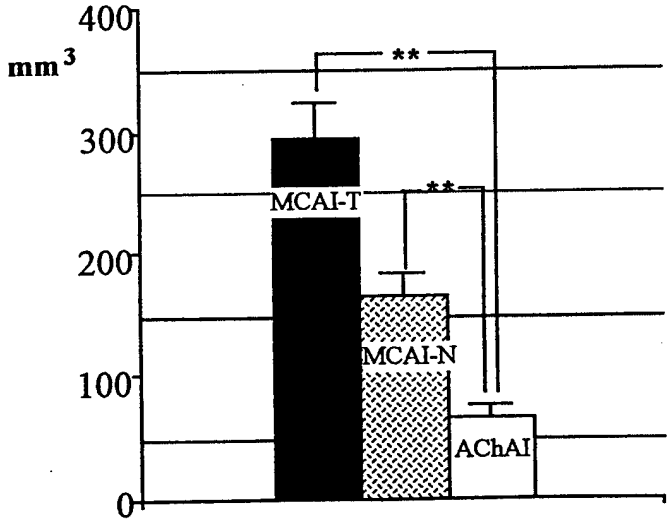
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(Left)
(Right)
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AChA
ChA Loop
LHipA-MCA
PCA
ICA
PcomA
PChA
BA
LHipA

a

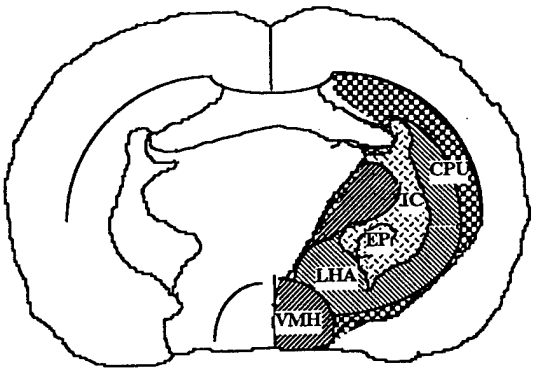

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MCAI-T
MCAI-N
AChAI



a



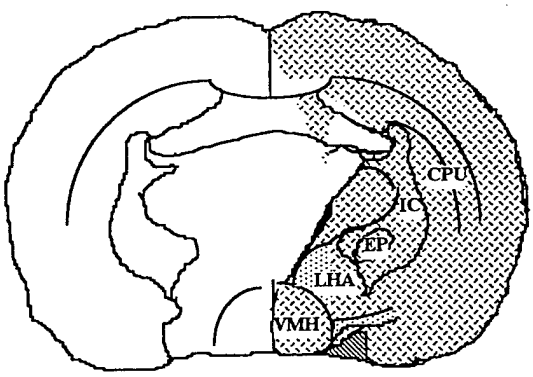
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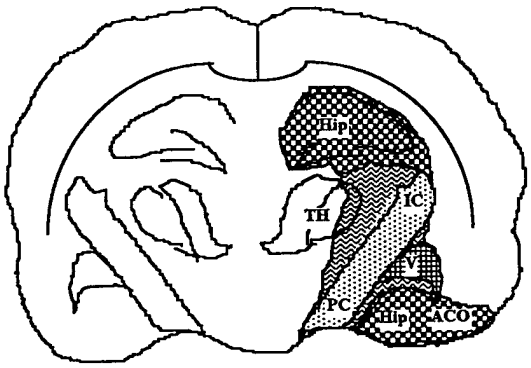
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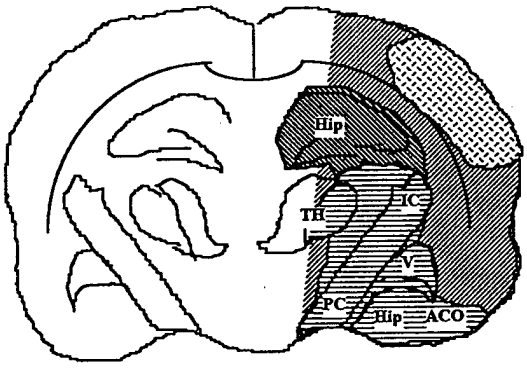
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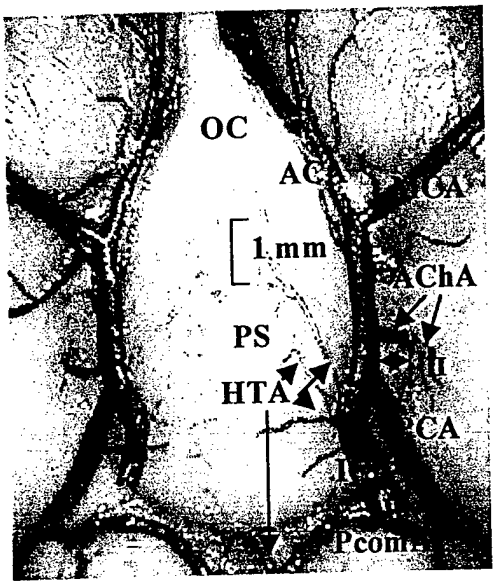
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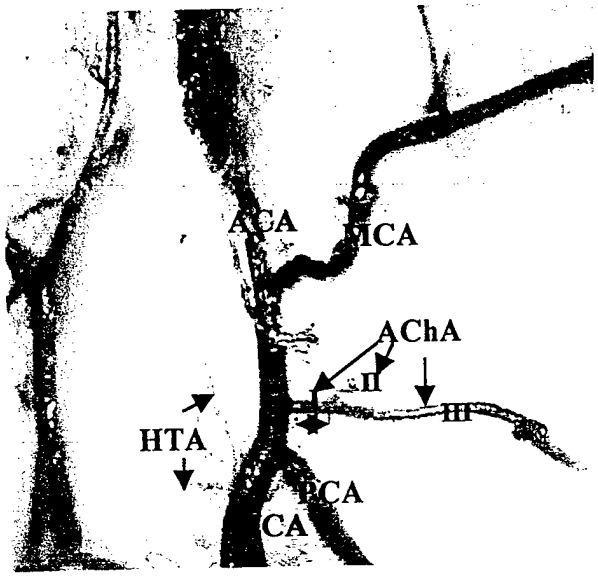
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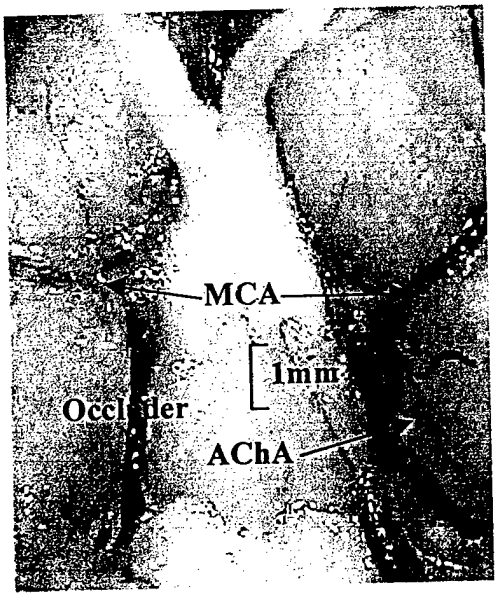
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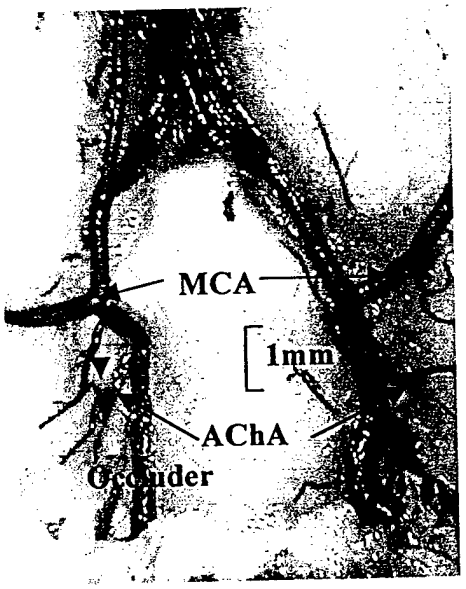
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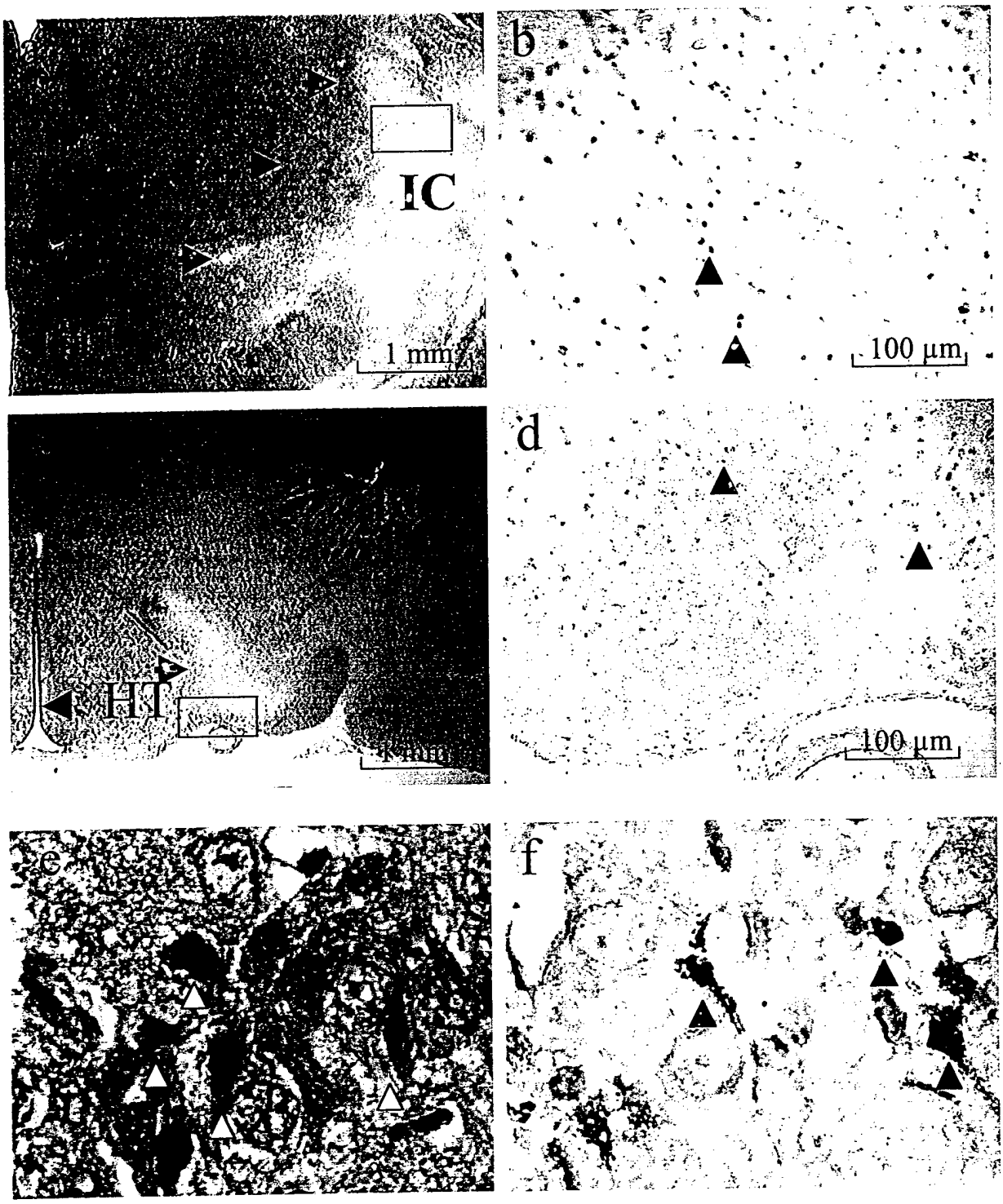

ACA
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HTA
PCA
CA

c


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Occluder
AChA

d


MCA
1mm
AChA
Occluder


IC
1 mm
b
100 µm
HT
1 mm
d
100 µm
c
f

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Brain Research 853 (2000) 1–4

Research report

Hypoperfusion induces overexpression of β-amyloid precursor protein mRNA in a focal ischemic rodent model

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Research report

Hypoperfusion induces overexpression of β-amyloid precursor protein mRNA in a focal ischemic rodent model

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Abstract

Silent stroke is one of the risk factors of dementia. In the present study, we used a novel focal ischemic animal model to investigate the effects of comparatively small changes of cerebral blood flow (CBF) on the expression of β-amyloid precursor protein (APP) mRNA. Focal ischemia was achieved by introducing a 4-0 monofilament to the bifurcation of anterior and middle cerebral arteries. Brain samples were harvested from ischemic core and penumbra of cortices at 1, 4 and 7 days following ischemia. The expression of APP mRNA was assessed by RT-PCR. The CBF was decreased to 50% for 1 day after stroke and recovered to 90% at the fourth day after stroke. The changes of CBF were accompanied by an increase in the expression of APP mRNA. APP mRNA increased to 208% and 152% in the penumbra and core ischemic regions, respectively, on the fourth day after MCAO and remained high through the seventh day of ischemia. This study suggests brain hypoperfusion enhances APP mRNA expression and may contribute to the progression of cognitive impairment after silent stroke. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cerebral blood flow; Amyloid precursor protein; Focal cerebral ischemia; Stroke

1. Introduction

Epidemiology studies suggest that cerebral stroke is one of the risk factors of dementia [7]. Patients with previous stroke events are more likely to develop dementia than age- and sex-matched subjects. In animal studies, we and others have demonstrated an overexpression of β-amyloid precursor protein (APP) and its mRNA in both global and focal ischemic animals [4,5,9]. We previously used a middle cerebral artery occlusion (MCAO)-induced transient focal ischemic rat model and decreased cerebral blood flow (CBF) to 20% of its basal level [11,13]. MCAO induced a 60% increase in APP mRNA in the penumbra of the lesion side as early as 1 h after ischemia and about 50% increase in APP mRNA in both core and penumbra regions 24 h later [11]. To further investigate the effects of chronic and less severe hypoperfusion on APP expression, we used a new focal ischemic rat model. In the new model, CBF is decreased by 50% and then spontaneously recovers to its basal level. This model allows us to assess the effects of silent stroke, in which the patient suffers the defects in CBF without the symptoms of stroke, on the later development of dementia.

2. Materials and methods

2.1. Animals

Sprague–Dawley female rats (200–225 g b.wt.) were purchased from Charles Rivers Laboratories (Wilmington, MA). They were housed in pairs in hanging, stainless steel cages in a temperature controlled room (25 ± 1°C) with daily light cycle (light on 0700 to 1900 h daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study.

2.2. Focal ischemic model

MCAO was achieved according to the methods described previously [13,14,19]. Briefly, following adminis-

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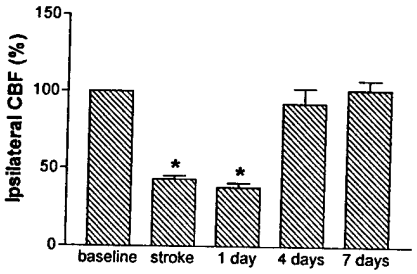


Fig. 1. Effects of blockade of the middle cerebral artery with a 4-0 monofilament thread on ipsilateral CBF in female rats. The observed declines in CBF immediately following occlusion and 1 day later were statistically different from baseline CBF values. $*p < 0.05$ vs. baseline CBF. Depicted are mean ± S.E.M. for five rats/group.

tration of anesthetics of ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) on the left side were exposed through a midline cervical incision and then gently dissected away from adjacent nerves. A 4-0 monofilament nylon suture was introduced into the left ECA lumen and gently advanced to the distal ICA until resistance was felt where the suture passed the bifurcation of MCA and anterior cerebral artery (ACA). Rectal temperature was monitored and maintained between 36.5°C and 37°C during the entire stroke procedure. The suture was left at the site afterwards.

2.3. Cerebral blood flow measurement

CBF was measured in all rats that underwent focal ischemia surgery [11]. A middle line section exposed the small area around bregma. Two symmetrical holes were drilled through the skull and adjacent to the dura. These two holes were located at 1.5 mm posterior to and 3.5 mm left/right of bregma. Two probes of a digital laser perfusion monitor (MICROFLO DSP, Oxford Optronix, Oxford, England) were placed on the dura to record the second to second change in CBF before, during, and after MCAO.

2.4. RT-PCR of APP mRNA

Rats were decapitated, the whole brains were dissected sampled for RT-PCR as described previously [11,12]. A neuroanatomical definition of the penumbra and core of

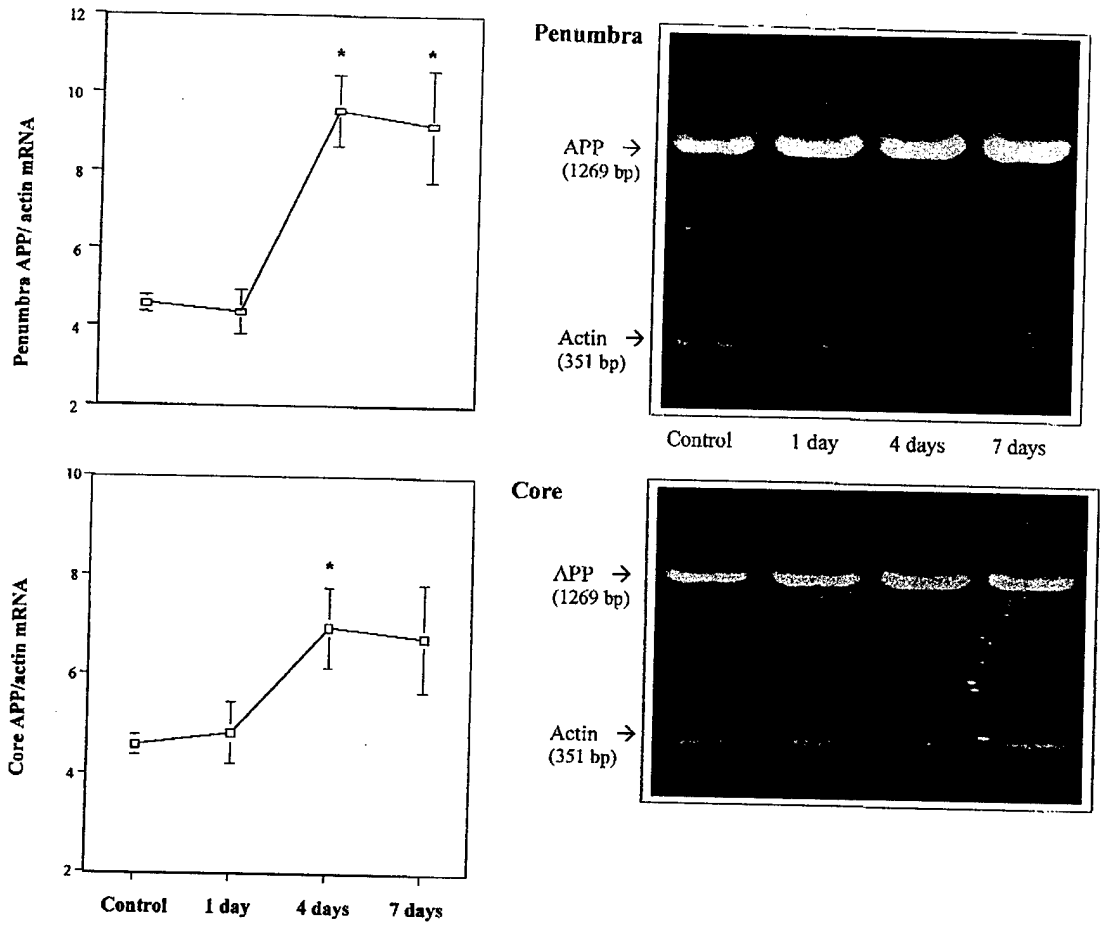


Fig. 2. (a) Effects of blockade of CBF through the middle cerebral artery on APP mRNA expression in the ipsilateral penumbra and core. $*p < 0.05$ vs. control values. Depicted are mean ± S.E.M. for four to six rats/group. (b) Representative blot of APP and actin mRNA from ipsilateral penumbra and core of animals subjected to MCAO.

the infarct was used and base upon the blood supplies to the regions. Briefly, the superior one-third of each cortical area from longitudinal cerebral fissure to lateral cerebral fissure was defined as the penumbral region, which is supplied predominantly by the ACA and, to a small extent, by the MCA. The inferior two-thirds was defined as the core region that is perfused exclusively by MCA. The application of this definition of the penumbra and core of the infarct is well-accepted and used by us [11,13,14].

RT-PCR was performed to assess the expression of APP mRNA according to methods described previously [13]. Two oligonucleotide primers for APP mRNA were designed to include a Kunitz protease inhibitor domain (KPI): sense primer 5′-TGCC ACCA CTAC CACA ACTA-3′ and antisense primer: 5′-GTTC TGCA TCTG CTCA AAG-3′. β-actin was used as an internal control of RT-PCR to be co-amplified with APP. This semi-quantitative measure of APP was expressed as ratios of APP/β-actin.

2.5. Statistics

The ratio of APP mRNA/actin mRNA were calculated. Kruskal–Wallis nonparametric analysis was used for statistical analysis. The Mann–Whitney *U*-tests were used when Kruskal–Wallis showed significance among groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. The effects of focal ischemia in the CBF

The introduction of a 4-0 monofilament to the bifurcation of ACA and MCA decreased the CBF to 44.8% of its basal level (Fig. 1). The CBF remained at 39.3% 1 day after ischemia. Despite persistent blockade of the MCA, CBF recovered to 87.9% by the fourth day, fully recovered to its basal level by the seventh day after ischemia.

3.2. The effects of focal ischemia in the expression of APP mRNA

The 4-0 monofilament-induced MCAO caused a time-dependent increase in APP mRNA expression in both penumbra and core ischemic regions (Fig. 2a and b). In contrast to our observations with a 3-0 monofilament-induced transient focal ischemic model [11], APP mRNA was not affected on the first day after MCAO. APP mRNA increased to 208% and 152% in the penumbra and core ischemic regions, respectively, at the fourth day after MCAO and remained high through the seventh day of ischemia.

4. Discussion

Epidemiological studies have suggested a strong link between stroke and cognitive impairment [2,5,6,10,16,17, 19]. Approximately 34% of patients with computed tomography (CT) identified brain ischemic lesions do not present any symptoms [8]. Most cases of asymptomatic cerebral infarction are lacunes and internal borderzone infarctions [7]. Patients with asymptomatic cerebral infarction have compromised cerebral blood supply, but not to the extent to cause stroke symptoms. Loeb et al. [7] recruited 108 patients with CT confirmed lacunar lesions. They followed these patients up to 4 years after their first stroke and found that 25% of these patients developed dementia, an incidence that is 4–12 times more frequently than age- and sex-matched normal control subjects. These clinical studies suggest that even small defects of CBF may cause later cognitive impairment.

Our in vitro studies have demonstrated that hypoglycemia causes time- and dose-dependent increases in APP mRNA expression in rat primary astroglia cells [12] and in human neuroblastoma cells (Shi and Simpkins, unpublished observation). As such, it is likely that both neurons and glia respond to the infarct with overexpression of APP. We [1,11,15] have reported enhanced APP and its mRNA expression in a variety of stroke animal models. In most of these models, severe blockade of CBF is produced. The focal ischemic model we used in the present study shows more subtle changes in CBF. CBF is decreased to 50% in this model compared with more than 70% decrease in CBF required to cause symptoms in stroke patients. In the present model, CBF recovers to the normal by the fourth day after stroke. These changes in CBF are similar to those encountered during silent stroke and transient ischemic attack.

A 50% decrease in CBF caused a delayed overexpression of APP mRNA to 208% and 152% in the penumbra and core ischemic regions, respectively. The persistent overexpression of APP mRNA along with the spontaneous recovery of CBF suggests that APP expression is not tightly coupled to ongoing blood flow in patients suffered from stroke. Triggered by the resulting disturbance of glucose metabolism and oxidative phosphorylation, as shown by clinical positron emission tomography studies, the increase in APP production could contribute to a cascade of cellular events that lead to neuronal dysfunction and death. The alternative possibility that overexpression of APP is an attempt to protect tissue from the effects of ischemia is less likely for several reasons. First, the form of APP produced contains a Kunitz protease inhibitor domain, indicating that the APP will be further processed to a neurotoxic form of β-amyloid [3]. Second, the response to infarction is slow in development in the present model. An attempted neuroprotection response would be expected within 1 day, during the time when the lesion is occurring. As significant neurodegeneration can be seen by

1 day [18], the observed APP response would appear not to be related to a process aimed at neuroprotection.

In summary, our study describes a novel focal ischemic animal model to study the effects of subtle changes in CBF on APP expression. We have demonstrated that a small decrease in CBF enhances APP mRNA suggesting that overexpression of APP may contribute to cognitive decline subsequent to stroke.

Acknowledgements

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Anterior Choroidal Artery Territory Ischemia Causes Weight Loss and Reduces Food and Water Intakes in Rats

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Abstract

Background and Purpose: Several brain regions play pivotal roles in governing dietary behavior. We conducted this study in rats to correlate changes in body weight and feeding behavior with cerebral ischemia in three different arterial territories. **Methods:** Focal ischemia was produced in the middle cerebral artery (MCA), anterior choroidal artery (AChA), and hypothalamic artery (HTA) territories, respectively, by manipulating the location of an occluder within the intracranial segment of the internal carotid artery. Body weight and food and water intakes were determined daily three days before and after ischemia onset. Ischemic damage was delineated by conventional and immunohistochemical methods. **Results:** Twenty-four rats had a small infarct in the HTA territory and 22 rats exhibited a larger infarct in the AChA territory (volume 5±1 vs 54±10 mm^3). The HTA territory infarction primarily involved the media hypothalamus, while the AChA territory covered the internal capsule (IC) and hypothalamus, including the far lateral hypothalamic area (fLHA). Rats with purely a HTA infarct showed little daily change in body weight or food and water intakes following ischemia, whereas rats with an AChA infarct exhibited progressive weight loss associated with reduction of food and water intakes. Occlusion of the MCA caused a huge hemispheral infarction of 375±34 mm^3, much more marked body weight loss, severe reduction of food and water intakes, and high mortality (12/26). **Conclusion:** Cerebral ischemia involved in the AChA territory causes weight loss and reduction of food and water intakes. Damage in the unilateral fLHA and IC may account for the syndrome.

KEY WORDS: Anterior Choroidal Artery, Hypothalamic Artery, Cerebral ischemia, Weight Loss, Feeding Behavior.

Introduction

In addition to disorders of consciousness, deficits of motor and sensorimotor function, and cerebral cortex dysfunction, cerebral ischemia may also result in body weight change. Several brain regions, especially the hypothalamus, play pivotal roles in governing dietary behavior, but the physiopathology remains sparse. Maintenance of stabile body weight suggests an active homeostatic control and regulation mechanism in the face of various pathophysiologic conditions.[1] A unique way to maintain stable body weight is to balance the daily intake of energy with its expenditure. Traditionally, food intake is regarded as a key controlled factor body weight regulation.

Body weight may serve as an indirect index of ischemic brain damage. Experimentally, weight loss has been proportionally correlated with infarct volume size induced by transient or permanent middle cerebral artery (MCA) occlusion.[2] Appetite loss was hypothesized to play an important role in body weight loss, and was thought due to ischemic injury to the anterior hypothalamus, although the mechanisms are not clear.[3] Any neuroprotective effects of anti-ischemia agents are accompanied by significant attenuation in body weight loss.[4,5]

Recently, we established a rat model that could selectively produce a deep infarct restricted to the hypothalamic artery (HTA) and/or anterior choroidal artery (AChA) territories.[6] Depending on intravascular filament placement, both the hypothalamus and internal capsule are involved or only the hypothalamus. Using this model, we tried to correlate functional deficits concerning body weight and feeding behavior with discrete brain lesions, hoping that a comparison of lesions in the different artery territories could

clarify the anatomic substrate and mechanisms of ischemia-induced changes in body weight and feeding behavior.

Materials and Methods

One hundred and eleven female Wistar rats were caged five/cage and acclimatized with a laboratory diet and tap water ad libitum under a fixed light-dark cycle for two-three weeks prior to experimentation. Sixty rats were employed to correlate body weight and food and water intakes following cerebral ischemia. All 60 animals underwent a 3-day pre-ischemic feeding process for determining baselines of daily dietary and drinking amounts and daily changes in body weight. Thereafter, the animals were randomly divided into three groups based on surgical procedures: (1) two sham-operated control groups (see below) including Control-1 (n=6) and Control-2 (n=6), (2) MCA occlusion (n=15), and (3) proximal suture placement attempting to produce deep infarct without MCA involvement (n=33). The three series were partially intermixed in time. The remaining 51 rats were used for examining the physiological parameters associated with the cerebral ischemia, and included 35 matched to proximal suture placement, 11 to MCA infarction, and 5 to the Control-1 group.

1. Determination of daily dietary and water intakes and body weight

Sixty rats were individually housed in cages and allowed free access to water (by bottle) and the laboratory diet (given in the form of chow alone or both chow and powder) for 6 days, 3 before and 3 following the onset of ischemia. Daily food and water

intakes were calculated by subtracting the weight of diet or volume of water remaining in a feeder or a water bottle from the amounts given every 24 hours.[7] Water and dietary powder were freshened daily, while the chow was provided once in a separate feeder in a quantity required for the whole 6 day feeding period. Body weights were determined the day before the rats were individually housed, and daily thereafter during the 6 days of the experimental interval. All data were obtained between 3:00 and 5:00 p.m.

2. Surgical procedures

To control for any diurnal variation in body weight or food and water intakes, all operations were performed between 3:30 and 6:00 p.m. Rats were anesthetized with 2% Isoflurane in a mixture of 30% oxygen and 70% nitrous oxide, delivered via a face mask. Body temperature was maintained during the operative period with a heating pad set at 37.0 °C. Cerebral ischemia was initiated within 25 minutes after anesthetic induction. Each rat was kept in a separate cage postoperatively, and allowed to recover from anesthesia at room temperature (21±1°C). To determine blood pressure, gases, pH and glucose concentrations, a catheter was temporarily inserted into the tail artery or femoral artery during the resting period, 24 hours and 72 hours following onset of the ischemia in 51 rats designated for physiologic parameter assessment.

In the deep infarction group (proximal placement of an occluder), the protocol was designed to induce an infarct in the HTA and/or AChA territories without affecting the MCA territory.[6] Briefly, using an operating microscope, a 3-0 nylon surgical suture coated with 0.1% of poly-L-lysine, was introduced through the right common carotid artery (CCA) into the ICA and advanced intracranially until resistance was encountered.

When the advanced distance from the CCA bifurcation was shorter than 18 mm, the occluder was withdrawn 2 mm. If the suture could be advanced equal to or greater than 18 mm without feeling resistance, the occluder was then withdrawn to a position 15, 15.5, or 16 mm from the CCA bifurcation, referring to a distance between the CCA bifurcation and the base of front teeth. To ensure cerebral ischemia production, rats not showing elevation of body temperature (equal to or more than 0.3 °C as compared with resting status) and/or abnormal postural reflexes within one hour following occluder placement were anesthetized again and the occluder advanced 0.5-1 mm further. [6]

The sham-operated control groups all underwent a surgical procedure identical to that used in the deep infarct group, in that the CCA, ICA and external carotid artery were ligated. In the Control-1 group, the occluder was advanced 13 mm from the CCA bifurcation (Control-1), while in the Control-2 group, no occluder was used at all. The occluder was left in place in all animals except in Control-2 group, and rats were then allowed to live for 72 hours. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

3. Confirmation of occluder tip location

Seventy-two hours after ischemia onset, each animal was reanesthetized with pentobarbital (50 mg/kg body weight) and sacrificed by decapitation, carefully avoiding changes in the catheter position. The position of the occluder tip relative to the MCA origin was verified by naked eye in all rats before the brains were harvested from the cranial base. The relationship was further confirmed in some rats by photography taken after an ipsilateral craniectomy was performed and the tip of the occluder directly

exposed.[6]

4. Histological Assessment

a. TTC staining. Each brain was coronally sectioned into seven 2-mm-thick slices, starting from the frontal pole, and stained by 2,3,5-triphenyltetrazolium chloride (TTC). Ipsilateral areas not stained red with TTC were recorded as infarcted.

b. H&E staining and in situ detection of apoptotic cells. Hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) were used to define the ischemic area more precisely and to prevent potential underestimation of infarct size and distribution.[6,8,9] All samples in the HTA and AChA infarct groups used for TTC staining ischemic lesion evaluation by, as well as three samples in Control-1 sham group were embedded in paraffin and cut into 5-μm slices, and evaluated for morphologic changes using H&E staining and the TUNEL method.

Any TUNEL-positive cells was assessed in situ by direct immunoperoxidase detection of digoxigenin-labeled 3'-OH DNA strand breaks, using the TUNEL method with the Apop Tag in Situ Apoptosis Detection Kit- Peroxidase (Oncor Technical Assistance Inc. MD 20877 U.S.A.).[6] Negative control slides were prepared using distilled water substituted for the TdT enzyme. This focal ischemia model enabled us to use anatomically match areas contralateral to those within the occluded vascular territory and served as internal controls for each brain slice.

c. Infarct distribution and measurement. Each experimental animal was

classified into MCA, AChA, and HTA infarct groups. A TTC stainless lesion involving the caudatoputamen and adjacent neocortex was defined herein as a MCA infarct, even though the lesion might cover non-MCA territory structures such as the thalamus, dorsal hippocampus, and those supplied by the AChA. All data from rats with partial MCA territory infarction (lesion involving the caudatoputamen but not adjacent neocortex) induced by erroneous occluder placement were excluded from further analysis. Lesions encompassing a core of the internal capsule without affecting the MCA territory were defined as AChA infarcts. An infarct limited to the hypothalamus, including the preoptic area, medial hypothalamus and lateral hypothalamus, was designated as a HTA infarct.

All TTC and H & E stained slices were correlated with changes in body weight and feeding behavior to generate overlap maps. Common infarct areas were identified by demarcating the infarct area and spatially overlapping this region with those of all rats within each subgroup, while the risk infarct area was generated by demarcating the area encompassing all infarct areas on the overlap maps.

The infarct size of each slice was determined by Epscan Mac 1.40 using the "NIH Image 1.54" computer program.[10] The total infarct volume was calculated as the numerical integration of infarct volume from all 2-mm brain slices, based on TTC staining. The infarct volumes in HTA and AChA infarct groups were also calculated using H&E staining.

5. Statistical analysis

Mean±SEM ANOVA (Fisher) statistical analysis was used to compare daily food and water intakes, body weight, and various physiologic parameters such as arterial blood

gases, glucose and blood pressure between groups. Paired t-test was also used to compare these factors before and after the onset of ischemia in the same group. For the sake of convenience, the body weight data analysis before group division, and daily food and water intakes at the third day of the pre-ischemic feeding period were considered as references. The Mann-Whitney test was used to compare the infarct volume between groups. The statistical software statview-J 4.1 (Abacus Concepts, Inc., Berkeley, CA) was also used, and a value of $p<0.05$ was considered significant.

Results

The mortality during 72 hours of permanent focal cerebral ischemia was about 7% (5/68) in rats subjected to the protocol for producing deep infarcts; the autopsies showed that the animals died with large infarctions of the MCA territory. Twelve rats in the MCA infarct group (n=26) died during the 72 hour post-ischemia interval. No deaths occurred in the sham-operation control groups during the experimental period, although five rats in the Control-1 group showed unilateral ocular ischemia (the ipsilateral eye lost its fresh red color and became darker compared with the contralateral eye). No signs of ischemia were observed in Control-2 group. The incidence of ocular ischemia in the other groups was not recorded, although it occurred frequently.

The relationship between the occluder tip position relative to the MCA bifurcation could be corroborated with cerebral infarct in different artery territories. Normal cerebral TTC and H&E staining, HTA or AChA territory infarct, or MCA territory infarct corresponded with catheter tip positions of more than 3 mm proximal to, 0.5-2 mm

proximal to, or 0.5-1mm across the MCA bifurcation in Control-1 group, HTA and AChA infarct groups, or MCA infarct groups. A proximal suture placement produced a HTA infarct in 24 rats and an AChA infarct in 22 (from a total 68 animals). Of the remaining 22 rats, eight had an MCA infarct, nine exhibited an incomplete MCA infarct involving the caudatoputamen with or without the overlying neocortical infarction, and five died.

There was no significant difference in resting arterial blood pH, CO_2, O_2, and blood pressure between groups (Table I). At 24 hours following sham-operation or cerebral ischemia, blood gas values were similar between groups, other than that O_2 levels in HTA and AChA infarct groups were significantly increased compared with controls. Mean arterial blood pressure (MABP) and diastolic pressure in the AChA and MCA infarct groups were significantly higher than in controls and the HTA infarct group, although systolic pressure was similar between groups. At 72 hours post-ischemia, all parameters were similar, except that O_2 in the AChA and MCA infarct group was higher than in the control and HTA infarct groups.

Rats of 12-14 weeks of age weighed 234-296 gm, with no difference between groups before individual housing (Fig. 1a). After the 3-day pre-ischemic feeding period, rats showed a slight increase in body weight (1-7 gm), of statistical significance ($p<0.05$, paired t-test) only in the Control-2 group. The body weights of the two control groups showed little change after sham surgery compared with those before surgery in the same group. All rats subjected to cerebral ischemia showed a decrease in their body weight ($p< 0.05$, respectively) one day after the onset of ischemia, regardless of infarct size, although no significant differences between the HTA infarct group and control groups

were noted during the 72 hour post-ischemic onset period. Body weight progressively decreased in the AChA and MCA infarct groups. Weight loss was much more severe in the MCA infarct group compared to the AChA infarct group ($p<0.05$ on the second and third days following cerebral ischemia, respectively).

Daily food intake averaged about 20 gm, without differences between groups during the pre-ischemic feeding period (Fig 1b). Sham operations induced a slight decrease in daily food intake. The two control groups showed different profiles of the intake following surgery: the amount in Control-1 group decreased on the first day, recovered on the second day, and decreased again on the third day, while it gradually recovered to be normal on the third day in Control-2 group. Food intake in HTA infarct group changed in a pattern similar to Control-2 group, whereas cerebral ischemia involving the AChA or the MCA plus AChA territories resulted in persistent reduction of food intake.

Daily water intake was about 60 ml, and was similar among groups during the preischemic feeding period (Fig 1c). Sham operation or cerebral ischemia was followed by a reduction of daily water intake ($p<0.05$) for 3 days, except that the Control-2 group recovered to that of the preischemic period by the second day following surgery. Water intake in HTA infarct group was lower than that in two control groups only at the first day following the onset of ischemia ($p<0.05$), whereas cerebral ischemia involving the AChA or the MCA territories resulted in persistent and severe reduction of water intake.

Blood glucose levels were similar among groups during the resting status (Fig.2). Cerebral ischemia involving MCA territory led to a slight reduction at 24 hours ($p<0.05$ compared with controls and HTA infarct groups, respectively), while there were no

significant differences between controls and AChA or HTA infarct groups. At 72 hours following the onset of cerebral ischemia, the glucose level in both AChA and MCA infarct groups was significantly lower than that in controls and HTA infarct group.

The total infarct volume was 5±1, 54±10, and 357±30 mm^3, respectively, in the HTA, AChA and MCA infarct groups, with statistically significant differences between each. A thread position designed for MCA occlusion produced infarction not only in the ipsilateral caudatoputamen and adjacent neocortex but also in the ipsilateral hippocampus, hypothalamus, thalamus and other structures including the internal capsule and cerebral peduncle, the latter structures are generally not supplied by the MCA (Fig.3, the lower two lanes).

A more proximal suture placement, namely an AChA infarct, produced an infarct limited to non-neocortical structures and centered on the internal capsule, (Fig.3, the middle two lanes). The AChA territory infarction was limited to the two slices, 8 and 10 mm from the frontal pole in 17 of 22 rat brains. The lesion involved the amygdaloid complex, internal capsule, part of the caudal caudatoputamen, and almost always included infarction in the hypothalamus. In the remaining rats, larger lesions were noted to extend to the slice 2 to 4 mm rostral to and 2 mm caudal to the 8 and 10 mm slices. The additional structures involved in the infarct included the rostral caudatoputamen, dorsal hippocampus, and part of the thalamus.

A suture position even more proximal (less than 2 mm proximal to the MCA bifurcation) produced an infarct fundamentally limited to the medial hypothalamus (Fig. 3, the upper two lanes). In some cases the lesion expanded to the preoptic area and globus pallidus (the first lane), or part of the internal capsule. In the longitudinal axis, the lesion

was limited to the 2 mm-thick slice 8-10 mm from the frontal pole in 21 of 24 rat brains. Twenty-four percent of rats with a HTA infarct had ischemic damage in the lateral hypothalamic area, but only two lesions invaded into the far lateral hypothalamic area. Two rats showed an infarction limited in the lateral hypothalamus associated with sporadic neuron death in the medial hypothalamus.

To directly link the changes in body weight and feeding behavior with the anatomic infarction distribution, overlap maps of infarct areas were constructed starting from 2mm-thick coronal slices 4 and 5 from the frontal pole in AChA infarct group (n=10, Fig. 4). This starting point was selected because all brain samples with an infarct in the AChA territory showed lesions on these two surfaces regardless of staining methods (TTC or H&E), and because an HTA infarction altered body weight and food and water intakes very little. The maps were primarily based on the TTC staining slices and modified slightly according to the H&E staining slices. A common (100%) infarct area was located only on slice 4, covering the internal capsule and fLHA. The fLHA was sparing from ischemic damage in two rats that were used for determining blood physiological parameters (data not shown).

TTC staining delineated the infarct region involving the MCA territory in all rats of MCA infarct group. It was technical difficulty to section 5μ-thick slices through the MCA infarcted region, and no samples in MCA infarct group were therefore analyzed for infarction size and distribution using H&E staining or the TUNEL method. In the AChA infarct group, 61% of rats showed a lesion involving the lateral hypothalamus using TTC staining in 2-mm-thick slice, while H&E staining on the 5-μm slice of the same samples demonstrated that an infarction frequency in this region of 96%. The far lateral

hypothalamic area (a region with a diameter about 0.5 mm, located 2.5 mm lateral to the parasagittal line including the lateral-most extremity of the lateral hypothalamic area or lateral components of the medial forebrain bundle) was at least partially damaged in most rats with an AChA infarct (Fig. 5a,b,c,d).

Discussion

In this study, we demonstrate a correlation between infarcts in three cerebral artery territories and daily changes in body weight and food and water intakes. Rats with ischemic damage encompassing the AChA territory showed progressive loss of body weight associated with reduction of daily food and water intakes. Rats subjected to ischemia involving the MCA territory displayed even more severe loss of body weight and reduction of daily food and water intake, and an associated higher mortality. Ischemic damage limited to the unilateral hypothalamus mainly involving the medial hypothalamus had little effect on body weight or daily food and water intake.

