Oxidative Modification of Peroxiredoxin Is Associated with Drug-induced Apoptotic Signaling in Experimental Models of Parkinson Disease*

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The aim of this study was to investigate changes in protein profiles during the early phase of dopaminergic neuronal death using two-dimensional gel electrophoresis in conjunction with mass spectrometry. Several protein spots were identified whose expression was significantly altered following treatment of MN9D dopaminergic neuronal cells with 6-hydroxydopamine (6-OHDA). In particular, we detected oxidative modification of thigalcohol-dependent peroxidases (peroxiredoxins; PRX) in treated MN9D cells. Oxidative modification of PRX induced by 6-OHDA was blocked in the presence of N-acetylcysteine, suggesting that reactive oxygen species (ROS) generated by 6-OHDA induce oxidation of PRX. These findings were confirmed in primary cultures of mesencephalic neurons and in rat brain injected stereotaxically. Overexpression of PRX1 in MN9D cells (MN9D/PRX1) exerted neuroprotective effects against death induced by 6-OHDA through scavenging of ROS. Consequently, generation of both superoxide anion and hydrogen peroxide following 6-OHDA treatment was decreased in MN9D/PRX1. Furthermore, overexpression of PRX1 protected cells against 6-OHDA-induced activation of caspase-3. In contrast, 6-OHDA-induced apoptotic death signals were enhanced by RNA interference-targeted reduction of PRX1 expression. Oxidative modification of PRX induced by 6-OHDA treatment was decreased in MN9D/PRX1. Taken together, our data suggest that oxidative modification of proteins, including peroxiredoxins (PRX), is associated with cellular responses to oxidative stress in dopaminergic neuronal cells. PRX1 overexpression may provide a molecular mechanism by which PRX1 exerts a protective role in experimental models of Parkinson disease.

Parkinson disease (PD)† is a common neurodegenerative disorder characterized by progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (1). Clinical manifestations, such as resting tremor, slowness of movement, stiffness, and postural instability in patients with PD are consequences of the loss of DA neurons and the resulting depletion of dopamine in the striatum. Most PD cases are sporadic, and its etiology is incompletely understood; however, an increasing body of evidence suggests that oxidative stress, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system may be involved in the pathogenesis of PD (2–5). Recent studies indicate that mutations in several genes appear to cause a familial form of PD (6), and mutations in these genes seem to share common pathways underlying the pathogenesis of a sporadic form of PD.

DA neurons are vulnerable to neurodegeneration because of their high levels of reactive oxygen species (ROS) (7); enzymatic metabolism of dopamine produces hydrogen peroxide and superoxide radicals, and intracellular autooxidation of dopamine induces the formation of dopamine-quinone, leading to the generation of hydroxyl radical when combined with iron (7, 8). Furthermore, there is a significant alteration in levels or activities of antioxidant enzymes in the substantia nigra (9–13). Among many potential consequences of oxidative stress, surges in ROS render DA neurons susceptible to oxidative modification of proteins. Oxidized α-synuclein exhibits an increased tendency to be misfolded and appears to be responsible for neurodegeneration (14). LaVoie et al. (15) demonstrated a mechanism by which dopamine covalently modifies parkin, decreases its solubility, and inactivates E3 ubiquitin ligase function. Oxidative modification of several other proteins, including DJ-1, ubiquitin carboxyl-terminal hydrolase L1, and Cu/Zn-superoxide dismutase, was also identified (16–18). Since the tissue content of oxidized proteins tends to be higher in the human substantia nigra compared with other brain regions (19), we were particularly interested in the identification of protein targets for oxidative damage and evaluating their functional relevance during DA neuronal death.

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‡ The abbreviations used are: PD, Parkinson disease; 2-DE, two-dimensional gel electrophoresis; 6-OHDA, 6-hydroxydopamine; DA, dopaminergic; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MPP+, 1-methyl-4-phenylpyridinium; NAC, N-acetylcysteine; PRX, peroxiredoxin(s); ROS, reactive oxygen species; TH, tyrosine hydroxylase; E3, ubiquitin-protein isopeptidase ligase; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Het, dihydroethidium; MAPK, mitogen-activated protein kinase; NL, non-linear.
Neurotoxin-based experimental models have been used to study biochemical changes reminiscent of those occurring in patients with PD (1). In our previous study (20, 21), we demonstrated that ROS-mediated apoptotic signaling increases within a few h after treatment with 6-hydroxydopamine (6-OHDA) and is responsible for degeneration of DA neurons. In the present study, we used integrated technologies, including two-dimensional gel electrophoresis (2-DE) and mass spectrometry, to identify proteins that are altered during 6-OHDA-mediated death. In the present study, we found that expression of 17 protein spots was significantly altered following 6-OHDA treatment (at least 4-fold increase/decrease with a p value of less than 0.05). Among these identified proteins, five spots corresponded to thioredoxin-dependent peroxidases (peroxiredoxins; PRX) that belong to an expanding family of antioxidant enzyme present in a large variety of organisms (22, 23). It has been proposed that PRX may be involved in a wide variety of cellular functions, including apoptosis (24, 25). Oxidative modification of PRX was confirmed in 6-OHDA-treated culture and rat brain models of PD. From subsequent study using cultured models of PD, we also found that (i) PRX proteins were oxidatively modified during 6-OHDA- but not 1-methyl-4-phenylpyridinium (MPP⁺)-induced DA neuronal death; (ii) co-treatment of antioxidant blocked 6-OHDA-induced oxidation of PRX; and (iii) overexpression of PRX1 prevented 6-OHDA-induced activation of p38 MAPK and caspase-3 through scavenging of ROS, whereas knockdown of PRX1 enhanced most of these events. Our data indicate that the redox state of PRX may play an important role in regulating 6-OHDA-induced apoptotic signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The MN9D DA neuronal cell line was established by somatic fusion between embryonic mesencephalic neurons and N18TG cells (26, 27). MN9D cells were plated at a density of 1 × 10⁶ on a 25 μg/ml poly-D-lysine-coated P-100 culture plate and cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 6.0 g/liter glucose at 37 °C in a humidified 5% CO₂ atmosphere. After 5 or 6 days of culture, cells were washed with minimal essential medium and trypsinized as described previously (20). Briefly, dissociated cells were plated at 1.0 × 10⁵ cells/8-mm diameter Aclar embedding film (Electron Microscopy Sciences) and maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 6.0 g/liter glucose at 37 °C in a humidified 5% CO₂ atmosphere. After 5 or 6 days of in vitro culture, cells were washed with minimal essential medium and treated with 20 μM 6-OHDA or 3 mM MPP⁺ for the times indicated in the presence or absence of 1 mM NAC.

