Molecular mechanisms of 6-hydroxydopamine-induced cytotoxicity in PC12 cells: Involvement of hydrogen peroxide-dependent and -independent action

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Received 8 September 2006; revised 7 December 2006; accepted 8 December 2006

Abstract

The neurotoxin 6-hydroxydopamine (6-OHDA) has been widely used to generate an experimental model of Parkinson’s disease. It has been reported that reactive oxygen species (ROS), such as the superoxide anion and hydrogen peroxide (H2O2), generated from 6-OHDA are involved in its cytotoxicity; however, the contribution and role of ROS in 6-OHDA-induced cell death have not been fully elucidated. In the present study using PC12 cells, we observed the generation of 50 μM H2O2 from a lethal concentration of 100 μM 6-OHDA within a few minutes, and compared the sole effect of H2O2 with 6-OHDA. Catalase, an H2O2-removing enzyme, completely abolished the cytotoxic effect of H2O2, while a significant but partial protective effect was observed against 6-OHDA. 6-OHDA induced peroxiredoxin oxidation, cytochrome c release, and caspase-3 activation. Catalase exhibited a strong inhibitory effect against the peroxiredoxin oxidation, and cytochrome c release induced by 6-OHDA; however, caspase-3 activation was not effectively inhibited by catalase. On the other hand, 6-OHDA-induced caspase-3 activation was inhibited in the presence of caspase-8, caspase-9, and calpain inhibitors. These results suggest that the H2O2 generated from 6-OHDA plays a pivotal role in 6-OHDA-induced peroxiredoxin oxidation, and cytochrome c release, while H2O2- and cytochrome c-independent caspase activation pathways are involved in 6-OHDA-induced neurotoxicity. These findings may contribute to explain the importance of generated H2O2 and secondary products as a second messenger of 6-OHDA-induced cell death signal linked to Parkinson’s disease.

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Keywords: 6-Hydroxydopamine; Hydrogen peroxide; p-Quinone; Caspase; Cytochrome c; Peroxiredoxin; Catalase; Glutathione

Introduction

Parkinson’s disease (PD) involves an irreversible degeneration of the nigrostriatal dopaminergic pathway, resulting in a marked impairment of motor control. Although the etiology of PD remains unknown, recent studies have suggested that oxidative stress is an important mediator in its pathogenesis [1]. It is thought that nigral dopaminergic neurons are rich in reactive oxygen species (ROS), because metabolism of dopamine itself (both enzymatic and nonenzymatic) leads to the generation of ROS, including the superoxide anion, hydrogen peroxide (H2O2), and hydroxyl radicals [2]. Indeed, there are several observations, such as the increased levels of the oxidation products of lipids, proteins, and nuclear acids in nigral cells, that are indicative of the role of oxidative stress in PD [2,3].

In order to elucidate the molecular pathways of neuronal death and to develop neuroprotective strategies, a number of in vitro and in vivo models have been characterized. 6-Hydroxydopamine (6-OHDA) is a selective catecholaminergic neurotoxin that has been widely used to produce PD models in
vitro and in vivo, which is accepted to induce a toxicity that mimics the neuropathological and biochemical characteristics of PD [4]. It has also been reported that neonate 6-OHDA-lesioned rats exhibit behavioral hyperactivity similar to that observed in attention-deficit hyperactivity disorder [5,6].

6-OHDA has been demonstrated to induce toxicity in a wide range of neuronal in vitro models, including human neuroblastoma cell lines [7,8], primary neuronal cultures [9,10], and the rat adrenal pheochromocytoma cell line, PC12 [11–13]. It is known that in PC12 cells 6-OHDA induces apoptotic cell death, and it has also been reported that mitochondrial dysfunction initiated by 6-OHDA induces release of cytochrome c, and the activation of caspase-3 [11,14–16]. Furthermore, it has been reported that 6-OHDA induces caspase-8 activation, which is related to extramitochondrial activation [9,17]; however, the detailed molecular mechanism of 6-OHDA-induced apoptosis remains to be elucidated.

It has been reported that 6-OHDA is oxidized rapidly by molecular oxygen to form the superoxide anion, hydrogen peroxide, and 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (p-quinone) as follows [18]:

\[
6\text{-OHDA} + \text{O}_2 \rightarrow \text{p-quinone} + \text{H}_2\text{O}_2.
\]

It is thought that the ROS generated from 6-OHDA initiate cellular oxidative stress. On the other hand, it has been reported that p-quinone mediates 6-OHDA-induced cell death [19]. However, the exact mechanisms of ROS production and neurotoxicity induction, particularly in relation to caspase activation, are less clearly defined.

