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13. ABSTRACT (Maximum 200 Words)

Parkinson's disease is characterized by the depletion of glutathione (GSH) in the substantia nigra and the degeneration of nigral dopamine neurons. In our study we examined the relationship between cellular GSH depletion and neuronal degeneration. Using rat mesencephalic cultures as a model, we found that GSH depletion results in phospholipase A_2 (PLA_2)-dependent release of arachidonic acid and increase in lipoxygenase (LOX)-dependent arachidonic acid metabolism. These events generate reactive oxygen species, which accumulate in the cells and result in oxidative stress and cell death. Cell death can be prevented by interrupting different steps of this process, including replenishment of GSH, inhibition of PLA_2 activity, inhibition of LOX activity and increase in the antioxidant defenses of the cells (up-regulation of superoxide dismutase, addition of ascorbic acid). Our studies provide information, which may be important in the understanding of the etiology of Parkinson's disease and could offer insights for the design of medication to prevent the progress of the disorder in Parkinson's patients.

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Table of Contents

INTRODUCTION

Oxidative stress is believed to contribute to the pathogenesis of Parkinson's disease (PD). One of the most abundant antioxidant molecules in the brain is glutathione (GSH). In brains from of PD patients the levels of GSH are low in the substantia nigra, the area which is predominantly affected in PD (Perry, Godin et al. 1982; Jenner, Dexter et al. 1992). In our earlier studies (Mytilineou, Kokotos Leonardi et al. 1999) we had shown that in rat mesencephalic cultures, which contain the neurons of the substantia nigra, depletion of glutathione causes cell death, which can be prevented by antioxidants, suggesting an association with oxidative stress. Low levels of GSH, however, were not toxic to the neurons if glial cells were not present (Mytilineou, Kokotos Leonardi et al. 1999). Based on these data, we proposed to investigate whether, during conditions of reduced GSH content, interactions between reactive oxygen species (ROS) and pro-inflammatory cytokines could contribute to neurodegeneration in PD. Specifically we proposed to examine: (1) The contribution of glial cells to the events that lead to neuronal degeneration during GSH depletion; (2) the role of glial pro-inflammatory responses (release of cytokines, nitric oxide, excitotoxins, arachidonic acid) in cell death, during GSH depletion; and (3) the contribution of ROS to the events that lead to cell death during GSH depletion.

Based on the results from studies conducted during the grant period, we have reached the following overall conclusions:

Depletion of GSH in mesencephalic cultures causes neuronal cell death associated with the formation of ROS and oxidative stress. Oxidative stress is caused at least in part by the increased release and metabolism of arachidonic acid, as a consequence of activation of phospholipase A_2 (PLA_2) and increased lipoxygenase (LOX) activity in GSH depleted cells. Protection from cell death can be achieved by the use of antioxidants and up-regulation of the mitochondrial antioxidant enzyme, Mn-superoxide dismutase (Mn-SOD). Protection can also be achieved by inhibition of PLA_2 activity and even more potently by inhibition of LOX, the arachidonic acid metabolizing enzyme. No direct evidence for a connection between inflammation and GSH depletion-induced neurodegeneration could be observed, although addition of the cytokine interleukin-1β (IL-1β) increases toxicity. However, anti-inflammatory agents do not provide protection.

BODY

This is a summary of the accomplishments from our research efforts during the grant period as they relate to the Statement of Work. The four yearly reports already submitted provide more detailed information on individual experiments.

Year 1: *Study of the contribution of glial cells to neuronal degeneration and mechanisms of glial mediated cell death during GSH depletion.*

Our previous studies (Mytilineou, Kokotos Leonardi et al. 1999) showed that GSH depletion, to levels that cause total cell loss in cultures containing neurons and glial cells, has no effect on cell viability in enriched neuronal cultures. An increase in the plating cell density sensitized glia-containing cultures to GSH depletion-induced toxicity, suggesting that cell death is the consequence of events that are induced by GSH depletion and are mediated by glial cells. The observation that the LOX inhibitor nordihydroguaiaretic acid (NDGA) provides full protection from toxicity suggested that arachidonic acid metabolism, through the LOX pathway, and the generation of reactive oxygen species may play a role in the loss of cell viability.

Low GSH levels as well as glial activation have been reported in the substantia nigra in PD (Perry, Godin et al. 1982; Jenner, Dexter et al. 1992) (Hirsch, Hunot et al. 1998). We, therefore, examined whether glial activation would increase the sensitivity of mesencephalic cultures to GSH depletion. To study the role of glial activation in the toxicity of GSH depletion, we exposed cultures to the bacterial endotoxin lipopolysaccharide (LPS) prior to threatment with the GSH depleting agent l-buthionine sulfoximine (BSO). The studies reported below have been published (Kramer, Yabut et al. 2002) and a reprint of the publication is attached with this report. Treatment of mesencephalic cultures with LPS increased the number of astrocytes as well as the number and IL-1β content of microglia. Independent experiments showed that the cytokine IL-1β increases the toxicity of GSH depletion (although the cytokines interferon-γ and tumor necrosis-α were ineffective). In spite of increased glial content and up-

regulation of IL-1β, LPS treatment protected the GSH-depleted cultures from neurodegeneration (Kramer, Yabut et al. 2002). This unexpected finding prompted us to examine further the mechanisms involved in this protection in order to gain more insight in the mechanisms glial interactions in cell degeneration and neuroprotection in conditions of GSH depletion.

LPS had been shown to up-regulate the antioxidant enzyme SOD in glial cultures (Del Vecchio and Shaffer 1991; Mokuno, Ohtani et al. 1994). Based on these finding we tested whether LPS treatment affected SOD and catalase activity in mesencephalic cultures. Treatment with LPS caused a greater than 2-fold increase in Mn-SOD but had no significant effect in the activity of Cu/Zn-SOD or catalase. Western blots from lysates from the cultures showed a significant increase in Mn-SOD protein after treatment with LPS, but no change in Cu/Zn-SOD, suggesting the possibility that protection from GSH depletion was provided by the up-regulation of the mitochondrial antioxidant enzyme Nn-SOD. This was further supported by the finding that SOD added to the culture medium provided protection from GSH depletion. Both forms of the enzyme were protective.

We then examine the cellular localization of the different forms of SOD and their response to LPS treatment of the cultures. Double label immunofluorescence showed that Cu/Zn-SOD was mostly localized in the neurons and there was no apparent change following treatment of the cultures with LPS. In contrast Mn-SOD was localized in both neurons and glial cells and

there was a big increase in the astrocyte content of this enzyme following treatment with LPS (Figures 8 and 9; (Kramer, Yabut et al. 2002).

These studies point to the complex involvement of glial cells in both toxicity and protection of neurons. In addition, the stong protective effect of the up-regulation of Mn-SOD suggests that superoxide generated as a result of GSH depletion and increased metabolism of arachidonic acid is an important component of the events that lead to cell death.

Year 2: *Study of the role of arachidonic acid and metabolites of the cyclooxygenase (COX) and LOX pathways in cell death caused by GSH depletion.*

The protection provided by the inhibition of arachidonic acid metabolism indicated that arachidonic acid plays a major role in the toxic events that follow GSH depletion. In the studies described below we examined (1) whether GSH depletion results in increased release of arachidonic acid, (2) whether this release is associated with PLA_2 activity and (3) whether arachidonic acid or any of the metabolites of the LOX pathway are toxic to GSH depleted cells. Independent experiments indicated that the COX metabolic pathway of arachidonic acid metabolism did not contribute significantly to the toxicity of GSH depletion. The results of these studies have been published and a reprint of this manuscript is appended to this report (Kramer, Yabut et al. 2004).

To determine whether GSH depletion promotes the release of arachidonic acid, we labeled membrane phospholipids with [^{3}H]arachidonic acid before GSH

depletion by treatment with the GSH synthesis inhibitor BSO. A significant increase in arachidonic acid release was observed in the BSO treated cultures, which could be prevented by the specific $cPLA_2$ inhibitor MAFP, indicating the involvement of $cPLA_2$ in the release of arachidonic acid by BSO.

To determine whether the release of arachidonic acid may be associated with the toxicity caused by GSH depletion, we exposed cell cultures to arachidonic acid in the presence or absence of BSO. arachidonic acid was not toxic to mesencephalic cultures with normal GSH levels. However arachidonic acid increased the toxicity of GSH depletion. Inhibition of LOX activity prevented the damage caused by combined arachidonic acid treatment and BSO, indicating that the metabolism of arachidonic acid by LOX is an important component of the events that lead to loss of viability.

Protection from GSH depletion by inhibition of LOX activity suggests that the products of arachidonic acid metabolism by LOX may account for the observed toxicity. We examined the effect of 12-HPETE and 12-HETE, the major products of 12-LOX reaction with arachidonic acid (Katsuki and Okuda, 1995) in mesencephalic cultures with normal or depleted GSH. 12-HPETE caused a small reduction of cell survival when GSH levels were normal and it augmented the toxicity of GSH depletion by 3 to 5-fold. 12-HETE caused no significant damage and did not modify the toxicity of BSO.

If, as shown above, arachidonic acid release and metabolism contribute to the toxicity of GSH depletion, reducing the release of arachidonic acid by inhibiting $cPLA_2$ activity should afford protection. Indeed the $cPLA_2$ inhibitors ATK

and MAFP prevent the toxicity of BSO in mesencephalic cultures, further supporting the involvement of arachidonic acid in toxicity.

Although complete protection is achieved when $cPLA_2$ inhibitors are added together with BSO, delayed exposure reduces their effectiveness. In contrast, inhibition of arachidonic acid metabolism or addition of antioxidants can rescue mesencephalic cells, even if treatment begins quite late, just prior to the onset of cell breakdown. The reason for this difference is not clear, but it implies that activation of PLA_2 is an early event in the process that leads to cell death.

Collectively our data suggest the following scenario concerning the events that lead to cell death after exposure of mesencephalic cultures to BSO: Depletion of cellular GSH causes increased release of arachidonic acid likely through the activation of PLA_2. Concomitant increase in LOX activity, also resulting from GSH depletion, drives the metabolism of arachidonic acid through this pathway. Oxygen free radicals generated during this metabolic process accumulate within the GSH depleted cells overwhelming existing defense mechanisms contributing to cell death.

Year 3: *Assessment of peroxide formation and its role in cell death during GSH depletion.*

The data from these experiments have been published and a reprint of this manuscript is appended to this report (Kramer, Yabut et al. 2004). The intracellular accumulation of H_2O_2 and/or other ROS was examined during the course of GSH depletion by loading the cells with H_2DCF-DA, which is converted

to a fluorescent derivative by ROS. Cultures were treated with BSO and then exposed to H_2DCF-DA at 4, 8, 24, 30 and 48 h later. No significant increases in fluorescence could be observed in the cultures up to 30 h post treatment. High intensity fluorescence began accumulating almost simultaneously with the appearance of damaged cells (Fig. 6 in manuscript attached). Fluorescence appeared initially within well-defined cellular organelles, resembling mitochondria, and in cell processes and eventually filled the entire cell. Some cells appeared ballooned and to be detaching themselves from the culture dish. ROS formation using this assay was blocked by PLA2 and LOX inhibitors, indicating the involvement of arachidonic acid. It is interesting that accumulation of reactive oxygen species, in amounts sufficient to be visualized with H_2DCF-DA microscopy, occurs only during the last stages before cell loss. This suggests that a critical threshold of toxic by-products of arachidonic acid metabolism is needed for the initiation of cell death pathway and, as our data above show, preventing this accumulation can rescue the cells.

Year 4: *Study of cellular events associated with neuronal cell death during GSH depletion.*

For the fourth year of the grant period we proposed to determine whether apoptosis was the cell death pathway following GSH depletion and assess the expression of pro- and anti-apoptotic genes during the process of cell death and as a result of antioxidant protection. Studies using TUNEL in combination with DNA staining demonstrated that apoptosis was not the cell

pathway followed during GSH depletion. Examination of cultures treated with the GSH synthesis inhibitor, BSO, showed complete lack of apoptotic morphology at 3, 8, 12, 24 and 48 hr after treatment with BSO. Some apoptotic morphology was observed in cells labeled with the astrocytic marker glial acidic fibrillary protein (GFAP), but never associated with cells stained positively with neuronal markers (neuron specific enolase, microtubule associated protein-2 or tyrosine hydroxylase).

Because of remaining time and resources we examined the possibility that GSH depletion and oxidative stress can combine with inhibition of proteasomal activity to induce the selective vulnerability of dopamine neurons observed in PD. Recent studies (published after the submission of the grant application) suggest that defects in the capacity of the ubiquitin-proteasome system to clear unwanted and misfolded proteins is also a common factor underlying both the familial and sporadic forms of PD (McNaught and Jenner, 2001; McNaught et al., 2001; McNaught et al., 2002). The results of these studies are being prepared for submission for publication and have been reported in detail in the 4th year report.

In summary we found the dopamine neurons show selective sensitivity to inhibition of proteasome activity. GABA neurons showed significantly lower sensitivity to inhibition of proteasome activity, as determined by measurement of [^{3}H]dopamine and [^{14}C]GABA uptake and by comparing the numbers of total (MAP2 stained) with the tyrosine hydroxylase stained neurons.

We also showed that the damage to dopamine neurons caused by inhibition of proteasome activity was greater in cultures under oxidative stress caused by depletion of GSH and exposure to L-DOPA or hydrogen peroxide.

KEY RESEARCH ACCOMPLISHMENTS

We have been able to identify several components of the events that occur when brain cells are depleted of GSH, which leads to cell death. We have also been able to determine means of rescuing the GSH depleted cells by interrupting different steps of this process and to compare their effectiveness. More specifically we have found that:

- Depletion of glutathione in brain cells causes the initiation of a chain of events that result in oxidative damage and cell death, which can be prevented by treatment with antioxidants and by up-regulation of superoxide dismutase activity.
- Following GSH depletion there is an increase in PLA_2-dependent release of arachidonic acid. The metabolism of arachidonic acid by lipoxygenase generates toxic free radicals that result in cell death.
- Inhibition of PLA_2 or lipoxygenase activity protect from the toxicity of GSH depletion.
- Intracellular accumulation of reactive oxygen species is a late event, which begins in mitochondria-like structures and before it extents to cell soma and neuronal processes.

- Antioxidants and lipoxygenase inhibitors can completely prevent cell death even if they are administered late in the course of GSH depletion. However, PLA_2 activity has to be inhibited early in order to afford full protection.

- Neuronal cell death caused by the depletion of GSH is not associated the with apoptotic pathway.

REPORTABLE OUTCOME

1. Kramer, B.C., Yabut, J.A. Cheong, J. Jnobaptiste, R., Robakis, T., Olanow, C.W. and Mytilineou, C. Lipopolysaccharide prevents cell death caused by glutathione depletion: Possible mechanisms of protection. Neuroscience, 114, 361-372, 2002.

2. Mytilineou, C., Kramer, B.C. and Yabut, J.A. Glutathione depletion and oxidative stress. Parkinsonism and Related Disorders, 8, 385-387, 2002.

3. Kramer, B.C., Yabut, J.A. Cheong, J. Jnobaptiste, R., Robakis, T., Olanow, C.W. and Mytilineou, C. Toxicity of glutathione depletion in mesencephalic cultures: A role for arachidonic acid and its lipoxygenase metabolites

4. McNaught, K. St P., Mytilineou, C., Jnobaptiste, R., Yabut, J., Shashidharan, P. Jenner, P. and Olanow, C.W. Impairement of the ubiquitin-proteasome system causes dopaminergic cell death and

inclusion body formation in ventral mesencephalic cultures. J. Neurochem. 81, 301-306, 2002.

5. Mytilineou, C., Walker, R.H., Jnobaptiste, R. and Olanow, C.W. Levodopa is toxic to dopamine neurons in an *in vitro* but not an *in vivo* model of oxidative stress. J. Pharmacol. Exp. Ther. 304, 792-800, 2003.

6. Kramer, B.C. and Mytilineou, C. Alterations in the cellular distribution of bcl-2, bcl-x and bax in the adult substantia nigra following striatal 6-hydroxydopamine lesions. J. Neurocytol., 33, 213-223, 2004.

The publications not directly related to the statement of work acknowledge the partial support of the grant.

Meeting Presentations:

Society for Neuroscience, 2000 Mytilineou, C., Kramer, B.C. and Yabut, J.A. Arachidonic acid release and toxicity in glutathione depleted mesencephalic cultures.

Oxygen Radical Society, 2001 Kramer, B.C. Yabut, J.A. and Mytilineou, C. Up[regulation of Mn-SOD by lipopolysaccharide protects mesencephalic cultures from glutathione depletion.

Society for Neuroscience 2001 Lipopolysacharide treatment protects mesencephalic cultures from glutathione depletion.

Doctorare Award: to Brian C. Kramer, PH.D. in Neurobiology, New York University Graduate School, June, 2001.

CONCLUSIONS

Our studies confirm the significance of oxidative stress in neuronal damage and suggest that it is likely to play a role in the degeneration of dopamine neurons in Parkinson's disease. We have demonstrated that depletion of the antioxidant GSH can cause changes in cellular functions which can lead to cell death, if sufficient protective mechanisms are lacking. The changes that are induced by GSH depletion include increased PLA_2 dependent release as well as increased metabolism of arachidonic acid, a process which results in the generation of reactive oxygen species, oxidative stress and cell degradation. The cells can be protected from damage by inhibiting the different steps that follow GSH depletion, including inhibition of the release of arachidonic acid or suppression of its metabolism. In addition, treatment with antioxidants or increase in SOD activity can rescue the cells from GSH depletion induced damage. These findings may be relevant to Parkinson's disease, where depletion of GSH in the substantia nigra has been described and could help devise strategies for the prevention or treatment of this disorder.

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APPENDICES

Copies of 6 published manuscripts supported by the grant.

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Review

Glutathione depletion and oxidative stress

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Dedicated to the memory of Gerald Cohen, a valued colleague and friend

Abstract

Oxidative stress is believed to contribute to the pathogenesis of Parkinson's disease. One of the indices of oxidative stress is the depletion of the antioxidant glutathione (GSH), which may occur early in the development of Parkinson's disease. To study the role of GSH depletion in the survival of dopamine neurons we treated mesencephalic cultures with the GSH synthesis inhibitor L-buthionine sulfoximine. Our studies have shown that the depletion of GSH causes a cascade of events, which ultimately may result in cell death. An early event following GSH depletion is a phospholipase A_2-dependent release of arachidonic acid. Arachidonic acid can cause damage to the GSH-depleted cells through its metabolism by lipoxygenase. The generation of superoxide radicals during the metabolism of arachidonic acid is likely to play an important role in the toxic events that follow GSH depletion.

Oxidative damage is believed to contribute to brain aging and neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. The substantia nigra in Parkinson's disease brains shows signs of oxidative stress, including damaged proteins and DNA [17,22,36], excess accumulation of iron [12,34] and lipid peroxidation [11], reduced complex I activity [31,37,38] and depletion of glutathione (GSH) [32,39]. Neurons in general are more exposed to the toxic by-products of oxygen metabolism than other cells, because they depend primarily on oxidative phosphorylation for energy production. A further compromising factor is that the brain is deficient in antioxidant molecules and free radical scavenging enzymes compared to other tissues, which makes neurons vulnerable to oxidative stress [10,14]. Dopaminergic neurons, the cells that degenerate in Parkinson's disease, are subjected to additional oxidative stress because of H_2O_2 produced during the metabolism of dopamine by monoamine oxidase (MAO) [7,9]. H_2O_2 can react with transition metals to form the highly reactive hydroxyl radical (OH·) known to cause damage to lipids, proteins and DNA [5,6].

GSH is among the most abundant soluble antioxidant molecules in the brain. GSH is a tripeptide comprised of cysteine, glycine and glutamate and is synthesized in the brain by both neurons and glial cells, although it is more abundant within astrocytes [33,35,40]. GSH, with the enzymes GSH peroxidase and GSSG reductase, serve to detoxify H_2O_2 to water and molecular oxygen and help maintain the cysteinyl-thiols ($R\text{-}CH_2\text{-}SH$) groups of proteins in the reduced state, which is often necessary for their functional integrity. Depletion of GSH could significantly affect the survival of dopamine neurons, particularly if they are under oxidative stress. The possibility that GSH loss may be an early event in Parkinson's disease, occurring even in pre-symptomatic patients [13], suggests that GSH may play a crucial role in the pathogenesis and progression of this disorder [21].

Several studies have examined the response of brain cells to depletion of GSH. Inhibition of GSH synthesis with L-buthionine (*S*,*R*)-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase [18] causes depletion of GSH in the brain and damage to mitochondria, suggesting that GSH dependent reactions are important for the detoxification of H_2O_2 in the mitochondria [20]. The same treatment was shown to increase lipid peroxidation and cause loss of catecholamine fluorescence and formation of dystrophic processes in murine nigrostriatal neurons [1]. In cell culture GSH protects neurons from the toxicity of 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium ion [41] and from dopamine-induced apoptosis [16,42]. GSH is also

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important for the survival of dopamine neurons with impaired energy metabolism [45].

To gain an understanding of the role of GSH depletion in the degeneration of dopamine neurons in Parkinson's disease, we initiated experiments using primary rat mesencephalic cultures containing dopamine neurons of the substantia nigra. Our studies show that treatment of mesencephalic cultures with BSO causes depletion of GSH and results in cell death [27,29]. The loss of GSH rather than BSO treatment itself is the cause of cell death, because it can be prevented by replenishing the GSH stores with GSH esters (unpublished results). Furthermore, the protective effect of ascorbic acid [27] and superoxide dismutase [44] indicates that cell death by GSH depletion is associated with oxidative stress. It is interesting, however, that severe depletion of neuronal GSH may not necessarily lead to cell death. When neurons are grown in culture without a significant amount of glial cells, reduction in GSH content to levels that would otherwise cause severe loss of cells fails to affect cell viability [27]. This finding suggests that depletion of GSH initiates a chain of events which eventually result in oxidative damage sufficient to cause cell death and that glial cells are an important participant in this pathway. Although astrocytes are commonly associated with trophic support of neurons [30,43], increased concentrations of astrocytes in the vulnerable areas of the substantia nigra pars compacta in Parkinson's disease suggests a role for glial cells in the degeneration of dopamine neurons [19].

An important pathway in the events that follow GSH depletion is the release of arachidonic acid and its metabolism by lipoxygenase. We have shown that during the early stages of GSH depletion, before any indication of cellular damage, there is an increase in phospholipase A_2 (PLA_2)-dependent release of arachidonic acid in mesencephalic cultures [28]. A study by Li et al. [25], as well as studies in our laboratory [27], show that inhibition of lipoxygenase (LOX) activity protects cells from the toxicity of GSH depletion. The protection afforded by inhibition of PLA_2 or LOX activity suggests that the higher levels of arachidonic acid and the products of its metabolism are likely responsible for the damage caused by GSH depletion. Arachidonic acid becomes very toxic when added to GSH-depleted cultures and this toxicity is prevented if LOX activity is inhibited [28]. The metabolism of arachidonic acid by LOX leads to the generation of reactive oxygen species, including superoxide and hydroxyl radicals [24], and can cause oxidative damage [23]. It has been demonstrated that when astrocytes are exposed to arachidonic acid they generate superoxide radical [2]. A role for superoxide in the toxicity caused by GSH depletion is supported by our recent finding that up-regulation of SOD in mesencephalic cultures can prevent BSO-induced cell death [44].

The interactions between neurons and glial cells during oxidative stress are not well understood at present but it is evident that they are complex and they include both protective and toxic components. In the brain, astrocytes are enriched in PLA_2 [15] and PLA_2 activity increases during oxidative stress [3]. Excess arachidonic acid increases H_2O_2 levels in mitochondria and depresses respiratory activity [4]. The arachidonic acid cascade is known to cause oxidative damage [24], which would be exacerbated in cells with decreased GSH content and reduced mitochondrial complex I activity, as is the case in Parkinson's disease.

The environment of the substantia nigra in Parkinson's disease is supportive of free radical chain reactions and oxygen radical pathology [8]. The possibility that a defect in the clearing these oxidatively damaged proteins in Parkinson's disease [26] may be the ultimate insult that results in the degeneration of dopamine neurons.

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LIPOPOLYSACCHARIDE PREVENTS CELL DEATH CAUSED BY GLUTATHIONE DEPLETION: POSSIBLE MECHANISMS OF PROTECTION

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Abstract—Glutathione is an important cellular antioxidant present at high concentrations in the brain. We have previously demonstrated that depletion of glutathione in mesencephalic cultures results in cell death and that the presence of glia is necessary for the expression of toxicity. Cell death following glutathione depletion can be prevented by inhibition of lipoxygenase activity, implicating arachidonic acid metabolism in the toxic events. In this study we examined the effect of glial activation, known to cause secretion of cytokines and release of arachidonic acid, on the toxicity induced by glutathione depletion. Our data show that treatment with the endotoxin lipopolysaccharide activated glial cells in mesencephalic cultures, increased interleukin-1β in microglia and caused depletion of glutathione. The overall effect of lipopolysaccharide treatment, however, was protection from damage caused by glutathione depletion. Addition of cytokines or growth factors, normally secreted by activated glia, did not modify L-buthionine sulfoximine toxicity, although basic fibroblast growth factor provided some protection. A large increase in the protein content and the activity of Mn–superoxide dismutase, observed after lipopolysaccharide treatment, may indicate a role for this mitochondrial antioxidant enzyme in the protective effect of lipopolysaccharide. This was supported by the suppression of toxicity by exogenous superoxide dismutase. Our data suggest that superoxide contributes to the damage caused by glutathione depletion and that up-regulation of superoxide dismutase may offer protection in neurodegenerative diseases associated with glutathione depletion and oxidative stress. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: L-buthionine sulfoximine, mesencephalic cultures, superoxide dismutase, oxidative stress, Parkinson's disease.

Oxidative stress is believed to contribute to the degeneration of dopamine neurons in Parkinson's disease (PD). Hydrogen peroxide (H_2O_2) is a major oxidative species produced normally within neurons during respiration. Within the dopamine neurons H_2O_2 is also formed during the metabolism of dopamine by monoamine oxidase. The high-energy requirements of brain function coupled with the metabolism of dopamine could result in concentrations of H_2O_2 sufficient to cause oxidative stress in dopamine neurons (Cohen and Kesler, 1999). Glutathione (GSH), an important soluble antioxidant in the brain, detoxifies H_2O_2 and lipid hydroperoxides (Meister, 1991). GSH is depleted in the substantia nigra in PD (Perry et al., 1982; Sofic et al., 1992) and it has been suggested that this loss may be an early event in its pathogenesis (Dexter et al., 1994).

Inhibition of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis, by L-buthionine sulfoximine (BSO) causes cell death in primary neuronal cultures and cell lines (Li et al., 1997; Mytilineou et al., 1999; Wullner et al., 1999). Inhibition of lipoxygenase activity prevents cell death, implicating arachidonic acid metabolism in the toxicity of GSH depletion (Li et al., 1997; Mytilineou et al., 1999). We have recently shown that damage from GSH depletion becomes greater in mesencephalic cultures with increased glia to neuron ratios (Mytilineou et al., 1999). The contribution of glia to the toxicity of GSH depletion is not well understood and, in view of the well-known neuroprotective role of astrocytes (Desagher et al., 1996; O'Malley et al., 1994; Takeshima et al., 1994; Wilson, 1997), such an effect may appear counter-intuitive. Several findings, however, could implicate astrocytes in arachidonic acid-mediated toxicity in primary cell cultures: (1) Among brain cells, astrocytes are exclusively responsible for the synthesis of arachidonic acid (Katsuki and Okuda, 1995). (2) Neurons depend on astrocytes for arachidonic acid

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Abbreviations: ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; BSO, L-buthionine sulfoximine; EDTA, ethylenediaminetetra-acetate; GFAP, glial fibrillary acidic protein; GSH, reduced glutathione; IGF, insulin-like growth factor; IL-1β, interleukin-1β; INF-γ, interferon-γ; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAP, microtubule associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PD, Parkinson's disease; PLA_2, phospholipase A_2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-α; TTBS, 20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween 20, pH 7.6.

transport, because they cannot perform the fatty acid desaturation steps necessary for its synthesis (Katsuki and Okuda, 1995; Moore et al., 1991). (3) The cytosolic form of phospholipase A_2 (PLA_2), the enzyme primarily responsible for the release of arachidonic acid in brain, is present within astrocytes (Stephenson et al., 1999; Stephenson et al., 1994) and microglial cells (Clemens et al., 1996).

Activation of glial cells causes secretion of cytokines, increases PLA_2 activity (Oka and Arita, 1991) and induces release of arachidonic acid in astrocytes (Minghetti and Levi, 1998; Stella et al., 1997) and microglia (Minghetti and Levi, 1998). Theoretically, glial activation combined with GSH depletion, which also increases lipoxygenase activity (Li et al., 1997; Shornick and Holtzman, 1993), should result in excess free radical generation and create additional oxidative challenge to GSH depleted cells.

To study the role of glial activation in the toxicity of GSH depletion, we exposed mesencephalic cultures to a bacterial endotoxin (lipopolysaccharide; LPS), prior to treatment with BSO. We show that LPS activated microglia and astrocytes, reduced the levels of GSH and increased the cytokine interleukin-1β (IL-1β), which is toxic to GSH depleted cells. However, the overall effect of LPS was a significant protection from the toxicity of GSH depletion. Our data suggest that LPS-induced up-regulation of Mn-dependent superoxide dismutase (SOD), the mitochondrial enzyme responsible for detoxification of superoxide (O_2^-), may play a significant role in the LPS-induced protection.

