Therapeutic Effect of Neuronal Nitric Oxide Synthase Inhibitor (7-Nitroindazole) against MPTP Neurotoxicity in Mice

Y. Muramatsu,¹ R. Kurosaki,¹ T. Mikami,¹ M. Michimata,¹ M. Matsubara,¹ Y. Imai,¹ H. Kato,² Y. Itoyama,² and T. Araki¹;³

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Effects of neuronal nitric oxide synthase (nNOS) inhibitor (7-nitroindazole), nonselective NOS inhibitor (NG-nitro-L-arginine methyl ester; L-NAME), and monoamine oxidase inhibitor (pargyline) were studied on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. The mice received four intraperitoneal injections of MPTP at 1-h intervals. A significant depletion in dopamine and DOPAC concentration was observed in the striatum from 1 day after MPTP treatment. The pretreatment of 7-nitroindazole and pargyline, but not L-NAME, dose-dependently protected against MPTP-induced depletion in dopamine content 3 days after MPTP treatment. Our histochemical study also showed that 7-nitroindazole and pargyline can prevent a marked decrease in the nigral cells and a marked increase in astrocytes in striatum 7 days after MPTP treatment. The protective effect of 7-nitroindazole against MPTP-induced dopamine and DOPAC depletion in the striatum was not attenuated by intraperitoneal pretreatment with L-arginine. Furthermore, the posttreatment of 7-nitroindazole or pargyline protected against MPTP-induced depletion of dopamine content. These results demonstrate that the protective mechanism by which 7-nitroindazole counteracts MPTP neurotoxicity in mice may be due not only to inhibition of nNOS, but also to MAO-B inhibition. Furthermore, our study suggests that the posttreatment of 7-nitroindazole and pargyline can prevent a significant decrease in dopamine levels in the striatum of MPTP-treated mice. These findings have important implications for the therapeutic time window and choice of nNOS or MAO inhibitors in patients with Parkinson’s disease.

Key words: MPTP; dopamine; 7-nitroindazole; L-NAME; pargyline; immunohistochemistry; mice.

INTRODUCTION

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is well known to produce extensive, relatively selective destruction of dopaminergic neurons in the substantia nigra. This neurotoxin also leads to a decrease of dopamine content in the striatum and a loss in the number of nigrostriatal dopaminergic neurons in several species, including monkeys (Burns et al., 1983; Ferrante et al., 1999; Hantraye et al., 1996) dogs (Johannessen et al., 1991; Rapisardi et al., 1990), cats (Schneider and Markham, 1986), and mice (Arai et al., 1986).
1990; Heikkila et al., 1984). The changes observed in animals after MPTP administration are similar to those found in humans with Parkinson’s disease (Burns et al., 1983). The neurotoxic effects of MPTP are thought to be initiated by MPP⁺, which is a major metabolite formed by the monoamine oxidase (MAO) B-mediated oxidation of MPTP (Tipton and Singer, 1993). MPP⁺ is taken up by high-affinity dopamine and noradrenaline uptake systems and is subsequently accumulated within mitochondria of nigrostriatal dopaminergic cells. There it disrupts oxidative phosphorylation by inhibiting Complex I of the electron transport chain (Gluck et al., 1994). This can lead to a number of deleterious effects on cellular function, resulting in neuronal cell death.

Nitric oxide (NO) has emerged as a key endogenous modulator of brain function. NO is an intracellular and short-lasting second messenger molecule that is synthesized from L-arginine in several tissues by a reaction catalyzed by nitric oxide synthase (NOS) (Dawson et al., 1991; Ignarro, 1990). NOS is not only located in the endothelium, but also in neurons, perivascular nerves, and astrocytes (Bredt et al., 1990; Murphy et al., 1993). The family of NOS, the enzymes that produce NO, consists of two different classes: the inducible and constitutive forms (Dawson and Snyder, 1994; Marletta, 1994). The inducible NOS (iNOS) is not regulated by calcium concentration but is regulated transcriptionally, and is primarily expressed in astrocytes, microglia, and inflammatory cells (Monte et al., 1997; Nathan and Xie, 1994). In contrast, there are two distinct constitutive calcium-dependent NOS (cNOS) isoforms, that is, neuronal NOS (nNOS) form in neurons and endothelial NOS (eNOS) form in pyramidal cells and endothelial cells (Moncada et al., 1991). Thus, NOS is well known to be abundant in brain tissues (Araki et al., 1998, 1999; Kidd et al., 1995; Marletta, 1994).