Stereotaxic Surgery and Drug Injection—All procedures were performed in accordance with approved animal protocols and guidelines established by Yonsei University. Male Sprague-Dawley rats (260–290 g; Orient, Suwon, Korea) were anesthetized by injection of chloral hydrate (360 mg/kg, intraperitoneally), positioned in a stereotaxic apparatus (David Kopf Instruments), and prepared for surgery as previously described with minor modifications (29). Briefly, rats received a unilateral injection of 5 μl (6 μg) 6-OHDA in the presence or the absence of 2 mM NAC at a rate of 0.5 μl/min into the right substantia nigra (anteroposterior, −5.3; mediolateral, +2.3; dorsoventral, −7.6 mm from the bregma), according to the atlas of Paxinos and Watson (30). Two or three rats were assigned to each condition in one experiment. All injections were performed with a Hamilton syringe equipped with a 26 S gauge beveled needle and attached to a syringe pump (KD Scientific, New Hope, PA). After injection, the needle was left in place for an additional 5
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...for the times indicated. Following treatment, cells were washed with ice-cold phosphate-buffered saline containing 2 mM EDTA and lysed on ice for 10 min in a buffer containing 50 mM Tris, pH 7.0, 2 mM EDTA, 1.0% Triton X-100, and protease inhibitor mixture. Total protein content was measured using the Bio-Rad protein assay kit. An equal amount of soluble proteins was separated on 12.5% SDS-polyacrylamide gels, blotted onto prefrosted polyvinylidene difluoride membranes, and processed for immunoblot analysis as described previously (21). Cellular lysates were also processed for 2-DE in 7-cm Immobiline DryStrips (pH 3–10 NL) and subjected to immunoblot analysis. Primary antibodies used were as follows: rabbit polyclonal antibody against PRX1 (1:3000; Labfrontier, Seoul, Korea); rabbit polyclonal antibody that recognizes both the sulfenic (Cys₅₁-SO₂H) and sulfenic form (Cys₅₁-SO₃H) of PRX1 to -4 (1:3000; Labfrontier) (31); and rabbit polyclonal antibodies that recognize the phosphorylated form of p38 or the cleaved forms of caspase-3 (1:1000; Cell Signaling). Rabbit polyclonal anti-actin antibody (1:3000; Sigma) was used as a loading control. Peroxidase-conjugated secondary antibodies (1:2000; Amersham Biosciences) were used as appropriate. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Immunocytochemistry—Double immunocytochemical labeling of TH and oxidized forms of PRX was performed in primary cultures of mesencephalic neurons as described previously with some modifications (20). Briefly, cultures were fixed with 100% methanol for 20 min at −20 °C and blocked for 1 h in phosphate-buffered saline containing 5% normal goat serum and 0.1% Triton X-100. Cells were then incubated with a mouse monoclonal anti-TH (1:7500; Pel-Freez) and a rabbit polyclonal antibody specific for the oxidized forms of PRX (1:400) in blocking solution at 4 °C overnight. After extensive washes with phosphate-buffered saline, cultures were further incubated at room temperature for 1 h with a mixture of Alexa Fluor 546-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200). Cells were then examined with an Axiovert 100 microscope equipped with epifluorescence and a digital image analyzer (Carl Zeiss). Using a ×20 objective lens, TH-positive neurons that were positive for oxidized forms of PRX were counted by examining 16 consecutive fields across the Aclar embedding film (approximately 170–200 TH-positive DA neurons were scored in the untreated control culture). Cells were counted as positive for the oxidized forms of PRX when the fluorescence intensity was strongly increased compared with that typically seen in cells processed without primary antibody. Manual counts were performed from the images captured in a digital image analyzer by an individual who was blind to the treatment conditions. For immunofluorescent labeling of oxidized forms of PRX in MN9D cells, cells were fixed, blocked, and further processed as described above. Alexa Fluor 488-conjugated goat anti-rabbit IgG was used as a secondary antibody.