In the present study using PC12 cells, we first determined the H2O2 and p-quinone generated from lethal concentrations of 6-OHDA. We then characterized the role and contribution of H2O2 and its secondary products, including p-quinone, on cell viability, peroxiredoxin oxidation, caspase activation, cytochrome c release, and mitochondrial injury induced by 6-OHDA.

**Materials and methods**

**Chemicals**

Dulbecco’s modified Eagle medium/nutrient mixture F-12 Ham, 1/1 (D-MEM/F-12), and horse serum were obtained from Gibco BRL (Rockville, MD); fetal bovine serum from JRH Biosciences (Lenexa, KS); glutathione (GSH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Nacalai Tesque (Kyoto, Japan); 6-OHDA (H4381, 97% > titration) and N-acetylcysteine (NAC) from Sigma-Aldrich (St. Louis, MO); H2O2 was from Wako Pure Chemical Industries (Osaka, Japan); rabbit anti-caspase-3 polyclonal antibody (pAb) from Cell Signaling Technology (Beverly, MA); mouse anti-actin monoclonal antibody (mAb) (clone C4) from Chemicon International (Temecula, CA); and mouse anti-cytochrome c mAb (BV-3026-3) and caspase inhibitors, such as z-DEVD-fmk, z-LEHD-fmk, and z-IETD-fmk, were obtained from Medical and Biological Laboratories (Nagoya, Japan). Rabbit anti-peroxiredoxin 3 and 6 pAbs were obtained from Lab Frontier (Seoul, Korea). Undifferentiated PC12 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan. Other chemicals were of the highest quality commercially available.

**Analysis of 6-OHDA autoxidation**

The autoxidation of 6-OHDA was measured spectrophotometrically [20]. The assay was conducted in a cell free system under conditions corresponding to cellular 6-OHDA treatments. Stock solutions of 6-OHDA (50 mM) were prepared in phosphate-buffered saline (PBS) solution and used for all experiments. PBS or serum containing DMEM/F12 (phenol red free) with or without catalase (50 U/ml), GSH (2 mM), or NAC (2 mM) was thermostatically maintained at 37°C during the experiment. The experiment was initiated by the addition of 6-OHDA to give a final concentration of 100 μM. Maximum absorption of p-quinone (490 nm) was monitored every 10 s.

**Determination of H2O2 generation**

Oxygen consumption by 6-OHDA was studied on a biological oxygen monitor (Yellow Springs Instrument, OH) connected to a recorder. The temperature of the system was kept at 37°C by a circulating water pump. Oxygen consumption was measured in 10 ml of PBS or serum containing DMEM/F12 (phenol red free). The reaction was started by the addition of variable amount of 6-OHDA in cuvettes. Catalase was added in cuvettes at certain periods.

Hydrogen peroxide concentrations were also measured by using the ferrous oxidation of xylene orange (FOX) assay [21] and scopoletin assay [22]. In the former, reaction mixtures were added to the FOX reagent consisting of 100 μM xylene orange, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H2SO4 at specific intervals. An increase in absorption at 560 nm was measured, and concentration was calculated with a standard curve generated with reagent H2O2. For the latter analysis, 1 ml of PBS, 400 μl of 10 μM scopoletin, and 400 μl of 32.5 U/ml horseradish peroxidase were then added to 200 μl of reaction mixture. After 5 min at room temperature, fluorescence was measured at Ex 366 nm and Em 460 nm.

**Cell culture and determination of cell viability**

Undifferentiated PC12 cells, rat pheochromocytoma cell line, were routinely maintained in D-MEM/F-12 medium containing 10% heat-inactivated fetal bovine serum and 5% heat-inactivated horse serum at 37°C under an atmosphere of 95% air and 5% CO2. In order to analyze the toxicity of 6-OHDA, the PC12 cells were grown on plates at a density of 2 × 105 cells/ml. After the cells were attached (16–18 h), they were treated with 6-OHDA at different concentrations for the indicated times. For the determination of cell viability, MTT assay and lactate dehydrogenase (LDH) release assay were conducted for the indicated periods. In the former, the cells were incubated with 0.5 mg/ml MTT in fresh medium at 37°C for 2 h.
Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, by volume), and they were mixed by pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland). For the latter analysis, LDH release was measured by using iodotetrazolium chloride according to the protocol outlined by the manufacturer in the Cytotoxic Detection Kit (Roche Diagnostics, Penzberg, Germany). The amount of formazan formed was measured at 490 nm using a Multiskan Ascent plate reader. It was confirmed that contaminated compounds derived from 6-OHDA had no influence on this assay with cell-free serum medium (data not shown). Maximum LDH release was determined by incubation of cells with 1% Triton X-100. Data are expressed as a percentage of maximum LDH release, after subtraction of background determined from serum medium alone.

