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Extracellular Accumulation of Nitric Oxide, Hydrogen Peroxide, and Glutamate in Astrocytic Cultures following Glutathione Depletion, Complex I Inhibition, and/or Lipopolysaccharide-Induced Activation

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ABSTRACT. Dopaminergic neuronal death in substantia nigra in Parkinson's disease is accompanied by depletion of reduced glutathione levels and inhibition of complex I activity which occur partially in normal or activated cells. The relationship between neuronal death and altered glial function is not known, but this may involve the release of toxic mediators from astrocytes and microglia, which in turn cause neuronal injury. We have examined the effects of L-buthionine-[*S*,*R*]-sulfoximine (L-BSO)-induced glutathione depletion, inhibition of complex I activity by 1-methyl-4-phenylpyridinium (MPR^+), and/or lipopolysaccharide (LPS)-induced activation on the extracellular accumulation of nitric oxide (NO), hydrogen peroxide (H_2O_2), and glutamate in primary cultures of rat forebrain astrocytes. Depletion of glutathione levels by up to 90% did not alter NO, H_2O_2, or glutamate levels in cultured astrocytes. Inhibition of complex I activity by up to 43% had no effect on extracellular NO accumulation, but increased H_2O_2 and glutamate levels. LPS-induced activation of cultured astrocytes increased extracellular levels of NO, H_2O_2, and glutamate. Extracellular accumulation of NO and H_2O_2 caused by LPS was markedly less in glutathione-depleted or complex I-inhibited astrocytic cultures compared to normal astrocytic cultures. In conclusion, complex I inhibition or activation of glial cells, alone or in combination with glutathione depletion, results in the extracellular accumulation of glutamate and the formation of NO and H_2O_2, which in turn may form highly toxic peroxynitrite and hydroxyl radicals. Thus, altered glial function leading to oxidative stress and excitotoxicity may contribute to the initiation or progression of neuronal death in substantia nigra in Parkinson's disease. BIOCHEM PHARMACOL **60**;7:979–988, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. astrocytes; 1-methyl-4-phenylpyridinium; nitric oxide; glutamate; hydrogen peroxide; Parkinson's disease

Degeneration of the dopaminergic nigrostriatal pathway, accompanied by the appearance of Lewy bodies and a reactive gliosis, constitutes the primary pathology of PD† [1, 2]. The cause of nigral cell death remains unknown, but current concepts suggest a genetic predisposition to a toxic process involving oxidative stress and mitochondrial dysfunction [3]. The involvement of free radical-mediated neuronal injury in substantia nigra in PD is supported by post-mortem findings of an early decrease in the levels of GSH [4] and an increase in iron levels [5] and mitochondrial manganese-dependent SOD activity [6]. Oxidative damage is evident from an increase in lipid peroxidation [7], reactive protein carbonyls [8], and DNA oxidation [9]. Impaired mitochondrial function in PD is apparent from a decrease in complex I activity [10] and reduced immunostaining for the α-ketoglutarate dehydrogenase complex [11] in substantia nigra. The degenerative process may also involve NO and glutamate-mediated excitotoxicity since NOS mRNA is increased in brain in PD [12] and inhibition of mitochondrial function by NO leads to glutamate release and excitotoxic cell death [13–15].

Previous studies have focused on the alteration in indices of oxidative stress occurring in nigral dopaminergic neurones as the cause of their degeneration. However, since dopaminergic neurones make up only 1–2% of the total nigral cell population in normal brain, and even less in PD, the extent of changes in GSH levels (40%) and SOD

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† *Abbreviations:* AAS, antibiotic and antimycotic solution; D/L-Val MEM, D-valine/L-valine MEM; D-Val MEM, D-valine MEM; EBSS, Earle's balanced salt solution; FBS, foetal bovine serum; H_2O_2, hydrogen peroxide; IL, interleukin; iNOS, inducible nitric oxide synthase; L-BSO, L-buthionine-[*S*,*R*]-sulfoximine; LPS, lipopolysaccharide; MEM, minimum essential medium; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, nitric oxide; PD, Parkinson's disease; and SOD, superoxide dismutase.

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(33%) and complex I (37%) activity measured in homogenates of post-mortem PD nigral tissue are too great to take place solely in dopaminergic neurones. This suggests that oxidative stress and mitochondrial dysfunction occur primarily, if not exclusively, in glial cells (astrocytes and microglia). Indeed, most GSH in brain is present in glial cells [16], and only glial cells stain positive for elevated non-haem iron in substantia nigra in PD [17]. In addition, SOD and complex I activity are markedly higher in astrocytes compared to neurones [18, 19]. Thus, the occurrence of oxidative stress and mitochondrial dysfunction may be more relevant to glial cells and contribute to the pathophysiology of nigral neuronal death in PD.

The relationship between the alteration in indices of oxidative stress and mitochondrial function in glial cells and neuronal death in substantia nigra in PD is unknown. One possibility is that glial dysfunction causes neuronal death by the withdrawal of normal trophic support for dopaminergic neurones [20]. Alternatively, altered glial function may cause the release of reactive oxygen/nitrogen species and glutamate, which then induce neuronal injury. Indeed, such a mechanism may play a role in the nigral degenerative process in PD, since glial cells proliferate during the disease process and are activated [21]. The levels of proinflammatory cytokines such as IL-1β, IL-6, and tumour necrosis factor-α are elevated in brain in PD [22, 23], and cytokine-activated glial cells secrete NO and reactive oxygen species which are toxic to cultured dopamine-containing and other neurones [24–28]. However, it is unclear whether glial activation also causes glutamate release or if depletion of GSH levels or inhibition of complex I activity in glial cells leads to the production and release of NO, reactive oxygen species, and glutamate.

Consequently, we studied the extracellular accumulation of NO, H_2O_2, and glutamate in primary cultures of rat forebrain astrocytes following depletion of glutathione levels with the glutathione synthesis inhibitor L-BSO, inhibition of mitochondrial function with the complex I inhibitor MPP^+, and/or activation of astrocytes with the bacterial endotoxin LPS.

