

Dextromethorphan Protects Dopaminergic Neurons against Inflammation-Mediated Degeneration through Inhibition of Microglial Activation

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ABSTRACT

Inflammation in the brain has increasingly been recognized to play an important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease. Inflammation-mediated neurodegeneration involves activation of the brain's resident immune cells, the microglia, which produce proinflammatory and neurotoxic factors, including cytokines, reactive oxygen intermediates, nitric oxide, and eicosanoids that impact on neurons to induce neurodegeneration. Hence, identification of compounds that prevent microglial activation may be highly desirable in the search for therapeutic agents for inflammation-mediated neurodegenerative diseases. In this study, we report that dextromethorphan (DM), an ingredient widely used in antitussive remedies, reduced the inflammation-mediated degeneration of dopaminergic neurons through inhibition of microglial activation. Pretreatment (30 min) of rat mesencephalic neuron-glia cultures with DM (1–10 μ M) reduced, in a dose-dependent manner, the microglia-mediated degeneration of dopaminergic neurons in-

duced by lipopolysaccharide (LPS, 10 ng/ml). Significant neuroprotection by DM was also evident when DM was applied to cultures up to 60 min after the addition of LPS. The neuroprotective effect of DM was attributed to inhibition of LPS-stimulated microglial activation because DM significantly inhibited the LPS-induced production of tumor necrosis factor- α , nitric oxide, and superoxide free radicals. This conclusion was further supported by the finding that DM failed to prevent 1-methyl-4-phenylpyridinium- or β -amyloid peptide (1–42)-induced dopaminergic neurotoxicity in neuron-enriched cultures. In addition, because LPS did not produce any significant increase in the release of excitatory amino acids from neuron-glia cultures and *N*-methyl-D-aspartate antagonist dizocilpine maleate failed to afford significant neuroprotection, it is unlikely that the neuroprotective effect of DM is mediated through *N*-methyl-D-aspartate receptors. These results suggest that DM may be a promising therapeutic agent for the treatment of Parkinson's disease.

Degeneration of dopaminergic neurons in the substantia nigra and dopamine-containing nerve fibers in the striatum is a pathological hallmark of Parkinson's disease (PD). The cause and underlying mechanism responsible for the progressive neurodegeneration of sporadic PD remain unclear (Olanow and Tatton, 1999). Inflammation in the brain, characterized by the activation of microglia and astroglia, has been closely associated with the pathogenesis of PD, as well as with several other degenerative neurological disorders, including Alzheimer's disease (McGeer et al., 1988; McGeer and McGeer, 1995; Dickson et al., 1993; Giulian 1999; Liu and Hong, 2003). Microglia, the resident immune cells in the brain, serve the role of immune surveillance under normal

conditions (Kreutzberg, 1996). Under pathological conditions, microglia become activated and produce a variety of factors, including cytokines and reactive oxygen and nitrogen species. Accumulation of these proinflammatory and cytotoxic factors is deleterious to neurons (Chao et al., 1992; Boje and Arora 1992; Jeohn et al., 1998; McGuire et al., 2001; Liu et al., 2002a). Dopaminergic neurons, in particular, are especially vulnerable to oxidative damage due to their reduced antioxidant capacity and potential defect in mitochondrial function (Jenner and Olanow, 1996; Greenamyre et al., 1999). Because the midbrain region that encompasses the substantia nigra is particularly rich in microglia (Kim et al., 2000), activation of nigral microglia and release of neurotoxic factors may be a crucial component of the degenerative process of dopaminergic neurons in PD.

In the mesencephalic mixed neuron-glia cultures, stimulation of microglia with an inflammagen lipopolysaccharide

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ABBREVIATIONS: PD, Parkinson's disease; LPS, lipopolysaccharide; TNF α , tumor necrosis factor- α ; IL, interleukin; NO, nitric oxide; MPP⁺, 1-methyl-4-phenylpyridinium; DA, dopamine; TH, tyrosine hydroxylase; ir, immunoreactive; SOD, superoxide dismutase; HBSS, Hanks' balanced salt solution; XO, xanthine oxidase; NMDA, *N*-methyl-D-aspartate.

(LPS) induces the production of factors that include tumor necrosis factor- α (TNF α), interleukin 1- β (IL-1 β), nitric oxide (NO), and superoxide. Studies have attributed the accumulation of those factors to the degeneration of dopaminergic neurons (Kim et al., 2000; Liu et al., 2000a, 2002a; Gayle et al., 2002; Gao et al., 2002a). Intranigral infusion or in utero administration of LPS in rats results in significant degeneration of nigral dopaminergic neurons and depletion of the striatal content of dopamine (Castano et al., 1998; Lu et al., 2000, 2002b; Gao et al., 2002b; Ling et al., 2002). Therefore, those in vitro and in vivo models of inflammation-mediated dopaminergic neurodegeneration are powerful tools for mechanistic studies and the identification of potential therapeutic agents.

