Edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one), a Radical Scavenger, Prevents 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Induced Neurotoxicity in the Substantia Nigra but Not the Striatum

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ABSTRACT

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes nigrostriatal dopaminergic neurotoxicity and behavioral impairment in rodents, and previous studies suggest that nitric oxide and reactive oxygen species are involved in MPTP-induced neurotoxicity. The present study examines the effect of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a radical scavenger, on MPTP-induced neurotoxicity in the striatum and substantia nigra pars compacta (SNc) of C57BL/6J mice. MPTP treatment (10 mg/kg s.c. with 2-h intervals) decreased dopamine levels and tyrosine hydroxylase immunostaining in the striatum and SNc. Pretreatment with edaravone (1 and 3 mg/kg i.p.) significantly reduced the neurotoxicity in the SNc but not striatum. An immunohistochemical study showed that MPTP caused microglial activation both in the striatum and SNc, whereas it increased 3-nitrotyrosine immunoreactivity, an in vivo biomarker of peroxynitrite production, in the SNc but not the striatum. Furthermore, MPTP increased lipid peroxidation product thiobarbituric acid reactive substance in the midbrain, but not the striatum. Edaravone inhibited activation of the microglia and the increased 3-nitrotyrosine immunoreactivity in the SNc but not the striatum, and it also inhibited thiobarbituric acid reactive substance levels in the midbrain. Behavioral analyses showed that edaravone improved MPTP-induced impairment of locomotion and Rotorod performance. These results suggest that edaravone protects against MPTP-induced neurotoxicity in the SNc by blocking the production of reactive oxygen species or peroxynitrite and imply that dopaminergic degeneration in the SNc may play an important role in MPTP-induced motor dysfunction of mice.
than the wild-type littermates. These findings suggest that reactive oxygen species and nitric oxide might participate in the dopaminergic neurotoxicity in MPTP-induced Parkinson's disease model. In this line, many antioxidants (radical scavengers) such as bromocriptine (Muralikrishnan and Mohanakumar, 1998), cytoines (Ferger et al., 1998), deprenyl (Ebadi et al., 2002), and salicylic acid (Mohanakumar et al., 2000) have been reported to protect against MPTP-induced neurotoxicity. However, there are discrepant reports concerning the effect of the antioxidant melatonin on MPTP-induced dopaminergic neurotoxicity; some authors have reported that the antioxidant protects against MPTP-induced dopaminergic neurodegeneration (Acuna-Castroviejo et al., 1997; Antolin et al., 2002; Thomas and Mohanakumar, 2004; Chen et al., 2005), whereas others have reported that it does not (Izhak et al., 1998; Morgan and Nelson, 2001).

Edaravone is a potent scavenger of hydroxy radicals and nitric oxide (Satoh et al., 2002), and it has been demonstrated to be beneficial for patients with acute ischemic stroke (Houkin et al., 1998; Yoneda et al., 2003). Banno et al. (2005) revealed that edaravone suppressed the production of nitric oxide and reactive oxygen species by activated microglia. We have recently reported that edaravone protected methamphetamine-induced striatal dopaminergic neurotoxicity by scavenging peroxynitrite (Kawasaki et al., 2006). These findings suggest that edaravone may be effective in improving reactive oxygen species- or nitric oxide-mediated neurodegenerative disorders. To further support this idea, we examined the effect of edaravone on MPTP-induced dopaminergic neurotoxicity in the substantia nigra pars compacta (SNc) and striatum and MPTP-induced behavioral impairment in C57BL/6J mice. This study shows that the mechanism of MPTP-induced neurotoxicity differs between the SNc and striatum and that edaravone protects against MPTP-induced neurotoxicity in the SNc but not the striatum.