MATERIALS AND METHODS

Materials

EBSS, FBS, BSA (fraction V) AAS (10,000 units penicillin, 10 mg streptomycin and 25 μg amphoceterin B per mL 0.9% NaCl), L-valine, poly-L-lysine, 0.25% trypsin–EDTA solution, bovine pancreatic trypsin (type III), yeast glutathione reductase (type III), β-FAD β-NADPH, *Aspergillus* nitrate reductase, sodium nitrite, β-NAD, yeast ADP, bovine liver glutamate dehydrogenase, L-glutamate, 30% (w/w) H_2O_2 solution, LPS (*Escherichia coli* 026:B6), 3,3′-dimethoxybenzimide (*o*-dianisidine), horseradish peroxidase, and L-BSO were obtained from Sigma Chemical Co. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from BDH Ltd. Bovine pancreatic DNase I was obtained from Boehringer Mannheim. D-valine containing MEM was obtained from GIBCO BRL. MPP^+ iodide was obtained from Research Biochemical Inc. Ubiquinone-1 was a gift from Eisai Pharmaceutical Co. Griess reagent (1 vol. 0.2% w/v napthylethylene diamine dihydrochloride in distilled water + 1 vol. 2% w/v sulfanilamide in 5% v/v phosphoric acid) was prepared on the day of use, and the components were obtained from Sigma Chemical Co. Cell culture plastics were obtained from Sigma and GIBCO BRL. All other reagents/materials were of analytical/cell culture grade and obtained from commercial sources. Cell culture reagents and materials were sterilised before use.

Cell Culture

Cultures of purified primary astrocytes were prepared from the forebrain of postnatal (1–2 days old) Wistar rats as previously described [29]. In each preparation, 7–10 rat pups were killed by decapitation, and the forebrain removed and cleared of meninges and blood vessels. The brain tissue was finely minced and incubated in 10 mL EBSS (containing 0.025% trypsin, 0.3% BSA, and 40 μg/mL of DNase I) for 30 min at 37° with gentle agitation. Trypsinisation was terminated by the addition of an equal volume of D/L-Val MEM (0.1 mg/mL of L-valine, 10% FBS, and 2.5% AAS, in MEM) and centrifuged at 250 *g* for 5 min. The pellet was triturated in EBSS (containing 0.3% BSA and 40 μg/mL of DNase I) about 20 times using a Pasteur pipette to form a dissociated cell suspension, then centrifuged as before. The pellet was suspended in D/L-Val MEM, divided equally, and plated in 6 poly-L-lysine-coated (5 μg/mL for 1 hr at 37°) 162 cm^2 vented cell culture flasks containing 20 mL D/L-Val MEM. The cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°. The culture medium was replaced with D/L-Val MEM after 1 and 3 days in culture. To retard the growth of fibroblasts and meningeal cells, the culture medium was replaced with D-Val MEM (10% FBS and 2.5% AAS, in MEM) after 7 days in culture, and every 3 days thereafter. When the astrocytes reached confluence (after approximately 14 days in culture), the cells were removed from the flasks by trypsinisation. The culture medium was aspirated and 10 mL 0.25% trypsin–EDTA solution was added and incubated for 3 min with gentle agitation, followed by the addition of an equal volume of D-Val MEM. The cell suspension was centrifuged at 250 *g* for 5 min and the pellet was suspended in D-Val MEM. The cells were counted on a haemocytometer using trypan blue exclusion, then plated at a density of 10^5 cells/cm^2 (approximately 0.3 mg total astrocytic protein) in poly-L-lysine-coated 24-well culture plates containing 1.0 mL D-Val MEM, and grown as described above. One day later, the astrocytes were used for experimentation. Immunocytochemical analyses have shown that this method produces cultures comprising >95% glial fibrillary acidic protein-positive astrocytes [29]. Although the contribution of microglial cells to these astrocytic cultures is very low, the possibility remains that they may contribute to some of

the findings in this study, but we believe that this would be negligible compared to astrocytes [26].

Glutathione Determination

Since glutathione is released from astrocytes following synthesis, the levels of total (reduced + oxidised) glutathione in the cell culture medium were measured spectrophotometrically as an indication of intracellular glutathione production [30, 31]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM K^+, EDTA, 0.3 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.4 mM NADPH, and 100 μL cell culture medium or glutathione standard, in a final volume of 1 mL. The reaction was initiated by the addition of 1 U/mL of glutathione peroxidase and initial reaction rates determined at 405 nm.

Complex I Assay

Astrocytic complex I activity was determined spectrophotometrically as previously described [10]. The reaction mixture contained 20 mM potassium phosphate (pH 7.2), 8 mM $MgCl_2 \cdot 6H_2O$, 150 μM NADH, 1.0 mM KCN, 2.5 mg/mL of BSA, and 50 μM ubiquinone-1, in a final volume of 1 mL. The reaction was initiated by the addition of 100 μL freeze/thawed cell suspension and initial reaction rates determined at 340 nm.

NO Measurement

NO is rapidly converted to nitrate and nitrite after synthesis and release from astrocytes. Therefore, the concentrations of NO metabolities in astrocytic culture medium were measured as an indication of NO production as described elsewhere [32]. An aliquot (10 μL) of cell culture medium or sodium nitrite standard (0–40 μM) was added to 96-well microplates containing 130 μL 50 mM HEPES–KOH (pH 7.4), 25 μM β-FAD, 5 μM NADPH, and 0.2 U/mL of nitrate reductase (final concentrations), and incubated for 30 min at 37°. Unreacted NADPH was oxidised by the addition of 10 μL 1.0 mM potassium ferricyanide for 10 min at room temperature. Griess reagent (50 μL) was added and allowed to react for 10 min at room temperature. Absorbances were read at 543 nm on a microplate reader, a nitrite standard curve was established, and the concentrations of nitrite + nitrate in the cell culture medium calculated.