Stable Transfection—To establish a stable cell line overexpressing PRX1 (MN9D/PRX1), MN9D cells were transfected with pCI eukaryotic expression vector (Promega) containing full-length human PRX1 cDNA using Lipofectamine 2000 (Invitrogen) as previously described with minor modifications (32). For gene silencing studies, MN9D cells were stably trans-
fect with pRNAT-U6.1 containing small hairpin RNA of PRX1 designed as a small hairpin RNA and green fluorescent protein as a reporter sequence (MN9D/shPRX1; Genescript). The sequence of the RNA was 5'-GTGATAGACCGATG- 
AATTATTTGATCCGTAATTTCTTGCTATCT- 
ACTTTTTT-3' (sense-loop-antisense-termination signal). Approximately 2 weeks after transfection, G-418-resistant cells (MN9D/PRX1 and MN9D/shPRX1) were selected and expanded in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 500 µg/ml G-418. MN9D/PRX1 and MN9D/shPRX1 cells were characterized by immunoblot analysis as described above. MN9D cells stably transfected with an equivalent plasmid vector alone (MN9D/Neo and MN9D/RNe) were used as controls.

Measurement of ROS—MN9D/PRX1 or MN9D/shPRX1 cells were treated with 100 µM 6-OHDA alone or in combination with 1 mM NAC, and ROS generation was measured and compared with control cells as previously described (21). Briefly, cells were incubated with 1 µM dihydroethidium for 30 min or 5 µM 2,7-dichlorofluorescin diacetate for 3 h at 37 °C (all reagents were from Molecular Probes, Inc. (Eugene, OR)) and washed twice with a buffer containing 144 mM NaCl, 10 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, 5 mM KCl, and 10 mM D-glucose. Images were taken using an Axiovert 100 microscope, and florescence intensity was quantified at 530-nm/590-nm excitation/emission wavelength for dihydroethidium and 488 nm/510 nm for 2,7-dichlorofluorescin diacetate using an FL 600 plate reader (Bio-Tek).

Statistics—Data are given as means ± S.E. Significance of difference was determined by one-way ANOVA and post hoc Student’s t test. Values of p < 0.001, 0.01, or 0.05 were considered statistically significant.

RESULTS

Proteome Analysis of 6-OHDA-treated MN9D Cells—We previously reported that 6-OHDA induces classic apoptosis, including cytochrome c release and caspase activation, whereas MPP+ induces caspase-independent cell death pathways in DA as well as cortical neurons (20, 32–34). We have also proposed that ROS are one of the initial triggers leading to activation of apoptotic signaling following 6-OHDA treatment, whereas ROS are not involved in MPP+-induced cell death in MN9D cells and primary cultures of mesencephalic neurons (20, 21). The objective of the present study was to identify specific target proteins of oxidative damage in DA neuronal cells through analysis of an array of proteins that are potentially altered during the early phase of the ROS surge induced by exposure to 6-OHDA. Following treatment of MN9D cells with 100 µM 6-OHDA for 1–12 h, proteome analysis of 6-OHDA-treated cells and matching untreated controls was performed using integrated technologies including protein separation by 2-DE and identification by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

A typical Coomassie Blue-stained protein pattern obtained after separation of a 1.5-mg protein sample from untreated MN9D cells is shown in Fig. 1A. More than 1500 matched protein spots ranging from ~10 to 100 kDa were typically detected in a PI 4–7 or 3–10 NL gel. Among 46 protein spots that were altered following 6-OHDA treatment for 1, 3, 6, and 12 h, expression of 17 protein spots was significantly changed at least 4-fold with a p value of less than 0.05 (n > 3). Fourteen representative regions boxed in Fig. 1A are compared in detail with and without 6-OHDA treatment in Fig. 1B. These altered protein spots were subjected to in-gel digestion with trypsin, MALDI-TOF mass spectrometry, and a data base search using either the NCBI or Swiss-Prot data base. Table 1 summarizes the identified proteins with accession number, their -fold change, MOWSE score (35), molecular weight/pI, matched peptides, and sequence coverage in MN9D cells treated with 100 µM 6-OHDA. Proteins that increased in their intensity following Coomassie Brilliant Blue staining included PRX1 (spot 1), proteasome subunit α type 1, glutathione S-transferase P1, PRX2 (spot 8), PRX6, eukaryotic translation initiation factor 4E, programmed cell death 5 protein, cofolin, and platelet-activating factor acetylhydrolase 1B β subunit. Proteins that decreased in their staining intensity included PRX1 (spot 2), glucose-6-phosphate 1-dehydrogenase X, calmodulin, proteasome subunit β type 1 precursor, PRX2 (spot 9), ubiquitin thiolesterase protein OTU B1, nucleotide-binding protein 1, and mitochondrial processing peptidase β subunit. In most cases, dramatic changes in expression levels occurred during the very early phase of cell death following 6-OHDA treatment (~1 h).