Materials and Methods

Animals. Male C57BL/6J 6-week old mice (Japan SLC, Shizuoka, Japan) were housed in cages (24 \times 17 \times 12 cm) in groups of five to six under controlled environmental conditions (22 ± 1°C; 12-h light/dark cycle, lighting at 8:00 AM; food and water ad libitum) for at least 1 week before use in the experiments. The procedures to handle the animals and their care were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Drugs and Treatments. The following drugs were used: MPTP (Sigma-Aldrich, St. Louis, MO) and edaravone (Mitsubishi Pharma Co., Osaka, Japan). The primary antibodies used were as follows: rabbit polyclonal antibodies against tyrosine hydroxylase, 3-nitrotyrosine (Chemicon, Temecula, CA), and rabbit polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1) (Wako, Osaka, Japan). Vectastain ABC standard kit (Vector Laboratories, Burlingame, CA) was used for immunodetection. All other commercially available chemicals used in the experiments were of superfine quality. Edaravone was dissolved in 1 N NaOH, adjusted to pH 7.4 with 1 N HCl, and diluted with saline to make 0.2 and 0.6 mg/ml solutions. MPTP was dissolved in saline to make a 2 mg/ml solution. These drugs were injected at a fixed volume of 5 ml/kg body weight. Seven- to 8-week-old mice were s.c. administered with 10 mg/kg MPTP four times at 2-h intervals. Edaravone (1 and 3 mg/kg) was i.p. administered 30 min before every MPTP dosing. The experiments of microglial activation, lipid peroxidation, peroxynitrite production, and motor functions were performed 1 day after the last MPTP dosing, and those of dopamine assay and tyrosine hydroxylase immunostaining were performed 3 days after the last MPTP dosing. Most mice used for behavioral experiments were used for the experiments of microglial activation and peroxynitrite production at 1 day after MPTP and for the experiments for dopamine assay and tyrosine hydroxylase immunostaining at 3 days after MPTP. Microglial activation and peroxynitrite production were measured in the same mice, and lipid peroxidation was measured in the separate mice.

Measurement of Dopamine Levels. The concentrations of dopamine were quantified by high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD-100; Eicom, Kyoto, Japan) (Kawasaki et al., 2006). The brains of mice were quickly removed and dissected on an ice-cold glass plate. The striatum and midbrain were individually isolated, frozen on dry ice, and stored at −80°C until assay. Tissue samples were homogenized in 0.2 M perchloric acid containing 100 μM EDTA and iso-proterenol as an internal standard. The homogenate was centrifuged at 15,000g for 15 min at 0°C. The supernatant was filtered through a 0.22-μm membrane filter (Millipore, Tokyo, Japan), and then a 10-μl aliquot of the sample was injected onto the HPLC column every 30 min for dopamine assay. An Eicom SC-5ODS column (3.0 mm i.d. × 150 mm; Eicom) was used, and the potential of the graphite electrode (Eicom) was set to +750 mV against an Ag/AgCl reference electrode. The mobile phase contained 0.1 M sodium acetate/0.1 M citrate buffer, pH 3.5, 190 mg/l octanesulfonic acid, 5 mg/l EDTA, and 17% (v/v) methanol. Values are expressed as nanograms per gram or micrograms per gram of tissue (wet weight).

Measurement of 1-Methyl-4-phenylpyridinium Ion Levels. The 1-methyl-4-phenylpyridinium ion (MPP+) levels were determined according to the HPLC with UV detection method devised by Przedborski et al. (1996). One and 3 h after a single dosing of MPTP (10 mg/kg s.c.), mice were sacrificed, and the striatum and midbrain were dissected. Tissue samples were homogenized in 0.1 M perchloric acid containing 100 μM EDTA. After centrifugation (15,000g for 15 min at 0°C), 10 μl of the supernatant was injected onto a C18-reverse-phase column (Symmetry C18, 5 μm, 4.6 × 150 mm; Waters, Milford, MA). The mobile phase [50 mM potassium phosphate buffer, pH 3.5, 190 mg/l octanesulfonic acid, 5 mg/l EDTA, and 17% (v/v) acetonitrile] was delivered at a flow rate of 1.0 ml/min. The UV detector was set to 295 nm. Data were calculated by an external standard calibration. Values are expressed as nanograms per milligram of tissue (wet weight).