Much attention has been focused the possible role of NO as a retrograde intracellular messenger mediating cell-to-cell interactions in the brain including the cell-mediated immune system, cerebral smooth muscle relaxation, inhibition of platelet aggregation, learning, and synaptic plasticity (Bredt and Snyder, 1994; Schulz et al., 1995). A number of experimental studies have also indicated that NO and NO donors can enhance the basal release of several neurotransmitters in the mammalian brain, including dopamine, glutamate, and acetylcholine (Lonart and Johnson, 1992; Nathan and Xie, 1994). These observations are of interest in regard to the role of NO as an intracellular messenger in the central nervous system (CNS).

Previous evidence has implicated both oxygen free radicals and NO for neuronal degeneration. The entry of calcium through N-methyl-D-aspartate (NMDA) receptors into cells stimulates NOS activity by binding to calmodulin, a cofactor for NOS (Bredt and Snyder, 1990). Experimental studies in cell cultures showed that NOS inhibitors can block NMDA-induced cell death (Dawson et al., 1991). Furthermore, NO may react with superoxide to generate peroxynitrite (Beckman et al., 1990), which may promote nitration of tyrosine (Beckman et al., 1992; Ischiropoulos et al., 1992) and produce hydroxyl radicals (Beckman et al., 1992; Tipton and Singer, 1993; van der Vliet et al., 1994). Thus, the generation of the free radical nitric oxide followed by production of peroxynitrite has been implicated in cell death (Dawson et al., 1991; Schneider and Markham, 1986).

Several studies previously showed that 7-nitroindazole, a relatively selective inhibitor of the nNOS, can protect against MPTP-induced neurotoxicity in mice or baboons (Bredt et al., 1990; Schneider and Markham, 1986). This was confirmed and extended by showing that mice deficient in the neuronal isoform of NOS are also resistant to MPTP neurotoxicity (Prast and Philippu, 1992). On the other hand, a recent interesting study suggests that the
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The mechanism by which 7-nitroindazole can protect against MPTP neurotoxicity is not solely due to inhibition of the neuronal NOS, but involves a decrease in MPP+ formation, indicating a competitive inhibitor of MAO-B (Castagnoli et al., 1997; Monte et al., 1997). Thus, the actual contribution of NO and the protective mechanism of 7-nitroindazole to MPTP neurotoxicity remain to be determined. In the present study, therefore, we investigated the effect of 7-nitroindazole against MPTP neurotoxicity in mice, in comparison with that of a nonselective NOS inhibitor (L-NAME; Nω-nitro-L-arginine methyl ester) and a MAO-B inhibitor (pargyline).

MATERIALS AND METHODS

Male C57BL/6 mice (Nihon SLC Co., Shizuoka, Japan) at 6 weeks of age were used in this study. The mice received four intraperitoneal injections of MPTP (Sigma) at a dose of 10 mg/kg at 1-h intervals, the total dose per mice being 40 mg/kg, as described previously (Araki et al., 2001a; Tanji et al., 1999). In the present study, there were no dead animals after MPTP treatment. All experiments were performed in accordance with Guidelines for Animal Expereriments of Tohoku University School of Medicine.

The mice were killed by cervical dislocation at 1, 3, and 7 days after MPTP treatment. After decapitation, brains were quickly removed and the two striata were rapidly dissected out freehand on an ice-cold glass Petri dish. Samples were immediately weighted, then frozen and stored at −80°C until assay. The dissection procedure was performed in less than 2 min. Striata were sonicated and homogenized in ice-cold 0.2 M perchloric acid containing 100 ng/mL isoproterenol as an internal standard. The homogenates were centrifuged at 20,000g for 20 min at 4°C. The supernatant was filtered (pore size 0.45 μm, Millipore filter), and a 30-μL aliquot of the supernatant was used for determination of the dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC), and isoproterenol (100 ng/mL) by high-performance liquid chromatography (HPLC), using an electrochemical detector (ECD) (Eicom, Japan). The mobile phase consisted of 0.1 M sodium citrate–0.1 M sodium acetate solution (pH 3.5), including 1.064 M octane sulfonic acid, 0.013 mM Na2 EDTA, and 15% (v/v) methanol. The recoveries of dopamine, DOPAC, and isoproterenol through the present procedures were >93%. Levels of dopamine and its metabolite were calculated from the comparison of simple peak area with an internal standard peak region and are expressed as μg/g tissue weight. Each group contained 6–9 mice. All values were expressed as means ± SE, and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test.