Analysis of PRX1 Expression following 6-OHDA Treatment—Since the initial identification of PRX1 proteins in yeast, six isoforms (PRX1 to -6) have been found in mammalian cells and demonstrated to have peroxidase activity (22–24, 36, 37). PRX1 has been shown to be inactivated under oxidative stress as a result of oxidation of a cysteine residue at the catalytic site to sulfenic (Cys51-SO2H) and sulfonic acids (Cys51-SO3H), a reaction that cannot be reversed by thioredoxin (38). Therefore, from our protein profiling data (Fig. 1 and Table 1), we were especially interested in two PRX1 protein spots (spots 1 and 2) that were differentially regulated following 6-OHDA treatment. To further investigate the drug specificity of these changes in PRX1 expression, MN9D cells were treated with two well known dopaminergic neurotoxins, 100 µM 6-OHDA or 50 µM MPP+, for the times indicated in Fig. 2. After treatment, total cellular lysates were subjected to 2-DE to compare expression profiles of the two PRX1 spots. As shown in Fig. 2A, PRX1 spot 2 was shifted to the acidic position of PRX1 spot 1 following 6-OHDA treatment. However, there was no discernible shift of PRX1 spot 2 to the acidic position in MN9D cells treated with MPP+ for 6–36 h, indicating that the change in the ratio between the two PRX1 spots is drug-specific. Based on mass spectra of the tryptic peptide obtained from MALDI-TOF mass spectrometry, the two PRX1 spots seemed to be identical (Fig. 2B), suggesting that the acidic shift of the PRX1 spot may represent 6-OHDA-induced modification of the protein. Previous reports, including those from our laboratory, have demonstrated that early generation of ROS and subsequent activation of ROS-mediated signal pathways play an essential role in cell death induced by 6-OHDA, but not by MPP+, in MN9D cells and primary cultures of cortical and mesencephalic neurons (20, 21, 32–34, 39). Therefore, we reasoned that ROS induced
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by 6-OHDA may contribute to the acidic shift of the PRX1 spot in MN9D cells.

To further investigate this hypothesis, we tested the effect of antioxidant on the 6-OHDA-induced changes in PRX1 expression. Cellular lysates from MN9D cells treated with 100 μM 6-OHDA in the presence or the absence of 1.0 mM NAC were subjected to conventional electrophoresis and mini-2-DE (7-cm strip), followed by immunoblot analysis using anti-PRX1 antibody. As shown in Fig. 3A, total expression levels of PRX1 remained the same regardless of treatment, indicating that the total amount of PRX1 is not altered following 6-OHDA treatment. Immunoblot analysis of mini-2-DE gels revealed three spots (Fig. 3B; designated spots a–c from the acidic to the basic position). Spots designated b and c in mini-2-DE gels correspond to spots 1 and 2 in Figs. 1B and 2A, respectively, whereas the spot designated spot a was apparently detected following immunoblot analysis. The relative ratio of the more acidic spots (spots a and b) to the spot at the more basic position (spot c) increased following treatment with 6-OHDA (Fig. 3, B and C). In untreated MN9D cells, spots a and b comprised 6.74 and 32.35% of the total PRX1 detected, respectively. Following 12 h of treatment with 6-OHDA, the relative ratio of these spots increased to 25.92 and 51.96%, respectively. This ratio reverted to the level of untreated controls in the presence of 1.0 mM NAC, suggesting that oxidative modification of PRX1 may be induced by treatment with 6-OHDA.

Oxidative Modification of PRX1 following 6-OHDA Treatment—To unambiguously determine oxidative modification of PRX1, total cellular lysates were obtained from MN9D cells following treatment with 100 μM 6-OHDA for the time period indicated and subjected to mini-2-DE followed by immunoblot analysis with an antibody that specifically recognizes the oxidized forms of PRX1 at the Cys51–SO2H or Cys51–SO3H epitope (31). Following 6-OHDA treatment for 9 h, three PRX1 spots designated a–c were detected with an antibody that recognizes all forms of PRX1 (Fig. 4A, second panel from the top). Only two protein spots at the more acidic positions designated a and b were detected with antibody recognizing the oxidized forms of PRX1; this staining pattern was largely reversed in the presence of 1 mM NAC (Fig. 4B). To further confirm whether dopamin-
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TABLE 1
Summary of the identified proteins altered after 6-OHDA treatment

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<tr>
<th>Spot number</th>
<th>Protein name</th>
<th>Accession number</th>
<th>Change</th>
<th>Protein mass/pI</th>
<th>MOWSE score</th>
<th>Matched peptide number</th>
<th>Sequence coverage</th>
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<td>1</td>
<td>Peroxiredoxin1</td>
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<td>22,177/8.3</td>
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<td>78</td>
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* Each protein spot in Fig. 1B was identified based on mass spectra of tryptic peptides obtained by MALDI-TOF mass spectrometry.
* Accession numbers of the identified protein spots were obtained from the NCBI or Swiss-Prot data base.
* Fold change was converted to log2 scale and expressed as a mean of relative intensity over the untreated control (n = 3, p < 0.05; ANOVA with post hoc Student’s t-test).
* Theoretical molecular mass (Da) and pI.
* MOWSE, molecular weight search; basically to compare the calculated peptide masses for each entry in the sequence database with the set of experimental data (35).