Immunohistochemistry. The brains were quickly removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 days and then transferred to 15% sucrose in 0.1 M phosphate buffer for 2 days. Serial 20-μm-thick coronal sections containing the midstriatum (+1.2 through −0.1 mm with respect to bregma) and midbrain (−3.4 through −3.64 mm with respect to bregma) were cut using a cryostat microtome at −20°C. The free-floating sections were preincubated for 15 min in 0.3% hydrogen peroxide in 100 mM phosphate-buffered saline containing 0.3% Triton X-100 (PBS-T). The sections were washed in PBS-T and then incubated with antibodies against tyrosine hydroxylase (1:1000), Iba-1 (1:5000), and 3-nitrotyrosine (1:100) in PBS-T overnight at room temperature. Subsequent primary incubation sections were washed in PBS-T and incubated in a secondary antibody solution containing biotinylated anti-rabbit IgG (Vector) in PBS-T for 2 h at room temperature. The sections were then incubated with the avidin-biotin peroxidase complex (1:2000) in PBS-T for 1 h at room temperature. Visualization was performed using 50 mM Tris-HCl buffer, pH 7.6, containing 0.02% 3,3′-diaminobenzidine and 0.005% hydrogen peroxide with 0.6% nickel ammonium sulfate. The sections were dehydrated with ethanol (70, 80, 90, and 100%, xylene) and then mounted and coverslipped. Immunostaining images in the dorsomedial regions of the midstratum sections were collected and analyzed using the NIH Image software package (Bethesda, MD). To quantify the optical density of tyrosine hydroxylase immunostaining and the number of Iba-1-immunoreactive microglia, samples from a 1.0-mm² area of the dorsomedial regions of the midstratum were counted from six inde-
dependent sections of the right hemisphere, generating an average count for each treated subject. The optical density of tyrosine hydroxylase-positive dopaminergic neurons in the striatum was expressed as percent change from basal levels, with 100% defined as the average count for vehicle-treated control mice. The number of tyrosine hydroxylase-immunopositive neurons in the SNc was quantified by counting blindly from six independent sections of the right hemisphere. To quantify the microglial activation in the SNc, we used a semiquantitative scoring system with scores from 1 to 3 to evaluate differences between the treatment groups, as previously reported (Pierri et al., 2005). A blinded observer performed the scoring. Score 1 was given when increased immunoreactivity was observed in the absence of morphologically activated microglia. Score 2 was given when in addition to the criteria for score 1, a number of individually distribution activated microglia were seen. Score 3 was given when in addition to the previous mentioned criteria (scores 1 and 2), clusters of activated microglia were observed.

**Measurement of Lipid Peroxidation.** Lipid peroxidation was assessed by determining the concentrations of thiobarbituric acid reactive substances (TBARS). The TBARS determination was performed spectrophotometrically (Munoz et al., 2006). The sample of brain tissue (i.e., striatum or ventral midbrain) was homogenized with three volumes of KCl [1.15% (w/v)] containing butylated hydroxytoluene (200 μM). An aliquot of the resulting sample was treated with SDS [8% (w/v)] followed by acetic acid (20%), and the mixture was vortexed for 1 min. Thiobarbituric acid (0.8%) was then added, and the resulting mixture was incubated at 95°C for 60 min. After cooling at room temperature, 3 ml of n-butanol was added, and the mixture was shaken vigorously. After centrifugation at 1500 g for 5 min, absorbance of the supernatant (organic layer) was measured at 532 nm using a Shimadzu UV-1650PC spectrophotometer (Kyoto, Japan).

**Rotarod Test.** The Rotarod (Neuroscience Inc., Tokyo, Japan) consisted of a rotating rod (2.8-cm diameter) and individual compartments for each mouse. Mice were trained for 2 consecutive days prior to MPTP dosing in an acceleration mode (2–16 rpm) for over 2 min. The training was repeated at a fixed speed (16 rpm) until the mice were able to stay on the rod for at least 600 s. On day 1 after the MPTP dosing, the mice were assessed for their coordination capability on the rod at 16 rpm for a maximum recording time of 600 s. The Rotarod test was performed twice every 30 min, and the results were averaged to obtain a single value for each mouse.