Effect of Pretreatment with L-NAME on MPTP-Treated Mice

The animals were divided into five groups: (1) saline-treated group (n = 6); (2) L-NAME (10 mg/kg)-treated group (n = 5); (3) MPTP- and saline-treated group (n = 6); (4) MPTP- and L-NAME (3 mg/kg)-treated group (n = 6); (5) MPTP- and L-NAME (10 mg/kg)-treated group (n = 6). The mice were injected intraperitoneally (ip) with L-NAME or saline 30 min before and 90 min after the first administration of MPTP (Groups (3)–(5)). For Groups (1) and (2), the saline-treated and L-NAME-treated animals were injected ip in the same manner with saline instead of MPTP. In addition, L-NAME (Sigma) was dissolved in saline.
Effect of Pretreatment with 7-Nitroindazole on MPTP-Treated Mice

The animals were divided into five groups: (1) peanut oil-treated group \((n = 6)\); (2) 7-nitroindazole \((50 \text{ mg/kg})\)-treated group \((n = 5)\); (3) MPTP- and peanut oil-treated group \((n = 6)\); (4) MPTP- and 7-nitroindazole \((30 \text{ mg/kg})\)-treated group \((n = 6)\); (5) MPTP- and 7-nitroindazole \((50 \text{ mg/kg})\)-treated group \((n = 6)\). The mice were injected ip with 7-nitroindazole or peanut oil 30 min before and 90 min after the first administration of MPTP (Groups (3)–(5)). For Groups (1) and (2), the saline-treated or 7-nitroindazole-treated animals were injected ip in the same manner with saline instead of MPTP. In addition, 7-nitroindazole (Sigma) was suspended in peanut oil.

Effect of Pretreatment with Pargyline on MPTP-Treated Mice

The animals were divided into five groups: (1) saline-treated group \((n = 6)\); (2) pargyline \((15 \text{ mg/kg})\)-treated group \((n = 5)\); (3) MPTP- and saline-treated group \((n = 6)\); (4) MPTP- and pargyline \((5 \text{ mg/kg})\)-treated group \((n = 6)\); (5) MPTP- and pargyline \((15 \text{ mg/kg})\)-treated group \((n = 6)\). The mice were injected ip with pargyline or saline 30 min before and 90 min after the first administration of MPTP (Groups (3)–(5)). For Groups (1) and (2), the saline-treated or pargyline-treated animals were injected ip in the same manner with saline instead of MPTP. In addition, pargyline (Sigma) was dissolved in saline.

Therapeutic Effect of 7-Nitroindazole or Pargyline with Posttreatment on MPTP-Treated Mice

Experiment 1. The animals were divided into five groups: (1) saline-treated group \((n = 6)\); (2) MPTP- and peanut oil-treated group \((n = 6)\); (3) MPTP- and 7-nitroindazole \((50 \text{ mg/kg})\)-treated group \((n = 6)\); (4) MPTP- and saline-treated group \((n = 6)\); (5) MPTP- and pargyline \((15 \text{ mg/kg})\)-treated group \((n = 6)\). The mice were injected ip with 7-nitroindazole, peanut oil, pargyline, and saline 2 and 100 min after MPTP treatments (Groups (2)–(5)). For Group (1), the saline-treated animals were injected ip in the same manner with saline instead of MPTP.

Experiment 2. The animals were divided into five groups: (1) saline-treated group \((n = 6)\); (2) MPTP- and peanut oil-treated group \((n = 6)\); (3) MPTP- and 7-nitroindazole \((50 \text{ mg/kg})\)-treated group \((n = 6)\); (4) MPTP- and saline-treated group \((n = 6)\); (5) MPTP- and pargyline \((15 \text{ mg/kg})\)-treated group \((n = 6)\). The mice were injected ip with 7-nitroindazole, peanut oil, pargyline, and saline 3 and 5 h after MPTP treatments (Groups (2)–(5)). For Group (1), the saline-treated animals were injected ip in the same manner with saline instead of MPTP.