EXPERIMENTAL PROCEDURES

Materials

Pregnant Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY, USA). MEM was purchased from Gibco-Life Technologies (Grand Island, NY, USA), horse serum from Gemini (Calabasas, CA, USA) and NU® serum from Collaborative Biomedical Products (Bedford, MA, USA). LPS (from *Escherichia coli* serotype 026:B6 and 0111:B4) and other chemicals were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies to glial fibrillary acidic protein (GFAP) were purchased from Sigma (St. Louis, MO, USA) and to tyrosine hydroxylase (TH) from Chemicon (Temecula, CA, USA). Monoclonal OX-42 antibodies against the rat microglial surface antigen complement receptor type 3 (Mac-1) (Perry et al., 1985) were purchased from Chemicon (Temecula, CA, USA). Polyclonal antibodies to IL-1β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), to Cu/ZnSOD from Chemicon and to MnSOD from StressGen Biotechnologies (Victoria, BC, Canada).

Cell cultures

The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Mesencephalic cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou et al., 1999). In brief, the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24- or six-well plates precoated with L-polyornithine (0.1 mg/ml) at a density of 300 000 cells/cm^2. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum and 10% NU® serum. Treatment began on the fifth or sixth day *in vitro*, at which time the medium was changed to MEM containing only 5% NU® serum. The method of McCarthy and de Vellis (1980) was used to prepare purified astrocytes. In brief, cortical cultures prepared from newborn rats were plated in 75 cm^2 flasks at 5×10^6 cells/flask. After seven days *in vitro* the flasks were shaken overnight on a rotary shaker at 250 r.p.m. and the following day the floating cells were removed and the remaining attached astrocytes were dislodged with Versene® and plated in 24-well plates. Treatment of astrocyte cultures began 24 h after plating.

Immunocytochemistry

Cells were plated on polyornithine coated glass coverslips in 24-well plates. They were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized and blocked with 0.3% Triton X-100 and 3% bovine serum albumin (BSA) for 30 min. Primary antibodies used were: anti-GFAP (1:1000), OX-42 (1:250), anti-microtubule associated protein (MAP-2 1:500), anti-IL-1β (1:250) and anti-TH (1:1000). Cultures were exposed to the primary antibodies overnight at 4°C. Secondary antibodies conjugated to Alexa fluorescent dyes (Molecular Probes, Eugene, OR, USA) were used at a dilution of 1:1000 for 30 min. The cultures were observed with an Olympus fluorescence microscope and the images recorded with a Spot video camera.

[^{3}H]dopamine uptake

Measurement of dopamine uptake was performed as described previously (Mytilineou et al., 1998). Cultures were rinsed with Kreb's phosphate buffer (pH 7.4) and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/ml ascorbic acid and 0.5 μCi/ml [^{3}H]dopamine (32.6 Ci/mmol; NEN, Boston, MA, USA). After rinsing, the radioactivity was extracted with 1 ml 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100). Cultures treated with the neuronal dopamine uptake blocker mazindol (10 μM) were used as blanks.

Cell viability assays – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined by the MTT reduction assay, as described previously (Han et al., 1996). In brief, 50 μl of a 5 mg/ml solution of MTT was added to each cell culture well containing 0.5 ml medium. After a 1-h incubation at 37°C, the medium was carefully removed and the formazan crystals were dissolved in 1 ml isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250, Molecular Devices Corporation, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) assay

A modification of the method by Bergmeyer et al. (1963) was used to determine LDH activity in the culture medium and the cells. Medium was collected, centrifuged to remove debris and frozen at −80°C until assay. Cells were freeze-thawed (×3) in 0.5 ml feeding medium, the medium was collected, centrifuged and the supernatant frozen at −80°C. 50 μl of supernatant and 100 μl of NADH (1.2 mg/ml H_2O stock) were added to 850 μl of buffer and the samples were vortex-mixed. 50 μl of feeding medium was used for blanks. Triplicate aliquots (250 μl) were placed into 96-well plates at room temperature and reaction was initiated by addition of 25 μl of sodium pyruvate (0.36 mg/ml H_2O stock). The rate of disappearance of NADH was measured at 340 nm using a plate reader.

GSH assay

GSH was quantified using a modification of a standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) with GSH reductase and NADPH (Tietze, 1969). In brief, the medium was carefully aspirated from the culture wells, 300 µl of 0.4 N perchloric acid was added and the plates were kept on ice for 30 min. The perchloric acid was then collected and stored at −80°C until assayed. Both oxidized (GSSG) and reduced (GSH) forms of glutathione are measured with this assay. However, because of the small amounts of GSSG present in mesencephalic cultures (~5% of total; Mytilineou et al., 1993), the values obtained were considered to represent GSH content.

The tissue attached to the bottom of the wells after removal of the PCA was dissolved in equal volumes of 20% SDS (sodium dodecyl sulfate) and 0.5 N NaOH and used for protein determination according to the method of Lowry (Lowry et al., 1951) with BSA as a standard.

SOD assay

Cells plated in six-well plates were collected in cold PBS (0.1 ml/well). Three to six wells were pooled for each sample. Cells were sonicated on ice, centrifuged at 4000×*g* for 10 min at 4°C and dialyzed overnight in PBS at 4°C (Slide-A-Lyzer dialysis cassettes, 10 K cut-off; Pierce, Rockford, IL, USA). SOD activity was assayed in 50 µl of the dialysate according to the assay developed by McCord and Fridovich (1969), modified for use with a microplate reader. In brief, 100 µl of xanthine solution (4 mM in 0.01 M NaOH) and 10 µl of cytochrome C (partially acetylated; 0.5 mg/ml) were added to 900 µl of 50 mM phosphate buffer. After the addition of 50 µl of sample, buffer for blanks or SOD for standards, triplicates of 250 µl were placed in the wells of a 96-well plate and the reaction was initiated by the addition of 25 µl/well xanthine oxidase (0.75 U/ml in 0.1 mM EDTA). The reduction rate of cytochrome c by superoxide radicals was monitored at 550 nm at 25°C for 10 min. Total SOD activity in the samples was determined from a standard curve and was corrected for protein content. MnSOD activity was determined after inhibition of the Cu/ZnSOD by a 5-min incubation with 2 mM KCN.

Catalase assay

Catalase activity was measured by a modification of the method described by Bordet et al. (2000). In brief, cells were plated in six-well plates and collected in PBS containing a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA). Three wells (0.1 ml/well) were pooled for each sample. The cells were freeze-thawed once, homogenized, centrifuged at 4000×*g* for 10 min and the supernatants stored frozen at −80°C until use. Cell extracts containing 50 µg protein were added to 250 µl of 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.8) and the disappearance of H_2O_2 was measured at 240 nm for 2 min an 5-s intervals in a plate reader.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The dialyzed cell homogenates used for SOD assays were also used for western blotting. Samples containing 20 µg of protein were mixed with 20 µl Laemmli buffer. The proteins were resolved on a 12% SDS–PAGE (Bio-RAD, Hercules, CA, USA) and transferred to Hybond™-P PVDF membrane (Amersham Pharmacia, Piscataway, NJ, USA) for 1 h at 15 V, using a Trans-Blot Semi-Dry Transfer Cell (Bio-RAD, Hercules, CA, USA). Membranes were incubated in blocking solution (5% milk, 5% newborn-calf serum) in TTBS (20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween 20, pH 7.6) at 4°C overnight and then with polyclonal antibodies to Cu/ZnSOD or MnSOD and with monoclonal antibody to TH in blocking solution for 2 h. They were then washed with TTBS and incubated with horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (whole molecule) antibodies (ICN, Aurora, OH, USA) in blocking solution for 1 h at room temperature. After washing with TTBS the membranes were visualized with ECL Plus western blot detection system (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analysis

All data are expressed as means ± S.E.M. Significance of differences between groups was determined using one-way analysis of variance (ANOVA) with Tukey–Kramer post-test for multiple comparisons.

RESULTS

LPS causes glial activation in mesencephalic cultures

LPS, an endotoxin used extensively to induce glial activation *in vivo* (Castano et al., 1998) and *in vitro* (Bronstein et al., 1995; Jeohn et al., 1998), was added to the medium of mesencephalic cultures at day 5 *in vitro*. Specific markers for astrocytes and microglia were used to test for glial activation. After 72-h exposure to 10 µg/ml LPS, immunocytochemistry with antibodies to GFAP revealed an increase in the number of astrocytic processes compared with the controls (Fig. 1A, B). The number and size of microglia cells labeled with OX-42 was also increased after LPS treatment (Fig. 1C, D). We also tested for the presence of the pro-inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α), which are secreted by activated glial cells (Benveniste, 1998). IL-1β was present exclusively in OX-42-labeled cells, indicating microglial localization of this cytokine (Fig. 1E, F). Labeling for IL-1β was weak in most microglia of control cultures (Fig. 1E). An increase in IL-1β immunoreactivity in OX-42-positive cells was observed after LPS treatment (Fig. 1F). Antibodies to TNF-α labeled both neurons and astrocytes and there was no apparent difference in intensity or distribution between control and LPS-treated cultures (not shown).

LPS provides protection against BSO-induced toxicity

To examine the effect of glial activation on GSH depletion-induced damage, cultures were pretreated with 10 µg/ml LPS for 24 h and then exposed to the GSH synthesis inhibitor, BSO, for an additional 48 h. LPS was present in the medium during BSO treatment. In agreement with our previous studies (Mytilineou et al., 1999; Mytilineou et al., 1998), BSO at 10 and 50 µM caused significant damage, as assessed by the MTT assay (Fig. 2A). Treatment with LPS significantly attenuated the BSO-induced damage (Fig. 2A). Cell viability was reduced to 42% and 9% of control values after a 48 h treatment with 10 and 50 µM BSO respectively, and LPS treatment improved survival to 65% and 56% of control with the same BSO treatment.

LPS concentrations ranging from 1 (the lowest effective concentration) to 50 µg/ml were equally protective from BSO toxicity (Fig. 2B). In the experiment presented

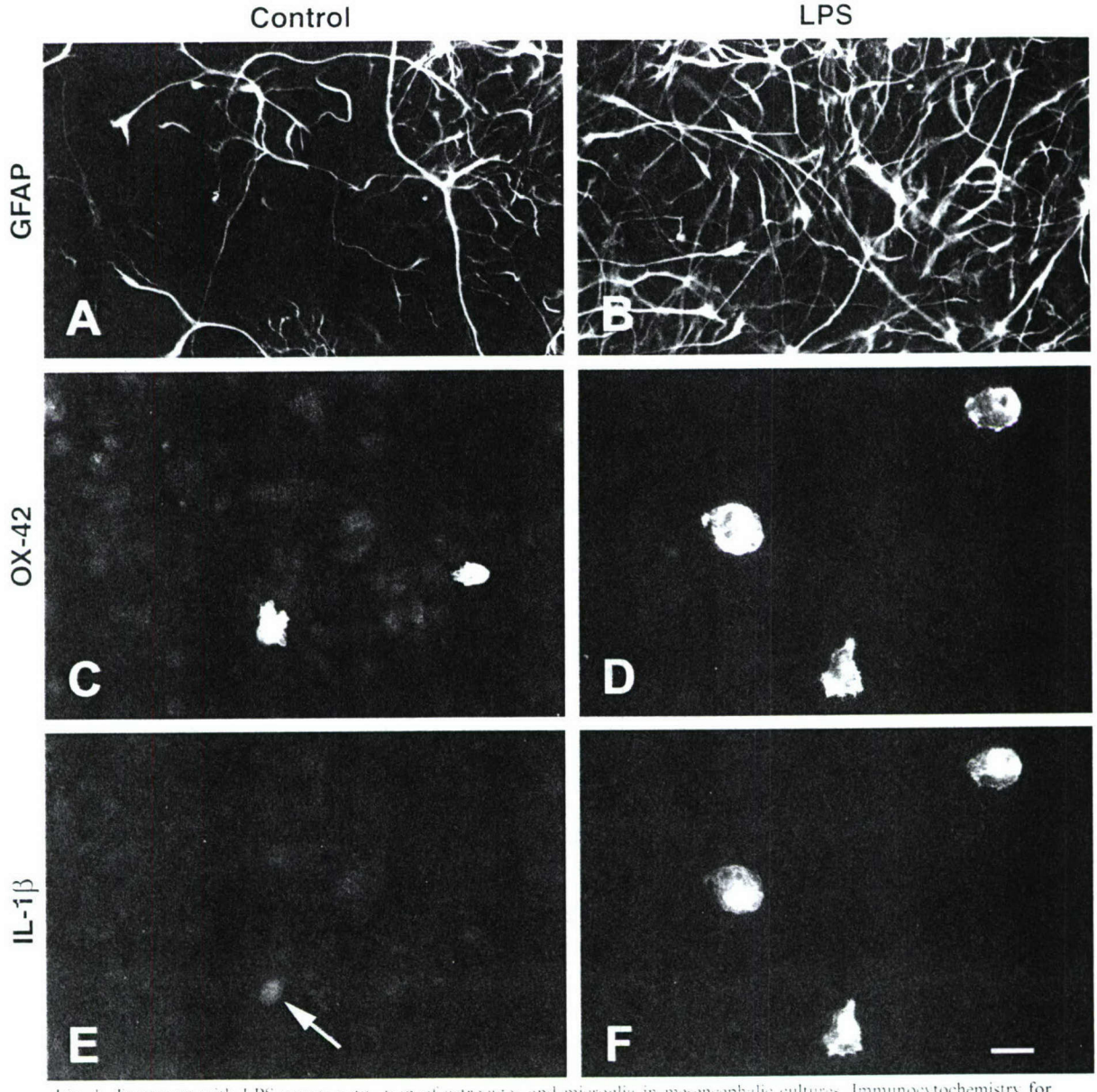

Fig. 1. Treatment with LPS causes activation of astrocytes and microglia in mesencephalic cultures. Immunocytochemistry for GFAP (A, B), OX-42 (C, D) and IL-1β (E, F) in control cultures (A, C, E) and cultures treated with 10 μg/ml LPS for 72 h. Increased labeling of GFAP-positive astrocytes was apparent in cultures treated with LPS (compare A and B). OX-42 labeling of microglia showed an increase in the size after treatment with LPS (compare C and D). The number of microglia was also increased after LPS treatment. Panels E and F show the same fields as C and D double labeled for IL-1β. In control cultures (E, some microglia (arrow) express low levels of the cytokine. After LPS treatment (F), all OX-42-labeled cells show increased immunoreactivity for IL-1β. Scale bar = 25 μm.

in Fig. 2B, 50 μM BSO caused only moderate loss of cell viability after 48 h (47% loss) and LPS prevented this damage at all concentrations tested. Differences in the extent of toxicity caused by BSO in some experiments are believed to be due to variations among primary cultures from different preparations.

It has been reported that LPS causes neuronal toxicity *in vitro*, which can be selective for dopamine neurons (Bronstein et al., 1995; Jeohn et al., 1998), and is sometimes achieved with ng/ml concentrations of LPS (Liu et al., 2000). To address the apparent conflict between these reports and our data, we compared the effect of LPS from *E. coli*, serotype 0111:B4 used by Liu et al. (2000), with the serotype 026:B6 used in our study. The concentrations used ranged from 0.1ng/ml to 10 μg/ml, which included the concentrations used in both studies. MTT assay to determine overall cell survival showed no detectable toxicity in our culture system at any LPS concentration (Table 1). Measurement of [^{3}H]dopamine uptake, which was used in the study by Liu et al. (2000) to assess for selective damage to dopamine neurons, also demonstrated no toxicity of LPS (Table 1). We speculate that the lack of LPS-induced damage in our experiments was likely due to the different cell culture conditions (feeding medium, cell density, etc.).

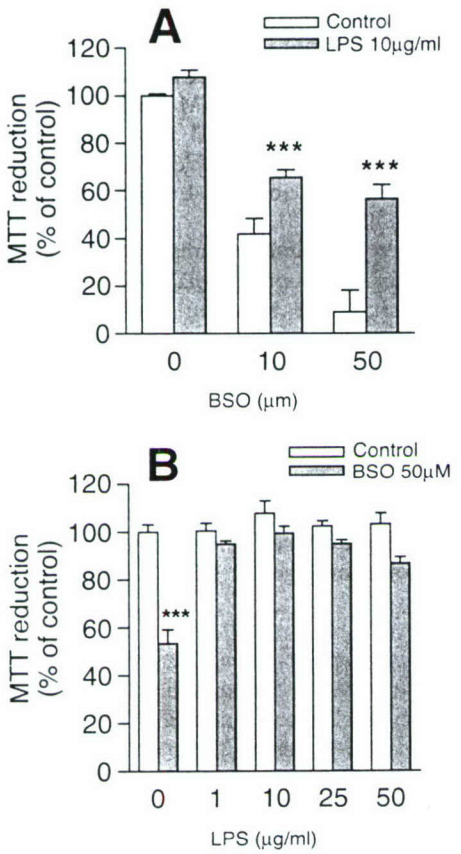

Fig. 2. LPS protects mesencephalic cultures from the toxicity of BSO treatment. (A) Cultures were exposed to 10 μg/ml LPS for 24 h and then treated with 10 or 50 μM BSO for an additional 48 h in the presence or absence of LPS. (B) Cultures treated with different concentrations of LPS for 24 h and then exposed to 50 μM BSO for 48 h. Bars show means ± S.E.M. ($n = 4$ per group; these experiments were repeated with similar results). ***$P < 0.001$ compared to the corresponding control; ANOVA followed by Tukey–Kramer multiple comparisons test.

LPS lowers GSH levels in astrocytes and mesencephalic cultures

We examined whether protection by LPS was the result of a direct effect on GSH levels. Since astrocytes, the major glial component in mesencephalic cultures, are important regulators of neuronal GSH (Sagara et al., 1993), we also tested the effect of LPS on GSH levels in cultures enriched in astrocytes. Astrocytes were treated with 1 or 10 μg/ml LPS for 24 h and then exposed to 10 μM BSO for an additional 48 h. After BSO treatment alone the levels of GSH were reduced to 43% of control values (Fig. 3A). In cultures treated with 1 or 10 μg/ml LPS, BSO caused an even greater decrease in GSH, to 14% and 13% of control levels respectively. Treatment with LPS alone caused significant reduction in GSH levels as well (to 73% and 76% of control with 1 and 10 μg/ml LPS, respectively). Neither LPS nor BSO treatment caused any loss in the viability of astrocytes as determined with the MTT assay (results not shown).

Treatment of mixed neuronal-glial cultures with LPS also caused a significant loss of GSH, reducing the levels to 77% and 25% of control values with 1 and 10 μg/ml, respectively (Fig. 3B). Treatment with 10 μM BSO for 48 h caused severe loss of cells in cultures not exposed to LPS and the GSH levels were only about 2% of controls. However, the GSH content in the few surviving cells may actually be underestimated when expressed in nmol/per mg protein, as protein measurement includes cell debris still attached to the culture dish. GSH levels in cultures treated with 10 μM BSO and 1 μg/ml LPS were 15% of control values, while 10 μg/ml LPS decreased GSH to levels not detectable by our assay, even though no apparent damage to the cells could be observed by phase contrast microscopy.

Possible mechanisms of LPS protection

Effect of growth factors and cytokines. Activated glial cells secrete both toxic and trophic substances. We tested the effects of insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF), known to be secreted by glial cells, on the toxicity of BSO in mesencephalic cultures (Fig. 4). In these experiments BSO-induced damage was determined by measuring the amount of LDH released into the culture medium. The concentration of growth factors used was 50 or 100 ng/ml and was selected for

Table 1. The effect of LPS obtained from different *E. coli* clones on cell survival (MTT reduction) and [^{3}H]dopamine uptake in mesencephalic cultures

LPS	MTT reduction		[^{3}H]dopamine uptake	
(ng/ml)	(% of control)		(% of control)	
	Clone 0111:B4	Clone 026:B6	Clone 0111:B4	Clone 026:B6
0	100.0 ± 1.7	100.0 ± 1.6	100.0 ± 3.8	100.0 ± 1.7
0.1	97.5 ± 1.5	98.1 ± 3.1	91.3 ± 1.8	99.2 ± 3.9
1	100.2 ± 0.2	98.6 ± 2.0	99.2 ± 3.4	97.8 ± 1.9
10	98.6 ± 1.9	99.7 ± 2.7	97.2 ± 1.4	93.8 ± 6.7
100	96.5 ± 0.8	92.5 ± 3.6	96.3 ± 4.4	93.1 ± 2.5
10 000	93.7 ± 0.5	98.5 ± 2.9	89.0 ± 1.4	98.0 ± 6.7

Mesencephalic cultures were exposed on the fifth day *in vitro* to LPS for 24 h and then treated again with LPS for an additional 48 h in order to duplicate the conditions used in the experiments examining the effect of LPS on the damage caused by GSH depletion. MTT assay and [^{3}H]dopamine uptake was performed at the end of LPS treatment. ANOVA showed no significant differences in MTT reduction or [^{3}H]dopamine uptake following LPS treatment.

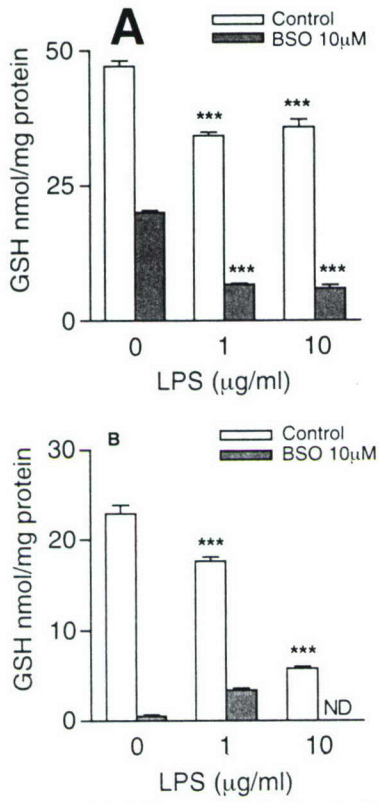

Fig. 3. Treatment with LPS causes reduction in GSH levels in purified astrocytes and mesencephalic cultures. Astrocytes and mesencephalic cultures were exposed to 1 or 10 μg/ml LPS for 24 h and then treated with 10 μM BSO for an additional 48 h. LPS decreased GSH levels in astrocytes (A) and potentiated the depletion caused by BSO. In mesencephalic cultures (B) LPS lowered GSH content significantly, particularly at the higher concentration. Mesencephalic cultures treated with BSO had minimal cell survival and very low GSH levels. In the presence of 10 μg/ml LPS and BSO GSH levels were not detectable (ND), although there was no apparent damage to the cells. Bars show means ± S.E.M. ($n = 4$ per group) $^{***}P < 0.001$; ANOVA followed by Tukey–Kramer multiple comparisons test.

maximal trophic activity towards neurons (Bouvier and Mytilineou, 1995). bFGF protected mesencephalic cells from toxicity at the lower concentrations of BSO (5 and 10 μM; Fig. 4A). With 50 μM BSO the damage was extensive (64% of total LDH was released into the medium) and bFGF was unable to confer any protection. Neither TGF-β nor IGF-I had any effect on BSO toxicity (Fig. 4B, C).

A number of pro- and anti-inflammatory cytokines are secreted upon activation of glial cells (Aschner, 1998; Giulian et al., 1993; Minghetti and Levi, 1998). We tested whether the pro-inflammatory cytokines IL-1β, interferon-γ (INF-γ) or TNF-α can modify BSO-induced toxicity. As shown in Fig. 5, exposure to IL-1β significantly increased BSO toxicity, while INF-γ (50 or 100 ng/ml) and TNF-α (20 ng/ml) had no effect (results not shown). The cytokines IL-6 and IL-10 (50 or 100 ng/ml), which can have anti-inflammatory properties and may be neuroprotective, were also tested but failed to provide protection against BSO toxicity (results not shown).

Up-regulation of antioxidant enzymes. LPS has been shown to up-regulate the antioxidant enzyme SOD in glial cultures (Del Vecchio and Shaffer, 1991; Mokuno et al., 1994). We examined the effect of LPS treatment (10 μg/ml for 48 h) on SOD and catalase activity in mesencephalic cultures. Treatment with LPS caused a greater than two-fold increase in MnSOD activity, but had no significant effect on Cu/ZnSOD (Fig. 6A). Catalase activity was not altered by LPS treatment (catalase activity after treatment with 10 μg/ml LPS was 106.7 ± 6.1% of control values, $n = 3$/group). MnSOD protein levels were also higher after LPS treatment (Fig. 6B), with no change in Cu/ZnSOD. To test the possibility that increased levels of SOD played a role in the protection from BSO toxicity we treated cultures with BSO in the presence or absence of SOD. Cu/ZnSOD or MnSOD added to the medium at 300 or 500 U/ml, provided significant protection from BSO toxicity (Fig. 7).

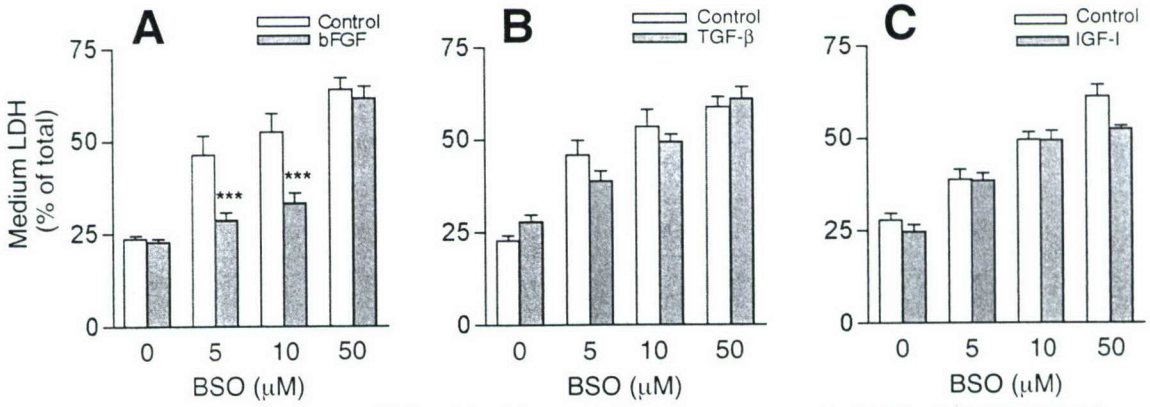

Fig. 4. The effect of growth factors on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml bFGF, TGF-β, or IGF-I for 24 h before exposure to BSO for an additional 48 h. Growth factors were present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium+cells). Bars show means ± S.E.M. ($n = 12$, from three separate experiments). $^{***}P < 0.001$; ANOVA followed by Tukey–Kramer multiple comparisons test. Similar results were obtained with 100 ng/ml growth factors.

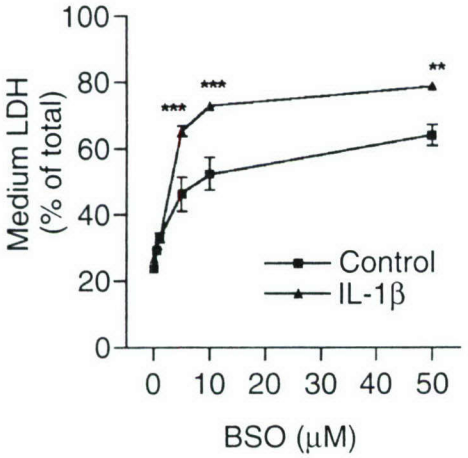

Fig. 5. The effect of IL-1β on BSO toxicity. Mesencephalic cultures were treated with 50 ng/ml IL-1β for 24 h before exposure to BSO for an additional 48 h. IL-1β was present during BSO treatment. LDH released in the medium was expressed as percent of total LDH (medium+cells). Bars show means ± S.E.M. ($n = 10$–12, from three separate experiments). $^{***}P < 0.001$; $^{**}P < 0.01$; ANOVA followed by Tukey–Kramer multiple comparisons test.

The localization of Cu/ZnSOD and MnSOD in mesencephalic cultures was examined using antibodies specific for the two enzymes. Labeling for Cu/ZnSOD was diffuse and was present at varying intensities mostly within neurons (Fig. 8A–C). No difference could be observed in Cu/ZnSOD labeling between control and LPS-treated cultures (Fig. 8A, D). Labeling for MnSOD was punctate, reflecting its mitochondrial localization (Fig. 9). In control cultures, MnSOD was present primarily within neurons, as demonstrated by its co-localization with MAP-2 (Fig. 9A–C). The intensity of MnSOD labeling in the neurons did not seem affected by LPS (Fig. 9D–F), though non-neuronal labeling was substantially increased. Under control conditions, little MnSOD immunoreactivity could be found in GFAP-positive astrocytes (Fig. 9G–I). After LPS treatment, however, very intense labeling appeared within GFAP-positive astrocytes (Fig. 9J–L). The somewhat weaker MnSOD label in the neurons of the LPS-treated cultures (Fig. 9D) is due to the very high intensity of the fluorescent label in the surrounding astrocytes, which prevents accurate photographic representation.