Body weight is hypothesized to be physiologically regulated, whereby coordinated adjustments in both the intake and expenditure of energy serve to stabilize the weight at a specific level .[1] Hypothalamic mechanisms appear to play a primary role in setting the level at which individuals regulate body weight. Dietary behavior may also be governed by the same mechanisms. In the present study, body weight in rats with a unilateral hypothalamus infarct was only slightly and transiently decreased. These results are in concordance with the previous reports, demonstrating that the unilateral lesion in the hypothalamus had only slight effect on body weight.[11] Most HTA infarcts produced

in the present study were located in the medial hypothalamus, which is generally considered as "satiety" center; lesioning in this area classically leads to obesity rather than aphagia. Rats with an AChA territory infarction, however, in which the HTA territory was also involved, had progressive body weight loss associated with persistent reduction of food intake for at least 3 days following the onset of ischemia. This divergence in the AChA infarct group suggests an imbalance between body weight and daily food intake during the early ischemic period.

The neuroanatomical and neurochemical basis for lesion-induced changes on body weight homeostasis observations remains unclear.[12] Available evidence indicates that symmetrical, bilateral lesions of the lateral hypothalamus cause body weight loss.[13] More medial lesions (but clearly in the lateral hypothalamic area) produced less severe effects on food and water intake, while damage in the far-lateral hypothalamus causes rapid weight loss and short survival times.[13] In contrast, electrical stimulation of the lateral hypothalamus increases food intake.[14] Neurons in this region fire spontaneously during naturally occurring feeding behavior and during hypoglycemia. [15,16]

Current investigations underlying feeding regulation within the lateral hypothalamus are focused on hypocretin/orexin, the excitatory peptides[17] that stimulate feeding when injected intracerebroventricularly.[18] The neurons producing hypocretin/orexin were localized in the lateral hypothalamus.[19] Aphagia may be due to damage to fibers traversing the lateral hypothalamus.[20] Cell-specific lesions of this region can also cause decreased food intake and body weight.[21] Consistent with those data, AChA infarction animals in the present study always showed damage in their far-lateral hypothalamus.

The hypothalamic mechanisms that control the set-points of many of the body's internal regulation systems were previously established with simultaneous bilateral treatments. For example, bilateral lesions of the medial or lateral hypothalamus led to obesity[11] or body weight loss,[22] respectively. Thus, the increase in lesion volume and involvement of the brain structures other than hypothalamus might be partly responsible for the manifestations of rats with an AChA or a MCA infarct in the present study. There is a feedback pathway for satiation involving the medial hypothalamus, lateral hypothalamus, nucleus of the vagus as well as afferent and efferent fibers.[23] Additional pathways that innervate higher cortical structures also must play a role in regulation of body weight and feeding behavior.

As shown in the present study and others, the internal capsule is constantly infarcted in the AChA infarct group.[6] Whether selective damage to the internal capsule causes weight loss needs further investigation. Certainly, intraluminal occlusion of the MCA produced an much larger infarction involving the MCA, AChA and HTA territories, with involvement of multiple brain structures, increased intracranial pressure, and disorder of various of brain functions including severe paralysis. The body weight loss associated with sharp reduction of food intake in rats with MCA infarction contributed to the high mortality in this group.

Water intake is similarly physiologically regulated, and given the wide range of homeostatic functions, hypothalamic mechanisms may also be involved. It is well-known that the region of the anteroventral third ventricle plays a crucial role in the central neural control of fluid balance. Brain structures including the subfornicial organ, hypothalamus and the solitary tract nucleus are linked to drinking behavior.[24,25] On the first day

following onset of ischemia, all rats, including those receiving sham operations, showed decrease in water intake. This finding suggests that experimental trauma alone or occlusion of one carotid artery (in which baroreceptor impulses from the carotid sinus region may be affected) may have a role in blood volume regulation, perhaps by influencing neuroendocrine control for water intake via release of atrial natriuretic peptide.[25] Two different patterns of water intake were observed during the consequent two-day period in the sham-operated groups: water intake returned to be normal ($p>0.1$ following surgery in rats receiving ligation of the carotid artery without occluder insertion, whereas it persisted at a reduced level in sham animals with the occluder advanced up to the position 13 mm from the carotid bifurcation. This observation suggests that ischemic damage rather than a disorder of the baroreceptors might play the greater role.

Unilateral medial hypothalamic ischemia affected water intake at least on the first day, since water intake in HTA infarct group was significantly lower than that in two sham-operated groups. Reduction of water intake was much more profound in the AChA or MCA infarct groups, and the severity was in proportion to infarct volume or extent of affected brain structures, in parallel with reduction of food intake. It is likely that the regulation processes of body weight and food and water intake are intertwined; the decrease in water intake might be simply associated with decrease in food intake.

Blood glucose in the AChA and MCA infarct groups decreased slightly compared with controls, suggestive a shortage of energy sources resulting from hypo- or aphagia. Conversely, diastolic blood pressure was higher in these two groups compared with the HTA infarct groups and controls, perhaps indicating a higher level of activity in the

sympathetic nervous system. Hormones that elevate blood glucose level, i.e. norepinephrine, might be high in plasma. On the other hand, high level of insulin secretion decreases blood glucose. Reportedly, bilateral medial hypothalamic damage significantly increases food intake and body weight associated with elevation of both blood glucose and insulin levels.[26] Blood determinations of circulating insulin and other hormones that elevate glucose levels may help clarify these mechanisms.

In conclusion, acute unilateral ischemia due to obstruction of the HTA has little effect on the regulation of body weight and food and water intakes, whereas a lesion involving the AChA territory causes progressive body weight loss associated with persistent reduction of food and water intakes. Damage in the far-lateral hypothalamus and internal capsule may account for this disorder.

Selected Abbreviations and Acronyms:

AChA, anterior choroidal artery; CCA, common carotid artery; fLHA, far lateral hypothalamic area; H&E, hematoxylin and eosin; HTA, hypothalamic artery; ICA, internal carotid artery; MCA, middle cerebral artery; TdT, terminal deoxynucleotidyl transferase; TTC, triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling.

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Legends for Figures

Fig.1. Daily changes in body weight (a), food (b) and water (c) intakes during the 3-day-pre-ischemic feeding period and the 3-day ischemic period relative to three different artery territories. All data were collected blinded to the extent and distribution of any ischemic injury. Rats (n=60) not subjected to cannulation for examination of blood physiology served for determination of changes in body weight, food and water intakes.

All animals underwent unilateral ligation of the common, internal and external carotid arteries after treatment. Control-1 group (n=6) includes those animals subjected to a sham-operation combined with advancement of the 3-0 coated surgical suture up to 13 mm from the carotid artery bifurcation. Contol-2 group (n=6) had no catheter placement. Ischemia in the middle cerebral artery (MCA) territory was produced by advancing an occluder across the MCA origin (n=15). Ischemia in the hypothalamic artery (HTA) territory or in the anterior choroidal artery (AChA) territory was produced by advancing an occluder proximal to the MCA origin to block the HTA or both of the AChA and HTA, respectively (the proximal suture placement group, n=33)

A lacunar-like infarct (n=12) in the hypothalamic artery (HTA) territory or a larger infarct (n=10) in the anterior choroidal artery (AChA) territory were produced in 33 rats, while the remaining, in which the middle cerebral artery (MCA) territory was involved, were excluded from the analysis. Nine of 15 rats subjected to MCA occlusion survived for three days. The data of the remaining, that died during the 3-day-cerebral ischemic period, were omitted.

HTAI (n=12) = hypothalamic infarct group; AChAI (n=10) = anterior choroidal

artery infarct group; MCAI (n=9) = middle cerebral artery infarct group. Means without sharing a common marked letter were significantly different ($p<0.05$). Bars represent S.E.M.

Fig.2 Changes in arterial blood glucose concentrations following the onset of cerebral ischemia in three arterial territories. HTAI (n=12) = hypothalamic infarct group; AChAI (n=12) = anterior choroidal artery infarct group; MCAI (n=5) = middle cerebral artery infarct group. Means not sharing a common marked letter were significantly different ($p<0.05$, n=4-11 for each point). Bars represent S.E.M.

Fig.3. Six representative rat brains showing cerebral infarcts in the hypothalamic artery (HTAI, the upper two lanes), anterior choroidal artery (AChAI, the middle two lanes) and middle cerebral artery (MCAI, the lower two lanes) territories. The sections are 6, 8, 10, and 12 mm from the frontal pole, respectively, as stained by 2,3,5-triphenyltetrazolium chloride immersion method. The areas without staining (white) represent infarction (pointed by arrow heads). CPU = caudatoputamen; Hipp = hippocampus; HT = hypothalamus; IC = internal capsule; Th = thalamus.

Fig.4. Infarct area overlap maps made from coronal slices (8 (a) and 10 mm (c) from the frontal pole) of 10 brains with anterior choroidal artery (AChA) infarct. The data of body weight and food and water intakes were documented in all these animals as shown in Fig.1. The common infarct area in AChA infarct was confined to the slice shown in (a) and involved the internal capsule (IC) and far lateral hypothalamic area (fLHA). ACO, cortical amygdaloid nucleus; CPU, caudoputamen; Hipp, hippocampus; HT,

hypothalamus; PC, cerebral peduncle; TH, thalamus; V, lateral ventricle.

Fig.5. Representative histological findings showing small infarcts in the anterior choroidal artery (AChA) territory. The square notes the regions of interest, the far lateral hypothalamic area (fLHA), in two rat brains stained by hematoxylin and eosin (a & c). Damage in the fHLA was reconfirmed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick labeling (TUNEL) method in the adjacent 5 μ-thick slices 5 (b & d) Small arrows indicate TUNEL-positive cells.

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Neuroprotective effects of estrogens: potential mechanisms of action

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Abstract

Epidemiological studies associate post-menopausal estrogen use with a reduction in risk of Alzheimer's disease, a reduction in risk of Parkinson's disease, and death from stroke. The neuroprotective efficacy of estrogens have been well described and may contribute to these clinical effects. Estrogen-mediated neuroprotection has been described in several neuronal culture model systems with toxicities including serum-deprivation, β-amyloid-induced toxicity, excitotoxicity, and oxidative stress. In animal models, estrogens have been shown to attenuate neuronal death in rodent models of cerebral ischemia, traumatic injury, and Parkinson's disease. Although estrogens are known to exert several direct effects on neurons, the cellular mechanisms behind the neuroprotective efficacy of the steroid are only beginning to be elucidated. In this review, we summarize the data supporting a neuroprotective role for estrogens in both culture and animal models and discuss neuronal effects of estrogens that may contribute to the neuroprotective effects. These effects include activation of the nuclear estrogen receptor, altered expression of bcl-2 and related proteins, activation of the mitogen activated kinase pathway, activation of cAMP signal transduction pathways, modulation of intracellular calcium homeostasis, and direct antioxidant activity. © 2000 ISDN. Published by Elsevier Science Ltd. All rights reserved.

1. Introduction

Estrogen replacement therapy is associated with numerous beneficial health effects including a reduction in risk for cardiovascular disease [37], decreased incidence of osteoporosis [37], and a significant reduction in all-cause mortality [13,55,58,67,90,119]. Estrogen use is also associated with a number of clinically relevant neurological benefits. Several studies indicate that estrogen use increases performance on some tests of memory/cognition, in particular those which involve verbal memory [106]. Epidemiological evidence supports a role for estrogen replacement therapy in reducing the incidence of Alzheimer's disease in post-menopausal women [56,61,88,123,133] and several small, clinical trials indicate that estrogen therapy improves cognitive functioning in Alzheimer's disease patients [30,57,86,87]. Both epidemiological evidence [73,101] and clinical reports [6,99] indicate a positive role for estrogen use in Parkinson's disease. Further, estrogen exposure may also decrease the extent of neuronal damage from stroke in humans [103] and in laboratory animals [15,26,53,108,109,128,149].

There are several possible explanations for estrogen-effects on memory and cognition, including modulation of neurotransmitter function [34,71,72,85,114] and increased synaptogenesis [9–11,29,82,83,124,125, 142,143,144]. Estrogens may also reduce the risk and/

Abbreviations: αE2, 17α-estradiol; βE2, 17β-estradiol; Aβ, amyloid β peptide; BDNF, brain derived neurotrophic factor; BSA, bovine serum albumin; CCA, common carotid artery; CREB, cAMP response element binding protein; ER, estrogen receptor; ERE, estrogen responsive element; GSH, glutathione; MCA, middle cerebral artery; NFκB, nuclear factor κB; NGF, nerve growth factor; PKA, protein kinase A; PKC, protein kinase C.

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or improve the outcome of neurodegenerative disease by directly enhancing the survival of neurons. In this review, we outline the evidence supporting a direct role of estrogen in neuronal survival and explore possible cellular mechanisms for the neuroprotective actions of estrogens.

2. Neuroprotective effects of estrogens

We first reported direct neuroprotective effects of β-estradiol (βE2) on SK–N–SH human neuroblastoma cells under conditions of serum deprivation [5]. Since then, protective effects of βE2 have been widely reported with neuroprotection reported in 12 different types of neuronal cells against 14 different toxicities (see Table 1) including serum-deprivation [5,35,39,40], oxidative stress [4,7,8,36,80,102,110], amyloid β peptide (Aβ)-induced toxicity [3,4,8,36,38,44,45,74,78,91,96], and excitotoxicity [36,94,102,110–112,136,147]. Further, demonstration of the neuroprotective effects of estrogens is not assay dependent as protection of neuronal cells have been demonstrated using morphological markers [11,12,36,78,102], dye exclusion techniques [3–5,7,35,38–41,98,136], vital dye techniques [91,147], lactate dehydrogenase release [94,96,110–112], and formazan dye conversion [3,4,8,80,96]. The assay-independence of estrogen-mediated neuroprotection is important as Lui and Schubert [70] have recently reported that high concentrations of estrogens (greater than 10 μM) can interfere with the MTT formazan dye assay in a manner which did not correlate with the neuroprotective effects.

Table 1
Summary of reported neuroprotection by β-estradiol in cultured neurons

Neuronal type	Species	Toxicity	Reference
SK–N–SH	Human	Serum-deprivation	[5,39,40]
		Aβ	[38]
SK–N–MC	Human	EAA	[80]
		H_2O_2	[80]
NT2	Human	EAA	[110]
		H_2O_2	[110]
B103	Human	Aβ	[78]
IMR32	Human	$FeSO_4$ and H_2O_2	[7]
PC12	Rat	Aβ	[8,74,96]
		H_2O_2	[8]
		EAA	[8]
		Serum-deprivation	[35]
HT-22	Murine	Aβ	[3,4,41]
		EAA	[3]
		H_2O_2	[3,4]
		Haloperidol	[98]
Neuro 2a	Murine	Aβ	[8]
Primary hippocampal	Rat	Aβ	[36,91]
		EAA	[36,136]
		$FeSO_4$	[36]
		Hypoglycemia	[36]
		gp-120	[12]
		Mast cell activation	[116]
Primary neocortical	Rat	Age in culture	[11]
		EAA	[111,112,147]
		Anoxia	[147]
		gp-120	[12]
		Haloperidol	[98]
Primary mesencephalic	Rat	EAA	[102]
		Superoxide	[102]
		H_2O_2	[102]
Primary neocortical	Murine	EAA	[94]
		Hypoxia	[94]
		Hemoglobin	[94]

The effective concentrations for βE2-mediated neuroprotection range from a low of near 0.1 nM [5,11,38,39] to a high of 50 μM [80,98,116,136]. A myriad of factors may contribute to the vast differences in neuroprotective βE2 concentrations. Different neuronal types and/or populations may possess differing sensitivities to estrogen-mediated protection. For example, Sawada et al. [102] report that a higher concentration of βE2 is required to attenuate glutamate toxicity in dopaminergic neurons than non-dopaminergic neurons. It is unknown if the type and relative abundance of estrogen receptors (ERs) present in the neuronal cells may contribute to the concentration of βE2 necessary for protection in various neuronal types. Differences in culturing conditions and media components may also contribute to the wide range in protective concentration as both reduced glutathione (GSH) [41] and nerve growth factor (NGF) [35] can potentiate estrogen-mediated neuroprotection. Additionally, while many studies do not require βE2-pretreatment to achieve neuroprotection [5,7,36,38–41,78,80,96,136], longer βE2 pretreatment times can increase the neuroprotective potency of the steroid [4,102]. Other factors which may alter the minimum concentration of βE2 necessary to achieve neuroprotection include the type of insult, severity of the insult, and endpoint used to define viability.

Estrogens are also potent neuroprotective agents in animal models of neuronal death. βE2 neuroprotection has been well studied in models of ischemia including middle cerebral artery (MCA) occlusion and common carotid artery (CCA) occlusion models. βE2 treatment results in a dramatic reduction in mortality in these models, reducing mortality by an average of 48% fol-

lowing transient MCA occlusion [109,149] and by 52% following CCA occlusion [132]. βE2 treatment has also been shown to improve neurological outcome following CCA occlusion [132]. A reduction in lesion size may underlie these beneficial effects of estrogen exposure as βE2 treatment has been reported to reduce ischemic lesion area and improve neuronal survival following ischemia induced by transient MCA occlusion [43,53,107–109,128,149], permanent MCA occlusion [26,146], and transient CCA occlusion [15,89,132]. Brain regions in which βE2 administration has been shown to reduce ischemic lesion area include the cortex [26], striatum [89], and hippocampus [15]. The magnitude of the βE2-mediated lesion reduction following MCA occlusion is consistently reported to be near 50% (Table 2) irrespective of the dose of estrogen with βE2 plasma levels ranging from low physiologic concentrations [26] to high pharmacological concentrations [109]. βE2 may also reduce lesion size when administered after onset of the ischemic episode [109,146,149] (Table 2). Although βE2 is known to have positive effects on the vasculature, it is unlikely that this is the sole mechanism for the beneficial effects of βE2 seen in these ischemic models as the βE2-induced reduction in lesion size is seen even in the absence of estrogen-induced changes in cerebral blood flow [26,107,132].

Estrogen-mediated protection has also been described in other animal models of neurotoxicity. βE2 treatment has been shown to improve neurological outcome following traumatic injury in male rats although no effect was seen in intact females [28]. βE2 administration has also been shown to preserve choline acetyltransferase immunoreactive neurons following fimbria–fornix transection [93]. Neuronal loss due to administration of dopaminergic toxins [21,22] and kainic acid [2] can be attenuated with βE2 treatment. Interestingly, Garcia-Segura et al. [32] have recently described increased activity of the enzyme aromatase, which synthesizes βE2, in reactive astrocytes in areas of brain injury caused by kainic acid treatment or physical injury suggesting the existence of physiologically mechanisms to increase local estrogen concentrations following neuronal damage.

In summary, estrogen exposure has been demonstrated to enhance the viability of neuronal cells both *in vitro* and *in vivo* under a wide range of toxic conditions including models of acute neuronal stress, as well as models of neurodegeneration. The protective effects of estrogens in these models has positive implications for role of the steroid in the treatment of neurodegenerative diseases, as well as acute neuronal death such as that associated with ischemic episodes.

3. Potential mechanisms of action for estrogen-mediated neuroprotection

Although estrogen is a well described neuroprotective agent, the mechanism by which estrogens exert their neuroprotective actions is unknown. Estrogens have a plethora of cellular effects including activation of nuclear ERs, increased expression of anti-apoptotic proteins, interactions with second messenger cascades, alterations in glutaminergic activation, maintenance of intracellular calcium homeostasis, and antioxidant activity. Several of these effects have been shown to enhance neuronal survival; however, the exact role of each of these pathways in estrogen-enhancement of neuronal survival remains to be elucidated.

3.1. Classical estrogen receptor activity

Estrogens classically exert their effects by a nuclear ER mechanism of action. In this model, the steroid enters the cell by passive diffusion and binds to the nuclear ER. Following a series of activation steps, the estrogen-ER complex associates with the estrogen re-

Table 2
Effects of estradiol administration on MCA occlusion-induced lesion size in female Sprague–Dawley rats

Time of E2 administration		Hemoglobin 95		
		Model	Reduction in lesion area (%)	Reference
Pre-occlusion	24 h	Transient	62–64	[109]
	2 h		44–60	[43,108]
	Chronic	Permanent	60	[26]
Post-occlusion	0.7 h	Transient	45–59	[109,149]
	1.5 h		37	[109]
	0.5 h	Permanent	71	[146]
	1 h		53	[146]
	2 h		46	[146]
	3 h		38	[146]

sponsive element (ERE) and functions as an enhancer for ERE-containing genes. Estrogen induction of ERE-containing genes may contribute to the neuroprotective effects of βE2 as βE2 exposure increases expression of the neurotrophin brain derived neurotrophic factor (BDNF) [113,117] most likely by an ER-mediated mechanism [117,126]. The anti-apoptotic bcl-2 gene contains an ERE in the promoter region [25], and the expression of bcl-2 protein is also increased with βE2 exposure [31,110]. In some [80,91,111], but not all [39,80,94,102,136], studies estrogen-mediated neuroprotection is attenuated by the ER antagonists tamoxifen or ICI 182,780. Supporting a role for ERα in neuroprotection, Gollapudi and Oblinger [35] have found that βE2 exposure attenuates serum-deprivation toxicity in PC12 cells transfected with ERα, but not those transfected with a control plasmid. The role of ERβ in neuroprotection has yet to be examined.

Interestingly, several lines of evidence suggests that these neuroprotective effects of estradiol are not solely mediated by a classical nuclear ER-mediated mechanism. First, ER antagonists do not attenuate the protective actions of βE2 in all models of neurotoxicity [39,80,94,102,136]. Similarly, estrogen-mediated neuroprotection can occur in the presence of mRNA [36] or protein synthesis inhibitors [36,94,102]. Furthermore, the structure activity relationship for estrogen-mediated neuroprotection [3,40] differs markedly from the structure activity relationship for steroid activation of the ER [65,139] resulting in many phenolic A ring estrogens, including αE2, which have low estrogenic potency, but are potent neuroprotective steroids in both culture [3,7,38–41,96,102,147] (Table 3) and rodent models [43,109] of neurotoxicity. Finally, estrogens have been shown to attenuate oxidative stress-induced neuronal death in HT-22 cells, a neuronal cell line which lacks nuclear ERs [4,41]. Although nuclear ERs may contribute to the neuroprotective effects, the above evidence indicates that estrogens can enhance neuronal survival even in the absence of classical ER activity.

3.2. Altered expression of Bcl-2-related proteins

The Bcl-2 family of proteins are important modulators of neuronal apoptosis and includes both inhibitors (bcl-2, bcl-X_L) and promoters (bax, bad, bcl-X_S) of apoptosis. The relative levels of these two competing classes of Bcl-family members appears to determine the vulnerability of an individual cell towards pro-apoptotic stimuli via complex protein–protein interaction [76]. Both bcl-2 [25] and bcl-X_L [91] have punitive EREs in their promoter regions. βE2 has been demonstrated to increase bcl-2 immunoreactivity in NT2 neuronal cells [110] and in neurons of the arcuate nucleus [31]. Similarly, βE2 can increase the levels of bcl-X_L mRNA in PC-12 cells transfected with ERα [35] and bcl-X_L immunoreactivity in primary rat hippocampal neurons [91]. βE2 exposure also causes a decline in the mRNA levels for the pro-apoptotic BAD in ERα transfected PC12 cells [35].

Of specific interest to ischemic models, Dubal et al. [27] report that MCA occlusion causes a decline in the message for bcl-2 in the penumbral region of the lesion which is attenuated with βE2 administration. The levels of bax, bcl-X_L, bcl-X_S, and bad mRNA was unchanged by βE2 treatment. Interestingly, the change in mRNA levels of ERβ paralleled bcl-2 levels while ERα mRNA levels were dramatically increased in the ischemic penumbra. This study does not address if the alteration in bcl-2 mRNA contributes to the neuroprotective effects of βE2 or if the attenuation of bcl-2 message levels is due to upstream attenuation of the insult.

3.3. Activation of the MAPK signal transduction pathway

The interaction between estrogens and neurotro-

Table 3
Comparison of relative neuroprotecive effect and relative potencies in ER binding, uterotrophic growth stimulation, and MCF-7 cell proliferation[a]

Steroid	Percent of βE2 activity			
	Neuroprotection [40]	RBA	Uterotrophic [65]	MCF-7 proliferation [139]
βE2	100	100	100	100
αE2	89	49 [65]	1	< 1
E-3-ol	102	79 [139]	ND	1
Estrone	88	66 [65]	29	2
Estriol	75	16 [65]	1	3

[a] Percent of βE2 neuroprotection refer to percentage protection of serum-deprived SK–N–SH cells with a 2 nM concentration. Percent RBA, uterotrophic growth stimulation, and MCF-7 cell proliferation are reported as relative potencies. Abbreviations used: RBA, relative binding affinity; ND, not determined.

phins BDNF and NGF is complex, with differential and reciprocal regulation of ERs and neurotrophin receptors (both p75 and trkA) by their ligands suggesting possible interactions between the two signal transduction pathways [126]. βE2 activation of the peptide growth factor signaling pathway, ras/b-raf/MAP kinase, has been described in neocortical explants [115] and primary neocortical neurons [27] while βE2 conjugated to bovine serum albumin (BSA) has been shown to activate this pathway in SK–N–SH neuroblastoma cells [120,135]. Activation of this pathway is rapid, with phosphorylation of the MAP kinase (ERK) occurring within 5–15 min after estradiol exposure [115]. The ER antagonist, ICI 182,780, did not attenuate βE2-induced ERK phosphorylation in neocortical explants [115] or SK–N–SH cells [135], but did in primary neocortical neurons [27].

The MAP kinase pathway is activated upstream of ERK phosphorylation, and most likely, activation of b-raf is cardinal [115]. Singh et al. [115] hypothesize that a multimeric complex consisting of an ER and b-raf mediates the βE2-mediated activation of the MAPK pathway. In support of this, they have demonstrated that βE2 treatment results in a rapid increase in b-raf activity which co-immunoprecipitates with the ER. Interestingly, treatment with the weakly estrogenic estrogen, αE2, can also activate ERK phosphorylation in cortical explants in the same concentration range as βE2 [127]. This suggests that αE2 can activate the ER-b-raf complex. Although αE2 is 100-fold less potent than βE2 at activating ER-ERE-dependent responses [65,139] (Table 3), αE2 has between 11 and 89% of the binding affinity as βE2 [50,65,129,139]. Together, these data indicate that estrogens which are weak agonists at stimulating ERE-mediated transcription may nevertheless potently stimulate non-classical ER-mediated events.

Singer et al. [112] have recently provided evidence that this pathway may mediate the neuroprotective effects of estrogens. They found that treatment with the MEK inhibitor, PD 98059, can prevent βE2-mediated protection in primary cortical neurons exposed to glutamate. However, the importance of this pathway in estrogen-mediated neuroprotection may differ with neuronal type and/or toxicity. For example, while βE2 has been shown to attenuate serum-deprivation- [5,39,40] and Aβ-induced toxicity [38] in SK–N–SH cells, free βE2 does not induce activation of this pathway in this cell line [120]. Further, BSA conjugated-βE2, which does induce ERK phosphorylation in SK–N–SH cells [120,135], does not attenuate the toxic effects of serum-deprivation in these cells [40].

3.4. Activation of cAMP–PKA–CREB pathway

The cAMP-PKA pathway is a well described signal transduction pathway in which increased adenylate cyclase activity results in increased cAMP concentrations and downstream activation of protein kinase A (PKA). Estradiol increases cAMP accumulation in hypothalmic neurons [49,138] and human neuroblastoma cells [134] and has cellular effects in neurons consistent with increased cAMP levels [48,77,83,134], including increased phosphorylation of cAMP response element binding protein (CREB) [47,134,151]. Interestingly, αE2 is equipotent to βE2 in inducing CREB phosphorylation in SK–N–SH cells [42]. The effects of βE2 on cAMP accumulation and CREB phosphorylation are rapid with the peak effect seen 30–60 min following exposure [47,49,134,138]. The effects of ER antagonists on these effects of βE2 are mixed [47,134].

It should be noted that CREB can be phosphorylated by pathways other than cAMP-PKA [18,104,105,145], and at least two of these pathways, the MAP kinase pathway [115] and the CAM kinase pathway [54], have also been shown to be activated by βE2 exposure. This raises the possibility that CREB is a convergence point for multiple estrogen-mediated signal transduction events (Fig. 1).

Activation of the cAMP signalling and/or increased CREB phosphorylation could contribute to the neuroprotective effects of estradiol, as activation of the cAMP pathway is associated with decreased susceptibility of neuronal cell to apoptotic signals [14,23,63,64,97]. Further, Hanson et al. [52] have demonstrated that elevation of cAMP is sufficient to enhance the survival of spinal motor neurons in culture. Increased CREB phosphorylation is also associated with increased resistance to ischemic injury [131]. Activation of the cAMP–PKA–CREB pathway may enhance neuronal survival by increased expression of the anti-apoptotic protein bcl-2 [59], activation of the MAP kinase, ERK [130], or inhibition of the pro-apoptotic raf-1 in non-neuronal cells [148].

The cAMP–PKA–CREB pathway is known to mediate several effects of estradiol, including depolarization of hypothalamic neurons [77], dendritic spine growth [83], increased expression of the neurotensin gene [134], and potentiation of kainate-induced currents [46,48]. It remains to be elucidated if the cAMP pathway and, in particular, CREB, is either necessary or sufficient to mediate the neuroprotective effects of estrogens.

3.5. Direct attenuation of glutamate receptor activation

Excitotoxic cell death occurs in several pathological disease states including stroke, Alzheimer's disease, Huntington's disease, and AIDS-related dementia [69]. This occurs when EAAs activate AMPA/kainate- and/or NMDA-type glutamate receptors and initiates sodium and calcium influx [17]. Estrogens may exert

neuroprotective effects by direct interaction with either AMPA/kainate or NMDA receptors. In electrophysiological studies, βE2 alters the depolarizing response to AMPA [141]. Weaver et al. [136] report direct attenuation of NMDA-induced calcium currents with concurrent βE2 exposure in cultured hippocampal neurons which correlates to neuroprotection by the steroid. As neuronal injury and energy depletion lead to toxic levels of extracellular glutamate [69], attenuation of excitotoxicity may confer protection from other toxicities. However, βE2 exposure has also been shown to increase hippocampal NMDA receptor binding [137,144]. Further, βE2 induction of dendritic spines in hippocampal neurons can be attenuated with NMDA receptor antagonist both *in vitro* [10,82] and *in vivo* [143] suggesting that βE2 exposure may increase NMDA activation. These opposing effects of βE2 on glutamate receptor activation may depend on the degree of glutaminergic activation. One report indicates that estrogen exposure increases the rise in intracellular calcium when exposed to physiologic glutamate concentrations, but attenuates the rise in intracellular calcium associated with toxic levels of glutamate in hippocampal neurons [16]. Although attenuation of glutaminergic receptor activation may contribute to the neuroprotective effects of estrogens, it cannot be the exclusive mechanism of estrogen-mediated neuroprotection. Neuronal cell types (SK–N–SH and HT-22 cells), which are not sensitive to AMPA/kainate or NMDA receptor-mediated toxicity [19,147], are nevertheless protected from other toxicities by estrogens [3–5,39–41,98].

3.6. Modulation of intracellular calcium concentrations

Increased intracellular calcium concentrations modulate numerous neuronal functions including cell survival, synaptic formation and strength, and calcium-mediated neuronal death [33]. Estrogens can modulate intracellular calcium levels through interactions with AMPA/kainate and/or NMDA receptors as discussed above [136,141]. Additionally, βE2 treatment of ovariectomized female rats decreased L-type calcium currents in neostriatal neurons [75], although voltage-gated calcium currents were increased in CA1 hippocampal neurons in a similar experimental paradigm [60]. In cultured hippocampal neurons, βE2 exposure is associated with a modest increase in intracellular calcium [82]. Further supporting βE2 modulation of intracellular calcium concentrations, βE2 treatment increases protein kinase C (PKC) activity in the preoptic area of female rats [1] and biphasically modulates camodulin kinase activity in rat cerebelli *in vitro* [54], two calcium-triggered enzymes.

Maintenance of intracellular calcium homeostasis has been shown to be a component of estrogen-mediated neuroprotection. βE2 treatment has been shown to attenuate the increase in intracellular calcium associated with Aβ- [7] and gp-120-mediated toxicity [12]. With gp-120 toxicity, βE2 reduced the number of hippocampal neurons which responded to gp-120 with increased intracellular calcium concentrations, but did not alter the degree of the calcium response in those neurons which responded [12]. In a gerbil model of global ischemia, βE2 slowed the rate of increase of intracellular calcium, but did not alter the final extent of intracellular calcium rise [15]. It remains to be determined if the ability of βE2 to blunt the toxin-induced increase in intracellular calcium concentrations is a cause or effect of the neuroprotective effect of the steroid.

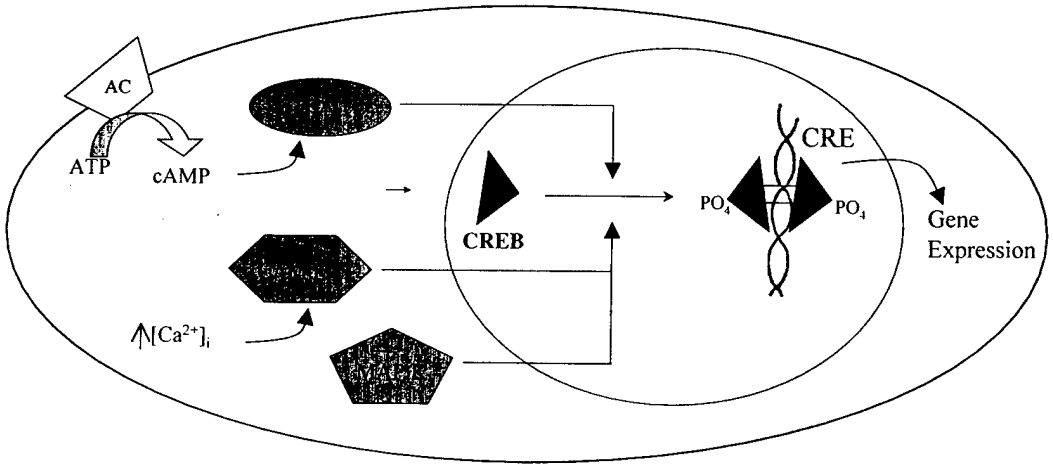


Fig. 1. A simplified, schematic representation of major pathways involved in the CREB phosphorylation including the cAMP–PKA pathway. Abbreviations used: AC, adenylate cyclase; PKA, protein kinase A; CAMK, Ca^{2+}-calmodulin-dependent kinase; MAPK, mitogen-activated protein kinase; CRE, cyclic AMP response element; CREB, CRE binding protein.

3.7. Antioxidant activity

Phenolic A ring estrogens have been shown to be potent inhibitors of lipid peroxidation in cell-free models [51,66,79–81,84,122]. The potency of these estrogens is equivalent to that of the well described antioxidant, α-tocopherol, tested under similar conditions [51,81,84,95,122]. Several studies provide a strong correlation between the neuroprotective effects of estrogens and the steroidal antioxidant activity [3,4,7,36,51,80,98]. βE2 can attenuate lipid peroxidation induced by Aβ exposure [36,44,62], glutamate toxicity [36], or $FeSO_4$ exposure [7,36], and can further attenuate the increase in intracellular peroxides induced by Aβ [3], haloperidol [98], and H_2O_2 [102]. These antioxidant effects are seen at the identical concentrations as the neuroprotective effects when examined in identical systems [3,7,36,44,80,98]. Further, the structure activity relationship for estrogen-mediated neuroprotection [3,40] is identical to the SAR for the antioxidant effects of the steroids [80,122].

Antioxidant activity of estrogens require concentrations of 1–10 μM in cell-free systems [51,66,79–81,84,122]. Similarly, antioxidant effects in cell culture models generally require more than 1 μM βE2 [3,7,36], and in these studies, the same concentration was required for the neuroprotective effects of βE2. Our laboratory has previously demonstrated diminished Aβ-induced lipid peroxidation with nM concentrations of βE2 which correlated with significant neuroprotection [44]. Interestingly, the neuroprotective potency of estrogens can be enhanced by the presence of GSH in the media [41]. The synergistic interaction could be due to redox cycling between estrogens and endogenous antioxidants such as occurs between α-tocopherol and ascorbate [100], or ascorbate and GSH [140].

Although the evidence supporting a role for the antioxidant effects of estrogens in neuroprotection is strong, antioxidant activity of estrogens may not account for neuroprotective effects in all systems. The antioxidant effects of estrogens generally require μM concentrations [51,66,79–81,84,95,122], while the neuroprotective effects can often be seen with significantly lower concentrations [1,5,11,12,35,38–41,78,91,110–112]. Further, Roth et al. [96] report attenuation of Aβ toxicity in PC12 cells by estrogenic compounds, but not other antioxidants including α-tocopherol, ascorbic acid, and melatonin. Singer et al. [112] have found that βE2 can attenuate glutamate toxicity in primary cortical neurons in the presence of concentrations of α-tocopherol (about 5 μM) sufficient to exert neuroprotective effects through antioxidant mechanisms. The presence of α-tocoperol in the model might isolate neuroprotective effects of estrogens which do not depend on antioxidant activity.

3.8. Other effects

Finally, there are several other, less well studied, neuronal effects of estrogen which may contribute to the neuroprotective efficacy. Both βE2 and αE2 can directly modulate the activity of the mitochondrial Na^+/K^+ ATPase derived from the rat brain [150]. βE2 treatment can attenuate the Aβ-induced decline in Na^+/K^+ ATPase activity in synaptosomal preparations [62]. Both isomers of estradiol can also modulate membrane fluidity in both neocortical and striatal membranes [20]. Activation of the transcription factor, nuclear factor κB (NFκB), is an important regulation of cell death [68] and βE2 can attenuate the toxin-induced activation of NFκB in glial cultures [24]. βE2 administration can also increase expression of apolipoprotein E in the brain [118,121] and apolipoprotein E treatment has been demonstrated to reduce the toxicity of Aβ in primary hippocampal neurons [92].

4. Summary

Estrogen is a multi-faceted hormone modulating many aspects of neuronal function and no single mechanism of action has yet to be elucidated for the neuroprotective effects of estrogens. Antioxidant effects of the steroid, ER activation, activation of the MAP kinase pathway, attenuation of NMDA receptor activation, and increased expression of anti-apoptotic bcl-2 family proteins have been implicated as mechanisms for the neuroprotective effects of estrogens. Additionally, estrogen exposure results in activation of the cAMP/PKA/CREB pathway and modulation of intracellular calcium concentrations and it is unknown to what extent, if any, estrogen neuroprotection is dependent on either of these effects.

It is likely that many of these effects of estrogens are coupled. For example, effects on membrane fluidity could modulate activity of membrane enzymes such as adenylate cyclase or ion channels such as NMDA receptors. Similarly, the antioxidant activity of estrogens could be responsible for the attenuation of toxin-induced NFκB activity. With the plethora of potentially neuroprotective pathways which are activated by estrogens, it is unlikely that a single pathway is solely responsible for the neuroprotective actions of the steroid. The importance of each individual pathway could vary with neuronal type, developmental stage, type of receptors expressed, extracellular environment, specific mechanism of the toxicity, or other factors. These factors may also alter the potency of estrogens in various models of neurotoxicity and neurodegeneration.

There is overwhelming data indicating that estrogens enhance survival of neurons both *in vitro* and *in vivo*. The applicability of this to human neurodegenerative

diseases and acute neuronal death require further clinical studies; however, this profusion of data indicated in a direct neuroprotective role for estrogens, as well as the proven clinical safety of estrogens, suggest that estrogens therapy may be useful in treating chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease or acute neurotrauma such as head injury and cerebral ischemia. Further, the efficacy of non-feminizing estrogens, such as αE2, in some of these models of neurotoxicity, suggest that these compounds may be clinically useful for treating neuronal death in men or women for whom estrogen therapy is contraindicated.

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Role of Estrogens and Estrogen-Like Non-Feminizing Compounds in the Prevention and Treatment of Alzheimer's Disease

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The present position paper is intended to provide evidence that estrogen deprivation contributes to the occurrence and course of Alzheimer's disease (AD) and that currently available estrogen preparations may be useful in the prevention and treatment of AD in women. Additionally, there is now substantial preclinical evidence to support the development of novel non-feminizing estrogens for use in male and female subjects for the protection of neurons from damage and death that underlies the neuropathology of AD. Estrogens and non-feminizing estrogen-like compounds may exert their beneficial effects in AD through a variety of mechanisms, including direct neuroprotective actions, and indirectly, through their neurotrophic effects. Inasmuch as estrogens are comparatively free of both acute and chronic toxicities, and non-feminizing estrogens are expected to be even safer, their use for years to decades for the prevention or treatment of AD is possible.

Estrogen replacement therapy (ERT) is associated with several neurological benefits. ERT has been shown to increase performance on some tests of memory/cognition.[1] Epidemiological evidence supports a role for ERT in reducing the incidence of AD in post-menopausal women .[2] This reduction in risk is dependent on the dose and duration of estrogen use and is seen irrespective of ethnic background or apolipoprotein E allotype. Further, several small clinical trials have found that estrogen use attenuates the decline of cognitive function in women with AD.[2] Recently, a large clinical trial of hysterectomized women treated for 12 months with Premarin was reported.[3] This trial failed to show any change is the 12 month course of the disease for women with a mean age of 74 to 77 years at the onset of the study. The reasons for the negative response are many and may relate to issues of study design, such as (i) the advance stage of the disease in the patent population evaluated, (ii) the choice of

hysterectomized (oophorectomized) women for the study, as this population has an earlier onset and a more aggressive form of the disease (Stanley Birge, personal communication), and (iii) the expected long duration of estrogen deprivation prior to onset of the trial.

Estrogens exert a plethora of actions which may contribute to these neurological effects including modulation of neurotransmitter function, enhanced neurite outgrowth and synaptic formation, direct neuroprotective effects, and modulation of amyloid precursor protein (APP) expression and processing.

