

Award Number: DAMD17-99-1-9552

TITLE: Opening of the Mitochondrial Permeability Transition
Pore by Reactive Oxygen Species is a Basic Event in
Neurodegeneration

PRINCIPAL INVESTIGATOR: John Savory, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Chapel Hill, North Carolina 27599-1350

REPORT DATE: July 2003

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20031212 155

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2003	3. REPORT TYPE AND DATES COVERED Final Addendum (1 Jul 02 - 30 Jun 03)	
4. TITLE AND SUBTITLE Opening of the Mitochondrial Permeability Transition Pore by Reactive Oxygen Species is a Basic Event in Neurodegeneration			5. FUNDING NUMBERS DAMD17-99-1-9552	
6. AUTHOR(S) John Savory, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia Chapel Hill, North Carolina 27599-1350 E-Mail: Js2r@virginia.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) This addendum concludes the work on the neurotoxicity induced by Aβ which was an extension of our original work involving the administration of aluminum maltolate into rabbit brain. The intracisternal injection of Aβ(1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These Aβ effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that Aβ-induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau. In addition, signaling pathways activated by aluminum maltolate and Aβ injections into rabbit brain, are also activated by the injection of MPP⁺ which is a neurotoxin that profoundly affects dopaminergic neurons. This MPP⁺ model is of considerable value in understanding neuronal injury in Parkinson's disease. The most valuable information that has been obtained in our studies is that the endoplasmic reticulum, along with mitochondria, are immensely important in regulating cell death or survival via apoptosis mechanisms, following neurotoxic injury. We suggest that agents that prevent oxidative damage and endoplasmic reticulum stress will provide improved protection from aluminum, Aβ or MPP⁺-induced neuronal injury.				
14. SUBJECT TERMS Neurotoxin				15. NUMBER OF PAGES 91
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	9
Appendices.....	10

INTRODUCTION:

Our grant entitled "Opening of the mitochondrial permeability transition pore by reactive oxygen species is a basic event in neurodegeneration" was originally funded for the period July 1, 1999 through June 30, 2002. On July 27, 2001 we received supplemental funds of \$179,228 to enable us to expand our research effort. We used some of these funds before June 30, 2002 and the results of our studies are given in the Final Report submitted on July 22, 2002. The remainder of the funds allowed us to continue our research for a few more months and the results are provided in this addendum to our final report.

BODY

OUTLINE OF EXPERIMENTS:

- Our work has continued with our original objective to study mechanisms of neuronal injury and death using the intracisternal administration of neurotoxins to New Zealand white rabbits. Our original work focused on aluminum maltolate as the neurotoxin administered. This toxic agent produced severe neurotoxic injury and we were able to provide some of the first results describing the endoplasmic reticulum as a target for this neurotoxin with subsequent cell death via an apoptotic mechanism. We also were able to modulate the toxicity by two agents, lithium or glial cell-line derived neurotrophic factor (GDNF). The mechanism by which these two agents appear to act is via protection against both mitochondrial and endoplasmic reticulum stress.
- After describing these novel pathways of *in vivo* neurotoxic action we extended our experiments to evaluate the intracisternal administration of the peptide A β on the apoptosis pathways. Some of this work was described in our Final Report and is relevant to human neurodegeneration, since A β forms the core of neuritic plaques that are seen in Alzheimer's disease. It is also known that A β is neurotoxic, albeit not as toxic as the aluminum compounds. We have extended this work on A β and the results are summarized in the present Report.
- We also realized the potential of our mechanistic studies to assess neuronal death in the substantia nigra, which is particularly relevant to Parkinson's disease. We chose to evaluate the neurotoxin MPP⁺ which has been used as a model for Parkinson's disease, particularly in rats, mice and primates. Its use derived from the illegal use of the precursor drug, MPTP, several years ago and a drug which produced a distressingly high incidence of Parkinson's disease in human users. MPP⁺ is the active metabolite of MPTP. Our exciting new results using this Parkinson's model system are also summarized in the present Report.

SUMMARY OF RESULTS:

Lithium inhibits A β -induced stress in the endoplasmic reticulum in rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation

This work was described in the Final Report but had not yet been published. Publication has now been accomplished in the **Journal of Neuroscience Research** and a reprint is attached as **Appendix I**.

A β (1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus.

We have demonstrated that the intracisternal injection of A β (1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These A β effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that A β -induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau.

This work has been submitted to **Molecular Brain Research** and a copy of the manuscript is attached as **Appendix II**.

Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain

Loss of neurons is a hallmark of neurodegenerative disorders and there is increasing evidence suggesting that apoptosis is a key mechanism by which neurons die in these diseases. Mitochondrial dysfunction has been implicated in this process of neuronal cell death but there is a growing body of evidence also suggesting an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondria or in concert with mitochondrial-initiated pathways. Studies in our laboratory have focused on neuronal injury resulting from the administration of aluminum maltolate, via the intracisternal route, to New Zealand white rabbits. Treatment of rabbits with aluminum maltolate induces both mitochondrial and endoplasmic reticulum stress. Agents such as lithium or GDNF have the ability to prevent aluminum-induced neuronal death by interfering with the mitochondrial and/or the endoplasmic reticulum-mediated apoptosis cascade. This animal model system, involving neurotoxicity induced by an aluminum compound, provides new information on the mechanisms of neurodegeneration and neuroprotection.

This work formed the basis of an oral presentation at the **Fifth Keele Meeting on Aluminum** held in Stoke-on-Trent, United Kingdom, February 23-25, 2003. A manuscript is now in press in **The Journal of Inorganic Biochemistry** and is attached as

Appendix III. The Abstract that was accepted for presentation at the Conference is attached as **Appendix IV.**

MPP⁺ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of the ATF-6 and NF- κ B signaling pathways.

Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie MPTP/MPP⁺-induced neurotoxicity, used extensively as a model for Parkinson's disease. Signaling pathways utilized within other subcellular organelles have been less extensively explored. In the present study we have examined the hypothesis that MPP⁺ targets other cell compartments, including the endoplasmic reticulum. Because rabbits possess more genetic similarities to primates than to rodents we have selected this animal model system for our MPP⁺ neurotoxicity studies. MPP⁺ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. We demonstrate that MPP⁺ in a dose-dependent manner induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by the activation of ATF-6 and NF- κ B, leads to activation of gadd 153 and caspase-3. These effects correlate with activation of the JNK kinase signaling pathway as well as phosphorylation of one of its substrates, c-jun. Our results demonstrate that MPP⁺ directly targets the endoplasmic reticulum and is neurotoxic to rabbits by mechanisms that involve the activation of the endoplasmic reticulum stress response and JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

This work has been submitted for publication in the **Journal of Neuropathology and Experimental Neurology** and the paper is attached as **Appendix V**; an **Abstract (Appendix VI)** has been accepted for presentation at the **33rd Annual Meeting of the Society of Neuroscience** to be held in New Orleans, LA, November 2003.

Cellular mechanisms of neurodegeneration and Alzheimer's disease

A review of some aspects of our work was presented at the **Annual Meeting of the Association of Clinical Scientists** held in St Petersburg, FL, 7-11 May, 2003. The **Abstract** of this presentation is attached as **Appendix VII.**

Is amyloid β -peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions?

As stated in our Final Report, the editor of **Neurobiology of Aging** invited the P.I. to write a commentary on this topic. In this article we present our hypotheses concerning the interaction of A β and Al. This commentary is especially relevant in light of the

recent publication on the enhanced neurotoxicity of A β in A β -producing transgenic mice (1). A preprint of our commentary was originally attached as an Appendix in our Final Report and we include a reprint attached to this Addendum as **Appendix VIII**.

KEY RESEARCH ACCOMPLISHMENTS:

Most of our Key Research Accomplishments from this project were included in our Final Report. Here we list additional Accomplishments for the past year.

- JNK and ERK kinase activation may mediate the A β -induced apoptosis; lithium and GDNF, although targeting these cellular signaling pathways in different ways, prevent the A β -induced caspase activation.
- MPP⁺ directly targets the endoplasmic reticulum and is neurotoxic to rabbits by mechanisms that involve the activation of the endoplasmic reticulum stress response and JNK kinase.
- We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

REPORTABLE OUTCOMES:

In the Final Report we have listed thirteen full-length papers and ten abstracts that resulted from the project supported by the Department of Defense. Here we list four additional full-length papers either published or submitted, together with four Abstracts. One full-length paper which was included in our Final Report is also included in this Addendum since we now have the final reprint.

Papers:

Ghribi O, Herman MM, Savory J. Lithium inhibits A β -induced stress in endoplasmic reticulum of rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation. *J Neurosci Res* 71: 853-862, 2003 (Appendix I).

Ghribi O, Herman MM, Pramoongago P, Savory J. A β (1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus. *Molecular Brain Research*, submitted (Appendix II).

Ghribi O, Herman MM, Savory J. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. *J Inorg Biochem*, in press (Appendix III).

Ghribi O, Herman MM, Pramoongago P, Savory J. MPP⁺ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF- κ B signaling pathways. *J Neuropathol Exp Neurol*, submitted (Appendix V).

Savory J, Ghribi O, Herman MM. Is amyloid β -peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions? (invited commentary) *Neurobiol of Aging*, 23: 1089-1092. 2002, (Appendix XIII). This paper was included in our Final Report as a preprint. The present Appendix XIII is a reprint.

Abstracts:

Ghribi O, Herman MM, Savory J. A β induces hyperphosphorylation of tau and apoptosis in rabbit brain, involving activation of the GSK-3 β and JNK pathways. Society for Neuroscience 32th Annual Meeting, Orlando, FL, November 2-7, 2002, (Appendix IX).

Savory J, Ghribi O, Herman MM. Aluminum-induction of endoplasmic reticulum-specific apoptosis in experimental neurodegeneration and its reversal by lithium. Presented at the 5th Keele Meeting on Aluminum, Stoke-on-Trent, United Kingdom, 22nd-25th February, 2003, (Appendix IV).

Ghribi O, Herman MM, Pramoonjago P, Savory J. MPP⁺ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF- κ B signaling pathways. To be presented at the 33rd Annual Meeting of the Society of Neuroscience to be held in New Orleans, LA; November 2003, (Appendix VI).

Savory J, Ghribi O, Herman MM. Cellular mechanisms of neurodegeneration and Alzheimer's disease. Presented at the Annual Meeting of the Association of Clinical Scientist held in St Petersburg, FL, 7-11 May, 2003, (Appendix VII).

CONCLUSIONS:

An extensive discussion of our conclusions was included in our Final Report. To these conclusions we now add that the intracisternal injection of A β (1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These A β effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. Treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that A β -induced apoptosis and the phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau. In addition, signaling pathways activated by aluminum maltolate and A β injections into rabbit brain are also activated by the injection of MPP⁺, which is a neurotoxin that profoundly affects dopaminergic neurons. This MPP⁺ model is of considerable value in understanding neuronal injury in Parkinson's disease. The most valuable information that has been obtained in our studies is that following neurotoxic injury, the endoplasmic reticulum, along with mitochondria, are immensely important in regulating cell death or survival via apoptosis mechanisms. We suggest that agents that prevent oxidative damage and endoplasmic reticulum stress will provide improved protection from neuronal injury induced by aluminum, A β or MPP⁺.

SO WHAT:

We now have a better understanding of how brain cells survive or die following the administration of three important neurotoxins. Aluminum is highly toxic and is the most common metal in the environment. A β is deposited as neuritic plaques in Alzheimer's disease and its neurotoxicity is almost certainly relevant to understanding the pathogenesis of this devastating neurodegenerative disorder. MPP⁺ is also highly neurotoxic and destroys dopaminergic neurons, a consequence of which are clinical symptoms of Parkinson's disease. We now know that both lithium and GDNF can ameliorate the toxicity induced by aluminum, A β , and probably by MPP⁺, although the latter studies have not been carried out in our animal model system. We have a solid basis of evidence to support the use of these treatments in neurotoxin-exposed personnel.

REFERENCES CITED:

1. Pratico D, Uryu K, Sung S, Tang S, Trojanowski JQ, Lee VM. Aluminum modulates brain amyloidosis through oxidative stress in APP transgenic mice. *FASEB J* 2002;16:1138-40.

Note: An extensive list of references is included in Appendices I, II, III and V.

Personnel receiving salary support from this grant:

John Savory, Ph.D.

Othman Ghribi, Ph.D.

Michael S. Forbes, Ph.D.

James C. Boyd, M.D.

Myxolydia Tyler

Natalie Spaulding

Lithium Inhibits A β -Induced Stress in Endoplasmic Reticulum of Rabbit Hippocampus But Does Not Prevent Oxidative Damage and Tau Phosphorylation

Othman Ghribi,¹ Mary M. Herman,³ and John Savory^{1,2*}

¹Department of Pathology, University of Virginia, Charlottesville, Virginia

²Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia

³IRP, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

The goal of this study was to assess the *in vivo* effect of A β on apoptosis pathways involving the endoplasmic reticulum and mitochondria, and its relationship to the induction of tau phosphorylation and DNA oxidative damage. In rabbits treated intracisternally with aggregated A β (1–42), clear evidence of endoplasmic reticulum stress was observed by the activation of caspase-12 and cleavage of caspase-3 in the endoplasmic reticulum. Mitochondrial injury was evident from the release of cytochrome c into the cytosol and the induction of oxidized mitochondrial DNA. Tau phosphorylation and nuclear translocation of NF- κ B and GSK-3 β were also observed. Treatment with lithium, an inhibitor of GSK-3 β , inhibited caspase activation but did not prevent mitochondrial DNA damage or tau hyperphosphorylation, suggesting that the translocation of GSK-3 β may represent an upstream event that leads to caspase activation but is unrelated to tau hyperphosphorylation or mitochondrial DNA oxidative damage. We propose that treatment by lithium alone is not sufficient to protect against the multiple adverse effects of A β , and the use of agents that prevent oxidative DNA damage and tau hyperphosphorylation, together with lithium, may provide better protection from the neurotoxic effect of A β .

© 2003 Wiley-Liss, Inc.

Key words: caspase 3; caspase 12; cytochrome c; GSK-3 β ; NF- κ B

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of extracellular neuritic plaques (NPs), the formation of neurofibrillary tangles (NFTs), and massive neuronal and synaptic loss. A β is the major component of NPs, with hyperphosphorylated tau being the primary constituent of NFTs; both of these aggregates are generally accepted as being key players in the pathogenesis of AD. Whether A β or tau actually represents the primary cause of neuronal loss in AD, and which of the two abnormal changes is generated first and is presumably the more important, remains a matter of

debate. Transgenic mice with mutated amyloid precursor protein, the precursor of A β , can develop A β deposits (Duff et al., 1996; Citron et al., 1997; Gau et al., 2002) but NFTs have not been detected (Games et al., 1995; Gau et al., 2002). Also, transgenic mice over-expressing mutant tau do not systematically exhibit NP aggregates (for review see Mudher and Lovestone, 2002). There is convincing evidence that NPs and NFTs have some association, however, because transgenic mice over-expressing both mutant amyloid precursor protein and mutant tau exhibit marked neurofibrillary degeneration (Lewis et al., 2001). In accord with these findings is the report that injection of A β fibrils in P301L tau transgenic mice enhances the formation of NFTs (Gotz et al., 2001).

In vitro studies using cultured cells exposed to synthetic A β have demonstrated evidence of tau phosphorylation and neurodegeneration (Takashima et al., 1993, 1998; Alvarez et al., 1999). These latter results suggest that A β is the trigger of tau accumulation and neuronal death. It has been suggested that the mechanism by which A β induces tau hyperphosphorylation involves the activation of glycogen synthase kinase-3 β (GSK-3 β), an enzyme that plays a crucial role in signaling processes involved in psychiatric diseases such as bipolar disorder, and in neurodegenerative diseases such as Alzheimer's (for review see Grimes and Jope, 2001). Lithium, a medication for bipolar disorder, is a direct inhibitor of GSK-3 β (Klein and Melton, 1996), and has been demonstrated to prevent hyper-

Contract grant sponsor: United States Department of the Army; Contract grant number: DAMD 17-99-1-9552; Contract grant sponsor: Alzheimer's and Related Diseases Research Award Fund, Virginia Center on Aging, Virginia Commonwealth University; Contract grant number: 02-3.

*Correspondence to: Dr. John Savory, Department of Pathology, University of Virginia Health Sciences Center, Box 800214, Charlottesville, Virginia 22908. E-mail: js2r@virginia.edu

Received 10 August 2002; Revised 3 October 2002; Accepted 7 October 2002

Published online 00 Month 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.10511

phosphorylation of tau (Munoz-Montano et al., 1997) and the A β -induced degeneration of cultured neurons (Alvarez et al., 1999).

In addition to plaque deposition and tangle formation, there are other possible pathological hallmarks of AD, including oxidative stress (for review see Smith et al., 2000; Butterfield et al., 2001) or perturbed endoplasmic reticulum function (for review see Mattson et al., 2001; Mattson and Chan, 2001). It has been shown in mouse cortical neurons that A β triggers an endoplasmic reticulum-specific apoptosis mediated by active caspase-12, which may activate effector caspases such as caspase-3 (Nakagawa et al., 2000). Additionally, the transcription factor NF- κ B seems to be linked to A β -evoked neuronal loss as well as to GSK-3 β pathways (Hoefflich et al., 2000; Javelaud and Besancon, 2001; Tang et al., 2001).

We have used an *in vivo* system, involving the administration of aggregated A β (1–42) peptide into rabbit brain via the intracisternal route, which we have observed to induce hyperphosphorylation of tau, oxidative stress, and perturbation of the endoplasmic reticulum. We have further treated these animals with lithium, an inhibitor of GSK-3 β (Bijur et al., 2000), which we have shown recently to inhibit aluminum-induced apoptosis in rabbit hippocampus (Ghribi et al., 2002b). We applied this treatment to determine if lithium inhibits A β -induced tau hyperphosphorylation and apoptosis.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies (mAbs) to caspase-3/CPP32 and calnexin were obtained from Transduction Laboratories (Lexington, KY). Caspase-12 mAb was a gift from Dr. Junying Yuan (Harvard Medical School, Boston, MA); cytochrome c, cytochrome c oxidase subunit IV, NF- κ B p50 and NF- κ B p65, GSK-3 β , p-Tyr²¹⁶ GSK-3 β , p-Ser⁹ GSK-3 β , and histone H1 were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and the anti-human PHF tau /AT8 mAb was supplied by Innogenetics (Ghent, Belgium).

Animals, Treatment Protocol, Clinical Monitoring and Tissue Collection

Adult female New Zealand white rabbits (3–4 years weighing 4–5 kg) received either intracisternal injections of 100 μ L normal saline (controls; $n = 6$), 100 μ L of 400 μ M A β (1–42) ($n = 6$; A β -treated group), or lithium and 100 μ L of 400 μ M A β (1–42) (lithium/A β -treated group; $n = 6$). A β (1–42), obtained from American Peptide Company (Sunnyvale, CA), was prepared by incubating freshly solubilized A β (1–42) in saline at 37°C for 3 days. The intracisternal injections were carried out over a period of 2 min under ketamine anesthesia as described previously (Ghribi et al., 2001a). Lithium carbonate (Sigma, St. Louis, MO) was administered orally by adjusting the drinking water to a concentration of 7 mM as described previously (Ghribi et al., 2002b). This concentration of lithium in the drinking water yields a serum concentration of 0.65 ± 0.35 mM (mean $\pm$ SEM), which is at the lower end of the therapeutic

range used in humans (Moyer, 1999). Lithium was started 14 days before A β (1–42) administration and continued until animal sacrifice. All rabbits were sacrificed 7 days after the intracisternal administration, and at necropsy were perfused with Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY) at 37°C. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Animal Care and Use Committee.

Western Blot Analysis

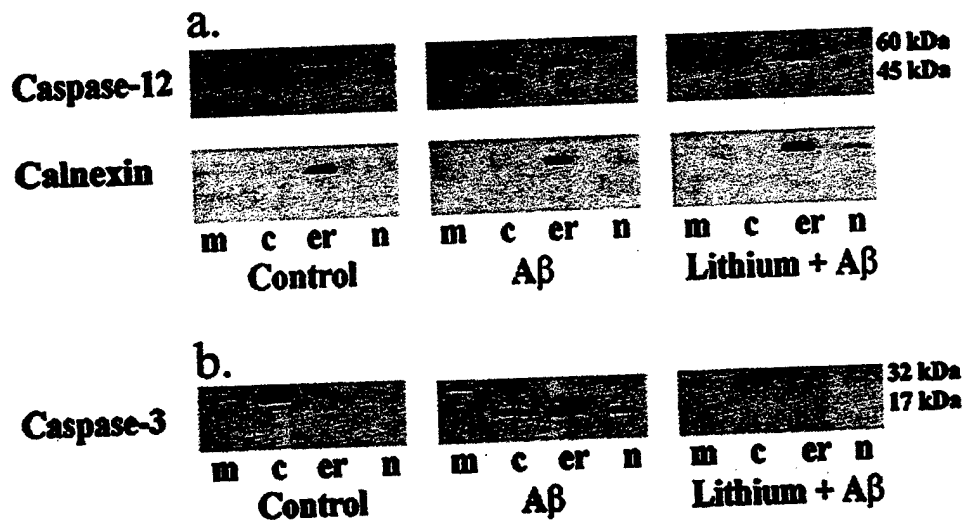
Proteins from enriched fractions of nuclei, mitochondria, cytosol and microsomes were extracted as described previously (Ghribi et al., 2001b). Tissue from the entire hippocampus was homogenized gently using a Teflon homogenizer (Thomas, Philadelphia, PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/ml pepstatin and 12.5 mg/ml of *N*-acetyl-Leu-Leu-Norleu-Al). The nuclear fraction was the first pellet (750 $\times$ g, 10 min) and the mitochondrial fraction was the second (8,000 $\times$ g, 20 min). The supernatant was further centrifuged at 100,000 $\times$ g for 60 min at 4°C to separate the cytosolic (soluble) from the microsomal (pelleted) fractions. The microsomal fraction is referred to as endoplasmic reticulum-enriched fraction, although vesicles and other membrane components are also included in this fraction, as confirmed previously by electron microscopy (Ghribi et al., 2002a). Protein concentrations in all fractions were determined with the BCA protein assay reagent (Pierce, Rockford, IL).

