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.

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 dismutase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 6-OHDA, 6-hydroxydopamine.
overactivity of surviving dopamine neurons with increased hydrogen peroxide (H₂O₂) 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, 1998). 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). [3H]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 Ca²⁺-, and Mg²⁺-free phosphate-buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24-well plates precoated with L-polynorhthine (0.1 mg/ml) at a density of 200,000 cells/cm². 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 which 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-μg/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 isopropanol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250; Molecular Devices, Sunnyvale, CA).

[3H]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 [3H]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-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]pipеразине hydrochloride (GBR-12909; 10 μM) were used as blanks. Blank values were less than 10% of untreated controls.

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, for cell cultures the medium was carefully aspirated from the culture wells removed and immersed in 4% paraformaldehyde in PBS at 4°C. Before analysis the samples were sonicated in 10 volumes of 0.4 M perchloric acid (PCA) 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 fluorometric 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, Chelmsford, MA).

Immunocytochemistry. Cells were plated on polyclarrhine-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 processed 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
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 [3H]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 (Mytileou et al., 1997). After 72 h of treatment, all concentrations of levodopa reduced [3H]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, [3H]dopamine uptake was reduced by 46% compared with control. Similarly, exposure to 100 and 200 μM levodopa reduced [3H]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.

Results

In Vitro Studies

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.

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
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-
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 (Table 2). 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.

**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 < 0.01; ***,** 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.
TABLE 1
Effect of levodopa and ascorbic acid treatment on the levels of GSH in mesencephalic cultures

<table>
<thead>
<tr>
<th>Levodopa</th>
<th>GSH</th>
<th>Control</th>
<th>BSO</th>
<th>Ascorbate</th>
<th>Ascorbate + BSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24.0 ± 1.0 (100)</td>
<td>3.0 ± 0.3 (12)</td>
<td>25.7 ± 0.5 (107)</td>
<td>4.4 ± 0.2 (18)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>31.3 ± 0.9 (131)**</td>
<td>2.2 ± 0.1 (9)</td>
<td>26.5 ± 0.5 (111)</td>
<td>3.3 ± 0.1 (14)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>37.7 ± 1.0 (157)**</td>
<td>0.0 ± 0.0 (0)</td>
<td>26.0 ± 1.4 (108)</td>
<td>1.6 ± 0.1 (7)</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>25.6 ± 0.7 (107)</td>
<td>0.0 ± 0.0 (0)</td>
<td>21.6 ± 1.6 (90)</td>
<td>0.0 ± 0.0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

*** 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).

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

* 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

**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 (Laia 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 (Men 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...
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 3

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

*** 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.
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 (Mená 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 (Kordover 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.

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