DISCUSSION

Our study shows that LPS protects mesencephalic cultures from damage caused by the inhibition of GSH synthesis with BSO. LPS treatment caused activation of glial cells, which was confirmed by the changes in morphology and the increase in the size of microglia (Kreutzberg,

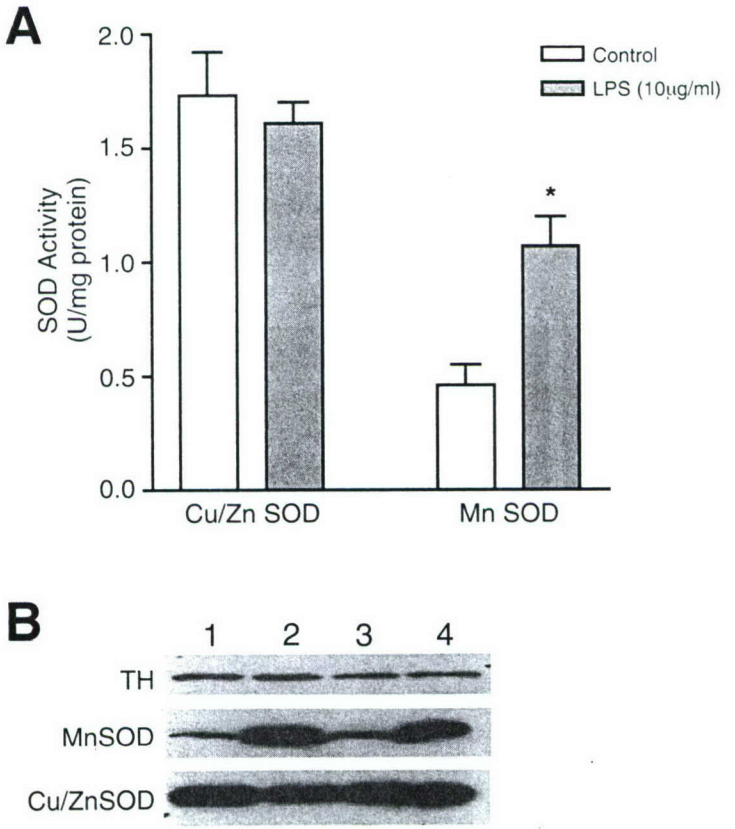

Fig. 6. LPS treatment up-regulates MnSOD in mesencephalic cultures. Cultures were treated with 10 μg/ml LPS for a total of 72 h. (A) SOD activity, measured in triplicates from pooled cells, from three separate experiments. Bars show means ± S.E.M. ($n = 3$). $^{*}P < 0.05$ Student's t-test. (B) Western blots of lysates from control (lanes 1 and 3) and LPS-treated (lanes 2 and 4) cultures from two independent experiments. Antibodies to TH and MnSOD were applied to the same blot. Separate blots from the same lysates were used for Cu/ZnSOD.

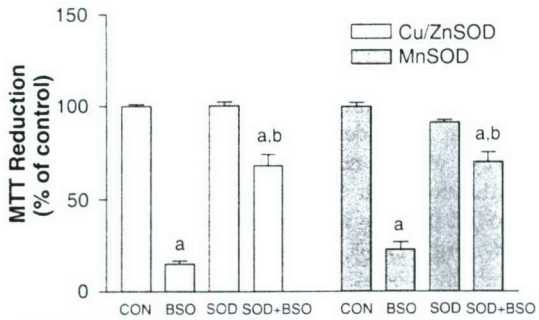

Fig. 7. Addition of SOD protects mesencephalic cultures from BSO toxicity. Cultures were exposed to 500 U/ml Cu/ZnSOD or 300 U/ml MnSOD with or without 50 μM BSO. MTT assay was performed 48 h after BSO treatment to determine cell viability. Bars show means ± S.E.M. ($n = 11$ for Cu/ZnSOD groups and $n = 8$ for MnSOD groups; for Cu/ZnSOD alone, $n = 7$); a differs from control, $P < 0.001$; b differs from BSO alone, $P < 0.001$. ANOVA followed by Tukey–Kramer multiple comparisons test.

1996) and by the increased number of GFAP-positive astrocytic processes (Aschner, 1998). The pro-inflammatory cytokine IL-1β was also increased in microglia after treatment with LPS, a further indication of an activated state (Giulian et al., 1994).

In our culture system, LPS treatment did not cause apparent cell death or reduction in the uptake of dopamine, although it has been previously reported that LPS is selectively toxic to dopamine neurons in mesencephalic cultures (Bronstein et al., 1995). Differences in the cell culture conditions are likely the cause of this apparent discrepancy. However, in spite of lack of cell death, LPS treatment resulted in oxidative stress, which was implied by the reduction in GSH content in both astrocytes and mixed neuronal cultures. Furthermore, LPS potentiated the effect of BSO on GSH depletion, which suggests that the inflammatory response of glial cells causes oxidative stress and has the potential to cause oxidative damage to the cultured cells. In addition, exposure of mesencephalic cultures to IL-1β increased BSO-induced damage, indicating that under conditions of oxidative stress IL-1β released by activated microglia could contribute to neurodegeneration. However, in spite of these apparently harmful effects, the overall result of LPS treatment was a significant protection from toxicity, suggesting that LPS may cause both pro- and antioxidant changes and that the protective events were predominant in our culture system.

Both astrocytes and microglia have the potential to provide support of neuronal survival *in vitro* (Engele et al., 1991; Hou et al., 1997; Nagata et al., 1993; O'Malley et al., 1992; Takeshima et al., 1994). Astrocytes stimulate neuronal growth, survival and regeneration by secretion of growth factors and extracellular matrix proteins (Fawcett, 1997; Muller et al., 1995). Following activation, astrocytes secrete both pro- and anti-inflammatory cytokines and growth factors (Aschner, 1998). We examined whether activated glial cells protected from damage caused by GSH depletion through the secretion of the growth factors bFGF, IGF-I and TGF-β. In a previous study we showed that bFGF reduced the damage caused by combined 6-hydroxydopamine and BSO treatment of mesencephalic cultures (Hou et al., 1997). In the present experiments bFGF, used at concentrations that produce maximum trophic effect in cultured neurons (Bouvier and Mytilineou, 1995), provided some protection from BSO toxicity when damage was not extensive, but it was substantially less effective than LPS. Therefore, it seems unlikely that secretion of bFGF by activated glia can by itself explain the protective effect of LPS treatment, although it may be contributory. TGF-β and IGF-I had no effect. LPS is

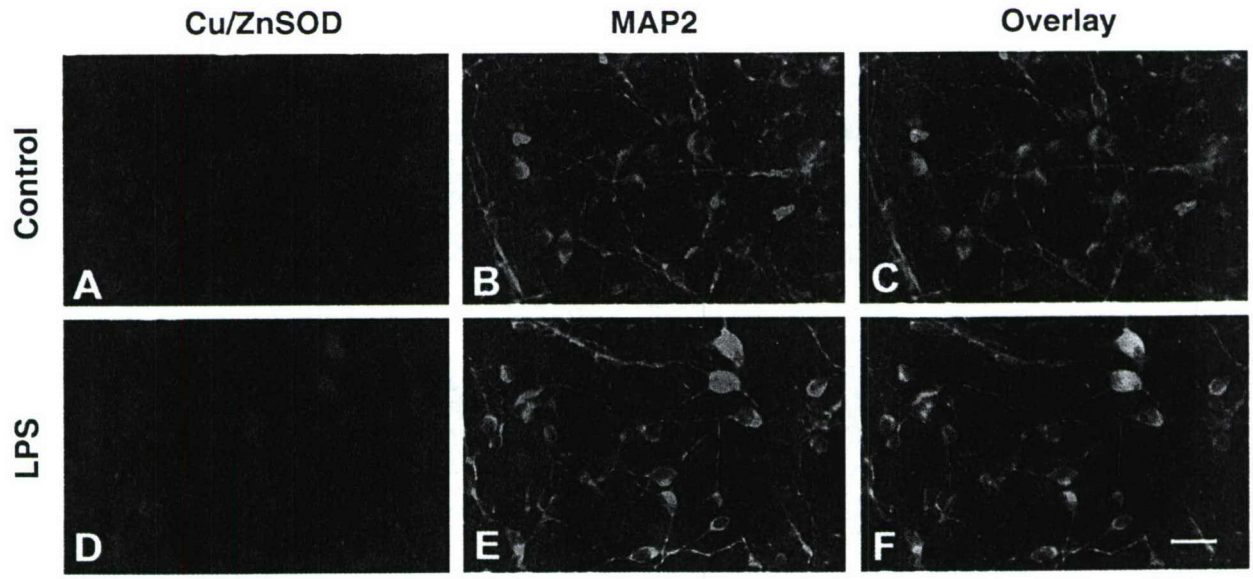

Fig. 8. Immunocytochemical localization of Cu/ZnSOD in mesencephalic cultures. Control (upper panel) and LPS-treated (10 μg/ml for 72 h; lower panel) cultures were double labeled for Cu/ZnSOD (A, D) and MAP-2 (B, E). Overlay of the two images (C, F) shows predominantly neuronal localization of Cu/ZnSOD and no apparent changes after treatment with LPS. Scale bar = 25 μm.

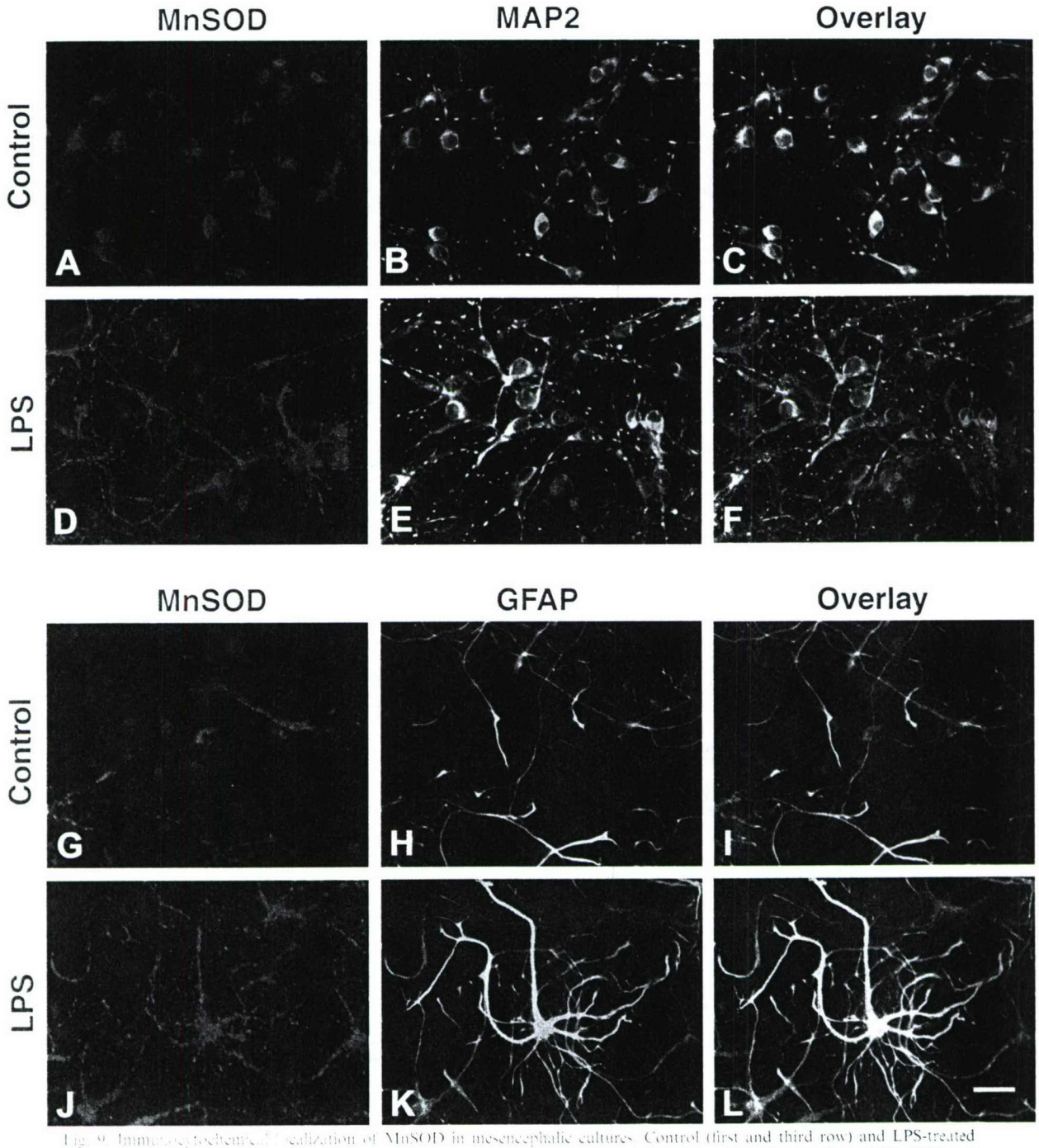

Fig. 9. Immunocytochemical localization of MnSOD in mesencephalic cultures. Control (first and third row) and LPS-treated (10 ng/ml for 72 h; second and fourth row) cultures were double labeled for MnSOD and MAP-2 (A–F) and MnSOD and GFAP (G–L). In control cultures MnSOD (A) was expressed strongly in neurons (B), as shown in the overlay of the two images (C). After LPS treatment, cells showed a strong increase in MnSOD immunoreactivity (D), which did not co-localize with MAP-2 (E, F). Double labeled with MnSOD (G) and GFAP (H) showed co-localization in some astrocytic processes (I). After LPS treatment, cells showed an increase in MnSOD (J), which was primarily within astrocytes (K, L). Scale bar = 25 μm.

a potent activator of IL-6 in astrocytes (Benveniste et al., 1990) and IL-6 is a key trophic and toxic effects on neurons (Gruol and Nelson, 1997). However, in our experiments IL-6 was neither toxic nor protective to GSH depleted cells. Some of the other cytokines IL-10, TNF-α and TNF-β did not modify the damage caused by BSO treatment.

Compared to neurons, astrocytes are enriched in antioxidant enzymes (Desagher et al., 1996; Makar et al., 1994). Support of neuronal survival by astrocytes is believed to be due, in part, to the scavenging of extracellular reactive oxygen species (Drukarch et al., 1998; Peuchen et al., 1997). Recently, it has been shown that priming with small doses of LPS can protect mice against

ischemia (Ahmed et al., 2000; Bordet et al., 2000; Dawson et al., 1999; Tasaki et al., 1997). The beneficial effect paralleled the induction of inflammation and was attributed to a compensatory activation of SOD by LPS (Bordet et al., 2000). Exposure of neuronal and glial cell cultures to LPS also up-regulates MnSOD, the inducible form of SOD present in the mitochondria (Kifle et al., 1996; Yu et al., 1999). In our study we found that LPS caused a significant increase in both protein content and activity of MnSOD in mesencephalic cultures. The increase in MnSOD protein occurred primarily in GFAP-positive astrocytes. There was no change in the protein content or activity of Cu/ZnSOD, the constitutive form of the enzyme present in the cytoplasm. In addition, we found no change in catalase activity, in agreement with the results obtained by Bordet et al. (2000), showing increased brain SOD activity but no changes in catalase after *in vivo* exposure to LPS. Activation of glial cells causes up-regulation and secretion of a number of pro- and anti-inflammatory cytokines, growth factors, as well as potential toxins (Minghetti and Levi, 1998), which makes it difficult to pinpoint at a single factor as the one responsible for the protective effect of LPS. However, a possible role for SOD in the LPS-induced protection was supported by the finding that addition of Cu/ZnSOD or MnSOD to mesencephalic cultures reduced the extent of BSO-induced damage. Both Cu/ZnSOD and MnSOD catalyze the dismutation of O_2^- and they would be expected to have a similar effect when added to the culture medium. Although the effect of exogenous SOD was likely extracellular, the protection observed probably resulted from scavenging of excessive O_2^- generated as a consequence of GSH depletion, which may have passed into the extracellular space. This is supported by a study showing that, in co-cultures of striatal glia and mesencephalic neurons, the depletion of GSH by BSO caused extracellular accumulation of ROS and cell loss, which was prevented by addition of SOD and catalase (Drukarch et al., 1998). Further experiments will be needed to identify the exact source of O_2^- in GSH depleted mesencephalic cultures, although damage by O_2^-, or its downstream ROS, would not necessarily be restricted to the cells of origin.

The selective increase in MnSOD in astrocytes compared to neurons is interesting and suggests that stimulation of astrocytes by LPS is likely involved in the up-regulation of MnSOD. It is also of interest that a selective increase in SOD within astrocytes results in the protection of all cells from oxidative damage. However, this concept is in agreement with numerous studies indicating that astrocytes can protect neurons from various oxidative insults (Desagher et al., 1996; Drukarch et al., 1998; Hou et al., 1997; Langeveld et al., 1995; Park and Mytilineou, 1992; Wilson, 1997).

There are several potential sources of O_2^- formation in cells, including oxidative phosphorylation used by the mitochondria for the generation of ATP. O_2^- is also formed during the metabolism of arachidonic acid by lipoxygenase, when hydroperoxyeicosetetraenoic acid (HPETE), the primary product of arachidonic acid metabolism, is converted to hydroxyeicosatetraenoic acid (HETE; Katsuki and Okuda, 1995). Our results suggest that O_2^- molecules play a very important role in the toxic events that follow GSH depletion. Merad-Saidoune et al. (1999) also reached a similar conclusion in a recent study showing that over-expression of MnSOD prevents mitochondrial damage caused by GSH depletion. Normally the concentration of O_2^- in the cells remains low as a result of the action of SOD. However, during GSH depletion SOD may not be able to handle the excess O_2^- generated from, among other sources, the metabolism of arachidonic acid by lipoxygenase. Arachidonic acid can also promote the generation of reactive oxygen species by directly inhibiting the mitochondrial respiratory chain (Cocco et al., 1999). When O_2^- levels become high, nitric oxide (NO) competes with SOD and combines rapidly with O_2^- to form peroxynitrite ($ONOO^-$). The damage to mitochondria caused by GSH depletion in neuronal and glial cultures has been shown to be the result of peroxynitrite production (Bolanos et al., 1995). Peroxynitrite can damage cells because it reacts with proteins acting as a selective oxidant and nitration agent (Bartosz, 1996). Nitrotyrosine is the product of the reaction of peroxynitrite with tyrosine and tyrosine residues (Reiter et al., 2000). The presence of nitrotyrosine in postmortem tissues in PD (Good et al., 1998) and other neurodegenerative disorders (Abe et al., 1995; Good et al., 1996; Sasaki et al., 2000), indicates that increased concentrations of O_2^- may contribute to their pathogenesis. Our data suggest that up-regulation of SOD activity may provide protection from oxidative stress and delay the progress of neurodegenerative diseases and in particular PD, where depletion of GSH may play a role in its pathogenesis (Jenner and Olanow, 1996; Sian et al., 1994).

Our study also illustrates the dependence of neurons on the surrounding glial cells. During conditions of oxidative stress, such as GSH depletion, the state of the surrounding glia can determine whether neurons will survive or die. Understanding of the relationships between neurons and glia should provide further insight into the process of neuronal degeneration, which contributes to the progression of neurological disorders.

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Toxicity of glutathione depletion in mesencephalic cultures: a role for arachidonic acid and its lipoxygenase metabolites

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Abstract

The contribution of arachidonic acid (AA) release and metabolism to the toxicity that results from glutathione (GSH) depletion was studied in rat mesencephalic cultures treated with the GSH synthesis inhibitor L-buthionine sulfoximine. Our data show that GSH depletion is accompanied by increased release of AA, which is phosholipase A2 (PLA_2) dependent. Exogenous AA is toxic to GSH-depleted cells. This toxicity is prevented by inhibition of lipoxygenase activity, suggesting participation of toxic byproducts of AA metabolism. Hydroxyperoxyeicosatetraenoic acid (HPETE), one of the primary products of AA metabolism by lipoxygenase is also toxic to GSH-depleted cells, whereas hydroeicosatetraenoic acid (HETE) is not. Cell death caused by GSH depletion is prevented by: (i) replenishment of GSH levels with GSH-ethyl ester; (ii) inhibition of PLA_2 activity; (iii) inhibition of lipoxygenase activity; and (iv), treatment with ascorbic acid. These data suggest that the following events likely contribute to cell death when GSH levels become depleted. Loss of GSH results in increased release of AA, which is PLA_2 dependent. Metabolism of arachidonic acid via the lipoxygenase pathway results in generation of oxygen free radicals possibly produced during conversion of HPETE to HETE, which contribute to cellular damage and death. Our study suggests that limiting AA release and metabolism may provide benefit in conditions with an existing depletion of GSH, such as Parkinson's disease.

Introduction

Glutathione (GSH) is a powerful antioxidant which controls the cellular redox state and protects neurons from the potential toxicity of reactive oxygen species (Meister, 1991; Jenner & Olanow, 1996; Schulz *et al.*, 2000). Oxidative stress is thought to play a role in the pathogenesis of several neurodegenerative disorders, including Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease (PD; Schulz *et al.*, 2000). In PD, loss of GSH appears early in the disease process (Dexter *et al.*, 1994; Sian *et al.*, 1994), which has led to the speculation that depletion of GSH could be an important pathogenic cause (Jenner & Olanow, 1998).

Several studies, including our own, have shown that depletion of GSH in primary neuronal cultures and neuronal cell lines results in cell death (Andersen *et al.*, 1996; Li *et al.*, 1997; Mytilineou *et al.*, 1998, 1999). The protective effect of ascorbic acid (Mytilineou *et al.*, 1999) and superoxide dismutase (Kramer *et al.*, 2002) indicates that oxidant stress is likely involved in cell death induced by GSH depletion. Recently it was shown that arachidonic acid (AA) appears to play an important role in GSH depletion-induced neuronal cell death (Li *et al.*, 1997; Mytilineou *et al.*, 1999). These studies demonstrated that inhibition of lipoxygenase (LOX), but not cyclooxygenase activity prevents the toxicity of GSH depletion, suggesting that the release and subsequent metabolism of AA by the LOX pathway contribute to the events that lead to cell death (Li *et al.*, 1997; Mytilineou *et al.*, 1999).

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Arachidonic acid, like other polyunsaturated fatty acids, is a structural component of membrane phospholipids bound at the *sn*-2 position. In the brain, it is liberated primarily by cytoplasmic phospholipase A_2 ($cPLA_2$; Piomelli, 1993). Normally free AA is maintained at low levels through recycling via energy dependent reactions, but its concentrations could increase in pathological conditions such as trauma or ischaemia (Farooqui *et al.*, 1997). Free AA exerts many biological actions either directly or through its active metabolites, but at high concentrations it also has the potential to cause toxicity and is thought to be involved in neurodegenerative disorders (Farooqui & Horrocks, 1991; Katsuki & Okuda, 1995). Arachidonic acid can be metabolized via the cyclooxygenase, LOX and cytochrome P450 pathways to form signalling molecules collectively known as eicosanoids (Needleman *et al.*, 1986). 12-LOX, the principal form of LOX in the brain (Wolfe & Poppius, 1984) produces 12-hydroxyperoxyeicosatetraenoic acid (12-HPETE), which leads to the formation of 12-hydroeicosatetraenoic acid (12-HETE). Highly reactive oxygen radicals are produced during the conversion of 12-HPETE to 12-HETE (Katsuki & Okuda, 1995).

The mechanism by which the release and metabolism of AA participate in the process of cell death during GSH depletion is not well understood at present. In order to gain further insight into the role of AA in oxidative stress-induced cell death, we examined the release and potential toxicity of AA and its LOX metabolites in GSH depleted mesencephalic cultures. The significance of these processes in free radical generation and cell death was further investigated by determining the effectiveness of inhibiting AA release and metabolism during the course of GSH depletion.

Materials and methods

Materials

Pregnant Sprague–Dawley rats were obtained from Taconic Farms (Germantown, NY, USA). Minimum essential medium (MEM) was purchased from Gibco-Life Technologies (Grand Island, NY, USA), horse serum from Gemini (Calabasas, CA, USA) and NU® serum from Collaborative Biomedical Products (Bedford, MA, USA). [^{3}H]AA (specific activity 217 Ci/mmol) was from New England Nuclear (Boston, MA, USA) and nonradioactive AA from Calbiochem (La Jolla, CA, USA). Arachidonyl trifluoromethyl ketone (ATK), methyl arachidonyl fluorophosphonate (MAFP), 12(S)-HPETE and 12(S)-HETE were obtained from Cayman Chemical (Ann Arbor, MI, USA), biacalein from Biomol (Plymouth Meeting, PA, USA), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) from Molecular Probes (Eugene, OR, USA) and glutathione monoethyl ester from Bachem Biosciences, Inc. (King of Prussia, PA, USA). All other chemicals were of the highest purity available and purchased from Sigma.

Preparation of cell cultures

The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou *et al.*, 1998). In brief, pregnant rats were killed by exposure to CO_2, the embryos removed, and the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). The tissue was dissociated mechanically into a single cell suspension and plated in 24- or 6-well plates precoated with L-polyornithine (0.1 mg/mL) at a density of 300 000 cells/cm^2, and at 150 000 cells/cm^2 for the experiments using the 12-LOX metabolites of AA. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum and 10% NU® serum. Treatment began on the 5–6th day *in vitro*, at which time the medium was changed to MEM containing only 5% NU® serum.

Cell viability assays

MTT assay

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, as described previously (Han *et al.*, 1996). In brief, 50 μL of 5 mg/mL MTT was added to each cell culture well containing 0.5 mL medium. After a 1-h incubation at 37 °C, the medium was carefully removed and the formazan crystals were dissolved in 1 mL isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250, Molecular Devices Corporation, Sunnyvale, CA, USA).

Lactate dehydrogenase assay

A modification of the method by Bergmeyer *et al.* (1963) was used to determine lactate dehydrogenase (LDH) activity in the culture medium and the cells. Medium was collected, centrifuged to remove debris and frozen at −80 °C until assay. Cells were freeze-thawed (× 3) in 1.0 mL feeding medium, the medium was collected, centrifuged and the supernatant frozen at −80 °C. Aliquots of supernatant (50 μL) and NADH (100 μL of 1.2 mg/mL H_2O stock) were added to 850 μL buffer and the samples were vortex-mixed. A total of 50 μL feeding medium was used for blanks. Triplicate aliquots (250 μL) were placed into 96-well plates at room temperature and reaction was initiated by addition of 25 μL sodium pyruvate (0.36 mg/mL H_2O stock). The rate of disappearance of NADH was measured at 340 nm using a plate reader.

Glutathione assay

Glutathione was quantified using a modification of a standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) with glutathione reductase and NADPH (Tietze, 1969). In brief, the medium was carefully aspirated from the culture wells, 300 μL 0.4 N perchloric acid (PCA) was added and the plates were kept on ice for 30 min. The PCA was then collected and stored at −70 °C until assayed. Both oxidized (GSSG) and reduced (GSH) forms of glutathione were measured with this assay. However, because of the small amounts of GSSG present in mesencephalic cultures (~5% of total; Mytilineou *et al.*, 1993), the values obtained were considered to represent GSH content.

The tissue attached to the bottom of the wells after removal of the PCA was dissolved in equal volumes of 20% sodium dodecyl sulphate and 0.5 N NaOH and used for protein determination according to the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard.

Measurement of [^{3}H]AA release

The method described by Stella *et al.* (1997) was used to measure the release of [^{3}H]AA with modifications. Mesencephalic cells were labelled with [^{3}H]AA (0.5 μCi/mL) for 24 h in feeding medium containing 1% fatty acid-free BSA. They were then washed three times with balanced salt solution and treated with the appropriate drugs

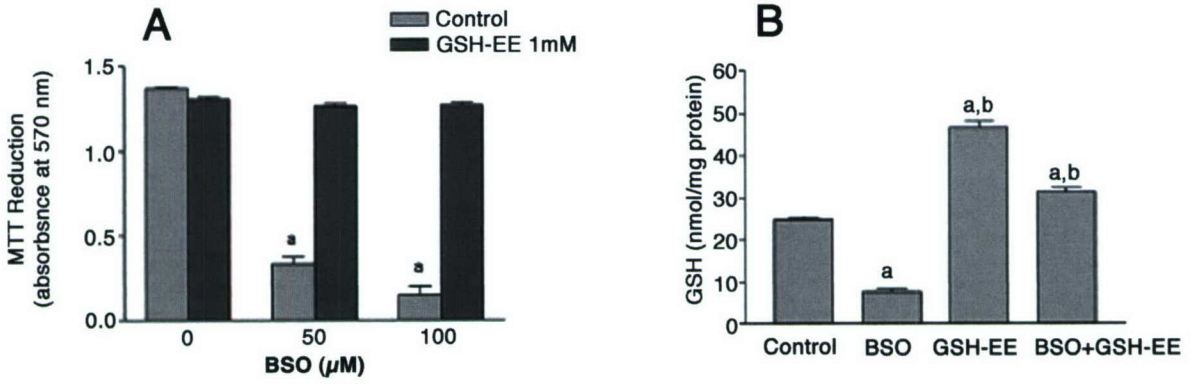

FIG. 1. Replenishment of GSH content prevents BSO-induced cell death.Mesencephalic cultures were treated with BSO in the presence or absence of 1 mM GSH-ethyl ester (GSH-EE) and were assayed 48 h later for viability with the MTT assay (A) and for GSH content (B). a, differs from control group $P < 0.001$; b, differs from group treated with BSO alone $P < 0.001$. ANOVA followed by Tukey–Kramer test. $n = 4$ cultures per group for (A) and six cultures per group for B.