Proteins (5 μ g) from the nuclear, mitochondrial, cytosolic and endoplasmic reticulum-enriched fractions were separated by SDS-PAGE (10% gel), then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and incubated with mouse mAb to cytochrome c at a dilution of 1:500, caspase-3 (1:200), caspase-12 (1:10), NF- κ B p65 and NF- κ B p50 (1:250), GSK-3 β , p-Tyr²¹⁶ GSK-3 β and p-Ser⁹ GSK-3 β (1:500). Cytochrome c oxidase, calnexin, and histone H1 were used as markers for mitochondria, endoplasmic reticulum, and nuclei, respectively, and were diluted at 1:250. The blots were developed with enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA) and bands were analyzed by densitometry with Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemistry

Oxidative DNA damage. We used the OxyDNA assay kit (Calbiochem, San Diego, CA), which is based on the direct binding of a fluorescent probe to 8-oxoguanine moieties in the DNA of fixed tissue. Frozen sections (14 μ m thick) at the mid-hippocampal level from control, A β (1–42)-treated and lithium/A β (1–42)-treated animals were air dried, fixed in ice-cold 4% paraformaldehyde in PBS for 15 min, washed briefly in TBS/Tween 20 (provided in the kit) and permeabilized in ice-cold 99% methanol for 30 min. After two washes, the sections were incubated with the provided blocking solution for

Fig. 1. A β induces endoplasmic reticulum stress. **A:** Caspase-12 predominantly resides in the endoplasmic reticulum fraction (er) as procaspase-12 (60 kDa) in control, A β -treated, and lithium/A β -treated animals. A β induces release of the active form of caspase-12 into the cytosol (c) and lithium prevents the A β -induced caspase-12 activation. Calnexin, used as an endoplasmic reticulum marker, stains predominantly the endoplasmic reticulum fractions (er). **B:** Pro-caspase-3 (32 kDa) is localized mainly in the cytosolic fractions (c) of control, A β -treated and lithium/A β -treated rabbits. Active caspase-3 (p17) is detected in the A β -treated animals only, and is localized in the endoplasmic reticulum (er) > cytosolic (c) > nuclear (n) > mitochondrial (m) fractions.



30 min at 37°C for 1 hr, washed twice in PBS, incubated overnight at 4°C with a 1:50 dilution of the FITC-conjugate concentrate that contains the binding protein conjugated to fluorescein, again washed twice in PBS, and incubated for 2 hr at 37°C at 1:500 dilution with a mitochondrial marker, Mito-Tracker red (Molecular Probes, Eugene, OR). After two washes in PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing the nuclear stain 4',6'-diamidino-2-phenylindole (DAPI), and observed under a fluorescent microscope using excitation and emission wavelengths of 365 and 490 nm, respectively (Olympus BH2, Melville, NY).

TUNEL assay. Frozen sections similar to those above were fixed and permeabilized as described previously (Henshall et al., 2000). Detection of DNA fragmentation was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique. In brief, tissue sections from control, A β (1-42)-treated and lithium/A β (1-42)-treated animals were dried for 15 min at room temperature and fixed in 10% formalin for 15 min, followed by a 10 min incubation in 1:2 vol/vol ethanol/acetic acid. Sections were washed three times in PBS for 5 min each, permeabilized with 3% Triton X-100 for 20 min and immersed in 3% hydrogen peroxide for 15 min. Sections were then washed three times in PBS buffer for 5 min each and processed for apoptosis detection using an Apoptosis Detection System, Fluorescein (Promega, Madison, WI). Five fields were captured from the CA1 region of the hippocampus from each animal, and results were compared between A β -treated and lithium/A β -treated animals. Results from the A β -treated rabbits were assigned a value of 100% and the lithium effect was expressed as the percent reduction in the number of positive neurons in the lithium/A β group when compared to the A β -treated rabbits.

Phosphorylated tau. Sections as above from control, A β (1-42)-treated and lithium/A β (1-42)-treated animals cut at the level of the hippocampus, were air-dried at room temperature, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of

1.5% normal serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse mAb against PHF tau (AT8) at 1:100 dilution. After washing with PBS and incubating with the biotinylated secondary antibody, sections were processed with a Vectastain Elite avidin-biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining.

RESULTS

Lithium Treatment Prevents A β -Induced Endoplasmic Reticulum Stress

Pro-caspase-12 (~60 kDa) was detected primarily in the endoplasmic reticulum from all rabbits (controls, A β -treated and lithium/A β -treated), as shown in Figure 1A. In A β -treated animals, a cleavage product band (~40 kDa), corresponding to activated caspase-12, was released from the endoplasmic reticulum into the cytosol; treatment by lithium fully prevented this release. Calnexin mAb, a specific marker for endoplasmic reticulum, stained this subcellular fraction only (Fig. 1A). Illustrated in Figure 1B is the finding that procaspase-3/p32 is expressed mainly in the cytosolic fractions from control, A β -treated and lithium/A β -treated animals. Caspase-3 p17, an activated form of caspase-3, although not detectable in controls, was present as an intense band in the endoplasmic reticulum and to a lesser extent in the cytosol and nuclear fractions of A β -treated animals. Treatment with lithium prevented the formation of active caspase-3 p17.

Lithium Treatment Inhibits A β -Induced Mitochondrial Cytochrome C Release

Cytochrome c, localized chiefly in the mitochondrial-enriched fractions and to a lesser extent in the endoplasmic reticulum fractions in the controls, was released into the cytosol after A β administration; lithium treatment inhibited this A β -induced translocation of cytochrome c (Fig. 2A).

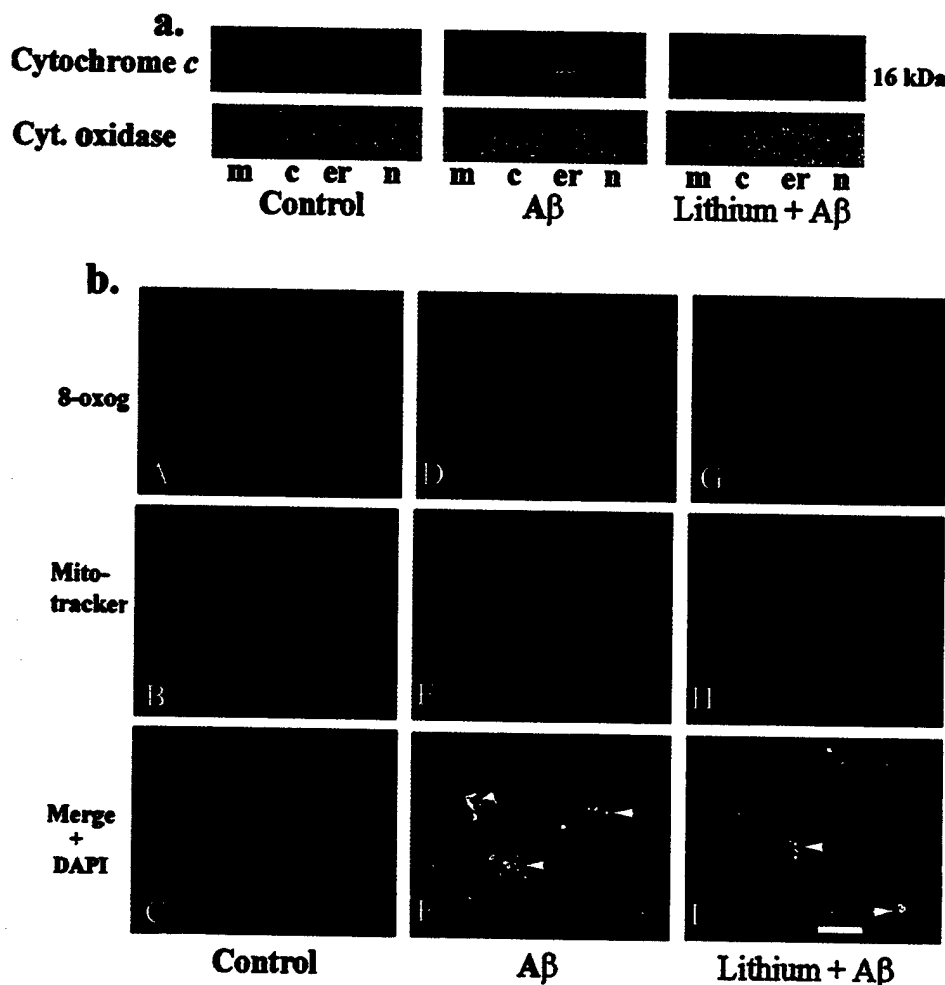


Fig. 2. A β induces mitochondrial stress. **A:** Cytochrome c, whereas detected in the mitochondrial (m) and to a lesser extent in the endoplasmic reticulum (er) fractions in controls, is present in the cytosolic (c) fraction after A β treatment; this translocation is largely prevented by lithium. Cytochrome c oxidase, used as marker for the mitochondria, stains only the mitochondrial fraction. **B:** Representative immunofluorescence photomicrograph at the mid-hippocampal level showing triple staining with an 8-oxoguanine mAb (green, A,D,G), which is a marker for oxidative DNA damage. MitoTracker (red, B,E,H) is used as a mitochondrial marker and DAPI (blue, C,F,I) for nuclei. Tissue from controls are depicted in the left panels (A–C), A β -treated in the center panels (D–F) and lithium/A β -treated in the right panels (G–I). In A β -treated animals, there is the emergence of positive staining for oxidative damage (D), an effect that is not prevented by lithium (G). Staining with 8-oxoguanine is primarily confined to mitochondria, represented as positive small round structures depicted by the arrows (F,I). Scale bar = 20 μ m.

Cytochrome c oxidase subunit IV, used as a specific marker for mitochondria, confirmed the integrity of the mitochondrial fractions.

Lithium Treatment Does Not Inhibit A β Induced Oxidative Stress in Mitochondria

Whereas sections from controls did not exhibit 8-oxoguanine immunoreactivity (Fig. 2B, panel A), sections from A β -treated animals showed high immunoreactivity to this antibody (Fig. 2B, panel D) and lithium treatment did not prevent the A β -induced oxidative DNA damage (Fig. 2B, panel G). MitoTracker, applied as a mitochondrial marker (Fig. 2B, panels B, E, H), and DAPI as a nuclear marker (Fig. 2B, panels C, F, I) demonstrated that 8-oxoguanine immunoreactivity was localized predominantly in the mitochondria (small round foci) in A β -treated (Fig. 2B, panel F) and in lithium-treated (Fig. 2B, panel I) animals.

Lithium Treatment Decreases A β -Induced TUNEL Positivity

Sections from control animals treated with DNase I (positive control) showed widespread TUNEL positivity

in the pyramidal layer (CA1) of the hippocampus (Fig. 3, panel A), whereas in a similar area in a representative section from the control animals there were no TUNEL-positive cells (Fig. 3, panel B). A β administration induced TUNEL positivity in this same region of the hippocampus (Fig. 3, panel C), which was reduced by lithium treatment (Fig. 3, panel D). Quantitative analysis of TUNEL positive cells at a magnification of 400 $\times$ showed that the A β -induced TUNEL staining was reduced markedly by lithium treatment (Fig. 3, graph E).

Lithium Treatment Inhibits A β -Induced Translocation of NF- κ B Into the Nucleus

The antibody to NF- κ B p65 yielded two bands corresponding to p100 and p65, and the antibody to NF- κ B p52 exhibited a single band; these were observed in the cytosolic fraction of controls, A β -treated and in lithium/A β -treated animals. Both gels exhibited all three bands, as seen in Figure 4. In A β -treated animals, the bands corresponding to p65 and p52 were also detected in the nuclear fractions, indicating NF- κ B activation and translocation into the nucleus. In lithium-treated animals,

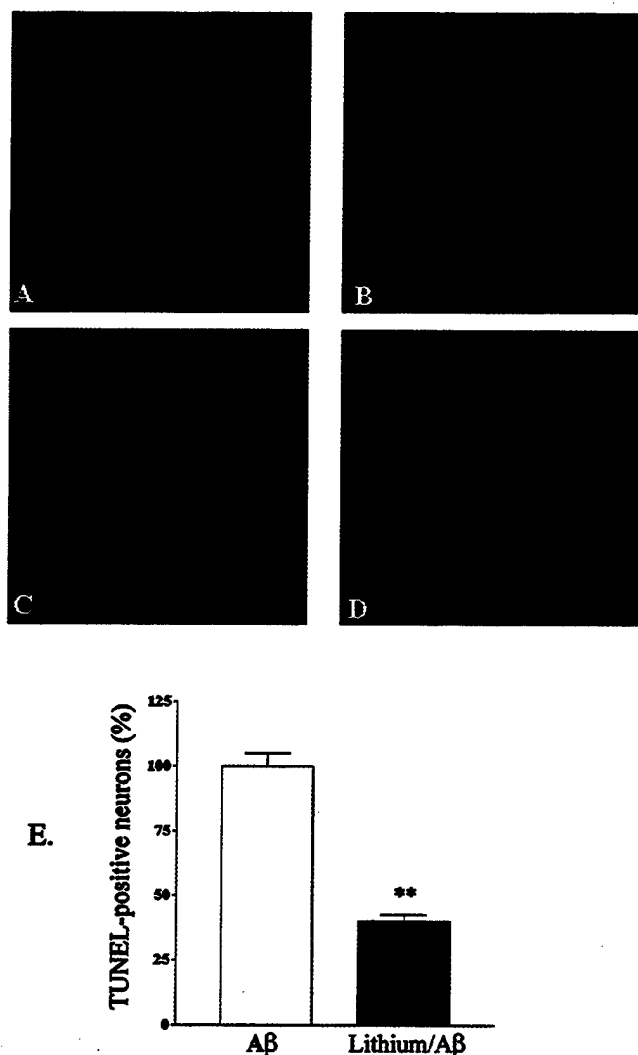


Fig. 3. A β -induced TUNEL positivity. A positive control section stained with DNase shows widespread TUNEL-positive staining in the pyramidal layer (CA1) of the hippocampus (A), whereas in a similar area in the control untreated group no TUNEL positive cells are seen (B). A β administration induces TUNEL-positive cells in the same region of the hippocampus (C) and lithium treatment reduces the emergence of this A β -induced TUNEL-positivity (D). Quantitative analysis (E), carried out on one section from each animal of the A β -treated and lithium/A β -treated animals, confirms the neuroprotective effect of lithium because there is a reduction in the number of TUNEL-positive neurons in comparison to A β -treated animals (panels A–D, 400 $\times$; ** P < 0.01, Student's t -test).

the p65 was barely detectable and the p52 band was reduced in comparison to A β -treated animals (Fig. 4).

Lithium Treatment Does Not Affect A β -Induced Phosphorylation of Tau

Immunohistochemical staining with the AT8 mAb demonstrated a lack of reactivity in control sections (Fig. 5A,D,G) but intense reactivity in the pyramidal cell layer of the hippocampus and the dentate gyrus after A β treat-

ment (Fig. 5B,E,H), an effect that was not prevented by treatment with lithium (Fig. 5C,F,I).

Lithium Prevents A β -Induced Translocation of GSK-3 Into the Nucleus

Western blot analysis of GSK-3 in controls demonstrated two intense bands in the cytosolic fraction and two faint bands in the nuclear fraction corresponding to α and β isoforms (Fig. 6). After A β treatment, the nuclear bands increased markedly; lithium prevented the translocation of GSK-3 β and fully abolished the presence of GSK-3 α in the nucleus (Fig. 6). These treatments had no effect on GSK-3 β phosphorylated at Ser⁹ or Tyr²¹⁶.

DISCUSSION

Injection of aggregated A β into the brain of experimental animals may represent a valuable tool for studying the direct neurotoxic effect of this peptide. Intracerebral injection of A β has been demonstrated to produce dose-dependent cortical lesions in primates (McKee et al., 1998), and tau phosphorylation and neuronal loss in the common marmoset (Geula et al., 1998); however, these A β effects were not observed in younger animals or in rat brain. The use of rabbits may be relevant to investigations of AD pathogenesis to enhance information obtained from transgenic animals, aged primates and cell culture, because rabbits more closely resemble primates than they do rodents (Graur et al., 1996) and, like AD patients, develop eye-blink conditioning impairment (Woodruff-Pak and Trojanowski, 1996).

In the present study, A β (1–42) produces a wide variety of events, namely stress in the endoplasmic reticulum, oxidative damage at the mitochondrial level, and hyperphosphorylation of tau. Lithium treatment, although preventing the endoplasmic reticulum stress-evoked caspase-12 release and activation of caspase-3, has no preventative effect on oxidative mitochondrial DNA damage or on the hyperphosphorylation of tau.

Disturbances of endoplasmic reticulum function have been shown to trigger a distinct apoptotic pathway involving the activation of caspase-12 (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). Upon specific endoplasmic reticulum stress, procaspase-12, which resides in the endoplasmic reticulum, is released into the cytoplasm as active caspase-12. Once activated, caspase-12 may trigger the activation of effector caspases, such as caspase-3, and may initiate apoptosis. Studies by Nakagawa et al. (2000) and Nakagawa and Yuan (2000) with mouse cortical neurons have shown that A β (1–40) activates caspase-12, and that reduction in caspase-12 provides protection from apoptosis.

Our results demonstrate that the administration of A β (1–42) peptide also activates caspase-12, and cleaves the apoptosis initiator, caspase-3, within the endoplasmic reticulum. These results represent the first report describing the endoplasmic reticulum as the main site for active caspase-3 after A β administration in vivo, although we have recently reported that the endoplasmic reticulum is the major site of localization of active caspase-3 after the

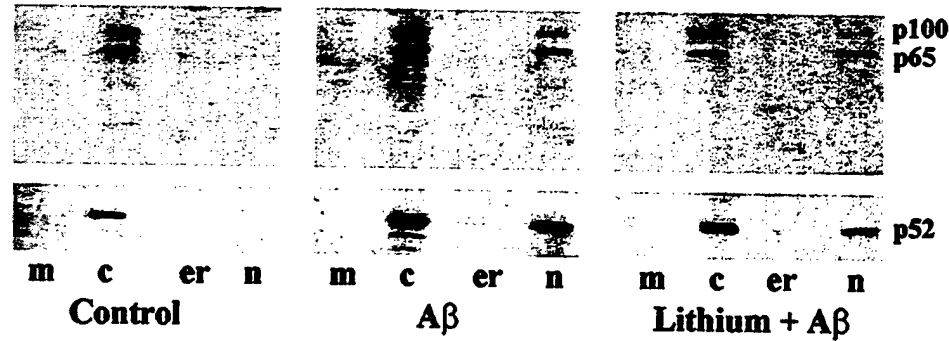
NF- κ B

Fig. 4. A β -induced NF- κ B activation. NF- κ B is composed of p100, p65, and p52, and the corresponding bands are positive in the cytosolic fractions (c) from control, A β -treated, and lithium/A β -treated animals. Upon activation by A β , p65 and p52 partially translocate to the nucleus (n); lithium reduces translocation of p65 and, to a lesser extent, p52.

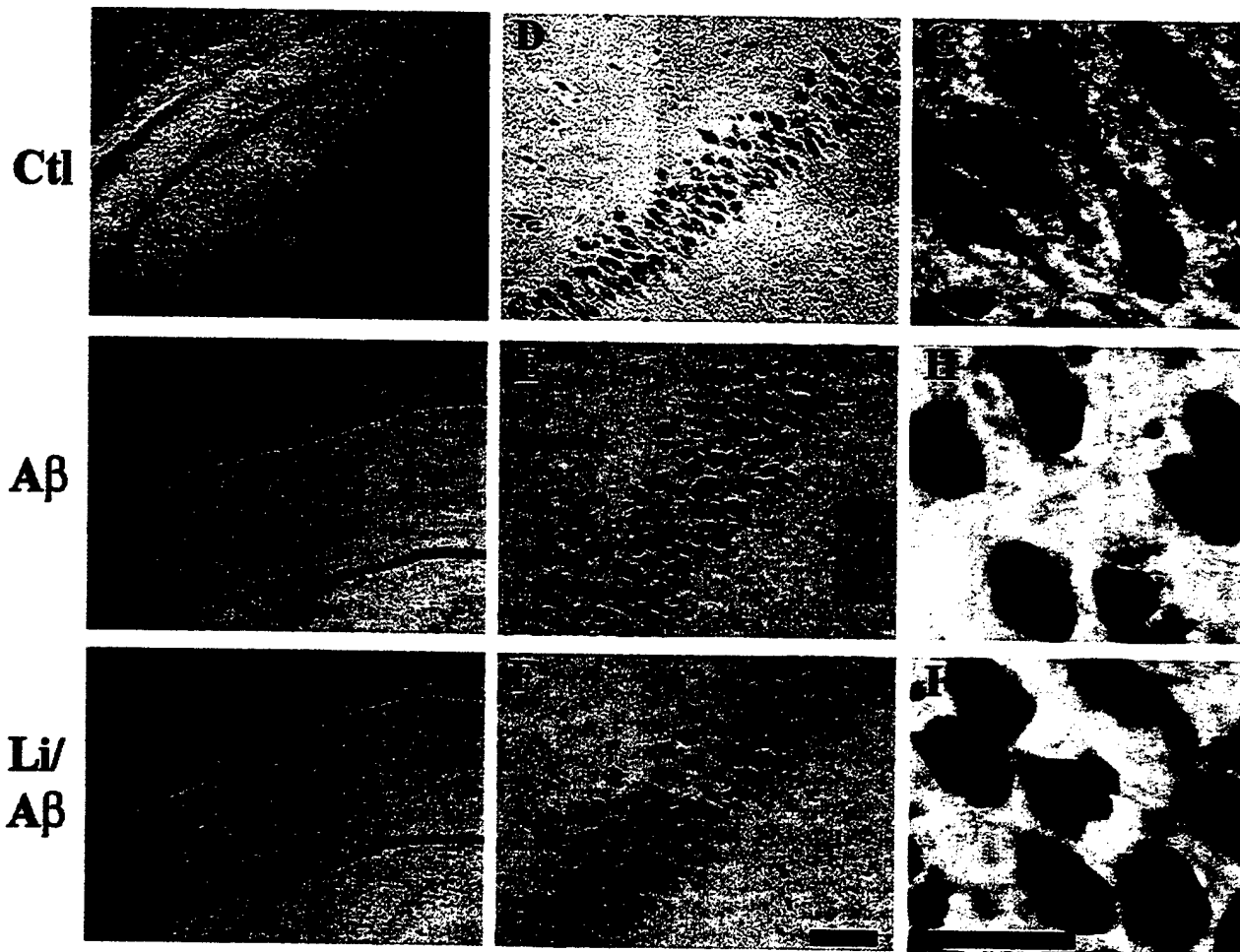


Fig. 5. A β -induces tau hyperphosphorylation. Photomicrographs of sections from the CA1 pyramidal cell layer of the hippocampus from control (A,D,G), A β -treated (B,E,H) and lithium/A β -treated (C,F,I) animals, stained with the AT8 mAb, which specifically detects hyperphosphorylated tau. No immunoreactivity is observed in the control; however, A β -treatment induces an intense immunoreactivity

for hyperphosphorylated tau in the CA1 layer and dentate gyrus (B), and lithium treatment does not prevent this hyperphosphorylation from occurring (C). D–F: Photomicrographs of the CA1 pyramidal cell layer. G,H,I: Higher magnifications of a segment of this area. Scale bar = 40 μ m (A–C); 50 μ m (D–F); 10 μ m (G–I).

intracisternal administration of the neurotoxin aluminum maltolate into rabbit brain (Ghribi et al., 2002a). In other studies, A β has been shown to induce caspase-3 activation and apoptosis in cerebral granule cells (Allen et al., 2001).