**Spontaneous Locomotor Activity.** Locomotor activity of the mice was measured using a digital counter system with an infrared sensor (Supermex; Muromachi Kikai, Tokyo, Japan) 1 day after MPTP dosing. Each mouse was placed in a plastic cage (24 × 17 × 24 cm), and then locomotor activity in each 10 min period was measured for 60 min.

**Statistical Analysis.** Data are presented as mean ± S.E.M. or median and range. Data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey-Kramer’s test or the Kruskal-Wallis test followed by a Mann-Whitney U test. Statistical analyses were performed using a software package Statview 5.0J for the Apple Macintosh computer (SAS Institute Inc., Cary, NC). Values of 5% or less were considered statistically significant.

**Results**

**Effect of Edaravone on MPTP-Induced Decrease in Dopamine Levels in the Striatum and Midbrain.** Figure 1 shows the effects of MPTP and edaravone on dopamine levels in the striatum and midbrain of mice. Repeated administration of MPTP (10 mg/kg, four times at 2-h intervals) caused a marked reduction in the striatal dopamine levels 3 days after the last MPTP dosing. Pretreatment with edaravone (1 and 3 mg/kg) did not attenuate MPTP-induced decrease in the striatal dopamine levels. Two-way ANOVA revealed the main significant effect of MPTP [F(1,42) = 327.217, P < 0.0001] but not of edaravone [F(2,42) = 0.654, N.S.]. There was no significant interaction between MPTP and edaravone [F(2,42) = 0.607, N.S.]. The effect of edaravone against MPTP-induced dopaminergic neurotoxicity in the ventral midbrain was also examined. The dopamine levels in the midbrain were significantly reduced in MPTP-treated mice 3 days after the last injection of MPTP. Edara-
vone at doses of 1 and 3 mg/kg significantly attenuated MPTP-induced reduction in dopamine levels in the midbrain, although edaravone alone did not affect the dopamine levels. Two-way ANOVA revealed the main significant effect of MPTP \( F(1,47) = 24.934, P < 0.0001 \) but not of edaravone \( F(2,47) = 2.290, \text{N.S.} \). There was a significant interaction between MPTP and edaravone \( F(2,47) = 4.939, P = 0.0113 \).

**Effect of Edaravone on MPTP-Induced Reduction of Tyrosine Hydroxylase Immunostaining in the Striatum and SNc.** We also examined nigrostriatal dopaminergic function by assessing the expression of tyrosine hydroxylase, an immunohistochemical marker for dopaminergic neurons (Figs. 2 and 3). Figure 2 shows the effects of MPTP and edaravone on tyrosine hydroxylase immunostaining in the striatum. Representative photomicrographs of tyrosine hydroxylase staining in the striatum 1 day after the last MPTP dosing are shown in Fig. 2A. Figure 2B shows the densitometric analysis of tyrosine hydroxylase immunostaining in the striatum. Repeated MPTP administration significantly reduced the tyrosine hydroxylase immunostaining in dopaminergic terminals. Pretreatment with edaravone (3 mg/kg) did not affect MPTP-induced reduction of tyrosine hydroxylase immunostaining. Two-way ANOVA revealed the main significant effect of MPTP \( F(1,20) = 269.782, P < 0.0001 \) but not of edaravone \( F(1,20) = 2.435, \text{N.S.} \). There was no significant interaction between MPTP and edaravone \( F(1,20) = 4.881, \text{N.S.} \). Figure 3 shows the effects of MPTP and edaravone on tyrosine hydroxylase immunostaining in the SNc. Representative photomicrographs of tyrosine hydroxylase immunostaining in the SNc 3 days after the last MPTP dosing are shown in Fig. 3A. Figure 3B shows the count of tyrosine hydroxylase-immunoreactive neurons in the SNc. Repeated MPTP administration caused a marked reduction in the number of tyrosine hydroxylase-immunoreactive neurons in the SNc, and this effect was attenuated by pretreatment with edaravone (3 mg/kg). Two-way ANOVA revealed the main significant effects of MPTP \( F(1,20) = 131.826, P < 0.0001 \) and edaravone \( F(1,20) = 17.240, P = 0.0005 \). There was a significant interaction between MPTP and edaravone \( F(1,20) = 19.403, P = 0.0003 \).