H_2O_2 Measurement

The concentration of H_2O_2 in astrocytic culture medium was determined as previously described [33]. An aliquot (100 μL) of cell culture medium or H_2O_2 standard (0–100 μM) was added to 200 μL 3,3′-dimethoxybenzimide (2 mM) and 200 μL horseradish peroxidase (240 U/mL). Absorbances were read at 500 nm, a H_2O_2 standard curve was established, and the concentrations of H_2O_2 in the cell culture medium calculated.

Glutamate Measurement

The concentration of L-glutamate in astrocytic culture medium was determined as previously described [34]. An aliquot (10 μL) of cell culture medium or glutamate standard (0–50 μM) was added to 96-well microplates containing 190 μL reaction mixture (50 mM Tris–HCl, 1 mM K^+, EDTA, 2.5% v/v hydrazine hydrate, 1.5 mM β-NAD, 1.0 mM ADP, and 12 U/mL of glutamate dehydrogenase; final concentrations) and allowed to react for 1 hr at room temperature. Absorbances were read at 340 nm on a microplate reader, an L-glutamate standard curve was established, and the concentrations of L-glutamate in the cell culture medium calculated.

Determination of the Effects of Altered Astrocytic Function on Extracellular Glutathione Levels and Complex I Activity in Astrocytic Cultures

The astrocytic culture medium was aspirated and replaced with fresh filter-sterilised D-Val MEM containing 100–500 μM L-BSO, 10–50 μM MPP^+, or 10–50 μg/mL of LPS. Control wells received D-Val MEM only. After 1 or 3 days of treatment, the cell culture medium was removed and 100 μL used for the determination of glutathione levels. Trypsin-EDTA (1 mL) was added to each well to detach and suspend adherent cells. The cell suspension was subjected to 3 cycles of freeze/thawing and 100 μL used for the determination of complex I activity. None of these treatments caused astrocytic death as determined by the release of lactate dehydrogenase (data not shown).

Determination of the Effects of Altered Astrocytic Function on Extracellular NO, H_2O_2, and Glutamate Levels in Astrocytic Cultures

The culture medium of primary astrocytes was aspirated and replaced with fresh filter-sterilised D-Val MEM containing 100–500 μM L-BSO, 10–50 μM MPP^+, 10–50 μg/mL of LPS, or a combination of these compounds. Control wells received D-Val MEM only. After 1 or 3 days, the cell culture medium was removed and assayed for NO, H_2O_2, and L-glutamate levels. None of these treatments caused astrocytic death as determined by the release of lactate dehydrogenase (data not shown).

Statistical Analyses

Results are presented as means ± SEM for 3 (glutathione depletion and complex I inhibition studies) or 6 cultures (NO, H_2O_2, and glutamate release studies) (6 culture wells per treatment for each culture). Results were analysed statistically using Mann–Whitney U-test (N = 3) or Student's *t*-test (N = 6) according to sample size, and one-way ANOVA, as indicated in the figure and table legends.

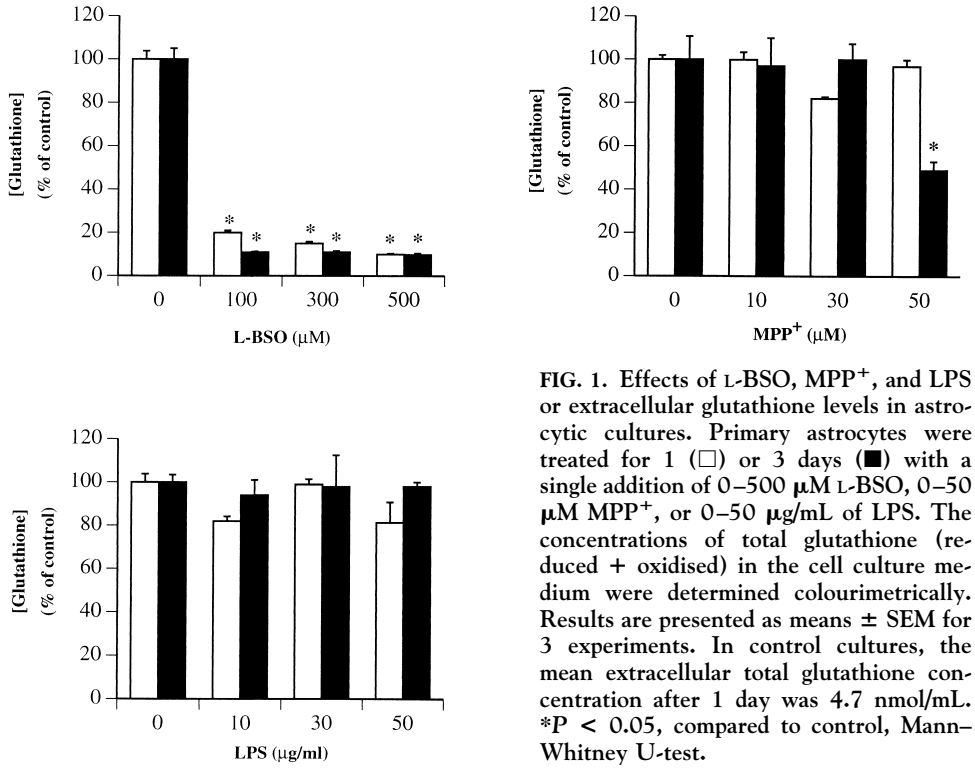

FIG. 1. Effects of L-BSO, MPP^+, and LPS or extracellular glutathione levels in astrocytic cultures. Primary astrocytes were treated for 1 (□) or 3 days (■) with a single addition of 0–500 μM L-BSO, 0–50 μM MPP^+, or 0–50 μg/mL of LPS. The concentrations of total glutathione (reduced + oxidised) in the cell culture medium were determined colourimetrically. Results are presented as means ± SEM for 3 experiments. In control cultures, the mean extracellular total glutathione concentration after 1 day was 4.7 nmol/mL. *$P < 0.05$, compared to control, Mann–Whitney U-test.