Telencephalic neurons deficient in caspase-3 are not protected from A β -induced death, however, indicating that caspase-3 does not mediate A β neurotoxicity (Selznick et al., 2000). In another study, although A β robustly activates

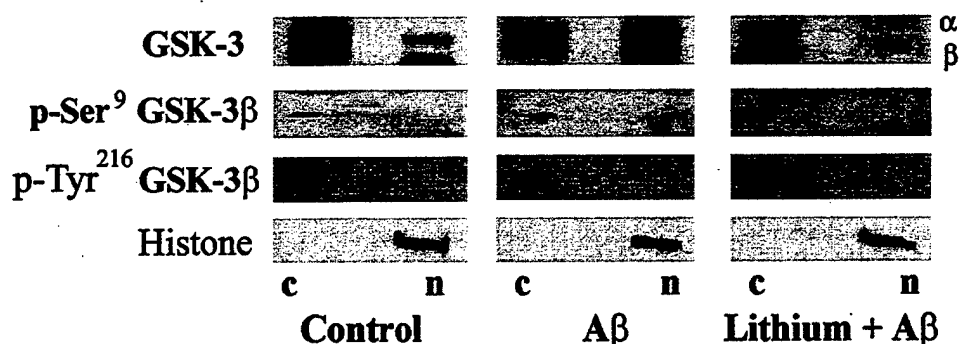


Fig. 6. A β induces translocation of GSK-3 into the nucleus. Cytosolic (c) and nuclear (n) fractions were separated from hippocampal tissue of control, A β -treated and lithium/A β -treated rabbits and Western blot analysis was carried out for GSK-3 β (the mAb used also recognizes the α isoform), and for Tyr²¹⁶ and p-Ser⁹ GSK-3 β . A β induces translocation of GSK-3 into the nucleus, which is fully prevented by lithium. In the control, p-Tyr²¹⁶ GSK-3 β and p-Ser⁹ GSK-3 β are confined to the cytosol and are not affected by A β or lithium treatment.

caspase-3 in rat cortical neurons, the A β neurotoxic effect is independent from this caspase activation, because caspase inhibitors fail to protect these neurons when exposed to A β (Saez-Valero et al., 2000).

The role of oxidative damage in the pathogenesis of AD has been examined in a number of studies (for review see Smith et al., 2000; Butterfield et al., 2001). Oxidative damage, as detected by an increase in the oxidized base 8-hydroxy-2'-deoxyguanosine (8-OHdG), has been found in postmortem AD brains (Gabbita et al., 1998; Lovell et al., 1999, 2000; Markesbery and Carney, 1999). Furthermore, mitochondrial DNA has been shown to be more sensitive to oxidative damage than nuclear DNA (Mecocci et al., 1994) and increased levels of 8-OHdG are accompanied by a decrease in mitochondrial 8-oxoguanine DNA glycosylase (hOGG1-2a), the enzyme that repairs 8-OHdG damage (Iida et al., 2002). Our results also demonstrate that damage to DNA occurs mainly in mitochondria.

The inducible transcription factor, NF- κ B, is an important mediator of the human immune and inflammatory response, and increased levels of NF- κ B activity have been observed in the brain of patients with various neurodegenerative disorders, including AD (Kaltschmidt et al., 1997). NF- κ B has been demonstrated to promote neuronal survival in some cases, and in other cases to promote neuronal death. Inhibition of NF- κ B may potentiate A β -mediated neuronal apoptosis (Kaltschmidt et al., 1999) whereas in other reports, NF- κ B activation has been demonstrated to mediate A β -induced neurotoxicity (Bales et al., 1998). In global ischemia and traumatic spinal cord injury, NF- κ B has been shown to promote neuronal death (for review see Mattson et al., 2000). Also, stress in the endoplasmic reticulum induced by a variety of stimuli may activate NF- κ B (Guerrini et al., 1995; Pahl and Baeuerle, 1995; Pahl et al., 1996). The present study demonstrates that NF- κ B is activated in response to the administration of A β (1-42). Whether translocation of NF- κ B into the nucleus represents a cellular defensive or

a deleterious mechanism after A β -induced ER stress and caspase-3 activation remains to be determined.

The controversy remains as to whether A β deposition or the accumulation of phosphorylated tau in NFTs is the primary cause of neurodegeneration in AD. The ability of A β to induce tau phosphorylation suggests that in AD, A β may lead to the generation of hyperphosphorylated tau and that tau accumulation may exacerbate the deleterious effect of A β ; however, it has been demonstrated that tau phosphorylation is not associated with apoptosis in SH-SY-5Y human neuroblastoma cells treated with A β (Ekinci et al., 2000). The mechanism by which A β induces tau hyperphosphorylation remains unclear. GSK-3 β is a physiological enzyme that has been considered as a candidate for tau phosphorylation and neurodegeneration in AD, and tau hyperphosphorylation in cultured cells induced by exposure to A β is attenuated by inhibition of GSK-3 β activity (for review see Mudher and Lovestone, 2002). GSK-3 β is subject to multiple regulatory mechanisms, among which phosphorylation has been the most widely studied. The activity of GSK-3 β is increased by phosphorylation of Tyr²¹⁶ (Wang et al., 1994; Hartigan and Johnson, 1999; Lesort et al., 1999); conversely, activity of GSK-3 β is decreased by phosphorylation at Ser⁹ (Plyte et al., 1992). In addition to regulation by phosphorylation, GSK-3 β function can be regulated by the ability of GSK-3 β to translocate from the cytoplasmic space into the nucleus. In the human neuroblastoma cell line, SH-SY-5Y, it has been demonstrated recently that nuclear levels of GSK-3 β are increased rapidly after exposure of cells to different apoptotic stimuli, such as serum-free media, heat shock and staurosporine (Bijur and Jope, 2001). Interestingly, these treatments increase nuclear levels of GSK-3 β , independent of phosphorylation at Ser⁹ or Tyr²¹⁶.

We demonstrate that A β induces translocation of GSK-3 β into the nucleus, and this translocation does not affect the p-Ser⁹ GSK-3 β or p-Tyr²¹⁶ GSK-3 β . Lithium

treatment completely prevents the nuclear translocation of GSK-3 β and has no effect on p-GSK-3 β . Interestingly, inhibition of GSK-3 β translocation by lithium is accompanied by inhibition of caspase-12 and caspase-3 activation at the endoplasmic reticulum level. These results are consistent with results obtained in the SH-SY5Y cell line showing that activation of caspases is a downstream event to the GSK-3 β nuclear translocation, because caspase inhibitors do not prevent GSK-3 β accumulation (Bijur and Jope, 2001). These results are relevant and may provide an explanation for the failure of caspase inhibitors to protect against the neurodegenerative effect induced by A β . Recently, a direct link between caspase-3 and GSK-3 β has been demonstrated. Indeed, MPP⁺ and rotenone, both inhibitors of mitochondrial complex I, induce caspase-3 activation; this effect was enhanced by increased GSK-3 β activity and attenuated by inhibiting GSK-3 β with lithium (King et al., 2001). In a second study in vascular smooth muscle cells and COS-7 cells, GSK-3 mediates hypoxia-induced apoptosis via the mitochondrial death pathway and inhibition of GSK-3 attenuated caspase-9 and caspase-3 activation (Loberg et al., 2002).

Inhibition of GSK-3 β translocation by lithium fails to prevent the tau hyperphosphorylation evoked by A β . The failure of lithium treatment to reduce or prevent tau hyperphosphorylation after A β administration may be due to one or more of the following factors. First, lithium has been demonstrated to inhibit tau hyperphosphorylation (as recognized by AT8 reactivity) in a dose-dependent manner. Indeed, in non-neuronal COS cells transfected transiently with tau, lithium reduces the GSK-3 β -induced tau phosphorylation only at a concentration of 1 mM or more (Sanchez et al., 2000). Therefore, the 7 mM lithium drinking water concentration used in the present study may yield a brain concentration less than the concentration needed to reduce tau phosphorylation; plasma concentrations of lithium with this dose are ~0.6 mM (Ghribi et al., 2002b). Because lithium has a narrow therapeutic index, higher concentrations of lithium may cause severe side effects, and administration of small molecules that directly inhibit GSK-3 β may represent an alternative to the use of higher doses of lithium. In addition, other candidates for tau phosphorylation have been proposed. In cortical neurons and in SH-SY-5Y neuroblastoma cells, A β induced tau phosphorylation, an effect inhibited by a MAP kinase inhibitor (Ekinici et al., 1999). Furthermore, in the latter study by Ekinici et al. (1999), inhibition of MAP kinase activity also attenuated A β -induced reactive oxygen species and apoptosis. The MAP kinase family member, JNK, has been demonstrated to phosphorylate tau in vitro, and JNK has been shown recently to colocalize with tau deposits in AD (Atzori et al., 2001). There is also recent evidence that A β -induced neuronal death involves the activation of the JNK pathway (Troy et al., 2001) and similar to GSK-3 β , JNK also phosphorylates tau (Reynolds et al., 1997, 2000). It is then possible that the JNK pathway activation plays a major role in tau phosphorylation. Further examination of the role of the JNK

pathway in A β -induced mitochondrial oxidative stress and tau hyperphosphorylation is required.

In summary, the results reported here demonstrate that the direct administration of synthetic A β into rabbit brain induces stress in endoplasmic reticulum and mitochondria and promotes hyperphosphorylation of tau. Translocation of GSK-3 β to the nucleus may represent an upstream event that leads to the activation of caspases, and may not be related to the A β -induced hyperphosphorylation of tau or to mitochondrial DNA damage; inhibition of GSK-3 β redistribution by lithium prevents only the A β -induced caspase activation and does not reverse the A β -related mitochondrial DNA damage or the hyperphosphorylation of tau. Therefore, further studies are needed to determine the mechanisms that underlie A β damage to mitochondrial DNA and the hyperphosphorylation and accumulation of tau. Protection against the effects of A β may require, in addition to lithium, the use of agents that inhibit subsequent oxidative damage and tau hyperphosphorylation.

REFERENCES

- Allen JW, Eldadah BA, Huang X, Knoblach SM, Faden FI. 2001. Multiple caspases are involved in beta-amyloid-induced neuronal apoptosis. *J Neurosci Res* 65:45–53.
- Alvarez G, Munoz-Montano JR, Satrustegui J, Avila J, Bogonez E, Diaz-Nido J. 1999. Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. *FEBS Lett* 453:260–264.
- Atzori C, Ghetti B, Piva R, Srinivasan AN, Zolo P, Delisle MB, Mirra SS, Migheli A. 2001. Activation of the JNK/p38 pathway occurs in diseases characterized by tau protein pathology and is related to tau phosphorylation but not to apoptosis. *J Neuropathol Exp Neurol* 60:1190–1197.
- Bales KR, Du Y, Dodel RC, Yan GM, Hamilton-Byrd E, Paul SM. 1998. The NF- κ B/Rel family of proteins mediates A β -induced neurotoxicity and glial activation. *Brain Res Mol Brain Res* 57:63–72.
- Bijur GN, De Sarno P, Jope RS. 2000. Glycogen synthase kinase-3 β facilitates staurosporine- and heat shock-induced apoptosis: protection by lithium. *J Biol Chem* 275:7583–7590.
- Bijur GN, Jope RS. 2001. Proapoptotic stimuli induce nuclear accumulation of glycogen synthase kinase-3 β . *J Biol Chem* 276:37436–37442.
- Butterfield DA, Drake J, Pocernich C, Castegna A. 2001. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. *Trends Mol Med* 7:548–554.
- Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, Johnson-Wood K, Lee M, Seubert P, Davis A, Kholodenko D, Motter R, Sherrington R, Perry B, Yao H, Strome R, Lieberburg I, Rommens J, Kim S, Schenk D, Fraser P, St. George HP, Selkoe DJ. 1997. Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nat Med* 3:67–72.
- Duff K, Eckman C, Zehr C, Yu X, Prada CM, Perez-Tur J, Hutton M, Buee L, Harigaya Y, Yager D, Morgan D, Gordon MN, Holcomb L, Refolo L, Zenk B, Hardy J, Younkin S. 1996. Increased amyloid- β 42(43) in brains of mice expressing mutant presenilin 1. *Nature* 383:710–713.
- Ekinici FJ, Linsley MD, Shea TB. 2000. Beta-amyloid-induced calcium influx induces apoptosis in culture by oxidative stress rather than tau phosphorylation. *Brain Res Mol Brain Res* 76:389–395.
- Ekinici FJ, Malik KU, Shea TB. 1999. Activation of the L voltage-sensitive calcium channel by mitogen-activated protein (MAP) kinase following exposure of neuronal cells to beta-amyloid. MAP kinase mediates beta-amyloid-induced neurodegeneration. *J Biol Chem* 274:30322–30327.

- Gabbita SP, Lovell MA, Markesbery WR. 1998. Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J Neurochem* 71:2034-2040.
- Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature* 373:523-527.
- Gau JT, Steinhilb ML, Kao TC, D'Amato CJ, Gaut JR, Frey KA, Turner RS. 2002. Stable beta-secretase activity and presynaptic cholinergic markers during progressive central nervous system amyloidogenesis in Tg2576 mice. *Am J Pathol* 160:731-738.
- Geula C, Wu C-K, Saroff D, Lorenzo A, Yuan M, Yankner BA. 1998. Aging renders the brain vulnerable to amyloid β -protein neurotoxicity. *Nat Med* 4:827-831.
- Ghribi O, Herman MM, DeWitt DA, Forbes MS, Savory J. 2001a. A- β (1-42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of *gadd* 153 and NF- κ B. *Brain Res Mol Brain Res* 96:30-38.
- Ghribi O, Herman MM, Forbes MS, DeWitt DA, Savory J. 2001b. GDNF protects against aluminum-induced apoptosis in rabbits by upregulating Bcl-2 and Bcl-XL and inhibiting mitochondrial Bax translocation. *Neurobiol Dis* 8:764-773.
- Ghribi O, Herman MM, Savory J. 2002a. The endoplasmic reticulum is the main site for caspase-3 activation following aluminum-induced neurotoxicity in rabbit hippocampus. *Neurosci Lett* 324:217-221.
- Ghribi O, Herman MM, Spaulding NK, Savory J. 2002b. Lithium inhibits aluminum-induced apoptosis in rabbit hippocampus, by preventing cytochrome c translocation, Bcl-2 decrease, Bax elevation and caspase-3 activation. *J Neurochem* 82:137-145.
- Gotz J, Chen F, van Dorpe J, Nitsch RM. 2001. Formation of neurofibrillary tangles in P301 τ transgenic mice induced by A β 42 fibrils. *Science* 293:1491-1495.
- Graur D, Duret L, Gouy M. 1996. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature* 379:333-335.
- Grimes CA, Jope RS. 2001. The multifaceted roles of glycogen synthase kinase 3 β in cellular signaling. *Prog Neurobiol* 65:391-426.
- Guerrini L, Blasi F, Denis-Donini S. 1995. Synaptic activation of NF- κ B by glutamate in cerebellar granule neurons in vitro. *Proc Natl Acad Sci USA* 92:9077-9081.
- Hartigan JA, Johnson GV. 1999. Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3 β -dependent pathway. *J Biol Chem* 274:21395-21401.
- Henshall DC, Chen J, Simon RP. 2000. Involvement of caspase-3-like protease in the mechanism of cell death following focally evoked limbic seizures. *J Neurochem* 74:1215-1223.
- Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. 2000. Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature* 406:86-90.
- Iida T, Furuta A, Nishioka K, Nakabeppu Y, Iwaki T. 2002. Expression of 8-oxoguanine DNA glycosylase is reduced and associated with neurofibrillary tangles in Alzheimer's disease brain. *Acta Neuropathol (Berl)* 103:20-25.
- Javelaud D, Besancon F. 2001. NF- κ B activation results in rapid inactivation of JNK in TNF α -treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF- κ B. *Oncogene* 20:4365-4372.
- Kaltschmidt B, Uhrek M, Volk B, Baeuerle PA, Kaltschmidt C. 1997. Transcription factor NF- κ B is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc Natl Acad Sci USA* 94:2642-2647.
- Kaltschmidt B, Uhrek M, Wellmann H, Volk B, Kaltschmidt C. 1999. Inhibition of NF- κ B potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci USA* 96:9409-9414.
- King, TD, Bijur GN, Jope RS. 2001. Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3 β and attenuated by lithium. *Brain Res* 919:106-114.
- Klein PS, Melton DA. 1996. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* 93:8455-8459.
- Lesort M, Jope RS, Johnson GV. 1999. Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3 β and Fyn tyrosine kinase. *J Neurochem* 72:576-584.
- Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E. 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* 293:1487-1491.
- Loberg RD, Vesely E, Brosius FC III. 2002. Enhanced glycogen synthase kinase-3 β activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism. *J Biol Chem* 277:41667-41673.
- Lovell MA, Gabbita SP, Markesbery WR. 1999. Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. *J Neurochem* 72:771-776.
- Lovell MA, Xie C, Markesbery WR. 2000. Decreased base excision repair and increased helicase activity in Alzheimer's disease brain. *Brain Res* 855:116-123.
- Markesbery WR, Carney JM. 1999. Oxidative alterations in Alzheimer's disease. *Brain Pathol* 9:133-146.
- Mattson MP, Chan SL. 2001. Dysregulation of cellular calcium homeostasis in Alzheimer's disease: bad genes and bad habits. *J Mol Neurosci* 17:205-224.
- Mattson MP, Culmsee C, Yu Z, Camandola S. 2000. Roles of nuclear factor κ B in neuronal survival and plasticity. *J Neurochem* 74:443-456.
- Mattson MP, Gary DS, Chan SL, Duan W. 2001. Perturbed endoplasmic reticulum function, synaptic apoptosis and the pathogenesis of Alzheimer's disease. *Biochem Soc Symp* 67:151-162.
- McKee AC, Kowall NW, Schumacher JS, Beal MF. 1998. The neurotoxicity of amyloid β protein in aged primates. *Amyloid* 5:1-9.
- Mecocci P, MacGarvey U, Beal MF. 1994. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 36:747-751.
- Moyer TP. 1999. Therapeutic drug monitoring. In: Burtis CA, Ashwood ER, editors. *Tietz textbook of clinical chemistry*. Philadelphia: PAWB Saunders Company. p 892.
- Mudher A, Lovestone S. 2002. Alzheimer's disease-do tauists and baptists finally shake hands? *Trends Neurosci* 25:22-26.
- Munoz-Montano JR, Moreno FJ, Avila J, Diaz-Nido J. 1997. Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS Lett* 411:183-188.
- Nakagawa T, Yuan J. 2000. Cross-talk between two cysteine protease families: activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 150:887-894.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature* 403:98-103.
- Pahl HL, Baeuerle PA. 1995. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF- κ B. *EMBO J* 14:2580-2588.
- Pahl HL, Sester M, Burgert HG, Baeuerle PA. 1996. Activation of transcription factor NF- κ B by the adenovirus E3/19K protein requires its ER retention. *J Cell Biol* 132:511-522.
- Plyte SE, Hughes K, Nikolakaki E, Pulverer BJ, Woodgett JR. 1992. Glycogen-synthase kinase-3—functions in oncogenesis and development. *Biochim Biophys Acta* 1114:147-162.
- Reynolds CH, Betts JC, Blackstock WP, Nebreda AR, Anderton BH. 2000. Phosphorylation sites on tau identified by nanoelectrospray mass spectrometry: differences in vitro between the mitogen-activated protein kinases ERK2, c-Jun N-terminal kinase and P38, and glycogen synthase kinase-3 β . *J Neurochem* 74:1587-1595.

- Reynolds CH, Utton MA, Gibb GM, Yates A, Anderton BH. 1997. Stress-activated protein kinase/c-jun N-terminal kinase phosphorylates tau protein. *J Neurochem* 68:1736-1744.
- Saez-Valero J, Angeretti N, Forloni G. 2000. Caspase-3 activation by beta-amyloid and prion protein peptides is independent from their neurotoxic effect. *Neurosci Lett* 293:207-210.
- Sanchez C, Perez M, Avila J. 2000. GSK3 β -mediated phosphorylation of the microtubule-associated protein 2C (MAP2C) prevents microtubule bundling. *Eur J Cell Biol* 79:252-260.
- Selznick LA, Zheng TS, Flavell RA, Rakic P, Roth KA. 2000. Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. *J Neuropathol Exp Neurol* 59:271-279.
- Smith MA, Rotkamp CA, Nunomura A, Raina AK, Perry G. 2000. Oxidative stress in Alzheimer's disease. *Biochim Biophys Acta* 1502:139-144.
- Takashima A, Honda T, Yasutake K, Michel G, Murayama O, Murayama M, Ishiguro K, Yamaguchi H. 1998. Activation of tau protein kinase I/glycogen synthase kinase-3 β by amyloid beta peptide (25-35) enhances phosphorylation of tau in hippocampal neurons. *Neurosci Res* 31:317-323.
- Takashima A, Noguchi K, Sato K, Hoshino T, Imahori K. 1993. Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity. *Proc Natl Acad Sci USA* 90:7789-7793.
- Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. 2001. Inhibition of JNK activation through NF- κ B target genes. *Nature* 414:313-317.
- Troy CM, Rabacchi SA, Xu Z, Maroney AC, Connors TJ, Shelanski ML, Greene LA. 2001. β -Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. *J Neurochem* 77:157-164.
- Wang QM, Fiol CJ, DePaoli-Roach AA, Roach PJ. 1994. Glycogen synthase kinase-3 β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J Biol Chem* 269:14566-14574.
- Woodruff-Pak DS, Trojanowski JQ. 1996. The older rabbit as an animal model: implications for Alzheimer's disease. *Neurobiol Aging* 17:283-290.

APPENDIX II

A β (1-42)-induced tau phosphorylation is not mediated by MAP kinase in rabbit hippocampus.

Othman Ghribi¹, Mary M. Herman³, Patcharin Pramoonjago¹, and John Savory^{1,2}

Departments of Pathology¹, Biochemistry and Molecular Genetics², University of Virginia, Charlottesville, VA, 22908, USA and IRP, NIMH, NIH³, Bethesda, MD .

Abstract

We demonstrate that intracisternal injection of A β (1-42) induces apoptosis and phosphorylation of tau in the hippocampus of New Zealand white rabbits. These A β effects correlate with the activation of JNK, ERK, c-jun and c-fos, but not of p38. treatment with 7 mM lithium inhibits apoptosis, prevents the activation of JNK and c-jun, increases the activation of ERK and c-fos, and does not affect tau. Our results suggest that A β -induced apoptosis and phosphorylation of tau occur through pathways that are distinct from each other, and that MAP kinases are not involved in the phosphorylation of tau.