**Effect of Edaravone on MPTP-Induced Microglial Activation in the Striatum and SNc.** Figure 4 shows the effect of edaravone on MPTP-induced activation of microglia in the striatum of mice. Microglial activation was assessed by staining fixed brain sections with an antibody against Iba-1, a marker of activated microglia. Representative photomicrographs and quantitative results of Iba-1 immunostaining in the striatum 1 day after the last MPTP dosing are shown (Fig. 4A). Edaravone (3 mg/kg) alone did not affect the number of activated microglia. Repeated MPTP administration caused a robust increase in the number of activated microglia. Two-way ANOVA revealed the main significant effect of MPTP \( F(1,20) = 176.977, P < 0.0001 \) but not of edaravone \( F(1,20) = 2.023, \text{N.S.} \) (Fig. 4B). Pretreatment with edaravone was administered i.p. 30 min before every MPTP dosing. Mice were sacrificed 1 day after the last MPTP dosing. A, representative photomicrographs of tyrosine hydroxylase staining in the SNc are shown. Scale bar, 200 \( \mu \text{m} \). B, tyrosine hydroxylase immunostaining in the SNc was quantified in each group. The number of tyrosine hydroxylase-immunopositive neurons is shown. Results are expressed as the mean ± S.E.M. of six mice. **, \( P < 0.01 \) compared with the vehicle/vehicle treatment group; ###, \( P < 0.01 \) compared with the MPTP/vehicle treatment group.

**Figure 3.** Effect of edaravone on MPTP-induced decrease in the immunoreactivity for tyrosine hydroxylase in the substantia nigra. Mice were administered 10 mg/kg MPTP s.c. four times at 2-h intervals. Vehicle or 3 mg/kg edaravone was administered i.p. 30 min before every MPTP dosing. Mice were sacrificed 3 days after the last MPTP dosing. A, representative photomicrographs of tyrosine hydroxylase staining in the SNc are shown. Scale bar, 200 \( \mu \text{m} \). B, quantitative results of Iba-1 immunostaining in the SNc were quantified in each group. The number of tyrosine hydroxylase-immunopositive neurons is shown. Results are expressed as the mean ± S.E.M. of six mice. **, \( P < 0.01 \) compared with the vehicle/vehicle treatment group.

**Figure 4.** Effect of edaravone on MPTP-induced activation of microglia in the striatum. Mice were administered with 10 mg/kg MPTP s.c. four times at 2-h intervals. Vehicle or 3 mg/kg edaravone was administered i.p. 30 min before every MPTP dosing. Mice were sacrificed 1 day after the last MPTP dosing. A, representative photomicrographs of Iba-1 immunostaining in the striatum are shown. Scale bar, 100 \( \mu \text{m} \). B, quantitative results of Iba-1 immunostaining in each group are shown. Results are expressed as the mean ± S.E.M. of six mice. **, \( P < 0.01 \) compared with the vehicle/vehicle treatment group.
vone (3 mg/kg) did not affect MPTP-induced activation of microglia in the striatum \( F(1,20) = 0.943, \text{N.S.} \). Figure 5 shows the effect of edaravone on MPTP-induced activation of microglia in the SNc. Representative photomicrographs of Iba-1 immunostaining are shown in Fig. 5A. We observed changes in cellular morphology, such as enlarged cell bodies and thickening of their processes. The microglial response was evaluated by the semiquantitative scoring system described by Pierri et al. (2005) (Fig. 5B) because it was difficult to count activated microglia in the SNc. Kruskal-Wallis test revealed the significant effect of the treatment \( P < 0.001 \). Few morphologically activated microglia were expressed in vehicle-treated mice (median, 1; range, 1–2). Administration of MPTP displayed clusters of activated microglia in the SNc (median, 3; range, 2–3; \( P < 0.01 \) versus vehicle-treated controls). Pretreatment with edaravone (3 mg/kg) attenuated significantly the MPTP-induced activation of microglia in the SNc (median, 1.5; range, 1–2; \( P < 0.01 \) versus MPTP-treated controls). Edaravone (3 mg/kg) alone did not cause a significant change in the morphology of the microglia (median, 1; range, 1–2).