Cholinergic impairment occurs early in AD and correlates with memory decline. Estrogens have been shown to augment cholinergic function in rats via increased choline acetyltransferase (ChAT) activity ,[4-6] acetylcholine synthesis,[7] and high affinity choline uptake.[6,7] Further, 17 β-estradiol (βE2) exposure attenuates the decline in ChAT immunoreactivity in the basal forebrain induced by fimbria-fornix lesions.[8]

Synaptic loss occurs in AD and may be a consequence of β-amyloid (βAP) containing plaques and neurofibrillary tangles. Estrogens promote neurite outgrowth,[9] enhance hippocampal synapse formation,[10] and strengthen long term potentiation.[11] The neurotrophic effects of estrogens may be mediated through increased expression of the neurotrophins NGF and BDNF, modulation of neurotrophin receptors, interactions with neurotrophin signal transduction cascades,[9] interactions with the NMDA receptor,[12,13] or activation of the transcription factor CREB.[14-16]

We first reported that βE2 is a potent neuroprotective agent in serum-deprived human neuroblastoma cells.[17] Since then more than 30 reports have described the neuroprotective effects of βE2 in cultured neuronal cells. βE2 has been shown to protect cultured neurons from at least 14 different toxicities including βAP exposure,[18-22] excitotoxicity,[20,23,24] and oxidative stress.[20,21,28] In animal models, βE2 attenuate neuronal death associated with ischemia,[29] fimbria-fornix lesions,[8] and age.[6] Further, βE2 has been demonstrated to increase the expression of the antiapoptotic proteins bcl-2 [30] and bcl-x_L.[31]

Several studies indicate that oxidative stress, lipid peroxidation in particular, is involved in AD pathology. Estrogens are effective inhibitors of lipid peroxidation in cellfree systems with both potency and efficacy equivalent to that of the well-described antioxidant, α-tocopherol.[32-34] In culture systems, βE2 prevents increased lipid peroxidation due to βAP exposure.[20] These antioxidant effects of estrogens require micromolar concentrations; however, we have described a synergistic interaction between glutathione and estrogens [22] which may increase the antioxidant potency of estrogens.

Estrogen use is associated with feminizing side effects, which limits its use in men and some women. However, at least some of these effects of estrogens are not mediated by a classical nuclear estrogen receptor (ER) mechanism allowing the possibility of designing estrogens with the beneficial neurological effects without unwanted feminizing effects. For example, α-estradiol (αE2) is more than 100-fold less potent than its optical isomer, βE2, in classical measures of estrogenic action including uterotrophic stimulation and MCF-7 cell proliferation. Nevertheless, αE2 is as potent and efficacious as βE2 in neuroprotection assays

[19,21,22,24-26,28] suggesting that the neuroprotective effects of estrogens are not mediated through a classical ER-mediated mechanism. An evaluation of the structure-activity relationship (SAR) of estrogen-mediated neuroprotection reveals that a phenolic A ring in the steroid structure is required for the neuroprotective effects of the compound [21,26] resulting in several nonfeminizing estrogens which possess neuroprotective potency without activating classical ER-mediated transcription. Several other lines of evidence similarly suggest that estrogen-mediated neuroprotection does not require nuclear ER activity. The neuroprotective effects of estrogens are not blocked by ER antagonists [23,25] or protein and mRNA synthesis inhibitors [20,23] and occur in neuronal cells devoid of nuclear ER activity. [18,22]

Evidence also suggests that the neurotrophic effects of estrogens may not require classical nuclear ER activity. The neurite promoting effects of βE2 in neocortical neurons is not blocked the ER antagonist ICI 182,780.[27] Further, the non-feminizing estrogen, 17α-dihydroequilenin, exerts neurotrophic effects in the rat hippocampus.[35] βE2 has been shown to interact with signal transduction cascades associated with neurotrophic effects including inducing phosphorylation and activation of ERKs [36] and phosphorylation of the transcription factor CREB.[15,16] αE2 induces phosphorylation of both ERK [36] and CREB [15] in the same dose range as βE2.

In summary, estrogens exert a myriad of effects on the neuronal tissue, and thus may interfere with the pathophysiological processes of AD at multiple points. Estrogens are widely used pharmacotherapeutic compounds with a thoroughly evaluated safety profile. In view of the excellent safety history of estrogen therapy, we believe that there is sufficient evidence to

support the chronic use of estrogen as a preventative measure for AD. The major limitations of estrogen use are their feminizing effects in men and the possibility of an increased risk of uterine cancer in women. In these cases, phenolic A ring estrogen-like compounds may offer several of the beneficial effects of estrogen therapy without the feminizing side effects.

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Protective Effect of Estrogens Against Oxidative Damage to Heart and Skeletal Muscle *In Vivo* and *In Vitro* (44463)

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Abstract. **Estrogen has been shown to protect skeletal muscle from damage and to exert antioxidant properties. The purpose of the present study was to investigate the antioxidant and protective properties of estrogens in rodent cardiac and skeletal muscle and H9c2 cells. Female Sprague-Dawley rats were separated into three groups, ovariectomized (OVX), ovariectomized with estrogen replacement (OVX + E2), and intact control (SHAM), and were assessed at two time periods, 4 and 8 weeks. Rodents hearts were analyzed for basal and iron-stimulated lipid peroxidation in the absence and presence of β-estradiol (βE2) by measuring thiobarbituric acid reactive species (TBARS). Isolated soleus (SOL) and extensor digitorum longus (EDL) were analyzed for creatine kinase (CK) efflux. Using H9c2 cells, the *in vitro* effects of βE2 and its isomer α-estradiol were investigated under glucose-free/hypoxic conditions. TBARS assay was also performed on the H9c2 in the presence or absence of βE2. The results indicate that OVX rodent hearts are more susceptible to lipid peroxidation than OVX + E2 hearts. OVX soleus showed higher cumulative efflux of CK than OVX + E2. Furthermore, H9c2 survival during oxidative stress was enhanced when estrogen was present, and both OVX hearts at 4 weeks and H9c2 cells particularly were protected from oxidative damage by estrogens. We conclude that estrogen protects both skeletal and cardiac muscle from damage, and its antioxidant activity can contribute to this protection.** [P.S.E.B.M. 2000, Vol 223]

The importance of estrogen in protecting tissues is currently receiving increasing attention. Estrogen is a fat-soluble hormone that can contribute to membrane fluidity by direct interactions with phospholipids. It has also been suggested that estrogen can suppress free radical–induced peroxidation chain reactions because of the similarity in structure to vitamin E, namely the presence of the hydroxyl group on the phenolic A ring (1, 2). By the age of 55, women normally pass through menopause resulting in ovarian exhaustion of follicles and a precipitous decline in ovarian steroids. Women can live more than a third of their lives in an estrogen-deficient state; however, overwhelming evidence suggests that postmenopausal estrogen loss can have negative effects on the brain (3, 4), bone (5, 6) and cardiovascular system (7). Despite this evidence, less than 25% of postmenopausal women receive estrogen replacement therapy (ERT) largely because of the fear that ERT increases the risk of uterine cancer and perhaps mammary tumors.

The normal endogenous production of free radicals creates both beneficial and detrimental effects. Recent evidence suggests a link in the over-production of free radicals and/or decreases in antioxidant capacity with the development of disease (e.g., cancer, atherosclerosis, and Alzhei-

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cumulative sum of the creatine kinase values determined at 30-min intervals from 30 to 240 min. This value is expressed as the cumulative release of creatine kinase (U/l) over the 240-min period.

Thiobarbituric Acid Reactive Substances (TBARS). Heart tissue or intact H9c2 cells were homogenized (PowerGen 125, Fischer Scientific, Pittsburgh, PA) in ice-cold 0.9% NaCl at pH 7 (20%w/v). TBARS were determined as previously described (21) by reacting the homogenate with 1.0% 2-thiobarbituric acid (TBA) solution in the presence of 12.5 *M* trichloroacetic acid (TCA) and 0.8 *M* HCl for 10 min at 100°C. For $FeCl_3$-stimulation of TBARS, the homogenate was incubated with 0.4 m*M* $FeCl_3$ at 37°C for 15 min in a water bath, and 1 m*M* desferrioxamine was added to the TBA reaction. In some studies, homogenates were incubated with either 10 μ*M* βE2, 0.1% ethanol (vehicle control), or normal saline for 1 hr prior to $FeCl_3$ treatment. Samples were centrifuged at 3000 r.p.m. at 4°C for 10 min, and the absorbance of the supernatant was read spectrophotometrically at 532 nm. The amount of TBARS was calculated from a standard curve using 1,1,3,3,-tetraethoxypropane in reagent-grade ethanol diluted with 0.9% normal saline and reacted with 0.8 *M* HCl/12.5% TCA and 1% TBA for 10 min at 100°C and absorbance read at 532 nm.

Plasma Creatine Kinase Activity (CK). Blood was centrifuged immediately, and the plasma fraction was frozen at −80°C until analysis. Plasma total CK levels and cumulative CK activity from isolated muscles were measured spectrophotometrically (Beckman DU-7400, Beckman Instruments, Fullerton, CA) at 340 nm using commercially available kits (Sigma Chemical Company, St. Louis, MO).

Circulating Estrogen and Progesterone. Serum concentrations of βE2 and progesterone were determined using solid phase radioimmunoassay kits (Diagnostics Products, Inc., Los Angeles, CA) according to the manufacturer's instructions.

Statistics. Mean and standard error of the mean (SEM) were calculated for all data. One-way analysis of variance (ANOVA) or Student's *t* test was performed to detect differences between treatments. A Tukey honest significant difference test was performed when significant differences were detected. Statistical significance was set at $P < 0.05$.

Table I. Physical and Hormonal Characteristics of 4-week and 8-week Old Animals According to Treatment Group

	SHAM	OVX	OVX + E2
Weight (g)			
4 weeks	252 ± 4 (7)	310 ± 5 (8)[a,b]	244 ± 2 (7)
8 weeks	285 ± 5 (8)	334 ± 8 (8)[a,b]	280 ± 15 (8)
Heart weight (g)			
4 weeks	0.92 ± 0.05 (8)	0.99 ± 0.03 (8)	0.91 ± 0.03 (8)
8 weeks	0.97 ± 0.03 (8)	1.1 ± 0.03 (8)[a,b]	0.95 ± 0.01 (8)
% heart mass			
4 weeks	0.37 ± 0.007 (7)	0.32 ± 0.006 (8)[a,b]	0.37 ± 0.004 (7)
8 weeks	0.34 ± 0.008 (8)	0.32 ± 0.01 (8)	0.34 ± 0.001 (8)
Soleus weight (mg)			
4 weeks	251 ± 59 (4)	306 ± 85 (4)	288 ± 45 (4)
8 weeks	211 ± 12 (4)	210 ± 15 (4)	207 ± 9 (4)
Soleus length (cm)			
4 weeks	2.5 ± 0.14 (4)	2.7 ± 0.29 (4)	2.8 ± 0.39 (4)
8 weeks	2.7 ± 0.13 (4)	2.8 ± 0.27 (4)	3.2 ± 0.49 (4)
EDL weight (mg)			
4 weeks	184 ± 46 (4)	219 ± 39 (4)	184 ± 29 (4)
8 weeks	207 ± 15 (4)	240 ± 31 (4)	197 ± 14 (4)
EDL length (cm)			
4 weeks	3.7 ± 0.38 (4)	3.9 ± 0.10 (4)	3.6 ± 0.37 (4)
8 weeks	4.0 ± 0.06 (4)	4.2 ± 0.28 (4)	4.1 ± 0.15 (4)
Estradiol (pg/ml)			
4 weeks	16.2 ± 2.5 (7)[c]	12.9 ± 1.4 (8)[a]	23.7 ± 1.3 (8)
8 weeks	12.3 ± 0.9 (8)	9.6 ± 0.80 (8)	29.1 ± 11.0 (8)
Progesterone (ng/ml)			
4 weeks	10.5 ± 3.0 (7)	9.4 ± 2.9 (8)	8.8 ± 3.0 (8)
8 weeks	44.2 ± 9.2 (8)[c]	9.4 ± 1.1 (8)[b]	8.6 ± 2.1 (8)
CK (U)			
4 weeks	103 ± 27 (8)	131 ± 43 (7)	59 ± 15 (8)
8 weeks	98 ± 19 (8)	62 ± 17 (7)	83 ± 26 (5)

Note. Data represented as means ± SEM with sample sizes in parentheses.
[a] $P < 0.05$ for OVX vs OVX + E2.
[b] $P < 0.05$ for OVX vs SHAM.
[c] $P < 0.05$ for SHAM vs OVX + E2.

dose, respectively. This appears to be cytoprotective rather than a mitogenic effect of the steroid as neither concentration of βE2 tested altered cell number in the absence of the toxicity (data not shown). The ability of αE2, an inactive isomer of βE2, was examined and found to attenuate the toxic effects of glucose-free/hypoxic conditions. αE2 similarly protected 29% of the cells at a 2-n*M* concentration and 50% of the cells at a 200-n*M* concentration (Fig. 4). Glucose deprivation under normal atmospheric conditions (10% CO_2/90% air) killed 32%–57% of the H9c2 cells (data not shown). This reduction in live cell number was almost completely blocked by concurrent treatment with 200 n*M* of either steroid. βE2 and αE2 blocked 74% and 67% of this cell death, respectively (data not shown). Hypoxia in the presence of glucose did not alter cell viability (data not shown).

Discussion

Estrogen and estrogen therapy have demonstrated beneficial effects on neurons, bone, cardiovascular system, and body composition. Estrogen's effects on lipid profiles have been documented by increasing circulating HDL and lowering LDL (23). In the presence of estrogen there is also decreased oxidation of LDL, a hypothesized starting point to atherosclerotic lesions. Various neuroneal cell types have been shown to be protected against β-amyloid-induced toxicity by the presence of estrogen (24). This protection may lower the incidence of Alzheimer's disease in postmenopausal women on ERT. Additionally, numerous reports show that loss of bone mineral density is slowed when estrogen therapy is given (25, 26). The protection of estrogen against skeletal muscle damage has also been shown using an exercise model (13), although the mechanism by which estrogens offer this protection is unclear. Furthermore, there is insufficient data to indicate estrogen's protective effect on cardiac tissue independent of the deleterious effects of atherosclerosis.

The present study provides evidence that supraphysiologic concentrations of estrogen are capable of reducing skeletal muscle damage and lipid peroxidation in the heart as well as increasing survival of a cardiac and skeletal muscle–related cell line when exposed to noxious conditions. These findings, along with the protective effects of estrogens on brain, bone, and cardiovascular system found by other investigators, provide compelling evidence of the benefits of ERT. The protective effects of estrogen can be mediated through genomic effects *via* the classical estrogen receptor (ER) or by its antioxidant properties. The findings from this study suggest that the antioxidant capabilities of estrogen may be, in part, responsible for the protection of skeletal and cardiac muscle.

When hearts from ovariectomized animals were preincubated with estrogen, there was a significant decrease in markers of lipid peroxidation. It is unlikely that there was ER-based protection due to the short time course of incubation and physical status of the cells. Furthermore, the antioxidant properties were demonstrated by the protection of α-estradiol (αE2) against cell death when the H9c2 cells were exposed to glucose deprivation and anoxia. The αE2 does not effectively activate genomic estrogen receptor mechanisms and may therefore offers its protection through its antioxidant structure, capable of absolving free radicals and stabilizing cell membranes. Moreover, Kume-Kick (4) found that after 3 weeks of gonadectomy, plasma levels of ascorbate and brain levels of ascorbate and glutathione did not change, lending further evidence that other antioxidants may remain intact without the influence of sex hormones.

Under the conditions used in this present study, estrogen treatment appears to be more protective in the slow-twitch, high oxidative muscles (e.g., the soleus). This muscle group showed greater CK efflux when estrogen was

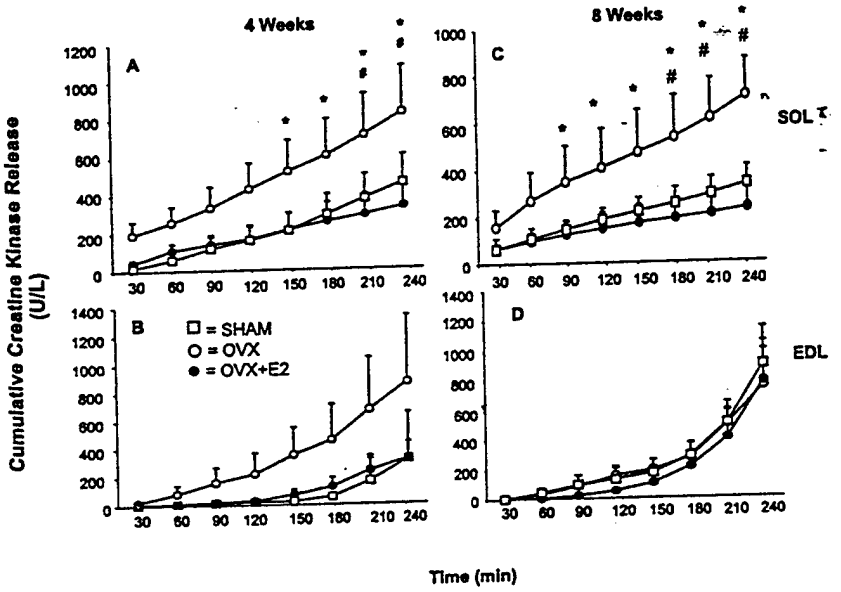


Figure 2. Cumulative creatine kinase release from 4-week treatment animals: (A) soleus and (B) extensor digitorum longus; and 8-week animals: (C) soleus and (D) extensor digitorum longus. Symbols represent: OVX (○), OVX + E2(●), SHAM (□). Statistical significance is expressed as follows: $^{*}P < 0.05$ for OVX vs OVX + E2; $^{\#}P < 0.05$ for OVX vs SHAM. Data are represented as mean ± SEM for $n = 4$ animals per treatment.

tracellular fatty acids have an ability to dissociate the F_0F_1 ATPase complex (34).

These interruptions in the mitochondria can cause mitochondrial swelling, loss of the electrochemical gradient, and changes in electron flux through the electron transport complexes causing uncoupling between electron flux and oxidative phosphorylation and leading to free radical production. The increased production of free radicals further compromises mitochondrial function by depressing aerobic enzyme activity (e.g., aconitase), damaging mitochondrial DNA, and damaging mitochondrial membranes. The leak of CK from the muscle following damage can further interrupt energy metabolism by decreasing the ability to regenerate ATP and phosphocreatine (PCr) stores. Loss of CK can also weaken muscle structure because it helps form the tight lattice in the M-region of sarcomeres, and loss of CK can deteriorate the stability of contracting filaments. A decrease in the ability to regenerate PCr and disruptions in sarcomere structure can alter the shortening capacity of muscle and decrease the time to fatigue.

In this study skeletal muscle, especially the high oxidative type 1 fiber-rich muscles, cardiac muscle, and cardiac-related cell line showed less indications of membrane damage in the presence of β-estradiol or its isomer α-estradiol. The preferential protection of highly oxidative tissues may be related to the amount of free radical production from oxidative metabolism. Further investigation into the area of skeletal muscle and cardiac muscle's protection *via* estrogen is needed to enhance the therapeutic usage of estrogen replacement therapy or possible alternatives that could enhance antioxidant capacity such as antioxidant supplementation or aerobic exercise.

We would like to thank Ms. Ilene Monck, Ms. Nancy deFibre, Sue Glennon and Sandeep Patel for their technical assistance.

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Experimental Model of Small Deep Infarcts Involving the Hypothalamus in Rats

Changes in Body Temperature and Postural Reflex

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Background and Purpose—Intraluminal middle cerebral artery (MCA) occlusion in rats has been reported to cause hyperthermia assumed to be caused by hypothalamic damage. To clarify the effects of hypothalamic ischemia on body temperature and to obtain a model simulating lacunar infarction, we attempted to produce small infarcts in deep structures (including the hypothalamus).

Methods—A surgical suture was advanced to occlude the origin of the hypothalamic (HTA) and/or anterior choroidal arteries (AChA) without compromise of the anterior or middle cerebral artery origins. After treatment, rectal temperature and postural reflex were examined repeatedly for 3 days under nonanesthetic conditions. The AChA and HTA and their link with small deep infarction were then confirmed by TTC, hematoxylin and eosin, and TUNEL stains and by microsurgical dissection after colored silicone perfusion into the cerebral arteries.

Results—Advancement of the suture near to but not occluding the MCA origin (0.5 to 1.9 mm proximal) produced small, deep, nonneocortical stokes in 25 of 36 animals without producing MCA ischemic changes. These infarctions mainly affected the hypothalamus in 13 animals (HTA area: infarct volume 6 ± 1 mm^3) and involved both the internal capsule and hypothalamus in 12 animals (HTA+AChA area infarct volume 48 ± 10 mm^3). Rats with HTA infarction alone exhibited persistent hyperthermia for 72 hours; some also had transient mild postural abnormality. The AChA+HTA infarct group showed a transient elevation of body temperature for 24 hours and definitive postural abnormality. In the remaining 11 animals, the suture was inadvertently advanced across the MCA origin, producing a large infarct that affected both the neocortex (MCA territory) and nonneocortical structures (volume 381 ± 30 mm^3, n=11). The MCA infarct group displayed a transient hyperthermia and severe postural abnormality.

Conclusions—When properly positioned, the intraluminal suture method permits selective AChA and/or HTA obstruction without inducing MCA territory ischemia. This model confirms that selective hypothalamic infarction produces significant and sustained temperature regulation abnormalities. The model also may be useful in investigating the pathophysiology of small, deep, end-vessel infarction. (***Stroke.*** **1999;30:2743-2751.)**

Key Words: cerebral ischemia, focal ■ choroid plexus ■ hypothalamus, internal capsule ■ middle cerebral artery

Fever is a common accompaniment of stroke and is more common in the acute stage and in patients with larger lesions.[1,2] Because even mild changes in body temperature can influence the extent of ischemic cerebral injury, most experimental stroke studies are performed under strict thermal control. This elevation of body temperature may be the result of an associated infection, but the possibility that the cerebral damage from the stroke itself causes the hyperthermia has not been excluded. To our knowledge, selective hypothalamic ischemia has never been intentionally produced to test this hypothesis experimentally.

Intraluminal middle cerebral artery (MCA) occlusion can cause persistent fever in rats lasting for at least 24 hours, and the hyperthermia can be correlated with ischemia in the hypothalamus and preoptic area.[3–6] The intraluminal thread has heretofore been thought to obstruct the anterior cerebral and/or anterior choroidal artery (AChA), a branch of the internal carotid artery (ICA) that arises just proximal to the MCA bifurcation, thereby reducing flow within the arteries that supply the hypothalamus.[7,8] In contrast, the transorbital MCA occlusion method obstructs the MCA without compromise of these proximal branches, thereby avoiding the hypothalamic damage and making body temperature elevations less likely.[9]

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In rats, 2 small arteries arise from the distal ICA proximal to the MCA bifurcation: the hypothalamic artery (HTA) and the AChA. Intraluminal thread advanced toward the MCA bifurcation probably causes the obstruction of these 2 small arteries concomitant with MCA trunk obstruction. Studies in humans or dogs indicate that AChA occlusion does not cause hypothalamic damage in these species, but whether the AChA supplies the hypothalamus in rats is still unclear.[10–12] This study was designed to test whether one or both of these arteries could be selectively obstructed to produce focal hypothalamic ischemia and whether the production of an isolated lesion could help clarify the anatomic substrate and mechanisms of ischemia-induced hyperthermia. Additionally, the development of a small, penetrating, artery infarction model could be useful in studying lacunar infarction.

Materials and Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee of National Cardiovascular Center, Osaka, Japan, and the University of Florida, Gainesville. Eighty 10-week-old rats, purchased from Oriental Yeast Co (Kyoto, Japan) and Charles Rivers Laboratories (Wilmington, Mass), were caged in groups of 5 and acclimatized with a laboratory diet and tap water ad libitum under a fixed light-dark cycle for 2 to 3 weeks before experimentation. Sixty rats were then randomly divided into 3 groups according to the type of intraluminal procedure, including (1) proximal suture placement attempting to produce deep infarction without MCA involvement (n=36), (2) sham-operated control (no occlusion of the HTA, AChA, or MCA, n=11), and (3) MCA occlusion (n=13). As described later, the 3 groups were partially intermixed during later portions of the experiment. The remaining 20 rats were used to measure arterial blood gases and blood pressure for any changes after isolated HTA and/or AChA territory infarctions and for perfusion studies.

Surgical Procedure and Infarction Techniques

All operations were performed between 3:30 and 6:00 PM to control for any diurnal variations in temperature. Rats were anesthetized with 2% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide delivered by face mask. During the operation, rats were warmed by a heating pad set at 37.0°C. Introduction of cerebral ischemia was initiated within 25 minutes after anesthetic induction. Each rat was kept in a separate cage after surgery and allowed to recover from anesthesia at room temperature (21±1°C).

In the deep infarction (proximal) group, the protocol was designed to induce an infarct in the HTA or AChA territories without affecting the MCA territory. The occluder tip was aimed to reach a position 1 to 2 mm proximal to the MCA bifurcation, a point chosen because the distance between the AChA and the MCA was >1 mm in female rats,[7] whereas the distance between the HTA and the MCA was not always clear. Briefly, with the use of an operating microscope, a 3-0 nylon suture coated with 0.1% poly-L-lysine[13] was introduced through the right common carotid artery (CCA) into the right ICA and advanced intracranially until resistance was encountered. When the advanced distance from the CCA bifurcation was shorter than 18 mm, the occluder tip was presumed to have reached the MCA bifurcation. The suture was then withdrawn 2 mm to allow the MCA origin to remain unobstructed and free to receive blood retrograde from the anterior cerebral artery. If the suture could be advanced ≥18 mm from the CCA bifurcation without feeling resistance, it was assumed that the occluder tip had crossed beyond the MCA origin from the ICA and was now within the anterior cerebral artery. The suture was then withdrawn to a position 15 to 16 mm from the CCA bifurcation.

In the sham-operated control group, the occluder was advanced 13 mm from the CCA bifurcation, thereby avoiding compromise of none of the deep penetrating vessels or the MCA. In the MCA-infarction group, the occluder was advanced until resistance was felt or >18 mm from the CCA bifurcation. Once proper placement was attained, the suture was secured in this position and left in place for the remainder of the experiment. At 72 hours, the animals were euthanized and investigated for catheter position, infarct size and location, and associated microscopic changes.

Since the induction of isolated deep cerebral ischemia in the proximal occlusion group was done blindly, at least 4 results could be predicted: no infarction or strokes in the HTA, AChA, or MCA territories. If rectal temperature elevation by >0.3°C and/or abnormal postural reflexes did not appear within 1 hour after surgery (supposing that the manifestations indicate HTA or AChA territory ischemia), the rat was subjected to a second operation, advancing the occluder 0.5 to 1 mm further. Six of 36 rats received the second operation.

In a parallel experiment, 3 groups of 5 rats each (proximal, sham-operated, and MCA occlusion) were randomly selected for determination of arterial blood gases and blood pressure. In these rats, the femoral artery was cannulated and used to continuously record arterial blood pressure until 10 minutes after the onset of cerebral ischemia. Arterial blood sampling for measurements of pH, $PaCO_2$, and PaO_2 was performed twice, once at rest before surgery and the other at 10 minutes after the onset of ischemia.

Body Temperature Measurement and Postural Reflex Test

Body temperature was measured by a probe temporarily inserted 2 cm into the rectum. Measurements were recorded before and 1, 3, 24, 48, and 72 hours after surgery in hand-handled, nonanesthetic rats. The resting level temperature before surgery was measured at 3:00 to 3:30 PM in all animals.

To evaluate sensorimotor function, the postural reflex test developed by Bederson et al[14] was carried out at the same time as body temperature determination. The degree of abnormal posture was estimated by suspending rats with their tails 20 cm above the floor. Intact rats extended both forelimbs toward the floor. Rats displaying this behavior were recorded as a score 0. Abnormal posture included flexing the contralateral limb toward the body and/or rotating the contralateral shoulder and limb medially. If the abnormal posture was observed, the rat was placed on a sheet of soft plastic-backed paper that could be gripped by its claws. Lateral pressure was applied from behind the shoulders so that the forelimbs slid gently to the left and then to the right. Rats that resisted sliding in both directions were graded as 1, more severely affected animals exhibiting a decreased resistance to the lateral push were scored as 2, and those that circled toward the paretic side consistently were graded as 3.

Confirmation of Occluder Tip Location

Seventy-two hours after ischemia onset, each animal was reanesthetized with pentobarbital (50 mg/kg body wt) and killed by decapitation; changes in the catheter position were carefully avoided. An ipsilateral craniectomy was performed, and the tip of the occluder was exposed by slightly elevating the temporal cortex with fine forceps. The tip of occluder relative to the MCA origin was confirmed in most rats with photography. Establishment of the catheter relation to the MCA was handicapped in MCA occlusion animals because of difficulties in retracting infarcted neocortical tissue. In sham controls, photography was not done because the occluder tip was located deep under the brain base and was not visible. Instead, the distance between the tip of the occluder and the coronal bone crevice of the cranial base underneath the optic chiasm was measured after brain removal.

To further clarify the relation between HTA and/or AChA occlusion and small deep infarcts, 5 rats subjected to each type of surgical procedure were perfused with colored silicone after perfusion of 100 mL of saline and 10% phosphate-buffered formalin just before decapitation. The circle of Willis was exposed by use of microsurgical technique and photographed for evidence that the occluder was obstructing the HTA and/or AChA proximal to the

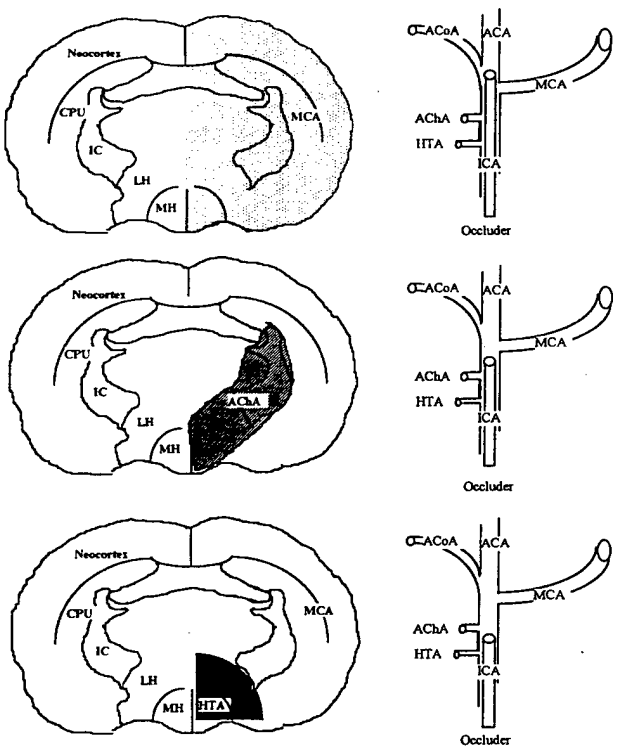


Figure 1. Schematic maps showing cerebral infarcts (left side) in 3 different territories of cerebral arteries, the MCA, AChA, and HTA, correlated to 3 different locations (right side) of the occluder tip. MCAI indicates MCA infarct; AChAI, AChA infarct; HTAI, HTA infarct; CUP, caudatoputamen; IC, internal capsule; LH, lateral hypothalamus; and MH, medial hypothalamus.

MCA. Brain samples were then submitted for histological analysis with hematoxylin and eosin and TUNEL staining methods.

Histological Assessment

TTC Staining

Each brain was coronally sectioned into seven 2-mm-thick slices starting from the frontal pole and stained with TTC. Areas ipsilateral to the occlusion not stained red with TTC were recorded as infarcted. All samples (n=13) with an infarct limited to the hypothalamus or those with AChA infarct without showing TTC lesion in the hypothalamus (n=5) were further analyzed by hematoxylin and eosin staining. and some were corroborated by the TUNEL method.

Hematoxylin and Eosin Staining and In Situ Detection of TUNEL-Positive Cells

Hematoxylin and eosin staining and the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate–biotin nick labeling (TUNEL) method were used to define the ischemic area more precisely and to prevent a potential underestimation of infarction size and distribution. All coronal sections in the HTA infarct group, the 3 slices located 4 to 6, 6 to 8, and 8 to 10 mm from the frontal pole in the AChA infarct group (which were used for evaluating ischemic lesion by TTC), and those samples perfused with colored silicone were paraffin-embedded and cut into 5-μm slices. Adjacent 5-μm slices were evaluated for morphological changes with the use of hematoxylin and eosin staining and the TUNEL method.

The presence of TUNEL-positive cells was assessed in situ by direct immunoperoxidase detection of digoxigenin-labeled 3′-OH DNA stand breaks with the TUNEL method[15,16] (the Apop Tag in Situ Apoptosis Detection Kit- Peroxidase). The 5-μm slices were washed twice with xylene for 5 minutes, 95% and 70% ethanol for 3 minutes each wash, and double-distilled water. They were then

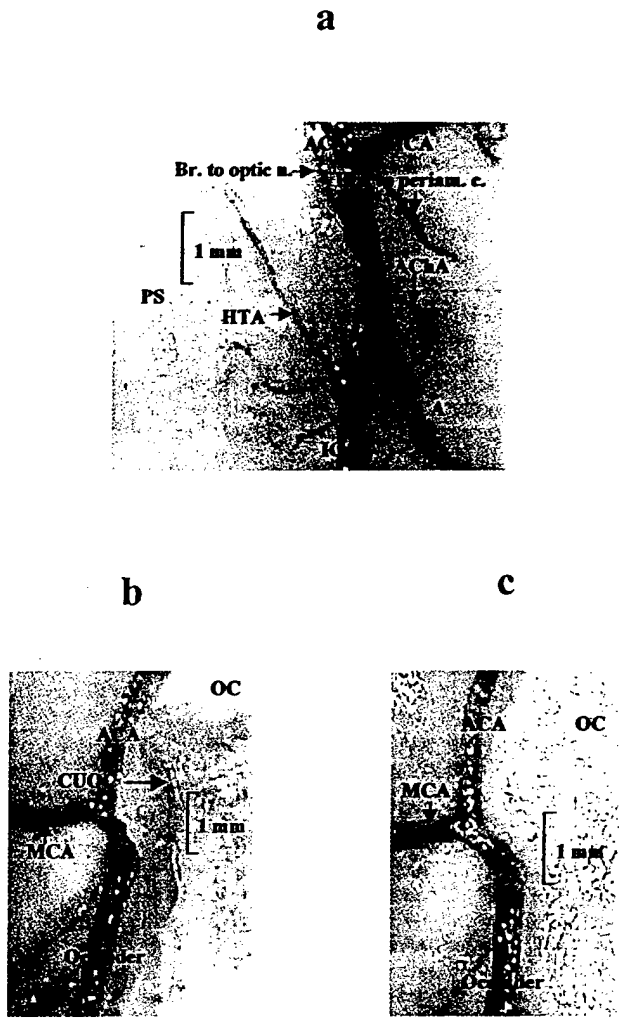


Figure 2. a, The HTA and AChA. Five rats subjected to the experimental surgery (see text for details) were perfused with colored silicone after perfusion with 100 mL of saline and 10% phosphate-buffered formalin, respectively, after onset of cerebral ischemia. The circle of Willis was exposed by microsurgical technique. The HTA or at least 1 branch to the hypothalamus and the AChA stemming from ICA proximal to the MCA was identified in the nonischemic side. ACA indicates anterior cerebral artery; Br. to Optic n., branch to optic nerve; Brs. to periam. c., branches to periamygdaloid cortex; PCA, posterior cerebral artery; PS, pituitary stalk. b and c, Occluder tip and the MCA. The occluder was advanced proximal to the MCA, resulting in obstruction of both the AChA and HTA. CUOC indicates coronal bone crevice of the cranial base underneath the optic chiasm; OC, optic chiasm.

treated with 20 μg/mL proteinase K (Sigma Chemical Co) for 15 minutes at room temperature and washed 4 times with distilled water for 2 minutes. Endogenous peroxidase was inactivated by covering the sections with 2% H_2O_2 for 5 minutes at room temperature. The sections were rinsed with PBS and immersed in TdT buffer for 15 minutes at room temperature. TdT enzyme was then added to incubate at 37°C for 60 minutes. The reaction was terminated by stopping buffer for 30 minutes at 37°C. After washing 3 times with PBS for 5 minutes, the sections were treated by anti-digoxigenin-peroxidase for 30 minutes at room temperature, washed again with PBS, and visualized by DAB Chromagen. After methyl green staining, the sections were washed again, dried, and mounted. Negative control slides were prepared, with distilled water substituted for the TdT enzyme. The unilateral focal cerebral ischemia model used in this study enabled us to use anatomically matching

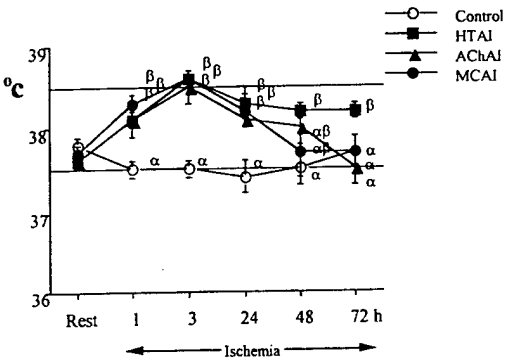


Figure 3. Changes in body temperature during focal cerebral ischemia regarding 3 different artery territories. MCAI indicates MCA infarct group (n=11); AChAI, AChA infarct group (n=12); HTAI, HTA infarct group (n=13); and Control, sham-operated control group (n=11). Means without sharing a common marked letter were significantly different ($P<0.05$). Bars represent SEM.

contralateral areas to the occluded vascular territory (HTA or AChA) as internal controls for each brain slice.

Infarct Distribution and Measurement

Infarctions were classified into MCA, AChA, and HTA subgroups. For convenience of analysis, the cerebral cortex above the rhinal fissure was considered as neocortex, whereas the piriform cortex was defined as a nonneocortical structure. As shown in Figure 1, we defined a TTC stainless lesion involving the caudatoputamen and adjacent neocortex as an MCA infarct even though the lesion might cover some non-MCA territory structures such as those supplied by the AChA. Similarly, we named lesions encompassing a core of the internal capsule without affecting the MCA territory as an AChA infarct. An infarct fundamentally limited to the hypothalamus including the preoptic area, anterior hypothalamus, and medial and lateral hypothalamus was designated as a HTA infarct.

Infarct size was determined by Epscan Mac 1.40 with the use of the NIH Image 1.54 computer program.[17] The total infarct volume

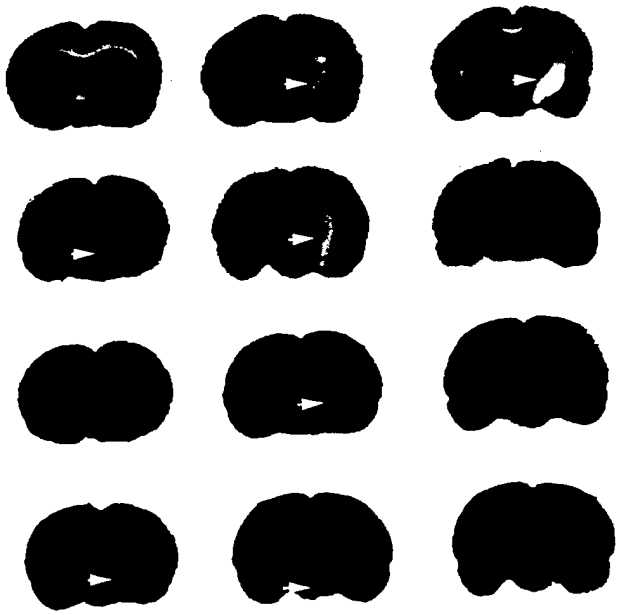


Figure 4. Four representative rat brains showing small deep infarcts in the anterior choroidal artery (upper 2 lines) and hypothalamic artery (lower 2 lines) territories on sections 6, 8, and 10 mm from the frontal pole, respectively, as stained by TTC-immersion method. The areas without staining (white) represent infarction (arrows).

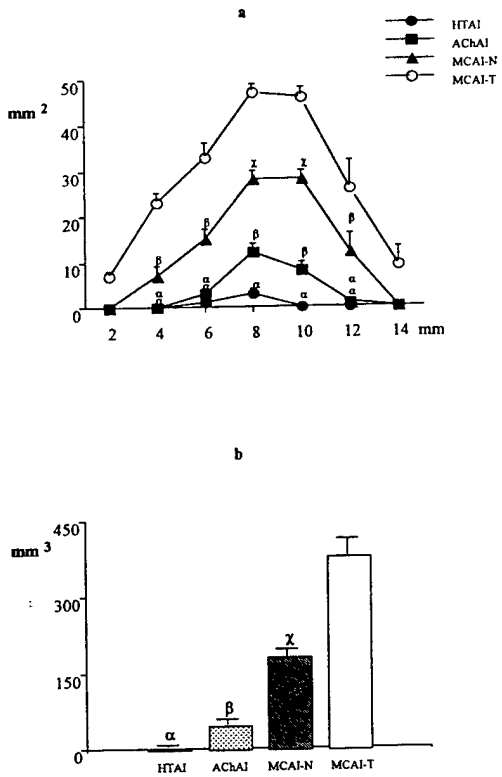


Figure 5. Infarct area (a) on 2-mm-thick coronal slices, starting from the frontal pole, and the infarct volume (b) after 72 hours of focal cerebral ischemia in 3 different artery territories. Cerebral ischemia was induced by advancing a 3-0 nylon surgical suture coated with 0.1% poly-L-lysine. MCAI indicates MCA infarct group (n=11); AChAI, AChA infarct group (n=12), and HTAI, HTA infarct group (n=13). MCAI-T represents the total infarct area or total infarct volume including both neocortex and nonneocortical structures. MCAI-N indicates the infarct area or infarct volume of the nonneocortical structure. Means without sharing a common marked letter were significantly different ($P<0.05$). Bars represent SEM.

was calculated by the numerical integration of infarct volume from all 2-mm thick brain slices, based on TTC staining in all rats. The infarct volume in the HTA infarct group was calculated again by use of hematoxylin and eosin staining. Similarly, the calculation of the infarct volume in the nonneocortical structures was derived by subtraction of the value of the neocortex from the total area.

Statistical Analysis

Fisher's ANOVA and paired *t* test were used for comparison of blood physiological parameters, blood pressure, and body temperature between groups and between those before and after ischemia in the same group. The Mann-Whitney test was used for comparison of the infarct size and postural reflex scores between the groups. The statistical software Statview-J 4.1 (Abacus Concepts, Inc) was used. The value of $P<0.05$ was considered significant.

Results

In the proximal occlusion group, the mortality rate during 72 hours of permanent focal cerebral ischemia was 8% (3 of 36). Autopsy showed that the rats died from pressure effects associated with MCA territory infarction. In the MCA occlusion group, 5 (38%) of 13 rats died before evaluation of ischemic outcome at 72 hours. No death occurred in the sham-operated controls.