Effect of L-Arginine on MPTP-Treated Mice Subjected to the Treatment with 7-Nitroindazole

The animals were divided into six groups: (1) saline-treated group \((n = 6)\); (2) L-arginine \((300 \text{ mg/kg})\)-treated group \((n = 5)\); (3) MPTP- and saline-treated group \((n = 6)\); (4) MPTP- and L-arginine \((300 \text{ mg/kg})\)-treated group \((n = 6)\); (5) MPTP- and
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7-nitroindazole (30 mg/kg)-treated group (n = 6); (6) MPTP- and 7-nitroindazole (50 mg/kg)-treated group (n = 6). The mice were injected ip with L-arginine or saline 30 min before and 90 min after the first administration of MPTP (Groups (3)–(5)). For Groups (1) and (2), the saline-treated or pargyline-treated animals were injected ip in the same manner with saline instead of MPTP. In addition, L-arginine (Sigma) was dissolved in saline.

In all experiments, the mice were killed by cervical dislocation at 3 days after MPTP treatment. After decapitation, brains were quickly removed and the two striata were rapidly dissected out freehand on an ice-cold glass Petri dish. Samples were immediately weighed, then frozen and stored at −80°C until assay. As described above, striatal extracts were prepared for monoamine measurements by HPLC. All values were expressed as means ± SE, and statistical significance was evaluated using an analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test.

Immunohistochemistry

The animals were divided into four groups: (1) saline-treated group (n = 5); (2) MPTP- and saline-treated group (n = 5); (3) MPTP- and 7-nitroindazole (50 mg/kg)-treated group (n = 5); (4) MPTP- and pargyline (15 mg/kg)-treated group (n = 5). The mice were injected ip with 7-nitroindazole, saline, or pargyline at 30 min before and 90 min after the first administration of MPTP (Groups (2)–(4)). For Group (1), the saline-treated animals were injected ip in the same manner with saline instead of MPTP.

The mice were anesthetized with sodium pentobarbital (50 mg/kg, ip) at 7 days after MPTP treatments, and the brains were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), following a heparinized saline flush. The brains were removed 1 h after perfusion-fixation at 4°C and were immersed in the same fixative until they were embedded in paraffin. Paraffin sections, 5 μm in thickness, were used for immunohistochemistry. The paraffin sections were also stained with hematoxylin–eosin and cresyl violet.

Tyrosine Hydroxylase (TH) Immunostaining

For TH immunostaining, a polyclonal anti-TH antibody (Chemicon International, Temecula, USA) and a Vectastain elite ABC kit (Vector Lab., Burlingame, USA) were used. The paraffin sections were washed for 5 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 0.3% hydrogen peroxidase in 10% methanol. The paraffin sections were then washed three times for 5 min in 0.01 M PBS, followed by 30 min of preincubation with 10% normal horse serum. The brain sections were then incubated with anti-TH antibody (1:200) including 0.3% Triton X-100 overnight at 4°C. After a 15-min rinse in changes of PBS, the sections were incubated with biotinylated secondary antibody for 2 h and then with avidin–biotin peroxidase complex for 30 min at room temperature. Immunoreactions were visualized using 0.05% diaminobenzidine and 0.01% hydrogen peroxidase in 0.05% Tris-HCl buffer (pH 7.6). Negative control sections were treated in the same way as described above, except that the antibody against anti-TH was omitted. The TH-positive neurons were observed under light microscopy at a magnification of 400×.
Glial Fibrillary Acidic Protein (GFAP) Immunostaining