![Drug-specific expression pattern of PRX1 spots in MN9D cells following treatment with 6-OHDA or MPP+](image)

![Immunoblot analysis of PRX1 expression following treat-](image)

ergic neurotoxins trigger oxidative modification of PRX in primary cultures of mesencephalic neurons, cultures were prepared from the ventral mesencephalon of embryonic day 14.5 rat embryos and grown in the presence or the absence of 20 μM 6-OHDA or 3 μM MPP+… As we described previously (20), DA neurons routinely comprise 5–6% of the total population of neurons routinely comprise 5–6% of the total population of
cells in untreated control cultures, as defined by immunocytochemical staining for TH, a rate-limiting enzyme of dopamine biosynthesis. The remaining TH-negative neuronal cells are mostly GABA-positive neurons.

Following 6-OHDA treatment, the appearance of the oxidized forms of PRX was evident as early as 3 h, and this level was maintained thereafter (Fig. 5A). Although it has not been determined unambiguously, based on the known molecular weights of PRX isoforms, the lower band is thought to represent the oxidized forms of PRX1 and PRX2, and the upper band is thought to represent the oxidized forms of PRX3 and PRX4. In control cultures, a small but discernible level of the potentially oxidized forms of PRX1 and PRX2 were detected (Fig. 5A, upper panel, lower band). As shown in Fig. 5, B and C, double immunofluorescent labeling of TH and the oxidized forms of PRX revealed that more than 80% of DA neurons were positive for the oxidized forms of PRX following 9 h of treatment with 6-OHDA. The oxidized forms of PRX were also evident in many non-DA cells following 6-OHDA treatment, indicating that the drug-induced appearance of the oxidized forms of PRX is not specific for DA neurons (Fig. 5B). The generation of the oxidized forms of PRX was completely reversed in cells co-treated with 1 mM NAC (Fig. 5B and C). In contrast, MPP⁺ did not induce oxidation of PRX (Fig. 5B and C), even after treatment for up to 36 h. In rats that were stereotaxically injected with 6-OHDA (6 μg) into the substantia nigra for 3 days, the total number of TH-positive DA neurons in the substantia nigra pars compacta was reduced to ~37% over the sham control group (n = 3; average of 15,226 in sham control versus 5,590 in the 6-OHDA-injected group), as determined by unbiased stereological cell counts (Fig. 6, A (upper panels) and B). The number of TH-positive DA was partially

FIGURE 4. Characterization of the oxidation state of PRX1 following 6-OHDA treatment. MN9D cells were treated with 100 μM 6-OHDA for 9 h in the presence or the absence of 1 mM NAC. A, total cellular lysates (10 μg) loaded on 7-cm ministrips were separated by 2-DE and subjected to immunoblot analysis using antibody that recognizes all forms of PRX1 (upper two panels) or antibody specific for the oxidized forms of PRX (lower three panels) (38). Three specific PRX1 protein spots (indicated by arrows and designated by α-c from the acidic side of gels) were visualized by enhanced chemiluminescence. B, immunocytochemical localization of the oxidized forms of PRX in MN9D cells treated with 6-OHDA in the presence or absence of NAC was performed as described under “Experimental Procedures.” Scale bar, 50 μm.

FIGURE 5. Oxidative modification of PRX in primary cultures of mesencephalic neurons following treatment with 6-OHDA. A, primary cultures derived from the ventral mesencephalon of embryonic day 14.5 rat embryos were treated with 20 μM 6-OHDA for the times indicated. Total cellular lysates (10 μg) were subjected to 2-DE and immunoblot analysis using polyclonal antibodies that recognize PRX1 or the oxidized forms of PRX. B, following treatment with 20 μM 6-OHDA or 3 μM MPP⁺ for 9 h in the presence or the absence of 1 mM NAC, cultures were double immunolabeled with a mouse monoclonal anti-TH and a rabbit polyclonal antibody specific for the oxidized forms of PRX, followed by Alexa Fluor 546-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibodies. The white arrows indicate TH-positive neurons. Scale bar, 20 μm. C, TH-positive neurons that were positive for the oxidized forms of PRX were counted as described under “Experimental Procedures.” Data represent the mean ± S.E. from three or four independent experiments (*, p < 0.01; ANOVA with post hoc Student’s t test).
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restored up to ~57% by co-treatment with 2 mM NAC (n = 3; average of 8672). As an independent assessment of survival, we also examined for the number of neurons in the substantia nigra pars compacta region by cresyl violet staining. As shown in Fig. 6, A (bottom panels) and C, values of cresyl violet analysis were quite similar to those of TH assessment (n = 3, 40.0% in the 6-OHDA-injected group and 66.4% in the NAC cotreatment group over the sham control group), ensuring that loss of neurons by 6-OHDA injection is not simply due to loss of TH expression. Although the basal level of the oxidized forms of PRX appeared in the substantia nigra pars reticulata (below the white dotted areas) of both the sham control and experimental group, double immunofluorescent staining revealed that, in this rat model, the level of immunopositive staining for the oxidized forms of PRX in TH-positive DA neurons increased on 6-OHDA-injected ipsilateral side of the substantia nigra pars compacta (Fig. 6D, middle panels, white dotted area). Interestingly, increase of the oxidized forms of PRX was very obvious among the shrunken TH-positive neurons (white arrows). This phenomenon was largely blocked in the presence of NAC. To unambiguously assess selectivity of this phenomenon in the substantia nigra pars compacta, TH-positive DA neurons or TH-negative cells co-localized with the increased level of the oxidized forms of PRX were counted using the confocal images acquired as described under “Experimental Procedures.” As shown in Fig. 6E, virtually no TH-positive neurons showed positive for the oxidized forms of PRX. In contrast, average of 14.7 TH-positive neurons per section demonstrated increased levels of the oxidized forms of PRX. In the group cotreated with 2 mM NAC, only 2.7 TH-positive neurons/section turned out to be positive for the oxidized forms of PRX. In all conditions, an average of 2.5–3.8 non-TH-positive cells/section demonstrated increased levels of the oxidized forms of PRX. Taken together, these data support the proposal that ROS induced by 6-OHDA...
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FIGURE 7. Effect of PRX1 overexpression on MN9D cell death induced by 6-OHDA. A, MN9D cells were stably transfected with a eukaryotic expression vector containing human-PRX1 cDNA (MN9D/PRX1). Total cellular lysates (5 μg) were subjected to immunoblot analysis using anti-PRX1 antibody. MN9D cells transfected with a eukaryotic expression vector alone (MN9D/Neo) were established as controls. A duplicate blot was probed with polyclonal anti-actin antibody to confirm equal loading. B, MN9D/Neo and MN9D/PRX1 cells were treated with 100 μM 6-OHDA for 12 h. Phase-contrast photomicrographs were taken under an Axiovert 100 microscope. Scale bar, 50 μm. C, cell viability following 24-h treatment with 6-OHDA was measured by the MTT reduction assay. Cell viability data from each experiment were expressed as a percentage of the untreated control. Each bar represents the mean ± S.E. from three independent experiments performed in triplicate (*, p < 0.05; ANOVA with post hoc Student’s t test).