Key words: A β , lithium, JNK, ERK, c-jun, tau.

Correspondence to: Dr. John Savory, Department of Pathology, University of Virginia Health Sciences Center, Box 800214, Charlottesville, Virginia 22908 U.S.A.

(434) 924-5682; FAX : (434) 924-2574; Email: js2r@virginia.edu

1. Introduction

The mechanisms by which neurons die in Alzheimer's disease (AD) are not fully understood. The observation that high levels of the soluble form of A β are found in the brain of AD patients suggests that the accumulation of this peptide plays a key role in the neuronal death that characterizes AD. However, the underlying mechanisms of this A β -induced neuronal death are still unclear. There have been a number of reports suggesting that activation of the MAP kinase signaling pathway plays an important role in mediating apoptotic neuronal death in AD (see for review [1]). In cultured rat microglia, treatment with 5 nM A β (25-35) is found to evoke the activation of the three MAP kinase subtypes, p38, ERK and JNK [2]. In mice transgenic for human amyloid precursor protein or containing a mutated presenilin-1 gene, both JNK and p38 are significantly activated in the cortex [3]. Mildly to severely affected AD patients also show activated JNK, ERK and p38 in the brain [4]. In addition to their putative role in regulating cell survival, there is mounting evidence that MAP kinases also phosphorylate tau protein (see for review [1]), the accumulation of which is a hallmark of AD. Taken together, these results indicate that the MAP kinase pathway is actively involved during the course of AD or experimentally *in vivo* or *in vitro* following the administration of synthetic A β .

We have recently shown that the intracisternal administration of A β (1-42) induces neuronal apoptosis and hyperphosphorylation of tau in the hippocampus of New Zealand white rabbits [5]. In this same study, we demonstrated that lithium inhibits apoptosis and

fails to prevent the hyperphosphorylation of tau. Lithium is an inhibitor of GSK-3 β , the activation of which is suggested to mediate A β -induced neurotoxicity. However, neither the mechanisms underlying the anti-apoptotic effect of lithium nor those triggering the phosphorylation of tau following A β treatment, are fully understood.

In the present study, we have tested the hypothesis that A β -induced apoptosis and phosphorylation of tau in rabbit hippocampus require activation of the MAP kinase signaling pathways. We have also examined the correlation of the anti-apoptotic effect of lithium, and its failure to prevent the phosphorylation of tau, to its effect on these signaling pathways.

2. Material and Methods:

Animals and treatment.

Adult (2-3 years and 4-5 kg) female New Zealand white rabbits received either intracisternal injections of 100 μ L normal saline (n=6; controls), 100 μ L of 2 mg/ml A β (1-42) (n=6; A β -treated group), 100 μ L of 2 mg/ml A β (1-42) plus lithium (n=6; lithium/A β -treated group) or lithium alone (n=6; lithium-treated group). Aggregated A β (1-42) (American Peptide Company, Sunnyvale, CA) was prepared by incubating freshly solubilized A β (1-42) at a concentration of 2 mg/ml in saline at 37°C for 3 days. Lithium carbonate (Sigma Chemical Co, St. Louis, MO) was administered orally by adjusting the drinking water to a concentration of 7 mM as described previously [6].

Lithium was started 14 days prior to A β (1-42) administration and continued until animal sacrifice. Serum lithium concentrations were determined in blood specimens obtained from an ear vein at sacrifice, using an AVL model 9180 clinical analyzer (Roche Diagnostics, Roswell, GA). This oral lithium treatment resulted in serum lithium concentrations of 0.68 ± 0.32 mM/L (mean $\pm$ SEM). These levels are close to the range of 0.6-1.2 mM/L, concentrations that are achieved and maintained during lithium therapy in human subjects [7]. Rabbits were sacrificed 7 days after the intracisternal administration of A β (1-42) or saline, and at necropsy were perfused with Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY) at room temperature. All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Western blot analysis.

Tissue from the entire hippocampus was gently homogenized using a teflon homogenizer (Thomas, Philadelphia PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/mL pepstatin and 12.5 mg/mL of N-acetyl-Leu-Leu-Norleu-Al). Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Illinois). Proteins (10 μ g) were separated by SDS-PAGE (10 % gel), followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and were incubated overnight at 4° C with mouse monoclonal antibody (mAb) to JNK1/2, p-JNK1/2, ERK1/2, p-ERK1/2, c-fos, p-c-jun (Santa Cruz Laboratories, Santa Cruz, CA), p38 and p-p38 (Cell Signaling,

Beverly, MA), and to caspase-3 (Active Motif, Carlsbad, CA). Phosphorylation of the microtubule-associated protein tau (tau) was assessed using either a 1:1000 dilution of the AT8 mAb (Innuogenetics, Ghent, Belgium) which recognizes a doubly phosphorylated epitope including S¹⁹⁹, S²⁰², and T²⁰⁵. We also used mAb PHF-1 at a 1:2500 dilution which also reacts with tau (gift from Dr. Peter Davis, Albert Einstein College of Medicine). β -actin (Sigma, Saint Louis, MI) was applied as a gel loading control at a 1:500 dilution. The blots were developed with enhanced chemiluminescence (Immun-Star goat anti-mouse IgG detection kit, Bio-Rad, Hercules, CA) and bands were analyzed by densitometry with Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

TUNEL assay.

Apoptosis detection was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique on frozen coronal brain sections (14 μ m thick) from the mid-hippocampal level of control, A β -treated, lithium/A β -treated, and lithium-treated animals. Detection of DNA fragmentation was performed using the Apoptosis Detection System (Fluorescein, Promega, Madison, WI) as we previously have reported [5;6]. Sections were counterstained with propidium iodide, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a fluorescent microscope using excitation/emission wavelengths of 365/490 nm (Olympus BH2, Melville, NY).

3. Results:

Lithium inhibits A β -induced apoptosis

Western blot analysis with caspase-3 mAb (Figure 1a) shows a 32 kDa band that corresponds to pro-caspase-3 in control, A β -treated, lithium/A β -treated, and lithium-treated animals. Caspase-3 p17 and p12, two activated forms of caspase-3, are present in the A β -treated animals only. Treatment with lithium completely inhibits cleavage of pro-caspase-3 to the active caspase-3 p 17 and p12.

Application of the TUNEL assay (Figure 1b), examined in the pyramidal layer (CA1) of the hippocampus, shows that while sections from control animals (panel A) exhibit no TUNEL-positive neurons, the A β -treated animals have numerous TUNEL-positive neurons (panel B); administration of lithium greatly reduces the TUNEL positivity (panel C). Treatment by lithium alone does not induce TUNEL positivity (panel D).

A β (1-42) induces phosphorylation of JNK and ERK, but not of p38, effects that are differentially regulated by lithium

The JNK mAb used in these studies reacts with both JNK1 and 2 and yields 54 and 46 kDa bands; the mAb recognizing phosphorylated JNK (p-JNK) also is immunoreactive with both p-JNK1 and p-JNK2 (Figure 2a). In all animal groups, levels of the 46 kDa JNK are higher than those of 54 kDa JNK. Administration of A β peptide significantly raises the levels of p-JNK; lithium inhibits the A β -induced increase in p-JNK and has no significant effect when administered alone (Figures 2a and b). While p-c-jun is not

detected in controls, it is expressed following A β (1-42) administration; lithium treatment prevents p-c-jun expression (Figure 2a).

Both 42 and 44 kDa ERK (Figure 3a) are detected in hippocampal tissue from control, A β -treated, lithium/A β -treated, and lithium-treated animals. Administration of A β (1-42) induces activation of p-ERK and this activation increases following treatment by lithium. Treatment with lithium alone significantly increases the levels of p-ERK 42 kDa compared to untreated controls; this treatment induces activation of lower levels of the 44 kDa band (Figure 3a and b). The transcription factor c-fos is not detected in controls but is expressed following A β ; lithium increases the levels of c-fos either when combined with A β or when administered alone (Figure 3 a).

p38 protein is present at approximately the same level in the hippocampus of control, A β -treated, lithium/A β -treated, and lithium-treated animals (Figure 3c). Phosphorylated p38 (p-p38) is barely detectable in these various treatment groups and does not appear to be affected appreciably by any of these treatment regimes, as depicted in Figure 3c.

Lithium doesn't prevent A β -induced p-tau

A β treatment results in the phosphorylation of the microtubule-associated protein tau as demonstrated by mAb AT8 or PHF-1 immunostaining (Figure 4). This A β -induced phosphorylation is not affected by lithium treatment, and lithium alone does not induce phosphorylation of tau.

4. Discussion:

The present study demonstrates that the intracisternal administration of synthetic A β (1-42) peptide into rabbit brain induces apoptosis, as indicated by the activation of caspase-3 and TUNEL, and by the phosphorylation of tau demonstrated by AT8 and PHF-1 immunoreactivity. These effects correlate with the activation of the MAP kinase members, JNK and ERK, and their respective substrates, c-jun and c-fos. A β does not activate the third MAP kinase family member, p38. Lithium treatment prevents the activation of apoptosis but not the hyperphosphorylation of tau. The ability of lithium to prevent apoptosis and its failure to prevent the phosphorylation of tau following A β administration correlates with the inhibition of JNK and its substrate c-jun, and with the increase in ERK phosphorylation and expression of its substrate c-fos. Interestingly, lithium administered alone activates ERK and c-fos, however, this activation is not associated with the induction of apoptosis or the phosphorylation of tau.

Cultured cells exposed to synthetic A β have demonstrated neurodegeneration and hyperphosphorylation of tau [8-10]. The mechanism by which A β induces tau hyperphosphorylation has been suggested to involve the activation of GSK-3 β [11];[9]. Lithium has been shown to be neuroprotective against a variety of experimental neurotoxic stimuli [12] and to prevent tau hyperphosphorylation [13] by mechanisms that are suggested to be mediated via the GSK-3 β signaling pathway. However, as we have shown recently [5] and in the present study, the failure of lithium to prevent

hyperphosphorylation of tau suggests that GSK-3 β phosphorylation is not involved in the A β -induced accumulation of tau in our model system. Many other enzymes have been suggested to phosphorylate tau. Apart from regulating apoptosis, MAP kinases also can phosphorylate neurofilaments and proteins including tau protein [14-16].

Our results, showing that A β activates JNK and ERK, raise the possibility that JNK or ERK may be responsible for the A β -evoked phosphorylation of tau. However, inhibition of JNK by lithium is not sufficient to prevent the phosphorylation of tau. Furthermore, activation of ERK by lithium alone does not induce the phosphorylation of tau. These results strongly suggest that phosphorylation of tau by A β in our model system is independent of the activation of the MAP kinase members.

The anti-apoptotic effect we report in the present study with lithium is in accordance with recent data showing that pretreatment for 7 days with 0.5-2 mM of lithium has prevented the glutamate-induced activation of JNK and c-jun, and the induction of apoptosis in granule cells [17]. However, in another study, lithium has been demonstrated to increase p-c jun and p-JNK in SH-SY5Y cells and in rat brain [18]. Valproic acid, which like lithium is a mood stabilizer, promotes neurite growth and cell survival by mechanisms that correlate with the activation of ERK kinase [19]. Activation of JNK and its substrate c-jun following A β treatment, and their inhibition by lithium, suggest that JNK and c-jun may be involved in regulating apoptosis in our animal system. In contrast, the observation that lithium increases ERK activation and c-fos levels without the induction of caspase-3 cleavage may suggest that ERK activation is not involved in regulating apoptosis. The mechanism that underlies c-fos activation and the significance of this

activation remain to be determined. The role of ERK in A β toxicity and phosphorylation of tau has been controversial. Activated ERK has been shown to be associated with early tau deposition in neurodegenerative diseases [20], and the downregulation of ERK has been associated with elevated A β [21]. In another study, however, it was demonstrated that ERK activation did not mediate A β neurotoxicity in rat cortical neurons [22]. Our results demonstrate that an increase in ERK activity by lithium alone does not correlate with the induction of apoptosis or the phosphorylation of tau, suggesting that ERK does not mediate the process of A β toxicity in our animal model. Further studies using specific inhibitors of JNK and ERK will better define the role of these MAP kinase family members in the phosphorylation of tau and the regulation of apoptosis following A β administration.

Acknowledgment: Supported by the United States Department of Defense and the Virginia Center on Aging. We thank Dr. Peter Davis for his gift of the PHF antibody.

References.

- [1] Mielke,K.and Herdegen,T. (2000) Prog. Neurobiol. 61, 45-60.
- [2] Pyo,H., Jou,I., Jung,S., Hong,S.and Joe,E.H. (1998) Neuroreport 9, 871-874.
- [3] Savage,M.J., Lin,Y.G., Ciallella,J.R., Flood,D.G. and Scott,R.W. (2002) J. Neurosci. 22, 3376-3385.
- [4] Zhu,X., Castellani,R.J., Takeda,A., Nunomura,A., Atwood,C.S. and Perry,G., Smith,M.A. (2001) Mech. Ageing Dev. 123, 39-46.
- [5] Ghribi,O., Herman M.M. and Savory J. (2003) J. Neurosci. Res.71, 853-862.
- [6] Ghribi,O., Herman,M.M., Spaulding,N.K. and Savory, J. (2002) J Neurochem. 82, 137-145.
- [7] Moyer,T.P. (1999) in: Therapeutic drug monitoring (Burtis, C.A.and Ashwood, E.R., Eds.)Tietz Textbook of Clinical Chemistry, pp. 892, W.B. Saunders Company Philadelphia, PA.
- [8] Takashima,A., Noguchi,K., Sato,K., Hoshino,T. and Imahori,K. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7789-7793.
- [9] Takashima,A., Honda,T., Yasutake,K., Michel,G., Murayama,O., Murayama,M., Ishiguro,K. and Yamaguchi,H. (1998) Neurosci. Res.31, 317-323.

- [10] Alvarez,G., Munoz-Montano,J.R., Satrustegui,J., Avila,J., Bogonez,E. and Diaz-Nido,J. (1999) FEBS Lett. 453, 260-264.
- [11] Tomidokoro,Y., Ishiguro,K., Harigaya,Y., Matsubara,E., Ikeda,M., Park,J.M., Yasutake,K., Kawarabayashi,T., Okamoto,K. and Shoji,M. (2001) Neurosci. Lett. 299, 169-172.
- [12] Jope,R.S. and Bijur,G.N. (2002) Mol. Psychiatry 7 Suppl 1, S35-S45.
- [13] Munoz-Montano,J.R., Moreno,F.J., Avila,J. and Diaz-Nido,J. (1997) FEBS Lett. 411, 183-188.
- [14] Reynolds,C.H., Betts,J.C., Blackstock,W.P., Nebreda,A.R. and Anderton,B.H. (2000) J. Neurochem. 74, 1587-1595.
- [15] Reynolds,C.H., Utton,M.A., Gibb,G.M., Yates,A. and Anderton,B.H. (1997) J. Neurochem. 68, 1736-1744.
- [16] Reynolds,C.H., Nebreda,A.R., Gibb,G.M., Utton,M.A. and Anderton,B.H. (1997) J. Neurochem. 69, 191-198.
- [17] Chen,R.W., Qin,Z.H., Ren,M., Kanai,H., Chalecka-Franaszek,E., Leeds,P. and Chuang,D.M. (2003) J. Neurochem. 84, 566-575.
- [18] Yuan,P., Chen,G. and Manji,H.K. (1999) J. Neurochem. 73, 2299-2309.
- [19] Yuan,P.X., Huang,L.D., Jiang,Y.M., Gutkind,J.S., Manji,H.K. and Chen,G. (2001) J. Biol. Chem. 276, 31674-31683.

Best Available Copy

- [20] Ferrer,I., Blanco,R., Carmona,M., Ribera,R., Goutan,E., Puig,B., Rey,M.J.,
Cardozo,A., Vinals,F. and Ribalta,T. (2001) Brain Pathol.11' 144-158.
- [21] Dineley,K.T., Westerman,M., Bui,D., Bell,K., Ashe,K.H. and Sweatt,J.D. (2001)
J. Neurosci. 21, 4125-4133.
- [22] Abe,K. and Saito,H. (2000) Neurosci. Lett. 292 1-4.

Legends for Figures:

Figure 1. a. A β induces cleavage of the pro-caspase-3 (32 KDa) to p17 and p12. This cleavage is prevented by lithium. **b.** Immunofluorescence images of TUNEL labeling in the hippocampal CA1 area. Control section shows no TUNEL-positive neurons (panel A) while a section from an A β -treated animal shows widespread DNA fragmentation (arrows, panel B). Treatment with lithium reduces the number of neurons exhibiting DNA fragmentation (panel C) and no TUNEL labeling is seen with lithium alone (panel D). Sections were counterstained with propidium iodide (red).

Figure 2. a. Representative Western blots of JNK, p-JNK and p-c-jun from hippocampal tissue. JNK is detected as a 54 kDa band and a more intense band at 46 kDa in the various groups. p-JNK is also detected as 54 and 46 bands and is increased by A β and reduced by lithium. Lithium alone does not increase p-JNK. p- c-jun is not detected in the control but is expressed following A β (1-42) administration and is markedly reduced by lithium. **b.** Densitometric scanning analysis showing that lithium markedly reduces the A β -induced increase in p-JNK **p<0.01 vs control, ++p<0.01 vs A β (ANOVA followed by Student *t* test).

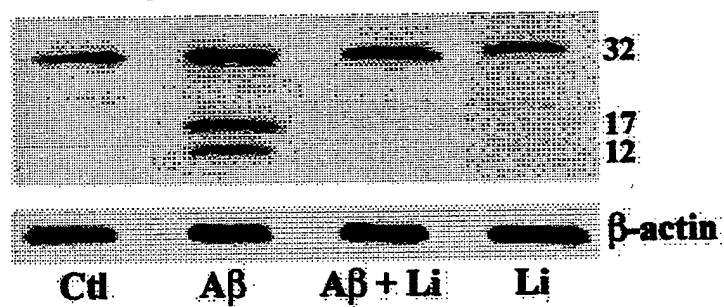
Figure 3. a. Representative Western blots of ERK, p-ERK and c-fos. ERK is detected as a double band at 44 and 42kDa. The active p-ERK is also detected as a double band at 44 and 42 kDa, which is barely seen in controls, but is significantly elevated following A β (1-42). Lithium increases the intensity of p-ERK when administered with A β (1-42) or alone. No c-fos is detected in the control but is induced by A β (1-42) treatment. Lithium

slightly increases the level of c-fos in comparison to the A β (1-42)-treated animal. b.

Densitometric analysis of p-ERK from hippocampal tissue shows that lithium with A β (1-42) increases the levels of p-ERK in comparison to the animal treated with A β (1-42) alone, or to the control. * $p < 0.05$ vs control, + $p < 0.05$ vs A β . c. p38 is distributed at similar levels in all of the groups, while the phosphorylated form (p-p38) is barely detectable.

Figure 4. AT8 and PHF-1 mAbs only faintly stain hippocampal tissue in a control but in the A β -treated animal there is intense immunoreactivity which is not affected by lithium treatment. No AT8 or PHF-1 positive bands are seen following treatment with lithium alone.

a. Caspase-3



b. TUNEL

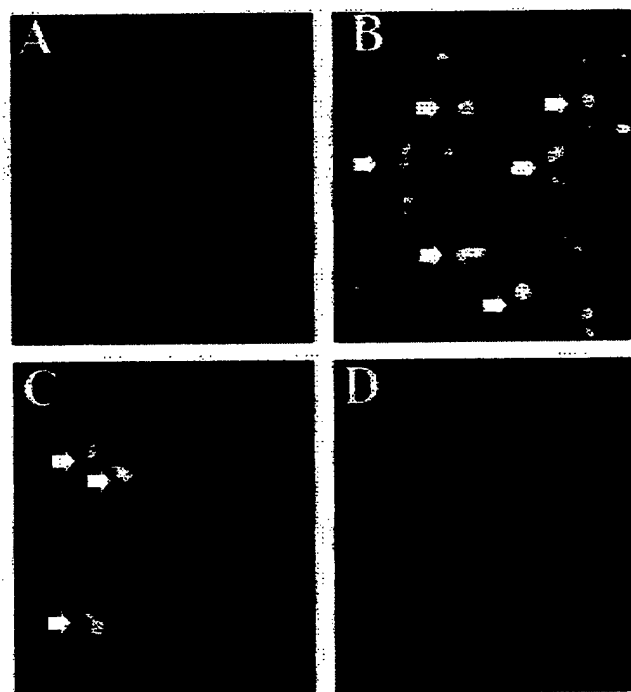


Fig 1.

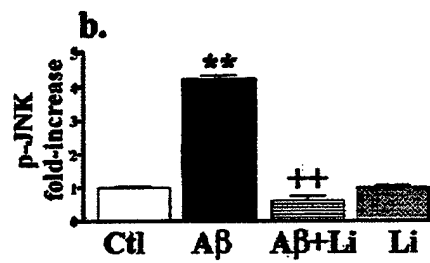
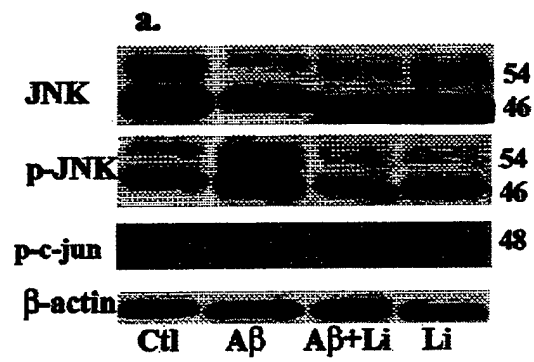


Fig.2

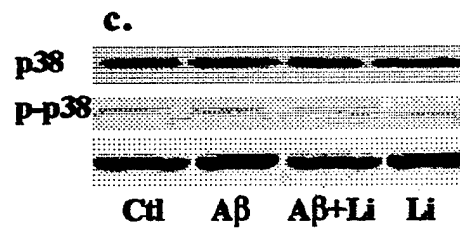
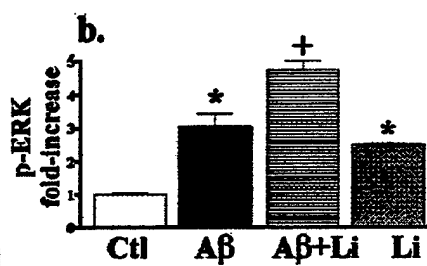
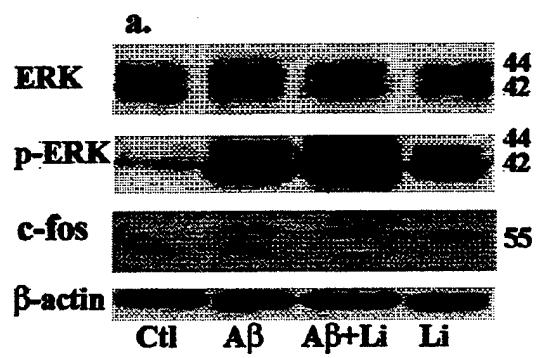


Fig.3

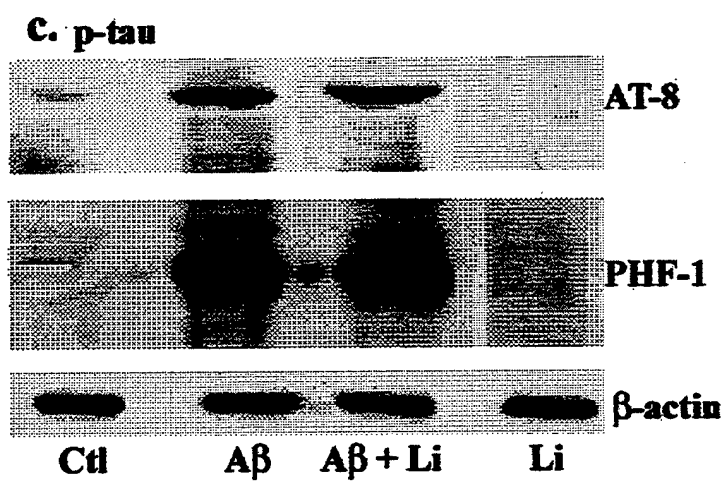


Fig.4

APPENDIX III

Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain.