DM is a dextrorotatory morphinan and is widely used as a nonopioid cough suppressant in a variety of over-the-counter remedies (Tortella et al., 1989). The exact mechanism of action of its antitussive activity, however, remains unclear. Nevertheless, studies using animal models of cerebral ischemia and hypoglycemic neural injuries have demonstrated that DM possesses neuroprotective activity (George et al., 1988; Monyer and Choi, 1988; Prince and Feeser, 1988; Steinberg et al., 1988; Britton et al., 1997; Tortella et al., 1999). Attempts to attribute the neuroprotective activity of DM to antagonism of glutamate receptors have been complicated by conflicting reports about its ability to prevent glutamate excitatory toxicity (Choi, 1987; DeCoster et al., 1995; Lesage et al., 1995; Berman and Murray, 1996; Haberecht et al., 1997). Nevertheless, the effect of DM on the degeneration of dopaminergic neurons has not been studied.

In this study, using primary mesencephalic neuron-glia cultures, we demonstrated that DM significantly reduced the LPS-induced degeneration of dopaminergic neurons. The neuroprotective effect of DM was related to its inhibition of the LPS-induced activation of microglia and the production of TNF α , NO, and superoxide.

Materials and Methods

Reagents. DM hydrobromide and 1-methyl-4-phenylpyridinium (MPP⁺) were purchased from Sigma-Aldrich (St. Louis, MO). Amyloid- β peptide (1–42) was obtained from American Peptide Co., Inc. (Sunnyvale, CA). Cell culture ingredients were obtained from Invitrogen (Carlsbad, CA). [³H]Dopamine (DA, 30 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA). The monoclonal antibody against the CR3 complement receptor (OX-42) was obtained from BD Pharmingen (San Diego, CA). The polyclonal anti-tyrosine hydroxylase (TH) antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA).

Rat Mesencephalic Neuron-Glia Cultures. Primary mesencephalic neuron-glia cultures were prepared from the brains of embryonic day 14/15 Fischer 344 rats, following our previously described protocol (Liu et al., 2002b; Qin et al., 2002). Briefly, the ventral mesencephalic tissues were removed and dissociated by a mild mechanical trituration. Cells were seeded at 5×10^5 /well to 24-well culture plates precoated with poly-D-lysine (20 μ g/ml) and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in 0.5 ml/well maintenance medium. The medium consisted of minimum essential medium containing 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum, 1 g/l glucose, 2 mM [SCAP]L-glutamine, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Three

days after the initial seeding, 0.5 ml of fresh maintenance medium was added to each well. For superoxide assays, 10⁵ cells in 0.1 ml of maintenance medium were seeded to each well of poly-D-lysine-coated 96-well culture plates with 0.1 ml/well fresh maintenance medium supplemented 3 days later. Seven-day-old cultures were used for treatment. The composition of the cultures at the time of treatment was approximately 48% astrocytes, 11% microglia, 40% neurons, and 1 to 1.5% TH-immunoreactive (ir) neurons.

Primary Mesencephalic Neuron-Enriched Cultures. Mid-brain neuron-enriched cultures were established as described previously (Gao et al., 2002a; Qin et al., 2002). Briefly, 24 h after seeding the cells, cytosine β -D-arabinofuranoside was added to a final concentration of 10 μ M to suppress glial proliferation. Three days later, cultures were changed back to maintenance medium and were used for treatment 7 days after initial seeding.

Primary Microglia-Enriched Cultures. Rat microglia-enriched cultures, with a purity of >98%, were prepared from whole brains of 1-day-old Fischer 344 rat pups, following our described protocol (Liu et al., 2002b; Qin et al., 2002). For superoxide assays, 10⁵ cells/well/0.2 ml medium were grown overnight in 96-well culture plates before use.

Analysis of Neurotoxicity. Degeneration of dopaminergic neurons was assessed by measuring the ability of cultures to take up [³H]DA, counting the number of TH-ir neurons after immunostaining. In addition, the average dendrite length of TH-ir neurons was measured as described previously (Liu et al., 2002b).

Uptake Assay. [³H]DA uptake assays were performed as described previously (Liu et al., 2002b). Cultures were incubated for 20 min at 37°C with 1 μ M [³H]DA in Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold Krebs-Ringer buffer, cells were collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific DA uptake observed in the presence of mazindol (10 μ M) was subtracted.