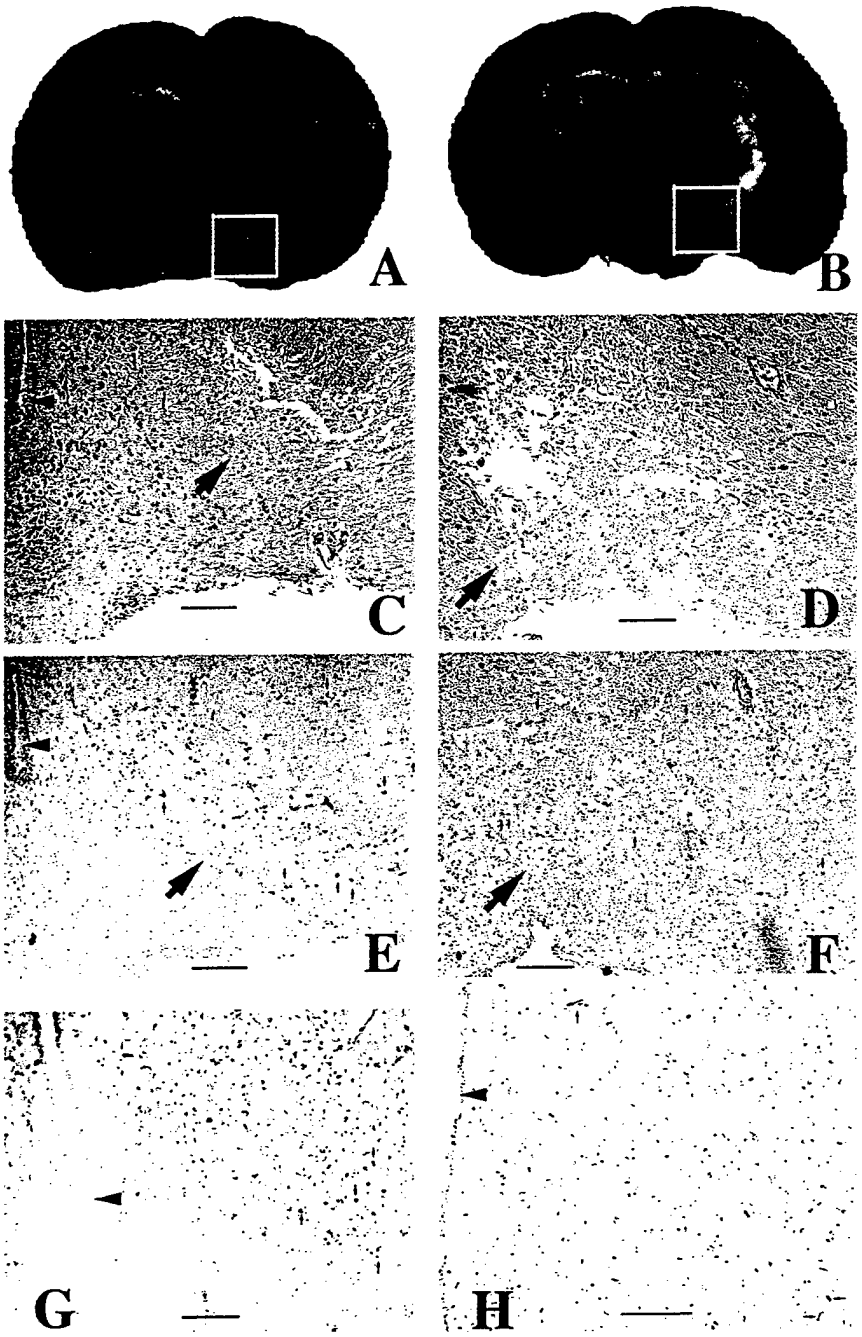


Figure 6. Representative histological findings showing small infarcts in the HTA or the AChA territory. The square notes the regions of interest in 2 rat brains in which TTC stained normal in the HTA infarct group (A) and in the AChA infarct group (B), respectively. From the same 2 samples, the hematoxylin and eosin staining in 5-μm-thick slices adjacent to the TTC-stained slices shown on A and B demonstrates a small infarct in the medial hypothalamus (C) and in the lateral hypothalamus (D), which were corroborated by the terminal TUNEL method in the closely adjacent slices (E and F). In 2 other rat brains from the AChA infarct group, the TUNEL method demonstrates scattered (g) and clustered (h) TUNEL-positive cells in the medial hypothalamus, whereas it appeared to be normal on the closely adjacent slices stained by TTC and hematoxylin and eosin (data not shown). Arrowhead indicates the wall of the third ventricle (C, E, G, and H). Large arrow indicates infarction in (C, D, E, and F). Small arrows indicate TUNEL-positive cells. Bars represent 100 μm in (C, D, E, F, G, and H).

No significant differences were noted between rats representing the proximal, sham-operated, and MCA occlusion groups in arterial pH, Pa_{O_2}, and Pa_{CO_2}. Mean values were 7.36 to 7.41, 189 to 214 mm Hg, and 36 to 45 mm Hg, respectively, at resting and 10 minutes after sham-operation or ischemia. The blood pressure in all groups increased 10 minutes after sham operation or ischemia (systolic pressure mean 140 to 145 mm Hg; diastolic pressure mean 92 to 97 mm Hg) versus resting state (systolic/diastolic pressure means 122 to 129/81 to 88 mm Hg), but the rise did not reach statistical significance. Animals used for blood gas and blood pressure evaluation were not used for infarct volume assessment.

Infarct Distribution and Suture Position

Positioning the suture proximal to the MCA origin produced an HTA infarct in 13 rats and an AChA infarct in 12 rats, findings confirmed by the occluder tip location relative to the MCA bifurcation and the distribution of ischemic changes with TTC and hematoxylin and eosin staining. Of the remaining 11 rats, 3 had a large (but survivable) MCA infarct, 5 had a smaller MCA infarct involving the caudatoputamen with or without partial neocortical infarction, and 3 died. The absolute success rate for production of isolated deep cerebral infarcts was 69% (25 of 36). The data from rats with MCA infarction resulting from erroneous MCA occlusion in the proximal occlusion group (n=3) and survivable infarction

No. of Rats With an Infarct in HTA, AChA, or MCA territories, respectively, in 4 Grades of the Postural Reflex Test

	1 h†				3 h				24 h			
	Control$^{\alpha}$	HTAI$^{\alpha\beta}$	AChAI$^{\beta}$	MCAI$^{\chi}$	Control$^{\alpha}$	HTAI$^{\alpha\beta}$	AChAI$^{\beta\chi}$	MCAI$^{\chi}$	Control$^{\alpha}$	HTAI$^{\alpha}$	AChAI$^{\beta}$	MCAI$^{\beta}$
Grade 3	0	0	1	6	0	0	1	6	0	0	1	4
Grade 2	0	2	4	2	0	1	3	4	0	0	1	2
Grade 1	0	3	2	3	0	4	5	3	0	4	9	5
Grade 0	11	8	5	0	11	8	3	0	11	9	1	0

HTAI indicates HTA infarct group; AChAI, AChA infarct group; MCAI, MCA infarct group. Groups without sharing a common superscript letter were significantly different from each others ($P<0.05$).

Cerebral ischemia was induced by advancing a 3-0 nylon surgical suture coated with 0.1% poly-L-lysine up to a position 0.5 to 1.9 mm proximal to the MCA bifurcation in a blind fashion in 25 of 36 rats, which caused a deep infarct in the HTA (n=13) or in the AChA (n=12) territories. The fate of the remaining 11 rats was MCA infarct in 3, partial MCA infarct in 5, and death in 3 rats, respectively. All data in these 36 rats were recorded before awareness of group division. The data from rats with the partial MCA infarct and those that died were omitted. Rats serving as positive controls for intraluminal occlusion of the MCA were made by advancing the occluder cross the MCA. The data from random occlusion of the MCA (n=3) and positive controls (intraluminal occlusion of the MCA, n=8) were pooled as 1 group (MCAI group), since there was no significant difference between these 2 groups. Rats in the control group (n=11) were subjected to a sham operation by advancing the 3-0 coated surgical suture up to 13 mm from the carotid artery bifurcation.

†Ischemic time. Square with dotted line indicates that among rats (totally, n=29) with the score grade ≤2, >82% of them might have a deep infarct.

animals in the MCA occlusion group (n=8) were pooled as one group for statistical analysis because there were no structural differences between the two. All sham controls showed normal TTC and hematoxylin and eosin staining in their brains. Data from dead rats or those with partial MCA infarction were excluded from the study analysis.

The occluder tip and the MCA bifurcation were usually not on the same plane, and the arteries were not fully filled with blood after the rats were killed, making adequate well-focused photography showing both areas simultaneously technically difficult. Colored silicone perfusion facilitated demonstration of the relation much better (Figure 2, b and c) and defined the AChA and HTA quite well (Figure 2a). Linking AChA occlusion with a small deep infarct was established by histological identification of the ischemic lesion after perfusion of colored silicone into cerebral arteries.

The cerebral histology stained by TTC and hematoxylin and eosin correlated well with occluder position. When the suture tip was located >3 mm proximal to the MCA bifurcation (sham-operated control), the histology was normal in all animals. In those animals exhibiting HTA and AChA infarctions (proximal occlusion group), the distance between occluder and the MCA bifurcation was 0.7 to 1.9 mm (mean±SEM 1.4±0.1 mm, n=12) in the AChA infarct group and 1.5±0.2 mm (n=9) in the HTA infarct group. Those with MCA infarctions had suture tips 0.5 to 1 mm across the MCA origin.

The distance between the occluder tip and the coronal bone crevice of the cranial base beneath the optic chiasm (refer to Figure 2) was longer in the sham-operated control group (4.3±0.2 mm) than in the others ($P<0.01$, respectively), whereas no significant difference between the AChA (2.5±0.2 mm) and the HTA infarct groups (2.7±0.2 mm) was detected. The distance in the MCA infarct group was the shortest among groups (0.6±0.2 mm, $P<0.01$, respectively).

Body Temperature

Body temperature was identical (37.6 to 37.8°C) in all groups before surgery (Figure 3). In the sham group, the temperature diminished at 3 hours after surgery ($P<0.05$) and returned to the baseline level during 24 to 72 hours after surgery. In the HTA infarct group, the temperature increased significantly to 38.1°C at 1 hour ($P<0.05$) and remained elevated thereafter and constantly higher than that in the sham-operated group throughout the duration of ischemia. In the AChA infarct group, the temperature also increased significantly at 1 hour after ischemia ($P<0.05$), remained elevated until 24 hours after ischemia, and then returned to normal. In the MCA infarct group, the temperature showed a similar significant increase from 1 to 24 hours ($P<0.05$), and then normalized.

Postural Reflex

All rats showed normal postural reflex (score=0) before intervention. The sham procedure produced no postural reflex test abnormality afterward. In the HTA infarct group, 2 of 13 rats showed early but transient grade 2 abnormality, subsequently followed by the tendency toward recovery. In the AChA infarct group, 5 of 12 rats manifested early grade 2 or 3 abnormalities that tended to decrease with time; all rats in this group exhibited a least grade 1 or 2 abnormality at the end of the experiment. In the MCA infarct group, 8 of 11 rats demonstrated early grade 2 or 3 abnormalities that persisted for 72 hours in the majority of animals.

Infarct Size

In the AChA infarct group, an infarct was located only in the nonneocortical structure (Figure 4, upper 2 rows). In 8 of 12 rats, the lesion was limited to 2 slices, that is, 8 and 10 mm from the frontal pole. In the remaining 4 rats, lesions expanded to 2 to 4 mm rostral to and 2 mm caudal to the 2 centrally affected slices. All AChA infarcts had discoloration of TTC-staining in the internal capsule. Additional structures involved in AChA infarction were the rostral caudatoputamen, dorsal hippocampus, and part of the thalamus. In the HTA infarct group, the lesion was fundamentally limited to the medial hypothalamus (Figure 4, lower 2 rows), although the lesion expanded to the preoptic area in 2 rats and part of the internal capsule in 5 rats.

As shown in Figure 5a, infarct area in MCA infarct group extended to all the slices examined and was significantly larger than that in the AChA infarct and HTA infarct groups in most slices ($P<0.05$). The volume in the HTA infarct group was the smallest among the 3 groups ($P<0.05$). The

Continued

48 h				72 h			
Control[α]	HTAI[α]	AChAI[β]	MCAI[β]	Control[α]	HTAI[α]	AChAI[β]	MCAI[β]
0	0	1	4	0	0	1	4
0	0	2	2	0	0	1	2
0	3	9	5	0	3	10	5
11	10	0	0	11	10	0	0

area in the AChA infarct group was significantly larger than that in the HTA infarct group on slices 8 and 10 mm from the frontal pole ($P<0.05$). The infarct volume in the MCA infarct group, AChA infarct group, and HTA infarct group was 381 ± 30 mm^3, 48 ± 10 mm^3, and 4 ± 1 mm^3, respectively, determined on the TTC staining slices. Because infarct area on most slices stained by hematoxylin and eosin was larger than that on their corresponding slices stained by TTC, the infarct area presented on Figure 5a was based on hematoxylin and eosin staining. Using hematoxylin and eosin staining slices in the HTA infarct group, the infarct volume was 6 ± 1 mm^3 (Figure 5b), which was larger than that calculated by TTC staining slices ($P=0.20$).

Hematoxylin and Eosin and TUNEL Staining

In the HTA infarct group (n=13), TTC staining delineated the HTA territory lesion in 11 of 13 rats. Combinations of hematoxylin and eosin staining methodology confirmed the lesion in all animals, 4 of which were proved by the TUNEL method only (Figure 6, A, C, and E).

In the AChA infarct group (n=12), infarction was identified by TTC staining in all rats, but the lateral hypothalamus showed a discoloration in only 7 of 12 rats. Hematoxylin and eosin staining corroborated with the TUNEL method showed that 11 of 12 rats had the ischemic damage in this region (Figure 6, B, D, and F). Ischemic changes in the medial hypothalamus and/or preoptic area were found by TTC staining in only 7 of 12 rats. The positive frequency in these regions was elevated to 8 of 12 rats by hematoxylin and eosin staining. In 2 of the remaining 4 rats judged normal by TTC and hematoxylin and eosin stainings (data not shown), scattered (Figure 6G) or clustered (Figure 6H) TUNEL-positive cells were detected in the medial hypothalamus, thus increasing the frequency further to 10 of 12 rats.

Discussion

We report herein that the advance of a coated 3-0 surgical suture to the position 0.7 to 1.9 mm proximal to the MCA bifurcation could cause infarction in the AChA or HTA territory in female rats. The brain regions affected by this maneuver are fundamentally limited to nonneocortical structures, mainly the medial hypothalamus (HTA infarct) and the internal capsule (AChA infarct). The present protocol provided a 69% rate in successful production of isolated deep infarcts, including 36% in the HTA territory alone and 33% in the AChA distribution. Because the internal capsule was constantly (100%) affected in an AChA infarct, this region appears to be part of the ischemic core in rat AChA occlusion. That HTA infarct could be produced independent of the AChA area indicate that these 2 regions are supplied by different arteries. An AChA infarct was almost always (91% frequency) accompanied by a HTA infarct. These observations and the results of cerebral artery perfusion with colored silicone (Figure 2) indicate that the HTA or at least 1 branch to the medial hypothalamus stems from the ICA more proximal to the MCA bifurcation than the AChA.

Using intraluminal filament model in rats, hyperthermia was formerly thought to be related to anterior hypothalamus and preoptic area damage associated with obstruction of the anterior cerebral artery and AChA.[3] In this study, however, a small HTA infarct in the medial hypothalamus constantly caused elevation of body temperature. These results indicate that a lesion involving the medial hypothalamus alone can cause hyperthermia irrespective of obstruction of the anterior cerebral artery or the AChA.

The hypothalamus appears to play a pivotal role in body temperature regulation. This region senses changes in local brain temperature, integrates information of temperature from other parts of the body, and sends efferent signals to various regions. Warm-sensitive neurons in the optic area of rats have been recently demonstrated to send excitatory signals to vasodilative neurons in the caudal part of the lateral hypothalamus, ventrolateral periaqueductal gray matter, and the reticular formation and send inhibitory signals to vasoconstrictive neurons in the rostral part of the ventral tegmental area.[18] Damage in the former regions or stimulation in the latter regions probably causes vasoconstriction.

Our observations that hyperthermia follows hypothalamic ischemia is strong evidence that infarction damages those mechanisms that regulate temperature. First, all supratentorial brain regions in the HTA infarct group were thoroughly examined with the use of hematoxylin and eosin staining, and no abnormality was identified, arguing against a possibility that damage in some other cerebral structure had occurred. Second, in a parallel experiment with the use of the transorbital method for MCA occlusion (HTA and AChA are exempt from occlusion), neither proximal (n=4) nor distal (n=10) permanent occlusion of the MCA caused rectal temperature to increase significantly (our unpublished data). Finally, direct injury of the hypothalamus by acute mechanical puncture[19,20] or the electrolytic method[21] causes hyperthermia. Neurogenic hyperthermia induced by mechanical puncture is hypothesized to affect the pyrogenesis mechanism on the basis of a set point shift, mediated by prostaglandins released from injured tissue and acting on surviving hypothalamic tissue.[19]

We found body temperature to be an accurate indicator predicting deep cerebral infarcts involving the hypothalamus. Special care, however, must be taken to accurately assess body temperature changes because anesthesia induction or a cool environment inhibits body temperature elevation.[3] We used isoflurane at the time of intraluminal arterial occlusion, an anesthetic agent usually accompanied by rapid recovery. Room temperature was maintained constant at 21±1°C. Such management permitted early detection of rises in body temperature in ischemic groups. Hyperthermia persisted for at least 3 days in rats with HTA infarction but lasted for lesser times in those with AChA or MCA territory damage. The mechanisms for this discrepancy are not clear and need further investigation.

The postural reflex test has been used for evaluating sensory-motor integration after focal cerebral ischemia. Striatum and overlying neocortex lesions caused by proximal MCA ligation[16] or rostral forelimb area of cortex damage induced by distal MCA occlusion[22] are both linked with deficits as measured by this test. However, damage involving the striatum and/or overlying neocortex does not necessarily correlate with deficit. Instead, sensory motor dysfunction appears to depend largely on the volume of infarct. In the original report of Bederson et al,[16] the sensitivity of the test was 88%. In their study, rats with small infarction limited to the caudatoputamen and dorsolateral cortex had no abnormality in the test. In our current study, the majority of rats with HTA infarct exhibited no abnormality or mild abnormality in the postural reflex test. On the other hand, all rats with AChA infarct involving the internal capsule had a definitive postural deficit irrespective of infarct volume. The results indicate the important role of the internal capsule in the manifestation of postural reflex abnormality. The positive test was a good indicator distinguishing AChA infarct from HTA infarct. Likewise, severe deficit in the early period of ischemia was suggestive of MCA infarct rather than AChA infarct. Thus the HTA, AChA, or MCA pattern can be predicted by the results of early postural reflex testing. (Table 1, 82% of all rats in ischemic groups exhibiting grade ≤2 at 1 hour after ischemia had a deep infarct).

With the use of the india ink double-perfusion technique, Ambach et al[23] confirmed that the preoptic area is supplied by arteries emerging from the anterior communicating artery and anterior cerebral artery, whereas the medial hypothalamus is supplied by the anterior, middle, and posterior tuberal arteries, which generally stem from the ICA in rats.[24] The arteries supplying the medial hypothalamus are terminal, with minimum of overlap in their supplying territories. The medial and central parts of the ventromedial nucleus are poorly vascularized. The infarcts localized in the ventral-medial hypothalamus in the current study was consistent with the characteristics of the arterial supply reported by Ambach and Palkovits.[24] In our study, it was unclear whether 1 or more of 3 tuberal arteries were involved in the HTA infarct production. Perfusion with colored silicone delineated arteries to the hypothalamus at the section of the ICA proximal to the AChA (Figure 2). Frequently, a recurrent branch stemming from the branch to the optic nerve arises in addition to the HTA shown in Figure 2, supplying the hypothalamus and being closely adjacent to the AChA (data not shown). An occluder advanced to the position to selectively obstruct the AChA may simultaneously occlude the recurrent branch.

The HTA infarct volume produced by the present protocol was ≈6 mm^3. From the viewpoint of small size and deep localization, the HTA infarct is comparable with lacunar infarction.[25] TTC staining of viable 2-mm-thick sections, however, tends to underestimate the extent of infarct, since the diameter of HTA infarcts was <2.4 mm in the majority of rats. A lesion confirmed by hematoxylin and eosin and TUNEL method was overlooked by TTC staining in 2 rats with HTA infarct. Similar ischemic changes were also dismissed in the medial hypothalamus (3 rats) and the lateral hypothalamus (4 rats) in animals with AChA infarct. To evaluate the extent of ischemic changes in small infarction, at least hematoxylin and eosin staining should be used concomitantly with TTC staining. Focal permanent[26–28] and transient[29,30] cerebral ischemia as well as transient global ischemia[31,32] can all induce apoptosis, a well-known mechanism of cell death. Reportedly, groups of apoptotic cells were localized in the inner boundary zone of the infarct after transient MCA occlusion in rats.[29] Neuronal apoptosis was present 3 days after mild ischemia in the peri-infarct area, where true infarction might evolve later.[33] Apoptosis could be detected as early as 30 minutes after reperfusion by the TUNEL method,[29] whereas detection of apoptotic body by hematoxylin and eosin staining took 12 hours of reperfusion.[34] Therefore ischemic changes in small deep infarction may be evaluated best by hematoxylin and eosin staining collaborated with TUNEL method.

In conclusion, a variation of the intraluminal suture MCA occlusion technique can produce ischemia in deep cerebral structures, either a small infarct fundamentally limited in the medial hypothalamus or a larger lesion involving the internal capsule and medial hypothalamus. Both infarcts are accompanied by hyperthermia, suggesting that damage in the medial hypothalamus is responsible for the rise of body temperature. The present model may facilitate research concerning various cerebral functional changes caused by small deep infarction.

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Estrogen attenuates over-expression of β-amyloid precursor protein messager RNA in an animal model of focal ischemia

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BRAIN RESEARCH

Research report

Estrogen attenuates over-expression of β-amyloid precursor protein messager RNA in an animal model of focal ischemia

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Abstract

Cerebral ischemia is a risk factor for late onset Alzheimer's disease. Since estrogen replacement therapy benefits the outcome of cerebral stroke in post-menopausal women, we designed the present study to investigate the effects of estrogen on the expression of β-amyloid precursor protein (APP) mRNA following focal ischemia in female rats. Female rats were ovariectomized (OVX) for two weeks. A single dose of 17 β-estradiol (E_2) (100 μg/kg) was injected s.c. two hours before a unilateral middle cerebral artery (MCA) occlusion. Brain samples were harvested from ischemic core and penumbra of cortices at one hour and twenty-four hours following MCA occlusion. The expression of APP mRNA was assessed by RT-PCR. At one hour after MCA occlusion, OVX rats had a 67.9% ($p < 0.05$) increase in APP mRNA in the penumbra. E_2 treatment reduced this APP mRNA over-expression by 26.3% at that region. At twenty four hours following MCA occlusion, OVX rats had increases in APP mRNA of 52.9% and 57.0% ($p < 0.05$) in the core and penumbra, respectively. E_2 treatment reduced the APP mRNA over-expression by 61.0% and 48.6% ($p < 0.05$) in these two regions, respectively. These effects appeared to reflect an interaction between hormonal environment and ischemia, since in the absence of MCA occlusion, there were no significant differences in APP mRNA expression among OVX, OVX-E_2 treated and intact female rats. The present study demonstrates that estrogen may have an important role in reducing the over-expression of APP mRNA following focal ischemia. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Middle cerebral artery; Estradiol; Cerebral blood flow; Hypometabolism; β-Amyloid precursor protein

1. Introduction

Several epidemiological studies have shown a strong relationship between cerebral stroke and consequential dementia. The incidence of dementia in the first year following a cerebral infarct is nine times greater than expected and after the first year, a 50% increase is observed in Alzheimer's disease (AD) [11]. Amyloid β-protein (Aβ) is the major component of neuritic plaque which is the characteristic feature of AD. APP can be cleaved by secretases into soluble or insoluble form of Aβ. The accumulation of the insoluble form of Aβ seeds the core of neuritic plaques in AD. We have previously reported an overexpression of APP mRNA induced by hypoglycemia in cultured primary astroglial cells [21]. In animal studies, APP expression was shown to increase in response to a variety of insults, including needle stab injury [17], kainic acid injection [16], head trauma [6] and persistent MCA occlusion [23].

Estrogen replacement therapy (ERT) in postmenopausal women has beneficial effects on the mortality and morbidity associated with cerebral stroke [2,9,12,18]. The Austrian Stroke Prevention Study [5] has demonstrated that postmenopausal women receiving ERT perform better on demanding cognitive tests when compared to postmenopausal women without ERT. Using MRI, they further demonstrated that this beneficial effect on cognition was associated with an estrogen-related prevention of silent ischemic brain damage.

In the present study, we used a transient focal ischemic animal model to investigate the effects of estrogen in the ischemia-associated expression of mRNA that encodes β-amyloid precursor protein.

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2. Materials and methods

2.1. Animals

Sprague–Dawley female rats (200–225 g body weight) were purchased from Charles Rivers Laboratories, (Wilmington, MA). They were housed in pairs in hanging, stainless steel cages in a temperature controlled room (25 ± 1°C) with daily light cycle (light on 0700 to 1900 h daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Two weeks before the focal ischemia surgery, all rats in the OVX and OVX + E_2 groups were ovariectomized to eliminate endogenous estrogens. Rats in OVX + E_2 group were administrated a single dose of 17 β-estradiol (E_2) (100 μg/kg) two hours before focal ischemia surgery. Control rats that were not ovariectomized were allotted into an intact-non-lesioned group or an intact-lesioned group.

2.2. Focal ischemic model

Middle cerebral artery (MCA) occlusion was achieved according to the methods described previously [20,22]. Briefly, following administration of anesthetics of ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) on the left side were exposed through a midline cervical incision and then gently dissected away from adjacent nerves. The distal ECA and its branches, the CCA and pterygopalatine arteries on the left side were coagulated completely by cautery. A 3-0 monofilament nylon suture was introduced into the left ICA lumen and gently advanced to the distal ICA until resistance was felt. The operating procedure was performed within 20 min with little bleeding. Rectal temperature was monitored and maintained between 36.5 and 37.0°C with a heating lamp during the entire stroke procedure. The suture was withdrawn from the left ICA after 40 min to allow MCA reperfusion.

2.3. Cerebral blood flow measurement

Cerebral blood flow (CBF) was measured in all rats that underwent focal ischemia surgery. The small area around bregma was exposed by a middle line section. Two symmetrical holes were drilled through the skull and adjacent to the dura. These two holes were located at 1.5 mm posterior to and 3.5 mm left/right of bregma. Two probes of a digital laser perfusion monitor (MICROFLO DSP, Oxford Optronix, Oxford, England) were placed on the dura to record the second to second change of CBF before, during, and after MCA occlusion.

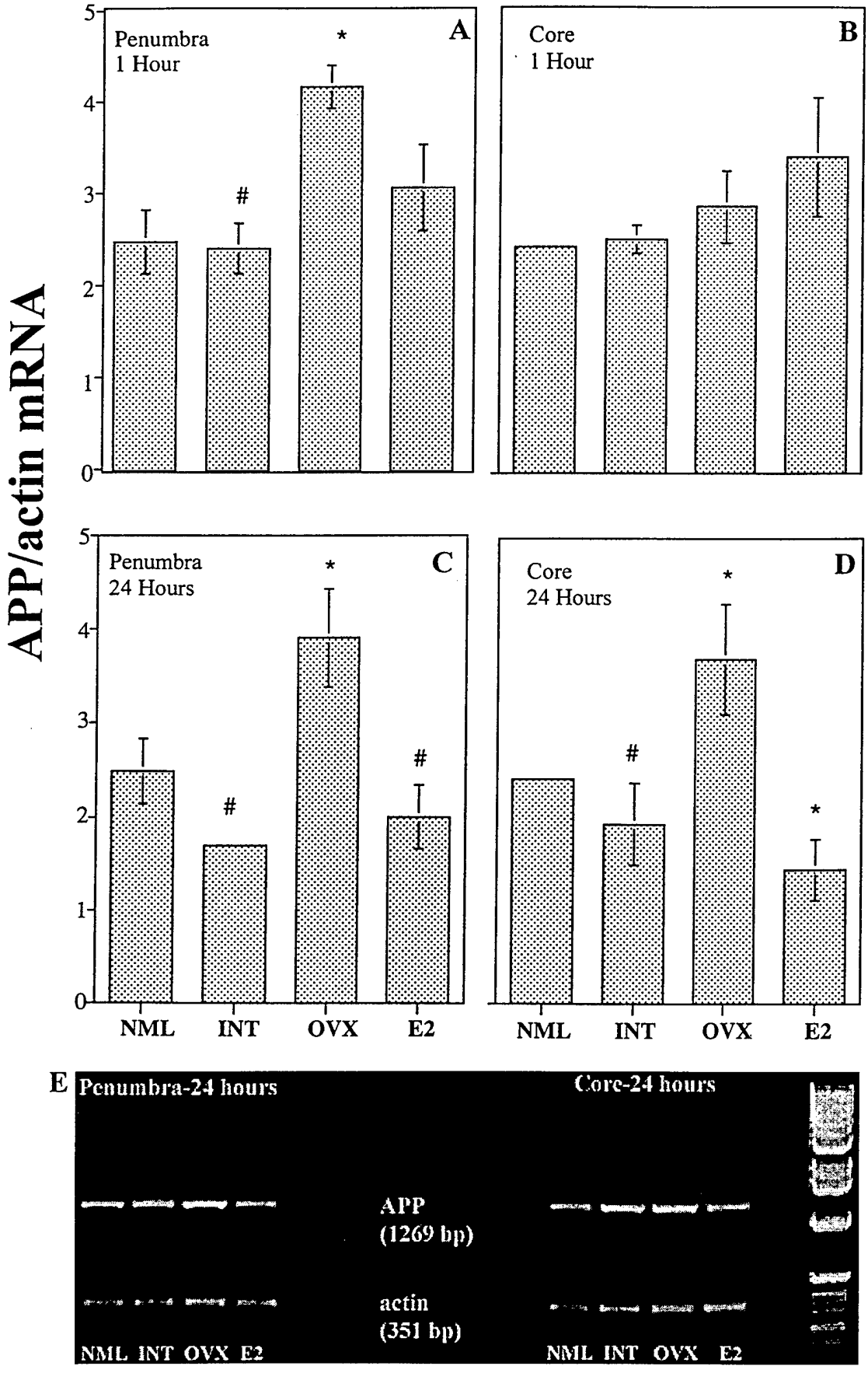
2.4. RT-PCR of APP mRNA

Rats were decapitated, the whole brains were dissected coronally from the olfactory bulb to the cerebellum in a metallic brain matrix (model RBM 4000C, ASI Instruments, Warren, MI). The sections that were at 7 to 11 mm posterior to the tip of olfactory bulb was sampled for RT-PCR. The superior one third of each cortical area from longitudinal cerebral fissure to lateral cerebral fissure was defined as the penumbral region, which is supplied predominantly by the anterior cerebral artery (ACA) and, to small extent, by the middle cerebral artery (MCA). The inferior two thirds was defined as the core region that is perfused exclusively by MCA. We have previously reported that in the occlusion-reperfusion model of stroke, the core region is consistently affected while the penumbra responsive to estrogen treatment [20,22].

RT-PCR was performed to assess the expression of APP mRNA according to methods described previously [21]. Two oligonucleotide primers for APP mRNA were designed to include a Kunitz protease inhibitor domain (KPI): sense primer 5′ TGCC ACCA CTAC CACA ACTA 3′ (nucleotide base 816–835) and antisense primer: 5′ GTTC TGCA TCTG CTCA AAG 3′ (nucleotide base 2085–2067). The numbers of nucleotide base were according to the rat APP genetic sequence numeration from Genebank (access number X07648). β-actin was used as an internal control of RT-PCR to be coamplified with APP. This semi-quantitative measure of APP was expressed as ratios of APP/β-actin.

cDNA was synthesized using 2 μg of total RNA prepared in an aliquot of 19 μl RNA reverse transcriptase cocktail. The mixture was incubated at 37°C for 30 min to destroy endogenous DNA, then heated at 75°C for 5 min to destroy DNase and followed by a 4°C soak. RT reaction was achieved by adding 1 μl of M-MLV reverse transcriptase to the 19 μl mixture at 42°C for 60 min. cDNAs of APP were amplified by using PCR (GeneAmp PCR Sys-

Fig. 1. The effects of E_2 administration on the expression of APP mRNA following MCA occlusion. All female rats were allotted to one of the four groups: NML = intact female rats without MCA occlusion; INT = intact female rats with MCA occlusion; OVX = OVXed female rats with MCA occlusion; E_2 = OVX + E_2 treated female rats with MCA occlusion. The cortical samples of rat brain cortices were collected from penumbra or core at 1 or 24 h after MCA occlusion. Total cellular RNAs were extracted for RT-PCR. The primers of actin were used as an internal control in the same RT-PCR procedure. Ratios of APP mRNA/actin mRNA are presented. Mean ± S.E.M. are depicted ($n = 6$ in each group). * $P < 0.05$ vs. NML; # $P < 0.05$ vs. OVX. When S.E.M. is not depicted, it was too small to be shown. 1E: Shown is a typical gel picture of RT-PCR results from samples taken from penumbra and core at 24 h after MCA occlusion. NML = intact female rats without MCA occlusion; INT = intact female rats with MCA occlusion; OVX = OVXed female rats with MCA occlusion; E_2 = OVX + E_2 treated female rats with MCA occlusion.


APP/actin mRNA
Penumbra
1 Hour
A
Core
1 Hour
B
Penumbra
24 Hours
C
Core
24 Hours
D
NML
INT
OVX
E2
E
Penumbra-24 hours
Core-24 hours
APP
(1269 bp)
actin
(351 bp)
NML INT OVX E2
NML INT OVX E2

tem 9600, Perkin-Elmer) with primers described above. Cycling conditions were programmed as 1 min at 94°C, followed by 94°C for 30 s, 54°C for 30 s and 72°C for 1 min for 30 cycles. After 30 cycles, extension was done at 72°C for 5 min followed by a 4°C soak. Ten microliters of PCR product were mixed with 2 μl DNA dye (30% glycerol, 0.25% bromophenol blue) and loaded to 1.3% agarose gel. The gel was visualized with ethidium bromide staining and analyzed using Kodak Digital Science™ 1D Image Analysis System (Eastman Kodak, Rochester, NY). All reagents used in RT-PCR were purchased from Promega (Madison, WI).

2.5. Statistics

The ratio of APP mRNA/actin mRNA were calculated. Kruskal–Wallis nonparametric analysis was used for statistical analysis. The Mann–Whitney U tests were used when Kruskal–Wallis showed significance among groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. The effects of E_2 administration on the expression of APP mRNA following mca occlusion

At 1 h following MCA occlusion, we observed a 67.9% increase in the expression of APP mRNA in penumbra of the cortices of OVX rats vs. intact-non-lesioned rats ($p < 0.05$) and a blunted over-expression of APP mRNA to 23.7% vs. intact-non-lesioned rats ($p > 0.05$) by E_2 treatment (Fig. 1A). We did not observe any significant differences in APP mRNA expression in ischemic core of the cortices of rats among these four groups at the 1 h sampling time (Fig. 1B). At 24 h following MCA occlusion, we observed increases in APP mRNA by 57.0% ($p < 0.05$) and by 52.9% ($p < 0.05$) vs. intact-non-lesioned rats in penumbra (Fig. 1C, E) and in core of OVX rats (Fig. 1D, E), respectively. Treatment with E_2 reversed APP mRNA expression to 80.7% ($p < 0.05$) and 59.9% ($p < 0.05$) of intact-non-lesioned rats in penumbra and in core, respectively (Fig. 1C, D, E). Noteworthy, there were no significant differences between intact-lesioned and intact-non-lesioned rats in the penumbra or in the core at 1 or 24 h after MCA occlusion. Expression of β-actin did not change in response to ischemia.

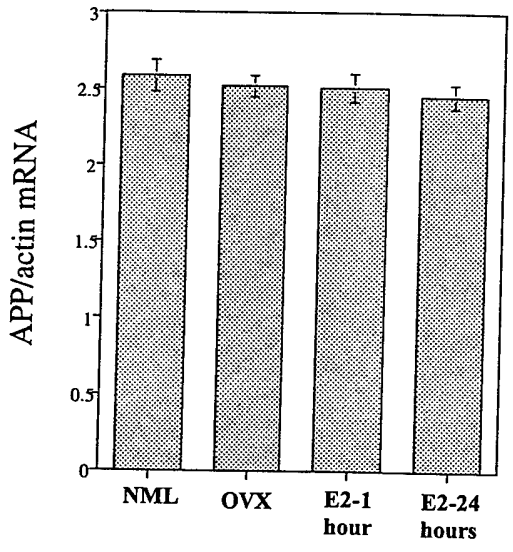


Fig. 2. The effects of E_2 administration alone on the expression of APP mRNA. All rats were placed into one of the four groups: NML, OVX, E_2-1 h that matched the time point of 1 h after MCA occlusion, E_2-24 h that matched the time point of 24 h after MCA occlusion. None of these rats underwent MCA occlusion. Cortical samples were collected for extraction of total cellular RNAs. RT-PCR was applied to measure the amount of cDNA in each sample. The primers of actin were used as an internal control in the same RT-PCR procedure. Mean ± S.E.M. are depicted ($n = 8$ in each group).

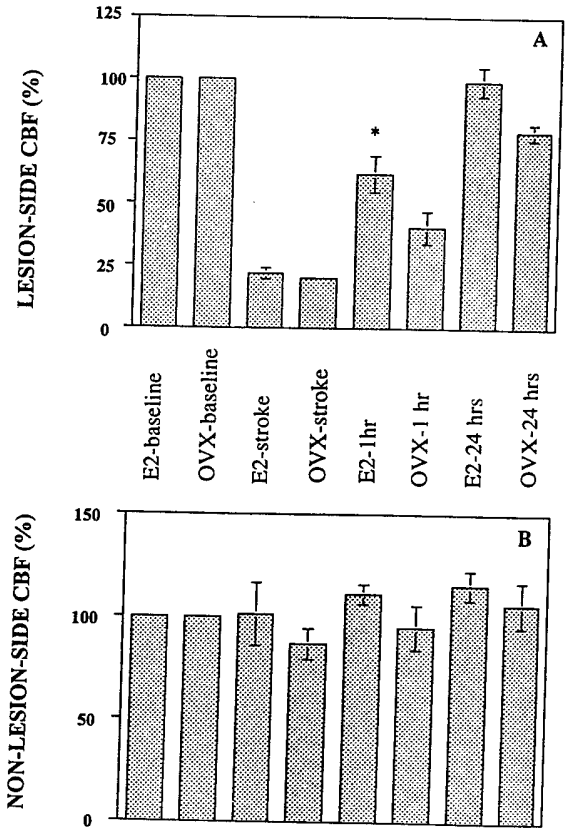


Fig. 3. The effects of E_2 administration on CBF prior to, during and following MCA occlusion. A digital laser perfusion monitor was used to measure the CBF. Two probes were placed symmetrically on the dura at the region that was supplied by MCA. CBFs were measured before, during, 1 h after and 24 h after MCA occlusion. The basal CBF in OVX and OVX + E_2 groups were taken as 100%. Mean ± S.E.M. of percentage in changes of CBF are depicted ($n = 8$ in each group). $P < 0.05$ vs. OVX at the same time point. The S.E.M. in OVX-stroke group was too small to be depicted.

3.2. The effects of E_2 administration alone on the expression of APP mRNA

The results above suggested that the changes in the expression of APP mRNA are associated with the change in estradiol levels. To determine if observed treatment-related changes in APP mRNA expression were the result of estradiol exposure alone or an interaction of the estradiol with the ischemic event, we evaluated four groups of non-lesioned rats: intact, OVX, OVX-E_2-1 h (sampled at 3 h 40 min after an infection of 100 μg/kg E_2 and matched with the 1 h time point) and OVX-E_2-24 h (matched with 24 h time point). We did not observe any significant difference in APP mRNA expression among these four groups (Fig. 2). Expression of β-actin did not change in response to estrogen administration.

3.3. The effects of E_2 administration on CBF following MCA occlusion

We measured CBF in OVX and OVX + E_2 rats to assess the effects of E_2 on CBF during and following MCA occlusion. MCA occlusion caused a reduction to 21.8% and 20.0% of basal CBF in the ipsilateral hemisphere in OVX + E_2 and OVX, respectively (Fig. 3A). Upon the removal of monofilament, a more rapid recovery of ipsilateral CBF to 61.75% of the basal level was observed in OVX-E_2 rats as compared to a 40% recovery in OVX rats ($p < 0.05$) (Fig. 3A). Twenty-four hours of reperfusion further increased ipsilateral CBF to 99% and 78.8% of basal levels in OVX + E_2 and OVX, respectively (Fig. 3A). In the non-lesioned, contralateral hemisphere, there were no significant changes in CBF over time in either OVX or OVX + E_2 groups (Fig. 3B).

4. Discussion

Accumulating evidences from cranial CT, MRI and other clinical studies suggest that cerebral stroke is a major risk factor of dementia, ranking only second to age [8]. Dementias that develop after stroke are associated with higher mean tissue loss [14], cerebral atrophy [24], infarct incidences [25], and to a less extent, with infarct locations [13]. Amyloid plaques, which result from an accumulation of insoluble APP cleavage fragments, are a characteristic pathological feature of AD that accounts for more than 90% of dementias. In animal studies, APP immuno-reactivity is increased at the boundary of the infarct at 4 days to 7 days after persistent MCA occlusion in male spontaneously hypertensive rats [23]. This enhanced APP expression is in the form of an increase in a Kunitz-type protease inhibitor domain-containing APP mRNA after persistent focal ischemia [1]. Together with our present study, these data suggest APP response to MCA occlusion-induced ischemia by mRNA overexpression and/or altered mRNA splicing.

We used an ischemia-reperfusion animal model that differs from the models mentioned above in that the ischemic regions were reperfused by CBF once the intralument monofilament was removed. We observed an 80% decrease in CBF during MCA occlusion and a recovery to 50%–100% of basal CBF over the first 24 h after removal of the monofilament. Reperfusion is viewed as a double-edged sword in the event of ischemia. While reperfusion may facilitate recovery of CBF, it is also able to introduce free radicals [3] to the vulnerable regions and thereby may hasten necrosis.