John Savory^{a,b}, Mary M. Herman^c and Othman Ghribi^a

^aDepartments of Pathology, ^bBiochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, and ^cIRP, NIMH, NIH, Bethesda, MD.

Correspondence to: Dr. John Savory, P.O. Box 800214, Department of Pathology,
University of Virginia Health Sciences Center, Charlottesville, Virginia 22908 U.S.A.
(434) 924-5682; FAX (434) 924-2574; Email: js2r@virginia.edu

Key Words: aluminum, apoptosis, caspase-3, caspase-12, gadd 153, endoplasmic reticulum, lithium.

Abstract

Loss of neurons is a hallmark of neurodegenerative disorders and there is increasing evidence suggesting that apoptosis is a key mechanism by which neurons die in these diseases. Mitochondrial dysfunction has been implicated in this process of neuronal cell death but there is a growing body of evidence suggesting also an active role for the endoplasmic reticulum in regulating apoptosis, either independent of mitochondria, or in concert with mitochondrial-initiated pathways. Investigations in our laboratory have focused on neuronal injury resulting from the administration of aluminum maltolate, via the intracisternal route, to New Zealand white rabbits. Treatment of rabbits with aluminum maltolate induces both mitochondrial and endoplasmic reticulum stress. Agents such as lithium or GDNF, have the ability to prevent aluminum-induced neuronal death by interfering with the mitochondrial and/or the endoplasmic reticulum-mediated apoptosis cascade. This animal model system involving neurotoxicity induced by an aluminum compound, provides new information on mechanisms of neurodegeneration and neuroprotection.

1. Introduction

Aluminum (Al) is ubiquitous in the environment and it has been speculated that its accumulation in human brain might be linked to neurodegenerative disorders including Alzheimer's disease, the parkinsonism-dementia complex of Guam and amyotrophic lateral sclerosis (see for review [1]). There are numerous reports both supporting and denouncing this link (see for review [2]) and further work is needed to clarify the possible role, if any, of Al in these devastating human disorders. Aluminum is unquestionably neurotoxic as was demonstrated by Alfrey et al., who discovered that Al accumulation was the cause of a fatal encephalopathy in many patients on longterm hemodialysis treatment for chronic renal failure [3]. There is also strong evidence implicating Al as a potent neurotoxin when it accumulates at a certain level in animal brain or in cultured cells and neurons. However, the molecular mechanisms by which Al interacts with cells and neurons and induces its neurotoxicity are poorly understood.

Some of the cellular process that are involved in the neurodegeneration induced by Al have been clarified in our laboratory by studies on New Zealand white rabbits treated with Al maltolate via the intracisternal route of administration. A review of our findings forms the basis of this present report.

2. Effect of Al on the mitochondrial-mediated apoptosis pathway

Apoptosis, or programmed cell death, plays a critical role in the normal development and maintenance of tissue homeostasis, and is also a process by which brain cells die in neurotoxic situations. Mitochondrial changes following cytotoxic stimuli represent a primary event in apoptotic cell death. The apoptogenic factor, cytochrome *c*, is released from mitochondria into the cytoplasm where it binds to another cytoplasmic factor, Apaf-1, and the formed complex activates the initiator caspase-9 that in turn activates the effector caspase, caspase-3. Release of cytochrome *c* from the mitochondria has been shown to involve three distinct pathways. One implicates the opening of the mitochondrial permeability transition pore (MTP), the second is triggered by the translocation to mitochondria of the pro-apoptogenic Bax which can form a channel by itself, and the third may result from the interaction of Bax with the voltage-dependent anion channel (VDAC) to form a larger channel which is permeable to cytochrome *c*. In contrast to Bax, the anti-apoptotic Bcl-2 has the ability to block the release of cytochrome *c* from mitochondria by mechanisms such as a direct blockade of the MTP opening, or by functioning as a docking protein [4,5].

Al has been demonstrated to accumulate in neurons following cell depolarization, where it inhibits $\text{Na}^+ / \text{Ca}^{+2}$ exchange and thereby induces an excessive accumulation of mitochondrial Ca^{+2} [6]. Increases in intramitochondrial Ca^{+2} levels lead to an opening of the MTP, with cytochrome *c* release and subsequent apoptosis resulting from activation of the caspase family of proteases.

We have shown that the intracisternal administration of Al maltolate results in

cytoplasmic cytochrome *c* translocation, Bcl-2 down-regulation and Bax up-regulation, as well as caspase-3 activation [7]. These results indicate that Al targets the mitochondria. Furthermore, the fact that we have demonstrated that the release of cytochrome *c* is inhibited by cyclosporin A, a specific inhibitor of the MTP opening, implicates opening of the MTP as the process by which cytochrome *c* translocates to the cytoplasmic space from mitochondria [8]. The mitochondria-regulated death pathway has also been shown to mediate asbestos-induced apoptosis in alveolar epithelial T cells [9], and iron has been demonstrated to alter Bcl-2 levels in mitochondria and to affect the MTP [10].

The use of pharmacological agents that prevent or reverse the apoptotic effects of Al can provide valuable mechanistic information on the effects of Al on cellular protein targets. We have demonstrated that the glial cell-line derived neurotrophic factor (GDNF) protects rabbit hippocampus from the neurotoxic effect of Al, but does not prevent the release of cytochrome *c* [11]. These results do not support the release of cytochrome *c* as the sole trigger of aluminum-induced apoptosis, at least in this animal model system. However, GDNF treatment increases the level of the anti-apoptotic protein, Bcl-XL, which when over-expressed, has the ability to sequester Apaf-1, and thereby to inhibit Apaf-1-dependent caspase-9 activation [12,13]. Formation of the cytochrome *c*-Apaf-1 complex, rather than the presence *per se* of cytochrome *c* in the cytoplasm, thus appears to be the determining factor in the activation of caspases and triggering of apoptosis.

The neuroprotective effect of GDNF treatment resulted from a single intracisternal injection of GDNF. We have shown that chronic treatment of rabbits with lithium in the drinking water results in inhibition of the A1-induced cytochrome *c* release, enhances levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL, prevents the redistribution of the pro-apoptotic protein Bax levels, and inhibits caspase-3 activation and DNA fragmentation [14]. The ability of lithium to block the release of cytochrome *c* might be attributed to either a direct effect on mitochondria by inhibiting the opening of the MTP, Bax translocation, or to an increase in the level of the anti-apoptotic Bcl-2. However, it is unlikely that lithium possesses the ability to directly block the opening of the MTP, as is the case for cyclosporin A which blocks this channel by specifically binding its matrix protein, cyclophilin D [15,16]. Rather, lithium may inhibit cytochrome *c* translocation by preventing (i) an A1-induced redistribution of Bax and (ii) a decrease in Bcl-2. Lithium possesses neuroprotective properties since it has been demonstrated to exert robust protective effects against diverse apoptotic insults induced by potassium deprivation [17], glutamate [18], β -amyloid [19,20], ischemia and anticonvulsant-induced apoptosis [21,22], and staurosporine and heat-shock [23]. One of the molecular mechanisms by which lithium protects against neuronal death has been attributed to the inhibition of glycogen synthase kinase-3 (GSK-3) [23], an enzyme which when activated promotes pro-apoptotic signaling [24]. Lithium has been shown to inhibit caspase-3 activation and to protect cerebellar granule cells from apoptosis induced by potassium deprivation [25] and to robustly increase levels of the anti-apoptotic Bcl-2 in various brain regions in rats and mice [18,26].

It appears from our studies of neurotoxicity in rabbits that Al induces perturbation of mitochondrial function, affecting the distribution and levels of the apoptosis regulatory proteins, and triggering signaling that leads to the initiation and expression of apoptosis. However, it remains unclear whether Al directly gains access to mitochondria or triggers mitochondrial stress through other signaling pathways.

Effect of aluminum on apoptosis-regulatory proteins that mediate endoplasmic reticulum stress

Although mitochondrial alterations may represent an important step in the mechanisms underlying neuronal cell death induced by Al, studies in our laboratory have provided evidence suggesting that the endoplasmic reticulum (ER) also plays an important role in regulating this cell death. The ER is an important subcellular site, since it is the major storage location for calcium and contains members of the *Bcl-2* family of proteins, Bcl-2 and Bcl-XL.

Endoplasmic reticulum stress-inducing agents have been shown to activate the expression of various genes, such as those coding for the gene *gadd 153*, important in growth arrest and DNA damage-induction, and the inducible transcription factor, NF- κ B, which plays an important role in the survival of neurons. Stress induced in the ER has also been shown to result in a specific type of apoptosis mediated by caspase-12 and is independent of mitochondrial-targeted apoptotic signals. Also, the ER can work in concert with

mitochondria to regulate cellular apoptosis. Indeed, it has been reported that the drug brefeldin induces ER dilatation and leads to cytochrome *c* release and caspase-3 activation [27]. This effect can be blocked by wild type Bcl-2 and, surprisingly, a Bcl-2 variant that is exclusively targeted to the ER, is also able to accomplish the same task. Bcl-2 may exert its protective effect by controlling calcium homeostasis in the ER as well as the mitochondria.

Studies performed in our laboratory have shown that Al-maltolate induces a redistribution of the apoptosis-regulatory proteins, with Bax being present at higher levels in the ER than in the cytosol and with decreased amounts of Bcl-2 in the ER [7]. We also have shown that Al induces stress in the ER, as demonstrated by the activation of *gadd 153* and its translocation into the nucleus. The *gadd 153* gene is specifically activated by agents that disturb ER function. We have also provided the first report of the *in vivo* activation of caspase-12 following Al treatment [28].

Although we have demonstrated an effect of Al on ER function, it remains unclear which signaling mechanisms lead to perturbation of ER homeostasis by Al. Aluminum may disturb Ca^{2+} homeostasis or protein processing in the ER. Stress signaling from the ER is suggested to be triggered by two distinct pathways, the unfolded protein response (UPR) and the overload response (EOR), for review see [29]. Severe insult that results in sustained depletion of Ca^{2+} stores or to prolonged ER stress leads to apoptotic cell death. In accordance with our hypothesis are data from cultured rat hippocampal cells treated with Al chloride showing the induction of cell death, a pathologic process diminished by

treatment with dantrolene, a known inhibitor of Ca^{2+} release from intracellular stores [30].

Conclusions

Speculation that apoptosis underlies Al-induced neuronal cell loss is gaining increased attention in the recent literature. Our earlier research primarily involved immunohistochemistry for demonstrating the toxic effects of the intracisternal administration of Al maltolate. We have extended this work by using subcellular fractionation of brain tissue and Western blot analysis to study markers of apoptosis. We have shown that the ER is a key organelle in regulating apoptosis and we have provided the first evidence for the *in vivo* activation of caspase-12. We have also demonstrated for the first time that activated caspase-3 is localized in the ER as well as in the cytoplasm and nucleus.

Our overall hypothesis (see Figure) is that Al induces ER stress with a response that leads to cross talk between the ER and mitochondria, eventually culminating in the activation of caspases and apoptosis. Stress in the ER results in perturbation of Ca^{2+} homeostasis, leading to down-regulation of the ER chaperones and anti-apoptotic proteins, up-regulation of the pro-apoptotic proteins, and activation of caspase-12. An increase in extracellular Ca^{2+} subsequent to ER stress leads to an increase in mitochondrial Ca^{2+} . The increase in mitochondrial Ca^{2+} results in depolarization of mitochondria, release of cytochrome c and activation of caspase-9. Activation of caspase-12 by ER stress and of

caspase-9 by mitochondrial stress in turn activates the effector caspase-3, with the eventual induction of apoptosis. We suggest that maintaining proper functioning of the ER, using pharmacological agents which prevent the perturbation of ER-Ca²⁺ mechanisms, represents an important novel approach for the prevention of Al-induced neuronal injury.

Acknowledgment.

Supported by Grant # DAMD 17-99-1-9552 from the US Department of the Army and the Virginia Center on Aging.

References

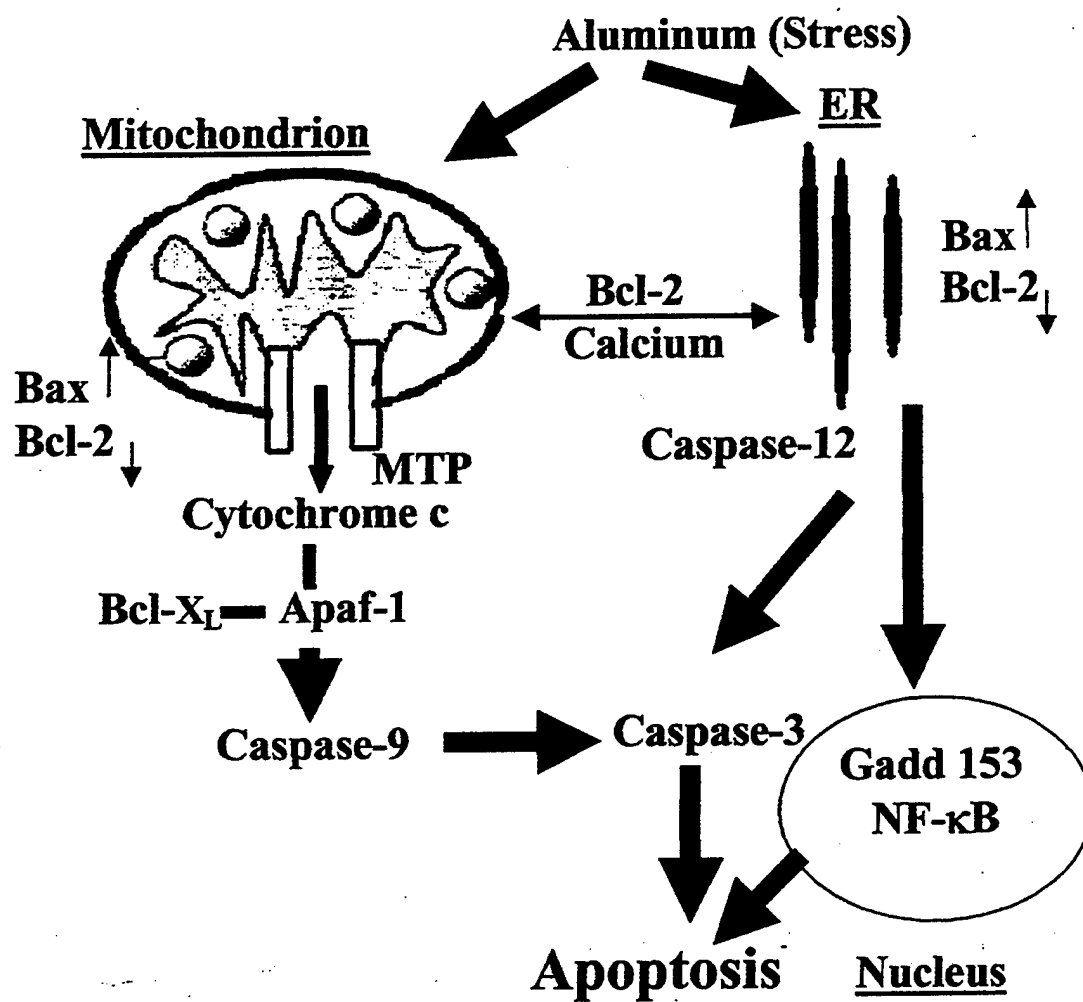
- [1] J.Savory, O.Ghribi, R.B.Martin, W.F.Forbes, M.M.Herman. in: C.Exley (Ed.), *Aluminum and Alzheimer's Disease: The Science that Describes the Link*, Elsevier, Amsterdam, 2001.
- [2] J.Savory, C.Exley, W.F.Forbes, Y.Huang, J.G.Joshi, T.Kruck, D.R.McLachlan, I.Wakayama. *J. Toxicol. Environ. Health*, 48 (1996) 615-635.
- [3] A.C.Alfrey, G.R.LeGendre, W.D.Kaehny. *N.Engl.J.Med.* 294 (1976) 184-188.
- [4] J.M.Adams, S.Cory. *Science* 281 (1998) 1322-1326.
- [5] R.Eskes, B.Antonsson, A.Osen-Sand, S.Montessuit, C.Richter, R.Sadoul, G.Mazzei, A.Nichols, J.C.Martinou. *J.Cell Biol.* 143 (1998) 217-224.
- [6] A.Szutowicz, H.Bielarczyk, Y.Kisielewski, A.Jankowska, B.Madziar, M.Tomaszewicz. *J Neurochem.* 71 (1998) 2447-2453.
- [7] O.Ghribi, D.A.DeWitt, M.S.Forbes, M.M.Herman, J.Savory. *Brain Res.* 903 (2001) 66-73.
- [8] O.Ghribi, D.A.DeWitt, M.S.Forbes, A.Arad, M.M.Herman, J.Savory. *J Alzheimers Disease* 3 (2001) 387-391.
- [9] V. Panduri, S.A Weitzman, N. Chandel, D.W. Kamp. *Am.J.Respir.Cell Mol.* 28 (2003) 241-248.
- [10] J. Kim, K.Y. Lee, E.Song. *Free radical Biology and Medicine* 33 (2002) 92
- [11] O.Ghribi, M.M.Herman, M.S.Forbes, D.A.DeWitt, J.Savory. *Neurobiol.Dis.* 8 (2001) 764-773.
- [12] Y.Hu, M.A.Benedict, D.Wu, N.Inohara, G.Nunez. *Proc.Natl.Acad.Sci.U.S.A.*, 95 (1998) 4386-4391.
- [13] G.Pan, K.O'Rourke, V.M.Dixit. *J.Biol.Chem.* 273 (1998) 5841-5845.
- [14] O.Ghribi, M.M.Herman, N.K.Spaulding, Savory. *J. Neurochem.* 82 (2002) 137-145.
- [15] A.P.Halestrap, A.M.Davidson. *Biochem. J.*, 268 (1990) 153-160.
- [16] M.Zoratti, I.Szabo. *Biochimica et Biophysica Acta* 1241 (1995) 139-176.
- [17] S.R.D'Mello, R.Anelli, P.Calissano. *Exp.Cell Res.* 211 (1994) 332-338.

- [18] R.W.Chen, D.M.Chuang. *J.Biol.Chem.* 274 (1999) 6039-6042.
- [19] J.R.Munoz-Montano, F.J.Moreno, J.Avila, J.Diaz-Nido. *FEBS Lett.* 411 (1997) 183-188.
- [20] G.Alvarez, J.R.Munoz-Montano, J.Satrustegui, J.Avila, E.Bogonez, J.Diaz-Nido. *FEBS Lett.* 453, (1999) 260-264.
- [21] S.Nonaka, N.Katsube, D.M.Chuang. *J.Pharmacol.Exp.Ther.* 286 (1998) 539-547.
- [22] S.Nonaka, D.M.Chuang. *Neuroreport* 9 (1998) 2081-2084.
- [23] G.N.Bijur, P.De Sarno, R.S.Jope. *J.Biol.Chem.* 275 (2000) 7583-7590.
- [24] M.Pap, G.M.Cooper. *J.Biol.Chem.* 273 (1998) 19929-19932.
- [25] A.Mora, G.Sabio, R.A.Gonzalez-Polo, A.Cuenda, D.R.Alessi, J.C.Alonso, J.M.Fuentes, G.Soler, F.Centeno. *J Neurochem.* 78 (2001) 199-206.
- [26] H.K.Manji, G.J.Moore, G.Chen. *J Clin.Psychiatry* 61 (2000) 82-96.
- [27] J.Hacki, L.Egger, L.Monney, S.Conus, T.Rosse, I.Fellay, C.Borner. *Oncogene* 19 (2000) 2286-2295.
- [28] O.Ghribi, M.M.Herman, D.A.DeWitt, M.S.Forbes, J.Savory. *Mol. Brain Res.* 96 (2001) 30-38.
- [29] R.J.Kaufman. *Biochim.Biophys.Acta* 1423 (1999) R13-R27.
- [30] S.Brenner. *Biol.Trace Elem.Res.* 86 (2002) 85-89.

Legends for Figures

Figure 1:

Aluminum induces neuronal apoptosis by its effects on the functioning of both the endoplasmic reticulum and mitochondria. At the endoplasmic reticulum level, Al leads to activation of caspase-12, which in turn can activate the effector caspase-3. Al-induced endoplasmic reticulum stress can also activate the transcription factors *gadd 153* and NF- κ B, leading to their translocation into the nucleus where they initiate apoptosis. Stress in mitochondria leads to opening of the mitochondria permeability transition pore, release of cytochrome *c* activation of caspase-9 and of caspase-3. Both stress in mitochondria and in the endoplasmic reticulum leads to down-regulation of the anti apoptotic proteins Bcl-2 and increase in the levels of the pro-apoptotic Bax and activation of the effector of apoptosis, caspase-3. Bcl-X_L, another anti-apoptotic protein, has the ability when overexpressed in the cytosol to sequester Apaf-1, and thereby to inhibit cytochrome *c*-Apaf-1 dependent caspase-9 activation. Stress in the endoplasmic reticulum may also lead to perturbation of the Ca²⁺ stores and of the correct folding of proteins resulting in an increase in cytosolic Ca²⁺ concentrations. Subsequent to this increase in cytosolic Ca²⁺ levels, mitochondrial Ca²⁺ also rises. As mitochondria cannot retain more Ca²⁺ than what is required, the excess Ca²⁺ is released back into the cytosol, a consequence of which is the opening of the MTP and release of cytochrome *c*. This cross-talk between mitochondria and endoplasmic reticulum involving Ca²⁺ is suggested to be regulated by Bcl-2.