Two-dimensional gel electrophoresis (2-DGE)

After treatment, the cells were washed with PBS and then dissolved in isoelectric focusing sample buffer consisting of 9 M urea, 2% (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulphonate) (Chaps), 65 mM dithioerythritol (DTE), and 0.5% carrier ampholyte (pH 4–7; Amersham Biosciences), as described previously [23]. The sample solution (50 μg protein) was applied to an immobilized pH gradient gel (7 cm, pH 4–7; Amersham Biosciences) and rehydrated for 12 h. Isoelectric focusing was performed for a total of 16,750 V h at a maximum voltage of 5000 V. Each strip was equilibrated in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 2% SDS, 30% glycerol, and 20 mM DTE, for 20 min. The second dimension was carried out by 12.5% SDS-PAGE. Proteins separated by 2-DGE were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA), and subsequently subjected to Western blot analysis, according to the previously described procedure [24].

Protein extraction, subcellular fractionation, and Western blot analysis

To obtain total cell extracts, treated cells were corrected, washed with ice-cold PBS, and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, and 1 mM Na3VO4 with a protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany)) at 4°C for 10 min. Indicated amounts of protein were loaded and separated by SDS-PAGE.

Following a centrifugation at 800g at 4°C for 10 min, the supernatant was separated from the pellet consisting of mitochondrial and cellular debris. The supernatant containing cytoplasmic proteins was purified by centrifugation at 13,000g at 4°C for 10 min. Indicated amounts of protein were loaded on separated by SDS-PAGE.

In all cases, the detection of specific proteins either in the total cell extracts or in the cell fractions was carried out by Western blot analysis as described above.

Caspase activity assays

Caspase activity was measured by cleavage of the Asp-Glu-Val-Asp (DEVD) peptide-conjugated p-nitroanilide (pNA), according to the protocol outlined by the manufacturer of the Caspase-3/CPP32 Colorimetric Protease Assay Kit (Medical & Biological Laboratories). Substrate cleavage, which resulted in the release of pNA (405 nm), was measured by using a Multiskan Ascent plate reader (Thermo Labsystems). Absorbance units were converted to picomoles of pNA using a standard curve generated with free pNA.

Statistical analysis

Data are expressed as means ± SD of at least three separate experiments. Statistical analyses were performed by an analysis of variance (ANOVA) using Dunnett and Tukey tests for multiple comparisons. The calculation method is described in each figure legend.

Results

Autoxidation of 6-OHDA and the formation of H2O2 and p-quinoine

It has been reported that 6-OHDA is readily oxidized in the presence of oxygen to yield H2O2 and p-quinone [18,20,26]. In order to determine the role of autoxidation in 6-OHDA-induced neurotoxicity, we first determined the formation of H2O2 and p-quinone from 6-OHDA in serum-containing medium without cells. An increase in the absorption at 490 nm, attributed to the production of p-quinone, was observed in a spectrophotometric assay and this reaction was completed within a few minutes (Fig. 1A). There was no difference between PBS solution and serum medium on the increase in the absorption at 490 nm (data not shown). Using a molecular extinction coefficient of 1892 M⁻¹ cm⁻¹ for p-quinone [20], the p-quinone generated from 100 μM 6-OHDA was calculated to be 103 μM (Fig. 1A).