For GFAP immunostaining, a polyclonal anti-GFAP antibody (Labsystems, Helsinki, Finland) and a Vectastain elite ABC kit (Vector Lab., Burlingame, USA) were used. The paraffin sections were washed for 5 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and treated with 0.3% hydrogen peroxidase in 10% methanol. The paraffin sections were then washed three times for 5 min in 0.01 M PBS, followed by 30 min of preincubation with 10% normal horse serum. The brain sections were then incubated with anti-GFAP antibody (1:200) including 0.3% Triton X-100 overnight at 4°C. After a 15-min rinse in changes of PBS, the sections were incubated with biotinylated secondary antibody for 2 h and then with avidin–biotin peroxidase complex for 30 min at room temperature. Immunoreactions were visualized using 0.05% diaminobenzidine and 0.01% hydrogen peroxidase in 0.05% Tris-HCl buffer (pH 7.6). Negative control sections were treated in the same way as described above except that the antibody against anti-GFAP was omitted. The GFAP-positive neurons were observed under light microscopy at a magnification of 400×.

RESULTS

Content of Dopamine and Its Metabolite (DOPAC)

Four injections of MPTP in mice caused a marked depletion in dopamine and DOPAC levels of the striatum. The striatal dopamine and DOPAC levels were significantly reduced from 1 day after MPTP treatment. Thereafter, the striatal dopamine and DOPAC levels were markedly decreased at 3 and 7 days after MPTP treatment as shown in Fig. 1. The depletion in striatal dopamine and DOPAC concentrations reached a maximal level at 3 days after MPTP treatment as described previously (Araki et al., 2000).

Figure 1. Levels of dopamine and DOPAC in the striatum after MPTP treatment. Control: saline-treated mice; 1 day: mice 1 day after MPTP treatment; 3 days: mice 3 days after MPTP treatment; 7 days: mice 7 days after MPTP treatment. Values are expressed as the mean ± SE *P < 0.05,** P < 0.01, compared with saline-treated group (Dunnett’s multiple range test). n = 6–9 mice.
Effect of Pretreatment with L-NAME, 7-Nitroindazole and Pargyline on the MPTP-Induced Decrements in the Striatal Levels of Dopamine and DOPAC

Pretreatment with 7-nitroindazole dose-dependently prevented the significant reductions in dopamine and DOPAC levels observed in the striatum of mice 3 days after MPTP treatment. In contrast, L-NAME did not affect the striatal dopamine and DOPAC depletion 3 days after MPTP treatment. Furthermore, L-NAME at a higher dose showed no significant change in dopamine and DOPAC levels in mice that received saline instead of MPTP, as compared with MPTP + saline group. Pargyline also prevented a significant decrease in dopamine levels in the striatum of mice 3 days after MPTP treatment in a dose-dependent manner. Pargyline at a higher dose also showed a significant increase in dopamine and DOPAC levels in mice that received saline injections instead of MPTP, as compared with MPTP + saline group (Table 1).

Therapeutic Effect of 7-Nitroindazole or Pargyline on the MPTP-Induced Decrements in the Striatal Levels of Dopamine and DOPAC

7-Nitroindazole at a dose of 50 mg/kg prevented a significant reduction in dopamine and DOPAC levels in the striatum of mice, when this compound was administered 2 and 100 min after MPTP treatment. Pargyline at a dose of 15 mg/kg also prevented a significant decrease in dopamine levels in the striatum of mice. However, pargyline showed no significant

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<tr>
<th>Table 1. Effects of Pretreatment with 7-Nitroindazole, L-NAME and Pargyline on Striatal Dopamine and DOPAC in MPTP-Treated Mice</th>
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<tbody>
<tr>
<td>Treatments</td>
</tr>
<tr>
<td>Effect of 7-nitroindazole</td>
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<tr>
<td>Vehicle (peanut oil)</td>
</tr>
<tr>
<td>7-Nitroindazole (50 mg/kg)</td>
</tr>
<tr>
<td>MPTP + vehicle (peanut oil)</td>
</tr>
<tr>
<td>MPTP + 7-nitroindazole (30 mg/kg)</td>
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<tr>
<td>MPTP + 7-nitroindazole (50 mg/kg)</td>
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<tr>
<td>Effect of L-NAME</td>
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<tr>
<td>Vehicle (saline)</td>
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<tr>
<td>L-NAME (10 mg/kg)</td>
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<tr>
<td>MPTP + vehicle (saline)</td>
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<td>MPTP + L-NAME (3 mg/kg)</td>
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<tr>
<td>MPTP + L-NAME (10 mg/kg)</td>
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<tr>
<td>Effect of pargyline</td>
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<td>Vehicle (saline)</td>
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<tr>
<td>Pargyline (15 mg/kg)</td>
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<tr>
<td>MPTP + vehicle (saline)</td>
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<tr>
<td>MPTP + pargyline (5 mg/kg)</td>
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<tr>
<td>MPTP + pargyline (15 mg/kg)</td>
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</table>

Note: Values were expressed as mean ± SE. n = 5–6 mice. Drug treatment schedules were described in Materials and Methods section.