Immunostaining. Dopaminergic neurons were recognized with the anti-TH antibody and microglia were detected with the OX-42 antibody, which recognizes the CR3 receptor as described previously (Liu et al., 2002b; Qin et al., 2002). Briefly, formaldehyde (3.7%) fixed cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution (30 min), primary antibody (overnight, 4°C), biotinylated secondary antibody (2 h), and ABC reagents (40 min). Color was developed with 3,3'-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN) operated with the MetaMorph software (Universal Imaging Corporation, Downingtown, PA). For visual counting of TH-ir neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100 \times magnification. To measure the average TH-ir dendrite, 50 TH-ir representative neurons in each well were selected and three wells for each treatment condition were selected.

Nitrite and TNF α Assays. The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent, and release of TNF α was measured with a rat TNF α enzyme-linked immunosorbent assay kit from R & D Systems (Minneapolis, MN), as described previously (Liu et al., 2002b; Qin et al., 2002).

Superoxide Assay. The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1 (Peskin and Winterbourn, 2000; Tan and Berridge, 2000). Neuron-glia or microglia-enriched cultures in 96-well culture plates were washed (2 \times) with Hanks' balanced salt solution without phenol red (HBSS). Cultures were then incubated at 37°C for 30 min with vehicle control (water) or DM in HBSS (50 μ l/well). Afterward, to each well was added 50 μ l of HBSS with and without SOD (50 U/ml, final concentration), 50 μ l of

WST-1 (1 mM) in HBSS, and 50 μ l of vehicle or LPS (10 ng/ml). Thirty minutes later, absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The difference in absorbance observed in the absence and presence of SOD was considered to be the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Xanthine Oxidase (XO) Activity. To determine whether DM acts as a superoxide scavenger, the superoxide-generating xanthine/XO system was used (McCord and Fridovich, 1968). Briefly DM, XO (10 mU; Sigma-Aldrich), and WST-1 (1 mM) were mixed in potassium phosphate buffer (50 mM, pH 7.6) in a quartz cuvette (1 cm). Xanthine (50 μ M, final concentration) was added to initiate the reaction (final volume, 1 ml). Absorbance at 450 nm was continuously monitored at 5-s intervals for 3 min. Results are expressed as a percentage of the increase in absorbance per minute observed with XO only.

Statistical Analysis. The data were expressed as the mean \pm S.E.M. Statistical significance was assessed with an analysis of variance followed by Bonferroni's *t* test using the StatView program (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Effect of DM on LPS-Induced Degeneration of Dopaminergic Neurons. Mesencephalic neuron-glia cultures were pretreated for 30 min with vehicle or 1 to 10 μ M DM before treatment with 10 ng/ml LPS. Seven days later, the degeneration of dopaminergic neurons was assessed. [³H]DA uptake assays indicated that LPS treatment reduced the uptake capacity to 37% of that of the vehicle-treated control cultures (Fig. 1A). DM significantly attenuated the LPS-induced decrease in DA uptake, in a dose-dependent manner. The lowest effective concentration of DM was 2.5 μ M, and DA uptake of cultures pretreated with 10 μ M before LPS stimulation was 71% of that of control cultures. DA uptake of cultures treated with 10 μ M DM alone did not differ significantly from that of control cultures (Fig. 1A). Counting the number of TH-ir neurons revealed that LPS treatment reduced the number of TH-ir neurons by 71% compared with vehicle-treated control cultures (Fig. 1B). DM (10 μ M) significantly attenuated the LPS-induced reduction in the number of TH-ir neurons (Fig. 1B). Morphologically, in addition to the reduction in abundance of TH-ir neurons, the dendrites of the remaining TH-ir neurons in the LPS-treated cultures was significantly less elaborate than that of the control cultures (Fig. 2). In cultures pretreated with DM (10 μ M) before LPS stimulation, TH-ir neurons were significantly more numerous with the TH-ir dendrites less affected compared with the LPS-treated cultures (Fig. 2). Although the average dendrite length of TH-ir neurons in the LPS-treated cultures was 5.2% of that of control cultures, that of the cultures pretreated with DM before LPS stimulation was 81% of control.

In addition to the determination of the effect of pretreatment with DM on the LPS-induced dopaminergic neurodegeneration, the effect of post-treatment was also examined. To this end, neuron-glia cultures were either treated with DM (10 μ M) and LPS (10 ng/ml) at the same time or DM (10 μ M final concentration) was added 30, 60, 120, or 180 min after the addition of LPS (10 ng/ml). Seven days later, DA uptake of the cultures was determined. Significant neuroprotection was observed in cultures with DM added up to 60 min after the addition of LPS (Fig. 3).