**Effect of Edaravone on MPTP-Induced Lipid Peroxidation and Peroxynitrite Production.** Figure 6 shows the effect of edaravone on MPTP-induced lipid peroxidation and peroxynitrite production in mouse brain. The level of TBARS is an index of lipid peroxidation (Fig. 6A), and for-

![Fig. 5. Effect of edaravone on MPTP-induced activation of microglia in the substantia nigra. Mice were administered with 10 mg/kg MPTP s.c. four times at 2-h intervals. Vehicle or 3 mg/kg edaravone was administered i.p. 30 min before every MPTP dosing. Mice were sacrificed 1 day after the last MPTP dosing. A, representative photomicrographs of Iba-1 immunostaining in the SNc are shown. Scale bar, 200 μm; inset, 100 μm. B, qualitative results of Iba-1 immunostaining in each group are shown. The microglial activation in the SNc was assessed using microglia score (see Materials and Methods). Horizontal lines, median values. **, \( P < 0.01 \) compared with the vehicle/vehicle treatment group; ##, \( P < 0.01 \) compared with the MPTP/vehicle treatment group.

![Fig. 6. Effect of edaravone on MPTP-induced lipid peroxidation and peroxynitrite production in the mouse brain. Mice were administered with 10 mg/kg MPTP s.c. four times at 2-h intervals. Vehicle or 3 mg/kg edaravone was administered i.p. 30 min before every MPTP dosing. Mice were sacrificed 1 day after the last MPTP dosing. Lipid peroxidation (A) was assessed by the formation of TBARS in the striatum and ventral midbrain. Results are expressed as the mean ± S.E.M. of six mice. **, \( P < 0.01 \) compared with the vehicle/vehicle treatment group; ##, \( P < 0.01 \) compared with the MPTP/vehicle treatment group. Representative photomicrographs of 3-nitrotyrosine immunostaining (B) in the striatum (a–c) and SNc (d–f) are shown. Scale bar, 200 μm; inset, 100 μm. Results are representative of three independent experiments.
mation of 3-nitrotyrosine is an index of peroxynitrite production (Fig. 6B). Both indices are indicated as markers of oxidative stress status. Repeated MPTP administration induced a marked increase in the production of TBARS in the ventral midbrain \[F(1,20) = 23.076, P = 0.0001\] but not the striatum \[F(1,20) = 1.361, N.S.] 1 day after the last injection of MPTP. Edaravone (3 mg/kg) significantly inhibited the TBARS production in the ventral midbrain. Two-way ANOVA revealed a significant interaction between MPTP and edaravone \[F(1,20) = 15.128, P = 0.0009\]. The 3-nitrotyrosine immunoreactivity was markedly increased in the SNc by MPTP treatment (Fig. 6Be), whereas edaravone did not affect the 3-nitrotyrosine immunoreactivity in the striatum (Fig. 6Bb). Pretreatment with 3 mg/kg edaravone inhibited the effect of MPTP on 3-nitrotyrosine formation in the SNc (Fig. 6Bd). In addition, edaravone alone did not alter 3-nitrotyrosine immunoreactive levels in the striatum and SNc (data not shown).