Furthermore, the formation of H2O2 in PBS and serum medium was quantified using three different methods. As shown in Fig. 1B, 6-OHDA consumed oxygen vigorously in a time- and concentration-dependent manner and this consumption was also completed within a few minutes in PBS solution. The addition of catalase at this point caused the formation of oxygen, equivalent to approximately one-half of the oxygen consumed. At 100 μM 6-OHDA, 50 μM oxygen was consumed and 25 μM oxygen was released by the addition of catalase,
suggesting that 50 μM H₂O₂ was formed from the 100 μM 6-OHDA in the PBS solution by reactions (1) and (2).

\[ \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + 1/2\text{O}_2. \]  

(2)

The generation of 50 μM H₂O₂ from 100 μM 6-OHDA, following incubation at 37°C for 5 min in PBS solution, was also observed by the methods of FOX and scopoletin assays. In serum medium, the generation of H₂O₂ from 6-OHDA was not determined by using either oxygen consumption or the scopoletin assay owing to H₂O₂ interference with the assay system. On the other hand, the formation of 50 μM H₂O₂ from 100 μM 6-OHDA was detected in serum medium using the FOX assay.

Effect of 6-OHDA and H₂O₂ on cell viability

To determine the effect of 6-OHDA, we next measured the cell viability using an MTT mitochondrial function assay. When the PC12 cells were cultured with variable amounts of 6-OHDA for 24 h, the cell viability decreased in a concentration-dependent manner (Fig. 2A). The cell viability was significantly decreased by 6-OHDA concentrations greater than 12.5 μM. Based on this result, we used 100 μM 6-OHDA in further analyses. A time-dependent study revealed that the mitochondrial function, as measured by the MTT assay, decreased linearly after the addition of 100 μM 6-OHDA (Fig. 2B). We also measured the viability using an LDH release assay, which is an index of membrane integrity. In contrast to the MTT assay, membrane integrity gradually decreased in a concentration- and time-dependent manner (Figs. 2C and D).

To clarify the role of H₂O₂ in 6-OHDA-induced cytotoxicity, the effect of H₂O₂ addition on the viability of PC12 cells was examined using the MTT and LDH release assays. As shown in Fig. 2, the cytotoxicity induced by H₂O₂ was similar to that induced by 6-OHDA; this suggests that not only the H₂O₂ generated from 6-OHDA but also secondary products such as p-quinone play a role in 6-OHDA-induced cytotoxicity. Further, we focused on the role of 50 μM H₂O₂ and secondary products generated from 100 μM 6-OHDA.

Protective effect of antioxidants against 6-OHDA- and H₂O₂-induced cell death

In order to understand the underlying mechanisms of 6-OHDA-induced cytotoxicity and the role of the H₂O₂ formed, we examined the effect of several antioxidants. In the case of thiol antioxidants such as GSH and NAC, two different protocols, namely the pre- and coincubation procedures, were used. In the former procedure, the cells were pretreated with antioxidants for 24 h and, subsequently, 100 μM 6-OHDA was added. In the latter procedure, the antioxidants and 100 μM 6-OHDA were added simultaneously and coincubated for 24 h. These thiol antioxidants effectively attenuated the cell death induced by 6-OHDA and H₂O₂ (Figs. 3A and B). In the case of GSH, the coincubation procedure tended to be more effective than preincubation for the attenuation of 6-OHDA. On the other hand, NAC was effective against H₂O₂-induced cytotoxicity in the preincubation procedure rather than by coincubation. In the case of 6-OHDA and NAC, a similar protective effect was observed using the two procedures. At an effective concentration against cell death induced by 6-OHDA, these antioxidants significantly attenuated the generation of p-quinone in spectrophotometric assays (Fig. 1A).

To clarify the role of the H₂O₂ formed from 6-OHDA, the protective effect of catalase, an H₂O₂-removing enzyme, was examined. The cell death induced by 50 μM H₂O₂ was completely abolished in the presence of 50 U/ml catalase (Figs. 4A and B). The two methods produced essentially similar results. In the case of 6-OHDA, a significant but partial protective effect of catalase was observed in both assay systems (Fig. 4). Since exogenously added H₂O₂ is known to diffuse rapidly into cells and react with cellular antioxidants [27], a
A washing experiment was conducted to estimate the effect of H$_2$O$_2$ and the products, including p-quinone, generated from 6-OHDA. In the washing experiment, PC12 cells were treated with 50 μM H$_2$O$_2$ or 100 μM 6-OHDA for 15 min, washed, and then cultured in fresh medium for 24 h. As shown in Fig. 4, the washing procedure had a significant protective effect against 6-OHDA, but not against H$_2$O$_2$. We discovered that there was no significant difference between the cytotoxic effects of washing experiment was conducted to estimate the effect of H$_2$O$_2$ and the products, including p-quinone, generated from 6-OHDA. In the washing experiment, PC12 cells were treated with 50 μM H$_2$O$_2$ or 100 μM 6-OHDA for 15 min, washed, and then cultured in fresh medium for 24 h. As shown in Fig. 4, the washing procedure had a significant protective effect against 6-OHDA, but not against H$_2$O$_2$. We discovered that there was no significant difference between the cytotoxic effects of thiol antioxidants against 6-OHDA and H$_2$O$_2$ toxicity. The cells were treated with 100 μM 6-OHDA or 50 μM H$_2$O$_2$ in the presence of different concentrations of GSH (A) and NAC (B) according to the pre- or cotreatment protocol described under Results. The viability was determined by MTT assay after 24 h. Mean ± SD of at least three experiments are shown. *P < 0.05 (Dunnett, ANOVA) when compared with controls (without antioxidant).
100 μM 6-OHDA and 50 μM H₂O₂ following the washing procedure, suggesting that the H₂O₂ formed from 6-OHDA immediately reacted with the cells and exhibited a cytotoxicity similar to that of H₂O₂ alone.