Ischemia-induced APP overexpression may provide more insoluble cleaved form of β-amyloid, which in turn could accumulate over time to form neuritic plaques of AD. On another hand, transgenic mice that overexpress APP are more vulnerable to focal ischemia-induced damages. This vulnerability may, at least to some extent, be due to a decrease in CBF and a blunted response to acetylcholine-induced vasodilation in APP transgenic mice [29]. Therefore, the overexpression of APP and ischemia may constitute a vicious cycle that leads to neurodegeneration.

While the high level of amyloidogenic forms of human APP can lead to formation of neuritic plaques and neurodegeneration, the low level of APP or exogenous soluble APP may exert neuroprotection. Smith-Swintosky et al. [28] have reported the administration of secreted forms of APP (APP 695 and APP 751) intra-cerebroventricularly protected CA1 neurons against ischemic injuries. Consistently, transgenic mice that express the 751 amino acids form of human APP showed significant protection against kainate-induced degeneration of presynaptic terminals and neuronal dendrites [27]. We have demonstrated in this study that E_2 reverses APP mRNA overexpression by more than 60% in both the penumbra and core of the ischemic area. The attenuation of APP overexpression by estrogen may serve to maintain a low level of APP and prevent APP accumulation. This low level of APP may in turn be able to protect focal ischemia-induced neuronal damages.

We and others have reported profound protective effects of estrogens on ischemic lesion sizes following transient MCA occlusion [4,22,26]. E_2 treatment of OVX rats causes a decrease in lesion sizes by about 50% compared with OVX rats [20,22]. Since stroke-induced mean tissue loss is closely associated with late onset AD [14], the neuroprotective effects of estrogen during and after attacks may contribute to a reduced incidence in AD following stroke. We have shown that estrogen has a more rapid effect on the recovery of CBF upon the removal of MCA occlusion, even though OVX and OVX + E_2 rats do not differ significantly in the recovery of CBF at 24 h following MCA occlusion. Dubal et al. [4] also reported a tendency in CBF increase following a low dose E_2 treatment (10 pg/ml) in a persistent focal ischemic animal model. Unfortunately, their sample size may have been too small ($n = 3$) to

detect a significant CBF change and they did not report the effect of high dose E_2 (60 pg/ml) on CBF following ischemia. This rapid and transient effect of estrogen on CBF recovery following MCA occlusion may contribute to the decreased ischemic area and consequential APP overexpression. Other of estrogen's biological functions may help to explain its protection against neurodegeneration. Estrogen interacts with neurotrophins and their receptors [7,15], enhances cerebral glucose utilization [19], and attenuates oxidative impairment of Na^+/K^+-ATPase activity and reduced glutamate transport induced by amyloid β-peptide in vitro [10].

In summary, we have shown an increase in APP mRNA expression associated with focal ischemia. We have also demonstrated that E_2 can attenuate APP mRNA overexpression in a manner that is related to estrogen's protective effects on lesion sizes and normalization of CBF during reperfusion. Therefore, our study suggests a profound effect of estrogen on central nervous system that may be able to interrupt a vicious cycle of ischemia and neurodegeration.

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A Novel, Synergistic Interaction Between 17 β-Estradiol and Glutathione in the Protection of Neurons against β-Amyloid 25–35-Induced Toxicity *In Vitro*

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ABSTRACT

The present studies were undertaken to investigate the possibility of an interaction between 17 β-estradiol (E2) and glutathione in protecting cells against the presence of β-amyloid 25–35 (βAP 25–35). We demonstrate that when evaluated individually, supraphysiological concentrations of either E2 (200 nM) or of reduced glutathione (GSH; 325 μM) can protect SK-N-SH human neuroblastoma cells from βAP 25–35 (20 μM) toxicity. This dose of βAP 25–35 was chosen based on the LD_{50} (28.9 μM) obtained in our earlier work. However, in the presence of 3.25 μM GSH, the neuroprotective EC_{50} of E2 was shifted from 126 ± 89 nM to 0.033 ± 0.031 nM, approximately 4000-fold. Similarly, in primary rat cortical neurons, the addition of GSH (3.25 μM) increased the potency of E2 against βAP 25–35 (10 μM) toxicity, as evidenced by a shift in the EC_{50} values of E2 from 68 ± 79 nM in the absence of GSH to 4 ± 6 nM in its presence. The synergy between E2 and GSH was not antagonized by the addition of the estrogen receptor antagonist, ICI 182,780. Other thiol-containing compounds did not interact synergistically with E2, nor were any synergistic interactions observed between E2 and ascorbic acid or α-tocopherol. Based on these data, we propose an estrogen-receptor independent synergistic interaction between glutathione and E2 that dramatically increases the neuroprotective potency of the steroid and may provide insight for the development of new treatment strategies for neurodegenerative diseases.

An increasing body of evidence indicates that oxidative damage plays a key role in the pathological events occurring in AD (Good *et al.*, 1996; Yan *et al.*, 1996), with use of antioxidants [i.e., any substance that under physiological conditions significantly delays or inhibits oxidation when present in low concentrations compared with those of an oxidizable substrate (Halliwell and Gutteridge, 1989)] emerging as a possibly useful therapy. Substances that fit this description and that are currently used in clinical trials include vitamin E and estrogens. A role for estrogens in the prevention of AD has been implicated by a retrospective epidemiological study, showing a dose- and duration-dependent relationship of ERT with a reduction in the incidence of AD (Paganini-Hill and Henderson, 1994). Recent studies revealed that ERT delays the onset of AD symptoms regardless of the ethnic origin of the subjects (Tang *et al.*, 1996b). As such, there is much to be gained in elucidating the mechanism by which ERT alters the course of AD.

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In some cases of familial AD, genetic mutations result in the increased production of βAP (Selkoe, 1997), a 39–43 amino acid peptide which upon aggregation (Pike *et al.*, 1991) and contact with the plasma membrane (Mattson *et al.*, 1993) is toxic to neurons in culture. The toxic portion of this peptide seems to be an 11-amino acid sequence (βAP 25–35) (Yankner *et al.*, 1989), and increased amounts of H_2O_2 (Behl *et al.*, 1994) and lipid peroxides (Behl *et al.*, 1992; Goodman *et al.*, 1996; Gridley *et al.*, 1997; Keller *et al.*, 1997) result from a βAP 25–35 challenge. We have observed (Green *et al.*, 1996) that this fragment rapidly aggregates, causing cell death with a dose-dependence and time-course similar to that reported for in primary neurons and B12 cells (Behl *et al.*, 1994). This provides an *in vitro* model system for studying the mechanism by which estrogens ameliorate βAP-induced toxicity on a variety of cell types.

Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* models, and evidence is emerging to suggest that their antioxidant activity is involved in their neuroprotective capacity. Structure-activity relationship studies revealed that a phenolic A ring (Behl *et al.*, 1997; Green *et al.*, 1997b) and at least three rings of the steroid

ABBREVIATIONS: AD, Alzheimer disease; βAP, β-amyloid peptide; DMEM, Dulbecco's modified Eagle's medium; E2, 17 β-estradiol; ERT, estrogen replacement therapy, GSH, reduced glutathione; ICI, ICI 182,780; RPMI-1640, Roswell Park Memorial Institute-1640 Medium; PDHS, plasma-derived horse serum; ANOVA, analysis of variance.

structure (Green *et al.*, 1997b) are required for the molecule to demonstrate neuroprotection. Additionally, estrogens reduce the cellular toxicity of βAP and other oxidative insults (Behl *et al.*, 1995; Goodman *et al.*, 1996; Green *et al.*, 1996) and we have recently demonstrated that this correlates to a decrease in the amount of generated lipid peroxidation (Gridley *et al.*, 1997). The mechanism by which estrogens act as an antioxidant at higher concentrations may directly relate to their ability to scavenge free radicals (Mooradian, 1993). We hypothesized that estrogens at physiologically relevant concentrations may be participating in a cycle whereby estrogens are regenerated by other endogenous antioxidants. Because reparation of lipid membranes relies on the glutathione peroxidase enzyme system (Meister and Anderson, 1983), and low concentrations of estrogen can reduce lipid peroxidation (Gridley *et al.*, 1997), we targeted glutathione as a likely candidate for this interaction. The present studies were undertaken to investigate the possibility of an interaction between E2 and glutathione in protecting cells against the presence of βAP 25–35.

Materials and Methods

Materials. Lyophilized βAP 25–35 (1 mg; Bachem, Torrance, CA) was initially dissolved in 200 μl of double deionized H_2O and with the addition of 800 μl of PBS, rapid aggregation was observed. E2 (Steraloids, Wilton, NH) was initially dissolved at 10 mg/ml in absolute ethanol (Fisher Scientific, Orlando, FL) and diluted in cell culture media to obtain the necessary concentrations. ICI (Zeneca, Chesire, England) was dissolved in absolute ethanol and spiked into individual cell culture wells to obtain the 200 nM concentrations. α-Tocopherol acetate was initially dissolved in 200 μl of absolute ethanol and diluted in cell culture media to the appropriate concentrations. Lipoic acid (thiotic acid), taurine (2-aminoethanoic acid), and ascorbic acid were initially dissolved in cell culture media and used at the concentrations indicated. Unless otherwise noted, materials were obtained from Sigma Chemical Corp (St. Louis, MO).

SK-N-SH neuroblastoma cell culture. SK-N-SH neuroblastoma cells were obtained from American Type Culture Collection (Rockville, MD). Cell cultures were grown to confluency in RPMI-1640 media (Fisher Scientific, Pittsburgh, PA) supplemented with 10% charcoal/dextran-treated FBS, (Hyclone, Logan, UT), 100 units/ml of penicillin G and 100 mg/ml of streptomycin (Sigma) in monolayers in Corning 150-cm^2 flasks (Fisher Scientific) at 37° under 5% CO_2, 95% air. Media was changed three times weekly. Cells were observed with a phase contrast microscope (Nikon Diaphot-300; Nikon, Tokyo, Japan).

SK-N-SH cells used in the following experiments were in passes 4 to 12. The growth media was initially decanted and the cells were rinsed with 0.02% EDTA for 30 min at 37. Cells were then counted on a Neubauer hemacytometer (Fisher Scientific) and resuspended in appropriate media. Studies were initiated by plating 1×10^6 cells per well in 24-well plates, allowing attachment in regular media and then decanting that media and replacing with the appropriate treatment after 4 hr. Cells were cultured in DMEM or RPMI-1640 without GSH (Life Technologies, Grand Island, NY), supplemented with 10% FBS and antibiotics, with absolute ethanol as a vehicle control, or supplemented with the addition of βAP 25–35 (20 μM), E2 (0.002–200 nM), GSH (0.0325–325 μM), α-tocopherol acetate (50 μM), ascorbic acid (100 μM), lipoic acid (10 μM), taurine (5 mM), ICI (200 nM), or a combination as indicated. The 20-μM concentration of βAP was selected as we have shown that it is near the LD_{50} for this peptide (Green *et al.*, 1996). Selection of antioxidant concentrations were made on the basis of preliminary dose-response evaluations used to identify the maximal concentration at which neuroprotection was not obtained (data not shown).

SK-N-SH cell viability was determined utilizing the trypan blue exclusion method (Black and Berenbaum, 1964). After 72 hr of incubation, treatment media was decanted and cells were lifted by incubating with 0.2 ml 0.02% EDTA for 30 min at 37°. Cells were suspended by repeated pipetting. Aliquots (100 μl) from each cell suspension were incubated with 100 μl aliquots of 0.4% trypan blue stain for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer within 10 min of addition of trypan blue stain. Two independent counts of live and dead cells were made for each aliquot.

Primary rat cortical cultures. Primary neuronal cultures were prepared according to methods described elsewhere (Chandler *et al.*, 1993). Briefly, Female Sprague-Dawley rats (Charles River Farms, Wilmington, MA) were housed and bred in our animal facility. Primary cortical neurons were prepared from 1-day-old rat pups as follows: brain tissue was removed from rat pups and placed in isotonic salt solution containing 100 units of penicillin G, 100 μg of streptomycin and 0.25 μg of amphotericin B (Fungizone; Life Technologies) per ml (pH 7.4). After removal of blood vessels and pia mater, the tissue was sectioned into approximately 2-mm chunks, suspended in 25 ml of 0.25% trypsin (weight/volume) in isotonic salt solution (pH 7.4), and placed in a shaking water bath for 10 min at 37° to dissociate the cells. The dissociated cell suspension was then removed and combined with 10 ml of DMEM containing 10% PDHS (Central Biomedia, Irwin, MO) and the undissociated chunks were mixed with 160 μg of DNase 1 and triturated until the cells dissociated. The cell suspensions were then combined, centrifuged at $1000 \times g$ for 10 min, and the resulting cell pellet washed with 50 ml of DMEM with 10% PDHS and plated on precoated poly-L-lysine 35-mm culture dishes at a density of 4×10^6 cells per dish and incubated in a humidified incubator containing 95% air and 5% CO_2 at 37°. On day 3, cells were treated with β-cytosine arabinoside (10 μM) for 48 hr and media was then aspirated and replaced with DMEM containing 10% PDHS and incubated for an additional 5 days before being used in experiments. At this time, cultures contain approximately 90% neurons and 10% astroglia. These appeared as many phase-contrast bright cells with characteristic neuronal morphology overlaying a number of flat phase dark cells that had typical astroglial morphology.

Treatments were made directly to primary cultures on culture day 10, maintaining a constant volume added regardless of treatment. Cultures were supplemented with the following treatments: Absolute ethanol and PBS as vehicle controls, βAP 25–35 (10 μM), E2 (0.02 nM-2 μM), GSH (3.25 μM), or combinations as indicated. The 10 μM concentration of βAP 25–35 was selected following preliminary studies aimed at causing a 40–60% cell death in 24 hr. Once treatments were added, primary cultures were incubated for an additional 24 hr and viability determined using the Live/Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions. Basically, the calcein AM (5 μM) and ethidium homodimer (5 μM) dyes were made fresh before use, and 300 μl were used to cover the bottom of the culture dish. Live cells were distinguished by the presence of intracellular esterase activity, which cleaves the calcein AM dye, producing a bright green fluorescence when excited. Ethidium homodimer enters cells with damaged membranes, and upon binding to nucleic acids, produces a red fluorescence. Both dyes are excited at 485 nm, and cultures plates were viewed with a fluorescent microscope (Nikon Diaphot-300). Three random fields were photographed, and the average number of live cells per field was determined by counting the number of bright green cells.

Statistics. The significant treatment effects on cell viability were determined using ANOVA followed by Scheffé's *post hoc* test, with significance determined at $p < 0.05$. For dose-response evaluations, EC_{50} values were calculated by randomly assigning cell counts at the indicated doses to generate 3–5 lines per treatment and determining the average value for those lines. Mann-Whitney rank sum nonparametric analyses were used on EC_{50} values because the variances for

the standard deviation were not equivalent. Comparisons between dose response relationships were calculated using two-way ANOVA to determine the significance of GSH or E2 presence or absence.

Results

The neuroprotective capability of physiologically relevant concentrations of E2 relies on the presence of glutathione in the cell culture milieu (Fig. 1). Using DMEM media, which lacks GSH in the cell culture recipe, and RPMI-1640 manufactured specifically without GSH, we demonstrated that the addition of βAP 25–35 reduced the number of viable cells by 41% and 47%, respectively, in these culture media. Concomitant treatment with E2 (2 nM) had no effect, which contrasted with our earlier work that showed these same concentrations of E2 to be neuroprotective in RPMI-1640 media (Green *et al.*, 1996). Based on our hypothesis that GSH may play a role in this system, we supplemented GSH (3.25 μM) to the cell culture milieu. The addition of GSH was not neuroprotective alone; when added with low concentrations of E2 (2 nM), however, it increased the number of viable cells by 88% and 47% in DMEM and RPMI-1640 lacking GSH, respectively.

To further evaluate the interaction noted between E2 and GSH, we assessed the neuroprotective capacity of GSH (Fig. 2). We noted that in the absence of estrogen, high concentrations of GSH were necessary to achieve neuroprotection, with a significant reduction (50%) in βAP 25–35-induced toxicity at the 325 μM dose. By contrast, in the presence of E2 (2 nM), the neuroprotective dose of GSH was reduced to 0.325 μM. Two-way ANOVA revealed a highly significant effect of E2 on the GSH induced neuroprotection (F, 41.4; $p < 0.001$). The neuroprotective EC_{50} value generated for GSH without E2 in the media was 82.6 ± 60 μM, contrasted by 0.04 ± 0.02 μM when E2 was present, an approximate 2000-fold increase in GSH potency in the presence of E2. Using this information, we analyzed the ability of E2 to increase the number of viable cells exposed to βAP 25–35 (Fig. 3). In the absence of GSH, high concentrations of E2 (200 nM) were necessary for significant neuroprotection (Fig. 3). However, in the presence of a nonprotective dose of GSH (3.25 μM), the neuroprotective dose of E2 shifted from 200 nM to 0.2 nM (Fig. 3). Again, two-way ANOVA revealed a highly significant effect of the presence of GSH on the neuroprotective effect of E2 (F, 44.33; $p < 0.001$). The calculated EC_{50} values likewise shift to the left, from 126 ± 87 nM in the absence of GSH to 0.033 ± 0.031 nM in the presence of GSH, a ~4000-fold increase in the potency of E2 in the presence of GSH.

To ensure that this synergy was not caused simply by cell origin or tumorigenicity, we performed similar experiments in rat primary cortical neurons. Again, the ability of E2 to protect neurons was evaluated in the presence and absence of GSH (3.25 μM) (Fig. 4). The addition of βAP 25–35 (10 μM) to

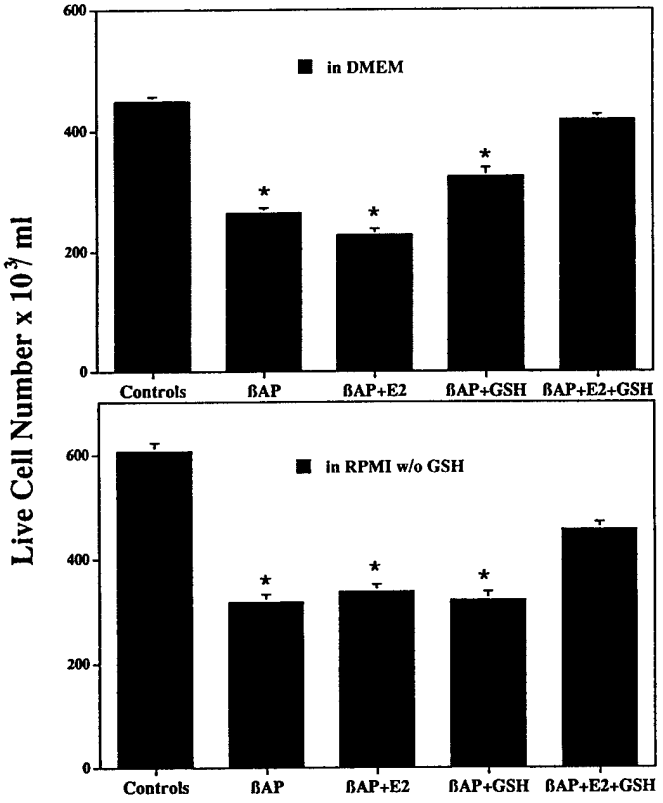


Fig. 1. Effects of 17-β estradiol (E2; 2 nM) and GSH (3.25 μM) on the toxicity induced by βAP 25–35 (20 μM) in different cell culture media that lack GSH in the cell culture recipe. Cells were plated at 10^6 cells/ml and were exposed to vehicle, βAP 25–35, E2, GSH, or a combination as indicated for 72 hr. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35) pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for four or five wells/group. *, $p < 0.05$ versus controls determined by ANOVA followed by Scheffé's *post hoc* test.

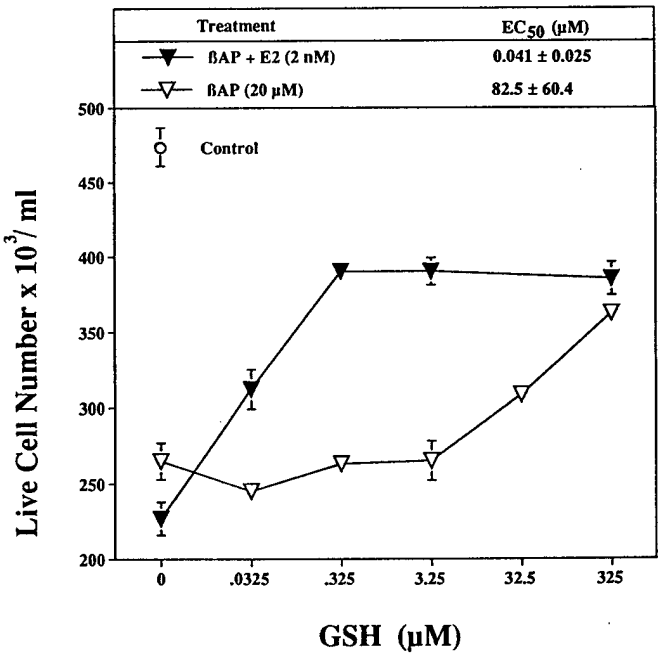


Fig. 2. Effects of GSH concentration on live SK-N-SH cell number subjected to a βAP 25–35 (20 μM) challenge in the presence and absence of a nonprotective dose of E2 (2 nM). Cells were plated at 10^6 cells/ml and exposed to treatments at the doses indicated. Controls represents cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for six wells per group. When error bars are absent, they are smaller than the symbol used to depict mean values. The effect of E2 on the response to GSH was highly significant (F, 41.48; $p < 0.001$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed $p = 0.036$.

primary cortical neurons resulted in a 39% to 40% reduction in the average number of viable cells per field in the absence and presence of GSH, respectively (Fig. 4). When increasing concentrations of E2 were evaluated against βAP 25–35, 200 nM E2 was the lowest concentration found to be protective (Fig. 4), which is in full agreement with our SK-N-SH cell line studies (Fig. 3). With the addition of GSH (3.25 μM), all concentrations of E2 of 2 nM or higher were neuroprotective (Fig. 4). Evaluation of EC_{50} values demonstrated similar changes in potency, from 68.1 ± 79 nM in the absence of GSH, to 4.3 ± 5.9 nM in the presence of GSH. Likewise, evaluation of the effect of GSH on the neuroprotective effect of E2 in rat primary cultures using two-way ANOVA demonstrated a significant effect (F, 8.53; $p < 0.005$).

We examined the specificity of this estrogen-antioxidant interaction for the GSH system by evaluating protection of SK-N-SH cells from βAP 25–35-induced toxicity. This was achieved by addition of a concentration of E2 (20 nM) that was at or near the protective threshold in the presence or absence of nonprotective concentrations of other well known antioxidants (Table 1). In four separate experiments, the reduction in viable cells ranged from 35% to 68% when subjected to a βAP 25–35 (20 μM) challenge. The 20 nM dose of E2 alone was either nonprotective or slightly protective (Table 1). No significant effect was observed on βAP 25–35-induced toxicity with the addition ascorbic acid (100 μM), α-tocopherol acetate (50 μM), taurine (5 mM), or lipoic acid (10 μM) alone or in combination with E2 (Table 1). However, the addition of GSH (3.25 μM) or the oxidized form of glutathione (1.5 μM) was enough to significantly reduce the cytotoxicity of βAP 25–35 when 20 nM E2 was present (Table 1).

The importance of an estrogen receptor in this interaction was determined by using ICI as an antiestrogen (Fig. 5). Again, βAP 25–35 (20 μM) was added to SK-N-SH cells in the presence and absence of E2 (2 nM), GSH (3.25 μM), and/or ICI (200 nM). βAP 25–35 reduced viable cells after 72 hr of exposure by 54% when compared with vehicle controls. Using concentrations of E2 with GSH that were not protective when administered alone but were neuroprotective when added together, the addition of ICI in 100-fold excess of the E2 concentration did not significantly alter the protective effects of E2 and GSH in combination. Indeed, ICI addition alone exerted neuroprotective activity.

Discussion

It is the purpose of this article to report a novel synergistic interaction between E2 and glutathione for neuroprotection. This interaction is independent of species origin and the tumorigenicity of the cells, as we have demonstrated protection in a human neuroblastoma cell line, in rat primary cortical neurons (present report), and the HT-22 transformed mouse hippocampal cell line (Green *et al.*, 1998). Furthermore, this effect seems to be independent of the type of cytotoxic insult used, as we have observed the same synergy with both serum deprivation and zinc toxicity (Gridley KE,

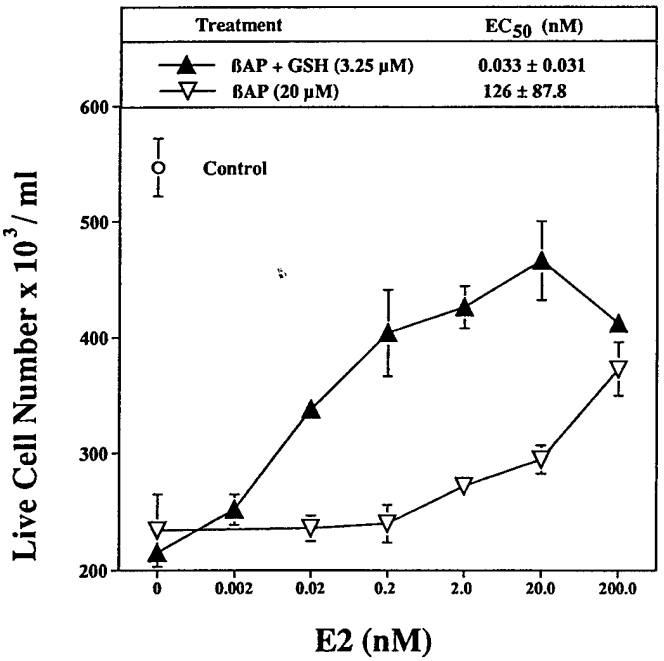


Fig. 3. Effects of E2 concentrations on live SK-N-SH cell number subjected to a βAP 25–35 (20 μM) challenge in the presence and absence of a nonprotective dose of GSH. Cells were plated at 10^6 cells/ml and exposed to treatments at the doses indicated. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± SEM for four wells per group. When error bars are absent, they are smaller than the symbol used to depict mean values. The effect of GSH on the response to E2 was highly significant (F, 44.33; $p < 0.001$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed a $p < 0.057$.

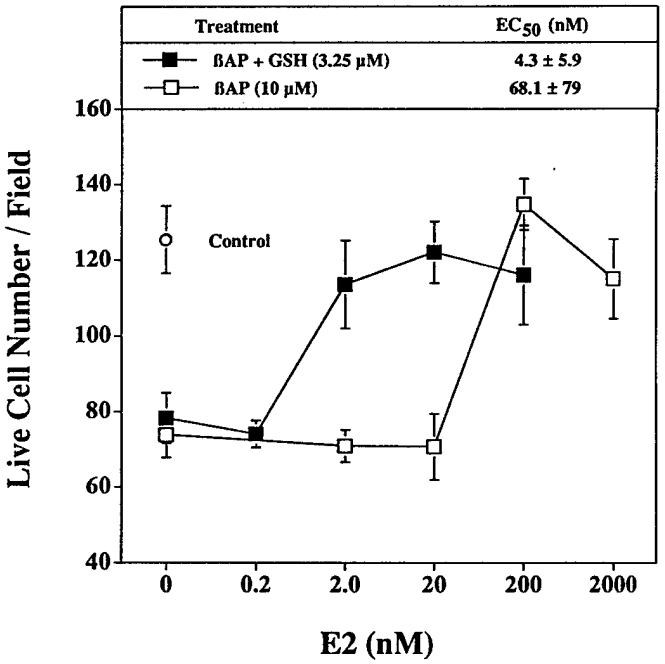


Fig. 4. Effects of concentrations of E2 on the average number of live rat primary cortical neurons per photographic field when subjected to a βAP 25–35 (10 μM) challenge in the presence and absence of a nonprotective dose of GSH. Cells were plated and exposed to treatments as previously indicated at the doses noted. *Controls*, cell numbers for the vehicle, GSH, E2, and GSH+E2 control groups (no βAP 25–35), pooled together after it was determined they were not statistically different from each other. Depicted are the mean values ± standard error for four to seven plates per group. The effect of GSH on the response to E2 was highly significant (F, 8.53; $p < 0.005$). A comparison of the EC_{50} values for the different dose response curves using the Mann-Whitney rank sum test showed $p < 0.057$.

Pariokar KS, Simpkins JW unpublished observations) as herein reported with βAP toxicity in SK-N-SH cells. This effect does not depend on the form of βAP used, as we have seen synergistic interactions against both βAP 1–40 and βAP 25–35 in HT-22 cells (Green *et al.*, 1998). Although differences exist in the glutathione-induced shift in the neuroprotective potency of E2 in these cell types, these may directly relate to the different cell culture techniques used to assess viability. Furthermore, intracellular concentrations of glutathione may play a role as we have determined that primary rat cortical neurons have higher intracellular glutathione concentrations (172 ± 12 μM) than SK-N-SH cells (15 ± 2 μM). In either case, we can resolve discrepancies with regard to differences reported between our work (Green *et al.*, 1996) and others (Behl *et al.*, 1995; Goodman *et al.*, 1996) for the neuroprotective concentrations of estrogens. In our previous work, we used a cell culture milieu containing GSH, whereas others used media that lacked GSH.

Mounting evidence supports the hypothesis that part of the neuroprotective activity of estrogens resides in their antioxidant capacity. Decreases in oxidative by-products are correlated with decreases in cellular toxicity in our previous studies (Gridley *et al.*, 1997), as well as those done by others (Behl *et al.*, 1997; Goodman *et al.*, 1996). Because the actions of most antioxidants are multifaceted, we expect that estrogens may be working through several mechanisms to provide this neuroprotection. Estrogen can participate in the nonenzymatic reduction of free radicals (Mukai *et al.*, 1990), further demonstrated in a cell-free system where peroxy-nitrite radicals were found to be reduced by estrogens (Mooradian, 1993) at the same concentration at which we see neuroprotection in the absence of glutathione. In addition, estrogens can participate in iron reduction (Ruiz-Larrea *et al.*, 1995), which may be paramount to decreasing the production of free radicals. Multiple studies have demonstrated that estrogens decrease lipid peroxidation in a variety of model systems (Sugioka *et al.*, 1987; Behl *et al.*, 1995; Lacort *et al.*, 1995; Goodman *et al.*, 1996; Tang *et al.*, 1996a; Behl *et al.*, 1997; Gridley *et al.*, 1997; Keller *et al.*, 1997). Estradiol has also been shown to reduce oxidative impairment of membrane transporters for ions and glucose resulting from βAP 25–35 exposure (Keller *et al.*, 1997). However, estrogens did not prevent impairment of membrane transport systems from toxic lipid peroxidation by-products (Keller *et al.*, 1997) providing additional support for the idea that their antioxidant nature is the basis for their activity in neuroprotection.

Likewise, GSH exerts its antioxidant activity through several mechanisms (Meister and Anderson, 1983). GSH can scavenge free radicals via a nonenzymatic mechanism (Meister and Anderson, 1983). In our system, high concentrations of GSH (325 μM) were necessary to protect cells from the βAP insult (20 μM). This neuroprotective concentration is a much higher concentration of GSH than is present in extracellular fluids (Smith *et al.*, 1996). Perhaps a more practical explanation involves the ability of GSH to act on intracellular peroxides via GSH peroxidases and GSH *S*-transferases (Meister and Anderson, 1983). This system functions in the defense against free radicals through the reduction of hydrogen peroxide. This action of GSH may be relevant to the βAP insult, because generation of increased amounts of H_2O_2 has been demonstrated in both βAP 25–35- and βAP 1–40-induced toxicity (Behl *et al.*, 1994) and increases in the activity of glutathione peroxidase are correlated with increases in cell survivability for both peptides (Sagara *et al.*, 1996).

The observed synergistic interaction between E2 and GSH seems to be mediated through an estrogen-receptor independent mechanism. Both the SK-N-SH human neuroblastoma and the HT-22 mouse hippocampal cell lines lack a functional estrogen receptor as determined by nuclear exchange assay (Green *et al.*, 1998). Further, a variety of estratrienes that act only transiently at the estrogen receptor exhibit neuroprotection equivalent to that of E2 (Green *et al.*, 1996; Green *et al.*, 1997a, 1997b; Green *et al.*, 1998). Additionally, we (Green *et al.*, 1997b) and Behl (Behl *et al.*, 1997) have demonstrated that an intact phenolic group is necessary for neuroprotection. The use of ICI as an antiestrogen is further support for this idea. The amount of ICI used, which in 100-fold excess of the E2 concentration satisfies the criteria required for demonstration of competitive inhibition, is at concentrations at which other phenolic A ring containing compounds demonstrate protection in the absence of glutathione (Green *et al.*, 1998).

Given the phenolic nature of estrogens, they could activate the antioxidant response element/electrophilic response element (Montano and Katzenellenbogen, 1997). The antioxidant response element/electrophilic response element has been shown to be activated by phenolic antioxidants to increase phase II enzyme production, which includes GSH *S*-transferase (Jaiswal, 1994). Yet another possibility involves the conjugation of glutathione to estrogens (Jellnick *et al.*, 1967). This action proceeds via glutathione *S*-transferases, where conjugation on the 4 position of the A ring of the steroid molecule provides bulky substituents (Elce and Harris, 1971), which has been shown *in vivo* to increase greatly the antioxidant potential (Miller *et al.*, 1996). Given that 20–30% of the cellular mitochondrial pool resides in the mitochondria in some cell types (Smith *et al.*, 1996), and estrogens stabilize mitochondrial function (Mattson *et al.*, 1997), the estrogen-glutathione synergy could function to protect mitochondria against oxidative damage.

TABLE 1

Effects of E2, a variety of antioxidant treatments, and their combination on the βAP 25-35-induced toxicity in SK-N-SH live cell number after 72 hr of treatment.

	Ascorbic acid (100 μM)	α-Tocopherol (50 μM)	Taurine (5 mM)	Lipoic acid (10 μM)	GSH (3.25 μM)	Oxidized glutathione (1.5 μM)
Control	365 ± 9	641 ± 25	628 ± 13	628 ± 13	657 ± 30	657 ± 30
βAP	118 ± 9[a]	366 ± 24[a]	408 ± 10[a]	408 ± 10[a]	331 ± 17[a]	331 ± 17[a]
βAP + E2	144 ± 9[a]	407 ± 21[a]	394 ± 3[a]	393 ± 3[a]	357 ± 19[a]	357 ± 19[a]
βAP + Treatment	124 ± 6[a]	448 ± 12[a]	402 ± 9[a]	406 ± 7[a]	369 ± 7[a]	350 ± 29[a]
βAP + E2 + treatment	188 ± 11[a]	456 ± 14[a]	439 ± 11[a]	416 ± 10[a]	534 ± 16[b]	513 ± 7[a,b]

[a] $p < 0.05$ versus vehicle-treated controls.
[b] $p < 0.05$ versus βAP-treated controls.

Lipophilic estrogens that partition to the plasma membrane should associate their phenolic A rings with the charged hydrophilic head groups of the membrane phospholipids. Conjugation of estradiol with bovine serum albumin at the 17- (Green *et al.*, 1997b) or 6- carbon positions (Green *et al.*, 1998), which prevents the appropriate orientation of the molecule into the plasma membrane, blocks the neuroprotective action of estradiol. Based upon the observation that βAP aggregates extracellularly and causes membrane lipid peroxidation (Behl *et al.*, 1995; Goodman *et al.*, 1996; Gridley *et al.*, 1997), we predict the hydroxyl hydrogen of estradiol is donated to prevent the cascade of membrane lipid peroxidation. Additionally, the enhanced potency of estrogens may result from its ability to donate hydrogen ions from several positions on the A ring (Jellnick and Bradlow, 1990). A relatively stable oxidized form of estradiol could result from this hydrogen ion donation and glutathione peroxidase could regenerate the reduced form of estrogen. This would operate by using GSH as a substrate for donation of the hydrogen group back to estrogen, and thus explain the synergy between the two molecules.

The specificity of estrogen's interaction for this glutathione system is supported by two lines of evidence. First, there are no apparent interactions noted between estrogen and the other thiols tested, lipoic acid or taurine, or any other antioxidants, including ascorbic acid or α-tocopherol. It is interesting to note that although α-tocopherol is a powerful antioxidant in its own right, estrogen has been argued to be even more powerful. Sugioka *et al.* (Sugioka *et al.*, 1987) postulate that this may be because of the ability of the tocopheroxyl radical to regenerate estrogen. We have not observed any such apparent interactions in our system. Second, the ability of oxidized glutathione to work in this system supports the argument that estrogens may be interacting with the glutathione peroxidase/reductase process. *In vivo* evidence supports this idea, in that oral contraceptive use has been correlated with an increase in glutathione peroxidase activity (Capel *et al.*, 1981; Massafra *et al.*, 1993).

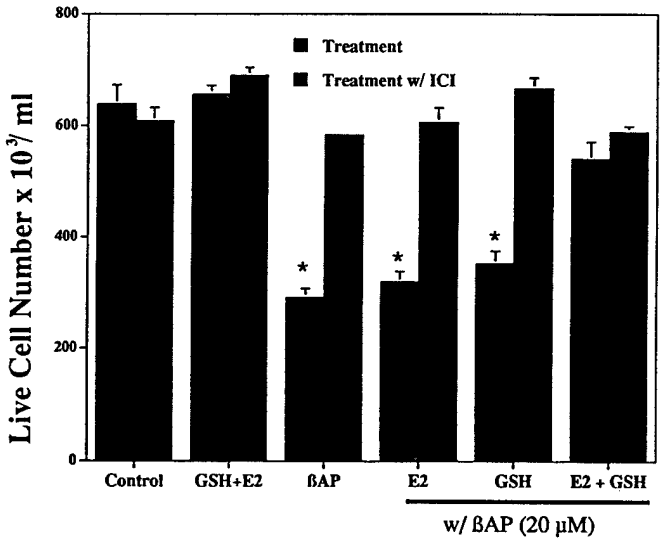


Fig. 5. Effects of the antiestrogen ICI (200 nM) on 20 μM βAP 25–35-induced toxicity in SK-N-SH cells using doses of E2 (2 nM) and GSH (3.25 μM), which alone are not protective but will exhibit protection when used in combination. Cells were plated at 10^6 cells/ml and were exposed to vehicle, βAP 25–35, E2, GSH, or a combination as indicated for 72 hr in the presence and absence of ICI. Depicted are the mean values ± standard error for three wells/group. *, $p < 0.05$ versus controls determined by ANOVA followed by Scheffé's *post hoc* test.

Finally, the identification of this synergistic interaction between estrogens and glutathione in neuroprotection has implications for the area of drug development for neurodegenerative diseases. Our observation that the potency of E2 was markedly affected by physiological concentrations of GSH in the cell culture milieu indicates that careful consideration for antioxidant defenses must accompany experimental design when assessing antioxidant drugs *in vitro*. Additionally, our data indicate that estrogen therapy can be improved by agents that enhance intracellular or extracellular glutathione concentrations.

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A system for the brain-enhanced delivery of estradiol: An assessment of its potential for the treatment of Alzheimer's disease and stroke

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1. Introduction
2. Chemical delivery system for 17β-estradiol
3. Effects of estrogens on cholinergic neurons following fimbria-fornix lesion
4. Effects of estrogens on focal ischemic lesion following middle cerebral artery occlusion
5. Effects of estrogen on the cerebral vasculature
6. Mechanism of the neuroprotective effects of estrogens
7. Conclusions

1. Introduction

One of the goals of efficient drug delivery to the central nervous system (CNS) is to deliver therapeutic levels of the active drug to a specific site while reducing unwanted peripheral side effects. This presents itself to be a formidable task for the routine delivery of pharmaceutical agents such as steroids, which may cause toxicity and potential adverse effects in the periphery. Estrogens equilibrate among all body tissues due to their high lipophilicity [1]. Moreover, when inside the CNS, there is no mechanism to prevent their redistribution back to the periphery as blood levels of the steroid decline [2, 3]. As a result, only a fraction of the administered estrogen dose accumulates at or near the site of action in the brain. These properties of the estrogens necessitate either frequent dosing or the administration of a depot form of the estrogens in order to maintain therapeutically effective concentrations in the brain [4].

We have previously described a novel chemical delivery system (CDS) for improving drug delivery through the blood-brain barrier (BBB) [5, 6]. The CDS is designed to increase the therapeutic index of the drug by increasing the concentration and/or residence time of the agent at its receptor and decreasing the concentrations of the drug at the peripheral sites. Furthermore, the CDS offers slow and sustained release of the drug when incorporated into the brain, thus offering a potential application to a variety of neurodegenerative diseases, which require chronic drug treatment. Given the evidence for a neuroprotective role of estrogens in a variety of *in vitro* studies [7], we investigated the effects of pre-treatment with 17β-estradiol (β-E_2) or the CDS for β-E_2 (E_2-CDS) on the vulnerability of cholinergic neurons following fimbria-fornix lesion, an animal model of neurodegeneration in the basal forebrain, and on the extent of ischemic lesion following middle cerebral artery (MCA) occlusion, an animal model of focal ischemia. Here we review the evidence for and against the use of enhanced brain delivery of estradiol for the treatment of Alzheimer's disease and stroke.

