Enhanced N-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Toxicity in Mice Deficient in CuZn-Superoxide Dismutase or Glutathione Peroxidase

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Abstract. Administration of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mammals causes damage to the nigrostriatal dopaminergic pathway similar to that observed in Parkinson disease (PD). Reactive oxygen species (ROS) are thought to be involved in the pathogenesis of MPTP-mediated dopaminergic neurodegeneration. To further clarify the role of superoxide anion radical ($O_2^-$) and to study the possible involvement of hydroperoxides in MPTP-mediated neurodegeneration, MPTP neurotoxicity was induced in mice deficient in either CuZn superoxide dismutase (SOD), a scavenger enzyme for $O_2^-$, or cellular glutathione peroxidase (GSHPx-1), a scavenger enzyme for hydroperoxides. Littermate control and homozygous deficient mice were injected intraperitoneally with a total cumulative dose of 0, 75, or 150 mg/kg of MPTP delivered over 5 d. All mice were killed 5 d after the last injection and the brains were processed for immunohistological analysis for tyrosine hydroxylase (TH) in the striatum and the substantia nigra pars compacta (SNc), as well as for direct measurements of dopamine concentrations in the striatum. The intensity of TH immunoreactivity in the striatum was evaluated by measuring the relative optical density (OD) with NIH IMAGE, and expressed as Log (OD of striatum)/Log (OD of white matter). Degeneration of TH-containing neurons was assessed by counting TH-positive neurons in the SNc. We found that this MPTP exposure protocol produced dose-dependent depletion of TH immunoreactivity and dopamine in the striatum in littermate control mice and both strains of knockout mice; however, reduction in TH immunoreactivity and dopamine content were significantly greater in CuZn-SOD or GSHPx-1 deficient mice compared with littermate controls. MPTP exposure did not significantly alter the number of TH-positive neurons in the SNc in littermate control or knockout mice. These data suggest that some of the deleterious effects of MPTP on striatal dopaminergic nerve terminals are mediated by both $O_2^-$ and hydroperoxides, and that they occur prior to dopaminergic neurodegeneration in the SNc. The similarity between the MPTP model and PD raises the possibility that both types of ROS may play a significant role in the early pathogenesis of dopaminergic neurodegeneration in PD.

Key Words: Glutathione peroxidase; Knockout mice; MPTP; Oxidative stress; Parkinson disease; Superoxide dismutase.

INTRODUCTION

Parkinson disease (PD) is characterized clinically by bradykinesia, resting tremor, and rigidity, and is due to progressive degeneration of dopaminergic neurons in the zona compacta of the substantia nigra pars compacta (SNc) (1). The causes of dopaminergic neurodegeneration in PD remain unclear. Because of the limitations associated with investigations of postmortem brain samples from PD patients, many investigators have focused their efforts on experimental models of PD such as the one produced by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP exposure causes an irreversible and severe parkinsonism that is clinically and pathologically similar to idiopathic PD, i.e. marked reduction in the dopaminergic neurons within the SNc, decrease in striatal tyrosine hydroxylase (TH) activity, and decreased dopamine (DA) levels in the striatum (2, 3). Although the precise mechanisms by which MPTP destroys dopaminergic neurons remain controversial, the toxic effects of MPTP appear to depend on its biotransformation to N-methyl-4-phenylpyridinium (MPP$^+$) by monoamine oxidase (MAO) (4, 5). MPP$^+$, a potent mitochondrial toxin, is thought to be the active neurotoxin since MAO inhibitors significantly decrease MPTP-induced toxicity in the brain (5) and mice deficient in MAO are resistant to MPTP-mediated neurotoxicity (6).

Damage to mitochondria by MPP$^+$ is thought to involve impairment of energy metabolism leading to increased free radical generation. Increased generation of superoxide anion radical ($O_2^-$) and hydroxyl radical ($OH$) are associated with MPP$^+$ in vitro and with MPTP in vivo (7–9). Furthermore, genetically altered mice with increased CuZn superoxide dismutase (CuZn-SOD) or Mn-SOD are resistant to MPTP-induced neurotoxicity (10, 11). Both CuZn-SOD and Mn-SOD detoxify $O_2^-$.