APPENDIX IV

Abstract

Aluminum-induction of endoplasmic reticulum-specific apoptosis in experimental neurodegeneration and its reversal by lithium. John Savory^{1,3}, Othman Ghribi¹, Mary M. Herman². *Depts. of Pathology¹ and Biochemistry and Molecular Genetics³, University of Virginia and IRP², NIMH, NIH.*

Although apoptosis under mitochondrial control has received considerable attention, mechanisms utilized within the endoplasmic reticulum (ER) and the nucleus in mediating apoptotic signals are not well understood. However, there is evidence in the literature suggesting that the ER plays an active role in regulating apoptosis. We have injected aluminum (Al) maltolate intracisternally into aged rabbits and have observed the induction of nuclear translocation of *gadd 153* and of the inducible transcription factor, NF- κ B. Translocation of these two proteins is accompanied by decreased levels of Bcl-2 in both the ER and nucleus. This treatment also induces caspase-12 activation which is a mediator of ER-specific apoptosis. These studies have been extended to assess neuroprotective strategies. Treatment with 7 mM of lithium carbonate in drinking water prevents the Al-induced translocation of cytochrome *c*, up-regulates Bcl-2 and Bcl-X_L, down-regulates Bax, abolishes caspase-3 activity and dramatically reduces DNA damage. The regulatory effect of lithium on the apoptosis-controlling proteins occurs in both the mitochondria and the ER. Thus, lithium offers promise for the treatment of neurodegenerative disorders since it is a relatively safe agent, can be administered orally, and has been used therapeutically for several decades.

APPENDIX V

MPP⁺ induces the endoplasmic reticulum stress-response in rabbit brain involving the activation of ATF-6 and NF- κ B signaling pathways.

Othman Ghribi¹, Mary M. Herman³, Patcharin Pramoonjago¹, and John Savory^{1,2}

Departments of Pathology¹, Biochemistry and Molecular Genetics², University of Virginia, Charlottesville, VA, and IRP, NIMH, NIH³, Bethesda, MD.

Correspondence to: Dr. John Savory, Department of Pathology, University of Virginia

Health Sciences Center, Box 800214, Charlottesville, Virginia 22908 U.S.A.

(434) 924-5682; FAX (434) 924-2574; Email: js2r@virginia.edu

ABSTRACT.

Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie the MPTP/MPP⁺-induced neurotoxicity, which has been used extensively as a model for Parkinson's disease. In the present study we have examined the hypothesis that MPP⁺ targets the endoplasmic reticulum. Because rabbits possess more genetic similarities to primates than to rodents we have selected this animal model system for our MPP⁺ neurotoxicity studies. MPP⁺ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. Here we demonstrate that MPP⁺ in a dose-dependent manner induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by activation of ATF-6 and NF- κ B, leads to activation of gadd 153. These effects correlate with the activation of caspase-3 and of JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

John Savory

KEY WORDS. MPP⁺, endoplasmic reticulum, ATF-6, NF- κ B, gadd 153, caspase-3,
JNK.

INTRODUCTION.

MPTP is a pro-toxin converted by monoamine oxidase B to the active toxin, MPP^+ . $MPTP/MPP^+$ is widely used to model Parkinson's disease in non-human primates and rodents, in which it induces a selective loss of dopaminergic neurons in the substantia nigra. MPP^+ is selectively accumulated by dopaminergic neurons and is concentrated within mitochondria where it acts to inhibit complex I of the electron transport chain. This effect results in a decline in the production of ATP and an increase in the formation of reactive oxygen species. These events are suggested, ultimately, to lead to cell death (for review see (1, 2). Although mitochondrial involvement in Parkinson's disease has received considerable attention, signaling pathways utilized within other subcellular organelles have been less extensively explored. A growing body of evidence now indicates that the endoplasmic reticulum (ER) plays a central role in cell signaling and mediates many neurodegenerative processes. Disturbances in the ER triggers stress signaling by two distinct pathways, the unfolded protein response (UPR) and the overload response (EOR) (for review see (3). The UPR components were first identified in yeast (see for review (4) and, to date, three signaling proteins have been identified in mammalian cells; these are PERK (PKR-like ER kinase), Ire1 (high inositol-requiring) α and β , and ATF-6 (activating transcription factor) α and β . It has not yet been determined what distinguishes the EOR from the UPR, but the signaling that mediates the EOR and UPR are different. The EOR leads to activation of NF- κ B and the UPR leads to activation of transcription factors which, in turn, activate ER stress responsive genes, such as grp 78(also called Bip) and grp 94 (see for review (5). These various

mechanisms are activated in order to deal efficiently and appropriately with encountered stress. However, severe or prolonged ER stress leads to cell death. The signaling by which the three sensors of the UPR, PERK, Ire 1 and ATF-6 lead to cell death is yet to be elucidated. All of the three sensors (i.e. PERK, Ire1 and ATF-6) converge to activate gadd 153 (also called CHOP), a transcription factor that has been implicated in the induction of apoptosis (for review see (5), by mechanisms that still remain to be determined. One possible explanation is that the ER stress response cascade activates JNK kinase; such activation plays a critical role in regulating apoptosis. Of interest in this regard is that CEP-1347/KT-7515, a JNK inhibitor, has been shown to inhibit cell loss in MPTP-treated mice (6).

In the present study, we have examined whether MPP^+ is toxic to the substantia nigra in the New Zealand white rabbit. We have administered MPP^+ directly into the cerebrospinal fluid using an injection into the cisterna magnum, and have focused our studies on assessing the effects of this treatment on the ER stress response. Our results demonstrate that MPP^+ is neurotoxic to rabbit, since it reduces the tyrosine hydroxylase-positive cell population in the substantia nigra. The apparent mechanism for this is that MPP^+ induces an ER stress response involving activation of the transcription factors, ATF-6 and NF- κ B, the activation of gadd 153 and the activation of caspase-3; the latter effect correlates with the activation of JNK kinase as well as one of its substrates, c-jun.

MATERIALS AND METHODS

Antibodies

The following antibodies were used for immunohistochemistry and Western blot analyses: mouse monoclonal antibodies (mAb) to tyrosine hydroxylase (Chemicon, Temecula, CA), ATF-6 and caspase-3 (Active Motif, Carlsbad, CA), goat anti-PERK and Ire1 polyclonal antibodies, and mAbs recognizing gadd 153, NF- κ B, JNK and p-JNK, p-c-jun and histone H1 (Santa Cruz Laboratories, Santa Cruz, CA), calnexin (Transduction Laboratories, Lexington, MD), mAbs to cytochrome c oxidase subunit IV and KDEL (detects both grp 94 and grp 78) (Stressgen, San Diego, CA).

Animals and treatment

Adult (2 years and 4-5 kg) female New Zealand white rabbits received intracisternal injections of 100 μ L of normal saline (n=6), 100 μ L of 4 mM (400 nmol) MPP⁺ iodide (Sigma Chemical Co., St Louis, MO) in saline (n=6), or 100 μ L of 10 mM (1 μ mol) MPP⁺ iodide in saline (n=6). The injections were carried out slowly over a period of 2 min under ketamine anesthesia. The animals were sacrificed at day 7. At sacrifice, rabbits were perfused at 37°C with Dulbecco's phosphate-buffered saline and the brains were promptly removed and cut to yield two symmetrical hemispheres, one for immunohistochemistry and the other for Western blot analysis. The respective sides chosen for these studies were alternated between successive animals. Each brain hemisphere intended for tissue sectioning was immediately frozen on a liquid nitrogen-

John Savory

cooled surface, placed into a zipper-closure plastic bag, and buried in dry ice pellets until transferring to -80°C for storage before sectioning and immunohistochemistry. For immunoblot analyses, the substantia nigra was isolated by a high cut through the most rostral midbrain in order to separate the brainstem from the cerebrum. The medial inferior portion of the midbrain was dissected out for homogenization. This area corresponds to the substantia nigral region containing the higher density of tyrosine hydroxylase-positive cells and processes.

All animal procedures were carried out in accordance with the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the University of Virginia Animal Care and Use Committee.

Immunohistochemistry

Tyrosine hydroxylase and p-c jun.

Coronal frozen sections ($14\text{ }\mu\text{m}$ -thick) at the substantia nigra level from control and MPP⁺-treated rabbits were air-dried, fixed in cold acetone for 10 min, treated with 1% hydrogen peroxide in PBS and incubated with a blocking solution of 1.5% normal horse serum, also in PBS. Subsequently, sections were reacted overnight at 4°C with a mouse mAb against tyrosine hydroxylase at a 1:1000 dilution or with phosphorylated c-jun at a 1:250 dilution. After washing with PBS and incubating with the biotinylated secondary

John Savory

antibody, sections were processed with a Vectastain Elite avidin-biotin complex technique kit (Vector Laboratories, Burlingame, CA) and visualized by diaminobenzidine/hydrogen peroxide, with light hematoxylin counterstaining. For negative controls, using similar sections, normal saline was substituted for the mAb. All procedures were performed at room temperature unless otherwise noted.

Oxidative DNA damage.

We used the OxyDNA assay kit (Calbiochem, San Diego, CA), which is based upon the direct binding of a mAb, fluorescently labeled, to 8-oxoguanine moities in the DNA of fixed tissue. Frozen sections (14 μ m thick) at the substantia nigra level from control and MPP⁺-treated animals were air-dried, fixed in ice-cold 4% paraformaldehyde in PBS for 15 min, briefly washed in TBS/Tween 20 and permeabilized in ice-cold 99% methanol for 30 min. After 2 washes, the sections were incubated with a blocking solution (provided in the kit) for 30 min at 37°C for 1 hr, washed twice in TBS/Tween 20, incubated overnight at 4°C with a 1:50 dilution of the FITC-conjugate concentrate that contains the binding protein conjugated to fluorescein, washed 5 times in TBS/Tween 20, and counterstained in the dark for 15 min with 1 μ g/ml propidium iodide (Sigma). After a 5 min wash in dH₂O, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a fluorescent microscope (Olympus BH2, Melville, NY) using excitation/emission wavelengths of 365/490 nm.

Western blot analysis

Proteins from whole homogenates or from the nuclear, mitochondrial, cytosolic and microsomal fractions, extracted as we have described previously(9, 10), were used when indicated. In brief, substantia nigra-enriched tissue was gently homogenized using a teflon homogenizer (Thomas Scientific, Philadelphia PA) in 7 volumes of cold suspension buffer (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 mg/ml aprotinin, 10 mg/ml leupeptin, 5 mg/mL pepstatin and 12.5 mg/mL of N-acetyl-Leu-Leu-Norleu-Al). The nuclear fractions were first isolated by centrifuging the homogenates at 750 g for 10 min at 4°C. The mitochondrial fractions were then isolated from the soluble (cytosolic) fraction at 8000 g for 20 min at 4°C. The 8000 g pellets were resuspended in cold buffer without sucrose and used as the mitochondrial fraction. The supernatant was further centrifuged at 100,000 g for 60 min at 4°C to separate the cytosolic from the microsomal fractions. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Illinois). Proteins (5 µg) from the nuclear, mitochondrial, cytosolic and microsomal fractions were separated by SDS-PAGE (10 % gel), followed by transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) and incubation with antibodies recognizing ATF 6, PERK, Ire1 and KDEL at a 1:100 dilution and gadd 153, NF-κB, caspase-3, JNK and p- JNK at 1:250. Calnexin mAb was applied as an ER marker at 1:500, histone H1 mAb as a nuclear marker at

1:500, and cytochrome c oxidase subunit IV mAb as a mitochondrial marker at a 1:1000 dilution. A mAb reacting with β actin (Sigma, Saint Louis, MI) was applied at a 1:250 dilution as a gel loading control. The blots were developed using an enhanced chemiluminescence detection kit (Bio-Rad, Hercules, CA).

RESULTS

Clinical symptoms.

The 1 μ mol MPP⁺ produces neurological symptoms, particularly gait difficulties and a partial loss of the righting reflex, and it was necessary to sacrifice the animals on day 7.

MPP⁺ reduces tyrosine hydroxylase (TH)-positive neurons and induces oxidative DNA damage.

Intracisternal administration of 1 μ mol (Figure 1a, panel C), but not 400 nmol MPP⁺ (Figure 1a, panel B), reduces TH immunoreactivity in the lateral substantia nigra (*pars compacta*) of New Zealand white rabbits in comparison to control (Figure 1a, panel A). While sections from a control (Figure 1b, panel A) or from a 400 nmol MPP⁺-treated rabbit (Figure 1b, panel B) do not exhibit 8-oxoguanine immunoreactivity, sections from a 1 μ mol MPP⁺-treated animal show intense immunoreactivity to this antibody (Figure 1b, panel C). Propidium iodide (P.I) is applied as a nuclear marker (Figure 1b, panels D-

F), and by merging the images it can be demonstrated (Figure 1b, panel I) that 8-oxoguanine immunoreactivity is predominantly localized in the nucleus (arrowheads) and to a lesser extent outside of the nucleus, probably in mitochondria (arrow, small round foci).

MPP⁺ activates the UPR and the EOR pathways.

While the ATF-6 pathway is activated by MPP⁺ treatment, as indicated by the Western blot analysis with ATF-6 mAb (Figure 2a), there is no apparent activation of the PERK and Ire1 pathways (data not shown). ATF-6 (90 kDa) is a constitutively synthesized ER protein and our result show that ATF-6 is exclusively localized in the ER in controls. In the 400 nmol MPP⁺-treated animals, the 90 kDa band is detectable in the ER and the cytosolic fractions. In the 1 μ mol MPP⁺-treated rabbits, ATF-6 is present as the 90 kDa band in the cytosol and as two intense bands (60 kDa and 50 kDa) in the nuclear fraction (Figure 2a).

The inducible transcription factor, NF- κ B, is normally sequestered in the cytoplasm by the inhibitory unit I κ B, and translocates to the nucleus following cytotoxic insults. Our results show that NF- κ B is detected as a 100 kDa band in the cytosolic fraction from the controls, 400 nmol MPP⁺-treated and 1 μ mol MPP⁺-treated animals; two additional bands of 100 and 65 kDa are detected in the nuclear fraction of the 1 μ mol-treated animals (Figure 2b).

John Savory

The purity of the different subcellular fractions used for these studies is shown to be satisfactory, as indicated by the subcellular markers (Figure 2c); cytochrome c oxidase subunit IV (Cyt.ox) stains mainly the mitochondrial fraction, calnexin stains the ER fraction and histone H1 the nuclear fraction.

MPP⁺ induces translocation of grp 94, grp 78 and gadd 153 to the nucleus.

In controls, the ER-resident proteins, grp 78 and grp 94, are detected in the ER fraction (Figure 3a). In the 400 nmol MPP⁺-treated rabbits, grp 78 and grp 94 are primarily localized in the nuclear fraction. In the 1 μ mol MPP⁺-treated rabbits, the grp 78 and grp 94 bands are less intense in the nuclear fraction than in the cytosolic (both bands) or ER fractions (grp 78) (Figure 3a and b). The grp 94 band is absent in the ER fraction (Figure 3b).

The protein gadd 153 is a non-ER-resident protein that is present in the cytosolic fractions in controls and in the 400 nmol MPP⁺-treated animals; following administration of 1 μ mol MPP⁺, gadd 153 is detected in both the cytosolic and the nuclear fractions (Figure 3b).

MPP⁺-induced ER stress correlates with the cleavage of caspase-3 and activation of JNK kinase.

Western blot analysis demonstrates the presence of a pro-caspase-3 band (32 kDa) in control and in MPP⁺-treated animals; caspase-3 p20 and p17, two activated forms of caspase-3, while not detectable in control or in 400 nmol MPP⁺-treated animals, are present in the 1 μ mol MPP⁺-treated rabbits (Figure 4a).

The mAb we used in these studies to detect JNK does not differentiate between JNK1 and JNK2. JNK is detected as a double band of 54 and 46 kDa in controls and in the 400 nmol and 1 μ mol MPP⁺-treated animals (Figure 4b). Also, the mAb employed for detecting active JNK (p-JNK) does not differentiate between phosphorylated JNK1 and JNK2. The active p-JNK is only slightly detectable as a double band in control and in the 400 nmol MPP⁺-treated animals. Administration of 1 μ mol MPP⁺ dramatically increases the levels of p-JNK (Figure 4c).

MPP⁺-induces the phosphorylation of c-jun.

Phosphorylated c-jun staining was examined in sections at the level of the substantia nigra (Figure 5, box in panel A). In sections from controls, cells are not stained with the phospho c-jun mAb (Figure 5, panel B). The 400 nmol MPP⁺ induces very few positive-scattered cells in the substantia nigra (Figure 5, panel C), whereas 1 μ mol MPP⁺ produces a marked phospho c-jun immunoreactivity of cells (Figure 5, panel D).

DISCUSSION.

Administration of MPP⁺ via the intracisternal route produces a dose-dependent loss of tyrosine hydroxylase immunostaining and oxidative DNA damage in the substantia nigra of New Zealand white rabbits. This treatment also induces activation of the two ER stress response pathways, UPR and EOR, as indicated by the translocation into the nucleus of gadd 153 and NF- κ B respectively. The neurotoxic effect of MPP⁺ correlates with cleavage of the effector of apoptosis, caspase-3, and the activation of the MAP kinase family member JNK, as well as activation of the transcription factor c-jun.

We have employed New Zealand white rabbits for our *in vivo* experiments as an alternative to the more commonly used rodent *in vivo* and *in vitro* systems, or to the less available primate. Rabbits may be a valuable resource for the study of neurotoxic agents since genetically they more closely resemble primates than rodents (11). We also have shown that this rabbit species is very susceptible to neurotoxin-induced neurodegeneration (12). Previous work has demonstrated differences in the susceptibility of different strains of rabbit to MPTP neurotoxicity (13). In the latter study, the intraperitoneal injection of MPTP causes a 40% reduction in striatal dopamine content in "little-silver-black" and no change in "chinchilla" rabbits. This difference in MPTP toxicity probably represents a difference in MPTP metabolism in these breeds. In our animal model system we have overcome these problems by injecting MPP⁺ directly into the cerebrospinal fluid of the brain via the cisterna magnum and, as shown in our results,

this mode of administration in New Zealand white rabbits is neurotoxic to dopaminergic neurons in the substantia nigra.

A growing body of evidence now indicates that the endoplasmic reticulum plays a central role in cell signaling and that it mediates many neurodegenerative processes. This intracellular organelle is of fundamental importance, since it serves as a dynamic Ca^{2+} store, with a high free Ca^{2+} concentration maintained within its lumen. Besides being a Ca^{2+} storage site, the ER lumen is the cellular compartment where surface and secreted proteins are synthesized, folded and assembled before being transported. Alterations in Ca^{2+} homeostasis lead to a disturbance in correct protein folding and result in the accumulation of misfolded proteins within the ER. Disturbances in Ca^{2+} homeostasis and protein processing trigger stress signaling from the ER by two distinct pathways, the UPR and the EOR (for review see (3)). This signaling is intended to provide cells with the capability to deal with changes in Ca^{2+} levels and with the toxic buildup of misfolded proteins. Although ATF-6, Ire-1 and PERK share the same protein and transcription factor targets, the relationship between the three proteins remains to be clarified. Furthermore, concomitant activation of the three signaling proteins is not required; activation of only one of the three is sufficient to activate the UPR cascade.

ATF-6 is a constitutively synthesized ER protein that binds directly to the cis-acting ER stress-response element (ERSE) responsible for the mammalian UPR (14,15). Upon ER stress, ATF-6 is first transported to the Golgi where it is cleaved in the nucleus and

activates the ER-resident chaperones, grp 94 and grp78, and gadd 153 (16, 17). However, up to the present time, not all the ATF-6-target genes have been fully identified. Our results show that 1 μ mol MPP⁺ clearly activates ATF-6, leading to cleavage in the nucleus. We also demonstrate that cleavage of ATF-6 correlates with activation of the target proteins, grp 94 and grp 78.

Increased levels of NF- κ B activity have been observed in the brain of patients with various neurodegenerative disorders including Parkinson's disease (18). NF- κ B, is an inducible transcription factor and is an important mediator of the human immune and inflammatory response. Exposure of cells to various pathological stimuli activates NF- κ B; activated NF- κ B dimer is rapidly released from the cytoplasm, where it is normally sequestered by the inhibitory unit I κ B, and then translocates to the nucleus. Various agents that induce stress in the ER have been shown to activate NF- κ B (19-21). The role of NF- κ B in regulating neuronal death is complex. In some cases it has been demonstrated to promote neuronal survival, and in other cases to induce neuronal death. It appears that whether NF- κ B acts as a promoter or inhibitor of neuronal loss depends on the cell type and the nature of the toxic stimuli. Our data show that the higher, but not the lower MPP⁺ concentration induces activation of NF κ -B, and that this activation correlates with the activation of caspase-3. Caspase-3 is an effector of apoptosis and may represent the last-step in the initiation of apoptosis prior to DNA fragmentation.

Grp94 and grp78 are ER-resident proteins that are activated in response to ER stress and serve as molecular chaperones. These proteins are involved in protein translocation, protein folding and assembly, and the regulation of protein secretion. Their expression is induced during cytotoxic stimuli including oxidative stress, chemical toxicity, treatment with Ca^{2+} ionophores and inhibitors of glycosylation (3). Activation of these chaperones during the UPR is necessary to alleviate ER stress, maintain ER function, facilitate protein folding and thus protect cells from toxic insults. However, mechanisms that underlie the cellular protection of these ER stress-chaperones are still unknown.

Activation of gadd 153 expression has been demonstrated following transient cerebral ischemia in the rat (22). It is a non-ER-resident protein that is specifically activated in response to stress in the ER. Upon activation, gadd 153 translocates from the cytoplasm to the nucleus where it may participate in induction of apoptosis. The gadd 153 gene is specifically activated by agents that disturb ER function such thapsigargin, an agent that perturbs intracellular free calcium. mRNA levels for gadd 153 has been demonstrated to be increased both during hypoxia and after exposure of cells to agents that elevate the levels of glucose-regulated proteins (23). The exact mechanism by which gadd 153 kills or participates in cellular death remains to be elucidated. Our results demonstrate that the non-toxic effect of the 400 nmol MPP^+ correlates with increased levels of grp 94 and grp 78 and with no gadd 153 in the nucleus. The marked translocation of gadd 153 to the nucleus, we show with the neurotoxic 1 μmol MPP^+ , is accompanied by a decrease in the level of grp 94 and grp 78 proteins in the nucleus.

John Savory

Caspase-3 is an effector caspase and its activation may occur through different pathways. This activation can result from cytochrome *c* release by the mitochondria and activation of caspase-9, which in turn activates caspase-3. Caspase-3 can also be activated by caspase 8 through death receptors. Perturbation of ER function and release of caspase-12 can also activate caspase-3 (24). The activation of caspase-3 that we observe with treatment with 1 μ M MPP⁺ may result from activation of one or more of these three different pathways.