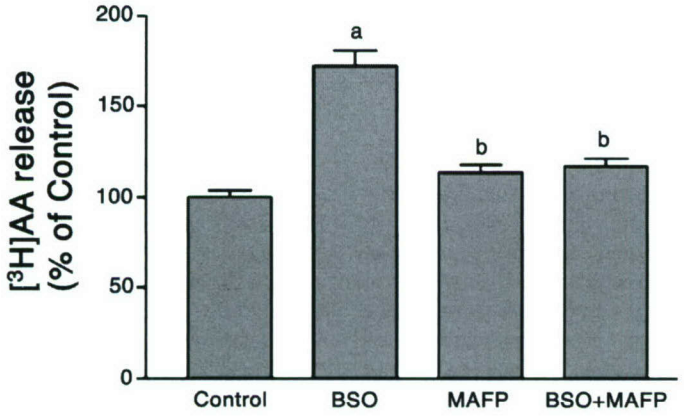

FIG. 2. BSO treatment increases the release of AA. Cultures were treated with [^{3}H]AA for 24 h to label membrane phospholipids and then treated with 50 μM BSO with or without 10 μM MAFP. Arachidonic acid release was measured 24 h after BSO treatment, a time point when no damage could be detected with the MTT assay. a, differs from control $P < 0.001$; b, differs from group treated with BSO alone; $P < 0.001$. ANOVA followed by Tukey–Kramer test. $n = 12$–14 cultures per groups except for the MAFP groups where $n = 6$.

[L-buthionine sulfoximine (BSO) and/or $cPLA_2$ inhibitors] in feeding medium containing 1% fatty acid-free BSA. Twenty-four hours after treatment, all cultures were exposed to 10 μM thimerosal for 10 min to inhibit AA reacylation (Stella *et al.*, 1994). The medium was then aspirated and 0.5 mL Kreb's phosphate buffer containing (in mM): NaCl, 120; KCl, 4.8; NaH_2PO_4, 15.6; $MgSO_4$, 1.2; $CaCl_2$, 1.0; and glucose, 33 at pH 7.4 and supplemented with 1% fatty acid-free BSA was added in each well. After 1 h at 37 °C, a 200-μL aliquot was collected and counted for radioactivity in a liquid scintillation spectrometer. MTT was then added to the medium remaining in the wells to test for cell viability.

Visualization of reactive oxygen species

H_2DCF-DA was used to visualize the generation of reactive oxygen species (ROS). H_2DCF-DA penetrates the cell membrane and is enzymatically hydrolysed by intracellular esterases to nonfluorescent H_2DCF. In the presence of ROS H_2DCF is oxidized to the highly fluorescent 2′7′-dichlorofluorescein (Tsuchiya *et al.*, 1994). Mesencephalic cultures were exposed to 1 μM H_2DCF-DA for 15 min at various time points after the beginning of BSO treatment. Cultures were then washed twice with physiological saline containing (in mM): NaCl, 135; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; and HEPES, 10 at pH 7.3, and were examined under a fluorescence microscope.

Statistical analysis

Values are expressed as means ± SEM. Significance of differences between two groups was determined by two-tailed Student's t-test. For multiple comparisons, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test was used.

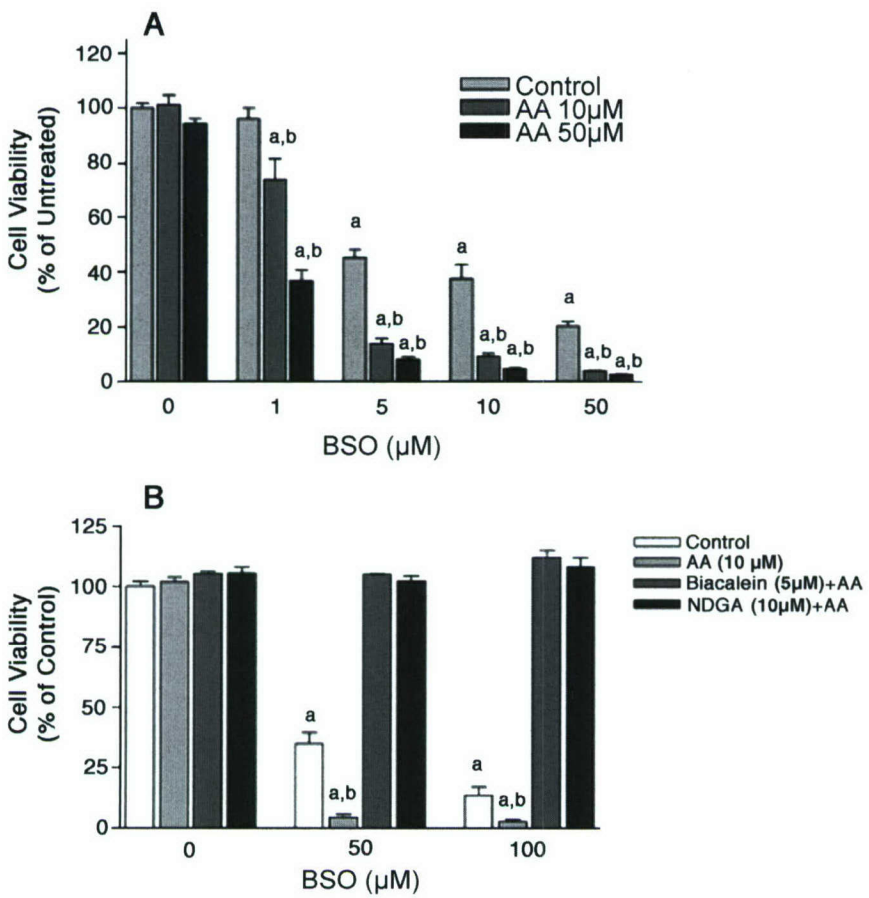

FIG. 3. Exogenous AA is toxic to GSH depleted cells. Cultures were exposed to increasing concentrations of BSO in the presence or absence of AA (10 or 50 μM) (A) or with 10 μM AA in the presence or absence of the LOX inhibitors biacalein and NDGA (B). All cultures were analysed for cell viability with the MTT assay 48 h after the initiation of treatment. a, differs from control, $P < 0.001$; b, differs from the corresponding BSO group not treated with AA, $P < 0.001$. ANOVA followed by Tukey–Kramer test. $n = 4$ per group for A and eight per group for B.

Results

Restoration of GSH levels prevents BSO toxicity

Our previous studies showed that inhibition of GSH synthesis by BSO induces depletion of GSH and cell death in mesencephalic cultures (Mytilineou *et al.*, 1998, 1999). To confirm that depletion of GSH is the primary cause of BSO toxicity, we treated mesencephalic cultures with GSH-monoethyl ester (GSH-EE), a GSH delivery agent that restores GSH levels (Martensson *et al.*, 1993). Figure 1A shows that in the presence of 1 mM GSH-EE the loss of cell viability caused by treatment with 50 or 100 μM BSO was prevented completely. The protection was accompanied by an increase in GSH to levels above control values (Fig. 1B). GSH-EE also increased the levels of GSH in cultures not treated with BSO (Fig. 1B).

To determine whether the MTT assay was an accurate index of loss of cell viability we compared the results of the MTT assay with results obtained from measurement of LDH release into the culture medium. The results were similar in all experiments. For example, in a similar experiment treatment with 50 μM BSO resulted in a 5.2-fold increase in the LDH activity in the medium and in a 77% reduction in cell viability as measured with the MTT assay.

The release of AA increases during GSH depletion

The metabolism of AA has been implicated in the toxicity of GSH depletion (Li *et al.*, 1997; Mytilineou *et al.*, 1999). To determine whether GSH depletion promotes the release of AA, we labelled membrane phospholipids with [^{3}H]AA before treating the cultures with BSO (50 μM). Spontaneous release of AA was measured 24 h after BSO treatment, a time point when there is severe GSH depletion without significant cell death (Mytilineou *et al.*, 1999). A significant increase in AA release was observed in the BSO-treated cultures, which could be prevented by the specific $cPLA_2$ inhibitor MAFP (10 μM), suggesting the involvement of $cPLA_2$ in the release of AA by BSO (Fig. 2). The nonspecific PLA_2 inhibitor mepacrine (1 μM) also prevented the BSO-induced AA release (not shown). MTT assay showed no decrease in cell viability at this time point.

Arachidonic acid is toxic to GSH-depleted cells

To test whether the release of AA could be associated with the toxicity caused by GSH depletion, we exposed cell cultures to AA in the presence or absence of BSO (Fig. 3). Arachidonic acid (10 or 50 μM) was not toxic to mesencephalic cultures if GSH synthesis was not inhibited (Fig. 3A). In the presence of BSO, however, AA caused loss of cell viability, which became greater at higher BSO concentrations. The toxicity of combined BSO and AA treatment was always greater than BSO alone. Inhibition of LOX activity with 5 μM biacalein or 10 μM NDGA prevented the damage caused by combined treatment with BSO and AA (Fig. 3B), suggesting that the metabolism of AA by LOX is an important component of the events that lead to loss of viability. We have previously shown that inhibition of LOX prevents damage related to BSO toxicity alone (Mytilineou *et al.*, 1999).

The effect of 12-LOX metabolites of AA on GSH depleted cells

Protection from BSO by inhibition of LOX activity suggests that the products of AA metabolism by LOX might account for the observed toxicity. The effect of 12-HPETE and 12-HETE, the major products of 12-LOX reaction with AA (Katsuki & Okuda, 1995), was examined in mesencephalic cultures in the presence or absence of BSO. In these experiments the toxicity of BSO was kept low by reducing the cell

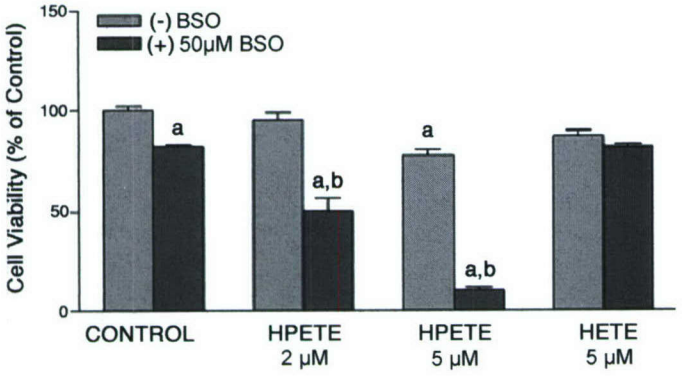

FIG. 4. Effect of 12-HPETE and 12-HETE on cultures with normal and reduced GSH levels. Mesencephalic cultures were exposed to the AA 12-LOX-metabolites 12-HPETE and 12-HETE for 24 h and then exposed to 50 μM BSO. MTT assay for cell viability was carried out 48 h later. A, differs from control, $P < 0.001$; b, differs from BSO alone, $P < 0.001$. ANOVA followed by Tukey–Kramer test. $n = 12$ cultures per group.

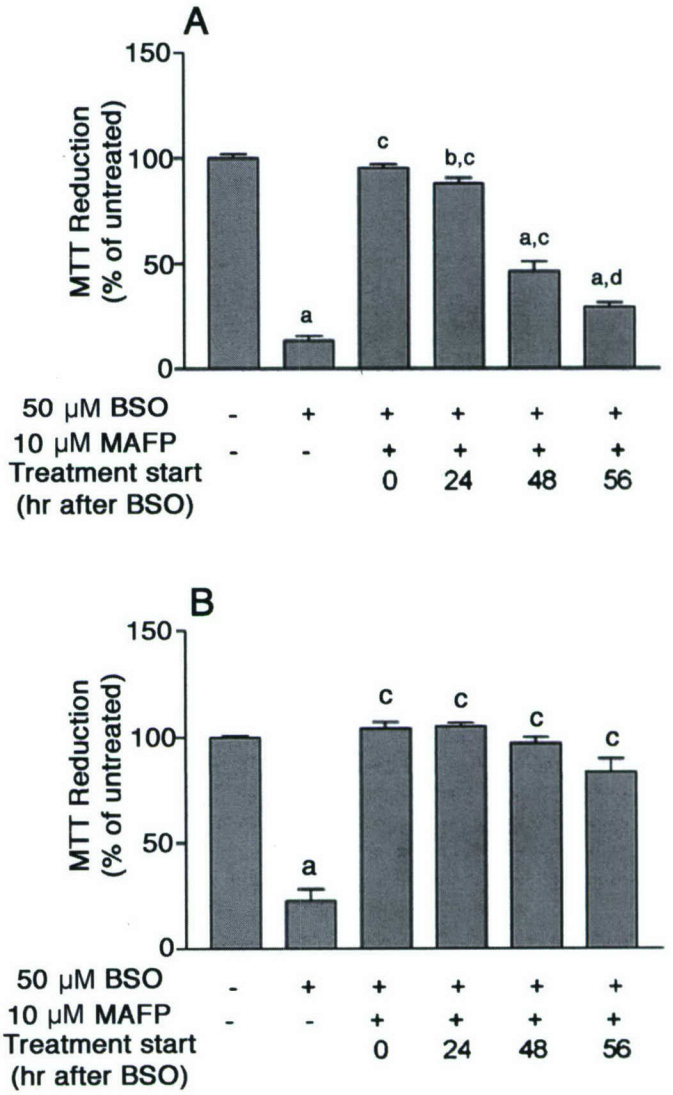

FIG. 5. Effectiveness of PLA_2 (A) and LOX inhibition (B) when applied at different times after exposure to 50 μM BSO. Cultures were treated with BSO and the inhibitors were added either at the same time with BSO (0) or at 24, 48 and 56 h after the beginning of BSO treatment. Cell viability was assayed with MTT 72 h after the beginning of treatment. Significantly different from control: a, $P < 0.001$; b, $P < 0.05$. Significantly different from BSO: c, $P < 0.001$; d, $P < 0.01$ ($n = 8$ per group). ANOVA followed by Tukey–Kramer test.

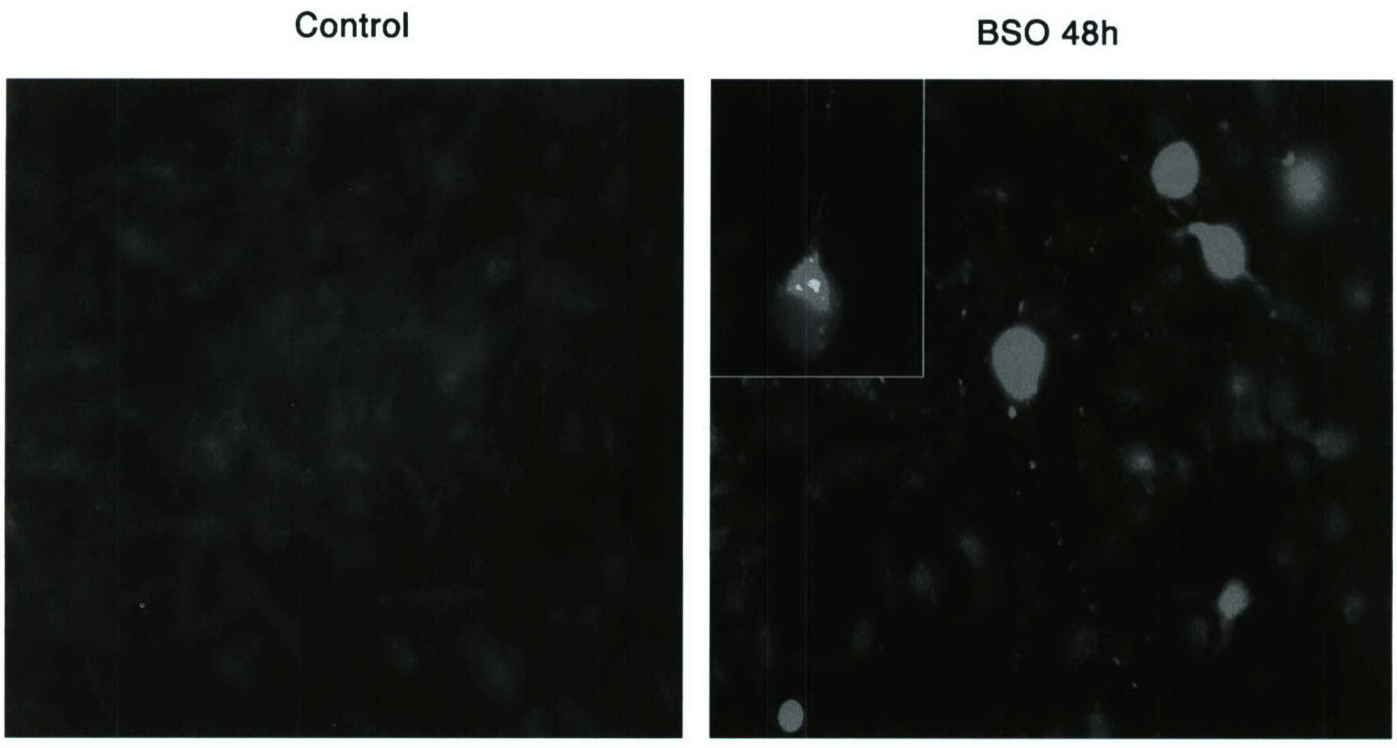

FIG. 6. ROS accumulation following treatment with BSO. Fluorescence photomicrographs from a control culture and a culture treated with 50 μM BSO for 48 h exposed to 1 μM H_2DCF-DA for 15 min. The accumulation of fluorescence indicates the presence of ROS. Intense fluorescence was seen localized within cellular organelles (inset).

density in the cultures (Mytilineou *et al.*, 1999). 12-HPETE caused a small reduction of cell survival in control cultures (22% loss at 5 μM) and it augmented the toxicity of BSO by threefold at 2 μM and fivefold at 5 μM (Fig. 4). In contrast, 12-HETE caused no significant damage and did not modify the toxicity of BSO.

cPLA$_2$ and LOX inhibitors prevent BSO toxicity; time course of protection

The cPLA$_2$ inhibitors ATK and MAFP prevented the toxicity of BSO in mesencephalic cultures. MTT assays showed that 10 μM ATK and MAFP added together with 50 μM BSO improved cell viability in mesencephalic cultures from $10 \pm 0.3\%$ of control with BSO alone to $98 \pm 4\%$ and $112 \pm 4\%$, respectively. Also, in agreement with previous studies (Li *et al.*, 1997; Mytilineou *et al.*, 1999), NDGA, a nonspecific LOX inhibitor and biacalein, a specific 12-LOX inhibitor, prevented BSO-induced damage (results not shown).

Because an increase in AA release appears relatively early in the course of GSH depletion (see Fig. 2A), we sought to determine the point in time beyond which inhibition of cPLA$_2$ or LOX could no longer rescue the cells from BSO toxicity. The inhibitors of cPLA$_2$ (MAFP; 10 μM) and LOX (biacalein; 5 μM) were added to the cultures either at the time of exposure to BSO, or 24, 48 and 56 h after the beginning of BSO treatment. All groups were analysed for cell survival with the MTT assay 72 h after the beginning of BSO treatment to allow for maximal damage by BSO in this group of cultures (Fig. 5). Inhibition of cPLA$_2$ fully protected from toxicity when added at the same time with BSO but protection diminished gradually when the inhibitor was added at later times (Fig. 5A). In contrast, inhibition of LOX fully protected from toxicity, even when biacalein was added just before the onset of damage (Fig. 5B). A similar course of protection was observed with the nonspecific cPLA$_2$ inhibitor mepacrine (1 μM) and the nonspecific LOX inhibitor NDGA (1 μM; results not shown). The antioxidant ascorbic acid, which was previously shown to protect from BSO toxicity (Mytilineou *et al.*, 1999), also protected from toxicity with similar efficacy even when added late after the beginning of BSO treatment. In an experiment where treatment with 50 μM BSO reduced MTT-determined cell viability to $26 \pm 7\%$ of control after 48 h, addition of 200 μM ascorbic acid restored cell survival to $81 \pm 1\%$, $84 \pm 1\%$ and $83 \pm 1\%$ of control levels, whether added together with BSO or 24 and 30 h later ($n = 4$; $P < 0.001$ compared with BSO alone).

ROS accumulation during the course of GSH depletion

We examined the intracellular accumulation of ROS during the course of GSH depletion by loading the cells with H_2DCF-DA, which is converted to a fluorescent derivative by ROS. Cultures were treated with BSO and then exposed to H_2DCF-DA at 4, 8, 24, 30 and 48 h later. No significant increases in fluorescence could be observed in the cultures up to 30 h post-treatment. High intensity fluorescence began accumulating almost simultaneously with the appearance of damaged cells (Fig. 6). Fluorescence appeared initially within well-defined cellular organelles (Fig. 6, inset) and in cell processes and eventually filled the entire cell. Some cells appeared ballooned and to be detaching themselves from the culture dish. ROS formation using this assay was blocked by PLA$_2$ and LOX inhibitors (results not shown).

Discussion

Mesencephalic cultures, which contain dopamine neurons of the substantia nigra, have been used widely to test hypotheses that may be relevant to the aetiology and treatment of PD. One of the defects characteristic of PD is a decrease in the GSH content of the substantia nigra (Perry *et al.*, 1982; Riederer *et al.*, 1989; Sian *et al.*, 1994). The loss of this antioxidant is thought to contribute to the pathogenesis and progression of the disease (Fahn & Cohen, 1992; Jenner *et al.*, 1992). In earlier studies we have used BSO, an inhibitor of the enzyme

γ-glutamylcysteine synthetase, to cause depletion of GSH in mesencephalic cultures and examine mechanisms of neuronal degeneration and neuroprotection (Mytilineou *et al.*, 1998, 1999). In the present study we carried out experiments to ascertain that GSH depletion is the cause of BSO-induced cell death. We have shown that restoration of GSH levels through the use of the cell permeable GSH-ethyl ester (Meister, 1991) prevents BSO-induced cell death in mesencephalic cultures, indicating that loss of GSH is the primary cause of BSO toxicity.

We therefore used BSO to cause depletion of GSH to study pathways that might lead to the generation of ROS and cell death in mesencephalic cultures. Our data indicate that an early event that can be detected following exposure to BSO is a PLA_2-dependent increase in the release of AA. We also demonstrate that exogenous AA becomes toxic to GSH depleted cells upon its metabolism by LOX. Although our experiments do not provide direct evidence that the AA released during the course of GSH depletion is responsible for the toxicity, the ability of PLA_2 inhibitors to prevent both the release of AA and cell death supports the hypothesis. It is of interest that AA, even at relatively high concentrations (10–50 μM), was not toxic to cells with normal GSH content. A similar result has been reported with a rat glioma cell line where AA was not toxic in the absence of BSO (Higuchi & Yoshimoto, 2002). These data suggest that two parallel mechanisms could be operating during the course of GSH depletion, one causing the release of AA and one increasing the toxicity of AA to the cells. The reason for the lack of AA toxicity in normal cells could be that the high levels of GSH (approximately 2.5 mM in our cultures), suppress LOX activity and prevent the accumulation of toxic by-products of AA metabolism. It has been shown that LOX is directly inhibited by GSH (Shornick & Holtzman, 1993) and that its activity increases in GSH-depleted cells (Li *et al.*, 1997; Chen *et al.*, 2000). In microsomal fractions from epithelial cells concentrations of 0.1, 1 and 10 mM GSH, which are within the range present in the mesencephalic cultures, reduced 12-LOX activity to 74, 38 and 16% of control values, respectively (Shornick & Holtzman, 1993). However, it is also possible that the sensitivity of GSH depleted cells to AA might be a result of additional oxidative events, caused by the depletion of GSH and not directly related to the release and metabolism of AA. A combination of oxidative insults could overwhelm the antioxidant potential of the cells and result in their demise.

The reaction of AA with molecular oxygen, catalysed by 12-LOX, generates several fatty acid radicals as intermediate by-products in the formation of 12-HPETE (Katsuki & Okuda, 1995). These radicals and the highly reactive oxygen species, which are produced during the conversion of 12-HPETE to 12-HETE, could contribute to the toxicity of AA (Katsuki & Okuda, 1995). In our study addition of 12-HPETE caused damage to mesencephalic cells, particularly when GSH levels were reduced by BSO. The lack of toxicity of 12-HETE suggests that the oxygen radicals produced during the conversion of 12-HPETE to 12-HETE might be contributing to cell damage. A recent study has shown that 12-HPETE, but not 12-HETE, can directly activate caspase-3 and cause apoptosis in a fibroblast cell line (Gu *et al.*, 2001).

Collectively our data suggest the following scenario concerning the events that lead to cell death after exposure of mesencephalic cultures to BSO: depletion of cellular GSH causes increased release of AA likely through the activation of PLA_2; a concomitant increase in LOX activity, also resulting from GSH depletion, drives the metabolism of AA through this pathway. Oxygen free radicals generated during this metabolic process accumulate within the GSH depleted cells overwhelming existing defense mechanisms and contributing to cell death. The capacity of PLA_2 inhibitors, LOX inhibitors and antioxidants to prevent death of GSH depleted cells supports this scenario.

How GSH depletion results in increased release of AA is not known. One possibility is that loss of GSH could disrupt cellular Ca^{2+} homeostasis and increase free intracellular Ca^{2+}. Physiological changes in the concentrations of free intracellular Ca^{2+} are able to cause translocation of the Ca^{2+}-dependent $cPLA_2$ to the membrane and increase its activity (Clark *et al.*, 1991). In addition GSH depletion and increased concentrations of H_2O_2 could cause peroxidation of membrane phospholipids, which makes them better substrates for PLA_2 (McLean *et al.*, 1993; Farooqui *et al.*, 1997). Activation of $cPLA_2$ might also occur in response to kinase-dependent enzyme phosphorylation (Lin *et al.*, 1993; Qiu *et al.*, 1998).

Although complete protection is achieved when $cPLA_2$ inhibitors are added together with BSO, delayed exposure reduces their effectiveness. In contrast, inhibition of AA metabolism or addition of antioxidants can rescue mesencephalic cells, even if treatment begins quite late, just before the onset of cell breakdown. The reason for this difference is not clear, but it implies that activation of PLA_2 is an early event in the process that leads to cell death. It is interesting that accumulation of reactive oxygen species, in amounts sufficient to be visualized with H_2DCF-DA microscopy, occurs only during the last stages before cell loss. This suggests that a critical threshold of toxic by-products of AA metabolism is needed for the initiation of cell death pathway and preventing this accumulation can rescue the cells.

Increased PLA_2 activity has been linked to neuronal injury in several neurodegenerative disorders, including cerebral ischaemia (Sapirstein & Bonventre, 2000; Arai *et al.*, 2001), stroke (Estevez & Phillis, 1997), neurotrauma (Bazan *et al.*, 1995) and Alzheimer's disease (Stephenson *et al.*, 1996). Recent studies have shown that inhibition of PLA_2 activity protects from loss of striatal dopamine in experimental models of PD (Tariq *et al.*, 2001), and that genetically induced decrease in $cPLA_2$ activity renders mice resistant to MPTP neurotoxicity (Klivenyi *et al.*, 1998). These results suggest that $cPLA_2$ might play a role in the pathogenesis and progression of PD. In the experimental model used in our study less than 5% of the cells represent dopamine neurons and GSH depletion results in generalized cell loss. However, the involvement of AA release and metabolism in oxidant stress and cell death caused by GSH depletion, supports a possible involvement of PLA_2 activity, as depletion of GSH and oxidative stress are prominent pathological features of PD.

Although potent specific inhibitors for the various isoforms of PLA_2 are not currently available for clinical use, their potential therapeutic value has made them subject of intense research efforts (Farooqui *et al.*, 1999; Cummings *et al.*, 2000). Further studies confirming an association between AA release and metabolism and the pathogenesis of PD will be required in order to determine whether such therapies have the potential to provide an effective treatment for PD patients.

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Abbreviations

AA, arachidonic acid; ATK, arachidonyl trifluoromethyl ketone; BSA, bovine serum albumin; BSO, L-buthionine sulfoximine; $cPLA_2$, cytoplasmic phospholipase A2; GSH, glutathione; GSH-EE, glutathione ethyl ester; H2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HETE, hydroeicosatetraenoic acid; HPETE, hydroxyperoxyeicosatetraenoic acid; LOX, lipoxygenase; LDH, lactate dehydrogenase; MAFP, methyl arachidonyl fluorophosphonate; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PCA, perchloric acid; PD, Parkinson's disease; ROS, reactive oxygen species.

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Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures

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Abstract

Mutations in α-synuclein, parkin and ubiquitin *C*-terminal hydrolase L1, and defects in 26/20S proteasomes, cause or are associated with the development of familial and sporadic Parkinson's disease (PD). This suggests that failure of the ubiquitin–proteasome system (UPS) to degrade abnormal proteins may underlie nigral degeneration and Lewy body formation that occur in PD. To explore this concept, we studied the effects of lactacystin-mediated inhibition of 26/20S proteasomal function and ubiquitin aldehyde (UbA)-induced impairment of ubiquitin *C*-terminal hydrolase (UCH) activity in fetal rat ventral mesencephalic cultures. We demonstrate that both lactacystin and UbA caused concentration-dependent and preferential degeneration of dopaminergic neurons. Inhibition of 26/20S proteasomal function was accompanied by the accumulation of α-synuclein and ubiquitin, and the formation of inclusions that were immunoreactive for these proteins, in the cytoplasm of VM neurons. Inhibition of UCH was associated with a loss of ubiquitin immunoreactivity in the cytoplasm of VM neurons, but there was a marked and localized increase in α-synuclein staining which may represent the formation of inclusions bodies in VM neurons. These findings provide direct evidence that impaired protein clearance can induce dopaminergic cell death and the formation of proteinaceous inclusion bodies in VM neurons. This study supports the concept that defects in the UPS may underlie nigral pathology in familial and sporadic forms of PD.