are responsible for oxidative modification of PRX in DA neurons of both in vitro and in vivo models of PD.

PRX1 Overexpression Protects against 6-OHDA-induced Neuronal Cell Death—To determine whether oxidative modification of PRX1 is associated with 6-OHDA-induced DA neuronal cell death, we established MN9D cells that were stably transfected with human PRX1 cDNA (MN9D/PRX1) or mock-transfected with vector (MN9D/Neo). As shown in Fig. 7A, the expression level of PRX1 was increased up to 2.4-fold in MN9D/PRX1 cells over the control level in MN9D/Neo. To investigate whether cell death induced by 6-OHDA was blocked by overexpression of PRX1, MN9D/Neo and MN9D/PRX1 cells were treated with 100 μM 6-OHDA for various times and subjected to phase-contrast microscopy and MTT reduction assay. Fig. 7B shows representative phase-contrast photomicrographs of MN9D/Neo and MN9D/PRX1 cells following treatment with 6-OHDA for 12 h. Following 6-OHDA treatment, short neurites were largely retracted, and cell bodies were shrunken in MN9D/Neo cells, whereas MN9D/PRX1 cells showed no obvious signs of these morphological changes. An MTT reduction assay revealed that overexpression of PRX1 significantly attenuated 6-OHDA-induced cell death; after 24 h of treatment, cell viability was 23.2 ± 1.7% for MN9D/Neo compared with 48.7 ± 9.3% for MN9D/PRX1 cells (Fig. 7C). These results were confirmed in two other N9D/PRX1 clones established independently (data not shown). Flow cytometric measurement of phosphatidylinerine externalization revealed that the percentage of annexin V-positive cells was reduced from 7.8% in MN9D/Neo cells to 2.5% in MN9D/PRX1 cells 12 h after 6-OHDA treatment (data not shown), suggesting that cells that overexpress PRX1 are less sensitive to 6-OHDA-induced cell death.

Mechanism of the Protective Effect Exerted by PRX1—To investigate how overexpression of PRX1 exerts its protective effect, we first compared ROS generation in MN9D/Neo and MN9D/PRX1 cells following 4 h of 6-OHDA treatment using the cell-permeable ROS-sensitive fluorescent dyes 2,7-dichlorodihydrofluorescein diacetate (a hydrogen peroxide indicator) and dihydroethidium (Het; a superoxide anion indicator). As shown in our previous study (21), only a background level of fluorescence was detected in untreated MN9D/Neo cells. However, intensity of fluorescence detected by 2,7-dichlorodihydrofluorescein diacetate or Het significantly increased in most MN9D/Neo cells following 6-OHDA treatment (Fig. 8A). This surge of ROS was largely blocked in the presence of 1 mM NAC. In contrast, the 6-OHDA-induced increase in ROS was significantly reduced in MN9D/PRX1 cells. As shown in Fig. 8, B and C, quantitative measurements of each ROS-sensitive dye revealed that the levels of ROS in MN9D/PRX1 following 6-OHDA treatment were comparable with those in MN9D/Neo cells co-treated with 1 mM NAC. In our previous study, we demonstrated that ROS-mediated activation of p38 MAPK and caspase-3 plays an important role in 6-OHDA-induced cell death in MN9D cells and in primary cultures of DA neurons (21). Therefore, we tested whether these cell death events are blocked in MN9D/PRX1 cells. As shown in Fig. 8D, phosphorylation of p38 increased following 6-OHDA treatment in MN9D/Neo cells, whereas activation of p38 was highly suppressed in MN9D/PRX1 cells. Similarly, 6-OHDA-induced activation of caspase-3 was significantly attenuated in MN9D/PRX1 cells (Fig. 8E). To confirm these results in a knockdown model, we stably transfected MN9D cells with PRX1 small hairpin RNA (MN9D/shPRX1) or with an empty vector alone (MN9D/RNeo). As shown in Fig. 9A, the expression level of PRX1 in MN9D/shPRX1 cells was reduced by 60% compared with that in MN9D/RNeo cells. MN9D/shPRX1 cells were more sensitive to 6-OHDA-induced cell death for all treatment times tested (Fig. 9B). As expected, knockdown of PRX1 in MN9D cells increased both 6-OHDA-mediated generation of Het-sensitive ROS and activation of the ROS-mediated apoptotic cell death pathway (Fig. 9, C–E).