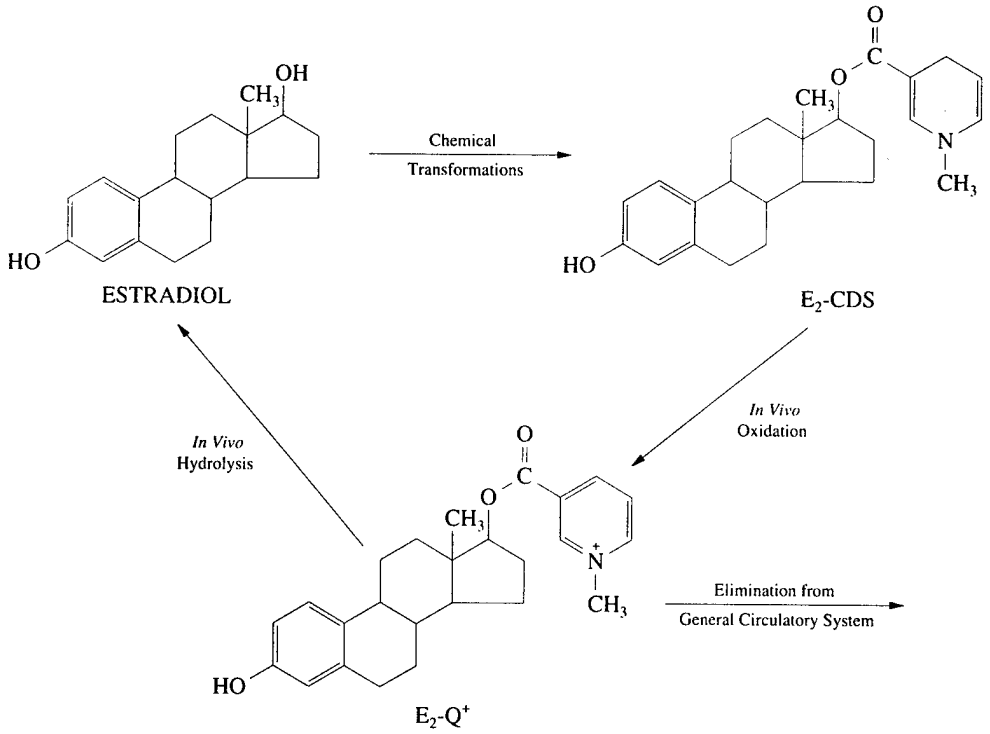
2. Chemical delivery system for 17β-estradiol

The CDS is an application of a dihydropyridine-pyridinium salt redox system to the delivery of drug specifically to the brain [8, 9]. The lipoidal dihydropyridine moiety is attached to the drug, thus increasing its lipid solubility and thereby enhancing its permeability through the BBB. The reduced dihydropyridine can be oxidized to the pyridinium ion in the brain parenchyma as well as in the systemic circulation. The charged pyridinium-drug complex is thus locked into the brain while the peripheral ionized pyridinium-drug complex can be cleared by renal or biliary processes due to its increased hydrophilicity. Sustained release of the active drug from the charged pyridinium-drug complex occurs in the brain as a result of the enzymatic hydrolysis of the ester linkage between the drug and the pyridinium moiety.

E_2-CDS (3-hydroxy-17β-{[(1-methyl-1,4-dihydropyridine-3-yl)carbonyl]oxy}estra-1,3,5-(10)-triene and the charged quaternary ion E_2-Q^+ (1-methyl-3-{[(3-hydroxyestra-1,3,5,-(10)-triene-17β-yl)oxy]carbonyl}pyridinium iodide) were synthesized as previously reported [6]. Briefly, the 3,17β-dinicotinate ester of β-E_2 was made by refluxing β-E_2 with nicotinoyl chloride or nicotinic anhydride in pyridine. This derivative was selectively hydrolyzed to the 17-monoester of β-E_2 with potassium bicarbonate in 95% methanol. The monoester of β-E_2 was then quaternized with methyl iodide. The delivery system, E_2-CDS, was then prepared by reduction of the obtained E_2-Q^+ with $Na_2S_2O_4$. The structure of each intermediate and the final product (E_2-CDS) was confirmed by NMR and elemental analysis. The yields at each step were 64–94%.

The brain-enhanced delivery of estradiol with E_2-CDS requires a series of chemical processes (Scheme), including oxidation of the dihydropyridine carrier of E_2-CDS to the corresponding quaternary pyridinium salt (E_2-Q^+), which provides the basis of locking the molecule in the brain, hydrolysis of the E_2-Q^+ by non-specific esterases at the C-17 position and the release of estradiol.

We conducted a series of studies to investigate the tissue distributions of both E_2-Q^+ and β-E_2 in intact male [10] as well as in ovariectomized female rats [11]. The E_2-CDS has demonstrated its predictive pharmacokinetic behavior, that is, the preferential retention of E_2-Q^+ and β-E_2 in the brain, with an apparent half-life of 8–9 days, with simultaneous elimination of these metabolites from the periphery. Regardless of the tissue evaluated, the levels of E_2-Q^+, the "locked-in" form of the E_2-CDS, were many fold higher than β-E_2 levels at each time point in a particular tissue, indicating a slow rate of hydrolysis of E_2-Q^+ to β-



Schematic representation of the *in vitro* synthesis and *in vivo* distribution of the chemical delivery system for 17β-estradiol (E_2-CDS). E_2-Q^+, the quaternary form of the E_2-CDS, is locked into the brain and quickly eliminated from the peripheral tissues. Hydrolysis of this charged, depot form of the delivery system results in the sustained release of estradial in the brain. (Reprinted with permission)

E_2. For instance, E_2-Q^+ reached a 52-fold higher concentration in the brain than plasma by 1 day after treatment with E_2-CDS and these brain-blood ratios increased to 170-fold by 14 days [10]. The increased brain/plasma ratios of E_2-Q^+, as well as β-E_2, confirmed that locking of the charged moiety, E_2-Q^+, into the brain had occurred, and E_2-Q^+ is retained in brain tissue but is rapidly cleared from peripheral tissues, an observation which is predicted by the properties of the CDS.

To demonstrate the preferential deposition and retention of estrogen in the CNS with the E_2-CDS, one dose of β-E_2 (equimolar to 1.0 mg E_2-CDS dose) was studied [11]. Estradiol concentrations in the CNS tissues of rats treated with β-E_2 were slightly increased on day 1 and were just above the detection limits of the assay at 7 days. In contrast, the 1.0 mg E_2-CDS dose resulted in brain estradiol concentrations that were 81- and 182-fold greater than those achieved following β-E_2 injection at 1 and 7 days, respectively. This demonstrates that E_2-CDS is much more effective than β-E_2 itself in delivering and retaining the estrogen in the brain.

The application of the CDS to estrogens are of particular interest since these hormones have broad therapeutic applications, and in most cases, the steroids are used primarily for their central actions [12]. Among these actions are the reproductive-related applications, including fertility regulation, sexual dysfunction, and the replacement therapy in postmenopausal patients, and the non-reproductive applications, including treatment of postmenopausal depression and cancer therapy. Our previous studies with the E_2-CDS indicate that after a single dose, prolonged pharmacological effects are observed. Specifically, in rats, E_2-CDS causes a dose- and time-dependent suppression of gonadotropin (luteinizing hormone and follicle-stimulating hormone) secretion following ovariectomy [13], long-term suppression of luteinizing hormone following castration [14], suppression of testosterone secretion for 2–3 weeks [15], stimulation of masculine sexual behavior in castrated males for 28 days [16], reduction in weight of androgen-responsive tissue [17], and body-weight suppression for 36 days in males [18] following a single i.v. administration of the E_2-CDS.

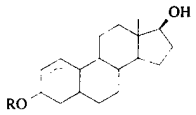
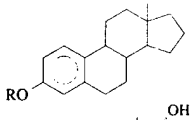
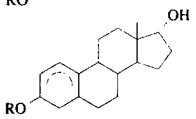
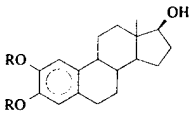
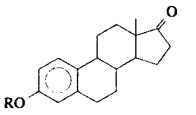
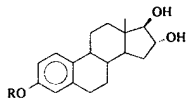
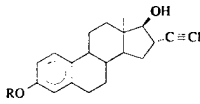
3. Effects of estrogens on cholinergic neurons following fimbria-fornix lesion

In rats, cholinergic neurons respond to peripheral administration of estrogen by increasing the activity of choline acetyltransferase (ChAT) [19], acetylcholine synthesis [20] and high affinity choline uptake [20]. Estrogens may, therefore, serve a modulatory role in the function of cholinergic neurons and thereby improve memory and cognitive performance in demented subjects. Indeed, it has been known since 1954 [21] that estrogen replacement therapy improves cognitive function in demented women. However, tests of the ability of estrogen replacement to specifically protect basal forebrain cholinergic neurons had not been done previously. Therefore, we evaluated the effects of estrogens on medial septal cholinergic neurons of the basal forebrain, a neuronal system known tobe dysfunctional in Alzheimer's disease.

The majority of cholinergic neurons that project to the hippocampus lie within the medial septal nucleus (MSN) and travel by the fimbria-fornix pathway. A lesion of this pathway causes a loss of MSN neurons labeled for ChAT [22, 23] and acetylcholine esterase [24]. Moreover, a fimbrial lesion causes degeneration of MSN neurons, creating a useful modul for studying disorders which destroy this cholinergic pathway, such as Alzheimer's disease.

We investigated the neuroprotective effects of β-E_2 on the cholinergic neurons of the MSN following partial unilateral fimbria-fornix lesion [25]. Adult female rats under-

Table: Phenolic A ring requirement for the neuroprotectivity of estratrienes

	Structure		Name	% of 17β-Estradiol Neuroprotection
1		R=H	3,17β-Estradiol	100[a]
		$R=CH_3$	3,17β-Estradiol 3-O-ME	−1.5
2		R=H	Estratriene-3-ol	103[a,c]
3		R=H	3,17α-Estradiol	81[a]
		$R=CH_3CO$	3,17α-Estradiol 3-acetate	−20
4		R=H	2-Hydroxy-17β-estradiol	70[a]
		$R=CH_3$	17β-Estradiol 2,3-O-ME	7
5		R=H	Estrone	58[a]
		$R=CH_3$	Estrone 3-O-ME	−11
6		R=H	Estriol	46[a]
		$R=CH_3$	Estriol 3-O-ME	2
7		R=H	Ethinyl Estradiol	41[a]
		$R=CH_3$	Mestranol	−6

Statistical analysis was performed on raw data. Data were then normalized using the following: [(Test group − Serum-free group)/(β-E_2 group − Serum-free group)] X 100
[a] $p < 0.05$ versus serum-free control groups
[b] trivial name is indicated here
[c] No 3-O-conjugates were available for evaluation

went bilateral ovariectomy using a dorsal approach and, 5 days later, treated with a single i.v. injection of E_2-CDS or the vehicle 2-hydroxypropyl-β-cyclodextrin (HPCD). All rats were then subjected to partial unilateral electrolytic fimbrial lesion the following day. In brief, a concentric bipolar electrode was lowered 4.5 mm from the dura and a current of 750 mA was passed through the electrode for 30 s to make the fimbrial lesion. At 20 days postlesion, brain slices from treated animals were assessed for ChAT immunoreactivity. ChAT-positive cells on the lesioned and nonlesioned sides of the MSN were manually counted from microscope images of the mounted brain sections.

Animals treated with HPCD showed a 44% decrease in ChAT immunoreactivity on the lesioned side compared to the nonlesioned side of the MSN at 21 days following fimbrial lesion. By contrast, rats treated with E_2-CDS had a 4% decrease in ChAT-positive neurons on the lesioned side when compared to the nonlesioned side.

In a second study using the same lesioning procedure, adult ovariectomized rats received either a s.c. β-E_2 pellet implant that produced physiological concentrations of estradiol, or, 5 days postovariectomy, a single i.v. injection of E_2-CDS or HPCD. At twenty days following fimbrial lesion, ChAT-immunoreactive neurons were determined on both the lesioned and nonlesioned sides of the MSN. Images were viewed using a microscope as in the first study; however, computer-assisted cell counts were conducted instead of a manual counting method.

Animals treated with HPCD showed a 55% decrease in ChAT immunoreactivity on the lesioned side compared to the nonlesioned side of the MSN at 21 days following fimbrial lesion. By contrast, rats treated with E_2-CDS or β-E_2 pellet had a 14 or 13% decrease, respectively, in ChAT-positive neurons on the lesioned side when compared to the nonlesioned side. The lesion-induced decrease in neuronal number for all groups is shown in Fig. 1.

Our study suggests that cholinergic neurons in the MSN are protected from lesion-induced degeneration by treatments, which increase brain β-E_2 levels. Using two different means of achieving enhanced brain concentrations of 17β-estradiol, we observed an attenuation of the loss of ChAT-positive MSN cells which occurred in β-E_2-deprived animals following lesion of the fimbria-fornix fiber bundle. To our knowledge, this is the first study to report a protective effect of estrogens on ChAT-immunoreactive neurons following fimbria-fornix lesion. This specific action of estrogens may help explain the observation that estrogen replacement therapy in postmenopausal women reduces the incidence of AD [26]. Thus, β-E_2 may play a neuroprotective role in the basal forebrain cholinergic system.

It is of interest that an equivalent protection of MSN cholinergic neurons are observed with both s.c. implantation of β-E_2 and following E_2-CDS administration. The dose of E_2-CDS used produced sustained increases in brain estradiol concentrations of greater than 1 ng/gm brain tissue [11], while the s.c. implant increased brain and plasma estradiol concentrations to 50 pg/ml [27]. The efficacy of the s.c. implant indicates that even low levels of exposure of the brain to estradiol can provide substantial protection

to basal forebrain cholinergic neurons. By inference, then, the large increases in brain estradiol observed by administration of E_2-CDS are not needed to protect basal forebrain cholinergic neurons.

4. Effects of estrogens on focal ischemic lesion following middle cerebral artery occlusion

Stroke is the second and fourth leading cause of death for women and men, respectively [28]. During stroke, depletion of cellular glucose and oxygen causes a rapid drop in membrane potential to almost 0 mV. Following membrane depolarization, the levels of extracellular excitatory amino acids increase to toxic concentrations, causing calcium influx, free radical formation and lactacidosis, leading to cellular damage [29–31]. The neuronal excitotoxic effect of certain amino acids has been of great interest, especially in ischemia models. An increased presynaptic release of excitatory neurotransmitters, especially glutamate, may induce injury of pyramidal neurons through an influx of electrolytes, such as chloride and calcium, which ultimately leads to destruction of postsynaptic neurons [32].

Both retrospective and prospective epidemiological studies have demonstrated the beneficial effects of estrogen as well as estrogen-progestin hormone replacement therapy in reducing the mortality that is associated with myocardial infarction and stroke in postmenopausal women [33–36]. Estrogen replacement therapy may also play a role in increasing the overall quality of life for postmenopausal women. On the negative sude, however, some studies show a duration- and dose-related risk of breast cancer with estrogen use and a significant increase in endometrial cancer; the latter is virtually eliminated with the addition of a progestin to the regimen. A woman's potential risks associated with estrogen replacement therapy must be weighed against her lifetime risks of developing cerebrovascular disease and stroke [33].

In animal studies, we and others have demonstrated that estrogens exert protective effects against events associated with ischemia. In particlar, our laboratory has demonstrated the importance of circulating estrogens in attenuating ischemic damage. Estrogen concentrations were inversely related to ischemic area caused by MCA occlusion [37]. Low circulating estradiol concentrations in ovariectomized rats were associated with the largest lesion area; average β-E_2 concentrations in intact female rats [38] with an intermediate lesion area; and elevated β-E_2 concentrations in β-E_2 replacement-treated rats with the smallest lesion area. As such, plasma β-E_2 concentrations may be a predictor of the extent of brain damage associated with MCA occlusion.

The treatment of various forms of estrogens at a variety of time points exerts neuroprotective effects against the damage caused by MCA occlusion in the female rat. Twenty-four hour pretreatment with E_2-CDS or β-E_2 caused decreases in mortality from 65% in ovariectomized rats to 16% in E_2-CDS-treated rats and 22% in β-E_2-treated rats. This reduction in the ischemic area of the brain observed in the E_2-CDS-treated rats and β-E_2-implanted rats (Fig. 2) may underlie a reduction in mortality. Twenty-four hour pretreatment with β-E_2 in ovariectomized rats consistently caused a reduction in ischemic area from 24.8% to 11.8%. Similarly, when administered 40 or 90 min after MCA occlusion, E_2-CDS continued to cause a reduction in ischemic area [39].

Low levels of circulating β-E_2 are sufficient to achieve maximal neuroprotection afforded by estrogens. This is evident by our observation that s.c. implantation of β-E_2 using a sustained release formulation, which produces circulating levels of estradiol of about 54 pg/ml, provides

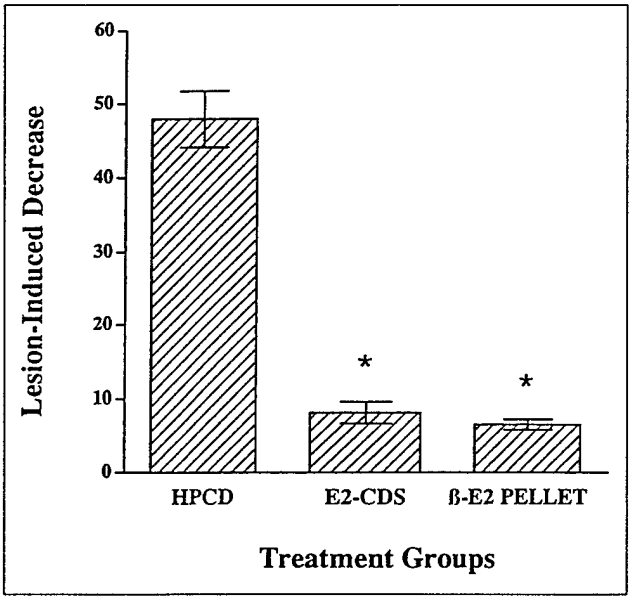


Fig. 1: Effects of pretreatment for 24 h with a hydroxypropyl-β-cyclodextrin (HPCD) or a chemical delivery system for 17β-estradiol (E_2-CDS) injection, or 6 days with a 17β-estradiol s.c. implant (β-E_2 Pellet) on the difference of choline acetyltransferase (ChAT)-positive neurons between the nonlesioned and lesioned sides of the medial septal nucleus (MSN) induced by partial unilateral fimbria-fornix lesion. Average difference ± SEM was obtained by counting the lesioned and nonlesioned sides of approximately five to six slices from sections containing the MSN. The difference is shown for all groups (n = 6 rats for HPCD and β-E_2 Pellet; n = 8 rats for E_2-CDS). *$p < 0.001$ versus HPCD control

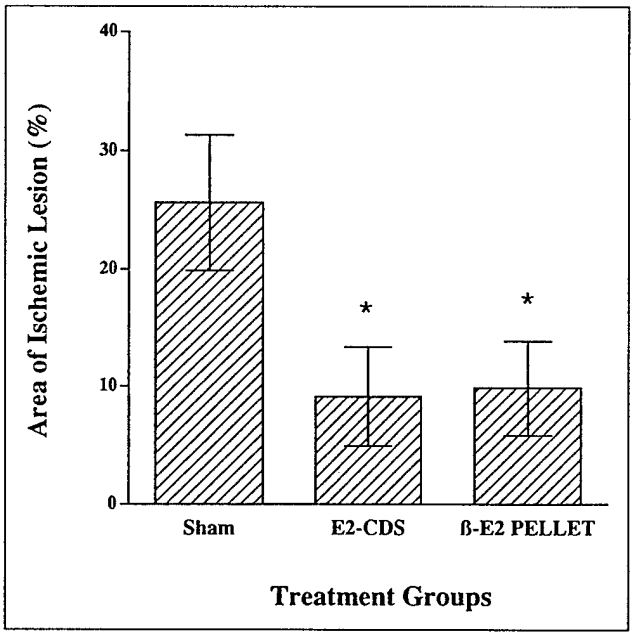


Fig. 2: Effects of pretreatment for 24 h with a Sham pellet (or injection), a chemical delivery system for 17β-estradiol (E_2-CDS), or a 17β-estradiol s.c. implant (β-E_2 Pellet) on the area of ischemic damage induced by middle cerebral artery occlusion. Values represent the mean ± SEM for the percentage of cross-sectional area occupied by the ischemic lesion in three brain slices (n = 6 rats for Sham, n = 8 rats for β-E_2 Pellet and E_2-CDS). *$p < 0.05$ versus Sham control

neuroprotection identical to E_2-CDS administration, which provides brain concentrations of the steroid of greater than 1,000 pg/ml. Indeed, a recent report indicates that near maximal neuroprotection can be achieved by a s.c. implant which increases serum estradiol concentrations to only 20 pg/ml [40]. Collectively, these data suggest that low levels of circulating estrogens are sufficient to achieve maximal neuroprotection against the ischemic damage of stroke.

5. Effects of estrogens on the cerebral vasculature

The mechanism of estrogen's protective effects against stroke is not clear, although several hypotheses are postulated. Estrogens may act indirectly on the vascular system to reduce platelet aggregation, to reduce the thrombotic and vasoconstrictive effects of thromboxane, to modulate serum lipid levels [41–43] or to reduce post-ischemic hyperemia [44]. Besides its well-documented systemic vasoactivity, estrogen may cause an effect on cerebral blood flow (CBF). In an incomplete global ischemic rabbit model, Hurn et al. investigated the effect of chronic pharmacological doses of β-E_2 on CBF by the radiolabeled microsphere technique [44]. Baseline regional blood flow was not changed by exogenous β-E_2 treatment in any brain region. CBF decreased in all animals during vessel occlusion but to a lesser extent in β-E_2-treated female rabbits when compared to normal female and normal male rabbits. After 10 min of reperfusion, hyperemia was more evident in normal female rabbits than in β-E_2-treated female rabbits. Since postischemic hyperemia may contribute to ischemic damage, the β-E_2 protection could have a hemodynamic component.

Estrogens could reduce ischemic damage during stroke by protecting cerebral microvessels. To assess this effect of estrogens, we exposed primary rat brain capillary endothelial cells (BCEC), the major component of the BBB, to ischemic and hypoglycemic insults *in vitro*. When incubated with BCEC, β-E_2 reduces cell loss by 35.9%, 28.4% and 23.5% when glucose in culture media was reduced to 50%, 20% and 10% of normal, respectively; and by 28.4% and 18.4% following 1 h or 4 h of anoxia, respectively [45]. These data indicate that exposure of endothelial cells to estrogens helps to maintain their viability during an ischemic episode.

Alternatively, estrogen could reduce ischemic damage by enhancing glucose uptake across the BBB during MCA occlusion [46]. Bishop et al. reported that β-E_2 benzoate enhances brain glucose uptake and transport across the BBB by 40% [47]. Additionally, Shi et al. [45] demonstrated that β-E_2 causes an increase in glucose transporter 1 (GLUT-1) protein and mRNA expression in the BBB, and this effect may contribute to an increase in ^{14}C-2-deoxy-D-glucose uptake with a similar time- and dose-dependent profile and magnitude. During MCA occlusion induced-ischemia, β-E_2 induces an increase in expression of GLUT-1 in the penumbral cortical regions but not in the cortical regions that are exclusively supplied by the MCA and therefore damaged by the occlusion [45].

GLUT-1 facilitation of glucose uptake across the BBB is the first step in brain glucose utilization. The phosphorylation and oxidation of glucose in the mitochondria is the rate-limiting step in the glucose-turnover-to-energy process as long as there is a sufficient glucose and oxygen supply. In the face of ischemic insult, the normal function of mitochondria is compromised and the demand for glucose is increased. Under this condition, GLUT-1 transport of glucose across the BBB becomes the rate-limiting step in glucose utilization. As such, the observed estrogen-induced blunting of the MCA occlusion-related loss of GLUT-1 transporters could provide the penumbral area, the region surrounding the core ischemic area, with glucose and thereby serve to increase neuronal survival following the ischemic insult.

The small magnitude of the effects of β-E_2 on brain microvessel glucose is in contrast to the effect of insulin on glucose transport in peripheral tissues, where increases of 20-fold in glucose transport and in glucose transporter concentrations have been reported [48]. These data support the contention that while in peripheral tissues insulin is required for glucose transport, in the brain, estrogens serve to modify ongoing glucose transport processes. As such, the absence of estrogens does not lead to overt expression of signs of glucose starvation, but rather to symptoms of neuroglucopenia, such as compromised cognitive function [49], weakness [50], irritability [51], and hot flushes [51, 52]. Indeed, we have shown that hot flushes in an animal model can be induced by hypoglycemia or by cellular neuroglucopenia and prevented by elevation of glucose [53, 54]. The ability of estrogens to treat the symptoms of menopause may in part be related to the observed increase in BBB extraction of glucose.

6. Mechanism of the neuroprotective effects of estrogens

Our laboratory first demonstrated that physiological doses of the potent estrogen, β-E_2, exert direct cytoprotective effects on neurons using a human neuroblastoma cell line, SK-N-SH, under the conditions of serum deprivation [55]. This is a cytoprotective effect of β-E_2 rather than a mitogenic effect as treatment with β-E_2 does not increase ^{3}H-thymidine uptake in these cells [55]. Several lines of evidence suggest that these neuroprotective effects of β-E_2 are not a result of the classical estrogen receptor (ER)-mediated mechanism.

First, tamoxifen, a mixed ER agonist/antagonist, does not block the neuroprotective effect of β-E_2 in serum-deprived SK-N-SH neuroblastoma cells [56]. Exposure to 2 nM β-E_2 during 48 h of serum deprivation increases live cell number by 2–3 fold over vehicle controls, and concurrent treatment with an one hundred fold excess of tamoxifen does not significantly alter the degree of protection conferred by β-E_2. Tamoxifen alone has no effect on cell viability in this assay system. This indicates that antagonism of the ER in this model of cell death does not antagonize the protection conferred by β-E_2.

Second, the structure-activity relationship for the neuroprotective effects of estrogens is different from the structure-activity relationship for binding to the ER. We have demonstrated that 17α-estradiol (α-E_2) has neuroprotective efficacy and potency similar to β-E_2, the potent, naturally occurring estrogen [56], although α-E_2 binds only weakly to the ER and the α-E_2-ER complex binds only transiently to the estrogen responsive element [57, 58]. Both we [59, 60] and Behl et al. [61] have demonstrated that estrogens with a hydroxyl group in the C3 position of the A ring are neuroprotective (Table). If the phenolic nature of the A ring is removed by a 3-O-conjugation, then the neuroprotective effects are abolished. The necessity of the phenolic A ring is demonstrated by the diphenolic estrogen mimic, diethylstilbesterol (DES). DES is neuroprotective, and retention of a single hydroxyl function on an aromatic ring is sufficient to retain neuroprotective activity [59, 60]. The

di-O-methyl ether of DES does not demonstrate protective activity. Furthermore, steroids that lack a phenolic A ring such as testosterone, progesterone, and cholesterol, do not demonstrate protective effects. This suggests that it is the possession of a phenolic A ring and not binding to the ER that confers neuroprotective potential.

Finally, estrogens have been shown to protect a neuronal cell line that lacks known estrogen receptors [62, 63]. HT-22 cells, derived from a mouse hippocampal cell line, do not demonstrate specific ^{3}H-β-E_2 binding in crude nuclear extracts or whole cell preparations [63]. Further, when HT-22 cells are transfected with an estrogen responsive element-reporter plasmid construct, no increase in reporter plasmid expression is seen with estrogen exposure [62]. High concentrations of estrogens have been shown to protect these neuronal cells from the toxic effects of β-amyloid peptide (β-AP), glutamate, buthionine sulfoximine, and hydrogen peroxide [61–63]. Further, we have shown that a physiologically relevant dose of estrogens (2 nM) can significantly protect HT-22 cells from the toxic effects of β-AP [63]; however, the protection of these cells with low nM doses of estrogens requires the presence of reduced glutathione in the extracellular environment [63], suggesting an antioxidant mechanism in the neuroprotection observed with low concentrations of estrogens.

Phenolic A ring estrogens may exert their neuroprotective actions through an antioxidant mechanism since lipophilic phenols are well known to be antioxidants [64]. Further, Sugioka et al. [65] demonstrated that estrogens, specifically phenolic A ring estrogens, are potent antioxidants. Also, β-E_2 not only protects neurons from oxidative insults such as hydrogen peroxide [62] and β-AP [62, 66, 67] toxicity but prevents the increase in lipid peroxidation that accompanies these toxicities [66, 68]. This is significant because increased lipid peroxidation is associated with a variety of neurodegenerative diseases including stroke-related ischemic/anoxic insults [69, 70] and Alzheimer's disease [71, 72].

Estrogens may exert their neuroprotective effects through modulation of calcium homeostasis in the neurons. It has been documented that loss of calcium homeostasis can lead to cell death [73]. Although the exact mechanism by which estrogens modulate calcium is currently unknown, it is known that estrogen reduces calcium currents in the rat neostriatal neurons [74] and also has calcium channel blocking properties in peripheral tissues [75, 76]. It is plausible that estrogens, by inhibiting the mobilization of calcium, may prevent calcium overload in the cells, thus maintaining calcium homeostasis.

Additionally, estrogens may exert neuroprotective effects by interactions with neurotrophins, their receptors and intracellular signaling pathways. Toran-Allerand et al. demonstrated not only regulation of ER expression by nerve growth factor (NGF) [77], but also estrogen up-regulation of NGF preferring trkA mRNA [77, 78] and differential regulation of mRNA for the low-affinity p75 receptor with estrogen treatment [78]. Further, estrogen increases both brain-derived neutrotrophic factor mRNA levels [79, 80] and NGF mRNA levels [81] in the cortex and hippocampus of adult female rats. Interestingly, β-E_2 can also activate growth factor signaling pathways in neurons; specifically, β-E_2 induces phosphorylation of both extracellular signal-regulated kinase 1 (ERK-1) and extracellular signal-regulated kinase 2 (ERK-2), which are also known as mitogen-activated protein kinases, within 5 min of exposure [82, 83]. This appears to be non-ER-mediated effect of β-E_2 as the membrane impermeable complex of E_2 bound to bovine serum albumin is also effective in inducing ERK phosphorylation [83].

Estrogen's neuroprotective effects may also be mediated via interactions with cyclicAMP (cAMP) response element-binding protein (CREB), a constitutively expressed transcription factor which is activated by phosphorylation. Zhou et al. have recently demonstrated that estrogen treatment of ovariectomized rats rapidly increases CREB phosphorylation in the preoptic area and bed nucleus of the stria terminalis [84]. This may be due to increased cAMP production as β-E_2 has been shown to activate adenylyl cyclase, thereby increasing cAMP levels in neurons [85] and other tissues [86]. β-E_2 may also modulate the level of CREB expression. We reported that hypoglycemic seizure results in a selective reduction of CREB immunoreactivity which is attenuated with β-E_2 treatment [87]. This decline in CREB immunoreactivity does not appear to be due to cell loss as the rats were sacrificed 90 min post-seizure. Interestingly, however, the regions of CREB decline correlate with regions that have previously been shown to have massive cell loss at one week following hypoglycemic seizure [88]. Collectively, these findings suggest that the neuroprotection offered by estrogens may be mediated in part by CREB.

7. Conclusions

Estrogens can be delivered to the brain by peripheral administration of the free steroid or through the CDS method which causes a brain depot for local release of estrogens. Both systems effectively deliver the steroid to the brain and both are associated with maximal protection of brain neurons from the effects of electrolytic lesions or ischemic events in animal models. In view of our observations and those of others that very low levels of circulating estrogens (20 to 50 pg/ml) are associated with maximal neuroprotection, either method of delivery of estrogens to the brain can achieve the desired effects of protection from the neurodegeneration of Alzheimer's disease or ischemic damage associated with stroke.

This surprising observation of the potent neuroprotection with low concentrations of estrogens likely relates to the pleotrophic effects of the steroid, operating through ER-independent mechanisms that include antioxidation, calcium flux modulation, alterations in the response to cytotoxic levels of glutamate and the modification of signal transduction pathways, including CREB, ERK-1 and ERK-2.

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Synthesis and antiviral activity of 7-*O*-(ω-substituted)-alkyl-3-*O*-methylquercetin derivatives

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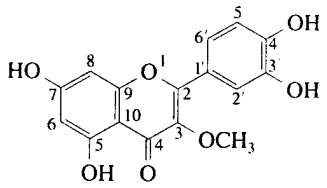
3′,4′-Di-*O*-benzyl-3-*O*-methylquercetin (**2**), the precursor in the synthesis of an important antivirally active flavone 3-*O*-methylquercetin (**1**), was regioselectively alkylated at the 7-OH position by a series of 1,ω-dihaloalkanes and ω-bromoalkanols. Dimerization of the flavone had to be avoided by applying strict reaction conditions. Subsequent debenzylation was carried out by catalytic transfer hydrogenolysis, affording quantitatively the 7-*O*-(ω-haloalkyl)-3-*O*-methylquercetin (**11**–**14**) and 7-*O*-(ω-hydroxyalkyl)-3-*O*-methylquercetin derivatives (**15**, **16**). All compounds were tested for their antiviral activity against poliomyelitis- and HIV-viruses.

Synthese und antivirale Aktivität von 7-*O*-ω-substituierten-alkyl-3-*O*-methylquercetin-Derivaten

3′,4′-Di-*O*-benzyl-3-*O*-methylquercetin (**2**), eine Vorstufe in der Synthese des bedeutenden antiviral aktiven Flavons 3-*O*-methylquercetin (**1**), wurde an der 7-OH-Position regioselektiv durch eine Reihe von 1,ω-Dihaloalkanen und ω-Bromoalkanolen alkyliert. Eine Dimerisierung der Flavone sollte durch die Einhaltung strikter Reaktionsbedingungen vermieden werden. Die anschließende Debenzylierung wurde durch katalytische Transferhydrogenolyse ausgeführt, welche quantitativ zu den 7-*O*-(ω-Haloalkyl)-3-*O*-methylquercetinen **11**–**14** und 7-*O*-(ω-Hydroxyalkyl)-3-*O*-methylquercetin-Derivaten **15**, **16** führte. Alle Verbindungen wurden auf antivirale Aktivität gegen Poliomyelitisviren und HIV geprüft.

1. Introduction

In the last decade, several 3-methoxyflavones isolated from *Euphorbia* sp., were found to be highly active against a wide range of picornaviruses, such as poliomyelitis-, coxsackie- and rhinoviruses [1–7]. In this series, 3-*O*-methylquercetin (**1**; 3-*O*-MQ; 3′,4′,5,7-tetrahydroxy-3-methoxyflavone; 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-1-benzopyran-4-one) was chosen as a natural prototype with promising properties. Modification of the 7-OH group of **1**, which does not seem necessary for the antiviral activity (SAR) [8, 9], enables a further investigation of the antiviral characteristics. In this paper the synthesis of some 7-*O*-(ω-substituted)-alkyl-3-*O*-methylquercetin derivatives and their antiviral activity against poliovirus and HIV are presented.



1

2. Investigations, results and discussion

Recently, an improved synthesis of the anti-picornavirus flavone 3-*O*-methylquercetin (**1**) has been developed in our laboratory [10], as was a general and practical synthesis of 3-methoxyflavones [11]. An important precursor in the synthesis of **1** is 3′,4′-di-*O*-benzyl-3-*O*-methylquercetin (**2**). The presence of two benzyloxygroups at C-3′ and C-4′ is essential for a highly regioselective O-alkylation at the C-7-OH group. The introduction of ω-hydroxy-, ω-bromo- and ω-chloroalkyl substituents is presented in Scheme 1. Relative amounts of the starting materials, reaction times and results of the *O*-alkylation reactions are shown in Table 1. In the alkylation reactions, problems such as regioselectivity, dialkylation, and, in the case of 1,ω-dihaloalkanes, dimerization had to be solved. First the

Table 1: Alkylation reactions

Compd.	Alkylating reagent (equiv.)	K_2CO_3 (equiv.)	Reaction time (h)	Yield (%)	Cryst. solvent	M.p. (C)	DCI MS[a]
3	1.1	85	2.5	85	C_2H_5OH–H_2O	117–118	511
4	10	1.1	52	88	C_2H_5OH	137	603, 605[b]
5	10	1.1	26	93	C_2H_5OH	92–93	659, 661[b]
6	5	1.1	50	96	C_2H_5OH	133–134	559, 561[c]
7	5	1.1	40	89	C_2H_5OH	112–113	587, 589[c]
8	5	1.1	72	92	C_2H_5OH	134–135	541
9	5	1.1	72	97	C_2H_5OH	123	597
17	1.1[d]	40	96	48	$CHCl_3$–C_2H_5OH	152	1019[f]
	1[e]	1.1	116	54	$CHCl_3$–C_2H_5OH	152	1019[f]

[a] m/z of $[MH]^+$. [b] ^{79}Br, ^{81}Br. [c] ^{35}Cl, ^{37}Cl. [d] The alkylating reagent is 1,2-dibromoethane. [e] The alkylating reagent is compound **4** in the presence of 0.7 equivalents of KI. [f] A FAB mass spectrum was recorded on a VG 70-SEQ hybrid mass spectrometer.

Pergamon

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Letter to Neuroscience

NUCLEAR ESTROGEN RECEPTOR-INDEPENDENT NEUROPROTECTION BY ESTRATRIENES: A NOVEL INTERACTION WITH GLUTATHIONE

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Post-menopausal estrogen replacement therapy is associated with a reduction in the risk of Alzheimer's disease and has been reported to improve cognitive functioning in several small clinical trials. The present study evaluates the dependence of estrogenic neuroprotection on the presence of estrogen receptors using the murine neuronal cell line, HT-22, exposed to the neurotoxic β-amyloid peptide. These cells lack functional estrogen receptors. The amyloid peptide killed 50–60% of these cells and concurrent treatment with either of three estratrienes, β-estradiol, α-estradiol, or estratrien-3-ol, resulted in a dose-dependent protection. The potency of this estrogen neuroprotection was dependent on the presence of glutathione in the culture media. The presence of reduced glutathione in the media increases the neuroprotective potency of estrogens by an average of 400-fold. These results demonstrate that a nuclear estrogen receptor is not necessary for the neuroprotective actions of estrogens; however, the presence of an appropriate antioxidant in the extracellular milieu is needed for estratriene neuroprotection at physiologically and pharmacologically relevant doses. These data suggest the possibility of combined estrogen-antioxidant therapy for neurodegenerative diseases such as Alzheimer's disease.
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Recent evidence suggests a role of ovarian steroids in normal maintenance of brain function and suggests that a loss of these hormones at menopause may play a role in the neurodegeneration associated with Alzheimer's disease (AD). Post-menopausal estrogen replacement therapy is associated with a 40% decline in the incidence of Alzheimer's disease[17] and a delay in the onset of the disease.[25] Further, estrogens are potent neuroprotective agents in a variety of *in vivo* model systems including fimbria–fornix lesions[19] and middle cerebral artery occlusion.[21] Additionally, we and others have shown that estrogens protect cultured neurons and neuronal cell lines from a variety of insults including serum-deprivation,[5,8] β-amyloid peptide (βAP) toxicity[2,6,7] and glutamate toxicity.[2,23]

Many effects of estrogens are mediated by the binding of the steroid to its nuclear estrogen receptor (ER) and the potent binding of the steroid–receptor complex to the estrogen responsive element (ERE) thereby activating transcriptional events. Increasingly, effects of estrogens are being described that occur by alternate mechanisms. Estrogens have direct effects on neuronal membranes that have been shown to involve specific interactions with membrane binding sites.[27] Further, estradiol potentiates excitatory postsynaptic potentials in hippocampal CA1 neurons within 2 min of its addition to the slice.[29] Estrogens have also been shown to modulate Ca^{2+} fluxes in multiple subtypes of neurons by a non-genomic mechanism.[13,16]

Several lines of evidence are emerging to suggest that the aforementioned neuroprotective effects of estrogens are not mediated through the classical ER pathway. First, tamoxifen, a mixed ER agonist/antagonist, does not prevent estrogen's protection of serum-deprived SK-N-SH neuroblastoma cells and has no protective ability of its own.[8] Second, the structure–activity relationship for the neuroprotective effects of estrogens differs markedly from their structure–activity relationship for binding to the ER with the result that many classically weak or inactive estrogens are potent neuroprotective agents.[3,8,9,22] Finally, Behl *et al.* have demonstrated estrogen protection against oxidative stress including βAP toxicity in HT-22 cells, a murine hippocampal cell line that lacks ERs.[2] This required a minimum estrogen dose of 10 μM to achieve significant neuroprotection whereas we demonstrate protection

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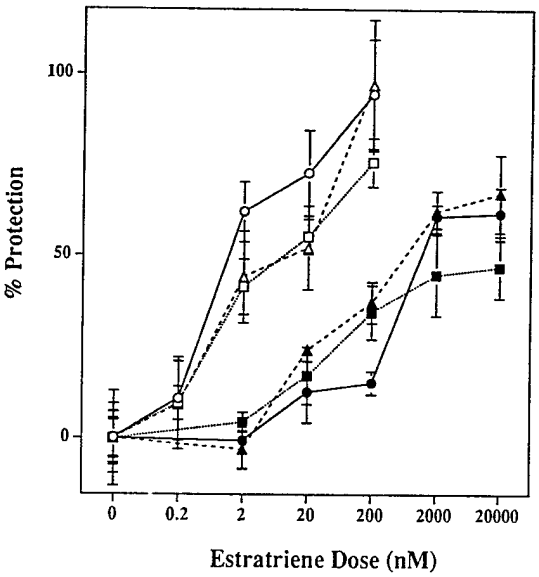
Abbreviations: AD, Alzheimer's disease. βAP, β-amyloid peptide. βE2, 17β-estradiol. BSA, bovine serum albumin. ER, estrogen receptor. αE2, 17α-estradiol. ERE, estrogen responsive element E-3-ol estratriene-3-ol. GSH, reduced glutathione. RPMI-1640, Roswell Park Memorial Institute media 1640.

against βAP toxicity with physiological estrogen doses of 0.2–2 nM.[7] The purpose of the present study is to evaluate the presence of glutathione in the culture media as the reason for the enormous discrepancy in effective protective doses of estrogens and to determine if physiological doses of estrogens could protect neuronal cells in the absence of an ER.

We present here a synergistic interaction between neuroprotective estrogens and the protection conferred by the intracellular antioxidant reduced glutathione (GSH). 17β-estradiol (βE2) the potent naturally occurring estrogen, protected HT-22 cells from cell death induced by the neurotoxic amyloid fragment, βAP (25–35) fragment with an ED_{50} of 5 nM and significant neuroprotection with 2 nM of the steroid (Fig. 1). However, this potency was dependent on the presence of 3.25 μM GSH in the culture media as the βE2 potency was reduced 628-fold when GSH was absent from the media. This estradiol–GSH interaction was also observed using βAP (1–40), one of the forms of amyloid found in AD plaques; a 200-nM βE2 dose offered 99.9% and 35.6% protection in the presence and absence of GSH, respectively (Table 1). This GSH effect may explain the discrepancy in protective βE2 doses seen in the literature. Goodman *et al.*[6] and Behl *et al.*[2,3] using culture medias lacking GSH, showed that micromolar doses of estradiol were required to protect primary neurons and HT-22 cells. The dose of GSH used in our studies is comparable to the low micromolar GSH concentrations found in the cerebrospinal fluid[1] suggesting that such an interaction may occur *in vivo*. This is further supported by the fact that significant neuroprotection is conferred by low nanomolar doses of estrogens in rodent models.[19,21]

We[9,21] and Behl *et al.*[2] have demonstrated that the neuroprotection by estrogens is dependent on the phenolic nature of the steroid A ring and not on the estrogenic potency. Therefore we also evaluated the effect of GSH on the protection conferred by two classically inactive estrogens, αE2 and E-3-ol. Both of these estrogens behaved similarly to βE2 with 17α-estradiol and E-3-ol protecting neurons with an ED_{50} of 6 nM and 14 nM, respectively, in the presence of GSH and an ED_{50} of 1014 nM and 3683 nM, respectively, in the absence of GSH (Fig. 1). Phenolic A ring estrogens are most likely exerting their effects by an antioxidant mechanism as the compounds have been shown to be potent inhibitors of oxidation *in vitro*.[24] The interaction between these estrogens and GSH could be due to redox cycling between endogenous antioxidants. Such interactions are known to occur between other antioxidants such as ascorbate and GSH[28] as well as ascorbate and α-tocopherol.[20]

We tested estradiol conjugated to BSA to determine if estradiol could protect HT-22 cells from βAP if the estrogen was restricted to the extracellular environment. Immobilization of the steroid by BSA conjugation abolished the ability of the steroid to protect as βE2-6-(carboxy-methyl)oxime:BSA containing 20 μM βE2 was unable to protect HT-22 cells from βAP toxicity in the presence of GSH (Table 2). We have previously observed that BSA conjugation at the 17 position of βE2 abolished the neuroprotective ability in a human neuroblastoma cell model (Green and Simpkins, unpublished observations). Collectively, these data suggest that the estratrienes must be to able to interact freely with the cell membrane or the intracellular space to exert their neuroprotective effects.