Animals treated with antioxidants such as vitamin E and desferrioxamine are resistant to MPTP-induced neurotoxicity (12). Finally, pretreatment of animals with diethyl dithiocarbamate (DDTC), an inhibitor of SOD, enhances MPTP toxicity (13), although DDTC has other effects beyond inhibition of SOD.

While the above findings secure a role for reactive oxygen species (ROS) in the pathogenesis of MPTP-induced neurotoxicity, they do not differentiate the relative significance of different ROS. For example, MPTP toxicity may be mediated by $O_2^-$ reacting with nitric oxide (NO$^-$) to form peroxynitrite, leading to protein nitration...
involvement of hydroperoxides such as $H_2O_2$ in the path-
from Sod1
sence of CuZn-SOD or GSHPx-1 activity in brain homogenates
zygous knockout mice and identi®ed by Southern blot analysis
homozygous knockout mice were produced by breeding hetero-
dismutate to hydrogen peroxide ($H_2O_2$), which produces
• OH in the presence of transitional metals such as iron
through Fenton chemistry. • OH is a highly reactive mole-
cule that can inhibit protein function, damage nucleic
acids, and initiate lipid peroxidation (15). It has been
shown that MPTP mediates • OH production in animals
(9) and that PD is clearly associated with an increased
level of nigral iron (16). Finally, although ROS produc-
(17), and not causally
related to MPTP-induced neurodegeneration.
To evaluate further the role of •O$_2^-$ and the possible
involve ment of hydroperoxides such as $H_2O_2$ in the path-
ogenesis of MPTP toxicity, we performed experiments
using homozygous Sod1 de®cient (Sod1$^{-/-}$) mice (Sod1
encodes CuZn-SOD) and homozygous Gpx1 de®cient
(Gpx1$^{-/-}$) mice (Gpx1 encodes cellular glutathione per-
oxidase). CuZn-SOD and glutathione peroxidase
(GSHPx-1) are scavenger enzymes for •O$_2^-$ and hydro-
peroxides, respectively. Both littermate control and
knockout mice were injected intraperitoneally with in-
creasing doses of MPTP, and their brains examined for
loss of TH-containing nerve terminals in the striatum,
striatal dopamine levels, and TH-containing neurons in
the SNC. Mice of different strains or ages show marked
differences in developing neurological and neuropatho-
logical effects after exposure to MPTP (2, 3). However,
the development of significant loss of dopaminergic neu-
rons in the SNC after MPTP administration usually does
not occur for at least 2 wk. To minimize potential ROS
generation from widespread cell death (17), we chose a
less severe MPTP model and harvested brain tissue at an
earlier time in this study.

MATERIALS AND METHODS

Animals

Generation of Sod1$^{-/-}$ and Gpx1$^{-/-}$ mice has previously been
described in detail (18, 19). In brief, wild-type (C57BL/6) and
homozygous knockout mice were produced by breeding hetero-
zygous knockout mice and identified by Southern blot analysis
of tail DNA. Pervious experiments have demonstrated the ab-
sence of CuZn-SOD or GSHPx-1 activity in brain homogenates
from Sod1$^{-/-}$ or Gpx1$^{-/-}$ mice, respectively (18, 19).

MPTP Administration

Wild-type and homozygous de®cient mice (25–30 g) at 10
wk of age were injected intraperitoneally with MPTP (Aldrich)
at 0, 15, 30, or 60 mg/kg·d for 5 d for a total cumulative dose
of 0, 75, 150, or 300 mg/kg of MPTP, and killed following
pentobarbital anesthesia on day 5 after the last injection. Mouse
brain was perfusion ®xed through the heart with 4% parafor-
maldehyde in phosphate buffered saline (PBS, 10 mM).

Immunohistochemistry

Eight-micron axial sections were cut from paraf®n-embedded
blocks first at the levels containing the basal ganglia, nucleus
anteromedialis thalami, and pars anterior hippocampus (Fig. 1).
Immunohistological analysis for tyrosine hydroxylase (TH) in
the striatum and the SNC was performed with a commercially
available antibody (Novocastra) at a dilution of 1:20. All sec-
tions were deparaf®nnized with xylene, endogenous peroxidase
activity was blocked by incubation in 0.3% methanol peroxide,
and the slides were hydrated through graded ethanol. Antigen
retrieval was performed by heating for 25 min in a citrate buff-
er, pH 6, followed by a 15-min cooling period at room tem-
perature. Staining was then carried out using a histomouse kit
(BioGenex) speci®cally designed for use with a mouse mono-
clonal antibody against mouse tissue. Negative controls omitted
the primary antibody.