**Effects of Edaravone on MPTP-Induced Behavioral Deficits.** Figure 7A shows the effect of edaravone on MPTP-induced locomotor deficits in a novel environment. Administration of MPTP significantly reduced spontaneous locomotor activity in mice \[F(1,92) = 26.496, P < 0.0001\]. Pretreatment with edaravone (3 mg/kg) significantly attenuated MPTP-induced hypolocomotion \[F(2,92) = 5.028, P = 0.0085\], whereas edaravone alone did not alter basal motor activity \[F(2,92) = 2.682, N.S.] (Fig. 7B). Figure 7B shows the effect of edaravone on MPTP-induced loss of motor coordination in the Rotarod test. The Rotarod test is widely used to measure coordinated motor skills that have been employed in the MPTP mouse model (Sedelis et al., 2001). All mice were trained and were able to stay on the rotating rod at a fixed speed of 16 rpm for 600 s. One day after MPTP dosing, the mice spent significantly less time overall on the rotating rod, indicating a loss of motor coordination \[F(1,87) = 8.208, P = 0.0052\]. This Rotarod performance was improved by edaravone at doses of 1 and 3 mg/kg. Two-way ANOVA revealed a significant interaction between MPTP and edaravone \[F(2,87) = 8.739, P = 0.0003\]. Edaravone alone did not affect Rotarod performance.

**Effect of Edaravone on MPP⁺ Levels in the Brain.** To exclude the pharmacokinetic effects of edaravone due to differences in MPTP metabolism, uptake, or elimination, we measured the MPP⁺ levels at different time points after a single administration of MPTP (10 mg/kg) (Table 1). Edaravone (3 mg/kg) did not affect the MPP⁺ levels in these regions at 1 and 3 h after treatment.

**Discussion**

The present study demonstrates that MPTP caused dopaminergic neurodegeneration in the SNc and striatum. MPTP decreased dopamine levels and tyrosine hydroxylase immunostaining in the SNc and striatum and increased Iba-1 immunoreactivity, a marker of activated microglia. Although the degrees of decrease in tyrosine hydroxylase immunostaining and activation of microglia were similar between the SNc and striatum, the degree of decrease in dopamine levels was higher in the striatum than the midbrain, including SNc. In this regard, Kurosaki et al. (2004) reported that MPTP-induced decrease in tyrosine hydroxylase immunostaining was observed in the striatum faster than the SNc. These results suggest that MPTP causes degenerative loss of nigrostriatal dopamine neurons, and the progression of striatal damage worsens to that of nigral damage after MPTP treatment. MPTP increased TBARS levels and 3-nitrotyrosine immunoreactivity, a marker of peroxynitrite, in the SNc but not the striatum. These changes in the SNc were reduced by the radical scavenger edaravone. In addition, edaravone attenuated decreases in dopamine levels and tyrosine hydroxylase immunostaining in the SNc but not the striatum. These findings suggest that reactive oxygen species and nitric oxide

![Fig. 7. Effect of edaravone on MPTP-induced behavioral deficits in mice. Mice were administered with 10 mg/kg MPTP s.c. four times at 2-h intervals. Vehicle or edaravone at doses of 1 and 3 mg/kg was administered i.p. 30 min before every MPTP dosing. One day after the last MPTP dosing, spontaneous locomotor activity in a novel environment (A) and motor coordination on the rotating rod (B) were assessed. n, number of mice used. Results are expressed as the mean ± S.E.M. of 7 to 23 mice. ++, P < 0.01 compared with the vehicle/vehicle treatment group; ##, P < 0.01 compared with the MPTP/vehicle treatment group.](image)

**TABLE 1**

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<th>Treatment</th>
<th>MPP⁺ Levels (ng/mg wet tissue weight)</th>
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<tr>
<td></td>
<td>Striatum: 1 h</td>
<td>Striatum: 3 h</td>
<td>Midbrain: 1 h</td>
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<tr>
<td>MPTP + vehicle</td>
<td>7.26 ± 0.42</td>
<td>2.26 ± 0.11</td>
<td>3.91 ± 0.48</td>
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<tr>
<td>MPTP + edaravone</td>
<td>7.39 ± 0.69</td>
<td>2.19 ± 0.14</td>
<td>4.00 ± 0.48</td>
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play a key role in MPTP-induced dopaminergic degeneration in the SNc, but not in the striatum. The finding that edaravone protects against only the nigral neurotoxicity seems to contrast with the previous observation that the drug blocks the striatal neurotoxicity in methamphetamine-treated mice (Kawasaki et al., 2006). This may be explained by the difference in the mechanism for neurotoxicity between methamphetamine and MPTP models. We have found in the separate experiments that methamphetamine increased TBARS levels in the striatum and edaravone blocked methamphetamine-induced increases in TBARS levels and 3-nitrotyrosine immunoreactivity. Regarding MPTP-induced striatal neurotoxicity, Sriram et al. (2006) reported that tumor necrosis factor-α may play a role in MPTP- or methamphetamine-induced selective degeneration of striatal dopaminergic nerve terminals. The exact mechanism for MPTP-induced neurodegeneration in the SNc and striatum remains to be determined. It should be noted that edaravone attenuated microglial activation in the SNc but not the striatum. In this regard, Hayley et al. (2004) reported that Fas deficiency attenuated MPTP-induced microglial activation in the SNc but not the striatum. These results suggest that MPTP causes microglial activation via different mechanisms in the SNc and striatum.