**Peroxiredoxin (Prx) oxidation induced by 6-OHDA and the protective effect of catalase**

We have previously reported that the H₂O₂-reducing enzyme Prx family of proteins and the protein DJ-1 were oxidized when cells were exposed to oxidants such as H₂O₂ [8,23,25]. It is thought that Prx oxidation is one of the stress markers indicating the oxidative insults induced by oxidative stress. We therefore examined the protein oxidation induced by 6-OHDA using Western blot analysis combined with 2-DGE. As shown in Fig. 5, an acidic spot shift of Prx 3 and 6 was observed 15 min after 100 μM 6-OHDA exposure. Almost all the Prx 6 and one-half of the Prx 3 were shifted to acidic satellites during this incubation time. It has been demonstrated that these acidic pI shifts are due to a posttranslational process that is caused by the oxidation of cysteine residues. The results are consistent with the idea that 6-OHDA causes Prx oxidation in a similar manner to that of H₂O₂ alone.

Fig. 4. Protective effect of catalase against 6-OHDA and H₂O₂ toxicity. The cells were treated with 100 μM 6-OHDA or 50 μM H₂O₂ in the presence or absence of catalase (50 U/ml). Washing experiment was conducted according to the protocol described under Results. The viability was determined by MTT assay (A) and LDH assay (B) after 24 h. Mean ± SD of at least three experiments are shown. *P < 0.05 (Turkey, ANOVA). NS, not significant.

Fig. 5. Prx oxidation induced by 6-OHDA and H₂O₂ and protective effect of catalase. Cell samples (50 μg protein) obtained from the cells treated with 0 μM, 50 μM H₂O₂, and 100 μM 6-OHDA for 15 min were separated by 2-D gel electrophoresis and subsequently subjected to Western blot analysis using antibodies specific to Prx 3 and Prx 6, as described under Materials and methods. The cells treated with catalase (50 U/ml) and 100 μM 6-OHDA were also analyzed.
oxidation of a cysteine residue to form cysteine sulfenic acid (Cys-SO\(_2\)H) or cysteine sulfonic acid (Cys-SO\(_3\)H); these are inactivated forms of Prx [23,28]. We found that a similar oxidation of Prx proteins occurred at 15 min after 50 μM H\(_2\)O\(_2\) exposure. To determine the role of the H\(_2\)O\(_2\) formed from 6-OHDA, we examined the oxidation of these proteins by 6-OHDA in the presence of 50 U/ml catalase. As shown in Fig. 5, the protein oxidation induced by 6-OHDA was completely abolished by the addition of catalase.

Characterization of caspase activation induced by 6-OHDA

It has been demonstrated in PC12 cells that mitochondrial dysfunction initiated by 6-OHDA induces the release of cytochrome c, caused the consequent activation of caspase-9 with apoptosome formation in the presence of ATP, and promotes the activation of caspase-3 [11,14-16]. Using Western blot analysis, we observed that 100 μM 6-OHDA induced the proteolytic activation of caspase-3. Activated caspase-3 was obvious at 6 h after 6-OHDA exposure (Fig. 6A). In order to determine the roles of H\(_2\)O\(_2\) and secondary products such as p-quinone, we examined the effects of catalase addition and the washing procedure; these were compared with the effect of 50 μM H\(_2\)O\(_2\) alone. As shown in Fig. 6B, a slight increase in activated caspase-3 was observed in the cells treated with 50 μM H\(_2\)O\(_2\) for 6 h. The observed caspase-3 activation was completely abolished in the presence of catalase but was
not influenced by the washing procedure. On the other hand, treatment with 100 μM 6-OHDA for 6 h caused a relatively strong increase in activated caspase-3 when compared with the effect of 50 μM H2O2 alone. Contrary to the results observed with H2O2 exposure, catalase failed to inhibit the caspase activation induced by 6-OHDA. It was also notable that the washing procedure effectively attenuated the increase of activated caspase-3. These actions of caspase-3 activation were further confirmed using a DEVD peptide conjugated to the chromophore pNa. Similar results were obtained in the case of caspase enzyme activity (Fig. 6C).