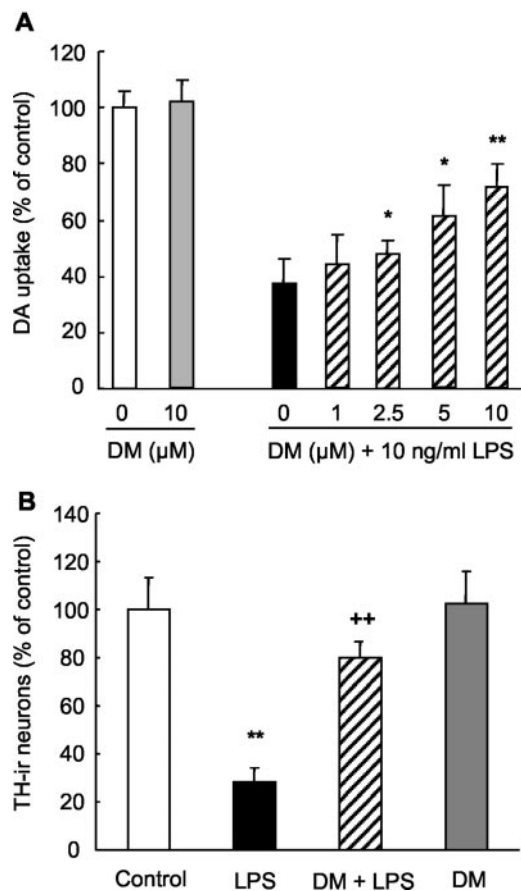


Fig. 1. Effect of DM on LPS-induced degeneration of dopaminergic neurons in mesencephalic neuron-glia cultures. Cultures were treated with vehicle alone, 10 μ M DM alone, or pretreated for 30 min with indicated concentrations of DM before treatment with 10 ng/ml LPS. Seven days later, neurotoxicity was assessed by DA uptake (A) or counting of TH-ir neurons after immunostaining with an anti-TH antibody (B) as described under *Materials and Methods*. Results in A are expressed as a percentage of the control cultures and are the mean \pm S.E.M. of five experiments performed in triplicate. **, $p < 0.001$; *, $p < 0.01$ compared with the control cultures. Results in B are the mean \pm S.E.M. of three experiments performed in triplicate. **, $p < 0.005$ compared with the control cultures; ++, $p < 0.005$ compared with the LPS-treated cultures.

Effect of DM on Neurodegeneration Induced by MPP⁺ or A β (1–42). To investigate whether the neuroprotective activity of DM was dependent on the presence of glial cells, we determined the effect of DM on the degeneration of dopaminergic neurons in neuron-enriched cultures after treatment with MPP⁺ or A β (1–42). As shown in Fig. 4, 5 days after treatment with 2 μ M MPP⁺ or 8 μ M A β (1–42), DA uptake was reduced by 92 and 60%, respectively, compared with the control cultures. Pretreatment of the neuronal cultures with 10 μ M DM before MPP⁺ or A β (1–42) did not significantly alter the magnitude of the MPP⁺- or A β (1–42)-induced reduction in DA uptake (Fig. 4). These results suggested that the neuroprotective effect of DM was mediated through the activity of glial cells.

Activation of glia, especially that of microglia, mediates the LPS-induced neurodegeneration (Bronstein et al., 1995; Araki et al., 2001; Gao et al., 2002b). Activated microglia secrete a variety of neurotoxic factors and TNF α , NO, and superoxide seem to be key mediators of dopaminergic neurodegeneration (Liu et al., 2000a; McGuire et al., 2001; Gao et al., 2002b; Gayle et al., 2002; Liu et al., 2002b; Qin et al.,

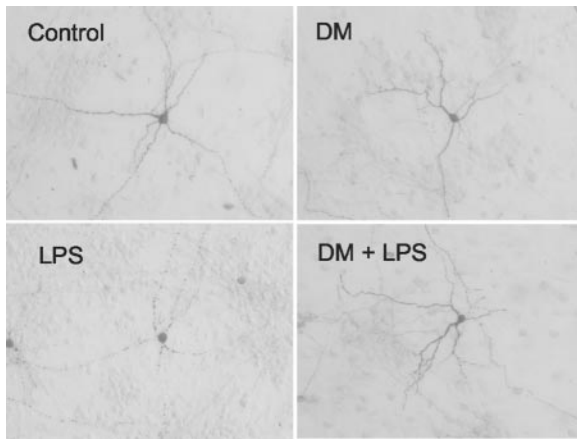


Fig. 2. Immunocytochemical analysis of the effect of DM on LPS-induced degeneration of dopaminergic neurons. Neuron-glia cultures were treated with vehicle alone, 10 μ M DM alone, 10 ng/ml LPS, or pretreated for 30 min with 10 μ M DM followed by treatment with 10 ng/ml LPS. Seven days later, cultures were immunostained with anti-TH antibody. Images shown are representative of three separate experiments.