Axial sections were then cut from paraf®n-embedded blocks
at deeper levels containing the SNC, substantia nigra pars retic-
ulata (SNr) and hippocampus (Fig. 2) for quantification of TH-
positive neurons. In the adjacent section, ApopTag (Intergen)
was used according to the manufacturer’s speci®cations to eval-
uate apoptotic cell death mediated by MPTP.

Quantification

Images (Fig. 1) were collected with a Spot 2 digital camera
(Diagnostic Instruments, Inc.). The camera white balance and
®at®eld correction were held constant as was the microscope illumination, and the gain manually set at 1. The digital output
from the camera was run through Adobe Photoshop 5.0 via the
Twin driver on a Macintosh G3 Power PC.

Data Analyses

Optical density (OD) was obtained with the software appli-
cation NIH-IMAGE (Wayne Rasband, NIMH). For each mouse,
pixel-weighted average OD of the reaction product (TH immu-
noactivity) was measured on bilateral striatum from an-
terioromedialis thalami, and pars anterior hippocampus (Fig. 1).

Dopamine Neurochemistry

In addition to quantifying the striatal OD of TH immuno-
activity, striatal dopamine concentrations were measured in
another set of mice. In these mice, the brains were removed rap-
idly without ®xation 5 d after the last MPTP injection, and the
striatum was dissected out and subsequently stored at −80°C.

Fig. 1. Photomicrographs (×10) of Axial Sections of Mouse Brains Stained for TH Immunoreactivity. The striatum and surrounding structures are shown bilaterally. All three mice were treated with MPTP at 75 mg/kg cumulative dose and killed 5 d later. Control, wild type; Sod1−/−, homozygous CuZn-SOD deficient; and Gpx1−/−, homozygous GSHPx-1 deficient.
Dopamine was measured using high performance liquid chromatography with electrochemical detection as described previously (20).

Quantification of Neurons
In addition to determining changes in striatal TH immunoreactivity and dopamine concentration, the TH-positive as well as TUNEL-positive neurons (ApopTag) in the right and left SNc were counted in 3 TH-immunostained and TUNEL-stained sections from the representative horizontal mesencephalic plane (plane number 141) (21) by two independent observers. The average numbers from each mouse were used for statistical analysis.

RESULTS
Mice
Male and female homozygous knockout mice grew normally and were healthy up to 20 months of age, although there was reduced fertility in the CuZn-SOD deficient mice (18). There is no difference in the basal levels of Mn-SOD, glutathione reductase, glucose-6-phosphate dehydrogenase, or lipid peroxidation in the major organs including the brain in either the Sod1−/− or Gpx1−/− mice (18, 19). At a dose of 30 mg/kg·d (150 mg/kg cumulative dose protocol), there were mild acute effects of MPTP, such as hypersalivation, which most of the mice recovered from within 30 min. However, one mouse in the Gpx1−/− group and five mice in the Sod1−/− group died between 2 and 5 d after the last injection in animals treated with 30 mg/kg·d. Most mice treated with MPTP at 60 mg/kg·d became acutely ill and died within 3 d of treatment.

Tyrosine hydroxylase Immunocytochemistry of Striatum
Littermate control mice given MPTP showed a dose-dependent degeneration of the dopaminergic terminal regions in the striatum as assayed by decreased TH OD score (Fig. 3). Relative TH OD scores in control mice were 1.56 ± 0.08, 1.38 ± 0.04 and 1.20 ± 0.04 for MPTP injections of 0, 75, and 150 mg/kg cumulative
was statistically significant ($p < 0.05$). However, repeated paired analysis with Bonferroni correction showed the decrease in striatal dopamine was significantly greater in CuZn-SOD deficient or GSHPx-1 deficient mice compared with littermate controls treated with either cumulative dose of 75 or 150 mg/kg MPTP.