Recently, evidence that ER stress contributes to neuronal death has been demonstrated in an *in vitro* model of Parkinson's disease where incubation of PC12 cells with MPP⁺ induces the phosphorylation of the endoplasmic reticulum kinases Ire1 α and PERK (8).

Treatment of SH-SY5Y cells with MPP⁺ has been demonstrated to increase the expression of gadd 153; rotenone or 6-hydroxydopamine, whose modes of action are via mitochondrial impairment and oxidative stress respectively, did not exhibit this effect (7). These latter results as well as our results suggest that MPP⁺ targets the ER directly.

Evidence has implicated the JNK kinase in the death of dopaminergic nigral neurons in the MPTP model of Parkinson's disease *in vitro* (25) and *in vivo* (26). In nigral cultures, MPP⁺ treatment induces JNK activation, an effect that precedes the maximal induction of apoptosis (27). MPP⁺ also activates JNK and NF- κ B in SH-SY5Y

neuroblastoma cells (28). However, in these studies no attempt was made to determine the possibility that ER stress is the inducer of JNK.

In the present report we find that MPP⁺-induced toxicity correlates with phosphorylation of c-jun in the substantia nigra. JNK activities are regulated in the cytoplasm by phosphorylation of threonine (Thr¹⁸³ for JNK1 and JNK2) and tyrosine (Tyr¹⁸⁵ for JNK1 and JNK2) residues. Some of the physiological and apoptotic actions of the JNKs include the phosphorylation of c-jun (see for review (29)).

Collectively, our results demonstrate that MPP⁺ is neurotoxic to rabbit brain by mechanisms that involve stress in the ER and activation of the JNK kinase signaling pathway. Prevention of ER stress or the inhibition of JNK kinase activity may thus be necessary to prevent MPP⁺ toxicity and may represent a new therapeutic approach in the prevention of neurotoxin-induced Parkinson's disease.

REFERENCES.

1. Cassarino DS, Parks JK, Parker WD, Bennett JP. The parkinsonian neurotoxin MPP⁺ opens the mitochondrial permeability transition pore and releases cytochrome c in isolated mitochondria via an oxidative mechanism. *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1999;1453:49-62.
2. Greenamyre JT, MacKenzie G, Peng TI, Stephans SE. Mitochondrial dysfunction in Parkinson's disease. *Biochem Soc Symp* 1999;66:85-97.
3. Kaufman RJ. Molecular chaperones and the heat shock response. Sponsored by Cold Spring Harbor Laboratory, 6-10 May 1998. *Biochim Biophys Acta* 1999;1423:R13-R27.
4. Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* 2001;13:349-55.
5. Cudna RE, Dickson AJ. Endoplasmic reticulum signaling as a determinant of recombinant protein expression. *Biotechnol Bioeng* 2003;81:56-65.
6. Saporito MS, Thomas BA, Scott RW. MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. *J Neurochem* 2000;75:1200-8.
7. Conn KJ, Gao WW, Ullman MD, McKeon-O'Malley C, Eisenhauer PB, Fine RE et al. Specific up-regulation of GADD153/CHOP in 1-methyl-4-phenyl-pyridinium-treated SH-SY5Y cells. *J Neurosci Res* 2002;68:755-60.
8. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosci* 2002;22:10690-8.
9. Ghribi O, Herman M.M, and and Savory J. Lithium inhibits Abeta-induced stress in the endoplasmic reticulum in rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation. *J Neurosci Res* 2003;71:853-862.
10. Ghribi O, Herman MM, Spaulding NK, Savory. Lithium inhibits aluminum-induced apoptosis in rabbit hippocampus, by preventing cytochrome c translocation, Bcl-2 decrease, Bax elevation and caspase-3 activation. *J Neurochem* 2002;82:137-45.

11. Graur D, Duret L, Gouy M. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). *Nature* 1996;379:333-5.
12. Ghribi O, Herman MM, DeWitt DA, Forbes MS, Savory J. A- β (1-42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of *gadd 153* and NF- κ B. *Mol Brain Res* 2001; 96:30-8.
13. Lermontova N, Drany O, Dobrota D, Mezesova V, Lombardi V, Bachurin S. Relative resistance of rabbits to MPTP neurotoxicity. *Mol Chem Neuropathol* 1995; 25:135-41.
14. Yoshida H, Haze K, Yanagi H, Yura T, Mori K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem* 1998;273:33741-9.
15. Roy B, Lee AS. The mammalian endoplasmic reticulum stress response element consists of an evolutionarily conserved tripartite structure and interacts with a novel stress-inducible complex. *Nucleic Acids Res* 1999;27:1437-43.
16. Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M et al. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol* 2000;20:6755-67.
17. Haze K, Okada T, Yoshida H, Yanagi H, Yura T, Negishi M et al. Identification of the G13 (cAMP-response-element-binding protein-related protein) gene product related to activating transcription factor 6 as a transcriptional activator of the mammalian unfolded protein response. *Biochem J* 2001;355:19-28.
18. Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M et al. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proc Natl Acad Sci U S A* 1997;94:7531-6.
19. Pahl HL, Baeuerle PA. The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci* 1997;22:63-7.
20. Pahl HL, Sester M, Burgert HG, Baeuerle PA. Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. *J Cell Biol* 1996;132:511-22.
21. Guerrini L, Blasi F, Denis-Donini S. Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons in vitro. *Proc Natl Acad Sci U S A* 1995; 92:9077-81.

22. Paschen W, Gissel C, Linden T, Althausen S, Doutheil J. Activation of gadd153 expression through transient cerebral ischemia: evidence that ischemia causes endoplasmic reticulum dysfunction. *Brain Res Mol Brain Res* 1998;60:115-22.
23. Price BD, Calderwood SK. Gadd45 and Gadd153 messenger RNA levels are increased during hypoxia and after exposure of cells to agents which elevate the levels of the glucose-regulated proteins. *Cancer Res* 1992;52:3814-7.
24. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000;403:98-103.
25. Sawada H, Ibi M, Kihara T, Honda K, Nakamizo T, Kanki R et al. Estradiol protects dopaminergic neurons in a MPP(+)-Parkinson's disease model. *Neuropharmacology* 2002;42:1056-64.
26. Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB. Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci U S A* 2001;98:10433-8.
27. Gearan T, Castillo OA, Schwarzschild MA. The parkinsonian neurotoxin, MPP+ induces phosphorylated c-Jun in dopaminergic neurons of mesencephalic cultures. *Parkinsonism Relat Disord* 2001;8:19-22.
28. Cassarino DS, Halvorsen EM, Swerdlow RH, Abramova NN, Parker WD, Jr., Sturgill TW et al. Interaction among mitochondria, mitogen-activated protein kinases, and nuclear factor-kappaB in cellular models of Parkinson's disease. *J Neurochem* 2000;74:1384-92.
29. Mielke K, Herdegen T. JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system. *Prog Neurobiol* 2000;61:45-60.

APPENDIX VI

MPP+ INDUCES THE ENDOPLASMIC RETICULUM STRESS-RESPONSE IN RABBIT BRAIN INVOLVING THE ACTIVATION OF ATF-6 AND NF- κ B SIGNALING PATHWAYS

O. Ghribi^{1*}; M.M. Herman²; P. Pramoonjago¹; J. Savory¹

1. Dept Pathology, Univ Virginia, Charlottesville, VA, USA 2. IRP, NIMH, NIMH, Bethesda, MD, USA

Inhibition of mitochondrial function and the subsequent generation of oxidative stress are strongly suggested to underlie the MPTP/MPP+-induced neurotoxicity, which has been used extensively as a model for Parkinson's disease. In the present study we examined the hypothesis that MPP+ targets the endoplasmic reticulum. Since rabbits are more genetically similar to primates than to rodents we selected this animal model system for our MPP+ neurotoxicity studies. MPP+ was administered directly into the brain of New Zealand white rabbits via the intracisternal route, and the effects on tissue from the substantia nigra were examined. MPP+, in a dose-dependent manner, induces the loss of tyrosine hydroxylase activity, oxidative DNA damage, and activation of the endoplasmic reticulum stress response. The endoplasmic reticulum response, mediated by activation of ATF-6 and NF- κ B, leads to activation of gadd 153. These effects correlate with the activation of caspase-3 and of JNK kinase. We propose that pharmacological agents that prevent the perturbation of endoplasmic reticulum function or prevent the activation of JNK may represent potential therapeutic approaches for the prevention of neurotoxin-induced Parkinson's disease.

Support Contributed By: US Department of the Army and the Environmental Protection Agency

Close

APPENDIX VII

Cellular mechanisms of neurodegeneration and Alzheimer's disease

John Savory^{1,2}, Othman Ghribi¹, and Mary M. Herman³

Departments of Pathology¹, Biochemistry and Molecular Genetics², University of Virginia, Charlottesville, VA, and IRP, NIMH, NIH³, Bethesda, MD.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the deposition of extracellular neuritic plaques (NPs), the formation of neurofibrillary tangles (NFTs), and massive neuronal and synaptic loss. A β is the major component of the NPs, with hyperphosphorylated tau being the primary constituent of NFTs; both of these aggregates are generally accepted as being key players in the pathogenesis of AD. Whether A β or tau actually represents the primary cause of neuronal loss in AD, and which of the two abnormal changes is generated first and is presumably the more important, remains a matter of debate. Furthermore, the mechanisms by which A β and/or tau kill neurons in Alzheimer's disease (AD) are not fully understood. Oxidative stress has been proposed as a major factor in neuronal injury and as an inducer of apoptosis. However, whether apoptosis or necrosis is the major route of cell death remains to be clarified, although an apoptotic mechanism is gaining increasing support.

We have developed a unique animal model where the intracisternal administration of aggregated A β (1-42) peptide into rabbit brain induces neuronal death and hyperphosphorylation of tau protein. Death of neurons is mediated by endoplasmic reticulum (ER)-specific apoptosis as is evident by the activation of caspase-12, a specific marker for ER stress, and by activation of the pivotal inducer of apoptosis, caspase-3, which we show to be primarily localized in the ER. These results implicate A β as the trigger of tau phosphorylation with the ER as a direct target for A β -induced neurotoxicity. We further demonstrate that neurotoxic effect of A β correlates with the activation of the JNK and ERK MAP kinases along with the activation of the transcription factors, c-jun and c-fos. Treatment of the experimental animals with lithium or glial cell-derived nerve growth factor (GDNF) prevents induction of ER stress but not hyperphosphorylation of tau. These results suggest that two distinct pathways are involved in the apoptosis cascade activation and in tau phosphorylation following A β administration. While the effects of lithium and GDNF on caspase-3 and tau are similar, they differentially regulate the MAP kinases, JNK and ERK pathways. The results of these experiments add extended insight into intracellular molecular signaling pathways that could form the basis underlying neurodegeneration particularly resulting from the neurotoxic effect of A β .



Commentary

Is amyloid β -peptide neurotoxic or neuroprotective and what is its role in the binding of metal ions?

John Savory^{a,b,*}, Othman Ghribi^a, Mary M. Herman^c

^a Department of Pathology, Health Sciences Center, University of Virginia, P.O. Box 168, Charlottesville, VA 22908, USA

^b Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, USA

^c IRP, NIMH, NIH, Bethesda, MD, USA

Received 23 January 2002; accepted 23 January 2002

Alzheimer's disease is a progressive neurodegenerative disorder with three characteristic neuropathological features, namely the extracellular deposition of amyloid β -peptide (A β) [28], the formation of neurofibrillary tangles (of which microtubule-associated protein tau (tau) is a primary component), and a selective loss of neurons. How these features interrelate and which, if any, is of primary importance is still unclear. Selective neuronal death involves vulnerable brain regions, in particular the hippocampus and cerebral cortex, and apoptosis may play a role in the process of cell loss (for review, see [15]).

Robinson and Bishop in their clearly presented and provocative article "A β as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease" [24], propose that A β accumulation is a neuroprotective response to an upstream adverse event that non-specifically leads to Alzheimer's disease and perhaps other types of neurodegeneration. The authors have developed their hypothesis on an extensive literature that fails to show that A β accumulation consistently correlates with neuronal loss and cognitive decline. Transgenic mice which accumulate large loads of A β -containing plaques show either no change or only a mild neurotoxic response to these loads. This endogenously produced A β may represent the optimal model system for studying aggregated A β neurotoxicity. However, as Robinson and Bishop state, "mice are not humans" and they could have a much different response to the presence of a neurotoxin. The other mode for creating a model for an increased burden of aggregated A β is to inject this material directly into the brain of experimental animals. As reviewed by Robinson and Bishop, the results of such experiments are equivocal but A β injections have been shown to produce neurotoxicity in aged monkeys [10]; however,

such treatment of rats does not appear to induce neurotoxicity [2]. Recent studies in our laboratory have employed rabbits as the experimental animal. Rabbits may be particularly relevant to the investigation of human disease since they belong to the mammalian order Lagomorpha, a group reported to more closely resemble primates than rodents [14]. The experiments performed in our laboratory involved the intracisternal injection of aggregated A β (1–42) into aged New Zealand white rabbits [12]. Although evidence of severe neurotoxicity was not observed, there was stress to the ER as indicated by reduced levels of the antiapoptotic protein, Bcl-2, in this organelle. Additional stress was suggested by the induction of nuclear translocation of *gadd153* and the inducible transcription factor, NF- κ B. It could be expected that any neuropathological response to this exogenous mode of administration of A β might be different from that induced by the A β produced endogenously, as is the case in transgenic mice. Exogenous administration certainly has more potential to introduce environmental contaminants such as toxic metals.

The neurotoxic effect of metal ions in transgenic mice carrying an increased burden of A β plaques has not been extensively studied. The hypothesis of Robinson and Bishop introduces the possibility that flocculant A β deposits possess neuroprotective properties by binding toxic materials, particularly toxic metals, rendering them inactive until they can be eliminated from the central nervous system. In regard to this latter hypothesis, another aspect of metal–A β interactions has focused on the possible intimate involvement of copper (Cu), zinc (Zn) and iron (Fe) in A β formation and/or biological activity. A β (1–42) binds Cu(II) with extremely high affinity [1] and Cu(II), Zn(II) and Fe(III) can induce aggregation of A β [3]. Furthermore, brain tissue levels of these metals are elevated in the neocortical region of patients with Alzheimer's disease, and are present in amyloid plaque deposits [19]. These findings can support the hypothesis that

* Corresponding author. Tel.: +1-434-924-5682; fax: +1-434-924-2574.
E-mail address: js2r@virginia.edu (J. Savory).

metal-induced aggregation of A β is an important step in the pathogenesis of Alzheimer's disease. However, in diseases where free Cu(II) or Fe(III) is available due to an inherited defect related to transport and cellular uptake of these metal species, there is no evidence of an increased formation of A β plaques. An example of this is in Wilson's disease, an inherited disorder of copper metabolism, in which there is increased free Cu(II) and considerable evidence of neurotoxicity, but not an increased formation of plaques [9]. Similarly, in neurodegeneration with brain iron accumulation-1 (formerly termed Hallervorden-Spatz disease) there are extracellular proteinaceous accumulations (spheroids), but again no evidence of excessive plaque formation [8]. Although Zn(II), Cu(II) and Fe(III) induce rapid aggregation of A β in vitro [3], and agents that specifically chelate Cu(II) and Zn(II) will solubilize A β in postmortem brain tissue obtained from Alzheimer's disease patients [7], there is no direct evidence that these metal ions are involved in the A β aggregation process in Alzheimer's disease. A recent report has shown that oral treatment of mice transgenic to APP 2567 with clioquinol, stated to be a Cu/Zn chelator, inhibits A β accumulation and significantly improves general health and body weight parameters in comparison to sham-treated animals [6]. Clioquinol is a derivative of 8-hydroxy quinoline and is not a specific chelator of Cu(II) and Zn(II), although there is some implication that this is the case by the authors of that article. In this regard, the order of stability constants for binding of 8-hydroxyquinoline derivatives is Fe(III) > Cu(II) $\gg$ Ni(II) > Al(III) > Zn(II) [21] and the affinity of clioquinol binding to Cu(II) and Zn(II) is much less (K_1 (Cu) = 8.9; K_1 (Zn) = 7.0) than the attomolar levels reported by Atwood et al. [1].

Robinson and Bishop review other features of A β aggregation that are important to the present commentary. *First*: patients with traumatic head injury can rapidly form A β plaques within a few days of the injury, but these lesions may eventually disappear [20]. *Second*: A β is constantly being produced in the healthy brain. Both of these events suggest that there has to be an additional component present to induce aggregation of A β and to render it relatively stable. If this other component is not Cu and Fe, as indicated by the lack of increased plaques in human diseases where these metals accumulate in the brain, then other metals or compounds must be considered. Zinc is still a possibility but this metal is ubiquitous and possesses many vital biological properties. It is unlikely, in our opinion, that Zn would suddenly assume a toxic role in the cerebral cortex of aged brains.

Robinson and Bishop mention aluminum (Al) as a possible toxic metal along with Cu, Zn and Fe. The hypothesis that aluminum is involved in the pathogenesis of Alzheimer's disease is anathema to most neuroscientists, probably due to conflicting data in the literature reflecting the complex chemistry and ubiquitous nature of this element. Aluminum is the most common metal in the earth's crust and is highly toxic at picomolar concentrations. As

with Cu and Fe, there is a human disorder that involves the accumulation of Al in the brain. This disease may result from long-term hemodialysis treatment and was quite common 20 years ago when Al-contaminated water was used to prepare dialysate solutions. In this iatrogenic disease, a fatal encephalopathy often developed, but was not associated with Alzheimer's-like pathology except in rare reports [4]. However, the renal-impaired patient is known to accumulate a plethora of abnormal metabolites, including conjugates of phenol, organic acids of the tricarboxylic acid cycle and others that undoubtedly are good Al ligands and would modify its toxic action [29]. Few investigators have attempted to detect Al within neuritic plaques, since the analytical challenges are considerable. A β has a molecular weight of $\sim$ 5000 kDa and since Al(III) has an atomic weight of 27 and is trivalent, it is possible that the ratio of A β on a weight to weight basis could be 550:1. Thus a microprobe method for detecting Al within plaques would have to be exquisitely sensitive. Candy et al. [5] reported the presence of Al within plaques in the brain of an Alzheimer's disease patient, although Landsberg et al. [18] failed to confirm this finding with the alternative microanalytical technique of particle-induced X-ray emission (PIXE). However, this nuclear microscopic technique is relatively insensitive below 15 μ g/g of Al, making the significance of this report questionable.

Arguments for or against the possible role of Al in neurodegenerative disorders have their strong supporters, but it is quite premature to discard Al as a possible toxic metal that could contribute to the stabilization of A β plaques and to their toxicity. The involvement of Al in the plaque hypothesis also provides a possible answer to the formation of the other classical neuropathological feature of Alzheimer's disease, namely the neurofibrillary tangle. It is difficult to conceptualize a mechanism whereby an insoluble mass of proteinaceous material might initiate intracellular events leading eventually to cell death. The fact that such materials, in the case of A β , may in one form be non-toxic but as a β -sheet become toxic supports the hypothesis that the injurious agent is bound to the plaque but can be released and gain entry to brain cells. In our laboratory, after the injection of Al maltolate intracisternally into rabbits, the numerous intensely argyrophillic intraneuronal neurofibrilamentous aggregates which result are immunopositive for hyperphosphorylated tau, abnormally phosphorylated neurofilament proteins, α_1 -antichymotrypsin and ubiquitin [16,25], all of which are present in the neurofibrillary tangles of Alzheimer's disease [17]. Interestingly, treatment with an Al chelating agent will cause the bulk of these intraneuronal protein accumulations to disappear [26,27]. Admittedly, the animal model system used in our studies reflects an acute neurotoxicity, and the neurofibrilamentous aggregates do not demonstrate paired helical filament structures, although the latter may well be a species-related phenomenon as only humans are known to display this structure. However, very few systems have come as close to mimicking the biochemical

composition of the Alzheimer's neurofibrillary tangle as has in this Al in vivo model.

One other piece of evidence supporting the hypothesis of the possible role of Al in the A β plaque is as follows: Nakagawa et al. have shown, using in vitro experiments with A β (1–40), that there is an endoplasmic reticulum-specific apoptosis mediated by a novel caspase, caspase-12 [23]. The endoplasmic reticulum (ER) is a subcellular organelle that plays a key role in protein folding and maintenance of calcium homeostasis. It also contains members of the Bcl-2 family of proteins such as Bcl-2, Bcl-X_L and Bax which play important roles in controlling neuronal viability and apoptotic death. The role of a perturbed ER function in the pathogenesis of Alzheimer's disease is discussed in a recent review by Mattson et al. where it is proposed that stress to the ER leads to altered proteolytic processing of amyloid precursor protein with a subsequent increase in the vulnerability of neurons to apoptosis [22]. We have shown that Al also induces stress in the ER of rabbit hippocampus as assessed by a decrease in the antiapoptotic and an increase in the proapoptotic proteins, caspase-3 activation and apoptosis [11,13]. As stated above, the administration of A β (1–42) via a similar intracisternal route also induces stress in the ER, however, no caspase-3 activation or apoptosis has been observed [12]. It is anticipated that the administration of higher doses of A β will induce a more severe neurotoxic response.

In summary, we generally support the hypothesis proposed by Robinson and Bishop that A β and its aggregates are non-toxic. As put forward in their excellent commentary, these aggregates (plaques) probably form constantly in the healthy brain but are cleared quickly. In traumatic brain injury, where there is an up-regulation of amyloid precursor protein and an increased production of A β , a large number of plaques may be deposited. The formation of the plaques is not dependent on the presence of Cu, Zn or Fe, but could be dependent on other toxic metals such as, but not limited to Al. However, the stabilization of the plaques for long periods of time, perhaps permanently, is dependent on the binding of metals, and again Al is a possibility although this has not been shown experimentally. Since patients with Wilson's disease or with neurodegeneration with brain-iron accumulation-1 do not have increased plaque formation, then Cu and Fe are not likely candidates for the stabilization of plaques, although they could be bound to these aggregates and thus could increase oxidative stress and neuronal injury. Sequestration of essential metals such as Cu, Zn, Fe, selenium and others could also contribute to cell injury. We believe it less likely, as proposed by Robinson and Bishop, that plaque formation is a specific mechanism for chelating toxic metals and perhaps other toxic agents, and in sequestering them for phagocytosis. Rather we favor A β aggregation as a physical property of this peptide, a process that may lead to neurotoxic complications if a toxic metal is available to bind and stabilize the aggregate. This process may also bind to other metals, inducing oxidative stress, and may de-

plete the brain cells of essential trace elements. A β aggregation may not be a tightly controlled physiological process; however, amyloid precursor protein itself is probably tightly regulated and A β production is largely a byproduct of its metabolism.

The excessive formation of A β plaques in transgenic mice overexpressing amyloid precursor protein is likely due to an over-production of A β , exceeding the cell's ability to metabolize and clear this peptide. Interesting future experiments could involve exposing these animals to toxic metals to ascertain whether the plaques can be stabilized and whether neurotoxicity ensues. A request for proposal to study the APP/PS1 transgenic mouse exposed to Al maltolate in the drinking water has been issued recently by Health Canada, and the results from such studies should help clarify the possible relationship between A β plaques and exposure to a neurotoxic metal.