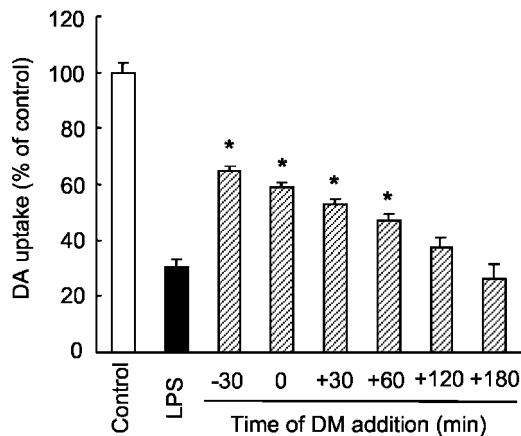


Fig. 3. Effect of post-treatment with DM on LPS-induced dopaminergic neurodegeneration. DM (10 μ M final concentration) was added 30 min before, at the same time, or 30 to 180 min after the addition of LPS (10 ng/ml final concentration). As controls, cultures were treated with vehicle alone or 10 ng/ml LPS alone. Seven days later, DA uptake was measured. Results are expressed as a percentage of the control cultures and are the mean \pm S.E.M. of three experiments. *, $p < 0.05$ compared with the control cultures.

2002). Therefore, we determined the effect of DM on the LPS-stimulated production of $\text{TNF}\alpha$, NO, and superoxide in neuron-glia- or microglia-enriched cultures. Release of $\text{TNF}\alpha$ from neuron-glia cultures was determined at 6, 24, and 48 h after LPS stimulation. As shown in Fig. 5A, the amount of $\text{TNF}\alpha$ in cultures pretreated with 10 μ M DM decreased by 22 to 24% compared with cultures treated with LPS alone (Fig. 5A). Accumulation of nitrite, an indicator of LPS-stimulated production of NO, was determined at 24, 48, and 72 h after LPS stimulation. As shown in Fig. 5B, in cultures pretreated with 10 μ M DM before stimulation with LPS, the level of NO was reduced by 30 to 41% between the 24- and 72-h time points (Fig. 5B).

The effect of DM on LPS-stimulated production of superoxide was determined in both the neuron-glia and microglia-enriched cultures. As shown in Fig. 6A, DM dose dependently inhibited the LPS-stimulated production of superoxide in neuron-glia cultures. Significant inhibition was observed in cultures pretreated with 2.5 to 10 μ M DM and a near com-

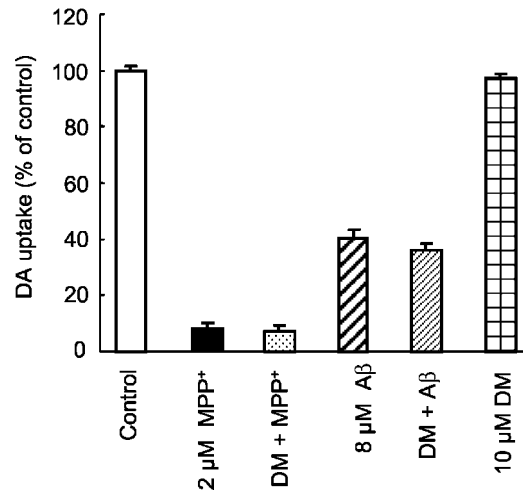


Fig. 4. Effect of DM on MPP^+ or $\text{A}\beta$ (1-42)-induced dopaminergic neurodegeneration in neuron-enriched cultures. Midbrain neuron-enriched cultures were pretreated for 30 min with 10 μ M DM followed by treatment with 2 μ M MPP^+ or 8 μ M $\text{A}\beta$ (1-42). Five days later, DA uptake assay was performed. Results are expressed as a percentage of the control cultures and are the mean \pm S.E.M. of four experiments. $\text{A}\beta$, $\text{A}\beta$ (1-42).