RESULTS

Glutathione Levels

The mean extracellular glutathione concentration in control astrocytic cultures after 1 day was 4.7 nmol/mL. This value is consistent with 10 nmol/3 mL (after 10 hr) reported elsewhere [30]. L-BSO depleted glutathione levels. Thus, after 1 day of treatment with 100 and 500 μM L-BSO, glutathione levels were reduced to 20% and 10% of control values, and after 3 days of treatment, glutathione levels decreased to 11% and 9.8% of control values, respectively (Fig. 1). Only 50 μM MPP^+ (3 days) reduced (by approximately 50%) glutathione levels in astrocytic cultures (Fig. 1). LPS had no effect on glutathione levels in cultures of actrocytes (Fig. 1).

Complex I Activity

The mean complex I activity in control astrocytic cultures after 1 day was 54 nmol/min/mg, comparing favourably with a value of 65 nmol/min/mg reported previously [24]. Treatment of cultured astrocytes with L-BSO or LPS had no effect on complex I activity (Fig. 2). MPP^+ inhibited complex I activity in cultured astrocytes (Fig. 2). After 1 day of treatment, 10 and 50 μM MPP^+ reduced astrocytic complex I activity to 94% and 75% of control values, respectively. Similarly, after 3 days of treatment, 10 and 50 μM MPP^+ reduced astrocytic complex I activity to 69% and 57% of control values, respectively.

NO Levels

In control cultures of astrocytes, the mean concentrations of nitrate + nitrite in the cell culture medium after 1 and 3 days were 9.3 and 9.7 μM, respectively. Depletion of glutathione levels (100–500 μM L-BSO) or inhibition of complex I activity (10–50 μM MPP^+) in astrocytes in culture for up to 3 days had no effect on extracellular NO levels (Fig. 3). In contrast, activation of cultured astrocytes with 10–50 μg/mL of LPS increased the levels of extracellular NO (Fig. 3). After 1 day, treatment with 10 and 30 μg/mL of LPS increased NO levels to 107% and 133% of control values, respectively. After 3 days, NO levels were increased to 127% and 143% of control values, respectively.

Activation of cultured astrocytes coupled to glutathione depletion or complex I inhibition resulted in a marked reduction in extracellular NO accumulation caused by LPS alone (Table 1). Thus, combined treatment of astrocytes in culture for 3 days with 30 μg/mL of LPS plus 300 μM L-BSO or 30 μM MPP^+ resulted in a 13–22% reduction in the levels of NO compared to treatment with 30 μg/mL of LPS alone (Table 1).

Extracellular H_2O_2

In control cultures of astrocytes, the mean concentrations of H_2O_2 in the cell culture medium after 1 and 3 days were 59.9 and 62.1 μM, respectively. These values are within the range of 30–100 μM reported previously [33]. Inhibition of

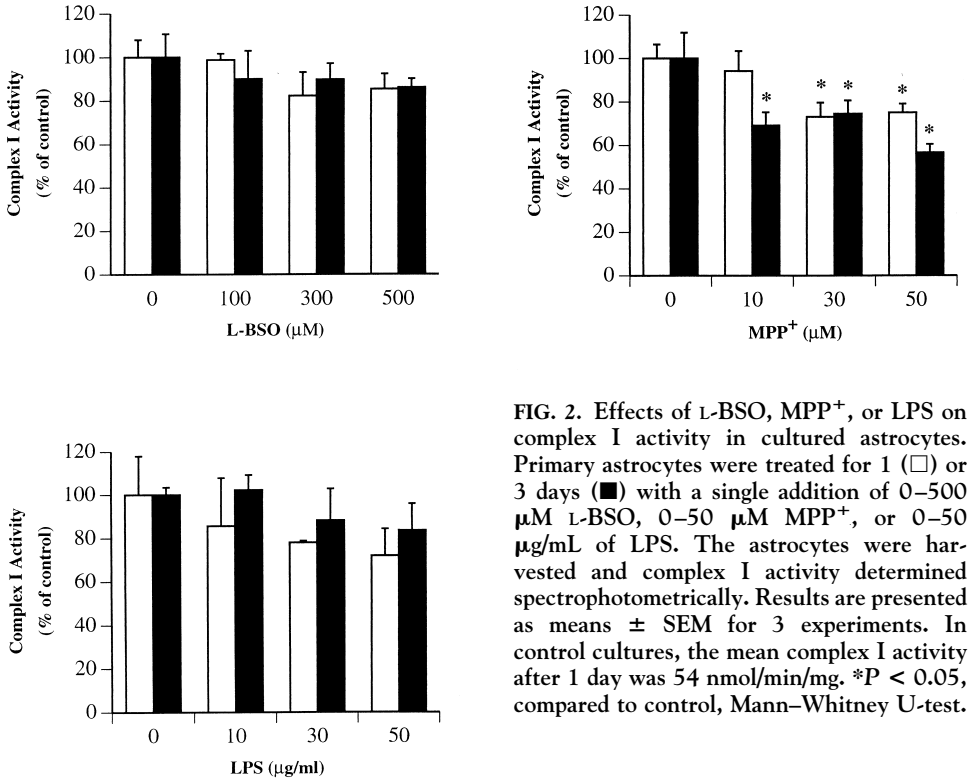

FIG. 2. Effects of L-BSO, MPP^+, or LPS on complex I activity in cultured astrocytes. Primary astrocytes were treated for 1 (□) or 3 days (■) with a single addition of 0–500 μM L-BSO, 0–50 μM MPP^+, or 0–50 μg/mL of LPS. The astrocytes were harvested and complex I activity determined spectrophotometrically. Results are presented as means ± SEM for 3 experiments. In control cultures, the mean complex I activity after 1 day was 54 nmol/min/mg. *P < 0.05, compared to control, Mann–Whitney U-test.

astrocytic complex I activity with 30–50 μM MPP^+ or activation of astrocytes with 30–50 μg/mL of LPS, but not depletion of glutathione levels with 100–500 μM L-BSO, stimulated the extracellular accumulation of H_2O_2 (Fig. 4). For example, after 1 day of treatment with 10 and 30 μM MPP^+, H_2O_2 levels were 134% and 147% of control values, but these levels did not change significantly thereafter (Fig. 4). Similarly, after 1 day of treatment, 10 and 30 μg/mL of LPS increased H_2O_2 levels to 159% and 175% of control values, respectively. However, after 3 days of treatment, 10 and 30 μg/mL of LPS increased H_2O_2 accumulation to 217% and 245% of control values, respectively.