*P < 0.05; **P < 0.01 [compared with MPTP + saline group or MPTP + peanut oil group (Dunnett's multiple range test)].
Table 2. Therapeutic Effects of 7-Nitroindazole or Pargyline on Striatal Dopamine and DOPAC in MPTP-Treated Mice

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dopamine (µg/g tissue)</th>
<th>DOPAC (µg/g tissue)</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (saline)</td>
<td>10.00 ± 0.73**</td>
<td>5.49 ± 0.93**</td>
</tr>
<tr>
<td>MPTP + vehicle (peanut oil)</td>
<td>4.24 ± 0.21</td>
<td>1.92 ± 0.15</td>
</tr>
<tr>
<td>MPTP + 7-nitroindazole (50 mg/kg)</td>
<td>8.43 ± 0.85**</td>
<td>4.57 ± 0.59**</td>
</tr>
<tr>
<td>Vehicle (saline)</td>
<td>10.00 ± 0.73**</td>
<td>5.49 ± 0.93**</td>
</tr>
<tr>
<td>MPTP + vehicle (saline)</td>
<td>4.47 ± 0.36</td>
<td>1.72 ± 0.15</td>
</tr>
<tr>
<td>MPTP + pargyline (15 mg/kg)</td>
<td>11.02 ± 0.75**</td>
<td>2.87 ± 0.20</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (saline)</td>
<td>9.77 ± 0.49**</td>
<td>3.79 ± 0.28*</td>
</tr>
<tr>
<td>MPTP + vehicle (peanut oil)</td>
<td>1.51 ± 0.61</td>
<td>2.03 ± 0.54</td>
</tr>
<tr>
<td>MPTP + 7-nitroindazole (50 mg/kg)</td>
<td>2.60 ± 0.20</td>
<td>2.41 ± 0.32</td>
</tr>
<tr>
<td>Vehicle (saline)</td>
<td>9.77 ± 0.49**</td>
<td>3.79 ± 0.93**</td>
</tr>
<tr>
<td>MPTP + vehicle (saline)</td>
<td>1.56 ± 0.62</td>
<td>1.58 ± 0.31</td>
</tr>
<tr>
<td>MPTP + pargyline (15 mg/kg)</td>
<td>3.40 ± 0.29</td>
<td>1.62 ± 0.11</td>
</tr>
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Note: Values were expressed as mean ± SE. n = 6 mice. Drug treatment schedules were described in Materials and Methods section.

* P < 0.05; ** P < 0.01 [compared with MPTP + vehicle group (Dunnett’s multiple range test)].

increase in the striatal DOPAC levels in mice. On the other hand, 7-nitroindazole and pargyline showed no significant effects in MPTP-induced dopamine and DOPAC depletion of mouse striatum, when each compound was administered 3 and 5 h after MPTP treatment (Table 2).

**Effect of L-Arginine on the Striatal Dopamine and DOPAC Levels of MPTP-Treated Mice Subjected to the Treatment with 7-Nitroindazole**

L-Arginine showed no significant change in dopamine and DOPAC levels in mice that received saline injections instead of MPTP, as compared with MPTP+saline group. In contrast, L-arginine exhibited no significant effect in dopamine and DOPAC levels in the striatum of mice 3 days after MPTP treatment. On the other hand, 7-nitroindazole significantly prevented a reduction in dopamine and DOPAC levels in the striatum of mice 3 days after MPTP treatment. The effect of 7-nitroindazole was not attenuated by L-arginine treatment (Table 3).