Keywords: α-synuclein, Lewy body inclusion, Parkinson's disease, 26/20S proteasome, ubiquitin *C*-terminal hydrolase L1 and parkin.

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Degeneration of the dopamine-containing neurons of the substantia nigra pars compacta (SNc) is the primary pathology occurring in Parkinson's disease (PD; Forno 1996). Neuronal death is accompanied by the appearance of cytoplasmic Lewy body inclusions which accumulate a wide range of proteins including α-synuclein, ubiquitin, neurofilaments, and oxidized/nitrated proteins (Pollanen *et al.* 1993; Good *et al.* 1998; Spillantini *et al.* 1998; Giasson *et al.* 2000). Nigral pathology in PD is associated with oxidative stress, mitochondrial dysfunction, and excitotoxicity but it is not clear how these events contribute to the neurodegenerative process (Jenner and Olanow 1998).

Recent evidence suggests that failure of the ubiquitin–proteasome system (UPS) leading to protein accumulation contributes to degeneration of dopaminergic neurons and Lewy body formation in the SNc in both familial and sporadic forms of PD (McNaught *et al.* 2001). Various deletions and point mutations in the parkin gene (6q15.2–27), which codes for a ubiquitin ligase, lead to a loss of enzyme activity and destruction of the SNc in cases of autosomal recessive juvenile parkinsonism (AR-JP; Shimura *et al.* 2001). In rare occurrences of autosomal dominant PD, missense mutations in the gene (4q21–q23) encoding α-synuclein have been shown to produce proteins that are prone to misfold and aggregate

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Abbreviations used: AR-JP, autosomal recessive juvenile parkinsonism; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; PD, Parkinson's disease; TH, tyrosine hydroxylase; UbA, ubiquitin aldehyde; UCH-L1, ubiquitin *C*-terminal hydrolase L1; UCH, ubiquitin carboxy-terminal hydrolase; UPS, ubiquitin-proteasome system; and, VM, ventral mesencephalic.

(Polymeropoulos *et al.* 1997; Goedert 2001). Mutant α-synucleins resist and inhibit proteolysis, and increase the sensitivity of cells to a variety of toxic insults (Bennett *et al.* 1999; Lee *et al.* 2001; Stefanis *et al.* 2001; Tanaka *et al.* 2001; Tofaris *et al.* 2001). These factors are thought to contribute to nigral dopaminergic cell death and Lewy body formation in α-synuclein-linked familial forms of PD (Goedert 2001; Spira *et al.* 2001). Additionally, a missense mutation in the gene (4p14) encoding for the de-ubiquitinating enzyme, ubiquitin *C*-terminal hydrolase L1 (UCH-L1), is associated with rare cases of autosomal dominant PD (Leroy *et al.* 1998). Further, we recently showed that all three proteolytic activities of 26/20S proteasomes are impaired in the SNc of patients with sporadic PD (McNaught and Jenner 2001; McNaught *et al.* 2002). The occurrence of impaired clearance of proteins in the SNc of PD patients is supported by evidence for an elevation in the levels of protein oxidation products and the aggregation of a variety of proteins (Yoritaka *et al.* 1996; Alam *et al.* 1997; Lopiano *et al.* 2000).

Thus, alterations in the UPS are associated with the development of nigral pathology in the various etiological forms of PD that have been discovered to date. Indeed, proteasomal inhibition can lead to degeneration of PC12 cells with inclusion body formation (Rideout *et al.* 2001). However, it has not been demonstrated that defects in the UPS can lead to degeneration and the formation of proteinaceous Lewy body-like inclusions in mesencephalic dopaminergic neurons. We therefore examined the effects of inhibition of 26/20S proteasomal function and UCH activity in fetal rat ventral mesencephalic (VM) cultures.

Materials and methods

Materials

Synthetic lactacystin and ubiquitin aldehyde (UbA) were obtained from Calbiochem (Beeston, Nottingham, UK). Cell culture plastics, media and reagents were obtained from Life Technologies (Paisley, UK) and Sigma–Aldrich (Poole, UK). Monoclonal mouse antibodies to tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD) were obtained from Boehringer Mannheim (East Sussex, UK). Polyclonal rabbit antibodies to α-synuclein (raised against an internal peptide of the human protein) and monoclonal mouse antibodies to ubiquitin were obtained from Chemicon International Ltd (Harrow, UK). Biotinylated goat anti-rabbit IgG, biotinylated goat anti-mouse IgG, avidin and biotin were components of the Vectastain ABC kit (Vectastain Laboratories Inc., CA, USA). [^{3}H]dopamine (32.6 Ci/mmol) and [^{14}C]γ-aminobutyric acid (GABA, 240 mCi/mmol) were obtained from NEN (Boston, MA, USA). All other reagents/materials were of analytical/cell culture grade and obtained from Sigma–Aldrich and other commercial sources.

Enriched fetal rat ventral mesencephalic neuronal cultures

Neuronal-enriched cultures containing dopaminergic neurons were prepared from the VM of fetuses (14–15 days gestation) obtained from Sprague–Dawley rats (Charles River, Kent, UK) as described elsewhere (Pardo *et al.* 1997). Cells were suspended in Dulbecco's modified Eagle medium (DMEM; supplemented with 15% fetal bovine serum, 1 mM sodium pyruvate and 4 mM L-glutamine) and plated at a density of 10^4 cells/cm^2 on 13-mm poly D-lysine-coated plastic coverslips in 24-well culture plates. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C for 1 day, after which the culture medium was changed to defined serum-free DMEM–F12 medium (supplemented with 25 μg/mL insulin, 100 μg/mL transferrin, 60 μM putrescine, 20 nM progesterone and 30 nM sodium selenite), and cells grown for a further 6 days before use. Immunocytochemical analyses showed that these VM cultures contained approximately 2% astrocytes and > 95% neurons.

Immunocytochemistry

Coverslips containing VM neurons were washed with 0.1 M phosphate-buffered slaine (PBS; pH 7.4) then fixed in 4% paraformaldehyde for at least 1 h before being immunostained using the Vector ABC method (Pardo *et al.* 1997). Primary antibody preparations for TH, GAD, α-synuclein, and β-synuclein were used at a dilution of 1 : 200, and the ubiquitin antibody preparation was used at a 1 : 500 dilution. Control experiments in which primary antibodies were omitted confirmed the specificity of these immunoreactions (data not shown). Stained coverslips were washed with 0.1 M PBS, dehydrated through ethanol, then mounted onto slides. Slides were examined under a Zeiss Axioskop (Carl Zeiss Inc., Thornwood, NY, USA) microscope equipped with a grid-containing eyepiece for determining neuronal count (approximately 10% of coverslip area counted) and morphology at ×200 magnification as previously described (McNaught and Jenner 1999).

[^{3}H]Dopamine and [^{14}C]GABA uptake

Determination of dopamine and GABA uptake into dopaminergic and GABAergic neurons, respectively, was conducted as described previously (Casper *et al.* 1991). VM cultures were rinsed with Kreb's phosphate buffer (pH 7.4) and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/mL ascorbic acid, 10 μM GABA, 0.5 μCi/mL [^{3}H]dopamine and 0.05 μCi/mL [^{14}C]GABA. After rinsing, the radioactivity was extracted with 1 mL 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100; Packard Bioscience, Meriden, CT, USA). VM cultures treated with the neuronal dopamine uptake blocker mazindol (10 μM) and the neuronal GABA uptake blocker diaminobutyric acid (1.0 mM) were used as blanks. For uptake studies, VM cultures were grown in minimum essential medium (MEM) containing serum.

Determination of the effects of inhibition of the UPS on dopaminergic cell viability, protein accumulation and inclusion body formation

The growth medium of VM cultures in 24-well culture plates was replaced with fresh filter-sterilized DMEM-F12 containing up to 10 μM lactacystin (a selective proteasome inhibitor; Fenteany and Schreiber 1998) or up to 10 μM UbA (an inhibitor of UCH; Melandri *et al.* 1996; Schaeffer and Cohen 1996) for a maximum of 48 h. To determine the extent of neuronal death in these cultures, cell counts were conducted following immunostaining. [^{3}H]Dopamine/[^{14}C]GABA uptake into the remaining plated neurons was

also measured as an indicator of cell viability. To determine alterations in morphology, protein accumulation and inclusion body formation in lactacystin/UbA-treated VM cultures, coverslips containing neurons were immunostained as described above.

Results

Inhibition of the UPS causes toxicity to dopaminergic neurons

Treatment of VM cultures with inhibitors of the UPS for up to 48 h produced a concentration-dependent decrease in the number of dopaminergic neurons as shown by a reduction in the number of TH-positive cells. After 24 h in culture, there was a loss of approximately 7% of TH-positive cells in control cultures compared to 40% and 55% reductions following exposure to 5 and 10 μM lactacystin, respectively (Fig. 1a). Similarly, in comparison to controls, the addition of 5 and 10 μM lactacystin inhibited [^{3}H]dopamine uptake into dopaminergic neurons by 55% and 58%, respectively (Fig. 1b). In contrast, 10 μM lactacystin had no significant effect on [^{14}C]GABA uptake after 24 h of exposure (Fig. 1b), and only a 22.9% ($p < 0.01$) reduction after 48 h of exposure. Treatment of VM cultures with 5 and 10 μM UbA caused approximately 70% and 90% loss of TH-positive cells, respectively (Fig. 1a).

Exposure of VM cultures to 10 μM lactacystin or 10 μM UbA for 24 h was associated with alterations in the morphology of the remaining TH-positive neurons. Both treatments caused destruction of neuronal processes and disintegration of dopaminergic neuronal membranes with ghosting (Fig. 2). Lactacystin caused enlargement of neuronal perikaryon while cell body size remained unchanged or only slightly increased following UbA treatment (Fig. 2).

Protein accumulation in VM neurons following inhibition of the UPS

Control VM cultures were immunoreactive for α-synuclein in axons, dendrites, and terminals but perikarya were poorly stained (Fig. 3a). Exposure for 24 h to 5 μM lactacystin (Fig. 3b) or 5 μM UbA (Fig. 3c), which produced preferential alteration in dopamine cell viability (Figs 1 and 2), caused an increase in the intensity of α-synuclein staining in perikaryon and processes. Immunostaining of control VM cultures for ubiquitin revealed staining in neuronal perikarya and processes (Fig. 3d). The intensity of staining in neuronal perikaryon and processes was increased following exposure to 5 μM lactacystin for 24 h (Fig. 3e) but was reduced following treatment with 5 μM UbA (Fig. 3f).

Inhibition of the UPS resulted in the formation of proteinaceous inclusions in the cytoplasm of VM neurons

VM cultures treated with low concentrations of lactacystin (5 μM) or UbA (5 μM) for 24 h, parameters that produced

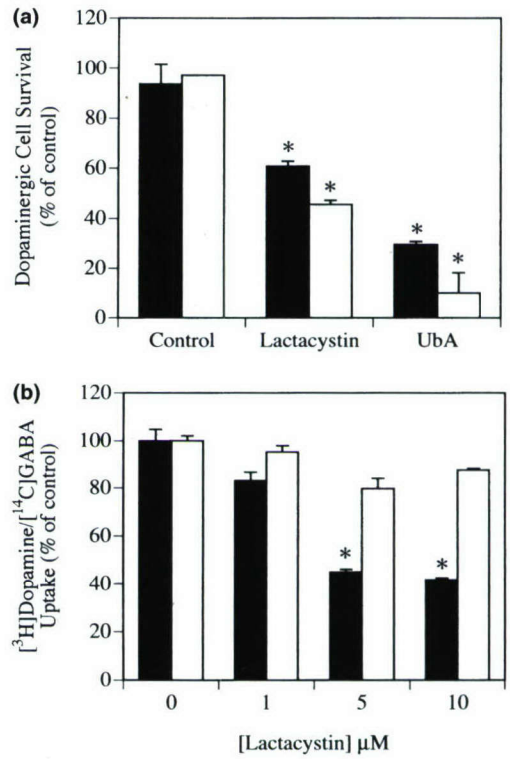

Fig. 1 Effects of inhibition of the UPS on dopaminergic and GABAergic cell viability. VM cultures in 24-well culture plates were exposed to lactacystin (5 or 10 μM) or UbA (5 or 10 μM) for 24 h. (a) Dopaminergic (TH-positive) cell survival was determined by neuronal count following immunocytochemistry. The lower and higher concentrations of lactacystin/UbA are represented by ■ and □, respectively. The control bars represent the mean number of TH-positive cells (approx. 400/cm^2) from six untreated cultures and are presented as 100%. (b) [^{3}H]Dopamine (■) and [^{14}C]GABA (□) uptake into respective neurons. Absolute control values for [^{3}H]Dopamine and [^{14}C]GABA uptake in VM cultures were 55337 ± 2600 and 20690 ± 419 DPM per culture well, respectively. Results, presented as mean ± SEM for six different culture preparations, were analyzed statistically using Student's *t*-test. *$p < 0.01$.

degeneration of dopaminergic neurons with little/no effect on [^{14}C]GABA uptake (Figs 1 and 2), caused cytoplasmic inclusions that were intensely immunoreactive for α-synuclein (Figs 4a and b) and lightly stained for ubiquitin (Fig. 4c). The α-synuclein/ubiquitin-immunoreactive inclusions varied in number and size from a single large inclusion (Fig. 4a) to several small cytoplasmic pebble-like structures (Fig. 4b) which tended to appear at an earlier time point. Inclusions were either round or oval and located in the middle of the cell body or displaced along the periphery of the cell (Figs 4a–c). The intense cytoplasmic staining for α-synuclein made it difficult to clearly delineate inclusion bodies in VM neurons following treatment with UbA although there was localized and concentrated immunoreactivity in perikarya (Fig. 4d).

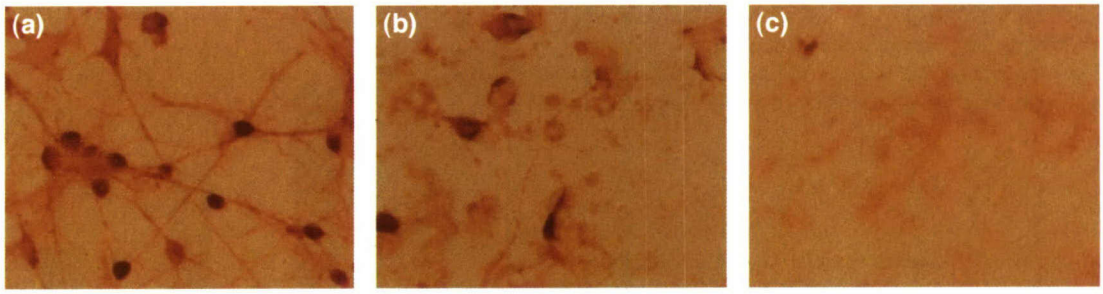

Fig. 2 Effects of inhibitors of the UPS on dopaminergic cell viability and morphology. VM cultures in 24-well culture plates were grown for 24 h in culture medium only (a), in culture medium containing 10 μM lactacystin (b) or in culture medium containing 10 μM UbA (c). Neurons were processed for TH-immunoreactivity.

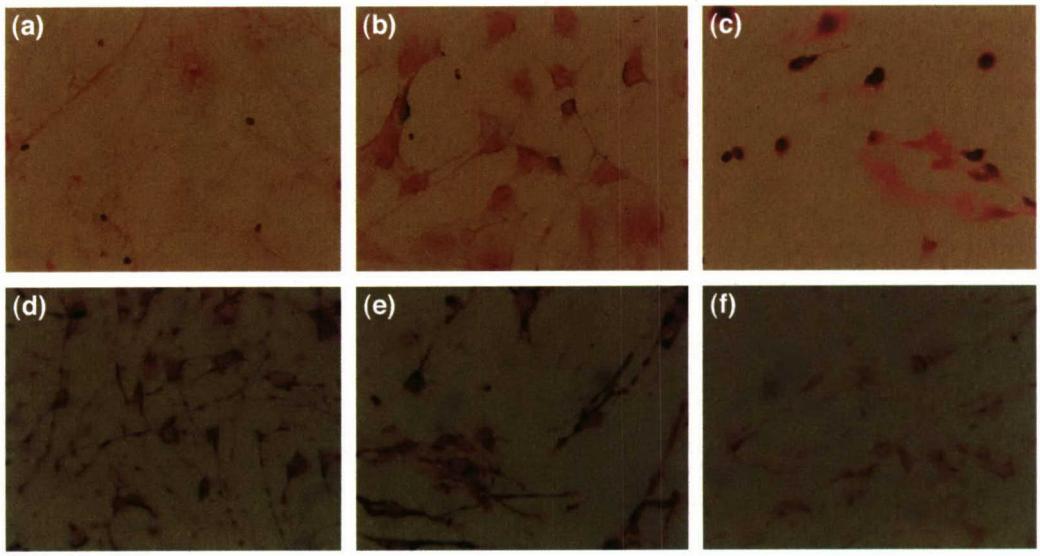

Fig. 3 Effects of inhibition of the UPS on α-synuclein and ubiquitin levels in VM neurons. VM cultures in 24-well culture plates were grown for 24 h in culture medium only (a,d), in culture medium containing 5 μM lactacystin (b,e) or in culture medium containing 5 μM UbA (c,f). These parameters were shown to produce dopaminergic toxicity with no significant effect on [^{14}C]GABA uptake (Figs 1 and 2). Neurons were processed for α-synuclein (a–c) or ubiquitin (d–f) immunoreactivity.

Discussion

We demonstrate in primary VM cultures that inhibition of normal proteasomal function with lactacystin induces a concentration-dependent degeneration of dopaminergic neurons and the formation of inclusion bodies that stain positively for α-synuclein and ubiquitin. Inhibition of UPS activity with UbA similarly induces a dose-dependent degeneration of dopamine neurons with an intense accumulation of α-synuclein in focal deposits that resemble inclusion bodies. These findings are consistent with the notion that failure of the UPS plays a role in the etiopathogenesis of PD (McNaught *et al.* 2001).

Lactacystin, an antibiotic derived from *Streptomyces*, selectively inhibits the proteolytic activities of 26/20S proteasomes (Craiu *et al.* 1997; Fenteany and Schreiber 1998; McNaught and Jenner 2001). Lactacystin-mediated impairment of 26/20S proteasomal activity has been shown to cause the accumulation of ubiquitinated proteins and to induce apoptosis in cerebellar granule cells (Canu *et al.* 2000). It has also been reported that lactacystin induces degeneration with inclusion body formation in cultured PC12 cells (Rideout *et al.* 2001). In the present study, we demonstrate that lactacystin causes dopaminergic cell death, alterations in protein handling and the formation of proteinaceous inclusion bodies in VM cultures. Interestingly, lactacystin caused swelling of axonal processes of VM neurons with increased staining for ubiquitin and α-synuclein as are found in nigral dopaminergic neurons in PD (Mezey *et al.* 1998). Taken together, these observations suggest that impairment of 26/20S proteasomal function in SNc neurons could play an important role in the neurodegenerative process occurring in patients with sporadic PD (McNaught and Jenner 2001; McNaught *et al.* 2002).

A mutation in the gene that encodes for UCH-L1, a de-ubiquitinating enzyme, has been reported in a small number of patients with familial PD, and expression of the mutant protein in *Escherichia coli* results in a 50% loss of

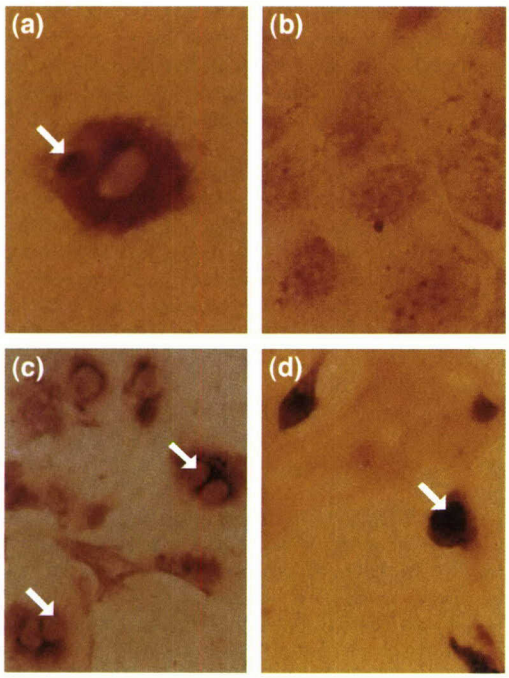

Fig. 4 Effects of inhibition of the UPS on the formation of cytoplasmic inclusions in VM cultures. VM cultures in 24-well culture plates were grown for 24 h in culture medium only or in culture medium containing 5 μM lactacystin or 5 μM UbA which, under these conditions, caused dopaminergic cell death, but had no effect on [^{14}C]GABA uptake (Figs 1 and 2). Neurons were processed for α-synuclein and ubiquitin immunoreactivity. (a) Five-micromolar (mM) lactacystin-treated cultures reveal a single α-synuclein-positive cytoplasmic inclusion body. (b) Five-micromolar lactacystin-treated cultures revealing several small α-synuclein-immunoreactive pebble-like inclusions in these cells. (c) Five-micrometer lactacystin-treated cultures revealing inclusion bodies lightly immunoreactive for ubiquitin. (d) Five-micromolar UbA-treated cultures revealing accumulation and localized immunoreactivity for α-synuclein.

enzymatic activity in comparison to wild-type controls (Leroy *et al.* 1998). UbA blocks UCH thereby reducing the availability of ubiquitin monomers for degradation of proteins via the UPS (Melandri *et al.* 1996; Schaeffer and Cohen 1996). We show for the first time that inhibition of UCH with UbA leads to a concentration-dependent degeneration of dopamine neurons in VM cultures. This was associated with focal cytoplasmic deposits which stain intensely for α-synuclein and appear to represent inclusion bodies. Thus, one may speculate that reduced availability of ubiquitin could impair activity of the ubiquitination cycle and inhibit ubiquitin-dependent protein degradation where UCH-L1 defects occur. Indeed, we found a marked reduction in ubiquitin staining in cultures exposed to UbA in this study. Post-mortem brain tissue from PD patients with the UCH-L1 mutation is not yet available for analysis and so it is unknown whether Lewy body formation occurs in this illness. However, in gracile axonal dystrophy in mice, mutations in UCH-L1 cause neuronal death and the presence of inclusion bodies that are immunoreactive for ubiquitin and other proteins (Saigoh *et al.* 1999). Thus, impairment of UCH-L1 could directly lead to protein handling dysfunction and nigral pathology in this familial form of PD.

Our study suggests that dopaminergic neurons are more susceptible than GABAergic neurons to alterations in viability following inhibition of the UPS with lactacystin, based on the significant differences in dopamine versus GABA uptake. This may relate to the highly oxidative environment within dopaminergic neurons which requires the UPS to clear a high level of damaged proteins that have the potential to be cytotoxic (Hirsch *et al.* 1997; Davies 2001; Sherman and Goldberg 2001). This may explain why nigral dopaminergic neurons preferentially degenerate in familial forms of PD despite alterations in protein expression and/or UPS function elsewhere in the brain (Shimura *et al.* 1999).

There is increasing evidence which points to interference with normal UPS function as an important etiopathogenic factor in PD. Gene defects in parkin and UCH-L1 that impair the ubiquitination of proteins necessary for 26/20S proteasomal degradation have been found in a small number of families with PD-like syndromes (Leroy *et al.* 1998; Shimura *et al.* 2001). Further, sporadic PD has been shown to be associated with impaired 26/20S proteasomal function (McNaught and Jenner 2001; McNaught *et al.* 2002). α-Synuclein, which is implicated in both sporadic and familial forms of PD, is degraded by 26/20S proteasomes (Bennett *et al.* 1999; Goedert 2001; Tofaris *et al.* 2001). Further, a glycosylated form of α-synuclein has been shown to be a substrate for parkin in SNc dopaminergic neurons in normal human subjects and its ubiquitination and proteasomal degradation are inhibited when parkin activity is impaired in AR-JP (Shimura *et al.* 2001). Thus, our findings support the concept that defects in the UPS with altered protein handling could play a significant role in the degeneration of dopaminergic neurons, protein accumulation and Lewy body formation that occur in patients with sporadic and familial PD.

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Levodopa Is Toxic to Dopamine Neurons in an in Vitro but Not an in Vivo Model of Oxidative Stress

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ABSTRACT

Levodopa is the "gold standard" for the symptomatic treatment of Parkinson's disease (PD). There is a theoretical concern, however, that levodopa might accelerate the rate of nigral degeneration, because it undergoes oxidative metabolism and is toxic to cultured dopaminergic neurons. Most in vivo studies do not show evidence of levodopa toxicity; levodopa is not toxic to normal rodents, nonhuman primates, or humans and is not toxic to dopamine neurons in dopamine-lesioned rodents or nonhuman primates in most studies. However, the potential for levodopa to be toxic in vivo has not been tested under conditions of oxidative stress such as exist in PD. To assess whether levodopa is toxic under these circumstances, we have examined the effects of levodopa on dopamine neurons in mesencephalic cultures and rat pups in which glutathione synthesis has been inhibited by L-buthionine sulfoximine. Levodopa toxicity to cultured dopaminergic neurons was enhanced by glutathione depletion and diminished by antioxidants. In contrast, treatment of neonatal rats with levodopa, administered either alone or in combination with glutathione depletion, did not cause damage to the dopamine neurons of the substantia nigra or changes in striatal levels of dopamine and its metabolites. This study provides further evidence to support the notion that although levodopa can be toxic to dopamine neurons in vitro, it is not likely to be toxic to dopamine neurons in vivo and specifically in conditions such as PD.

Levodopa (L-3,4-dihydroxyphenylalanine; L-dopa) is the most effective symptomatic treatment for Parkinson's disease (PD). However, there is an ongoing debate as to whether levodopa therapy might, independent of its symptomatic effects, promote the degeneration of dopamine neurons and thereby accelerate the rate of progression of PD (Fahn, 1996; Olanow and Stocchi, 2000). Levodopa is converted in the brain to dopamine; both levodopa and dopamine undergo oxidative metabolism and can thereby generate cytotoxic free radicals and other oxidizing species. Concerns for levodopa toxicity arose mainly from studies using embryonically derived or transformed cells in culture. Several laboratories, including our own, have shown that under certain conditions levodopa and dopamine can be toxic to a variety of neuronal cells in vitro and that dopamine neurons are especially sensitive to this toxicity (Mena et al., 1992; Mytilineou et al., 1993; Basma et al., 1995; Lai and Yu, 1997). In contrast, levodopa has not been convincingly shown to be toxic to dopamine neurons in vivo. Chronic levodopa treatment does not affect the survival of substantia nigra pars compacta (SNc) dopamine neurons in normal rodents (Hefti et al., 1981; Perry et al., 1984), nonhuman primates (Zeng et al., 2001), or humans (Quinn et al., 1986). Recent studies also indicate that levodopa is not toxic to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated rodents, and indeed may even have trophic effects (Murer et al., 1998; Datla et al., 2001). However, in these studies, oxidative defense mechanisms were preserved and there is no evidence to suggest that the SNc was in a state of oxidative stress. The situation may be different in PD, where there is evidence that the SNc is in a state of oxidative stress (Jenner and Olanow, 1996) and dopamine neurons may be vulnerable to oxidizing species.

In the SNc in PD, there is an increase in the concentration of iron, which promotes oxidative reactions, and a decrease in the concentration of reduced glutathione (GSH) (Perry et al., 1982; Sian et al., 1994), an important brain antioxidant. In addition, oxidative stress in the PD nigra could result from

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ABBREVIATIONS: PD, Parkinson's disease; SNc, substantia nigra pars compacta; GSH, reduced glutathione; MEM, minimal essential medium; TH, tyrosine hydroxylase; MAP-2, microtubule-associated protein-2; PBS, phosphate-buffered saline; BSO, L-buthionine sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PCA, perchloric acid; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance; SOD, superoxide dimutase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 6-OHDA, 6-hydroxydopamine.

overactivity of surviving dopamine neurons with increased hydrogen peroxide (H_2O_2) production (Cohen, 1990). Collectively, these changes could promote the formation of reactive oxygen species and induce oxidative damage. Indeed, there is evidence of oxidative damage to proteins, lipids, and DNA in the SNc of PD patients (Jenner and Olanow, 1996). The addition of levodopa under these conditions could add to the pro-oxidant environment in the SNc by way of the formation of reactive oxygen species formed during the autoxidation or metabolism of levodopa and/or dopamine (Graham, 1978). Furthermore, the quinone products of levodopa autooxidation can bind to and deplete GSH levels, thereby further reducing antioxidant defenses available to dopamine neurons. Indeed, increased levels of cysteinyldopa have been found in PD brains (Fornstedt et al., 1989; Spencer et al., 1998).

To determine the potential toxicity of levodopa in models in which oxidative defense mechanisms are impaired to more closely resemble the situation in PD, we examined the effect of levodopa on mesencephalic cultures and newborn rat pups with oxidative stress induced by reduced levels of GSH.

Materials and Methods

Materials. Time pregnant Sprague-Dawley rats and 2-day-old rat pup litters were purchased from Taconic Farms (Germantown, NY). MEM was purchased from Invitrogen (Carlsbad, CA), horse serum from Gemini (Calabasas, CA), and NU serum from Collaborative Research (Bedford, MA). [^{3}H]Dopamine (specific activity 32.6 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). Primary polyclonal antibodies to tyrosine hydroxylase (TH) were purchased from Protos Biotechnologies (New York, NY) and monoclonal antibodies to microtubule associated protein-2 (MAP-2) from Chemicon International (Temecula, CA). Secondary antibodies conjugated to Alexa fluorescent dyes were from Molecular Probes (Eugene, OR). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell Cultures. The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Mesencephalic cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou et al., 1999). In brief, the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24-well plates precoated with L-polyornithine (0.1 mg/ml) at a density of 200,000 cells/cm^2. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum, and 10% NU serum. Treatments began on the 3rd day in vitro, at which time the medium was changed to MEM containing only 5% horse serum. This preparation (low plating cell density and low serum content) results in cultures relatively enriched in neurons.