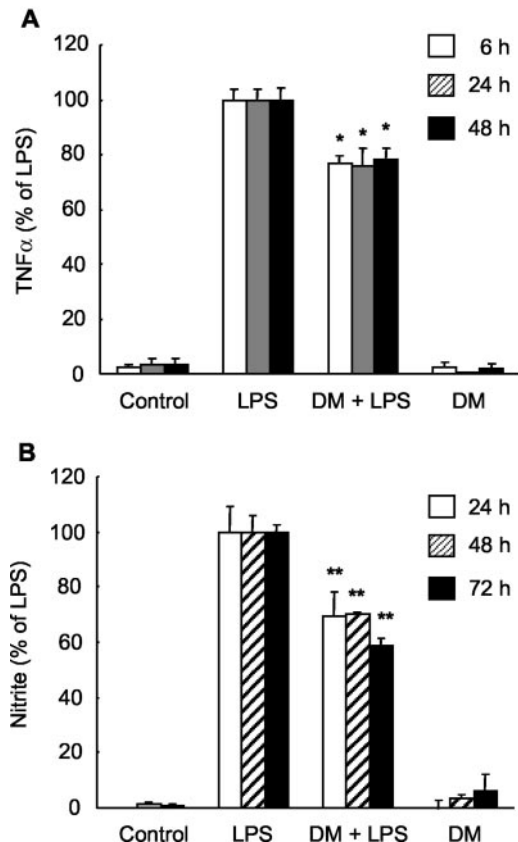


Fig. 5. Effect of DM on LPS-induced release of $\text{TNF}\alpha$ and production of NO. Neuron-glia cultures were pretreated for 30 min with vehicle or the indicated concentrations of DM before stimulation with 10 ng/ml LPS. Supernatants were removed at 6, 24, and 48 h for the measurement of $\text{TNF}\alpha$, and at 24, 48, and 72 h for NO. The results are expressed as a percentage of the control cultures and are the mean \pm S.E.M. of five experiments for $\text{TNF}\alpha$ and four experiments for nitrite. *, $p < 0.05$; **, $p < 0.005$ compared with the LPS-treated cultures.

plete inhibition of LPS-stimulated superoxide production was observed in cultures pretreated with 10 μ M DM (Fig. 6A). In microglia-enriched cultures, pretreatment with 1 to

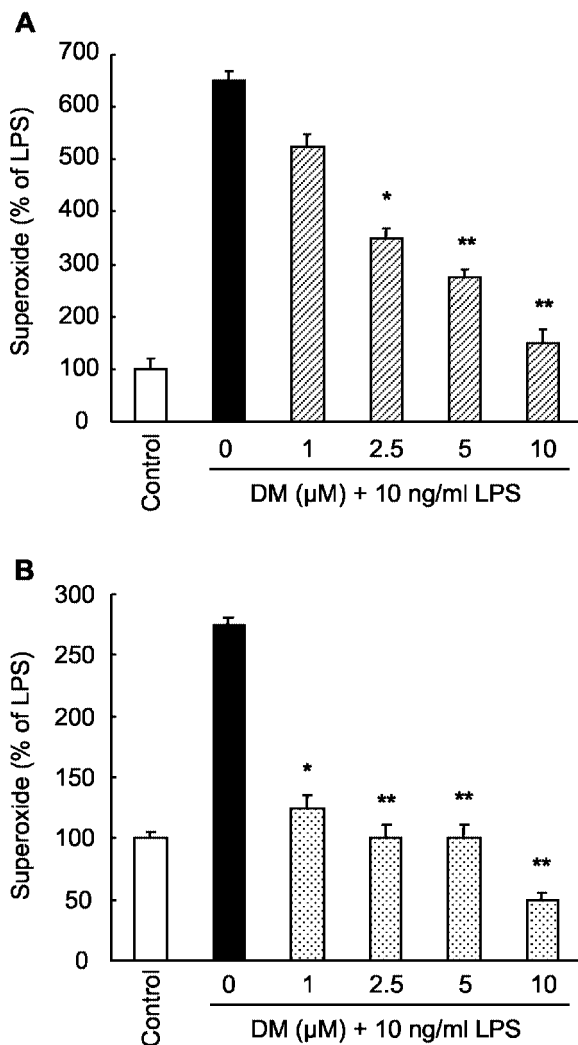


Fig. 6. Effect of DM on LPS-induced generation of superoxide. Mesencephalic neuron-glia cultures (A) or microglia-enriched cultures (B) were pretreated for 30 min with indicated concentrations of DM before stimulation with 10 ng/ml LPS. Production of superoxide was measured as SOD-inhibitable reduction of the tetrazolium salt WST-1 as described under *Materials and Methods*. The results are expressed as a percentage of the control cultures and are the mean \pm S.E.M. of three experiments in A and of two experiments in B. *, $p < 0.05$ compared with the control cultures; +, $p < 0.05$ compared with the LPS-treated cultures.

10 μ M DM significantly inhibited LPS-stimulated production of superoxide (Fig. 6B).

Effect of DM on Superoxide Generation by Xanthine/XO. The xanthine/XO superoxide-generating system has been widely used to determine the superoxide scavenging activity of agents of interest (Wang et al., 1999). The production of superoxide by the xanthine/XO system was very sensitive to the presence of SOD (Fig. 7A). However, DM (1–10 μ M) did not have any effect on the xanthine/XO-driven superoxide generation capacity (Fig. 7B), indicating that at 1 to 10 μ M, DM did not act as a superoxide scavenger.