Despite significant reduction in TH immunoreactivity and dopamine level in the striatum, this MPTP exposure protocol did not induce a significant dopaminergic neuronal loss in the SNc. The average number of TH-positive neurons in the SNc was 61 ± 5 in littermate control mice treated with vehicle and 52 ± 2 in control mice treated with MPTP at 150 mg/kg cumulative dose (not statistically different). One-way analysis of variance comparing TH-positive neurons in the SNc was not statistically different ($p > 0.05$) among control, CuZn-SOD deficient, and GSHPx-1 deficient mice treated with MPTP at 150 mg/kg cumulative dose (Fig. 6). In addition, we did not observe any TUNEL-positive neurons in the SNc in any of the groups (data not shown). Although we did not use nonbiased stereologic techniques to count nigral neurons, the lack of even a trend toward more neuronal loss in either knockout strain suggested that the MPTP dosing regimen and timing of sacrifice selected for this study were relatively selective for striatal over nigral damage. Loss of nigral neurons could have been significant if brain tissues were harvested at a later time after last MPTP injection.

**DISCUSSION**

Consistent with the observations of several laboratories (13, 22, 23), our results provide evidence that MPTP produces a marked reduction in dopamine content and TH immunoreactivity in the striatum of mice 5 d after exposure. Importantly, our study is the first to demonstrate that MPTP-induced dopaminergic neurodegeneration is significantly enhanced in mice deficient in either CuZn-SOD or GSHPx-1. This suggests that both $O_2^-$ and hydroperoxides are involved in the pathogenesis of MPTP-induced neurotoxicity.
Sod1<sup>−/−</sup> and Gpx1<sup>−/−</sup> mice were exposed to MPTP for 5 days, and the brains harvested 5 days later. Striatal TH immunoreactivity was quantified and expressed as optical density (OD) scores (n = 5 mice in all groups except n = 4 mice in the Gpx1<sup>−/−</sup> group exposed to 150 mg/kg MPTP). Two-way analysis of variance was significantly different (p < 0.05). Repeated paired analysis with Bonferroni correction showed a significant difference between littermate controls and CuZn-SOD deficient mice treated with MPTP at 75 or 150 mg/kg, and a significant difference between littermate controls and GSHPx-1 deficient mice treated with MPTP at 75 mg/kg. *: p < 0.01 for control mice treated with vehicle versus those treated with MPTP at 75 or 150 mg/kg. •: p < 0.05 for control littermate mice versus either Sod1<sup>−/−</sup> or Gpx1<sup>−/−</sup> mice treated with MPTP at 75 mg/kg. •: p < 0.05 for littermate control mice versus Sod1<sup>−/−</sup> mice treated with MPTP at 150 mg/kg.

**TABLE 1** Optical Density Scores of Control and Knockouts

<table>
<thead>
<tr>
<th>Dosage of MPTP (Cumulative Dose mg/kg)</th>
<th>Control</th>
<th>Sod1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Gpx1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.56 ± 0.08</td>
<td>1.47 ± 0.04</td>
<td>1.52 ± 0.02</td>
</tr>
<tr>
<td>75</td>
<td>1.38 ± 0.04*</td>
<td>1.20 ± 0.01*</td>
<td>1.22 ± 0.06*</td>
</tr>
<tr>
<td>150</td>
<td>1.20 ± 0.04*</td>
<td>1.09 ± 0.05*</td>
<td>1.08 ± 0.02</td>
</tr>
</tbody>
</table>

The increased sensitivity of mice lacking CuZn-SOD to MPTP-induced neurotoxicity observed in this study is consistent with earlier observations showing that mice with increased CuZn-SOD or Mn-SOD are resistant to MPTP-mediated neurotoxicity (10, 11). In combination, these three studies suggest that •O<sub>2</sub>− generation is a critical step in MPTP-induced neurodegeneration. The mechanism by which •O<sub>2</sub>− contributes to dopaminergic neurodegeneration is not fully clarified, but is thought to involve the formation of even more reactive radicals. One of those radicals is peroxynitrite formed through reaction between •O<sub>2</sub>− and nitric oxide (NO•). Peroxynitrite subsequently inhibits protein function by nitration. One of the common markers of this process is formation of 3-nitrotyrosine (10). It has been shown that MPTP administration is associated with increased 3-nitrotyrosine concentration in the striatum, and inhibition of nitric oxide synthase (NOS) not only inhibits formation of 3-nitrotyrosine, but also protects against MPTP toxicity both in mice and in primates (10, 14, 24). Nitrotyrosine also has been localized immunohistologically to Lewy bodies of the substantia nigra from patients with idiopathic PD (25). In addition, peroxynitrite can decompose to form •OH and nitrogen dioxide radicals (26), thereby initiating lipid peroxidation and DNA oxidation, phenomena well documented in nigral tissue from PD patients (27–29). Interestingly, the role of NO• in MPTP-induced toxicity has been challenged recently because 7-nitroindazole, a NOS inhibitor, may also act as a MAO inhibitor or as an atypical antioxidant (30–32).