Treatment of Rat Pups. Treatment of rats began on the 5th postnatal day. The animals were divided into four treatment groups who were injected subcutaneously with 1) 100 mg/kg L-dopa methyl ester plus 200 mg/kg L-buthionine sulfoximine (BSO), 2) 100 mg/kg L-dopa methyl ester alone, 3) 200 mg/kg BSO alone, and 4) saline controls. Injections were administered twice daily for three or five doses and the animals were sacrificed 24 or 72 h after the last injection. A group of animals injected as described above was sacrificed 2 h after the first injection to confirm entry of levodopa in the brain.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Cell viability was determined by the MTT reduction assay, as described previously (Han et al., 1996). In brief, 50 μl of a 5-mg/ml solution of MTT was added to each cell culture well containing 0.5 ml of medium. After 1-h incubation at 37°C, the medium was carefully removed and the formazan crystals were dissolved in 1 ml of isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250; Molecular Devices, Sunnyvale, CA).

[^{3}H]Dopamine Uptake. For measurement of dopamine uptake cultures were washed with Krebs' phosphate buffer (pH 7.4) to remove any drugs remaining in the incubation medium and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/ml ascorbic acid and 0.5 μCi/ml [^{3}H]dopamine (32.6 Ci/mmol; PerkinElmer Life Sciences). After rinsing, the radioactivity was extracted with 1 ml of 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100). Cultures treated with the neuronal dopamine uptake blocker 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine hydrochloride (GBR-12909; 10 μM) were used as blanks. Blank values were less than 10% of untreated controls.

Glutathione Assay. GSH was quantified using a modification of a standard recycling assay based on the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) with glutathione reductase and NADPH (Tietze, 1969). In brief, for cell cultures the medium was carefully aspirated from the culture wells, 300 μl of 0.4 N perchloric acid (PCA) was added, and the plates were kept on ice for 30 min. The PCA was then collected and stored at −80°C until assayed. Both oxidized [glutathione disulfide (GSSG)] and reduced (GSH) forms of glutathione are measured with this assay. However, because of the small amounts of glutathione disulfide present in mesencephalic cultures (~5% of total; Mytilineou et al., 1993), the values obtained were considered to represent GSH content.

The tissue attached to the bottom of the wells after removal of the PCA was dissolved in equal volumes of 20% SDS and 0.5 N NaOH and used for protein determination according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

To determine the GSH levels in the rat pups, the animals were anesthetized with a mixture of ketamine and xylazine (1 and 0.1 mg/kg, respectively), perfused intracardially with 50 ml of ice-cold saline to remove blood; quickly decapitated; and the brain removed, separated into forebrain and hindbrain, and frozen on dry ice. The brains were homogenized in 10 volumes of 0.4 M PCA.

HPLC Analysis. The levels of levodopa, dopamine, and dopamine metabolites were assayed by HPLC with amperometric detection as described previously (Kalir and Mytilineou, 1991). In brief, brain tissue was frozen on dry ice and stored at −80°C. Before analysis the tissues were sonicated in 0.4 M PCA (dilution 1:5 or 1:10), centrifuged, and the supernatants were injected into an HPLC (model 5000; ESA, Chelmford, MA).

Immunocytochemistry. Cells were plated on polyornithine-coated glass coverslips in 24-well plates. They were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized and blocked with 0.3% Triton X-100 and 3% bovine serum albumin for 30 min. Cultures were exposed to the primary antibodies overnight at 4°C at dilutions of 1:1000 for TH and 1:400 for MAP-2. Secondary antibodies conjugated to Alexa fluorescent dyes were used at a dilution of 1:1000 for 30 min. The cultures were observed with a fluorescence microscope (Olympus, Tokyo, Japan) and the images recorded with a Spot video camera. For cell counts after incubation with the primary antibodies the cultures were processed with the peroxidase-coupled avidin-biotin ABC kit with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. The number of dopaminergic neurons in cultures was determined by counting the cells positively immunostained for TH. Forty fields (1 × 1 mm) in two transverse strips across the diameter of the dish were counted using an inverted microscope (Nikon, Melville, NY) at 20× magnification.

For immunohistochemistry of brain sections, the animals were anesthetized with a mixture of ketamine and xylazine as described above, perfused intracardially with 50 ml of saline, followed by 4% paraformaldehyde in 0.01 M PBS. The brains were immediately removed and immersed in 4% paraformaldehyde in PBS at 4°C for

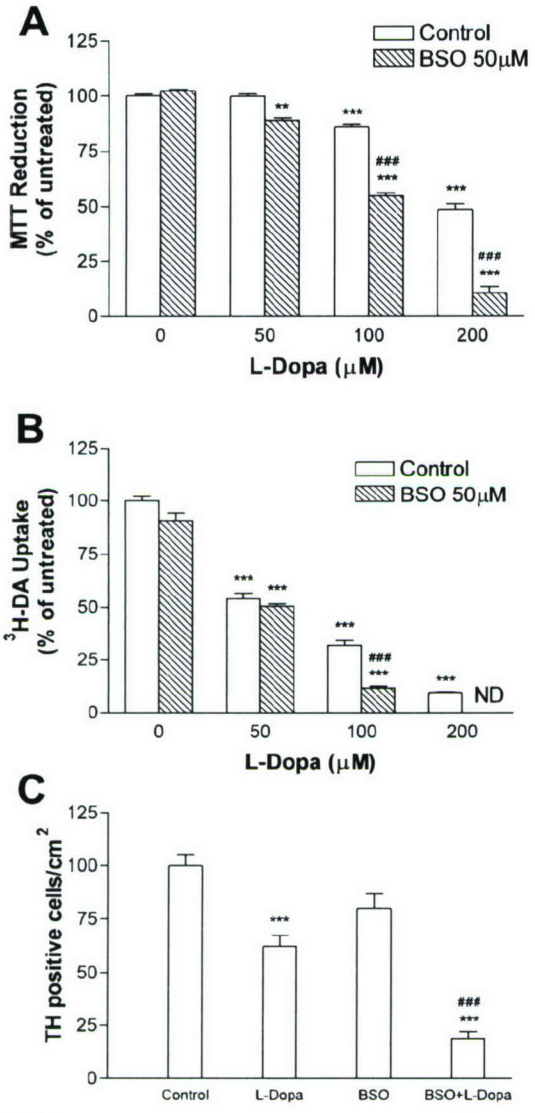

Fig. 1. GSH depletion increases levodopa toxicity in vitro. Mesencephalic cultures were treated with levodopa in the presence or absence of 50 μM BSO on the 3rd day in vitro and cell survival was determined after 72 h by the MTT assay (A), neuronal [^{3}H]dopamine uptake (B), and TH-positive cell counts (C). Levodopa concentration in C was 100 μM. ND, not detectable. Columns are means ± S.E.M. (A, $n = 15$; B, $n = 9$; and C, $n = 8$–9). ***, differs from control $p < 0.001$; **, $p < 0.01$. ###, differs from the corresponding group not treated with BSO $p < 0.001$. ANOVA followed by Tukey's test.

5 h, and then blocked, sunk in 30% sucrose in the same buffer, and cut on a cryostat into 50-μm sections.

Sections were washed three times in 0.1 M PBS for 5 min each, incubated with 5% normal goat serum in 0.01 M PBS with 0.2% Triton X-100 for 1 h, and then incubated overnight at 4°C with mouse monoclonal antibody to tyrosine hydroxylase at a concentration of 40 ng/ml, with 0.2% Triton X-100 and 2.5% normal goat serum in 0.01 M PBS. After washing three times in PBS, sections were incubated for 60 min in biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200, washed again three times in PBS, and incubated for 60 min in peroxidase-conjugated avidin (ABC) solution (Vector Laboratories). After washing, sections were developed in 3,3′-diaminobenzidine tetrahydrochloride (10 mg/10 ml of PBS) with 4.3 μl/10 ml of 30% hydrogen peroxide. Sections were mounted and dried overnight, dehydrated, defatted, and coverslipped using Permount (Fisher Scientific, Fair Lawn, NJ).

Statistical Assessment. For multiple comparisons, statistical analysis was carried out with ANOVA followed by Tukey's test. Significance between two groups was tested by independent two-tailed t test.

Results

In Vitro Studies

GSH Depletion Increases Levodopa Toxicity in Mesencephalic Cultures. The effect of levodopa (50, 100, and 200 μM) on cell viability was examined in mesencephalic cultures in the presence or absence of BSO, an inhibitor of GSH synthesis (Fig. 1). In cultures with normal GSH content (i.e., no BSO), 72 h of treatment with levodopa was associated with a dose-dependent reduction in cell viability, as determined by the MTT assay (Fig. 1A). Treatment with 50 μM BSO alone did not cause cell loss. However, when BSO was combined with levodopa, there was a dose-dependent reduction in cell survival that was greater than the cell loss caused by levodopa alone.

The sensitivity of dopamine neurons to levodopa and/or BSO treatment was examined by measuring [^{3}H]dopamine uptake (Fig. 1B) and by counting the number of cells labeled with antibodies to TH, the rate-limiting enzyme in the synthesis of dopamine (Fig. 1C). The combination of these two methods of analysis provides a close estimate of the extent of damage to dopamine fibers and dopamine neurons (Mytilineou et al., 1997). After 72 h of treatment, all concentrations of levodopa reduced [^{3}H]dopamine uptake, and this effect was enhanced by combined treatment with BSO. The effect of levodopa and/or BSO on dopaminergic neurons seems to be much greater than their effect on overall cell viability (compare Fig. 1B with Fig. 1A). Although 50 μM levodopa had no apparent effect on cell viability using the MTT assay, [^{3}H]dopamine uptake was reduced by 46% compared with control. Similarly, exposure to 100 and 200 μM levodopa reduced [^{3}H]dopamine uptake by 68 and 90% of control values, respectively, whereas cell viability was reduced by only 14 and 52% with the same concentrations of levodopa. This suggests preferential involvement of dopamine neurons.

The increased sensitivity of dopamine neurons to levodopa toxicity was confirmed by double-label immunocytochemistry with antibodies to TH and MAP-2, which labels all neurons (Fig. 2). After treatment with 100 μM levodopa, damaged TH-positive neurons can be seen in an area where other neurons labeled with MAP-2 seem to be intact (Fig. 2, C and D). BSO treatment alone (50 μM) did not affect neuronal survival (Fig. 2, E and F), whereas the combination of BSO and levodopa (100 μM) caused severe damage to neurons stained with both TH and MAP-2 (Fig. 2, G and H).

Antioxidants Protect from Levodopa Toxicity. The decrease in cell viability caused by levodopa and by the combination of BSO and levodopa was markedly decreased by the antioxidant ascorbic acid (200 μM) (Fig. 3A). The antioxidant enzymes catalase (300 U/ml) and superoxide dismutase (SOD; 300 U/ml) also provided some protection from levodopa toxicity (Fig. 3B). A combination of SOD and catalase was more effective.

Effects of Levodopa and BSO on GSH Levels. We also examined the effect of levodopa on GSH levels in mesencephalic cultures (Fig. 4). Levodopa did not alter GSH content after 24 h of treatment, but enhanced the loss of GSH that

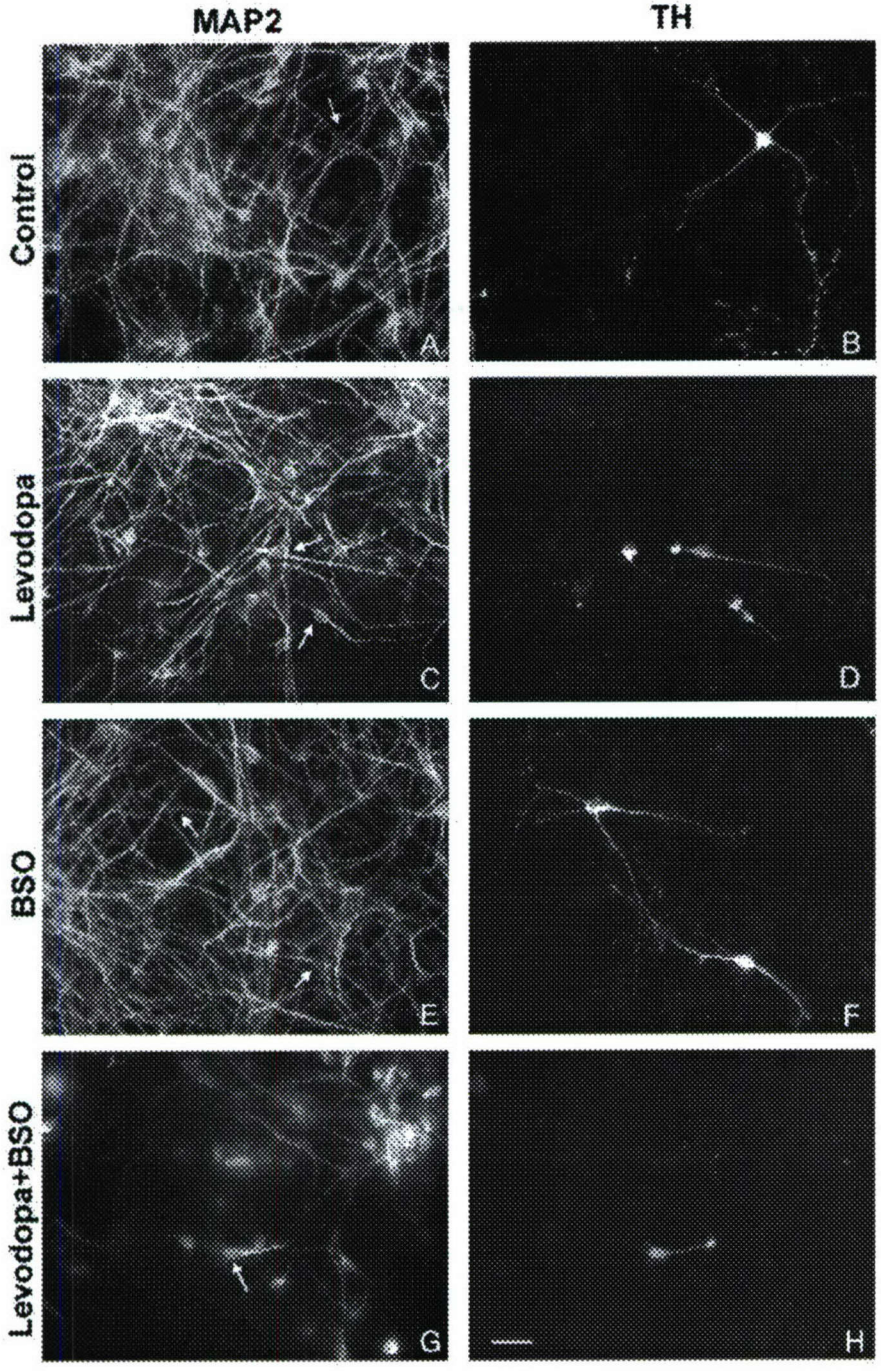

Fig. 2. Dopamine neurons are sensitive to levodopa toxicity in vitro. Photomicrographs from mesencephalic cultures double labeled with antibodies to MAP-2 (A, C, E, and G) and TH (B, D, F, and H). The same area is photographed with the two different antibodies. A and B, control culture. C and D, culture treated with 100 μM levodopa for 72 h. E and F, culture treated with 50 μM BSO for 72 h. G and H, combination of BSO and levodopa treatment. The arrows in the MAP-2-labeled photomicrographs indicate the TH-positive neurons shown in the right-hand panel. Scale bar, 25 μm.

occurred in the presence of BSO in a dose-dependent manner (Fig. 4A). After 72 h of exposure to 100 μM levodopa, there was a significant increase in GSH when levodopa was administered alone or in combination with 1 μM BSO (Fig. 4B). In contrast, GSH levels were significantly reduced when 100 μM levodopa was combined with 10 or 50 μM BSO. A higher concentration of levodopa (200 μM) had no effect on GSH levels in control cultures, and enhanced the depleting effect of BSO at all concentrations (Fig. 4B).

To determine whether the protection from levodopa toxicity by ascorbic acid was related to an effect on cellular GSH content, we examined levels of GSH in cultures treated with BSO and levodopa with and without ascorbic acid (Table 1). In agreement with the data shown in Fig. 4B, treatment of control cultures with 50 or 100 μM levodopa for 72 h resulted in significant increases in GSH content. Ascorbic acid prevented the up-regulation of GSH caused by levodopa but not the depletion induced by BSO (50 μM).

In Vivo Studies

In Vivo Effect of Levodopa and BSO on GSH Levels. The levels of GSH were measured in the brains of rat pups after treatment with levodopa alone or in combination with BSO. Neonatal rats were used because BSO does not effec-

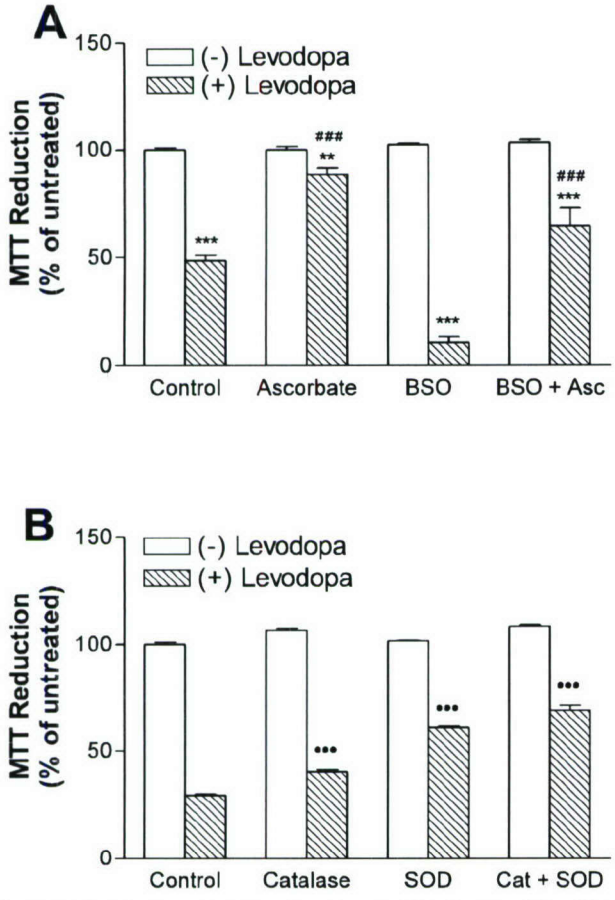

Fig. 3. Antioxidants protect from levodopa toxicity in vitro. The effect of ascorbic acid (200 μM; A) and catalase (300 units/ml) and SOD (300 units/ml; B) was studied in mesencephalic cultures treated on the 3rd day in vitro with 200 μM levodopa in the presence or absence of 50 μM BSO. Cell viability was determined with the MTT assay after 72 h. Columns are means ± S.E.M. n = 15/group for A and 8/group for B. ***, differs from the corresponding group not treated with levodopa $p < 0.001$; **, $p < 0.01$; ###, differs from the group not treated with ascorbate $p < 0.001$; •••, differs from the group treated with levodopa alone $p < 0.001$. ANOVA followed by Tukey's test.

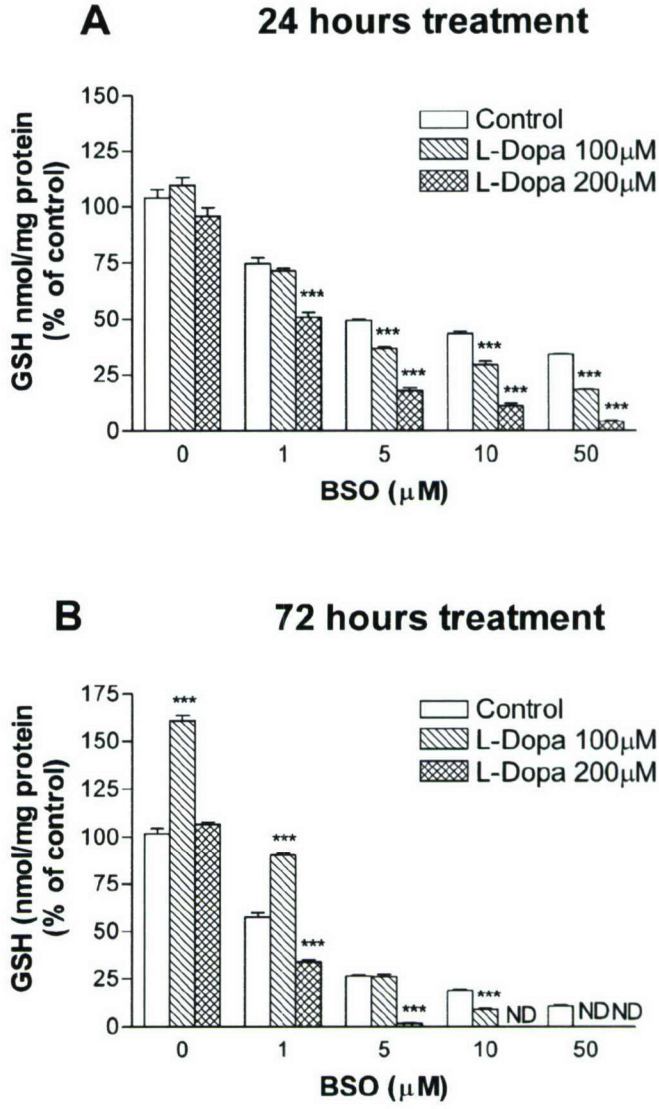

Fig. 4. Effect of levodopa on GSH levels in mesencephalic cultures. Cultures were treated with 100 or 200 μM levodopa in the presence of increasing concentrations of BSO. GSH was measured 24 or 72 h after treatment. Bars are means ± S.E.M. (n = 4/group). ***, $p < 0.001$ compared with the corresponding group not treated with levodopa. ANOVA followed by Tukey's test.

tively cross the blood-brain barrier in the adult rat and results in only minor GSH depletion (Slivka et al., 1988). A course of treatment with either three or five injections of BSO reduced GSH levels to 30 and 25% of control values, respectively, measured 24 h after the last injection (Table 2). Similar reductions were achieved in both the forebrain and hindbrain. This depletion, however, was not sustained and levels of GSH returned to almost 80% of control values after approximately 3 days. Injections of levodopa alone (100 mg/kg) did not cause significant changes in GSH levels and did not modify the depletion of GSH caused by BSO (Table 2). To ascertain that significant amounts of levodopa enter the brain and that there is no interference in levodopa entry by BSO, we injected 5-day-old rat pups with 100 mg/kg levodopa and/or 200 mg/kg BSO and assayed for levodopa and dopamine by HPLC 2 h after the injection. Levodopa could not be detected in the brain of control or BSO-treated animals, but was present in significant amounts after levodopa injection (Table 3). Injection of BSO did not interfere with the entry of levodopa in the brain. The levels of dopamine were more that 10 times higher after levodopa injection, indicating active metabolism of levodopa during this time. DOPAC and HVA levels were increased between 50 and 100 times during the 2 h after levodopa injection (Table 3).

Levodopa Does Not Damage Dopamine Neurons in Vivo. The survival of dopamine neurons after in vivo treatment with BSO and levodopa was examined by TH immunocytochemistry of midbrain sections from rat pups. No apparent damage to dopamine neurons could be observed 3 days after five injections of levodopa, even when GSH content was substantially reduced by coadministration of BSO (Fig. 5). To further assess the possibility of damage to substantia nigra dopamine neurons after levodopa and/or BSO injections, we determined the levels of dopamine and dopamine metabolites in the midbrain and striatum, 3 days after five injections. Table 4 shows that striatal and mesencephalic levels of dopamine and metabolites were not different when levodopa was administered to control or BSO-treated animals.

TABLE 1

Effect of levodopa and ascorbic acid treatment on the levels of GSH in mesencephalic cultures

Mesencephalic cultures were treated for 72 h with 50 μM BSO in the presence of 50, 100, or 200 μM levodopa, beginning on the 3rd day in vitro. Ascorbate (200 μM) was added at the same time with BSO. The values represent means ± S.E.M. (n = 9–12 from two independent experiments). The numbers in parentheses are the values expressed as percentage of untreated controls.

Levodopa	GSH			
	Control	BSO	Ascorbate	Ascorbate + BSO
μM	*nmol/mg protein*			
0	24.0 ± 1.0 (100)	3.0 ± 0.3 (12)	25.7 ± 0.5 (107)	4.4 ± 0.2 (18)
50	31.3 ± 0.9 (131)***	2.2 ± 0.1 (9)	26.5 ± 0.5 (111)	3.3 ± 0.1 (14)
100	37.7 ± 1.0 (157)***	0.0 ± 0.0 (0)	26.0 ± 1.4 (109)	1.6 ± 0.1 (7)
200	25.6 ± 0.7 (107)	0.0 ± 0.0 (0)	21.6 ± 1.6 (90)	0.0 ± 0.0 (0)

*** $p < 0.001$ compared with control cultures not treated with levodopa. All BSO-treated groups were different from corresponding controls $p < 0.001$; ANOVA followed by Tukey's post test.

TABLE 2

Effect of levodopa and BSO injections on the GSH content of the neonatal rat brain

Levodopa methyl ester (100 mg/kg) and BSO (200 mg/kg) were dissolved in saline and injected s.c. to rats beginning on the 5th postnatal day, in a volume of 10 μl/10 g body weight. The animals received one injection the 1st day of treatment and then two injections daily. The data are means ± S.E.M. from five to six animals per group from two separate experiments, except for the group assayed 72 h later (2–3 animals/group).

Treatment	GSH	
	Forebrain	Hindbrain
	% of untreated	
3 injections, assay 24 h later		
Saline	100.0 ± 3.8	100.0 ± 1.2
BSO (200 mg/kg)	29.3 ± 0.7***	26.9 ± 1.2***
Levodopa (100 mg/kg)	104.2 ± 3.1	101.0 ± 3.0
BSO + levodopa	29.4 ± 0.9***	24.3 ± 1.3***
5 injections, assay 24 h later		
Saline	100.0 ± 3.8	100.0 ± 3.9
BSO (200 mg/kg)	22.9 ± 1.7***	17.7 ± 1.6***
Levodopa (100 mg/kg)	102.5 ± 2.6	104.5 ± 2.8
BSO + levodopa	23.6 ± 1.7***	17.4 ± 2.7***
5 injections, assay 72 h later		
Saline	100.0 ± 3.0	100.0 ± 0.5
BSO (200 mg/kg)	80.5 ± 0.1*	74.9 ± 0.1
Levodopa (100 mg/kg)	95.3 ± 4.4	100.1 ± 11.5
BSO + levodopa	80.1 ± 0.9*	76.6 ± 3.1

* $p < 0.05$; *** $p < 0.001$ compared with the corresponding controls. ANOVA followed by Tukey's post test. The GSH values in control animals varied among different groups from 0.82 to 0.93 μmol/g wet weight in the forebrain and from 1.2 to 1.6 μmol/g wet weight in the hindbrain.

Discussion

In this study, we examined the in vitro and in vivo effects of levodopa on mesencephalic neurons exposed to oxidative stress through the depletion of GSH. GSH is a major soluble cellular antioxidant that has been shown to reduce levodopa toxicity in vitro (Lai and Yu, 1997), whereas its depletion causes cell death in mesencephalic cultures (Mytilineou et al., 1999). The in vitro toxicity of levodopa is well documented (Mena et al., 1992; Mytilineou et al., 1993; Basma et al., 1995; Melamed et al., 1998). Our data confirm these reports and further demonstrate that dopamine neurons in vitro become especially sensitive to levodopa when GSH levels are depleted. Levodopa in the presence of the GSH synthesis inhibitor BSO causes further depletion of GSH, which could contribute to the increased toxicity. The toxicity of levodopa, even under conditions of low levels of GSH, was significantly reduced by the antioxidant ascorbic acid at concentrations reported to be normally present in the extracellular space (Spector, 1989). Protection from in vitro levodopa toxicity by ascorbic acid has been reported previously (Mytilineou et al., 1993). The protection by ascorbic acid in our study was not due to sparing of cellular GSH, as was the case after in vivo administration of BSO (Martensson et al., 1991), indicating that ascorbic acid can serve as an essential antioxidant even under conditions of severe GSH depletion (Martensson et al., 1991). Levodopa toxicity was also diminished by the antioxidant enzymes catalase and SOD, which provides further support for the involvement of reactive oxygen species in levodopa toxicity.

A dual effect, both an increase and a decrease in the content of GSH, resulted from in vitro treatment with levodopa, with high concentrations further depleting GSH when administered in combination with BSO. Lower levodopa concentrations caused a delayed up-regulation of GSH content when administered alone or with low concentrations of BSO. In our previous studies, we have shown that mild oxidative stress caused by low-concentration levodopa treatment increases GSH content in mesencephalic cultures (Mytilineou et al., 1993; Han et al., 1996), possibly because of an up-regulation of defense mechanisms after sublethal injury. In support of this interpretation, the up-regulation of GSH was prevented by the antioxidant ascorbic acid (Han et al., 1996). Thus, levodopa can have opposing effects on GSH levels, causing up-regulation of GSH synthesis as a result of mild oxidative damage and reductions in GSH at higher concentrations. This latter effect is possibly related to both GSH consumption during oxidative stress and binding of levodopa-derived quinones to remaining GSH molecules. The net effect of levodopa on GSH content thus depends on the levodopa concentration.