DISCUSSION

Degeneration of the nigrostriatal dopaminergic pathway is a hallmark of PD. LPS-induced degeneration of dopaminergic neurons in mesencephalic neuron-glia cultures is a useful in vitro model for the identification of potential therapeutic

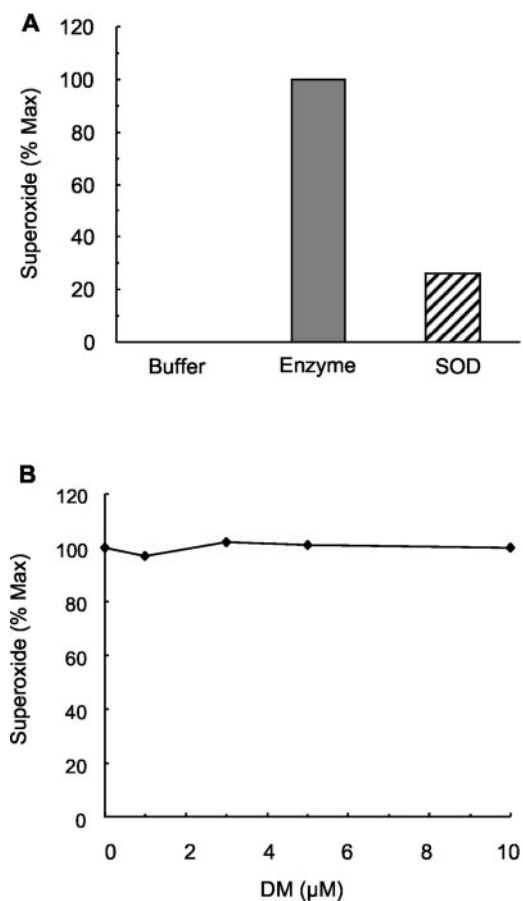


Fig. 7. Lack of effect of DM on superoxide generation by the xanthine/xanthine oxidase system. Xanthine oxidase was coincubated with SOD (A) or indicated concentrations of DM (B) and xanthine. Production of superoxide was measured using WST-1 as described under *Materials and Methods*. The results are expressed as a percentage of the activity observed in the presence of xanthine oxidase and are the mean of duplicate determination from one experiment. Similar results were obtained in a separate experiment.

agents for the treatment of inflammation-mediated neurodegeneration. In this study, we show that DM afforded significant protection of dopaminergic neurons against LPS-induced degeneration. The neuroprotective effect of DM might be related to its ability to reduce the production, by activated microglia, of neurotoxic factors such as $\text{TNF}\alpha$, NO, and especially superoxide.

Inflammation-related neurodegeneration is mediated through the activation of glial cells and the production of proinflammatory and cytotoxic factors. In the brain, two types of glial cells, astroglia and microglia, are the main players in the neuroinflammatory process (Aloisi, 1999). Astroglia serve to maintain homeostasis and secrete neurotrophic factors to promote neuronal survival. Microglia, on the other hand, play a role of immune surveillance under normal conditions, and become readily activated in response to infections and neuronal injuries under pathological conditions (Kreutzberg, 1996). Activated microglia produce a wide array of factors, including cytokines, reactive oxygen species, reactive nitrative species such as NO, and eicosanoids. Compared with activated microglia, both the repertoire and quantity of proinflammatory and cytotoxic factors produced by activated astrocytes are rather limited (Liu et al., 2002a). Neverthe-

less, the accumulative impact of these factors on neurons can eventually lead to neuronal death (Liu and Hong, 2003).

Of the numerous neurotoxic factors produced by activated microglia, the consequences of overproduction of $\text{TNF}\alpha$, NO, and superoxide free radicals have been relatively well studied. McGuire et al. (2002) demonstrated that $\text{TNF}\alpha$ was capable of inducing the death of cultured dopaminergic neurons. Excessive accumulation of NO has long been known to be toxic to neurons (Chao et al., 1992; Dawson et al., 1994; Bronstein et al., 1995; Jeohn et al., 2000; Liu et al., 2002a). The overproduction of free radicals is especially deleterious to neurons (Cadet and Brannock, 1998; Floyd, 1999). Moreover, oxygen free radicals such as superoxide can react with NO to form much more deadly intermediates such as peroxynitrite (Beckman et al., 1990; Estevez and Jordan, 2002). In fact, a recent study has identified peroxynitrite as a key mediator of neurotoxicity induced by LPS- or $\text{A}\beta$ (1–42)-activated microglia (Xie et al., 2002). Several studies have shown that factors produced by activated microglia may work together to induce neurodegeneration. For example, Chao et al. (1995) have demonstrated that $\text{TNF}\alpha$ and IL-1 exert a synergistic neurotoxicity (1995). Similarly, Jehon et al. (1998) have shown that the combination $\text{TNF}\alpha$, IL-1 β , and interferon- γ , but not comparable concentrations of these factors individually, was toxic to cultured cortical neurons. Therefore, neurodegeneration in such diseases as PD is, at least in part, induced by the combined impact of multiple factors generated from activated microglia, and to a lesser extent, activated astroglia. Hence, agents that are capable of inhibiting the production of multiple factors such as NO, $\text{TNF}\alpha$, and superoxide in activated microglia may be highly relevant to the development of potential therapeutic agents. In our studies, we have shown that the production of NO, $\text{TNF}\alpha$, and superoxide by LPS-activated microglia is significantly inhibited by DM and inhibition of the production of these factors confers significant protection to dopaminergic neurons against inflammation-mediated degeneration.