Characterization of the cytochrome c release induced by 6-OHDA

The release of cytochrome c from mitochondria into the cytosol is required for the assembly of the apoptosome and hence for the activation of the caspase cascade in the intrinsic pathway of apoptosis [29]. Western blot analysis was conducted in order to detect the presence of cytochrome c in cytosol extracts from cells treated with 100 μM 6-OHDA. As shown in Fig. 7A, a time-dependent study revealed that cytosolic cytochrome c was detectable after 30 min during 6-OHDA exposure and its level increased with incubation time. We next examined the effects of catalase addition and the washing procedure on the release of cytochrome c after incubations of 2 or 6 h. As shown in Fig. 7B, catalase effectively attenuated the cytochrome c release induced by H2O2; however, the washing procedure was not effective. On the other hand, a similar level of cytochrome c release with H2O2 was observed in the case of 6-OHDA after a 2-h incubation (Fig. 7B). Furthermore, catalase exhibited a protective effect against 6-OHDA-induced cytochrome c release. The protective effect of catalase was also evident with a 6-h incubation time. However, the washing procedure did not have a strong inhibitory effect on cytochrome c release.

Effect of inhibitors of caspase-8, caspase-9, and calpain on the caspase-3 activation induced by 6-OHDA

In order to determine the involvement of a cytochrome c-independent pathway on caspase-3 activation induced by 6-OHDA, the effects of caspase inhibitors were examined. As shown in Fig. 8A, the appearance of activated caspase-3 was effectively inhibited in the presence of z-LEHD-fmk (a caspase-9 inhibitor) and z-IETD-fmk (a caspase-8 inhibitor), suggesting that the activation of not only caspase-9 but also caspase-8 plays a role in the caspase-3 activation induced by 6-OHDA. We further examined the inhibitory effect of calpain, which is a Ca2+-responsive cytosolic cysteine protease associated with the cleavage of caspase-12, on the caspase-3 activation induced by 6-OHDA. The appearance of activated caspase-3 induced by 6-OHDA was significantly inhibited in the presence of MDL 28170 (calpain inhibitor III) (Fig. 8B).

Discussion

6-OHDA, a selective catecholaminergic neurotoxin, has been widely used to produce PD models. The toxic effect of 6-OHDA is thought to be mediated by its uptake into catecholaminergic nerve endings through the high-affinity catecholamine transporter system [4]. It has been postulated that 6-OHDA induces cell death by the following three main mechanisms: (I) ROS generation by autoxidation, (II) H2O2 generation after deamination by monoamine oxidase (MAO), and/or (III) direct inhibition of mitochondrial complexes I and IV [4]. However, a number of recent studies have demonstrated that 6-OHDA does not induce toxicity either by direct mitochondrial inhibition or by enzymatic deamination by MAO, but via an extracellular mechanism [11,30,31]. Using inhibitors of dopamine and noradrenaline transporters, such as GBR-12909 and nisoxetine, a study demonstrated that transporter-mediated uptake has a minimal role in the 6-OHDA toxicity in PC12 cells [11]. We also observed that these inhibitors failed to inhibit the cell death, cytochrome c release, and caspase activation induced by 6-OHDA (data not shown). These lines of evidence indicate that extracellular autoxidation, which occurs through the generation of H2O2 and p-quinone, plays an important role in 6-OHDA-induced cytotoxicity.