	ED_{50} Values (nM) ± SEM	
	With GSH	Without GSH
βE2	5 ± 2	3266 ± 408
E-3-ol	13 ± 4	3683 ± 964
αE2	7 ± 2	1014 ± 156

Fig. 1. Effects of estratrienes in the presence and absence of glutathione on the neurotoxicity induced by βAP (25–35) in HT-22 cells. Experiments were initiated by plating cells in Nunc® 24 well plate and cells were allowed to attach for 4 h before treatment. Cells were exposed to 20 μM βAP in the presence of the indicated dose of βE2, αE2, or estratriene-3-ol either in RPMI-1640 media containing 3.25 μM GSH or RPMI media without GSH. After 48 h, cells were suspended and viability determined using the Trypan Blue dye exclusion method.[26] Steroids and βAP were prepared as previously described.[7] ED50 values were log-transformed and the resulting values were analysed by two-way analysis of variance. The glutathione effect was highly significant with F=364 and $P<0.0001$ whereas the effect of estrogen type approached significance (F=3.4, $P<0.06$) and no interaction was found between estrogen type and glutathione interactions (F=2.47, $P<0.12$). Data were normalized to the βAP free control group as 100% protection and the βAP alone group as 0% protection. The mean ± S.E.M. for 3–5 wells per group are presented in the graph and the average ED_{50} values ± S.E.M. are presented in tabular form. The relationships shown were repeated in at least two separate experiments.

Table 1. Effects of 17β-estradiol in the presence and absence of glutathione on the neurotoxicity induced by β-amyloid peptide (1–40) in HT-22 cells

Treatment	Live Cell Number ($\times 10^3$ cells) ± S.E.M.
Control (no βAP)	627 ± 11
βAP (20 μM)	321 ± 12
βAP+200 nM βE2	424 ± 12**
βAP+3.25 μM GSH	312 ± 12
βAP+3.25 μM GSH+200 nM βE2	626 ± 27*

Experiments were carried out as described in Fig. 1 with the exception that βAP (1–40) was allowed to incubate for four days before dilution to 20 μM in the culture media. The aging of βAP is necessary to aggregate the peptide, a necessity for its toxicity.[18] Data are presented as mean ± S.E.M. for 3–4 wells per group. Data were analysed by one-way analysis of variance with $P<0.05$ being considered significant. The data are presented as the mean ± S.E.M. for four wells per group. *$P=<0.05$ versus the βAP (1–40) alone group. **$=P<0.05$ versus both the βAP (1–40) alone group and the control (no βAP) group.

Table 2. Effects of 17β-estradiol and 17β-estradiol-6-(carboxy-methyl)oxime:bovine serum albumin conjugate on β-amyloid peptide (25–35) induced toxicity in HT-22 cells

Treatment	Live Cell Number ($\times 10^3$ cells) ± S.E.M.
Control (no βAP)	427 ± 11*
βAP (20 μM)	189 ± 11
βAP+0.2 μM βE2	324 ± 14*
βAP+20 μM βE2:BSA	216 ± 17

Experiments were initiated by plating cells in Nunc® 24 well plate and cells were allowed to attach for 4 h before treatment. Cells were exposed to 20 μM βAP (25–35) in the presence of the indicated dose of βE2 or βE2:BSA in RPMI-1640 media containing 3.25 μM GSH. After 48 h, cells were suspended and viability determined using the Trypan Blue dye exclusion method.[22] Steroids and βAP was prepared as previously described.[7] Data were analysed by one-way analysis of variance and a Scheffe's *post hoc* analysis was used for planned comparisons between groups. Data are presented as mean ± S.E.M. for four wells per group. *$=P<0.05$ versus the βAP (20 μM) group.

The low dose of GSH used in these studies had no effect on βAP-induced toxicity by itself inasmuch as βAP (25–35) caused a 54 ± 4% and a 55 ± 3% decrease in cell viability in the absence and presence of 3.25 μM GSH, respectively. However, a GSH dose of 325 μM showed significant protection from βAP toxicity in another cell type, SK-N-SH neuroblastoma cells (Green, Gridley and Simpkins, unpublished observations). Similarly, Mark *et al.*[12] and Kruman[9] *et al.* protected primary hippocampal neurons and PC12 cells, respectively, from βAP-toxicity using a high dose (1 mM) of a membrane-permeable form of GSH. Further, exposure of HT-22 cells to 3.25 μM GSH alone, 200 nM βE2 alone, or two in combination did not cause an increase in cell number in the absence of an insult (data not shown) indicating that the increase in cell number is due to protection and not to a mitogenic effect of the compounds.

We evaluated HT-22 cells for the presence of specific ^{3}H-estradiol binding in both nuclear extracts and whole cell preparations by methods previously described.[14,15] HT-22 cells did not demonstrate specific binding in either assay with only 6 ± 3.93 fmol specific binding per million cells compared to 56 ± 6.5 for the ER positive MCF-7 cell line in the whole cell preparation and 0.05 fmol per million cells compared to 35.21 for the MCF-7 cells in the crude nuclear pellet. The dose of ^{3}H-estradiol used in these binding studies (2 nM) is sufficient to saturate binding in both ER subtypes[10] indicating that neither ERα nor ERβ is active in these cells. This is consistent with the observation of Behl *et al.*[2] who observed no increase in reporter gene activity with estradiol exposure when HT-22 cells were transfected with an estrogen responsive element–reporter gene construct. The protection conferred by physiological doses of estrogens on these cells confirms that a major portion of the protective effects of estrogens is independent of the ER.

Oxidative damage is a component of a variety of neurodegenerative diseases including AD and age is associated with a decreased antioxidant capacity of the brain.[4] The possibility that either estrogens or antioxidants such as vitamin C or vitamin E can attenuate the disease process is presently being explored in several clinical trials. Our data suggests the possibility that phenolic A ring estrogens could be used for their neuroprotective properties without the estrogenic side effects such as feminization or an increased risk of certain cancers which are ER mediated. Further, these data suggest that a combination of estrogen therapy with antioxidant therapy may prove more effective than either alone.

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Research report

Low concentrations of estradiol reduce β-amyloid (25–35)-induced toxicity, lipid peroxidation and glucose utilization in human SK-N-SH neuroblastoma cells

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Low concentrations of estradiol reduce β-amyloid (25–35)-induced toxicity, lipid peroxidation and glucose utilization in human SK-N-SH neuroblastoma cells

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Abstract

The present studies were undertaken to determine the role of physiologically relevant concentrations of estrogens on amyloid-induced changes in cell viability, metabolic demands, and lipid peroxidation in response to the toxic fragment of β-amyloid (βAP 25–35). To this end, SK-N-SH human neuroblastoma cells were exposed to βAP 25–35 or βAP 25–35 plus 17β-estradiol, and cell viability, media glucose use and lactate production were measured at time points ranging from 3 to 15 h for examination of acute effects, or at 48 and 72 h time points for chronic effects. Addition of βAP 25–35 to SK-N-SH cells decreased the number of viable cells from 5% at 3 h to 35% at 15 h when compared to vehicle controls. Chronic treatment for 48 and 72 h caused decreases in viable cell number of 70% and 65%, respectively. Paradoxically, both glucose utilization and lactate production were found to be increased for the βAP-treated cells. Concomitant estrogen treatment was found to be neuroprotective, as the severity of the insult on cell viability was decreased by 40% at 15 h and up to 71% at 72 h. Likewise, the addition of 17β-estradiol decreased both the glucose use and lactate production of the cells. Chronic treatment with βAP caused increases in lipid peroxidation over vehicle treated controls of 82% and 78% at 48 and 72 h, respectively, while decreases in peroxidation of 48% were seen with simultaneous estrogen treatment. These results indicate that the neuroprotective effects of estrogens against βAP-induced toxicity are due in part to their capability to decrease lipid peroxidation and may additionally be attributable to decreasing the metabolic load of the cell. © 1997 Elsevier Science B.V.

Keywords: β-Amyloid; Estradiol; Lipid peroxidation; Glucose utilization; Lactate production; SK-N-SH neuroblastoma cell

1. Introduction

The care of Alzheimer's Disease (AD) patients costs approximately $58 billion per year in the United States [22], and AD afflicts 5% of the population over age 60 [18]. Age-matched epidemiological studies reveal that women have an incidence of AD two to three times higher than men [1]. Retrospective studies suggest that estrogen replacement therapy (ERT) reduces the incidence of AD and this effect of estrogen is believed to be dose and duration dependent [31]. Recently, Tang et al. [42] have provided evidence that estrogen users demonstrate a lower incidence and delayed onset of symptoms of AD. Since an effective treatment for AD is currently not available, our understanding of the mechanism by which ERT alters the course of AD has profound implications.

One distinguishing feature in AD brains is the presence of senile plaques, the constitutive component being identified by Glenner et al. [12] as β-amyloid peptide (βAP). The genetic mutations reported to occur with some forms of familial AD have been linked with an overproduction of βAP from amyloid precursor protein (APP) [35], with an eleven amino acid fragment identified as the toxic portion of the molecule [43,44]. Another consistently observed feature in AD patients is an asymmetric decline in brain glucose metabolism [17] which has been noted early in the course of disease [17,19,33,39]. The hypometabolism associated with these brain regions may account for the non-memory language and visuospatial function impairments seen in AD [17]. The idea that glucose utilization may play a role in memory processes has merit; retention of passive avoidance tasks and operant conditioning paradigms in rats

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is facilitated by post-training glucose administration [13,25]. Moreover, Craft et al. [8] have provided evidence that regulation of glucose may be a factor in AD pathology as blood glucose levels in AD patients were shown to be elevated overall when compared to normal subjects. This is in contrast to an increased length of elevation of blood glucose correlating to improvement in memory performance in these same patients [8].

Mechanisms of cytotoxicity associated with βAP indicate that aggregation of the peptide [32,44] and contact with the plasma membrane are integral components [21] and that mitochondrial degeneration is an early event [2]. Equivalent cytotoxic concentrations have been shown for both the full length peptide and the neurotoxic fragment in both rat primary neuronal cultures and B12 cell lines [3]. To our knowledge, the role of glucose use in βAP-induced toxicity has not been determined. Behl et al. [3] have demonstrated that hydrogen peroxide and lipid peroxidation mediate βAP toxicity and it has been reported that extremely high doses of the ovarian steroid, 17β-estradiol (E2), can attenuate βAP-mediated toxicity by reducing the oxidative stress imposed by βAP [4,14]. More recently, we have demonstrated that physiological doses of estrogen, and those used in ERT, are protective against βAP 25–35-induced toxicity [16]. Because of the inconsistencies with regard to neuroprotective concentration and duration of treatment in these studies, we hypothesized that there may be a time dependence for the neuroprotective effects of estradiol in our system. The present study was undertaken to determine the effects of low doses of 17β-estradiol on the time course of βAP-induced toxicity, and additionally on cellular metabolic activity and lipid peroxidation.

2. Materials and methods

2.1. Cell culture

SK-N-SH neuroblastoma cells were obtained from American Type Tissue Collection (Rockville, MD). Cell cultures were grown to confluency in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin (all reagents obtained from Sigma Chemical Corporation, St. Louis, MO) in monolayers in plastic Corning 150 cm^2 flasks (Fisher Scientific, Inc. Orlando, FL) at 37°C under 5% CO_2 and 95% air. Media were changed three times weekly. Cells were observed with a phase contrast microscope (Nikon Diaphot-300).

Study 1 was designed to assess the acute effects of βAP, E2, and their combination on cell viability and glucose utilization. Experiments were initiated by lifting cells with 0.02% EDTA and plating at a density of 1×10^6 cells per ml, allowing attachment overnight in RPMI-1640 media. Media were then replaced with appropriate treatment media. Cells were cultured in RPMI-1640 with 10% FBS and absolute ethanol as a vehicle control, or supplemented with the addition of βAP 25–35 (Bachem, Torrance, CA), 17β-estradiol (E2; Steraloids, Wilton, NH), or βAP 25–35 and E2 together. Lyophilized βAP 25–35 (1 mg) was initially dissolved in 200 μl dd H_2O then 800 μl PBS was added. βAP 25–35 was then diluted to a final concentration of 20 μM in RPMI-1640. In our model system, we have shown a LD_{50} of 28.9 μM for the neurotoxic fragment [16], which is similar to the neurotoxic concentrations reported for the full length peptide [44]. E2 was initially dissolved in 1 mg/ml absolute ethanol and diluted in RPMI-1640 to a final concentration of 2 nM. This concentration of E2 exceeds the EC_{50} for protection against toxicity in SK-N-SH cells (Gridley, et al.; unpublished observations). Assessments of trypan blue excluding cells, glucose utilization, and lactate production were made at 3, 6, 9, 12, and 15 h of treatment.

Study 2 was designed to assess the effects of βAP 25–35, E2, and their combination on cell viability, membrane oxidation, and glucose utilization over the course of 2 to 3 days. In study 2, experiments were initiated by lifting cells with 0.02% EDTA as described above. Cells were suspended and then centrifuged at 1000 RPM for 5 min. The cell pellet was resuspended in the appropriate treatment media at a concentration of 1.0×10^6 cells/ml. Determinations were made at 48 and 72 h as indicated.

Study 3 was designed to assess the effects of pre-, co-, or post-estradiol treatment on βAP 25–35-induced toxicity. In study 3, experiments were initiated by lifting cells with 0.02% EDTA as described above. Cells were suspended in media and then centrifuged at 1000 RPM for 5 min. The cell pellet was resuspended and treated with E2, βAP 25–35, vehicle control, or their combination, as indicated for 24 h. After 24 h, initial treatment media were decanted and replaced with appropriate treatment media for a total of 48 h to assess pre-, post- or co-treatment conditions. Pre-treated cells received E2 upon initiation of experiment and βAP 25–35 after 24 h of incubation. Post-treated cells received βAP 25–35 upon initiation and E2 after 24 h of incubation. Co-treated cells received both E2 and βAP 25–35 upon initiation, and E2 after 24 h of incubation. βAP 25–35 treated cells received either vehicle control or βAP 25–35 upon initiation and the other after 24 h of incubation. E2 and vehicle treated controls received the appropriate treatment on both initiation and after 24 h of incubation.

2.2. Cell viability

Cell viability was determined utilizing the trypan blue exclusion method [7]. At the times indicated, treatment media were decanted and cells were lifted by incubating with 0.4 ml 0.02% EDTA for 30 min at 37°C. Cells were suspended by repeated pipetting. One-hundred μl aliquots from each cell suspension were incubated with 100 μl aliquots of 0.4% trypan blue stain (Sigma Chemical Corp.)

for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer within 10 min of addition of trypan blue. Two independent counts of live and dead cells were made for each aliquot.

2.3. Glucose / lactate determinations

Glucose and lactate determinations were assayed using a YSI STAT 3000 glucose analyzer (YSI Inc., Yellow Springs, OH) on initial and decanted treatment media. Utilization was determined per million trypan blue excluding cells.

2.4. Thiobarbituric acid reactive products (TBARs)

At the appropriate times, cell suspensions, as generated for cell viability, were subsequently tested for the production of malondialdehyde (MDA), a by-product of lipid peroxidation which can be assayed using a modified TBAR procedure based on methods used by Subbarao et al. [40] and Ohkawa et al. [30]. Two-hundred μl of cell suspension was added to 300 μl of 0.9% normal saline in glass tubes, and incubated with 0.5 ml 20% glacial acetic acid and 1.0 ml 0.8% thiobarbituric acid for 60 min (lightly stoppered) in a boiling water bath. Upon cooling, tubes were centrifuged for 10 min at 1500 rpm and then 200 μl pipetted into 96 well plates and read on the SLT 3000 tray reader at 530 nm. Thiobarbituric acid (Sigma Chemical Corp.) was initially made at 0.8%, and used in the assay to give a final concentration of 0.4%. 20% Acetic acid (Fisher Scientific) was adjusted to a pH of 3.5 with the addition of NaOH and used at a final concentration of 5%. External standards used in the thiobarbituric acid reactive product (TBAR) procedure were made from 1,1,3,3-tetramethoxypropane (TMP, Sigma Chemical Corp.) in reagent grade ethanol (Fisher Scientific) and diluted in 0.9% normal saline to give concentrations necessary for generation of standard curves.

2.5. Statistics

Statistical analysis was performed between groups at each time point using ANOVA followed by Scheffe's post hoc tests. $P < 0.05$ was considered to be significant. For each study $n = 3$–5 wells per group.

3. Results

3.1. βAP (25–35) and E2 effects: acute toxicity

The addition of βAP 25–35 reduced the number of trypan blue excluding SK-N-SH cells by 5% at 3 h to a maximum of 35% at 15 h when compared to vehicle treated controls (Fig. 1). Simultaneous exposure of cells to βAP and 17β-E2 (2 nM) resulted in a delay in the appearance of βAP-induced toxicity, as there was no change in cell number at 3 h when compared to vehicle-treated controls and only a 3% change at the 6 h time point. Additionally, E2 in combination with βAP 25–35 reduced the severity of the insult as viable cell loss was decreased 40% relative to the βAP 25–35-treated group at 15 h. E2 alone did not affect cell viability over the 15 h time course.

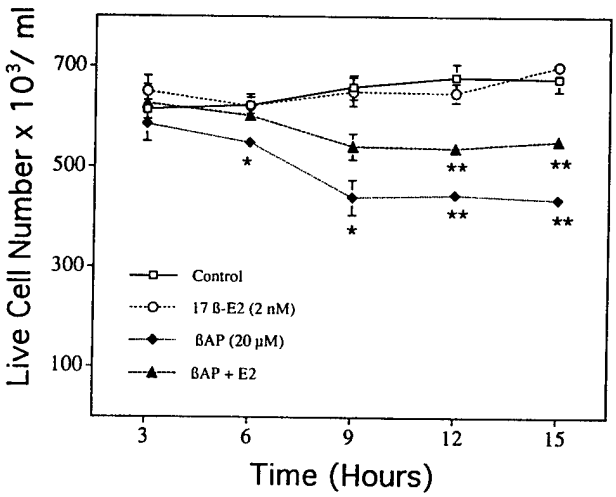


Fig. 1. Acute effects of simultaneous treatment with βAP 25–35 (20 μM; LOT #ZM513), 17β-estradiol (E2), or their combination on the number of live SK-N-SH cells. Data are expressed as mean ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus vehicle treated control groups, ** $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

3.2. Chronic toxicity

Chronic βAP 25–35 and E2 effects, assessed at the 48 and 72 h time points, demonstrated a βAP 25–35 induced cell loss of 70% and 65%, respectively, when compared to the vehicle controls (Fig. 2). Once again, simultaneous E2 exposure with βAP caused decreases in live cell loss of 69 and 71%, respectively, at the 48 and 72 h time points. E2 exposure alone had no significant effect on cell viability over the 15 h time course (Fig. 2).

3.3. Glucose utilization and lactate production

Acute E2 treatment alone had no effect on glucose utilization over the 15 h time course; however, addition of βAP 25–35 increased the amount of glucose used by SK-N-SH cells by 25 and 44% when compared to controls at the 3 and 6 h time points, respectively (Fig. 2). At 9, 12, and 15 h of treatment, glucose use was significantly increased in the βAP treatment group by 100, 72, and 76%, respectively, in comparison to vehicle treated controls (Fig. 2). E2 treatment with βAP 25–35 decreased glucose utilization at all time points, with reductions of 64% at 3 h

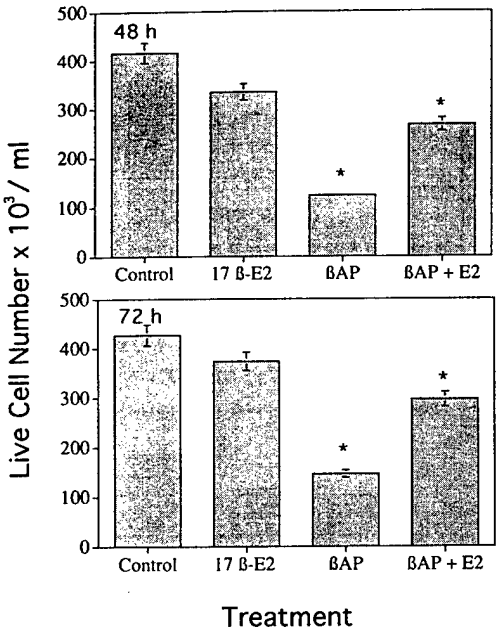


Fig. 2. Chronic effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # WM513), 17β-estradiol (E2), or their combination on the number of live SK-N-SH cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

to 83% at 15 h ($P < 0.05$) (Fig. 3). Acute effects on lactate production were similar (Fig. 4) as βAP 25–35 treatment increased the amount of lactate detected in the media,

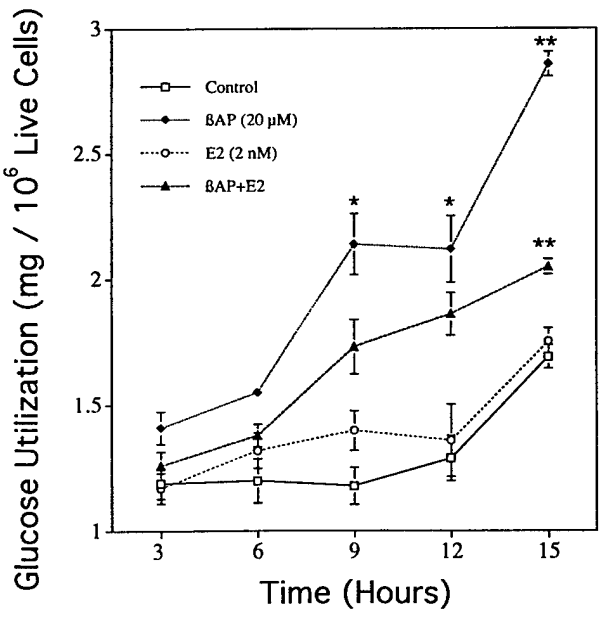


Fig. 3. Acute effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # ZM513), 17β-estradiol (E2), or their combination on glucose utilization. Media glucose was subtracted from initial glucose values and normalized per million cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus control groups, ** $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

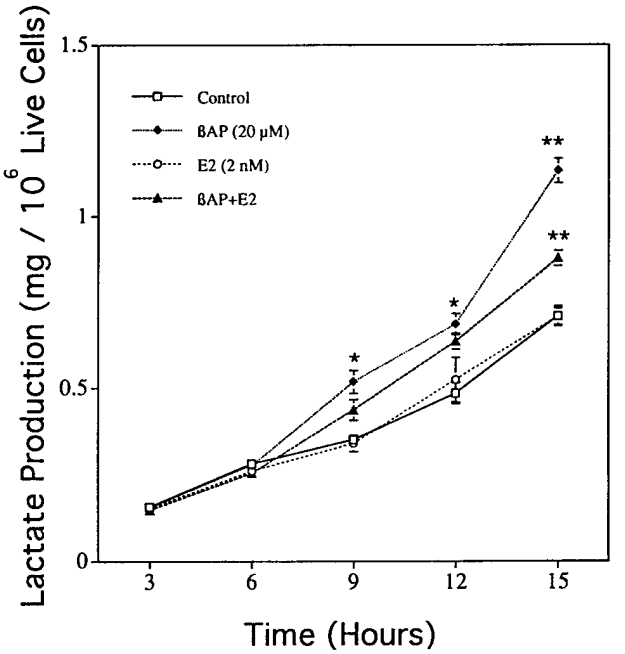


Fig. 4. Acute effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # ZM513), 17β-estradiol (E2), or their combination on the production of lactate SK-N-SH cells. Media lactate was subtracted from initial lactate values and normalized per million cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus control groups, ** $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

contrasted by those treated with βAP 25–35 and E2, where lactate production was decreased by 57% at 15 h.

Chronic treatment with βAP 25–35 resulted in similar changes in glucose utilization (Fig. 5). Increases in glucose utilization of 56% and 65%, were seen in the βAP group at the 48 and 72 h time points, respectively, when compared to vehicle treated controls (Fig. 5). Decreases in glucose use resulting from the addition of E2 with βAP 25–35 were 63% and 67% at the 48 and 72 h time points, respectively (Fig. 5). The chronic effects of βAP and E2 on lactate production were similar to those seen with acute treatment (Fig. 6). No difference was noted between controls and E2 treated cells (Fig. 6), and βAP treatment significantly increased the amount of lactate produced (Fig. 6). Concomitant exposure of E2 with βAP reduced the lactate production (Fig. 6).

3.4. Lipid peroxidation

Chronic exposure of cells to βAP 25–35, E2, and the combination was additionally evaluated for lipid peroxidation as short term decreases in lipid peroxidation have been previously demonstrated when βAP treated cells were pretreated with high doses of estradiol [14,15]. Increases in lipid peroxidation of 82% were observed with

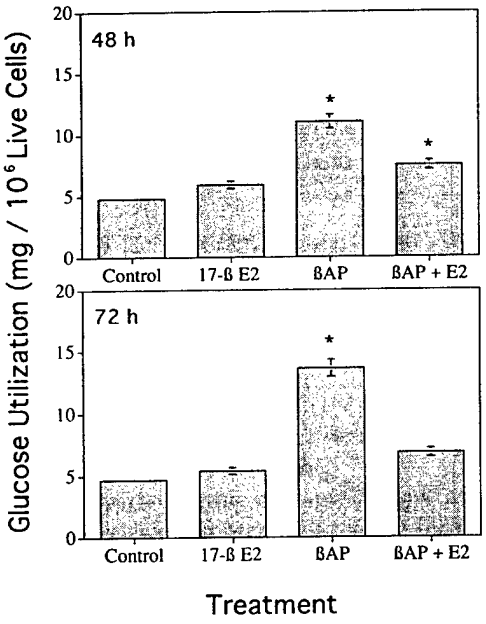


Fig. 5. Chronic effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # WM513), 17β-estradiol (E2), or their combination on glucose utilization. Media glucose was subtracted from initial glucose values and normalized per million cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

48 h of βAP 25–35 treatment when compared to vehicle treated controls (Fig. 7). Simultaneous E2 and βAP 25–35 treatment decreased lipid peroxidation by 48% at the 48 h

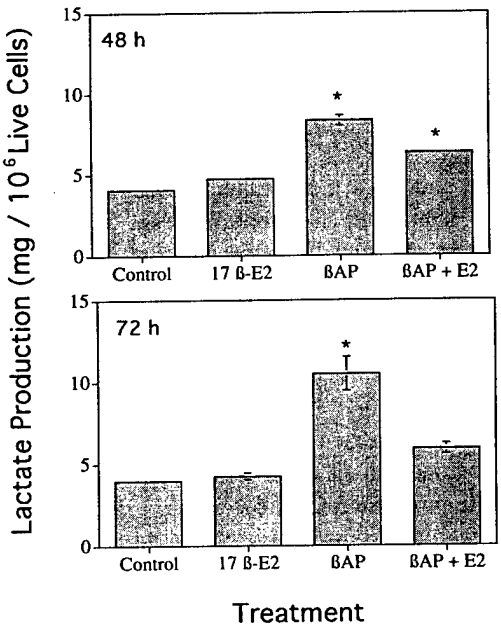


Fig. 6. Chronic effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # WM513), 17β-estradiol (E2), or their combination on production of lactate. Media lactate was subtracted from initial lactate values and normalized per million cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

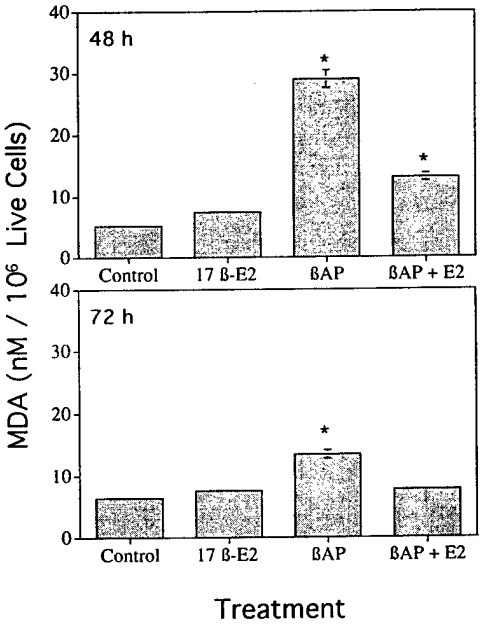


Fig. 7. Chronic effects of simultaneous treatment with βAP 25–35 (20 μM; LOT # WM513), 17β-estradiol (E2), or their combination on lipid peroxidation in SK-N-SH cells. Lipid peroxidation determined by the method of TBARs and expressed as amount of malondialdehyde (MDA) produced per million live cells. Data are expressed as means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

time point (Fig. 7). At 72 h, βAP 25–35-treated cells showed a 78% increase in lipid peroxidation, while in cells treated with βAP and E2, lipid peroxidation levels were not increased over the level of vehicle treated controls (Fig. 7).

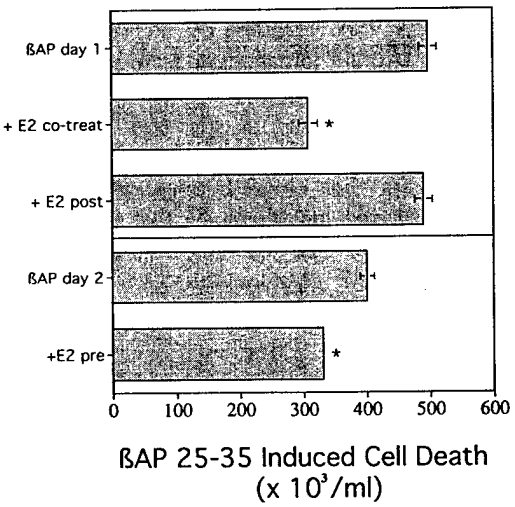


Fig. 8. Effects of pre-, co-, and post-treatment with 17β-estradiol (E2) on βAP 25–35 induced toxicity (20 μM; LOT #B01200) as determined by cell death in SK-N-SH cells. Data are expressed as the means ± S.E.M. If S.E.M. values are not shown, the error was too small to be depicted. * $P < 0.05$ versus all other groups as determined by ANOVA followed by Scheffe's post hoc tests.

3.5. E2 pre- and post-treatment

Finally, we characterized the effects of pre- and post-estradiol treatment with regards to βAP 25–35-induced toxicity. Cells pre-treated with E2 for 24 h, followed by 24 h of βAP 25–35 treatment showed a 17% decrease in βAP 25–35-induced toxicity when compared to cells treated with βAP 25–35 for the same time period (Fig. 8). Further, toxicity to cells co-treated with E2 and βAP 25–35 was significantly decreased by 38% when compared to cells treated with βAP 25–35 for the same time period; however, E2 added to cells post-βAP 25–35 treatment showed no neuroprotective effect (Fig. 8).

4. Discussion

The present study provides evidence that low concentrations of estradiol can attenuate βAP-induced toxicity using either pre- or co-treatment, and that co-treatment can reduce lipid peroxidation and decrease the metabolic load of the cell as demonstrated by a lowering in the rate of glucose use and lactate production. Behl et al. [4], as well as Goodman et al. [14], have demonstrated that μM concentrations of estradiol reduce lipid peroxidation with 2–20 h of steroid pretreatment and an additional 20 h of insult. Our earlier findings demonstrated that low doses of E2 can protect SK-N-SH cells from βAP-induced toxicity, but these studies used 96 h of concomitant exposure to βAP and E2 [16]. The present study, using both short and long term exposures to insult, bridges these findings by demonstrating that low doses of estrogen can protect cells from the acute effects of βAP and additionally that chronic low doses of E2 reduce lipid peroxidation and protect cells from βAP-induced toxicity. Inasmuch as the cell death and the magnitude of neuroprotection by E2 was not affected when we used the βAP 1–40 peptide (Green et al., unpublished observations), these results indicate that estrogens protect neurons from βAP toxicity.

There is much evidence supporting a decline in glucose metabolism early in AD [17]. Studies in humans using positron emission topography suggest that hypometabolism may be a component of AD pathogenesis, in as much as asymptomatic individuals at risk for AD show hypometabolism in brain regions that were reported to progress to pathology [19,33,39]. In addition, decreased concentrations of glucose transporters have been noted in brains of AD patients [37] and selective decreases in the expression of certain subunits of mitochondrial enzymes necessary for oxidative metabolism have been demonstrated [36]. Recently, mutations in mitochondrial DNA coding for cytochrome *c* oxidase have been linked to late-onset AD patients [9]. Consequently, we were surprised to observe that exposure of SK-N-SH cells to βAP increased glucose use in close association with cell death, with both acute and chronic responses to this neurotoxic peptide.

There are several plausible explanations for this paradoxical observation. The breakdown of the mitochondria, which has been reported to occur as early as 2 h after treatment with βAP 25–35 [2] would result in marked reductions in ATP generation by oxidative metabolism. In order to meet the metabolic demand of the cell during an βAP-induced insult, an increase in glucose use for anaerobic metabolism would be the likely result. Our observation of an acute increase in glucose utilization is consistent with a rapid βAP-induced mitochondrial compromise, as is the observed increase in the production of lactate. Alternatively, key lipid peroxidation repair mechanisms utilizing glutathione rely on the generation of NADPH [24]. NADPH is generated from the pentose phosphate pathway (PPP) at two places, the rate-limiting enzyme being glucose-6-phosphate dehydrogenase which uses glucose as its substrate, and 6-phosphogluconate dehydrogenase which acts on 6-phosphogluconate. With the observed increases in lipid peroxidation, glucose utilization would increase to allow for repair and maintenance of membrane integrity and it has been demonstrated that PPP activity is stimulated in response to H_2O_2 exposure in primary mixed cortical cultures [5]. Since Behl et al. [3] provide evidence for H_2O_2 production in response to a βAP 25–35 insult, the observed changes in glucose and lactate also support this hypothesis. Finally, the observed increase in glucose utilization with βAP exposure may represent the selective βAP toxicity of SK-N-SH cells that are metabolically less active. In this case, the cells more resistant to βAP 25–35 would appear to utilize more glucose. Presently, we are not able to distinguish between these explanations for the likely cause of enhanced glucose use.

The neuroprotective effect of estrogens observed in response to βAP 25–35 insult on cell viability may result from its ability to reduce membrane lipid peroxidation rather than its mitogenic effects. Our data show no effects of estradiol on cell proliferation as demonstrated by the lack of increase in live cell number in estrogen treated control cultures. Further, thymidine uptake in these cells is not affected up to 48 h of treatment in either a serum deprivation model or in the presence of serum [6]. Estrogens are reported to be potent antioxidants in a variety of in vivo and in vitro models [23,26,27,29,34,41] and high doses of this steroid have been shown to reduce lipid peroxidation caused by βAP 25–35 [4,14] and this additionally correlates with cell protection. Further, estrogens reportedly reduce excitatory amino acid-induced neuronal toxicity [38], an insult that has an oxidative stress component [28], and a variety of non-estrogenic estratriene compounds have been shown to reduce neuronal cell death induced by serum deprivation [15] an insult that causes an apoptotic-type cell death [11]. Since both necrosis [3] and apoptosis [20] have been linked to βAP 25–35-induced toxicity and oxidative stress may participate in apoptotic

signaling [10], the antioxidant activity of estrogens may largely contribute to their neuroprotective capacity.

In summary, βAP-induced neuronal death is associated with membrane lipid peroxidation and a persistent hypermetabolism both of which are ameliorated by simultaneous exposure to low concentrations of 17β-estradiol. These data indicate that the reported beneficial effects of estrogens in AD may be subsequent to their neuroprotective actions.

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Phenolic A Ring Requirement for the Neuroprotective Effects of Steroids

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Estrogens are reported to reduce the incidence of Alzheimer's disease and 17β-estradiol (βE2), the potent, naturally occurring estrogen, exerts neuroprotective effects in a variety of *in vivo* and *in vitro* model systems. The present study elucidates the structural requirements of steroids and related compounds for neuroprotectivity at low nM doses. All estrogens tested with an intact phenolic A ring protected SK-N-SH neuroblastoma cells from the toxic effects of serum-deprivation. All 3-O-methyl ether cogeners tested were inactive indicating the importance of a phenolic A ring. The diphenolic estrogen mimic diethylstilbesterol (DES) was neuroprotective and retention of a single phenolic function was sufficient to retain neuroprotective activity. The di-O-methyl ether of DES was inactive. The following steroids which lack a phenolic A ring were also inactive: testosterone; dihydrotestosterone; progesterone; corticosterone; prednisolone; 6 α-methylprednisolone; aldosterone; and cholesterol. Finally, phenol, lipophilic phenols, and tetrahydronapthol were inactive. These results suggest that a phenolic A ring and at least three rings of the steroid nucleus are necessary for the neuroprotective activity of estrogens. © **1997 Published by Elsevier Science Ltd. All rights reserved**

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INTRODUCTION

Postmenopausal estrogen replacement therapy (ERT) is associated with a 40% reduction in the incidence of Alzheimer's disease [1] and a delay in the onset of the disease [2]. Further, ERT has been reported to improve cognitive function in Alzheimer's patients in small, open clinical trials [3–5]. Estrogens may also be important in protecting patients from more acute brain damage as ERT reduces the incidence of and mortality from stroke in post-menopausal subjects in some [6–8], but not all, studies [9]. We have recently demonstrated that 17β-estradiol exerts neuroprotective effects at pM concentrations in both animal and tissue culture models [10–15]. These effects of estradiol appear to be independent of the estrogenic potency of the molecule as the weak estrogen, 17α-estradiol, was as effective as 17β-estradiol in protecting neuronal cells and this protection was not significantly antagonized by tamoxifen [12].

We developed an *in vitro* model for neuroprotection based upon survival of human SK–N–SH neuroblastoma cells in serum deprived conditions in order to assess structure–activity relationships (SAR) in steroid neuroprotection. This insult kills 80–90% of cells by 48 h. SK–N–SH cells were chosen because they exhibit numerous features necessary for screening of potential neuroprotective drugs. They are human in origin, exhibit a neuronal phenotype upon differentiation and are sensitive to environmental insults such as serum deprivation and hypoglycemia [10, 12]. Further, the neuroprotective effects of 17β-estradiol observed in SK–N–SH cells are seen also in rat primary neuronal cultures at similar doses of the steroid [16, Green and Simpkins, unpublished observations). The ease with which SK–N–SH cells are cultured, in contrast to other neuronal cell types, makes them especially suitable for the screening of large numbers of compounds in the present study.

MATERIALS AND METHODS

Cell cultures

SK-N-SH cells, obtained from American Type Tissue Collection (Rockville, MD), were grown to confluency in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G

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and 100 μg/ml streptomycin (Sigma, St. Louis, MO) at 37°C and under 5% CO_2 and 95% air. Cells used in the following experiments were in passes 7 to 12. All experiments were initiated by the backculturing of SK–N–SH cells at a concentration of 1.0×10^6 cells/well.

Experimental media

In all studies, cells were cultured in RPMI-1640 media (Serum Free, SF group), RPMI-1640 media supplemented with 10% FBS (FBS group), or RPMI-1640 media supplemented with one of the following compounds at a concentration of 2 nM: 17β-estradiol (1,3,5,(10)-estratrien-3,17β-diol); 17α-estradiol (1,3,5,(10)-estratrien-3β,17α-diol); 17β-estradiol 3-O-methyl ether (1,3,5,(10)-estratrien-3,17β-diol,3-O-methyl ether); 17α-estradiol 3 acetate (1,3,5,(10)-estratrien-3β,17α-diol 3-acetate); estrone (1,3,5,(10)-estratrien-3-ol-17-one); estrone-3-O-methyl ether (1,3,5,(10)-estratrien-3-ol-17-one 3-O-methyl ether); estriol (1,3,5(10)estratrien-3β,16α,17β-triol); estriol-3-O-methyl ether (1,3,5(10)estratrien-3β,16α,17β-triol 3-O-methyl ether); 17α-ethynyl estradiol (1,3,5,(10)-estratrien-17α-ethynyl-3β,17α-dio l); 17α-ethynyl estradiol 3-O-methyl ether (1,3,5,(10)-estratrien-17α-ethynyl-3β,17α-dio l 3-O-methyl ether); 2-hydroxy estradiol (1,3,5(10)estratrien-2,3, 17β-triol); 2,3-methoxyestradiol (1,3,5 (10)estratrien-2,3,17β-triol,2,3-O-methyl ether); estratrien-3-ol (1,3,5(10)estratrien-3-ol); pregnisolone (1,4-pregnadien-11β,17,21-triol-3,20-dione); methyl-prednisolone (6α-methyl-1,4-pregnadien-11β,17,21-triol-3,20-dion e); corticosterone (4-pregnan-11β,21-diol-3,20-dione); progesterone (4-pregnen-3,20-dione); aldosterone (4-pregnan-11β,21-diol-3,18,20-trione); phenol; 5,6,7,8 tetrahydronapthol; butylated hydroxytoluene; or butylated hydroxyanisol (all steroids from Steraloids, Wilton, NH; other chemicals from Sigma, St. Louis, MO). All steroids were initially dissolved in absolute ethanol and diluted in RPMI-1640 media to a final concentration of 2 nM. To control for possible ethanol effects in the treated wells, both the serum-free media (SF group) and FBS media (FBS group) were supplemented with 0.0001% V/V absolute ethanol.

Quantitation of cell viability

Cell viability was assessed at 48 h of treatment using the Trypan Blue dye-exclusion method [17]. After 48 h of treatment, cell suspensions were made by lifting the cells with 0.02% EDTA. Aliquots from each cell suspension was incubated with 0.4% Trypan Blue stain (Sigma, ST. Louis, MO) for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer within 15 min of addition of Trypan Blue. Two independent counts of live cells were made for each aliquot.