In addition to forming peroxynitrite, •O<sub>2</sub>− can produce H<sub>2</sub>O<sub>2</sub> by metal- or enzyme-catalyzed reactions. H<sub>2</sub>O<sub>2</sub> can then mediate •OH formation in the presence of transition metals such as iron, which is significantly increased in the nigral tissue of PD patients (16). H<sub>2</sub>O<sub>2</sub> is detoxified by catalase and GSHPx-1, but primarily by GSHPx-1 due to its lower Km (15). In this study, GSHPx-1 deficient mice were significantly more susceptible to striatal dopaminergic damage from MPTP administration compared with littermate control mice. The extent of enhancement of MPTP toxicity was comparable to the results obtained in Sod1<sup>−/−</sup> mice. These findings provide direct evidence that formation of hydroperoxides plays a significant role in the pathogenesis of MPTP-induced neurotoxicity.

How might increased ROS generation mediate neurodegeneration? The cellular targets of oxidative stress are not well understood, although mitochondria have been proposed as one of the major sites (33–35). Mitochondria consume over 90% of the cell’s oxygen, and the mitochondrial respiratory chain is a major source of •O<sub>2</sub>− under normal conditions (36). Inhibition of mitochondrial complex I, a well-known effect of MPP+, which is also observed in the SN from PD patients (35), may lead to generation of ROS, further inhibiting mitochondrial complex I. This negative cycle is likely to be exaggerated in mice deficient in either cellular CuZn-SOD or GSHPx-1 due to their compromised antioxidative capacity, resulting in enhanced MPTP toxicity. In addition, •O<sub>2</sub>− and H<sub>2</sub>O<sub>2</sub> can potentiate each other in mediating oxidative...
Fig. 4. Photomicrographs (×400) of Mouse Brain Striatum Stained for TH Immunoreactivity. Note the relatively sparse TH immunoreactivity in the terminal regions of the striatum in MPTP-treated mice (B) as compared with the same regions in the control mice (A).
of white matter). All three groups of mice were treated with PD further raises the possibility that both types of ROS peroxides. The similarity between the MPTP model and positive-neurons between the left and right SNc. SNc was used. There was no significant difference in the TH point. The average number of TH-positive neurons in the two effects of MPTP are mediated by both oxidative stress since \( \cdot O_2^- \) and \( H_2O_2 \) inhibit catalase and SOD respectively (15). Finally, enhancement of MPTP neurotoxicity in knockout mice could also be due to indirect effects on sites involved in the metabolism of MPTP, e.g. the dopamine transporter and vesicular monoamine transporters that have been shown to influence MPTP neurotoxicity (37). In either case, due to the dosing protocol of MPTP employed in these studies, the results suggest that MPTP-induced production of \( \cdot O_2^- \) and \( H_2O_2 \) are early events in dopaminergic neurodegeneration that precede nigral neurodegeneration. In summary, we observed significantly greater MPTP neurotoxicity in mice lacking either CuZn-SOD or GSHPx-1 compared with littermate control mice. These data provide direct evidence that some of the deleterious effects of MPTP are mediated by both \( \cdot O_2^- \) and hydroperoxides. The similarity between the MPTP model and PD further raises the possibility that both types of ROS may play a significant role in the etiology of dopaminergic neurodegeneration in PD.

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REFERENCES

12. Lan J, Jiang DH. Desferrioxamine and vitamin E protect against iron and MPTP-induced neurodegeneration in mice. J Neural Trans 1997;104:469–81

23. Tatton NA, Kish SJ. In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. Neuroscience 1997;77:1037–48
26. Beckman JS, Beckman TW, Chen J. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–24

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