Combined activation of astrocytes in culture with 30 μg/mL of LPS plus inhibition of complex I activity with 30 μM MPP^+ resulted in an approximate 20% reduction in the extracellular levels of H_2O_2 compared to 30 μg/mL of LPS activation alone (Table 1). Similarly, after 3 days, H_2O_2 levels following combined activation of astrocytes

TABLE 1. Extracellular accumulation of NO, H_2O_2, and glutamate in astrocytic cultures following treatment with L-BSO, MPP^+, and/or LPS

	$[NO_2^- + NO_3^-]$		$[H_2O_2]$		[Glutamate]	
Treatment	**1 Day**	**3 Days**	**1 Day**	**3 Days**	**1 Day**	**3 Days**
Control	9.3 ± 0.11	9.7 ± 0.11	59.8 ± 4.50	63.9 ± 5.6	17.7 ± 0.6	18 ± 0.01
300 μM L-BSO	9.8 ± 0.96	9.6 ± 0.06	63.6 ± 13.61	65.8 ± 5.6	20.7 ± 9.1	18.6 ± 0.15
30 μM MPP^+	9.0 ± 0.73	10.0 ± 0.23	109.1 ± 9.1*	100 ± 11*	22.4 ± 0*	22.4 ± 1.4*
30 μg/mL of LPS	13.9 ± 0.9*	14.9 ± 0.6*	87.5 ± 18*	110.4 ± 11*	21.8 ± 2*	21.0 ± 0*
30 μg/mL of LPS + 300 μM L-BSO	10.3 ± 2.30†	13.0 ± 0.22†	95.9 ± 13.6	89 ± 8.0†	24.5 ± 5.2	25.4 ± 0.18
30 μg/mL of LPS + 30 μM MPP^+	10.4 ± 0.11†	11.6 ± 1.35†	70.4 ± 0.5.9†	90.0 ± 6.0†	25.7 ± 1.5†	27.1 ± 2.1†
300 μM L-BSO + 30 μM MPP^+	08.9 ± 0.51	9.9 ± 0.22	136.4 ± 13.6‡	135 ± 13‡	20.1 ± 2.0	21.3 ± 3.4

Primary astrocytes were prepared from the forebrain of 1–2-day old Wistar rats, plated in poly-L-lysine-coated 24-well culture plates at a density of 10^5 cells/cm^2 containing 1.0 mL D-Val MEM, and grown under an atmosphere of 5% CO_2/95% air at 37°. Astrocytes were treated for 1 day or 3 days with a single addition of 300 μM L-BSO, 30 μM MPP^+, 30 μg/mL of LPS, or a combination of these compounds. The concentrations (μM) of NO, H_2O_2, and glutamate in the cell culture medium were determined spectrophotometrically. Results are presented as means ± SEM for 6 experiments.

*$P < 0.01$, compared to control, ANOVA.

†$P < 0.01$, compared to treatment with 30 μg/mL of LPS, ANOVA.

‡$P < 0.01$, compared to treatment with 30 μg/mL of MPP^+, ANOVA.

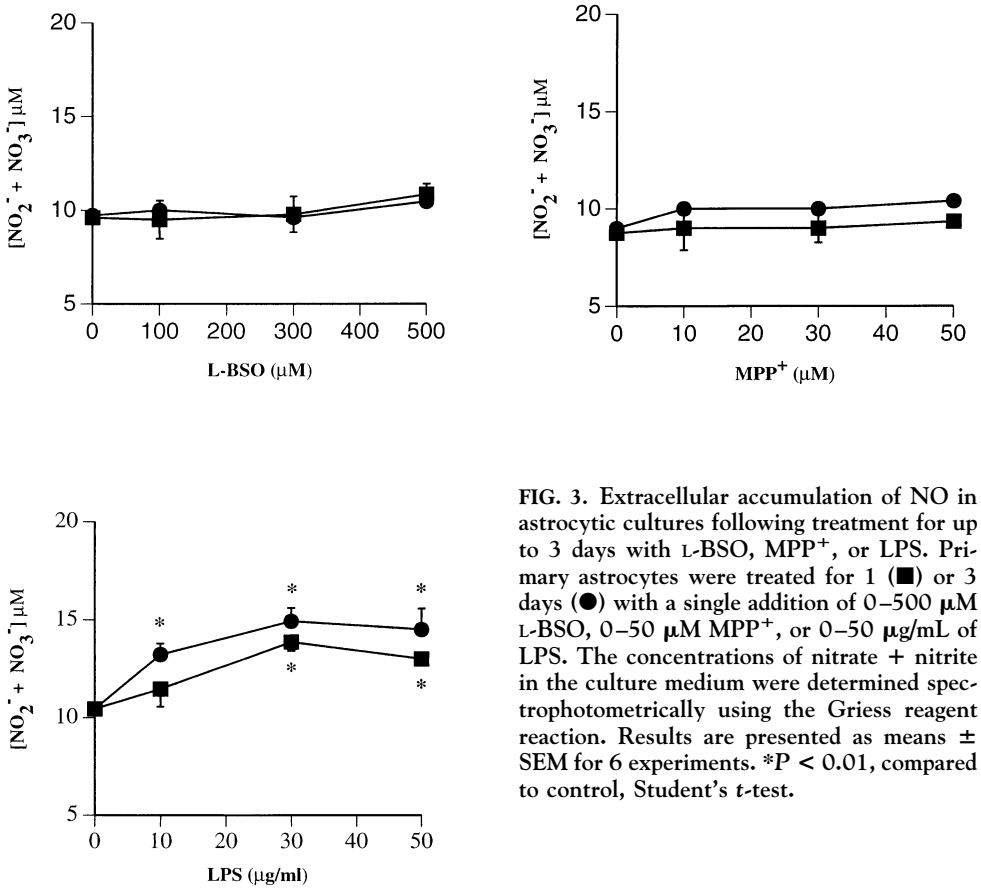

FIG. 3. Extracellular accumulation of NO in astrocytic cultures following treatment for up to 3 days with L-BSO, MPP^+, or LPS. Primary astrocytes were treated for 1 (■) or 3 days (●) with a single addition of 0–500 μM L-BSO, 0–50 μM MPP^+, or 0–50 μg/mL of LPS. The concentrations of nitrate + nitrite in the culture medium were determined spectrophotometrically using the Griess reagent reaction. Results are presented as means ± SEM for 6 experiments. *$P < 0.01$, compared to control, Student's *t*-test.