In this study, DM seems to be significantly more potent in inhibiting LPS-induced superoxide production than the production of NO and $\text{TNF}\alpha$ (Figs. 5 and 6). The DM-induced reduction in superoxide was due to an inhibition of production but not scavenging of the superoxide free radical (Fig. 7). This result may imply that LPS-induced superoxide generation may play a more critical role than other microglia-originated factors in the induction of dopaminergic neurodegeneration. In fact, in mesencephalic neuron-glia cultures stimulated with very low concentrations of LPS (<1 ng/ml), production of superoxide, but not NO and $\text{TNF}\alpha$, seems to mediate LPS-induced dopaminergic neurotoxicity (Gao et al., 2002a). Interestingly, both the pesticide rotenone and $\text{A}\beta$ (1–42) exhibited significantly enhanced neurotoxicity toward mesencephalic or cortical neurons in the presence of microglia. The elevated neurotoxicity was attributed to the activation of microglia and production of superoxide free radicals (Gao et al., 2002b; Qin et al., 2002). Furthermore, LPS or $\text{A}\beta$ (1–42) failed to stimulate astroglia to produce superoxide (Qin et al., 2002). It is possible that agents that have a preferential inhibitory activity toward free radical generation may prove to be very effective in providing neuroprotection in the context of inflammation-mediated degeneration. The inhibitory and neuroprotective profile of DM seems to be similar to that of naloxone stereoisomers. Naloxone is more

effective in the inhibition of superoxide generation than $\text{TNF}\alpha$, NO, or IL-1 β (Simpkins et al., 1985; Chang et al., 2000; Liu et al., 2000a, 2002b). The neuroprotective effect of naloxone has been observed in both the in vitro and in vivo models of inflammation-mediated neurodegeneration (Lu et al., 2000; Liu et al., 2000a,b, 2002b). It will be important to determine whether the neuroprotective effect of DM can be observed in animal models of inflammation-mediated neurodegenerative diseases.

Over the years, a number of studies have reported that DM has neuroprotective effects (Choi 1987; Steinberg et al., 1988; George et al., 1988; Prince and Feeser, 1988; Monyer and Choi, 1988; DeCoster et al., 1995; Lesage et al., 1995; Berman and Murray, 1996; Britton et al., 1997; Haberecht et al., 1997). The mechanism of action responsible for this neuroprotective activity has been attributed to DM's potential antagonistic effect on the NMDA receptor complex. However, the discovery of high (nanomolar) and low (micromolar) affinity binding sites for DM in the central nervous system has made it more difficult to explain many of its observed beneficial effects (Craviso and Musacchio, 1983a,b). In this study, the neuroprotective effect of DM was most likely mediated by the inhibition of LPS-induced microglial activation and production of neurotoxic factors, including $\text{TNF}\alpha$, NO, and superoxide. In other words, glutamate-mediated excitatory neurotoxicity probably played little role in the inflammation-mediated neurodegenerative process. High-performance liquid chromatography analysis indicated that the levels of excitatory amino acids such as glutamate and aspartate in the supernatants of LPS-treated neuron-glia cultures were $\leq 5 \mu\text{M}$ (data not shown). It is generally accepted that levels of glutamate as high as 100 to 1000 μM are required to induce significant neuronal death (Obrenovitch et al., 2000). Furthermore, dizocilpine maleate, an effective NMDA antagonist, failed to afford significant protection against LPS-induced dopaminergic neurodegeneration (data not shown). Therefore, it is unlikely that the neuroprotective effect of DM is mediated by a blockade of NMDA receptors.

In addition to the inhibition of superoxide generation, DM was also capable of reducing NO production. Preliminary studies indicated that the inhibitory effect on NO production seemed to be due to a direct effect of DM on the enzymatic activity of inducible nitric-oxide synthase but not on LPS-induced expression of iNOS gene in microglia (data not shown). Furthermore, investigation on the precise mechanism of action responsible for the inhibitory effect of DM on superoxide generation, NO synthesis, and $\text{TNF}\alpha$ release are certainly warranted. Nevertheless, the observation that DM is capable of inhibiting LPS-induced microglial activation and reducing the production of proinflammatory and cytotoxic factors may provide insight into the potential novel effect of DM as a neuroprotective agent.

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