**Immunohistological Changes of 7-Nitroindazole or Pargyline with Pretreatment on the Substantia Nigra of MPTP-Treated Mice**

Representative microphotographs of TH immunostaining in the substantia nigra are shown in Fig. 2. Dopaminergic neurons with the TH antibody were easily detectable in the substantia nigra in saline-treated mouse brains. In the saline-treated animals, bodies and fiber of dopaminergic cells were intensely stained with evident immunopositive processes.
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Table 3. Effect of L-Arginine on Striatal Dopamine and DOPAC Levels of MPTP-Treated Mice Subjected to the Treatment with 7-Nitroindazole

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dopamine (µg/g tissue)</th>
<th>DOPAC (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (saline)</td>
<td>10.22 ± 0.35**</td>
<td>3.78 ± 0.16*</td>
</tr>
<tr>
<td>L-Arginine (300 mg/kg)</td>
<td>12.51 ± 0.36**</td>
<td>2.43 ± 0.13</td>
</tr>
<tr>
<td>MPTP + vehicle (peanut oil)</td>
<td>2.43 ± 0.42</td>
<td>2.70 ± 0.62</td>
</tr>
<tr>
<td>MPTP + L-arginine (300 mg/kg)</td>
<td>3.85 ± 0.87</td>
<td>3.59 ± 0.53</td>
</tr>
<tr>
<td>MPTP + 7-nitroindazole (50 mg/kg)</td>
<td>8.39 ± 0.30**</td>
<td>5.63 ± 0.30**</td>
</tr>
<tr>
<td>MPTP + 7-nitroindazole (50 mg/kg) + L-arginine (300 mg/kg)</td>
<td>9.39 ± 0.35**</td>
<td>6.08 ± 0.42**</td>
</tr>
</tbody>
</table>

Note: Values were expressed as mean ± SE. n = 5–6 mice. Drug treatment schedules were described in Materials and Methods section.

* P < 0.05; ** P < 0.01 [compared with MPTP + vehicle group (Dunnett’s multiple range test)].

Figure 2. Dopaminergic neurons stained with antibodies for tyrosine hydroxylase (TH) in the substantia nigra of (a) saline-, (b) MPTP-, (c) MPTP + pargyline- and (d) MPTP + 7-nitroindazole-treated mouse. n = 5 mice. Bar = 100 µm. Drug treatment schedules were described in Materials and Methods section.
In contrast, a decrease in the number of these cells was noticed 7 days after MPTP treatment. On the other hand, pretreatment of 7-nitroindazole and pargyline prevented a marked reduction in dopaminergic cells 7 days after MPTP treatment.

**Immunohistological Changes of 7-Nitroindazole or Pargyline with Pretreatment on the Striatum of MPTP-Treated Mice**

Representative microphotographs of GFAP immunostaining in the striatum are shown in Fig. 3. GFAP-positive reactive astrocytes were not likely observed in the striatum of saline-treated mice. Seven days after MPTP treatment, GFAP-positive reactive astrocytes exhibited a ramified form with many fine processes in the striatum and markedly increased in this area. On the other hand, the pretreatment of 7-nitroindazole and pargyline prevented a marked increase in GFAP-positive reactive cells in the striatum 7 days after MPTP treatment.

![Figure 3. Astrocytes stained with antibodies for glial fibrillary acidic protein (GFAP) in the striatum of (a) saline-, (b) MPTP-, (c) MPTP + pargyline- and (d) MPTP + 7-nitroindazole-treated mouse.](image)

$n = 5$ mice. Bar = 50 μm. Drug treatment schedules were described in Materials and Methods section.
DISCUSSION

Recent studies demonstrate that the toxic effect of the parkinsonism-inducing MPTP is mediated through an excessive production of NO (Hantraye et al., 1996; Matthews et al., 1997; Schneider and Markham, 1986). In mice, 7-nitroindazole is known to prevent MPTP-induced striatal dopamine depletion and nigral cell loss (Schneider and Markham, 1986). This compound has also been found to counteract the neurochemical, histological, and behavioral effects of MPTP in baboons (Hantraye et al., 1996). Furthermore, MPTP neurotoxicity is reported to be significantly, but not completely decreased in mutant mice lacking the nNOS gene (Przedborski et al., 1996). These observations have attracted great interest for the contribution of NO to MPTP neurotoxicity. However, a more recent study demonstrates that protective effect of 7-nitroindazole against MPTP neurotoxicity may be mediated through the inhibition of MAO (Monte et al., 1997). Castagnoli et al. (1997) also suggested that 7-nitroindazole can inhibit the MAO-B catalyzed oxidation of MPTP. Therefore, it is conceivable that the inhibition of MAO-B may explain, at least in part, the neuroprotective action of nNOS inhibitor by reducing the MPP\textsuperscript{+} formation from MPTP. These observations seem to suggest that the precise mechanisms for the protection of neuronal NOS inhibitor on MPTP neurotoxicity have not yet been satisfactorily explained.