with 30 μg/mL of LPS plus depletion of glutathione levels with 300 μM L-BSO were reduced by approximately 20% compared to 30 μg/mL of LPS treatment alone (Table 1). Combined depletion of glutathione levels with 300 μM L-BSO plus inhibition of complex I activity with 30 μM MPP^+ resulted in a 25% (1 day) and 35% (3 days) elevation of extracellular H_2O_2 levels compared to treatment with 30 μM MPP^+ alone (Table 1).

Extracellular Glutamate

The mean concentrations of glutamate in the medium of control cultured astrocytes after 1 and 3 days were 18.3 and 18.4 μM, respectively. These values are within the range of <1.0–100 μM reported previously [35]. Inhibition of astrocytic complex I activity (10–50 μM MPP^+) or activation of astrocytes in culture (30–50 μg/mL of LPS), but not depletion of glutathione levels (100–500 μM L-BSO), increased extracellular glutamate levels (Fig. 5). Thus, 30 μM MPP^+ and 30 μg/mL of LPS increased glutamate levels to 128% and 121% of control levels, respectively, after 1 day of treatment. After 3 days of treatment, glutamate levels were not significantly different, being 123% and 116% of control values, respectively. Also, after 1 day of treatment with 10 and 30 μM MPP^+, and 10 and 30 μg/mL of LPS, extracellular glutamate levels were 126% and 129%, and 109% and 121% of control values, respectively.

Accumulation of extracellular glutamate caused by combined activation (30 μg/mL of LPS) of astrocytes in culture with depletion of glutathione levels (300 μM L-BSO) was not significantly different from either treatment alone (Table 1). However, activation (30 μg/mL of LPS) of cultured astrocytes in the presence of complex I inhibition (30 μM MPP^+) resulted in extracellular glutamate accumulation which was higher than accumulation caused by either treatment alone (Table 1).

DISCUSSION

In brain, glial cells outnumber neurones, with astrocytes representing the majority of all cells. Glial cells provide structural and metabolic support for neurones and secrete trophic factors to support neuronal growth and function. In neurological diseases, the morphology and secretory profile of astrocytes and microglia are altered [36, 12]. These activated glial cells release inflammatory mediators such as cytokines to initiate and maintain immunological defences to protect neurones [36]. However, activated glial cells may also cause neuronal death via the release of neurotoxic substances [26]. In this study, we showed that activation of cultured astrocytes by LPS caused the extracellular accu-

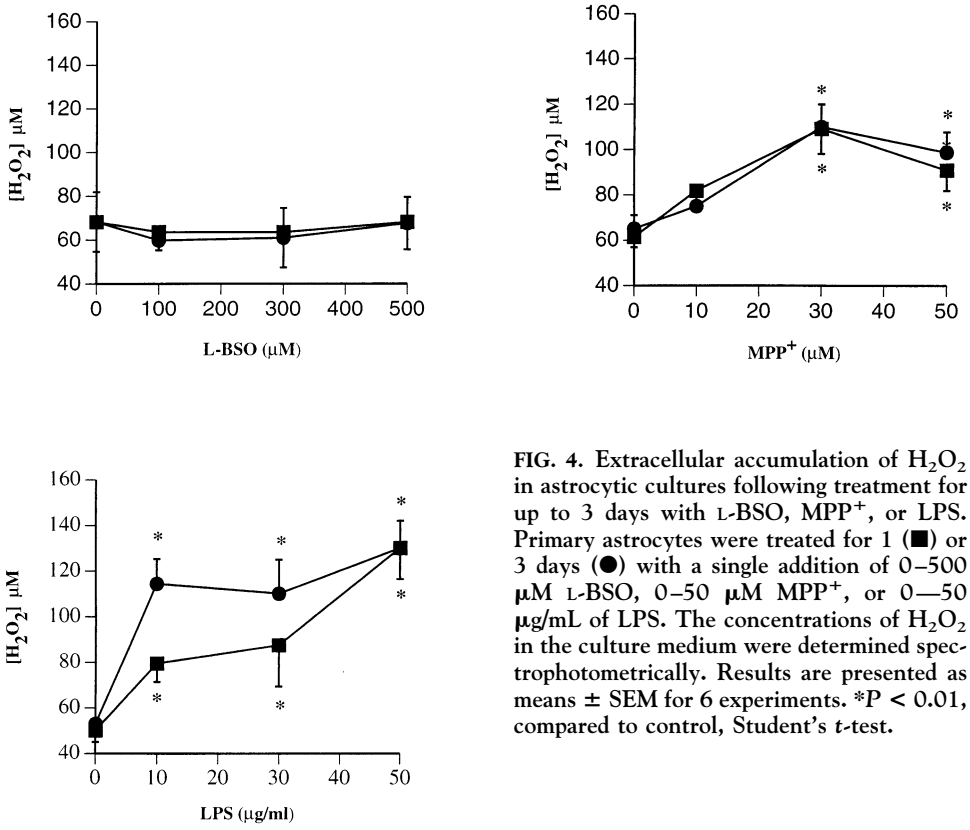

FIG. 4. Extracellular accumulation of H_2O_2 in astrocytic cultures following treatment for up to 3 days with L-BSO, MPP^+, or LPS. Primary astrocytes were treated for 1 (■) or 3 days (●) with a single addition of 0–500 μM L-BSO, 0–50 μM MPP^+, or 0—50 μg/mL of LPS. The concentrations of H_2O_2 in the culture medium were determined spectrophotometrically. Results are presented as means ± SEM for 6 experiments. *$P < 0.01$, compared to control, Student's *t*-test.