DISCUSSION

In this study, we used 2-DE and mass spectrometry to evaluate an array of proteins whose expression is regulated during
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the early phase of 6-OHDA-induced cell death in MN9D cells. Among the 17 protein spots whose expression changed at least 4-fold (statistically significant with a \( p \) value of less than 0.05), we found that five spots corresponded to altered expression of PRX. Using a specific antibody that recognizes 2-Cys PRX-SO\(_2\)H and PRX-SO\(_3\)H (31), we confirmed that the oxidized forms of PRX are generated during the early phase of 6-OHDA treatment in both MN9D cells and primary cultures of mesencephalic neuronal cells. Furthermore, an increase in the oxidized form of PRX is also detected in TH-positive neurons in the substantia nigra pars compacta following stereotaxic injection of 6-OHDA into the substantia nigra. Data presented in this paper demonstrate that 6-OHDA-induced generation of ROS is responsible for the generation of the oxidized forms of PRX. Based on our results obtained from MN9D/PRX1 (overexpression of PRX1) and MN9D/shPRX1 (RNA interference-targeted reduction of PRX1) cells, we propose that PRX1 plays a protective role in 6-OHDA-treated DA neuronal cells by scavenging ROS and thus preventing ROS-induced activation of p38 MAPK and caspase-3, which lead to cell death. Thus, oxidative inactivation of PRX results in activation of the ROS-induced cascade of apoptotic signaling in DA neuronal cells. Although acidic shift of PRX3 and -6 was observed in PC12 cells following 6-OHDA treatment (42), to the best of our knowledge, this is the first clear demonstration of oxidative modification of PRX and its close involvement in the regulation of 6-OHDA-induced apoptotic signaling in DA neuronal death.

PRX proteins belong to an expanding family of thiol-specific antioxidant proteins present in a large variety of organisms (22, 23). The six isoforms of mammalian PRX are divided into three classes: typical 2-Cys homodimers containing two identically conserved redox-active cysteine residues (PRX1 to -4); atypical monomeric 2-Cys (PRX5); and 1-Cys containing one conserved cysteine (PRX6) (37). Proposed functions of PRX include cellular proliferation, differentiation, immune responses, and apoptosis (24, 25). Overexpression of PRX5 prevents p53-dependent ROS generation and apoptosis (43), and overexpression of PRX1 and PRX2 leads to removal of H\(_2\)O\(_2\) and protects thyroid cells from apoptosis (44). Furthermore, depletion of PRX3 by RNA interference in HeLa cells or suppression of 1-Cys PRX by an antisense oligonucleotide in rat lung epithelial cells leads to sensitized cells susceptible to peroxide-induced apoptosis (45, 46). Our experiments involving overexpression and depletion of PRX1 in MN9D cells, together with in vitro and in vivo models, support the proposal that PRX plays a critical role in ROS-mediated DA neuronal apoptotic signaling. Although it has not been investigated extensively, several studies indicate that activation of mitogen-activated protein kinases (MAPKs) is regulated by PRX. For example, activation of c-Jun NH\(_2\)-terminal kinase in lung cancer cells and p38 MAPK in macrophages is suppressed by PRX1 (47, 48). Kang et al. (49) showed that cytosolic PRX attenuates the activation of both c-Jun NH\(_2\)-terminal kinase and p38 MAPK, but potentiates extracellular signal-regulated kinase in TNF-treated HeLa cells. Similarly, regulation of stress-activated MAPK by 2-Cys PRX has been demonstrated in yeast (50). In this regard, our demonstration that scavenging of ROS by PRX1 is responsible for blocking activation of p38 MAPK and caspase-3 further supports the role of PRX as an important regulator of the cell death pathway. Previous reports have shown that expression of PRX1 in colorectal cells and PRX5 in human tendon cells is up-regulated in response to oxidative stresses (47, 51, 52). In contrast to these reports, we found that total expression levels of PRX1 are not changed in response to 6-OHDA treatment in MN9D cells but that the ratio of the oxidized and the reduced form of PRX1...
seems to be more important in determining the extent of ROS-mediated death in DA neuronal cells. Although we have not determined whether the different isoforms of PRX perform distinct roles in this system, we postulate that the oxidative modification of various isoforms of PRX may be a critical event during the induction of apoptosis by 6-OHDA in DA neuronal cells.