There are conflicting results on the effect of the antioxidant, melatonin, on MPTP-induced neurotoxicity. The neuroprotective effect of melatonin was demonstrated in the SNc (Antolin et al., 2002; Thomas and Mohanakumar, 2004; Chen et al., 2005), whereas the result was negative in the striatum (Itzhak et al., 1998; Van der Schyf et al., 2000; Morgan and Nelson, 2001). Only one report has shown that melatonin has a protective effect against MPTP-induced neurotoxicity both in the SNc and striatum, but the degree of the protective effect in the striatum was small (Li et al., 2002). The discrepancy may be explained by the difference in the brain regions. This point may be explained by the present finding that the effect of edaravone on MPTP-induced neurotoxicity differs between the SNc and striatum. Edaravone protected MPTP-induced decreases in tyrosine hydroxylase immunostaining in the SNc and dopamine levels in the midbrain but had no effect on those in the striatum. It should be noted that edaravone did not affect MPP⁺ levels in the striatum and SNc. This suggests that the effect of edaravone is not related to the metabolism of MPTP.

Motor impairment is observed only after severe dopamine depletion (70–80%) in the striatum in Parkinson’s disease patients (Riederer and Wuketich, 1976). This is a basis for dopamine replacement therapies in Parkinson’s disease. The sites of action of l-dopa are not only striatal dopaminergic terminals but also the SNc (Robertson and Robertson, 1989; Crocker, 1997; Fox et al., 1998). Previous studies indicate the importance of dopaminergic function in the SNc being preserved in the improvement of MPTP-induced motor dysfunction. Crocker et al. (2003) showed that calpain inhibition attenuated MPTP-induced locomotor deficits without recovering striatal dopamine levels. In addition, Hayley et al. (2004) demonstrated that Fas deficiency attenuated MPTP-induced dopaminergic loss and microglial activation in the SNc but not the striatum. In view of the evidence that nigral dopaminergic neurons release dopamine not only from their axons projecting to the striatum but also their dendrites (Björklund and Lindvall, 1975; Cheramy et al., 1981), Park’s groups (Crocker et al., 2003; Hayley et al., 2004) hypothesized that preservation of the SNc may contribute to improving functional outcome through stimulation of dopamine turnover in the striatum and extrastriatal regulation of the basal ganglia. The hypothesis is consistent with the present finding that edaravone improves MPTP-induced behavioral changes (hypolocomotion and impaired Rotarod performance) and protects against MPTP-induced neurotoxicity in the SNc but not the striatum. Then, it is likely that dopaminergic activity in the SNc plays a key role in edaravone-induced improvement of MPTP-induced motor dysfunction.

In conclusion, the present study shows that MPTP increases the production of reactive oxygen species or nitric oxide and causes microglial activation in the SNc but not the striatum. In addition, the radical scavenger edaravone protects against MPTP-induced neurodegeneration in the SNc but not the striatum and improves MPTP-induced motor dysfunction. The present study in a MPTP-induced dopaminergic degeneration model, together with the recent finding in a methamphetamine-induced model (Kawasaki et al., 2006), suggest that edaravone may be useful for treating Parkinson’s disease and other oxidative damage-related neurodegenerative diseases, although the drug is used for the treatment of acute stroke (Houkin et al., 1998).

References


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