The present study showed that the pretreatment of 7-nitroindazole can protect against the striatal dopamine and DOPAC depletion in mice 3 days after MPTP treatment. However, the pretreatment of L-NAME did not show the neuroprotective effect on MPTP-induced striatal dopamine depletion (Table 1). On the other hand, the pretreatment of pargyline significantly prevented the striatal dopamine depletion in MPTP-treated mice. In DOPAC levels, however, this drug had no significant effect in MPTP-treated mice (Table 1). Our immunohistochemical study with TH staining also showed that 7-nitroindazole and pargyline can prevent a marked decrease in the nigral cells 7 days after MPTP treatment (Fig. 2). Furthermore, our study showed that 7-nitroindazole and pargyline prevented a marked increase in GFAP-positive cells in the striatum 7 days after MPTP treatment (Fig. 3). These results indicate that neuronal NOS inhibitor and MAO-B inhibitor but not nonselective NOS inhibitor can protect against MPTP-induced striatal dopamine depletion in mice. Furthermore, 7-nitroindazole or pargyline prevented a significant decrease in dopamine levels in the striatum of mice, when each compound was administered 2 and 100 min after MPTP treatment (Table 2). However, the neuroprotection was not apparent when 7-nitroindazole or pargyline was administered 3 and 5 h after MPTP treatment, suggesting there is a temporal window for therapeutic intervention. Therefore, the present findings seem to suggest important implications for the therapeutic time window and choice of nNOS inhibitor or MAO inhibitor in patients with Parkinson’s disease.

To clarify the mechanism of protective effect of 7-nitroindazole against MPTP neurotoxicity, we investigated whether 7-nitroindazole can block MPTP-induced striatal dopamine and DOPAC depletion in mice after L-arginine treatment. We recently reported that the motor deficit induced by 7-nitroindazole was significantly attenuated in mice by the treatment with L-arginine (Araki et al., 2001b). In the present study, however, the neuroprotective effect of 7-nitroindazole against MPTP-induced striatal dopamine and DOPAC depletion was not attenuated by pretreatment with L-arginine, suggesting that inhibition of NO formation may be implicated (Table 3). The results may suggest that nNOS does
not play a key role in neurotoxic processes of MPTP. Interestingly, a recent study demonstrated that the intraperitoneal administration of 7-nitroindazole or pargyline in rats can produce a marked increase in the striatal dopamine content and a significant decrease in the striatal DOPAC concentration. In contrast to 7-nitroindazole and pargyline, however, the intraperitoneal treatment with L-NAME did not change the striatal DOPAC concentration, suggesting a lack of inhibitory MAO properties for this NOS inhibitor, although this drug induced a mild increase in the striatal dopamine content (Desvignes et al., 1999). These observations are, at least in part, consistent with our present results. A recent interesting report has suggested that mutant mice lacking the iNOS gene were significantly more resistant to MPTP than their wild-type littermates (Liberatore et al., 1999). These observations seem to suggest that iNOS is important factor in the MPTP neurotoxic processes. Therefore, inhibitors of iNOS may be also useful in the treatment of Parkinson’s disease.

CONCLUSION

The present study demonstrates that the protective mechanism by which 7-nitroindazole counteracts MPTP neurotoxicity in mice may be due not only to inhibition of nNOS, but also to MAO-B inhibition. Furthermore, our study suggests that the posttreatment of 7-nitroindazole and pargyline can prevent a significant decrease in dopamine levels in the striatum of MPTP-treated mice. These findings have important implications for the therapeutic time window and choice of nNOS or MAO inhibitors in patients with Parkinson’s disease.

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