Acknowledgments

We gratefully acknowledge helpful discussions with Dr. R. Bruce Martin, Emeritus Professor in the Department of Chemistry at the University of Virginia. Supported by Grant # DAMD 17-99-1-9552 from the US Department of the Army and the Virginia Center on Aging.

References

- [1] Atwood CS, Scarpa RC, Huang X, Moir RD, Jones WD, Fairlie DP, et al. Characterization of copper interactions with Alzheimer amyloid β peptides: identification of an attomolar-affinity copper binding site on amyloid beta(1–42) *J Neurochem* 2000;75:1219–33.
- [2] Bishop GM, Robinson SR. β -Amyloid helps to protect neurons against oxidative stress. *Neurobiol Aging* 2000;21(Suppl 1S):S226.
- [3] Bush AI, Pettingell WH, Multhaup G, Paradis M, Vonsattel JP, Gusella JF, et al. Rapid induction of Alzheimer A β amyloid formation by zinc. *Science* 1994;265:1464–7.
- [4] Candy JM, McArthur FK, Oakley AE, Taylor GA, Chen CP, Mountfort SA, et al. Aluminium accumulation in relation to senile plaque and neurofibrillary tangle formation in the brains of patients with renal failure. *J Neurol Sci* 1992;107:210–8.
- [5] Candy JM, Oakley AE, Klinowski J, Carpenter TA, Perry RH, Atack JR, et al. Aluminosilicates and senile plaque formation in Alzheimer's disease. *Lancet* 1986;1:354–7.
- [6] Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, et al. Treatment with a copper-zinc chelator markedly and rapidly inhibits β -amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* 2001;30:665–76.
- [7] Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, et al. Aqueous dissolution of Alzheimer's disease A β amyloid deposits by biometal depletion. *J Biol Chem* 1999;274:23223–8.
- [8] Duchen LW, Jacobs JM. Nutritional deficiencies and metabolic disorders. In: Adams JH, Corsellis JAN, Duchen LW, editors. *Greenfield's neuropathology*. New York, NY: Wiley, 1984. p. 609–10.
- [9] Duchen LW, Jacobs JM. Nutritional deficiencies and metabolic disorders. In: Adams JH, Corsellis JAN, Duchen LW, editors. *Greenfield's neuropathology*. New York, NY: Wiley, 1984. p. 595–9.

- [10] Geula C, Wu C-K, Saroff D, Lorenzo A, Yuan M, Yankner BA. Aging renders the brain vulnerable to amyloid β -protein neurotoxicity. *Nat Med* 1998;4:827–31.
- [11] Ghribi O, DeWitt DA, Forbes MS, Herman MM, Savory J. Co-involvement of mitochondria and endoplasmic reticulum in regulation of apoptosis: changes in cytochrome *c*, Bcl-2 and Bax in the hippocampus of aluminum-treated rabbits. *Brain Res* 2001;903:66–73.
- [12] Ghribi O, Herman MM, DeWitt DA, Forbes MS, Savory J. A β (1–42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of *gadd 153* and NF- κ B. *Mol Brain Res* 2001;96:30–8.
- [13] Ghribi O, Herman MM, Forbes MS, DeWitt DA, Savory J. GDNF protects against aluminum-induced apoptosis in rabbits by upregulating Bcl-2 and Bcl-X_L and inhibiting mitochondrial Bax translocation. *Neurobiol Dis* 2001;8:764–73.
- [14] Graur D, Duret L, Gouy M. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies) *Nature* 1996;379:333–5.
- [15] Honig LS, Rosenberg RN. Apoptosis and neurologic disease. *Am J Med* 2000;108:317–30.
- [16] Huang Y, Herman MM, Liu J, Katsetos CD, Wills MR, Savory J. Neurofibrillary lesions in experimental aluminum-induced encephalomyelopathy and Alzheimer's disease share immunoreactivity for amyloid precursor protein, A β , α ₁-antichymotrypsin and ubiquitin-protein conjugates. *Brain Res* 1997;771:213–20.
- [17] Iqbal K, Grundke-Iqbal I. Neurofibrillary tangles, beta-protein fibrillogenesis and neuritic plaques. In: Calne DB, editor. *Neurodegenerative disease*. Philadelphia: Saunders, 1994. p. 71–95.
- [18] Landsberg JP, McDonald B, Watt F. Absence of aluminium in neuritic plaque cores in Alzheimer's disease. *Nature* 1992;360:65–8.
- [19] Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* 1998;158:47–52.
- [20] Macfarlane DP, Nicoll JA, Smith C, Graham DI. APOE ϵ 4 allele and amyloid β -protein deposition in long term survivors of head injury. *Neuroreport* 1999;10:3945–8.
- [21] Martell AE, Smith RM. *Critical stability constants*. New York, NY: Plenum, 1974.
- [22] Mattson MP, Gary DS, Chan SL, Duan W. Perturbed endoplasmic reticulum function, synaptic apoptosis and the pathogenesis of Alzheimer's disease. *Biochem Soc Symp* 2001;151:62.
- [23] Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature* 2000;403:98–103.
- [24] Robinson SR, Bishop GM. A β as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease. *Neurobiol Aging*, 2000, in press.
- [25] Savory J, Huang Y, Herman MM, Reyes MR, Boyd JC, Wills MR. Abnormal tau accumulation and partial reversal by desferrioxamine in Al-induced neurofibrillary degeneration in rabbits. In: Iqbal K, Mortimer JA, Winblad B, Wisniewski HM, editors. *Research advances in Alzheimer's disease and related disorders*. New York, NY: Wiley, 1995. p. 543–51.
- [26] Savory J, Huang Y, Herman MM, Reyes MR, Wills MR. Tau immunoreactivity associated with aluminum maltolate-induced neurofibrillary degeneration in rabbits. *Brain Res* 1995;669:325–9.
- [27] Savory J, Huang Y, Wills MR, Herman MM. Reversal by desferrioxamine of tau protein aggregates following 2 days of treatment in aluminum-induced neurofibrillary degeneration in rabbit: implications for clinical trials in Alzheimer's disease. *NeuroToxicology* 1998;19:209–14.
- [28] Selkoe DJ. The molecular pathology of Alzheimer's disease. *Neuron* 1991;6:487–98.
- [29] Wills MR. *Metabolic consequences of renal failure*. 2nd ed. Tring, UK: Maund & Irving Ltd., 1978. p. 13–5.

APPENDIX IX

A β induces hyperphosphorylation of tau and apoptosis in rabbit brain, involving activation of GSK-3 β and JNK pathways.

Othman Ghribi¹, Mary M. Herman³, Natalie K. Spaulding¹, and John Savory^{1,2}.

Departments of Pathology¹, Biochemistry and Molecular Genetics², University of Virginia, Charlottesville, VA, and IRP³, NIMH, NIH, Bethesda, MD.

Alzheimer's disease is characterized by deposition of A β , formation of neurofibrillary tangles involving hyperphosphorylation of tau protein, and neuronal loss in vulnerable brain regions. There is evidence that accumulation of A β aggregates is an upstream process that leads to tau hyperphosphorylation and accumulation, and eventually neuronal death, although the mechanisms underlying these processes is unclear. We have developed an animal model system to study the effect of A β by its direct (intracisternal) injection into rabbit brain. Using this system we have investigated the effect of A β on GSK-3 β and JNK pathway signaling, activation of which is known to interfere with apoptotic neuronal death cascade and to induce hyperphosphorylation of tau. Adult (2.5-3 years and 4-5 kg) female New Zealand white rabbits received either intracisternal injections of 100 μ L normal saline (n=6; controls) or 100 μ L of 2 mg/ml A β (1-42) (n=6; A β -treated group). Rabbits were sacrificed after 7 days and perfused with Dulbecco's phosphate buffered saline. The brains were promptly removed, and a coronal section cut and bisected to yield two symmetrical hippocampal segments, one for Western blot analysis and the other for immunohistochemistry. Our results show that A β induces activation of GSK-3 β , by its translocation into the nucleus, phosphorylation of JNK, and hyperphosphorylation of tau at Ser 202 as measured by the AT8 mAb. This A β treatment triggers cytochrome *c* release from mitochondria to the cytoplasm, decreases the levels of the antiapoptotic Bcl-2 protein, and activates caspase-3 together with the transcription factor NF- κ B. Thus, we have demonstrated that A β triggers tau hyperphosphorylation and apoptosis by mechanisms involving simultaneous activation of GSK-3 β and JNK pathways. We suggest that treatment with agents that prevent activation of GSK-3 β and JNK pathways would represent a therapeutic strategy for the prevention of A β -induced neurodegeneration.

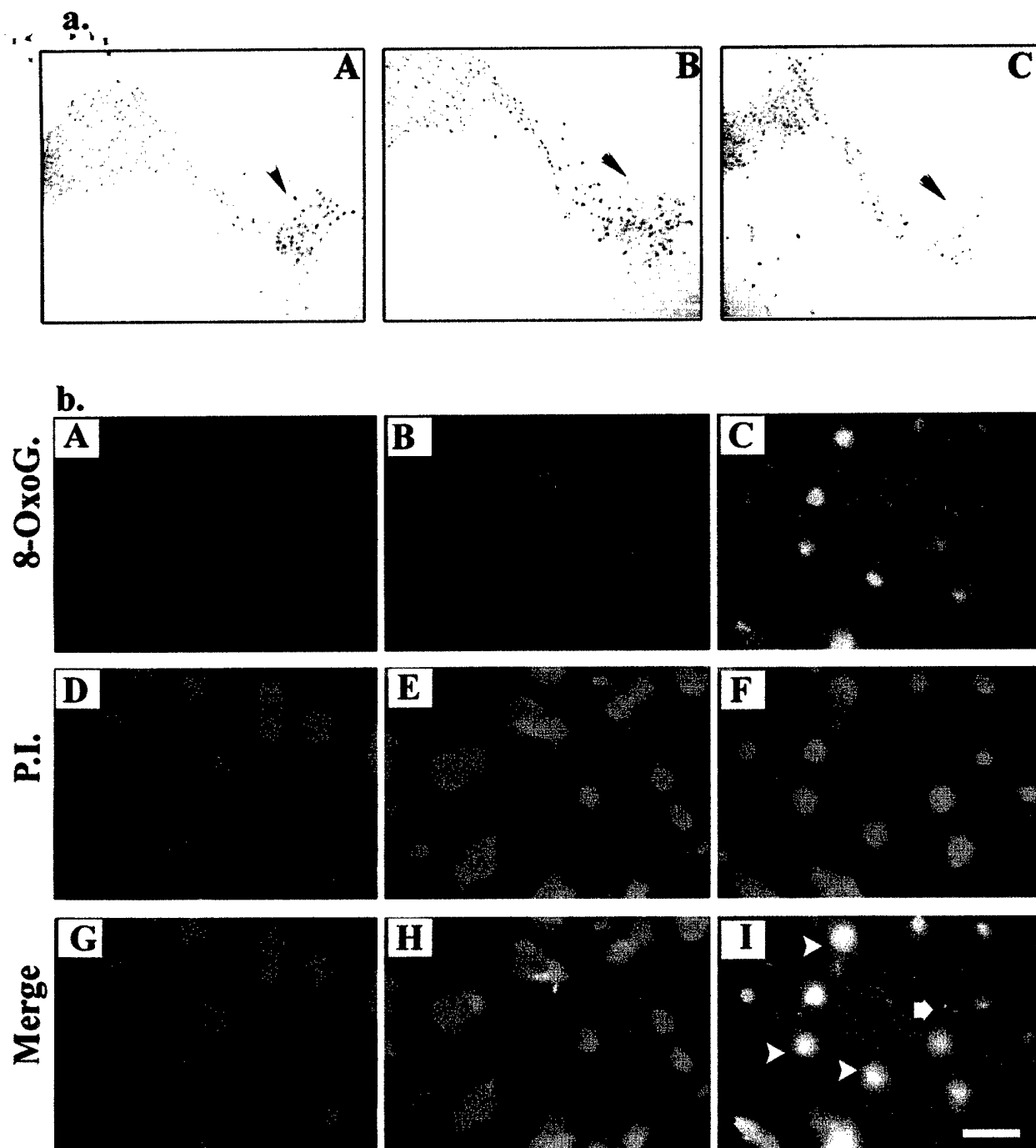
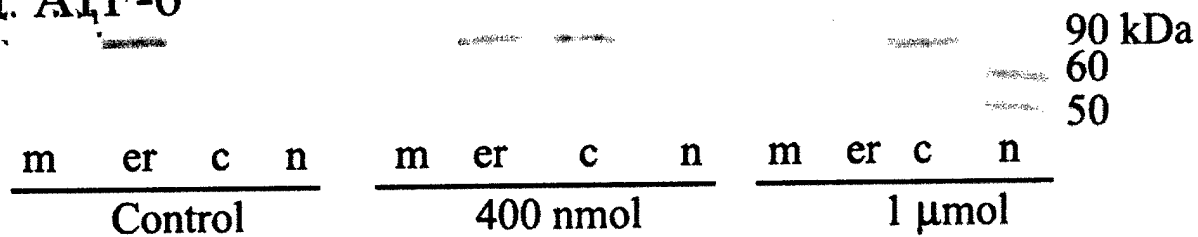


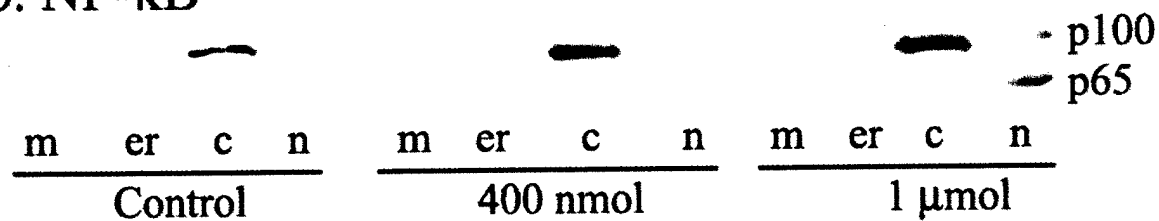
Figure 1.

Best Available Copy

a. ATF-6



b. NF-κB



c. Subcellular Markers

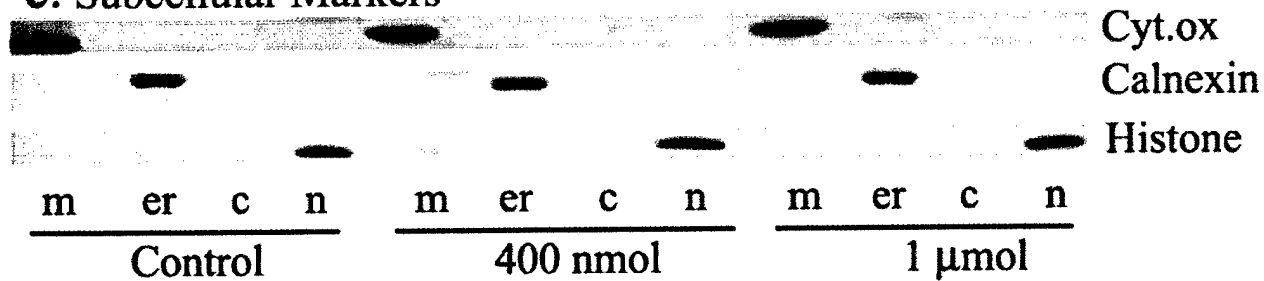
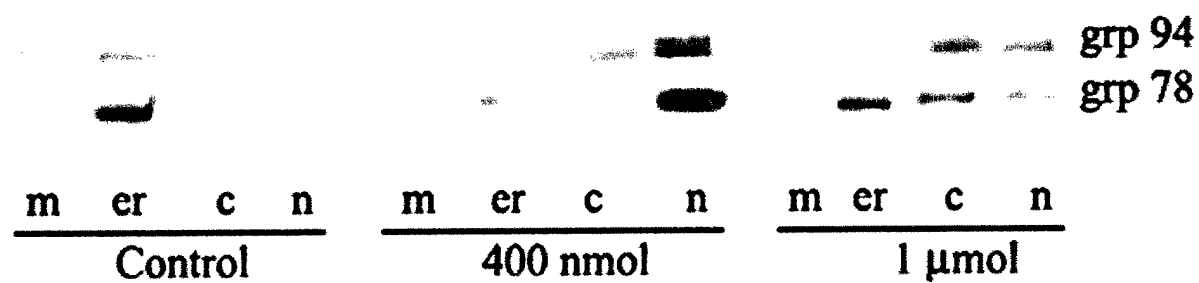


Figure 2.

a. KDEL



b. Gadd 153

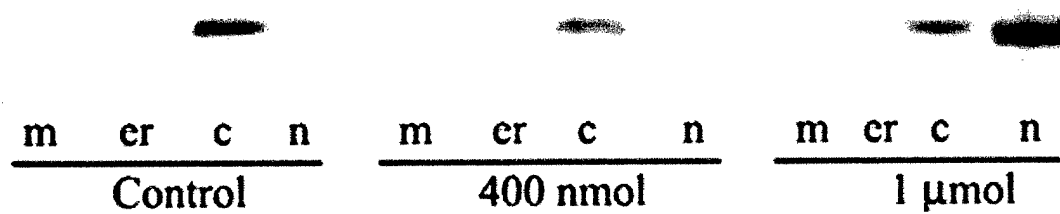
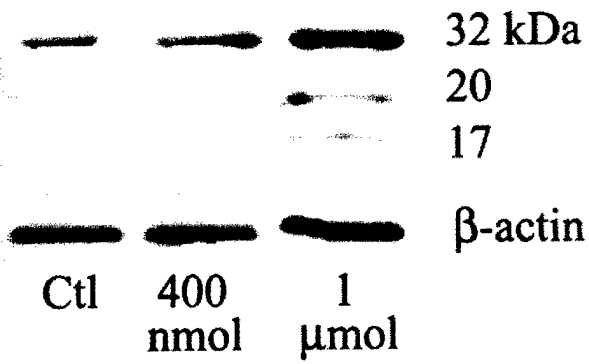
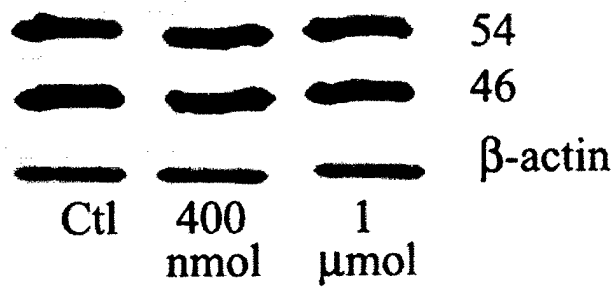


Figure 3.

a. Caspase-3



b. JNK



c. p-JNK

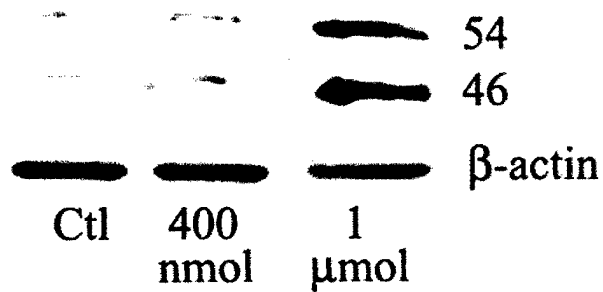


Figure 4.

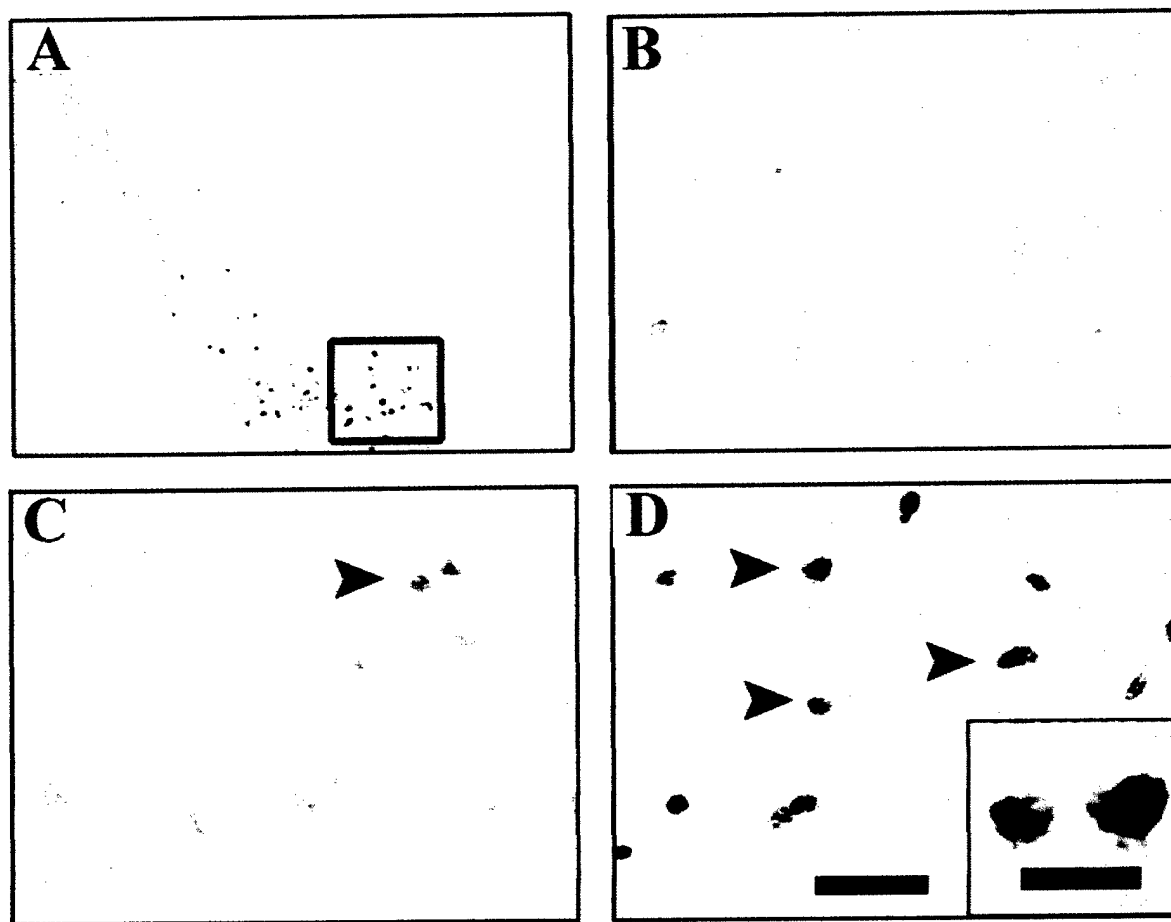


Figure 5.