mulation of NO, H_2O_2, and glutamate. The characteristics of NO release were consistent with previous studies showing that activation of glial cells by cytokines leads to expression of iNOS mRNA, up-regulation of iNOS, and NO production [27, 28]. The mechanism of extracellular glutamate accumulation in astrocytic cultures was not examined in this study, but glutamate may be released from astrocytes secondary to impairment of the respiratory chain by LPS-generated NO. Indeed, NO inhibits mitochondrial respiration in cultured astrocytes at the level of complex IV, and this causes a rapid time-independent but concentration-dependent release of glutamate from rat brain synaptosomes [37, 15]. NO also inhibits complex II–III activity, and previous studies have shown that inhibition of complex II by malonate causes rapid glutamate release *in vivo* [13, 24]. Glutamate release following mitochondrial dysfunction is attributed to the depletion of ATP levels, impairment of the Na^+/K^+-ATPase resulting in decline of the Na^+ gradient, and reversal of the Na^+-glutamate transporter. Consequently, there is efflux of the metabolic pool of glutamate from the cytoplasm [15]. In addition to its other roles, astrocytes participate in maintaining low extracellular glutamate levels *in vivo* by virtue of their ability to readily accumulate extracellular glutamate released by neurones and subsequent metabolism to glutamine [35]. However, recent studies have shown that high levels of H_2O_2 and peroxynitrite impair the ability of astrocytes to perform this function [38]. Thus, the extracellular accumulation of H_2O_2 and possibly peroxynitrite from glial cells following activation with LPS may also contribute to the elevated levels of glutamate measured in cell culture medium in this study. Increased extracellular levels of H_2O_2 in astrocytic cultures following activation by LPS may be attributed to observations that stimulation of NOS activity produces superoxide radicals and H_2O_2 as well as NO [39, 40]. Alternatively, H_2O_2 or superoxide ions (followed by dismutation by SOD) may be released from the respiratory chain following inhibition of complex II–III activity by LPS-generated NO. This is consistent with findings that inhibition of complex II–III activity by antimycin A in isolated submitochondrial particles results in the release of superoxide radicals from the electron transport chain [41]. It is not known if glial activation is a cause or consequence of neuronal degeneration in substantia nigra in PD. Either way, the release of reactive oxygen/nitrogen species and glutamate from activated glial cells may at least contribute to the progression of nigral cell death in PD by a number of mechanisms, including oxidative stress, mitochondrial dysfunction, and excitotoxicity. H_2O_2 is not a free radical, but in the presence of high concentrations of transition metals is converted via Fenton reactions to highly reactive hydroxyl radicals [42]. This observation is particularly relevant to the hypothesis of oxidative stress- and free radical-mediated molecular damage in substantia nigra, since iron levels are also increased in this brain region in PD [5].

GSH serves as a non-enzymatic scavenger for hydroxyl

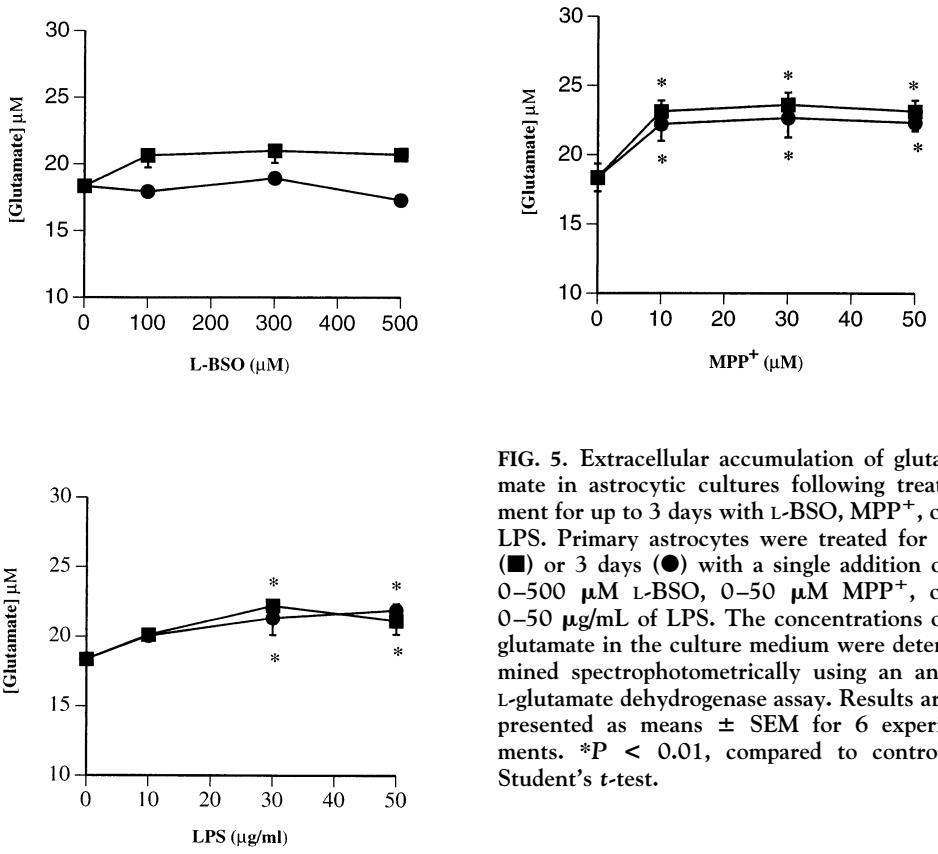

FIG. 5. Extracellular accumulation of glutamate in astrocytic cultures following treatment for up to 3 days with L-BSO, MPP^+, or LPS. Primary astrocytes were treated for 1 (■) or 3 days (●) with a single addition of 0–500 μM L-BSO, 0–50 μM MPP^+, or 0–50 μg/mL of LPS. The concentrations of glutamate in the culture medium were determined spectrophotometrically using an and L-glutamate dehydrogenase assay. Results are presented as means ± SEM for 6 experiments. *P < 0.01, compared to control, Student's *t*-test.