Estrogen receptor binding

Cells were maintained for 24 h in phenol red free RPMI-1640 supplemented with 10% charcoal stripped FBS. Cells were then lifted and 1×10^6 cells were plated per well and allowed to attach for 4 h. Cells were incubated with 2 nM ^{3}H-estradiol alone for total binding and 2 nM ^{3}H-estradiol plus 2 μM unlabeled estradiol for nonspecific binding for 1 h. Cells were then rinsed with ice cold DPBS five times before lysis with 0.1 N NaOH. Liquiscint™ scintillation solution was then added to the cell lysate and the mixture was counted on a Beckman LS5000 counter.

Statistical analysis

The significance of differences among groups was determined by one way Analysis of Variance. Planned comparisons between groups were done using Scheffe's F–test. For all tests, $p < 0.05$ was considered significant. All statistical analyses were performed on raw data.

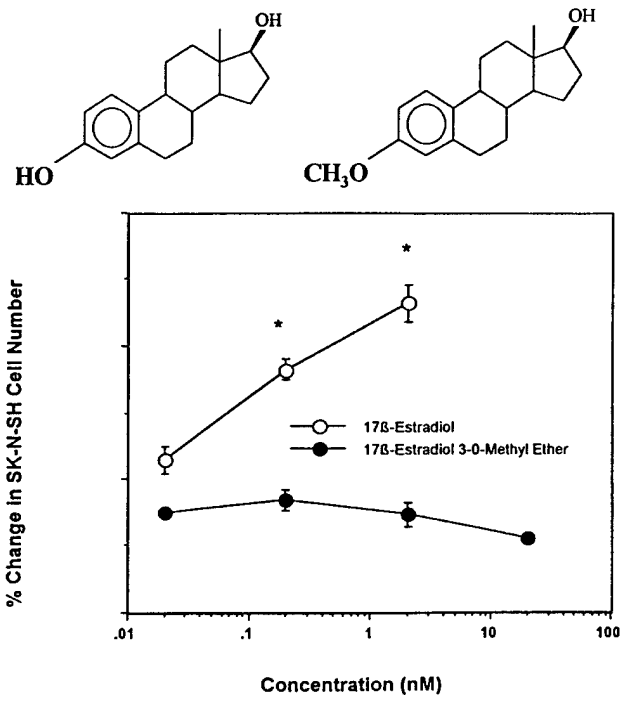


Fig. 1. The hydroxyl function in the C3 position is necessary for neuroprotective activity as 17β-estradiol but not its 3-O-methyl congener protects SK–N–SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult and live cell number was determined 48 h later. Raw data were compared to the respective serum-free control group by analysis of variance and Scheffe's F test and data were then normalized to the serum free group (=100%). * = $p < 0.05$ serum-free controls. Data are expressed as mean ± SEM for 4 to 6 wells/group. The structures shown are 17β-estradiol and 17β-estradiol-3-O-methyl ether, respectively..

RESULTS

17β-estradiol caused a dose-dependent protection of serum-deprived SK–N–SH cells with an ED_{50} of 0.13 nM and significant neuroprotection at the 0.2 nM dose (Fig. 1). This effect was robust with the 2 nM concentration of 17β-estradiol showing neuroprotection in eight separate trials (Figs 1–3 and Tables 1–3). 17α-estradiol, 1,3,5(10)-estratrien-3-ol and 2-hydroxy-17β-estradiol were equivalent to 17β-estradiol in their neuroprotectivity. (Fig. 2). Estrone, estriol and 17α-ethynyl-17β-estradiol were significantly neuroprotective but appeared to be less active than 17β-estradiol (Fig. 2). The dephenolic estrogen mimic, diethylstilbesterol (DES) was an active neuroprotectant and retained nearly full neuroprotectivity when one but not both of the phenolic hydroxyl functions were replaced with an O-methyl ether function (Fig. 3). Similarly all steroids were rendered inactive when the 3-hydroxyl group was replaced with an O-methyl ether group (Figs 1 and 2), a substitution that eliminates the acidic hydrophilic properties of the A ring. The 3-O-methyl ether of 17β-estradiol was inactive even at concentrations as high as 20 nM (Fig. 1).

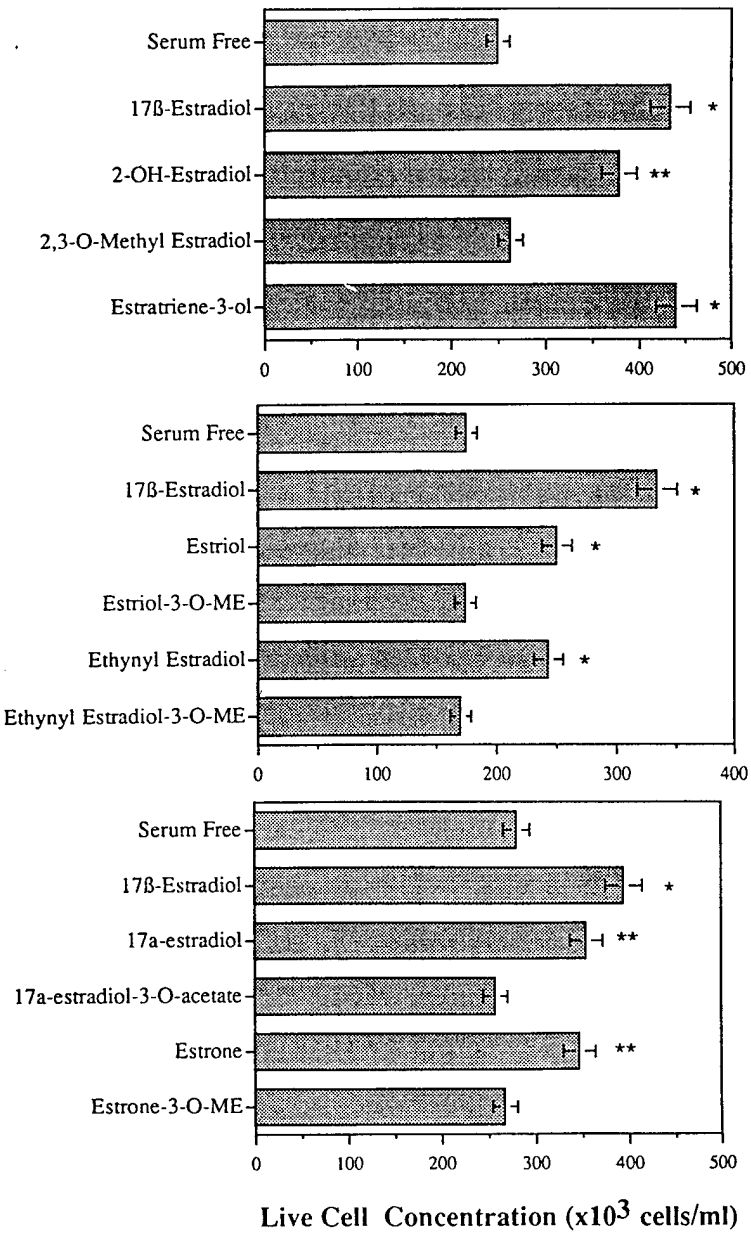


Fig. 2. Phenolic A ring estrogens but not their 3-O-conjugates protects SK–N–SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult and live cell number was determined 48 h later. * = p <0.05 vs serum free controls. ** = p <0.05 vs serum free controls and the respective 2,3-O-methyl estradiol. Data are expressed as mean ± SEM for 5 wells/group. The three panels represent three individual experiments..

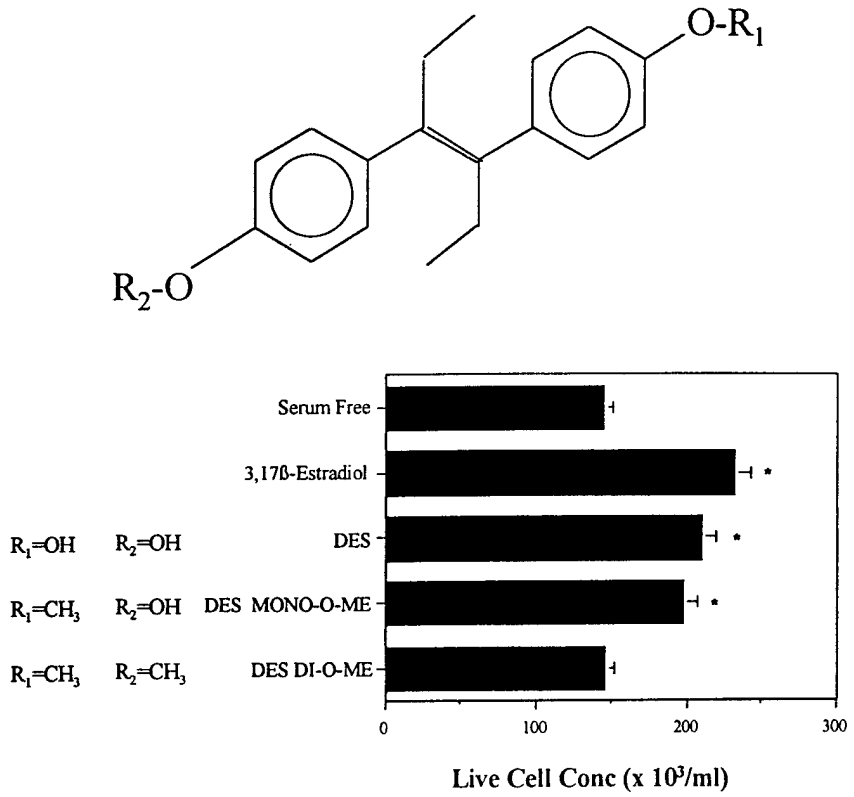


Fig. 3. Diethylstilbesterol (DES), DES Mono-O-Methyl Ether (DES Mono-O-ME) and DES Di-O-Methyl Ether (DES DI-O-ME) protect SK–N–SH cells from the toxic effects of serum deprivation. Steroids or vehicle controls were added concurrent with the insult and live cell number was determined 48 h later * = p <0.05 vs serum free controls and DES di-O-methyl ether groups. Data are expressed as mean ± SEM for 6 wells/group. Shown is the core structure of DES and its ether conjugates..

Further, immobilization of the steroid also abolished neuroprotective effects as β-estradiol-17-hemiacetate:BSA was not significantly protective at concentrations as high as 200 nM (Table 1).

Two 19-carbon androgens and five 21-carbon steroids were also evaluated for neuroprotection in our assay (Table 2). The two androgens containing a C-3 ketone, namely testosterone with a partially unsaturated A ring and dihydrotesterosterone with a saturated A ring, were both inactive. Similarly, all five of the 21-carbon pregnane derivatives that were tested contained a C-3 ketone function; the three Δ^4-steroids, corticosterone, progesterone and aldosterone, and two $\Delta^{1,4}$-steroids, prednisolone and 6-methylprednisolone, were inactive (Table 2). Finally, cholesterol was tested because it has a 3-hydroxyl function on a completely saturated A ring and was inactive (Table 2).

Table 1. *Effect of β-estradiol-17-hemiacetate:BSA on live SK–N–SH cell number under serum-free conditions*

Treatment	Live cell number (mean ± SEM × 10^3/ml)
Serum Free Controls	153 ± 12
17β-estradiol (2 nM)	314 ± 23*
βE2:BSA (2 nM)	171 ± 15
βE2:BSA (20 nM)	206 ± 12
βE2:BSA (200 nM)	162 ± 13

*p <0.05 vs serum-free control groups

Further, we evaluated phenol and 5,6,7,8 tetrahydronapthol which represent the A and AB ring of the estratrien structure, respectively. Phenol was toxic, resulting in a 22% decrease in live cells from the serum-free group (data not shown). Tetrahydronapthol had no effect on live cell number (data not shown). Finally, we assessed the activity of two lipophilic phenols, butylated hydroxytoluene and butylated hydroxyanisol which resulted in a 14% and a 10% decrease in live cell number from the serum-free group (Table 3).

Finally, we evaluated the SK–N–SH cells for the presence of specific ^{3}H-β-estradiol binding in whole cell preparations. SK–N–SH cells did not demonstrate specific binding with only 1 ± 0.9 fmol of 3-H-estradiol bound per 10^6 cells as compared to the estrogen-receptor containing MCF-7 cells with 56 ± 6.5 fmol specific binding per 10^6 cells in this binding protocol.

Table 2. Effects of a variety of non-phenolic A ring steroids on live SK–N–SH cell number under serum-free conditions

Treatment	Live cell number (Mean ± SEM × 10^3/ml)	Treatment	Live cell number (mean ± SEM × 10^3/ml)
Study 1		Study 2	
Serum Free Controls	94 ± 7	Serum Free Controls	177 ± 18
Testosterone (2 nM)	87 ± 6	17β-estradiol (2 nM)	329 ± 33*
Dihydrotestosterone (2 nM)	90 ± 7	Prednisolone (2 nM)	187 ± 16
Cholesterol (2 nM)	65 ± 4	6α-Methylprednisolone (2 nM)	173 ± 13
Progesterone (2 nM)	58 ± 6	Aldosterone (2 nM)	132 ± 18
Corticosterone (2 nM)	69 ± 7		

*p <0.05 vs serum-free control groups

DISCUSSION

This study represents the first demonstration of SAR associated with low doses of neuroprotective steroids. A hydroxyl function on the aromatic ring of estratrienes is required for their neuroprotectivity. However, a phenolic ring is not neuroprotective in the absence of a steroid-like structure. Simple enhancement of the lipophilicity of the phenolic group was not sufficient to impart neuroprotectivity to this moiety as two highly lipophilic phenols, 2, [6]-di-tert-butyl-*p*-cresol and 2, [3]-*t*-butyl-4-hydroxy-anisol were both inactive in our assay. The conformational shape of the flat, phenolic ring and/or the enhanced acidity of phenols relative to cyclohexanols may be important in conferring the observed neuroprotective activity. However, it is clear from our data that a steroid structure is inactive in the absence of a phenolic A ring as is a phenolic ring in the absence of the steroid backbone.

Immobilization of the estrogen molecule by conjugation to a BSA molecule attenuates the protective effect of the steroid. We evaluated the 17-conjugate in order to decrease interference with the 3-OH group. Additionally, we have evaluated the 6-conjugated estradiol:BSA at estradiol concentrations as high as 20 μM and observed no protection in another neuronal cell line, HT–22, from βAP toxicity (Green and Simpkins, unpublished observations). The BSA conjugation allows exposure of the extracellular space to the steroid but prohibits the diffusion of the estradiol to the intracellular spaces. The lack of protection actions of the estradiol:BSA conjugates indicates that the estrogen must be free to pass into or through the neuronal membranes. The untethered steroid structure may be needed to interact with cellular membranes or to properly localize the neuroprotectant.

Table 3. Effects of Lipophilic phenols on live SK–N–SH cell number under serum-free conditions

Treatment	Live cell number (Mean ± SEM × 10^3/ml)
Serum free controls	203 ± 16
17β-estradiol (2 nM)	373 ± 11*
Butylated hydroxytoluene (2 nM)	175 ± 11
Butylated hydroxyanisole (2 nM)	182 ± 16

*p <0.05 vs serum-free control groups

The present study provides evidence for the dissociation of the estrogenic potency of steroids and their neuroprotectivity. Several of the active neuroprotective phenolic A ring steroids including 17α-estradiol, estriol, estra-2, 17β-triol and estratriene-3-ol have only weak or no estrogenic activity as assessed by binding to the estrogen receptor, binding of the steroid-estrogen receptor complex to the estrogen-responsive element or the mitotic response to acute or chronic exposure of uterine and mammary tissue to the steroid [18–23]. Several of these compounds, however, are as neuroprotective as 17β-estradiol in our assay system. This observation suggests that the neuroprotectivity of these compounds is not mediated primarily through the nuclear estrogen receptor. This conclusion is consistent both with our observation that the neuroprotectivity is preserved even in the face of a classical estrogen antagonist [12] and the lack of specific estradiol binding sites in these cells. Additionally, Behl *et al.* [24] has demonstrated neuroprotective activity of 17β-estradiol in a cell type that lacks the estrogen receptor as determined by the inability of estradiol to increase transcription of an ERE-reporter gene construct transfected into the cells.

Behl *et al.* [25] has recently reported a similar SAR for estrogen protection of the HT–22 hippocampal cell line and hippocampal slices from toxins that cause oxidative damage such as H_2O_2. Those studies required a minimum dose of 10 μM to achieve significant neuroprotection whereas we demonstrate significant protection of SK–N–SH cells with doses as low as 0.2 nM from either serum-deprivation [12] or βAP toxicity [15]. This 50,000 fold difference in effective doses may be due in part to different properties of the cell lines, different culturing conditions, or different toxic insults. However, the low nM estradiol doses at which we demonstrate neuroprotection *in vitro* are

also capable of neuroprotection *in vivo* using a rat focal ischemia model [26].

Phenolic A ring steroid compounds may exert their neuroprotective actions through an antioxidant mechanism. Lipophilic phenols are well known to be antioxidants [27]. Estrogens, specifically phenolic A ring estratrienes, have been shown to have antioxidant activity and to reduce membrane lipid peroxidation [28–30]. Also, 17β-estradiol protects neurons from oxidative insults such as hydrogen peroxide [24], iron sulfate [31] and β-amyloid peptide [15, 24, 31] toxicity. This is significant as increased lipid peroxidation is associated with a variety of neurodegenrative diseases including ischemic/anoxic insults [32–35] and Alzheimer's disease [36–38].

Although the structural requirement for estratrien neuroprotection is suggestive of an antioxidant mechanism, the doses with which we show neuroprotection complicates that view. The aforementioned antioxidant effects of estrogens required low μM doses of the steroid [28–31]. However, we achieve neuroprotection with 10,000-fold lower doses, demonstrating significant neuroprotection with as low as 0.2 nM of the steroid. Further, other compounds with antioxidant capacities were inactive in our assay system. Methylprednisolone, a neuroprotective steroid that has been shown to inhibit lipid peroxidation at high μM concentrations [39] is inactive at the low steroid doses used in our model. Two phenols with antioxidant properties, 2, [6]-di-tert-butyl-*p*-cresol and 2, [3],-*t*-butyl-4-hydroxy-anisol were also inactive.

We tested compounds at a 2 nM dose, a dose 10-fold higher than necessary to see the neuroprotective effects of 17β- and 17α-estradiol in our assay system [12]. This dose is physiologically relevant, only slightly above the levels of circulating estrogens in pre-menopausal women. The inactivity of the non-phenolic estrogens was not due to the dose tested as concentrations as high as 20 nM 17β-estradiol-3-O-methyl ether showed no neuroprotective activity (Fig. 1). Similarly corticosterone and progesterone at concentrations up to 200 nM were not neuroprotective in our assay system (Green and Simpkins, unpublished observations).

Chronic neurodegenerative diseases and acute neuronal injury are presently untreatable. The present report provides the first evidence for a structural requirement for the neuroprotectivity of an important class of compounds. The phenolic A ring requirement for the neuroprotective action of steroids may provide important clues to their mechanism of action and for the design of drugs with enhanced neuroprotective properties.

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17β-estradiol Attenuates CREB Decline in the Rat Hippocampus following Seizure

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ABSTRACT: **Cyclic AMP response element-binding protein (CREB) is a transcription factor that has been implicated in the activation of a number of genes. We reported that CREB levels decline following a severe hypoglycemic episode in the hippocampus and cortex in the male rat brain. The present experiment was undertaken to investigate whether 17β-estradiol prevents the decline in CREB-immunoreactive cells following seizure in female rats. Rats were divided into four groups: ovariectomized (OVX), ovariectomized and insulin-treated (OVX-I), estrogen-replaced (E2), and estrogen-replaced and insulin-treated (E2-I). Generalized seizures were induced by injections with insulin (12.5 IU/kg, intraperitoneally) and animals were recovered by administration of glucose within 5 min of the occurrence of seizure. Control animals were injected with saline instead of insulin. All animals were perfused 90 min after recovery and the brains were processed for CREB immunoreactivity. CREB-positive neurons were counted using a computer-assisted program. Insulin treatment of OVX rats caused a significant decline in CREB-positive neurons in the CA1, CA3, and dentate gyrus compared to OVX rats. Estrogen treatment of OVX rats significantly increased CREB-positive neurons in the CA1 and dentate gyrus and attenuated the insulin-induced decline of CREB-positive neurons in all three regions compared to OVX rats. In conclusion, estrogens appear to induce CREB expression and attenuate its decline in the hippocampus following a severe hypoglycemic episode.** © 1997 John Wiley & Sons, Inc. *J Neurobiol* 33: 961–967, 1997

Keywords: **CREB; estrogen; hippocampus; seizure**

INTRODUCTION

Cyclic AMP response element-binding protein (CREB) is a constitutively expressed transcription factor involved in the regulation of certain immediate early genes such as *c-fos* and *Jun*B (Herdegen et al., 1994) as well as neuropeptides including somatostatin (Montminy et al., 1986), proenkephalin (Hyman et al., 1994), and prodynorphin (Cole et al., 1995). Activation of CREB by phosphorylation occurs in response to stimuli that are mediated by distinct second-messenger pathways. Currently, it is believed that various protein kinases such as protein kinase A (PKA), Ca^{2+}/calmodulin kinase (CaM-kinase), protein kinase C (PKC), and CREB kinase can all phosphorylate CREB (Delmas et al., 1994; Vallejo, 1994) and thus lead to regulation of gene transcription. This transcriptional function can eventually lead to relative changes in neuronal function (Greenberg et al., 1986; Morgan and Curran, 1986).

Estrogen and estrogenic compounds are known

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to alter brain functioning (McEwen, 1981). We have shown the cytoprotective action of 17β-estradiol (E_2) on neuronal and glial cell types *in vitro* after insult (Bishop et al., 1994). E_2 treatment can prevent the hypoglycemia-induced death of C6 glioma cells (Bishop and Simpkins, 1994). In addition, estradiol can attenuate neuronal loss in response to a variety of insults, including serum deprivation, excitatory amino acid treatment, and β-amyloid (Behl et al., 1995; Goodman et al., 1996; Green et al., 1996, 1997; Singer et al., 1996). E_2 prevents the decrease of choline acetyltransferase (ChAT) activity in the hippocampus and frontal cortex following ovariectomy (Singh et al., 1994) and attenuates the decrease of ChAT-immunoreactive neurons in the medial septum following fimbria–fornix transection (Rabbani et al., 1997). Collectively, these data indicate that estrogens appear to have neuroprotective actions similar to those reported for neurotrophic agents.

We have reported that hypoglycemia-induced seizure results in a regionally selective reductions in CREB in the hippocampus and cortical brain regions (Panickar et al., 1995), and hypoglycemia-induced seizures have been shown to cause massive cell loss in these regions of CREB decline (Auer et al., 1985). In view of the reported neuroprotective effects of estrogens, we investigated the effects of 17β-estradiol on CREB-positive neurons in the rat hippocampus following hypoglycemia-induced seizure.

MATERIALS AND METHODS

Female Sprague–Dawley rats (280–350 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were individually caged in a light- and temperature-controlled environment with water and food available *ad libitum*. Food, but not water, was deprived for 24 h prior to seizure induction.

Animal Surgical Procedures

For all surgical procedures, animals were anesthetized with Metofane^R. All rats (n = 19) underwent bilateral ovariectomy (OVX) using a dorsal approach. Ten rats then received a pellet containing E_2, and nine rats received an empty pellet through a subcutaneous implant at the time of OVX. Pellets were made by packing approximately 3 mg crystalline 17β-estradiol (Pharmos, Alachua, FL) into 5-mm Silastic (Dow Corning, Midland, MI) tubes which were sealed on both ends with Silastic adhesive. Capsules were washed twice with ethanol in scintillation vials, dried, and placed in saline solution for 24–48 h before implantation.

Hypoglycemic seizures were induced 1 week after the surgery in the OVX and E_2 animals by an intraperitoneal (i.p.) injection of insulin (12.5 IU/kg) in sterile physiological saline. Control OVX and E_2 animals received vehicle injections (1 mL/kg). The resulting groups were: (a) OVX and insulin-treated (OVX-I, n = 6), (b) OVX and saline-treated (OVX, n = 3), (c) E_2-replaced and insulin-treated (E_2-I, n = 7), and (d) E_2-replaced and saline-treated (E_2, n = 3). The dose of insulin and the blood glucose levels reached after insulin administration were based on a pilot experiment conducted earlier in our laboratory (Rajakumar et al., 1995).

The level of generalized seizure invoked was recorded by two independent investigators according to the paradigm defined by Kosobud and Crabbe (1990). These investigators define clonic seizure as "a very dramatic and violent whole-body clonus, including episodes of running and explosive jumps, usually terminating in tonic hindlimb extensor convulsion." The animals that received insulin developed seizures approximately 90 min after the insulin injection. Within 5 min of seizure onset, the experimental rats were injected with 3 mL of 30% glucose (i.p.) which terminated the seizures. Control rats were injected with saline (1 mL/kg). All animals were given food pellets and water during the postictal recovery interval and were sacrificed 90 min following the onset of seizure (Gass et al., 1993).

Rats were anesthetized with Metofane^R and were perfused transcardially with 100 mL of saline (0.9%) followed by 400 mL of 0.1 *M* sodium phosphate-buffered 4% formaldehyde (pH 7.4). Following perfusion, the brains were postfixed *in situ* for 2 h at 4°C. The brains were then removed and equilibrated in 30% sucrose phosphate-buffered saline (PBS) (pH 7.4).

Immunohistochemistry

The brains were frozen and cut into 50-μm saggital sections, and 48 sections from each animal were collected in individual wells of 24-well polystyrene culture plates containing 300 μL PBS. Endogenous peroxidative activity was blocked by a 15-min incubation in 3% hydrogen peroxide-PBS, and nonspecific binding was reduced by incubation in 3% normal goat serum (NGS) in PBS. Sections were incubated overnight in rabbit anti-CREB antibody (Upstate Biotechnology; 1:1000 in PBS/0.1% Triton-X 100/3% NGS). We have shown that this titer of antibody optimally detects CREB, among titers ranging from 1:500 to 1:10,000. Slices were then washed twice in PBS and incubated overnight in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:1000; Sigma Chemical Corp., St. Louis, MO). Following overnight reaction with the secondary antibody, slices were washed, incubated overnight in extravidin–peroxidase (1:1000; Sigma Chemical Corp.), washed again, and then reacted with 0.005% hydrogen peroxide, 0.5 mg/mL diaminoben-

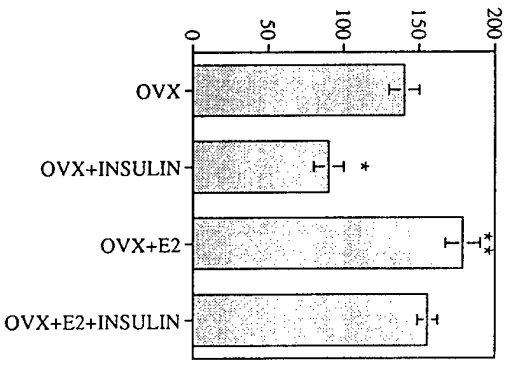


Figure 1 Number of CREB-immunoreactive neurons in the pyramidal cell layer of CA1 in ovariectomized and estrogen-replaced animals, with and without insulin treatment. The values expressed are means ± S.E.M. of total number of CREB-positive neurons from 5–6 slices/animal for each group. $*p < 0.001$, $**p < 0.05$ vs. OVX.

zidene (Sigma Chemical Corp.) in 0.05 *M* Tris-buffered saline (pH 7.6).

Control sections were obtained by omission of the primary antibody during incubation. Immunolabeled sections were mounted on glass slides, dehydrated, and coverslipped with Eukitt (Calibrated Instruments, Ardsley, NY).

Image Analysis

Images of the mounted brain sections were captured using a computerized program, "Capture" (TrueVision, Indianapolis, IN), and cells counts were conducted using "Image Pro Plus" (Media Cybernetics, Silver Springs, MD). Neurons were identified visually and images were segmented by stain intensity. CREB-positive neurons were identified visually using the methods similar to that described by West et al. (1991) as round objects containing a dark brown coloration. To aid the computer in identifying the immunoreactive cells, the threshold intensity was adjusted interactively by selecting the minimum and maximum image intensity cutoffs for each area in every slice after the area of interest was outlined. In the present experiment, the areas of interest were CA1, CA3, and dentate gyrus. Following adjustment of the intensity (range between 0 and 255), a threshold value was determined by the investigator for individual regions in every section. The threshold value was the value at which the image could be segmented into its constituent elements, thereby aiding the investigator in analyzing the individual shape and size when a neuronal shape was identified. This thresholding was optimized to maximize the number of separate neurons present while including as much of the cell body as possible. The number of cells were then counted automatically by the computer. The number of cells obtained by using an automated counting method has been shown to be consistent with the number of cells counted manually (Rabbani et al., 1997). From a total of approximately 24 sections in every animal where the hippocampus was discernible, every fourth slice was counted. There were approximately 5–6 slices counted/animal. The investigator who counted the cells was blinded to the treatment group of the animal.

Analysis was conducted on the total number of cells defined as CREB positive for the region of interest to determine between-group differences using analysis of variance, followed by Scheffé's test for planned post hoc comparisons of groups. Chi-square analysis was conducted to determine the significance of differences between groups in the number of animals that convulsed when treated with insulin.

RESULTS

Insulin-induced hypoglycemic seizure resulted in a 36% decrease in the number of CREB-immunoreactive cells in the CA1 pyramidal cell layer of the hippocampus of OVX rats ($p < 0.001$) (Fig. 1) compared to OVX animals. By contrast, in OVX + E_2 rats, insulin treatment reduced the number of CREB-positive neurons in the CA1 region by only 13%. Interestingly, in the absence of insulin treatment, estradiol increased the number of CREB-positive cells in CA1 by 22% ($p < 0.05$).

In the CA3 pyramidal cell layer of the hippocampus, there was a 39% decrease in CREB-positive cells in OVX + I rats ($p < 0.001$) (Fig. 2) compared to OVX animals. In the OVX + E_2 rats, the decrease was only 12% upon insulin treatment.

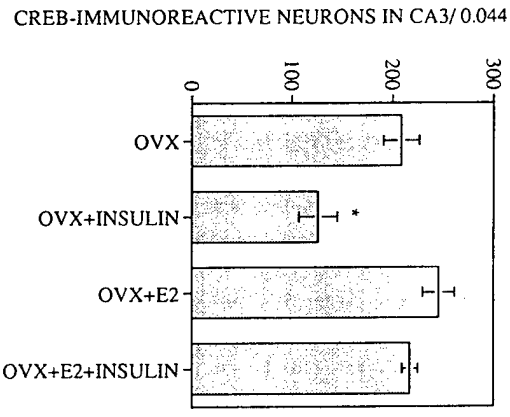


Figure 2 Number of CREB-immunoreactive neurons in the pyramidal cell layer of CA3 in ovariectomized and estrogen-replaced animals, with and without insulin treatment. The values expressed are means ± S.E.M. of total number of CREB-positive neurons from 5–6 slices/animal for each group. $*p < 0.001$ vs. OVX.

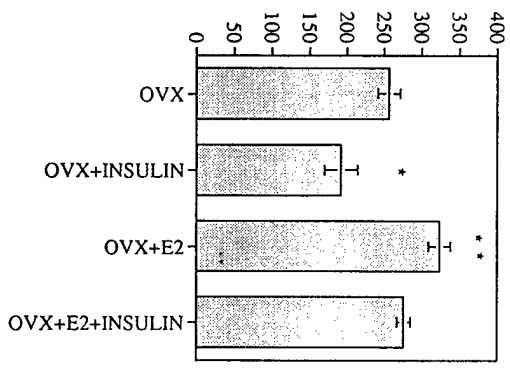


Figure 3 Number of CREB-immunoreactive neurons in the dentate gyrus in ovariectomized and estrogen-replaced animals, with and without insulin treatment. The values expressed are means ± S.E.M. of total number of CREB-positive neurons from 5–6 slices/animal for each group. $*p < 0.001$, $**p < 0.05$ vs. OVX.

There was a nonsignificant increase in CREB-positive cells of 18% in the E_2-treated compared to OVX rats.

In OVX rats, the granule cell layer of the dentate gyrus showed a decrease in CREB-positive cells of 25% upon insulin treatment ($p < 0.05$) (Fig. 3), whereas in the E_2 animals there was a 15% decrease. E_2 animals that were not treated with insulin showed a 26% increase in CREB-immunoreactive cells compared to the OVX rats ($p < 0.05$).

Of the regions of interest within the hippocampal formation, the intensity of staining of CREB appeared to be highest in the E_2 rats that were not treated with insulin (Fig. 4). In addition to the hippocampus and dentate gyrus, CREB immunoreactivity was also qualitatively diminished in intensity in the insulin-treated OVX rats but not in the E_2-treated rats, in cortical regions adjacent to the rhinal fissure along its entire extent (e.g., lateral orbital cortex). However, CREB was ubiquitously expressed in the thalamic nuclei (anteroventral thalamic nuclei, laterodorsal thalamic nuclei, and ventroposterior thalamic nuclei), superior colliculus, inferior colliculus, and the anterior pretectal area in all animals in both OVX and E_2 groups. There did not appear to be a decrease in the number or intensity of staining of CREB-positive cells in these regions in either group in response to insulin treatment.

CREB is a nuclear protein and, as such, the antibody labels predominantly the nucleus. However, at higher magnification, we observed cytoplasmic staining of CREB in some of the pyramidal cell neurons in CA1, CA3, and the granule cells of the dentate gyrus.

In the OVX group, all six animals treated with insulin developed clonic seizure, whereas in the E_2 group only two of seven ($p < 0.05$, χ^2 analysis) animals developed clonic seizure. The other five animals in the E_2 group did, however, show lethargic movement following insulin treatment.

DISCUSSION

Our findings suggest that E_2 can prevent CREB decline in the rat hippocampus following hypoglycemia-induced seizure. The decrease in CREB-immunoreactive cells in various regions of the hippocampal formation of the female rat is consistent with our earlier observations using male animals (Panickar et al., 1995). The present finding that CREB immunoreactivity was attenuated by pretreatment of the animals with estrogen is consistent with reports that estrogens exert protective effects on neurons (Behl et al., 1995; Bishop and Simpkins, 1994; Dluzen et al., 1996; Goodman et al., 1996; Green et al., 1996, 1997; Rabbani et al., 1997; Singer et al., 1996).

Currently, very little is known about the role of CREB in seizures. Moreover, expression of CREB proteins after neuronal injury paradigms including seizures, transection of nerves, and cortical impact injury appears to be complex. Moore et al. (1996), using pentylenetetrazol-induced seizure in rats, reported that the total amount of CREB protein or the number of CREB-positive cells did not change in the hippocampus or cortex at any of the time points tested up to 60 min following seizure. Inasmuch as we are unaware of any reports of cortical cell loss associated with pentylenetetrazol-induced seizures, this report suggests that maintenance of CREB concentrations and resistance of neurons to seizure-associated death are related. However, there are other differences between the present study and that reported by Moore et al. (1996), including the following. (a) We administered insulin and induced hypoglycemic seizure; (b) we sacrificed the animals 90 min following seizure; and (c) we used female instead of male rats. It is not likely that the sex of rats played a role, since we found a decrease in CREB following hypoglycemia-induced seizure at 90 min following seizure in male rats (Panickar et al., 1995). There are also reports that levels of CREB in the brain and spinal cord have remained unchanged, decreased, or increased after mechanical injury (Dash et al., 1995; Herdegen et al., 1992, 1994; Yoneda et al., 1994). It appears from these

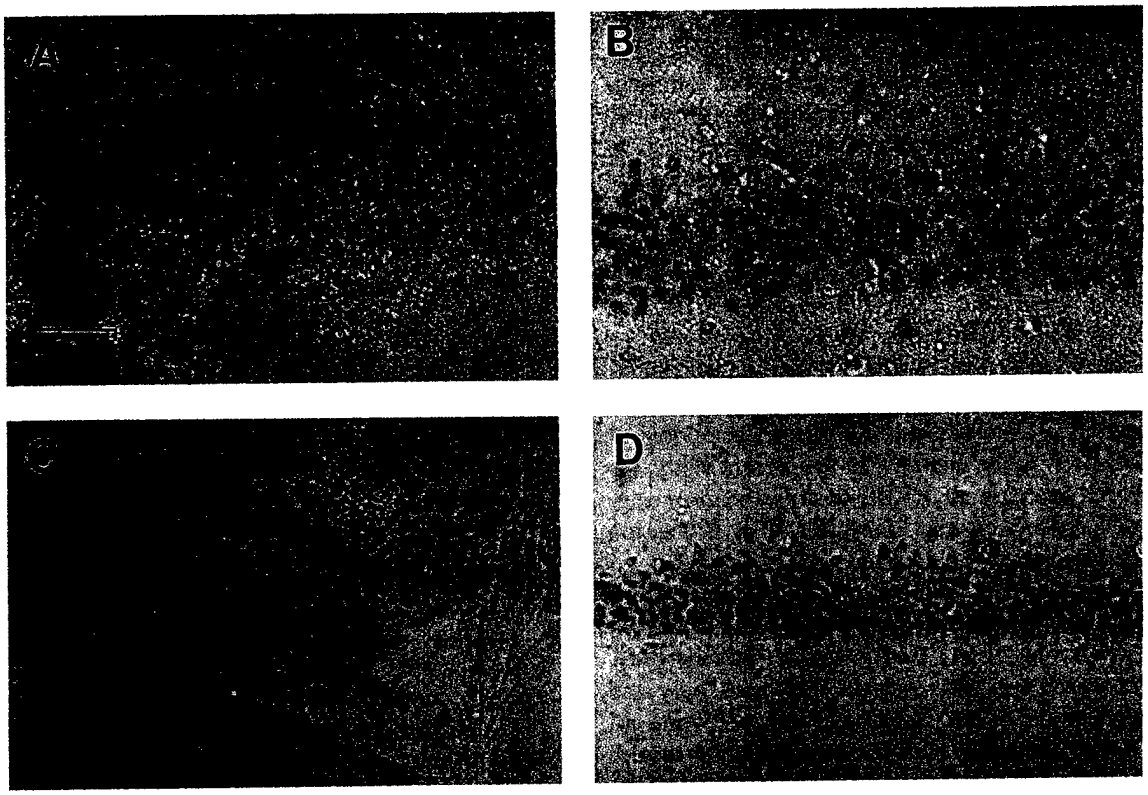


Figure 4 Photomicrographs from representative samples showing CREB immunoreactivity in stratum pyramidale of CA1 region in ovariectomized and estrogen-replaced rats, with and without insulin treatment. (A) Estrogen-replaced, (B) estrogen-replaced and insulin-treated, (C) ovariectomized, and (D) ovariectomized and insulin-treated. Scale bar = 100 μm.

studies that expression of CREB may vary depending on the paradigm used to study neuronal injury, the region of the nervous system studied, and the duration following injury, among other factors.

The decline in CREB-immunoreactive cells following hypoglycemic seizure in CA1, CA3, and dentate gyrus does not appear to be a result of cell loss, since the animals were sacrificed 90 min following seizure. Auer et al. (1985) reported that extensive cell death occurs in the hippocampus at 1 week following seizure. Thus, it appears that the observed decline in CREB-positive neurons precedes the loss of cells in the hippocampus.

The exact mechanism by which estrogen exerts its neuroprotective action is not known. In our study, E_2 attenuated the decrease of CREB-positive cells in the hippocampal formation when the animals were subjected to severe hypoglycemia. Moreover, in the absence of insulin treatment there was an increase in CREB-positive cells in the hippocampus of estrogen-treated animals. As such, an estrogen-induced activation of CREB could activate neuroprotective mechanisms in hippocampal neurons. It is tempting to speculate that some of the neuroprotective actions of estrogen might involve signaling pathways mediated by CREB. Several of the genes that are regulated by CREB, including BDNF and Trk*B* (Condorelli et al., 1994), would be expected to be overexpressed with estrogen treatment (Miranda et al., 1993; Singh et al., 1995).

Recently, Zhou et al. (1996) reported that estrogen treatment of OVX rats increases immunoreactivity for the phosphorylated form of CREB in the preoptic area and bed nucleus of the stria terminalis. Gu et al. (1996) reported that treatment of ovariectomized rats with estrogen caused a significant induction in the number of phosphorylated CREB-immunoreactive nuclei in the periventricular nucleus within 30 min that was maintained for at least 4 h. The antibody to CREB used in the present study recognizes both the phosphorylated and the unphosphorylated form of CREB. It is therefore difficult to determine from our results whether the estrogen-mediated increase in CREB was of the phosphorylated or the unphosphorylated form. In any case, an increase in CREB-positive cells following

estrogen treatment suggests that the CREB signaling pathway may mediate the action of estrogen.

There are several mechanisms by which estrogens could prevent CREB decline following hypoglycemic seizure. First, estrogens have been reported to reduce the influx of Ca^{2+} into neurons (Mermelstein et al., 1996). An estrogen-induced reduction in intracellular Ca^{2+} would be expected to reduce activation of CaM kinases and/or PKC. In this case, an E_2-related decline in CREB would be expected but was not observed in the present study. Alternatively, an E_2-induced activation of cAMP-PKA pathway (Petitti et al., 1992) would be expected to activate CREB phosphorylation and increase the transcription of CREB itself in the neurons. In the present study, the observed increase in CREB-positive neurons suggests that estrogen may increase CREB expression in the hippocampal pyramidal cells.

Estrogens have been reported to have anticonvulsant and proconvulsant actions (Morell, 1992; Schultz-Krohn et al., 1986). In the present study, we observed that with estrogen treatment, only two of seven rats developed clonic seizure, while all OVX rats developed clonic seizure with insulin treatment. A plausible explanation for the anticonvulsant activity of estrogen with insulin treatment is that estrogens influence the extent of hypoglycemia induced by insulin. However, this is unlikely, since we have shown that estrogen pretreatment does not influence the plasma glucose levels at 60 min after administration of the 12.5-IU/kg dose of insulin (Rajakumar et al., 1995). An alternative possibility is that estrogen treatment enhances the extraction of low levels of plasma glucose during insulin-induced hypoglycemia, thereby preventing seizures. We have shown that estrogen enhances brain extraction of glucose (Bishop and Simpkins, 1995; Shi and Simpkins, 1997), apparently by increasing the number of GLUT1-type glucose transporters in the endothelial cells of the blood–brain barrier (Shi and Simpkins, 1997).

In conclusion, our results suggest that severe hypoglycemic episodes result in a reduction of CREB-positive cells, but that estrogen pretreatment may ameliorate this decline in CREB-positive cells.

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