The results of the in vivo administration of levodopa to experimental animals have been conflicting. A potential for levodopa to induce in vivo toxicity is suggested in a study showing that levodopa treatment to rodents that had survived exposure to 6-hydroxydopamine (6-OHDA) caused further damage to dopamine neurons in the ventral tegmental area (Blunt et al., 1993). However, these results are not supported by more recent studies, where levodopa treatment after partial 6-OHDA lesions actually enhanced neuronal survival (Murer et al., 1998; Datla et al., 2001). Levodopa has also been reported to cause damage to embryonic dopamine neurons transplanted into the striatum of 6-OHDA-lesioned rats (Steece-Collier et al., 1990), although another study did not confirm these findings (Blunt et al., 1992).

These studies have provided the clinical community with some level of comfort that levodopa is not toxic to dopamine neurons in vivo, but no study has yet examined the effects of levodopa under conditions of increased oxidative stress, such

TABLE 3

Levels of L-dopa, dopamine, and dopamine metabolites in 5-day-old rat pups injected with levodopa and BSO

Rats were injected subcutaneously on postnatal day 5 with 100 mg/kg levodopa and/or 200 mg/kg BSO in a volume of 10 μl/10 g body weight. The control group was injected with the same volume of saline. Brains were dissected and frozen on dry ice 2 h after injection, and PCA extracts were assayed for catecholamines with HPLC. The values, expressed as nanograms per gram wet weight, represent means ± S.E.M. (n = 6 animal/group).

Treatment group	L-dopa	Dopamine	DOPAC	HVA
Control	0	148 ± 7	144 ± 7	161 ± 8
BSO (200 mg/kg)	0	152 ± 14	135 ± 11	184 ± 8
Levodopa (100 mg/kg)	90 ± 15***	1,588 ± 106***	12,228 ± 272***	7,365 ± 232***
BSO + levodopa	129 ± 11***	1,902 ± 159***	13,198 ± 278***	6,979 ± 129***

*** Differs from corresponding control group $p < 0.001$; ANOVA followed by Tukey's post test.

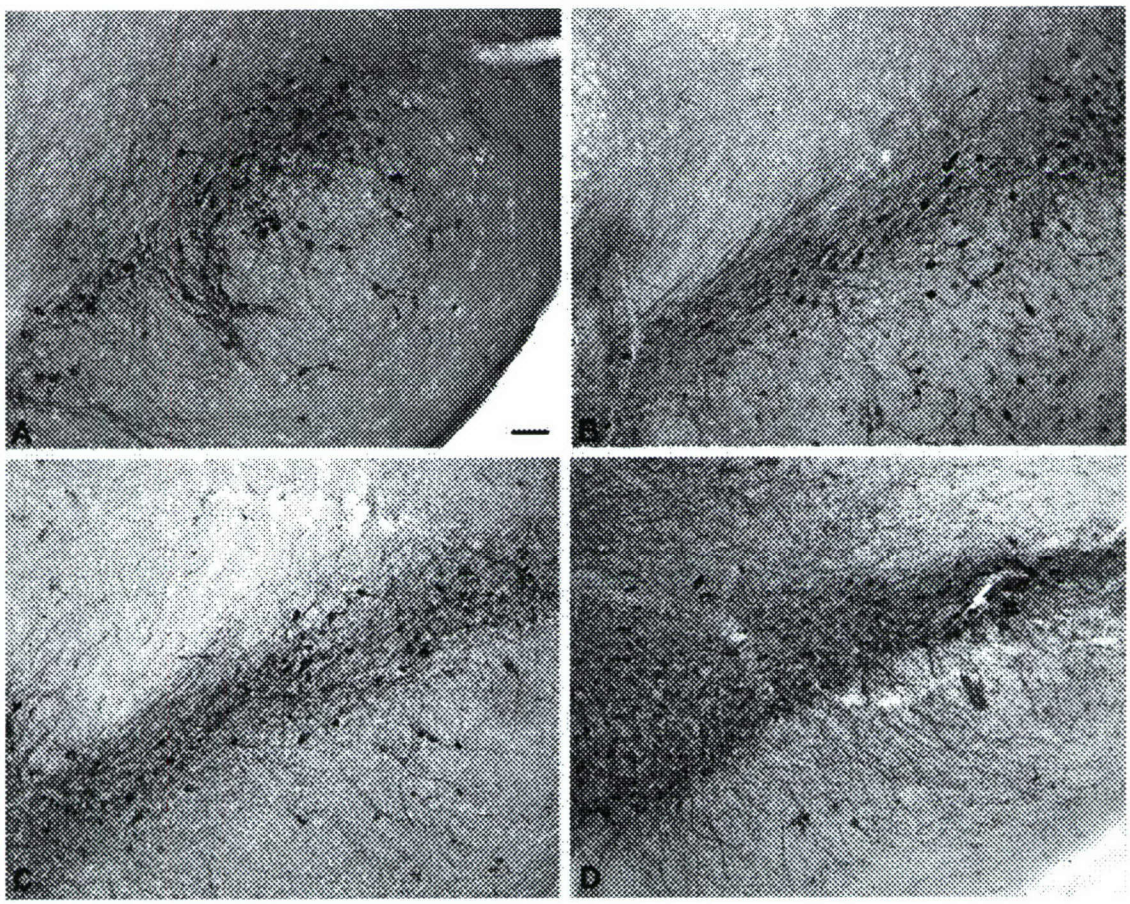

Fig. 5. In vivo levodopa does not damage substantia nigra neurons. Midbrain sections from rat pups showing TH-positive dopamine neurons in the substantia nigra. Treatment with levodopa and/or BSO caused no apparent loss of TH-positive neurons. A, control. B, levodopa (100 mg/kg, five injections). C, BSO (200 mg/kg, five injections). D, levodopa and BSO. Scale bar, 100 μm.

as occur in PD. In our study, oxidative stress due to GSH depletion synergistically enhanced cell death induced by levodopa in cell cultures. However, no change in TH staining and neuronal morphology was observed in the substantia nigra of rodent pups when levodopa was administered alone or in combination with BSO, at levels resulting in an extensive depletion of brain levels of GSH (>75% loss). In contrast to the in vitro results, levodopa had no effect on the GSH content of the brain of rat pups when administered either alone or in combination with BSO. In addition levodopa did not change the levels of dopamine and its metabolites in the rodent midbrain or striatum even when GSH levels were reduced by BSO.

Several technical issues, such as inability of BSO to penetrate the mature blood-brain barrier and its lethality to young animals (Martensson et al., 1991) forced us to adopt a model that has some notable limitations. It is possible that more prolonged exposure to levodopa would eventually cause damage to the nigrostriatal system in GSH-depleted animals and furthermore that different results might been attained in the adult brain. Establishing a model for intracranial infusion of BSO may allow for this issue to be examined in adult rats. However, neonatal animals do provide a good control for cultured dopamine neurons, in which the toxic effects of levodopa of concern to clinicians have been described, and illustrate that in vitro toxicity is not necessarily seen in the in vivo model.

There are several possible explanations for the different effects of levodopa in in vitro and in vivo models. Ascorbate levels are very low in mesencephalic cultures (Kalir and Mytilineou, 1991), and ascorbate protects against levodopa toxicity. In contrast, concentrations of ascorbic acid are high

TABLE 4

Dopamine and dopamine metabolite levels in the striatum and ventral midbrain of rat pups, 3 days after injection of levodopa and BSO

Rats received five subcutaneous injections of 100 mg/kg levodopa, 200 mg/kg BSO, or a combination of the drugs in a volume of 10 μl/10 g body weight, twice daily, beginning on postnatal day 5. The control group was injected with the same volume of saline. Seventy-two hours after the last injection (postnatal day 11) the striatum and ventral midbrain were dissected and frozen on dry ice. PCA extracts were assayed for catecholamines with HPLC. The values are means ± S.E.M. from 10 to 12 animals per group from two independent experiments expressed as percentage of controls. Control values in the two experiments varied for dopamine from 2040 to 5260 ng/g wet weight (striatum) and 400 to 1480 ng/g wet weight (midbrain), for DOPAC from 910 to 1010 ng/g wet weight (striatum) and 210 to 300 ng/g wet weight (midbrain), and for HVA from 410 to 800 ng/g wet weight (striatum) and 210 to 410 ng/g wet weight (midbrain). Analysis of variance showed no significant differences in the values of dopamine, DOPAC, or HVA among the different groups.

Treatment	Striatum			Ventral Midbrain		
	Dopamine	DOPAC	HVA	Dopamine	DOPAC	HVA
Control	100.0 ± 4.3	100.0 ± 5.6	100.0 ± 3.1	100.0 ± 4.8	100.0 ± 11.4	100.0 ± 3.8
BSO	105.8 ± 5.6	103.4 ± 7.5	102.9 ± 2.8	82.7 ± 6.4	120.5 ± 14.1	112.7 ± 4.0
Levodopa	87.5 ± 7.2	95.7 ± 5.3	93.7 ± 4.7	114.5 ± 15.0	103.6 ± 8.8	109.8 ± 6.7
BSO + levodopa	88.3 ± 7.8	109.0 ± 9.5	106.5 ± 7.4	82.9 ± 12.8	108.7 ± 9.3	110.7 ± 5.3

in the brain (Milby et al., 1982) and especially in the neonatal rat (Kratzing et al., 1985). Other antioxidant defense mechanisms may also contribute to the ability of in vivo neurons to withstand oxidative insults. Glial cells, which lie in close association with the neurons in the brain, could serve as a buffer against toxic substances. Glial cells also secrete growth factors and other trophic substances that can protect neurons from damage (Park and Mytilineou, 1992; O'Malley et al., 1994; Sullivan et al., 1998). Indeed, glial-secreted factors have been shown to protect from levodopa toxicity in vitro (Mena et al., 1997).

There is also evidence arguing against levodopa toxicity in humans. For example, we have observed robust survival of implanted dopamine neurons in levodopa-treated PD patients who had undergone fetal nigral transplantation (Kordower et al., 1998) and long-term treatment with high-dose levodopa did not cause apparent damage to SNc neurons in a patient with atherosclerotic parkinsonism (Quinn et al., 1986). Furthermore, clinical studies suggest that levodopa does not enhance the rate of disease progression or mortality rate of PD patients (Diamond and Markham, 1990; Uitti et al., 1993). Our in vitro study indicates that a decreased antioxidant capacity makes neurons more vulnerable to levodopa and suggests that the potential for toxicity exists, especially in the limited environment of cell culture. However, the finding that levodopa is not toxic to dopamine neurons in an in vivo model, in which oxidative stress had been induced by GSH depletion, is reassuring and provides additional support for the notion that levodopa is not likely to be toxic to dopamine neurons in PD.

Acknowledgments

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Alterations in the cellular distribution of bcl-2, bcl-x and bax in the adult rat substantia nigra following striatal 6-hydroxydopamine lesions

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Abstract

The proteins of the bcl-2 family play an important role during apoptosis and may also regulate cell death in response to oxidative stress, which has been implicated in Parkinson's disease. In this study we examined the localization of the pro-apoptotic protein bax, and the anti-apoptotic proteins bcl-2 and bcl-x_L in the substantia nigra (SN) of the adult rat and their response to oxidative stress caused by striatal injections of 6-hydroxydopamine (6-OHDA). Our data show that bcl-2, bcl-x and bax proteins are present in the SN. Bcl-2 and bax are localized primarily in neurons including all those positive for tyrosine hydroxylase (TH). The intraneuronal distribution of bcl-2 and bax were different. Bcl-2 was diffuse throughout the cell while bax was localized in well-defined structures around the nucleus and within processes. Bcl-x staining in neurons was weak, though it was strongly expressed in GFAP-positive astrocytes. 6-OHDA injections, which resulted in loss of dopamine neurons between 7–14 days post-lesion, altered the distribution of bax, bcl-2 and bcl-x proteins in the SN. Bcl-2 and bax were decreased in the TH-positive cells of the SN from 3 to 14 days post-lesion and many TH-positive neurons were bcl-2 negative. Neuronal bcl-x was initially unchanged after lesion, but increased in astrocytes between 3–7 days post-lesion before the increase in GFAP immunoreactivity, which was detectable at days 10–14. While the neuronal distribution of bcl-2 and bcl-x does not change following lesion, bax became evenly distributed thought the soma. Morphological features of apoptosis, including TUNEL labeling and chromatin condensation was not observed. These data suggest that striatal 6-OHDA lesions do not result in classical apoptosis in the SN of the adult rat, even though there are changes in the content and distribution of members of the bcl-2 family of proteins.

Introduction

Parkinson's disease (PD) is a neurological disorder, which results from the degeneration of dopamine neurons in the substantia nigra pars compacta (SNpc). While its etiology remains unknown, studies indicate that oxidative stress may be involved. Evidence of decreased mitochondrial complex I activity (Schapira *et al.*, 1992) and depletion of glutathione (GSH) (Sofic *et al.*, 1992; Sian *et al.*, 1994) in post-mortem brains from PD patients, both of which could lead to oxidative stress, supports this notion. Further support is provided by the presence of lipid peroxidation in the SNpc, a marker of oxidative stress (Dexter *et al.*, 1994, 1989).

Apoptosis, a genetically controlled process morphologically characterized by nuclear and cytoplasmic condensation, chromatin fragmentation and membrane blebbing (Kerr *et al.*, 1972), may also be involved in the degeneration of dopamine neurons during PD (Tompkins *et al.*, 1997; Anglade *et al.*, 1997; Kosel *et al.*, 1997; Jellinger, 2000; Banati *et al.*, 1998). The bcl-2 family plays a crucial role in mediating apoptosis in the brain (Merry & Korsmeyer, 1997; Gross *et al.*, 1999; Sastry & Rao, 2000). The presence of mRNA for bcl-2, bcl-x and bax in the adult central nervous system (Abe-Dohmae *et al.*, 1993; Castren *et al.*, 1994; Shimohama *et al.*, 1998; Gonzalez-Garcia *et al.*, 1995; Merry *et al.*, 1994), suggests that these genes and their protein products continue to play a role in neuronal survival beyond the developmental periods. Indeed, there is evidence that the bcl-2 family of proteins may be involved in the dopaminergic degeneration associated with PD (Tatton, 2000; Mogi *et al.*, 1996). These same proteins have also been shown toplay a role in mediating cell death in response to

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oxidative stress. For example, over-expression of bcl-2 in PC-12 cells decreases signs of lipid peroxidation following hydrogen peroxide treatment (Bruce-Keller *et al.*, 1998), whereas mice deficient in bcl-2 exhibit increased indices of oxidative stress (Hochman *et al.*, 1998).

6-Hydroxydopamine (6-OHDA), a neurotoxin specific to catecholamine neurons, is widely used to model the dopaminergic degeneration characteristic of PD (Sauer & Oertel, 1994; Jeon *et al.*, 1995; Ichitani *et al.*, 1991; Kramer *et al.*, 1999). Striatal 6-OHDA lesions result in a delayed degeneration of dopaminergic neurons in the SNpc. The majority of this degeneration occurs between seven and fourteen days, however dopaminergic death can still be observed for up to sixteen-weeks (Sauer & Oertel, 1994).

The delayed time course of nigral degeneration following striatal 6-OHDA lesions is consistent with the slow progression of apoptosis and provides an attractive model to explore the possible links between oxidative stress, apoptosis, and dopaminergic degeneration. In this study we have investigated the distribution of bcl-2, bcl-x and bax within the neurons and glia of the SN of naïve rats and then examined whether these proteins are altered following 6-OHDA lesions.

Materials and methods

ANIMALS AND SURGICAL PROCEDURES

Adult female Sprague-Dawley rats weighing 200–250 g were obtained from Taconic Farms (Germantown, NY). Animals were housed in a temperature-controlled vivarium (25°C) with a 12-hour light/dark cycle. Food and water were available *ad libitum*. All procedures, which related to the care, treatment, and euthanasia of experimental animals were approved by the Institutional Animal Care and Use Committees and conformed to the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Using Ketamine (100 μg/100 g-body weight) and Xylazine (10 ug/100 g-body weight) anesthesia, two stereotaxic injections of 6-OHDA were made into the right striatum using a 5 μl Hamilton microsyringe with a 24 gauge steel cannula. Two microliters of a 5 μg/μl solution of 6-OHDA (Sigma, St. Louis, MO) in 0.2 μg/μl L-ascorbate-saline (Sigma, St. Louis, MO) was injected at 0.5 μl/min into each site using the following coordinates with reference to bregma and dura: (1) anterior-posterior, 0.5; lateral, −2.5; and ventral, −5.0 and (2) anterior-posterior, −0.5; lateral, −3.9; and ventral, −5.0. Tooth-bar was set to 0 for each injection. Control animals received an injection of vehicle alone. Animals were sacrificed 1, 3, 7, 10, and 14 days after injections.

SAMPLE PREPARATION FOR RNA AND PROTEIN

Untreated, naïve, animals utilized for PCR and Western blots were deeply anesthetized with chloral hydrate and perfused transcardially with ice-cold PBS to remove contaminating blood. The brains were rapidly removed and a 3 mm thick block containing the midbrain was made using a rodent brain matrix (ASI Instruments, Inc. Warren, MI). The block was cut horizontally just below the aqueduct and the ventral lateral area (containing the cerebral peduncle, SN pars compacta and reticular) was carefully dissected and frozen at −80°C until processed into RNA or protein.

PCR

2.5 μg of RNA, extracted with TRIzol® Reagent, was reverse-transcribed to cDNA with Superscript™ (Gibco, Grand Island, NY) following manufacturer's protocol. PCR was performed using 2.5 units of Platinum® Pfx DNA polymerase and supplied buffer (Gibco, Grand Island, NY). Primers for the amplification of bcl-2 (product size, 284 bp; 5′atggcgcaagccgggagaacagggt3′/5′tcaggtgcagctgactggacatcta3′) bcl-$x_{L/S}$ (product size 397/210; 5′gcattcagtgatctaacatcccagc3′/5′tctggtcacttccgactgaagagtg3′) and bax (product size 402; 5′agaggatggctggggagacacctga3′/5′atgtgggcgtcccgaagtaggaaag3′) were used as appropriate. Samples were cycled 33 times (30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C) in a DNA thermal cycler PTC-100™ (MJ Research, Watertown, MA). Amplified products were resolved by electrophoresis.

WESTERN BLOTS

Whole cell lysate was prepared from untreated, control ventral midbrain as described above in ice-cold RIPA buffer (10 mM Tris-HCL, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, pH 7.4) that contained Complete™ protease inhibitor cocktail (Roche, Indianapolis, IN). Sonicated homogenates were centrifuged at 20,000g for 10 minutes at 4°C. Protein concentrations were determined using the Lowry assay (Lowry *et al.*, 1951) with BSA as a standard. 25 μg of protein was resolved on a 12% SDS-PAGE gel (Bio–RAD, Hercules, CA) and transferred to Hybond™-P PVDF transfer membrane (Amersham Pharmacia, Piscataway, NJ) for 1 hour at 15V using a Trans-Blot Semi-Dry Transfer Cell (Bio–RAD, Hercules, CA). Membranes were incubated with polyclonal antibodies to bax (P-19), bcl-2 (N-19), bcl-$x_{L/S}$ (S-18) (10 μg/ml; Santa Cruz, Santa Cruz, CA) in blocking solution (5% milk, 5% newborn-calf serum in TTBS) overnight at 4°C. Membranes were washed with TTBS and incubated with horseradish-peroxidase conjugated goat anti-rabbit IgG (whole molecule) antibody (ICN, Aurora, OH) in blocking solution for 1 hour at room temperature. The membranes were visualized with ECL Plus Western blot detection system (Amersham Pharmacia, Piscataway, NJ).

Immunocytochemical procedures

TISSUE PREPARATION

Animals were deeply anesthetized with chloral hydrate and perfused transcardially with ice-cold PBS (0.1 M; pH 7.4) for 1 minute, followed by 4% paraformaldehyde in PBS for 10 minutes. The brains were immediately removed and a 3 mm thick block containing the midbrain was made using a rodent brain matrix (ASI Instruments, Inc. Warren, MI) and post-fixed in 4% paraformaldehyde for 6 hours. Blocks were embedded in paraffin following standard procedures

(Sheehaw & Hpapchack, 1980). Sections were cut in the coronal plane at 5 μm, mounted onto gelatin-coated slides and stored at 4°C until used. In order to compare antibody label among the different animals used in this study ($n = 4-5$ per time point), every 100th section from each animal was stained for tyrosine hydroxylase (see below), counterstained with Nissl and numbered according to the diagrams from a rat brain atlas (Paxinos & Watson, 1998). Matched sections at different levels of the substantia nigra (every 100th section) were used for each antibody.

IMMUNOCYTOCHEMISTRY

Sections deparaffinized in Citro-Solve (Fisher Scientific, Agawam, MA) were rinsed with two changes of 100% ethanol followed by 3% hydrogen peroxide in methanol and washed in dH_2O for 15 minutes. Antigen retrieval reagent (Vector Laboratories, Burlingame, CA) was used following manufacturer's protocol. Briefly, mounted sections were heated in a microwave for 1 minute at high-power and 9 minutes at 70% power in retrieval reagent. They were allowed to stay in retrieval reagent for 20 minutes following microwave treatment and then washed in PBS (0.01 M; pH 7.4) for 15 minutes. Double labeled immunocytochemistry was used to examine the presence of bax, bcl-2 and bcl-x in the ventral midbrain. Slides were incubated overnight at 4°C with polyclonal antibodies to Bax (P-19), Bcl-2 (N-19), or Bcl-$x_{L/S}$ (S-18) (4 μg/ml; Santa Cruz, Santa Cruz, CA). Dopamine neurons were identified with monoclonal antibodies to tyrosine hydroxylase (TH; 40 ng/ml; Chemicon, Temeculat, CA) and astrocytes with monoclonal antibodies to glial fibrillary acidic protein (GFAP; 400 ng/ml G-A-5, Sigma, St. Louis, MO). For studies comparing the intracellular localization of bax and bcl-x or bax and bcl-2, a monoclonal antibody to Bax (4 μg/ml; Santa Cruz, Santa Cruz, CA) was used together with bcl-x and bcl-2 polyclonal antibodies. After incubation with primary antibodies, slides were washed for 30 minutes in PBS and incubated with Alexa-fluor 488-conjugated anti-rabbit IgG (H+L) and Alexa-fluor 594-conjugated anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR) for 1 hour at room temperature. All primary and secondary antibody incubations were done in blocking solution containing 3% goat serum and 0.3% triton X-100.

Following all primary and secondary antibody incubations, sections were incubated in 1 μg/ml Hoechst 33258 (Sigma, St. Louis, MO). After a 30-minute wash in PBS, slides were rinsed in dH_2O and coverslipped with Fluoromount-G (EMS, Ft. Washington, PA) Control sections were incubated in blocking solution that did not contain primary antibodies.

TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using the *In Situ* Cell Death Detection Kit-TMR red (Boehringer Mannheim, Indianapolis, IN) according to manufacturers protocol. A positive-control was produced by adding DNase (50 units/50 μl buffer, pH 7.6) to control sections for 15 minutes. Incubation of sections in reaction buffer that did not contain terminal

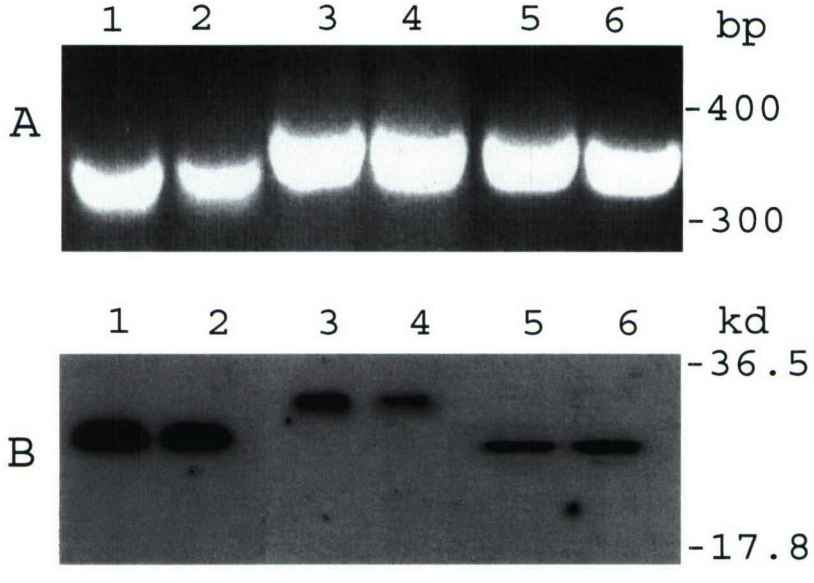

Fig. 1. mRNA and protein for bcl-2, bcl-x_L and bax are present in the ventral-midbrain. (A) RT-PCR for bcl-2 (lanes 1 and 2), bcl-x_L (lanes 3 and 4) and bax (lanes 5 and 6) (B) Western blots for bcl-2 (lanes 1 and 2, bcl-x_L (lanes 3 and 4) and bax (lanes 5 and 6). The duplicate lanes represent preparations from different naïve animals.

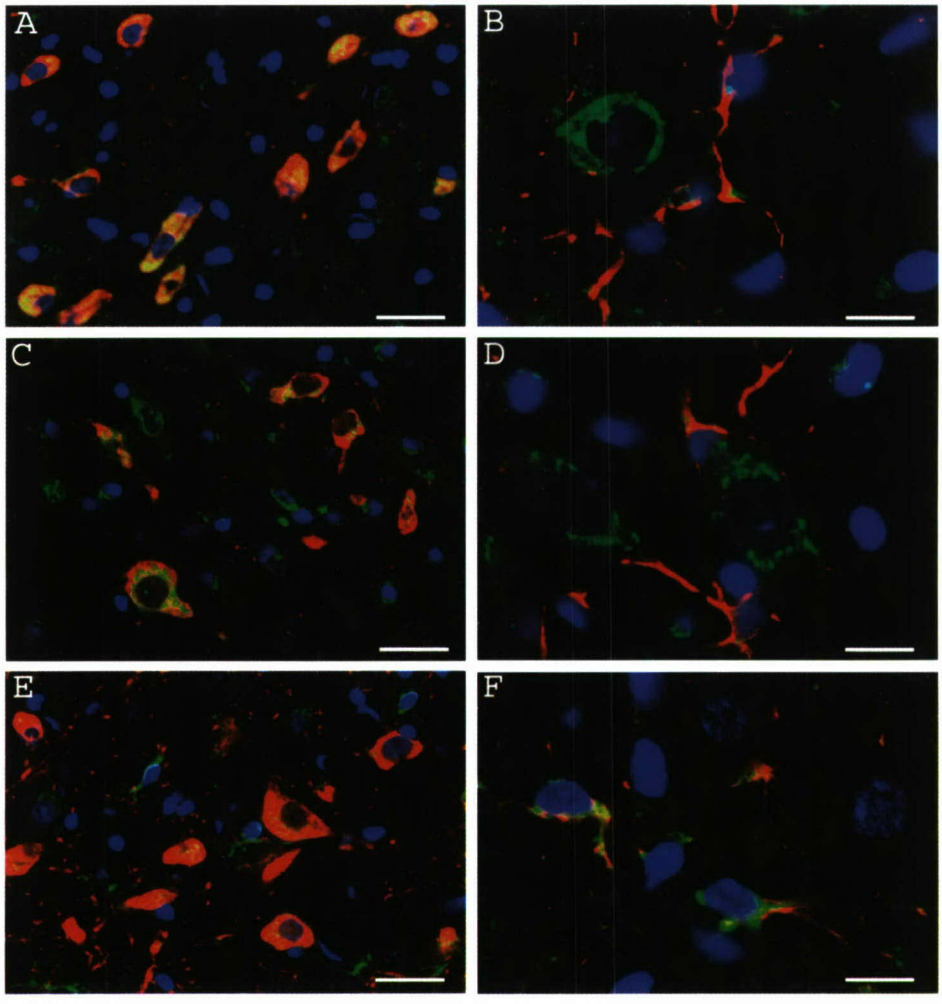

Fig. 2. Localization of bcl-2, bcl-x and bax in the SNpc of the naïve rat brain. Double label immunocytochemistry for: TH (red) and bcl-2 (green, A), bcl-x (green, C) and bax (green, E) and double labeling for GFAP (red) and bcl-2 (green, B), bcl-x (green, D) and bax (green, F). Scale bar = 10 μm.