radicals as well as a substrate in the enzymatic removal of H_2O_2 [42]. Cellular GSH levels are depleted by treatment with L-BSO following irreversible inhibition of γ-glutamylcysteine synthetase, the rate-limiting enzyme for GSH formation [43]. In this study, L-BSO decreased extracellular glutathione levels by up to 90%. This is consistent with reports that L-BSO depletes intracellular astrocytic glutathione levels, resulting in a decrease in glutathione secretion and hence reduced extracellular concentrations [44, 31]. The glutathione secreted by cultured astrocytes is mostly GSH with a negligible content of oxidised glutathione [45, 31]. In this study, depletion of glutathione did not alter extracellular NO, glutamate, or H_2O_2 levels. This observation is consistent with recent findings that catalase may be the most important means of metabolising H_2O_2 in astrocytes and that it is able to sustain antioxidant defences in the presence of low glutathione levels [33, 45]. Indeed, astrocytes retain their ability to detoxify exogenously applied H_2O_2 following depletion of glutathione levels to 14% of control values, but this ability is abolished by inhibition of catalase activity [45]. Since glutathione depletion in the present study did not alter NO, glutamate, or H_2O_2 levels, this implies that a reduction in glial GSH levels in substantia nigra in PD may not directly cause dopaminergic cell death. This is supported by studies showing that chronic infusion of L-BSO into the lateral ventricles of rats caused a high degree of GSH depletion, but did not cause nigral cell death [46]. However, in mixed glial/neuronal co-culture studies, depletion of glial glutathione content reduces neuronal viability [44]. This appears to be due to the low catalase activity in cultured neurones, which also rely on astrocytes to a major extent for the provision of GSH or its precursors [33, 44, 16]. Depletion of glial GSH levels may render neurones susceptible to oxidative stress, since culturing mesencephalic neurones with glutathione-depleted astrocytes results in increased neuronal susceptibility to H_2O_2 stress and reduced neuronal survival [44]. In addition, although GSH depletion *in vivo* does not cause neuronal death, it does potentiate the toxicity of neurotoxins, including MPTP/MPP^+ and 6-hydroxydopamine [46, 47]. Thus, based on this and previous studies, depletion of glial GSH levels in substantia nigra in PD may act as a susceptibility factor, predisposing neurones to oxidative stress and mitochondrial dysfunction.

MPP^+, the active metabolite of MPTP, inhibits complex I activity, thereby exerting its cytotoxicity. In this study, inhibition of complex I activity by MPP^+ caused accumulation of extracellular glutamate, an effect also caused by rotenone (data not shown). Glutamate accumulation may contribute to the neurotoxicity of MPTP and the neurodegenerative process in PD. Indeed, the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 attenuates neuronal death caused by MPTP in primates [48]. However, the role of glutamate-mediated excitotoxicity in the neurodegenerative process in PD is controversial, since glutamate levels are not elevated in the disease. Nevertheless, recent studies

suggest that physiological levels of glutamate are sufficient to cause neuronal death [14]. It is postulated that inhibition of mitochondrial function, as occurs in substantia nigra in PD, leads to ATP depletion, alteration in membrane function, loss of voltage-dependent Mg^{2+} blockade of the NMDA receptor, and neuronal supersensitivity to physiological levels of glutamate. Thus, inhibition of glial complex I activity and a continuous release of small amounts of glutamate may contribute to neuronal death in PD. Because glutamate also stimulates NO production, this suggests that a cycle of NO release, mitochondrial inhibition, and glutamate release may contribute to the neurodegenerative process in PD. We also showed that inhibition of complex I activity in astrocytes resulted in the extracellular accumulation of H_2O_2. This observation is consistent with findings that inhibition of complex I in isolated mitochondria leads to the leakage of superoxide radicals from the respiratory chain which may be converted by SOD to H_2O_2 [41]. Since SOD activity is increased in substantia nigra, this mechanism may play an important role in the nigral degenerative process in PD [6].

Since GSH depletion, complex I inhibition, and glial activation occur simultaneously in substantia nigra in PD, the effects of a combination of such alterations on the extracellular accumulation of NO, H_2O_2, and glutamate in astrocytic cultures were examined. LPS activation of GSH-depleted or complex I-inhibited astrocytes caused a reduction in NO and H_2O_2 levels, compared to LPS activation alone. The mechanism of this is unknown, but may result from the rapid reaction of NO with superoxide radicals to produce peroxynitrite, which occurs faster than the clearance of H_2O_2 by catalase [42]. This observation is important, since increased levels of 3-nitrotyrosine, the reaction product of peroxynitrite with tyrosine residues, occurs in Lewy bodies in substantia nigra in PD [49]. Interestingly, inhibition of complex I activity in glutathione-depleted astrocytes resulted in a marked elevation of H_2O_2 levels, which may reflect the inability of other antioxidant defence systems (e.g. catalase) to counteract increased free radical generation in the presence of MPP^+-induced mitochondrial dysfunction plus glutathione depletion.

In conclusion, the findings in this study suggest that glutathione-depleted, mitochondria-impaired, or activated glial cells may contribute to neuronal, death via the extracellular accumulation of reactive oxygen/nitrogen species and glutamate. Indeed, we showed recently in another investigation that culturing LPS-activated astrocytes with dopaminergic neurones caused neuronal death via the release of NO, H_2O_2, and glutamate from astrocytes in rat brain astrocytic/ventral mesencephalic co-cultures [50]. In addition, we showed that although L-BSO-induced glutathione depletion or MPP^+-mediated complex I inhibition did not cause neuronal death in co-cultures, these glial dysfunctions increased (via release of free radicals and glutamate) the vulnerability of neurones to toxicity caused by neurotoxins such as 6-hydroxydopamine and MPP^+ [50]. Thus, altered glial function may contribute to the development or progression of nigral dopaminergic death in PD.

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