Chevallet et al. (53) reported that oxidation of PRX can be reversed under certain conditions and that the regeneration rate varies between the different isoforms of PRX. Our data show that maximal oxidation of PRX in DA neuronal cells is achieved 1–3 h after exposure to 6-OHDA and is maintained for at least an additional 9 h. The same phenomenon is observed in MN9D cells even after removal of 6-OHDA following an initial treatment of 1–3 h (not shown). Although the reversibility of PRX oxidation in DA neuronal cells needs to be examined more carefully, we suggest that the reaction may be irreversible in this system. Reversal of the oxidized forms of PRX by sulfiredoxin has been demonstrated (54, 55). Sulfiredoxin was recently identified as an antioxidant protein and characterized by its role in the efficient reduction of conserved cysteines from the sulfenic to sulfenic acid form (56). In preliminary experiments using MN9D cells that overexpress sulfiredoxin, we have not seen any protective effect on 6-OHDA-induced cell death as determined by the level of oxidized forms of PRX and an MTT reduction assay (data not shown). Although the reversibility of PRX oxidation in DA neuronal cells needs to be examined more carefully, we suggest that the reaction may be irreversible in this system. Reversal of the oxidized forms of PRX by sulfiredoxin has been demonstrated (54, 55). Sulfiredoxin was recently identified as an antioxidant protein and characterized by its role in the efficient reduction of conserved cysteines from the sulfenic to sulfenic acid form (56). In preliminary experiments using MN9D cells that overexpress sulfiredoxin, we have not seen any protective effect on 6-OHDA-induced cell death as determined by the level of oxidized forms of PRX and an MTT reduction assay (data not shown). Moreover, immunoblot analysis indicates that levels of thioredoxin, which has been shown to regenerate the active form of PRX (57), are decreased following treatment with 6-OHDA (data not shown). Therefore, we speculate that a lack or inefficiency of sulfiredoxin and reduced levels of thioredoxin may be responsible for accumulation of the oxidized forms of PRX during 6-OHDA-induced DA neuronal death. Recently, Bai et al. (58) reported that DA neurons in the thioredoxin-1 transgenic mice are significantly resistant to MPTP administration. It is somewhat contradictory to our result indicating that MPP+ does not induce ROS-mediated modification of PRX in both MN9D cells and primary cultures of mesencephalic neurons. Since thioredoxin is demonstrated to regulate cell death through a wide variety of mechanisms (57), further examinations remain to determine whether and how the thioredoxin system may play a protective role in various PD models.

Relatively little information is available regarding the pathophysiological role of PRX in the nervous system. Previous studies using human, rat, and mouse tissues indicate that PRX is expressed in glial and neuronal cells of the central and peripheral nervous system (59–61). Proteome analysis or suppression subtractive hybridization have shown aberrant expression of PRX in brains of patients with neurological diseases, including Alzheimer disease, Down syndrome, Pick disease, and PD (62–

![FIGURE 9. Effect of PRX1 silencing on 6-OHDA-induced cell death. Small hairpin RNA of PRX1 was generated as a small hairpin RNA in pRNAT-U6.1 and used to establish a stable cell line (MN9D/shPRX1). An empty vector was used to establish control cells (MN9D/RNeo). A, total cellular lysates (2 μg) were subjected to immunoblot analysis using anti-PRX1 antibody. A duplicate blot was probed with polyclonal anti-actin antibody to confirm equal loading. B–E, cells were treated with 100 μM 6-OHDA for the times indicated. B, cell viability was measured by the MTT reduction assay. Cell viability data from each experiment were expressed as a percentage of the untreated control. Each point represents mean ± S.E. from three independent experiments performed in triplicate (*, p < 0.05; ANOVA with post hoc Student’s t test). C, following incubation with 1 μM Het, fluorescence intensity of MN9D/RNeo and MN9D/shPRX1 was measured using an FL 600 plate reader. Values are expressed as the fold increase over the untreated control. Each bar represents mean ± S.E. from three independent experiments performed in triplicate (**, p < 0.01; ANOVA with post hoc Student’s t test). Total cellular lysates (50 μg) were separated on 10% SDS-PAGE and subjected to immunoblot analysis using antibodies that recognize phosphorylated p38 MAPK (D) and the active form of caspase-3 (E). Duplicate blots were probed with polyclonal anti-actin antibody to confirm equal loading.](http://www.jbc.org/content/283/15/9996/F1.large.jpg)
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fications of such other modifications in neurological disorders,
cluding PD, remain to be extensively determined.

Although the exact cause of the neuronal degeneration in PD
is not known, studies of experimental models of PD and iden-
tification of genes implicated in familial forms of PD indicate
that oxidative stress is one of many potential causes (1). Reduc-
tion of the antioxidant system and ROS generation through
various mechanisms both contribute to DA neuronal cell death,
and thioredoxin is proposed to be an important regulator of
neuroprotection (71). Although the underlying molecular
mechanisms are not clearly understood, oxidative modifications
of important proteins, such as α-synuclein, DJ-1, and par-
kin, are associated with DA neuronal death (6). In addition,
oxidative modification at cysteine residues has been reported in
a growing number of enzymes, kinases, and transcriptional fac-
tors and provides an important way of regulating cellular func-
tions (24, 72–74). Further elucidation of the role of the PRX
catalytic cycle in the pathogenesis of PD could contribute to our
understanding of the pathogenesis of PD and the development
of more effective ways of preventing DA neuronal death.

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