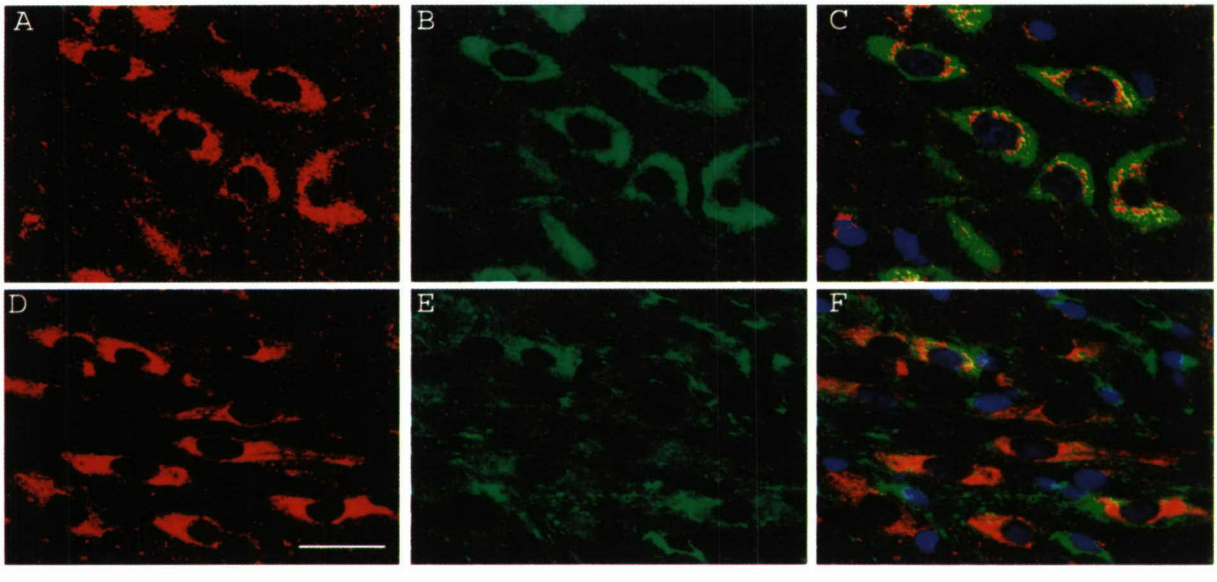

Fig. 3. Bax, bcl-x and bcl-2 are present in the same neurons in the adult rat SN. Top panel: Double label immunocytochemistry for: Bax (red, A) and Bcl-2 (green, B) and composite (C) in the SN. Bottom panel: Double label immunocytochemistry for: Bax (red, D) and Bcl-x (green.E) and composite (F) in the SN. Scale bar = 10 μm.

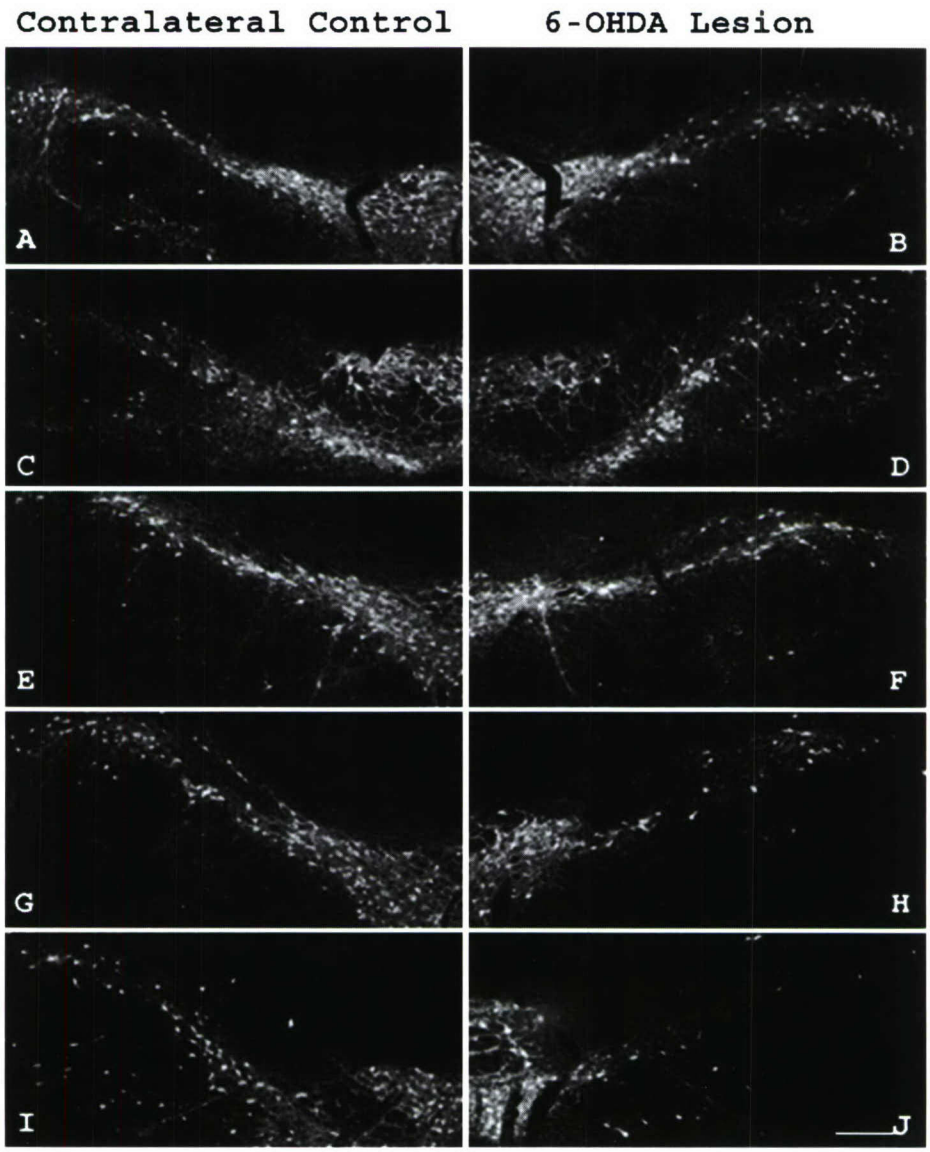

Fig. 4. Striatal 6-hydroxydopamine induced the loss of tyrosine hydroxylase-positive dopamine neurons in the substantia nigra pars compacta. Representative photomicrographs of coronal sections through the ventral-midbrain of animals at 1 (A and B), 3 (C and D), 7 (E and F), 10 (G and H) and 14 (I and J) days post-lesion. Figures B, D, F, H, and J represent the lesioned nigra and A, C, E, G, and I represent the contralateral control of the same section. Immunoreactivity for tyrosine hydroxylase demonstrates the time course of degeneration. Notice the severe loss of TH-positive dopamine neurons on days 10 (H compared to G) and 14 (J compared to I). Scale bar = 300 μm.

deoxynucleotidyl transferase provided a negative control. At the completion of TUNEL labeling, sections were labeled with TH and counterstained with Hoechst 33258 as described above.

IMAGE ACQUISITION

Images were acquired using a Spot RT Slider camera and software (v3.0) (Diagnostic Instruments, Sterling Hights, MI) attached to an Olympus X60-fluorescent microscope (Melville, NY). The illustrations presented in this manuscript were assembled using Adobe® Photoshop® 7.0. Only minor adjustments were made to the digital images obtained from the fluorescent microscope to insure optimal contrast and brightness of the resulting photomicrographs. This in no way altered the observations made. The plates were printed on Fujix Pictrography 3000 color printer (Fujifilm USA, Inc., Edison NJ).

Results

Bcl-2, Bcl-x AND Bax mRNA AND PROTEINS ARE PRESENT IN THE ADULT RAT MIDBRAIN

The presence of mRNA for bax, bcl-2 and bcl-x in the ventral midbrain (VMB) of untreated adult rats was identified by RT-PCR. Primers were designed

to produce a band at 355 bp for bcl-2 and 402 bp for bax. The primers used to identify bcl-x were designed to differentiate between bcl-x_L and its splice variant bcl-x_S, by producing a band at 397 bp for bcl-x_L and 210 bp for bcl-x_S. Based on the predicted size of the PCR products, we have concluded that the bands obtained represent bax, bcl-2 and bcl-x_L (Fig. 1A). No band corresponding to bcl-x_S was detected. When total cellular RNA or water was used as a template for PCR amplification with the above primers, no bands were observed (data not shown).

Western blots were performed separately with antibodies to bax, bcl-2 and bcl-x to identify whether the mRNA species identified above are actively translated into protein in the adult VMB. Bands were detected at approximately 21 kd for bax, 29 kd for bcl-2, and 30 kd for bcl-x_L (Fig. 1B). The primary antibody used to detect bcl-x does not differentiate between the long and the short form, however we did not detect a band at 21 kd, the expected molecular weight of bcl-x_S (Minn *et al.*, 1996). When Western blots were probed with antibodies that had been pre-absorbed with blocking peptide, no bands were observed (data not shown).

LOCALIZATION OF Bcl-2, Bcl-x AND Bax IN THE SUBSTANTIA NIGRA OF RAT BRAIN

Having confirmed the presence of mRNA and protein for bax, bcl-2 and bcl-x in the VMB, we used coronal sections from naïve rats to study the cellular distribution in the normal SNpc. Dopamine neurons were identified by their immunoreactivity to TH and astrocytes were identified by immunoreactivity to GFAP.

Intense Bcl-2 immunoreactivity was observed in the neurons of the SN, whereas it was rarely present in GFAP-postitive astrocytes (Fig. 2A and B). Bax immunoreactivty was also present the neurons of SN but was mostly absent from the astrocytes (2C and D). On the contrary, Bcl-x immunoreactivity was not as intense in neurons as it was in GFAP-positive astrocytes (Fig. 2E and F). Double labeling of cells with bax and bcl-2 (Fig. 3A–C) or bax and bcl-x (Fig. 3D and E) demonstrated the presence of these proteins in the same neuron.

EFFECT OF STRIATAL 6-OHDA LESIONS ON TH AND GFAP IMMUNOREACTIVITY

Striatal 6-OHDA injections were used to induce oxidative stress and subsequent dopaminergic cell death. Nigral sections were examined 1, 3, 7, 10, and 14 days post-lesion. There was no apparent loss of TH-positive neurons between 1 and 3 days (Fig. 4A–D). Loss of TH-positive neurons was apparent 7 days post-lesion and progressed throughout the course of the study (Fig. 4E–J).

GFAP immunocytochemistry revealed a higher concentration of astrocytes in the VMB as compared to the dorsal areas (Fig. 5A). Among the areas containing dopamine neurons, the greatest population of GFAP-positive cells was located in the ventral tegmental area (VTA) (Fig. 5B). In addition, substantial populations of GFAP-positive cells were distributed throughout the SNpc and substantia nigra pars lateralis (SNL) (Fig. 5C and D). Following 6-OHDA, no noticeable difference in GFAP-positive astrocytes was observed 1, 3 and 7 days post-lesion (data not shown). An increase in GFAP-positive astrocytes was apparent on the lesioned side at days 10 and 14, which coincides with the depletion of TH-positive cells (Fig. 6A and B).

Fig. 5. Distribution of astrocytes in the adult rat ventral-midbrain. Top panel: (A) Double label immunocytochemistry for: TH (green) and GFAP (red) which demonstrates the high level of GFAP positive cells in the ventral midbrain. Scale bar = 300 μm. Bottom panel: Enlargement of boxed areas of Figure 5A from left to right representing the ventral tegmental area (B), medial (C) and lateral (D) portions of the substantia nigra. Scale bare = 25 μm.

Fig. 6. GFAP immunoreactivity increases between 10–14 days post 6-OHDA lesion. Double label immunocytochemistry for TH (red) and GFAP (green) in the non-lesioned (A) and the lesioned (B) SNpc. Note the increase of GFAP immunoreactivity on the lesioned side as compared to the unlesioned. These photomicrographs are representative of observations made at days 10 and 14. Scale bar = 25 μm.

Fig. 7. Effect of striatal 6-OHDA lesions on bcl-2 immunoreactivity in the SNpc. Top panel: Double label immunocytochemistry for TH (red, A), bcl-2 (green, B) and composite (C) in the non-lesioned SN. Note the intense staining of bcl-2 in TH-positive dopamine neurons. Arrows and arrowheads indicate two examples of such cells. Bottom panel: Double label immunocytochemistry for: TH (red, D), bcl-2 (green, E) and composite (F), in the lesioned SN. Notice that some TH-positive cells appear to have little or no bcl-2 as compared to the non-lesioned control. Arrows and arrowheads indicate two examples of such cells. These photomicrographs are representative of observations made from days 3–14. Scale bar = 25 μm.

Fig. 8. Effect of striatal 6-OHDA lesions on bcl-x immunoreactivity in the SNpc. Top panel: Double label immunocytochemistry for TH (red, A), bcl-x (green, B) and composite (C) in the intact SN. Bottom panel: Double label immunocytochemistry for: TH (red, D), bcl-x (green, E) and composite (F), in the lesioned SN. Note the increase of bcl-x immunoreactivity on the lesioned side. This increase appears to be mostly in astrocytes and is seen between days 3–7. These photomicrographs are representative of observations made from days 3–7. Scale bar = 25 μm.

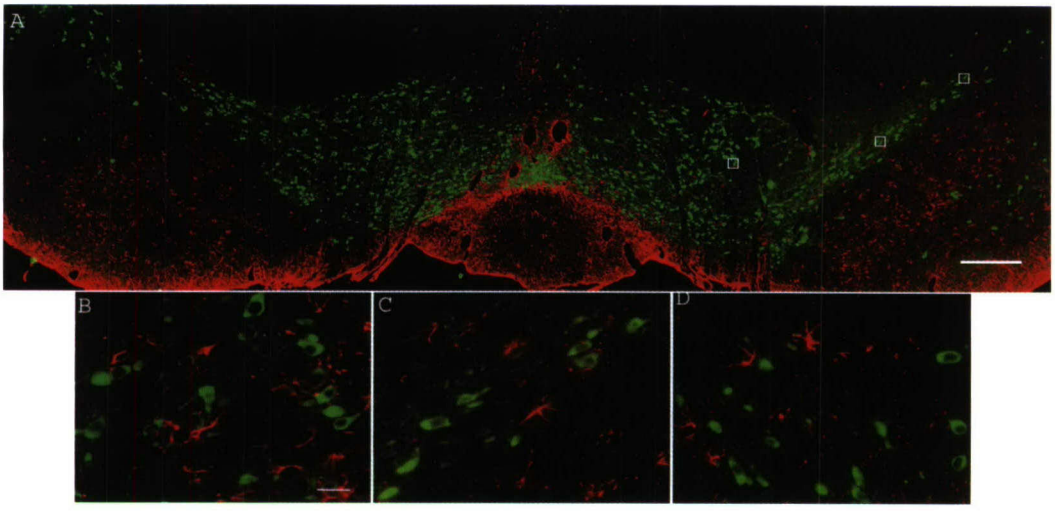

Fig. 5.

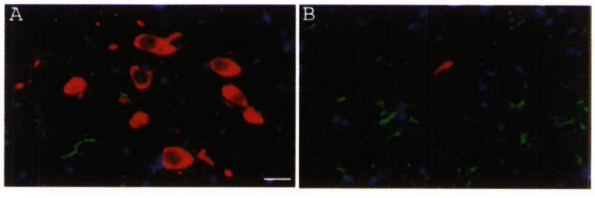

Fig. 6.

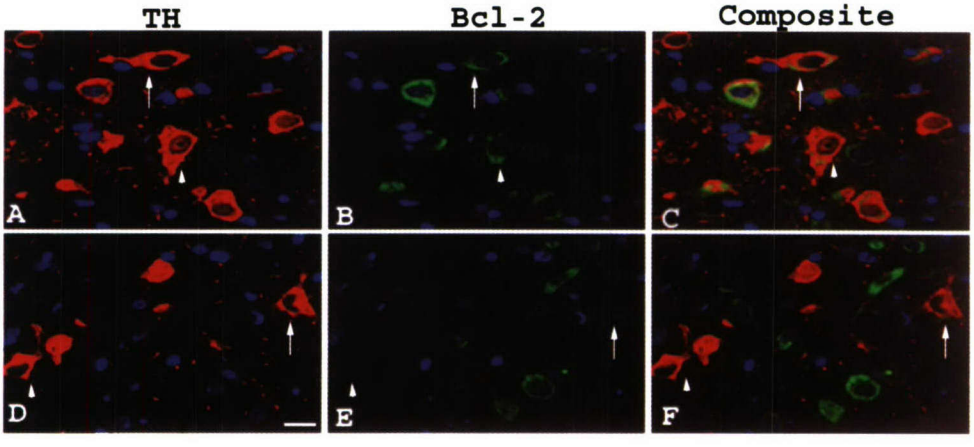

Fig. 7.

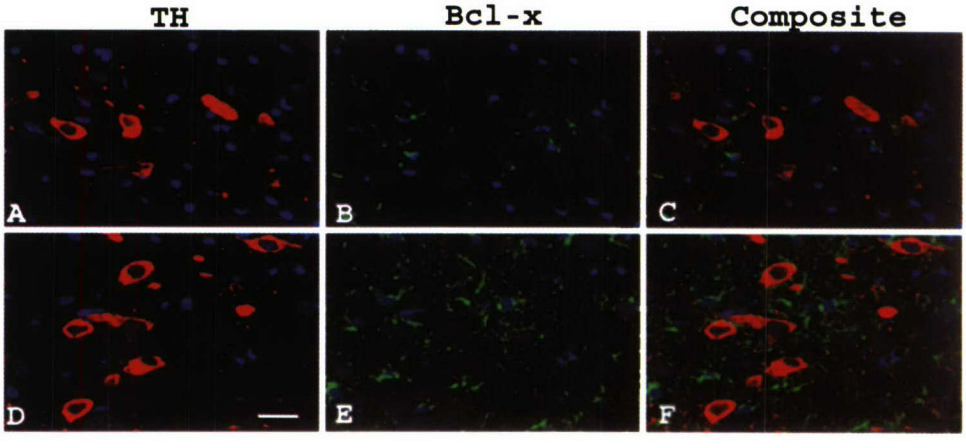

Fig. 8.

EFFECT OF STRIATAL 6-OHDA LESIONS ON Bcl-2, Bcl-x AND Bax IMMUNOREACTIVITY

A reduction of bcl-2 immunoreactivity in the TH-positive neurons was observed 3 days after 6-OHDA lesions and persisted throughout the study. In fact, TH-positive neurons with no bcl-2 immunoreactivity were often present on the lesioned side (Fig. 7A–F). 6-OHDA lesions had no effect on neuronal bcl-x immunoreactivity, however there was increased bcl-x staining in presumptive astrocytes in the lesioned SN on days 3 and 7 (Fig. 8A–F). This increase was no longer present on days 10 and 14 (not shown).

An overall reduction in bax immunoreactivity was observed in the lesioned nigra between days 3–7 (Fig. 9A–F), which was concomitant with a change in the cellular distribution of this protein from a well organized to defused pattern (Fig. 9G–L). Between days 10–14, surviving dopamine neurons on the lesioned side had varying intensities of TH immunoreactivity with some only lightly stained. Bax immunoreactivity was very intense in these cells, when compared to dopamine neurons that were strongly stained with TH (Fig. 9M–O).

INDICES OF APOPTOSIS

To determine if 6-OHDA lesions result in DNA fragmentation of dopamine neurons in the SN, we used TUNEL labeling to identify free 3'-OH termini, which characterize classical apoptosis. There was no evidence of TUNEL-labeled apoptotic nuclei in dopamine neurons on either the lesioned or non-lesioned sides at any of the time points examined (data not shown). There was also no evidence of condensed chromatin as determined by Hoechst staining (data not shown).

Discussion

Members of the bcl-2 family regulate cell death and survival through the initiation and inhibition of apoptosis, a process classically defined by its morphological characteristics (Kerr *et al.*, 1972), and their presence in the developing brain is well characterized (Shimohama *et al.*, 1998; Yamada *et al.*, 2000; Deckwerth *et al.*, 1996; Merry *et al.*, 1994). In this study we have documented the presence of bcl-2, bax, and bcl-x_L in the adult rat ventral-midbrain (VMB). Our data from RT-PCR and Western blot analysis indicate that these genes are actively transcribed and translated beyond the developmental period in the rat VMB. It also demonstrates that bcl-x_L, the anti-apoptotic splice variant of bcl-x (Boise *et al.*, 1993; Minn *et al.*, 1996), is the predominant form of bcl-x transcribed and translated in the VMB. Consistent with this observation, others have also reported that bcl-x_L is the predominant bcl-x species present in the brain (Gonzalez-Garcia *et al.*, 1994; Boise *et al.*, 1993). Within the SN, the region of the VMB enriched with the dopamine neurons that selectively degenerate during PD, immunocytochemistry revealed the presence of bax, bcl-2 and bcl-x in all dopamine neurons, however, there were differences in the intracellular distribution of neuronal bcl-2 and bax. Bax immunoreactivity was localized to well-defined regions in both the processes and in the perinuclear cytoplasm, a finding consistent with similar observations in Purkinje cells (Krajewski *et al.*, 1994), whereas bcl-2 was evenly distributed throughout the cytoplasm of dopamine neurons. These observations also confirm reports suggesting that pro- and anti-apoptotic members of the bcl-2 family may be localized in separate compartments in the absence of a death signal (for reviews see Gross *et al.*, 1999; Merry and Korsmeyer, 1997).

Following 6-OHDA lesions of the striatum, degeneration of TH-positive dopamine neurons of the SN occurred primarily between 7 to 14 days, in agreement with the findings of Sauer and Oertel (1994). Immunoreactivity for both the pro-apoptotic bax and the anti-apoptotic bcl-2 appeared initially decreased in TH-positive dopamine neurons of the lesioned side following 6-OHDA. This decrease is first observed three days post-lesion, before there is any apparent loss of dopamine neurons, suggesting an active response to 6-OHDA induced oxidative stress. It has been proposed that the ratio of pro- and anti-apoptotic proteins determines a cell's commitment to apoptosis (Gross *et al.*, 1999). The decrease in the anti-apoptotic protein bcl-2 in TH-positive dopamine neurons would support this finding. The apparent decrease in the pro-apoptotic protein bax, however, is counter-intuitive. One explanation for this might be that another pro-apoptotic member of the bcl-2 family is playing a role in dopaminergic degeneration following 6-OHDA lesions. For example, bad and bak are both able to displace bax from bcl-2 and promote apoptosis (Chittenden *et al.*, 1995; Yang *et al.*, 1995). Alternatively the redistribution of bax from a compact to a finely dispersed form may have resulted in an apparent loss of fluorescent intensity. During the period coinciding with the most active dopaminergic degeneration, the intensity of bax immunoreactivity varied among dopamine neurons depending on their TH content. Neurons strongly immunofluorescent for TH exhibited weak bax immunoreactivity, while neurons weakly immunofluorescent for TH had strong bax immunoreactivity. It is possible that the reduction in TH indicates damage and precedes cell death.

The uneven distribution of GFAP-positive astrocytes in the midbrain is intriguing. Similar observations have been reported previously (Hajos & Kalman, 1989; Zilles *et al.*, 1991). The apparent absence of GFAP-positive astrocytes in large areas of the midbrain might be explained by the presence of protoplasmic astrocytes that express little or no GFAP (for review see Walz, 2000). It has been suggested that the GFAP-positive astrocytes present in the VMB play a role in the pathophysiology

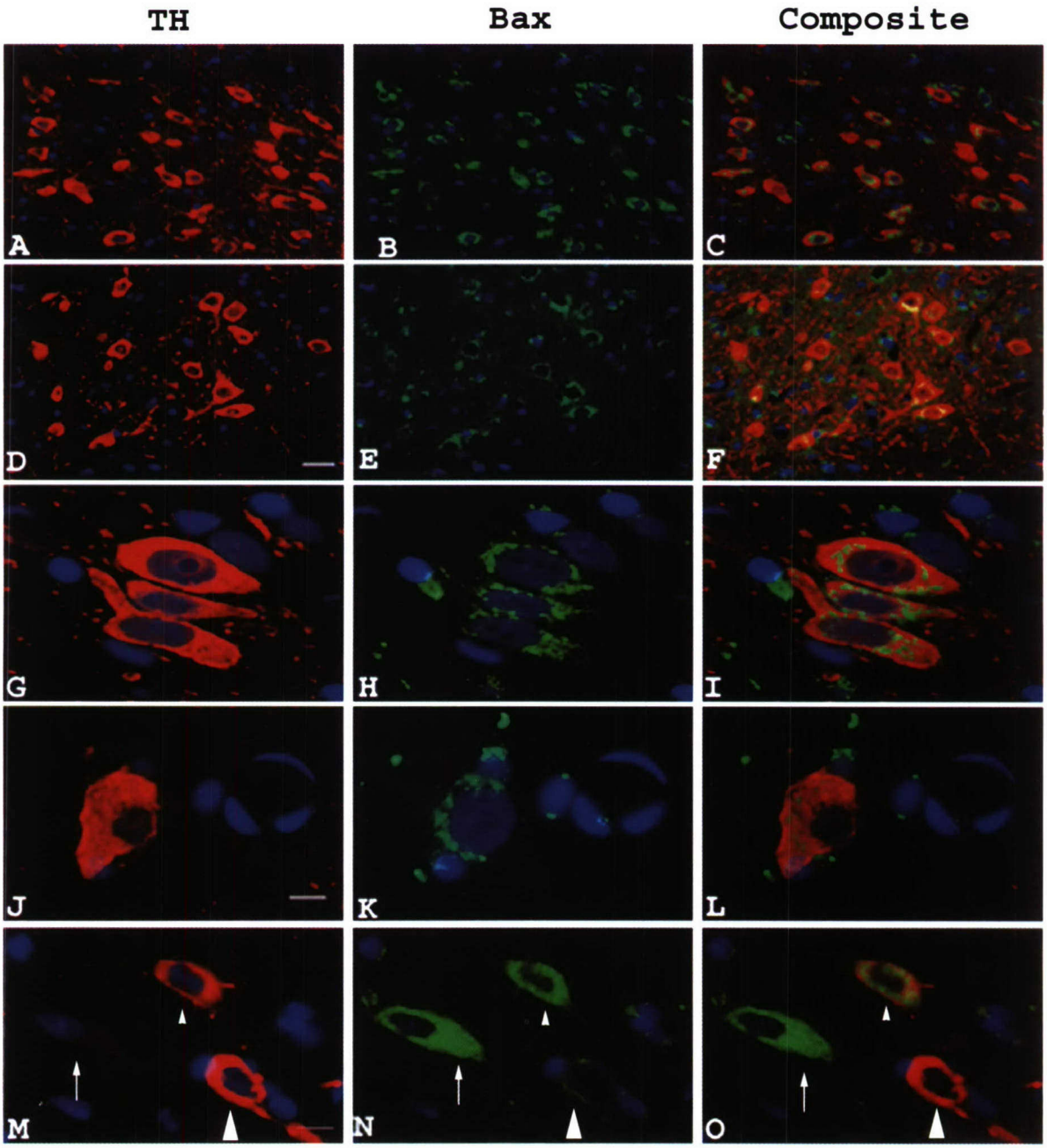

Fig. 9. Effect of striatal 6-OHDA lesions on bax immunoreactivity in the SNpc. Double label immunocytochemistry for TH (red), bax (green) and composites in the non-lesioned SN (A, B, C) and lesioned side of the same section (D, E, F). Note the intense staining of bax in TH-positive dopamine neurons of the control side as compared to the lesioned nigra. Scale bar = 25 μm. Higher magnification reveals that bax (green, H and K) appears to be more evenly distributed throughout the soma of TH neurons (red, G and J) in the lesioned (L) vs. non-lesioned side (I). These photomicrographs are representative of observations made from days 3–10 post-lesion. Immunocytochemistry for TH (red, M), bax (green, N) and composite (O) on the lesioned side days 10 and 14 demonstrate that there is an inverse relationship between TH and Bax immunoreactivity. Cells lightly stained for TH are strongly immunoreactive for bax (small arrow); those moderately stained for TH are moderately stained for Bax (small arrowhead); and cells strongly stained for TH are lightly stained for Bax (large arrowhead). Scale bar = 10 μm.

of PD (Hirsch *et al.*, 1999) and their presence in the VMB would support this notion. Indeed, studies indicate that glial cells play a role in the degeneration of dopamine neurons *in vitro*, following oxidative stress (Kramer *et al.*, 2002, 1999). We have observed an increase in bcl-x_L immunoreactivity in cells presumed to be astrocytes 3–7 days post-lesion, however an increase in GFAP immunoreactivity is not observed until days 10–14. Perhaps the increase of bcl-x occurs in protoplasmic astrocytes and the subsequent increase of GFAP

immunoreactivity observed on days 10 and 14 is the result of GFAP up-regulation in these cells.

The loss of dopamine neurons in Parkinson's disease (PD) is well documented; however the mechanism through which they die is unknown. While some groups report the presence of apoptotic morphology in the parkinsonian brain (Tompkins *et al.*, 1997; Anglade *et al.*, 1997; Hirsch *et al.*, 1999), others do not (Kosel *et al.*, 1997; Jellinger, 2000; Banati *et al.*, 1998). While apoptotic morphology was not present in the SN following 6-OHDA induced damage, we did observe changes in the distribution of bcl-2, bcl-x_L and bax, which indicates an active response to oxidative stress. Indeed, studies have suggested that cell death in response to oxidative stress include characteristics of both apoptosis and necrosis (Banati *et al.*, 1998) and that bcl-2 could attenuate cell death following GSH depletion, even though apoptotic morphology is not observed (Kane *et al.*, 1995).

In conclusion, our observations demonstrate that bcl-2, bcl-x_L and bax, which are present in the adult rat SN, are altered following 6-OHDA induced oxidative stress despite the absence of apoptotic morphology. These observations suggest that a connection between the bcl-2 family of proteins and oxidative stress may exist in PD. Further studies will be needed to confirm this possibility. In addition, our data support the notion that the link between classical apoptotic genes and classical apoptotic morphology may not be as absolute as once thought. Thus, we speculate that the bcl-2 family of proteins may also play a role in a mode of cell death that shares some, but not all of the characteristics of apoptosis.

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