As shown in Fig. 3, 6-OHDA neurotoxicity was inhibited in the presence of thiol antioxidants such as NAC and GSH. It has also been reported that these thiol antioxidants effectively attenuate the insults induced by 6-OHDA, not only in vitro but also in vivo [11,30]. NAC is readily taken up by cells and subsequently acts as the source of cellular GSH. On the other hand, it is known that GSH is barely incorporated into cells. It was observed that rather than preincubation, coincubation with GSH tended to be more effective against 6-OHDA-induced cytotoxicity (Fig. 3A). Since GSH (2 mM) prevented 6-OHDA autoxidation, it appears that the inhibitory effect of GSH against 6-OHDA-induced cytotoxicity is mediated via the suppression of 6-OHDA autoxidation in the extracellular fluid. It is known that the cellular concentration of GSH is approximately 2 mM, while the extracellular concentration is lower; this suggests that

Fig. 8. Effect of inhibitors of caspases and calpain on the caspase-3 activation induced by 6-OHDA. The cell samples treated with 100 μM 6-OHDA in the presence or absence of inhibitors of caspases (2 μM, A) and calpain (120 μM MDL 28170, B) for 6 h were subjected to Western blot analysis using an antibody against caspase-3.
6-OHDA is readily oxidized in the extracellular fluid rather than in intracellular fluid. Taken together, these findings support the importance of the extracellular events leading to the generation of H$_2$O$_2$ and p-quinone in the 6-OHDA-induced neurotoxicity.

In order to clarify the role of the generated H$_2$O$_2$ and p-quinone in 6-OHDA-induced neurotoxicity, the effects of catalase addition and a washing procedure were examined. Since extracellularly added catalase is barely incorporated into cells, the effect of catalase addition suggests that the effect of H$_2$O$_2$ occurs in the extracellular fluid. Other published studies have successfully demonstrated the protective effect of catalase against 6-OHDA [11,31,32], although one study failed to do so [19]. In these studies, the experimental conditions such as concentrations of 6-OHDA and catalase, incubation time, and cell strain differed as follows: 150 μM 6-OHDA and 30 U/ml catalase for 15 min in PC12 cells (protective) [11], 100 μM 6-OHDA and 800 U/ml catalase for 24 h in PC12 cells (protective) [31], 175 μM 6-OHDA and 2500 U/ml catalase for 24 h in N-2A cells (protective) [36], and 100 μM 6-OHDA and 100 U/ml catalase for 24 h in SH-SY5Y cells (not protective) [19]. With the exception of the results published by Hanrot et al. [11] who used a short-time exposure, it is thought that a large quantity of catalase is necessary for protection against 6-OHDA-induced cytotoxicity. Therefore, it appears that not only enzyme activity but also other factors such as the iron present in catalase might be related to the prominent protective effect of catalase against 6-OHDA. In order to understand these differences, it is important to measure the H$_2$O$_2$ generated from 6-OHDA under the given culture conditions and compare the sensitivity of cell strains against H$_2$O$_2$.

As shown in Fig. 5, protein oxidation was observed 15 min after the addition of H$_2$O$_2$ and 6-OHDA. We previously reported that antioxidant proteins, namely Prx and DJ-1, were oxidized in an H$_2$O$_2$ concentration-dependent manner and the susceptibility of Prx 6, 3, and 2 to oxidation was relatively high [23,25]. It has been reported that the oxidation of the active cysteine in Prx to Cys-SO$_2$H or Cys-SO$_3$H (inactivated forms in Prx) cannot be reversed by the reducing equivalents provided by thiol-containing proteins such as thioredoxin. The enzyme responsible for the reduction of sulfanylated Prx has been identified and named sulfiredoxin (Srx) [33]. Kinetic analysis of the reduction of the sulfenic forms of Prx by Srx revealed that this was a slow process ($k_{\text{cat}}=0.18 \text{ min}^{-1}$) [34]. We observed oxidized Prx 3 and 6 at 6 h after 6-OHDA exposure (data not shown). The oxidation of these proteins indicates that the effect of the H$_2$O$_2$-reducing system comprising Prx decreases, which is important for cellular antioxidant functions. Since catalase exhibited a complete protective effect against oxidation of Prx, it is considered that the H$_2$O$_2$ generated from 6-OHDA acts as a major player in the oxidation of cellular proteins.

It has been reported that 6-OHDA-induced cell death involves apoptotic features such as DNA fragmentation, PS exposure, and caspase activation [10,11,16]. In this study, we observed significant activation of caspase-3 in the cells treated with 6-OHDA. Interestingly, in contrast to Prx oxidation, the effect of only H$_2$O$_2$ on caspase activation was relatively low (Fig. 6). Catalase was less effective against the caspase activation induced by 6-OHDA, while the washing procedure effectively attenuated this activation to a level similar to that observed with H$_2$O$_2$ exposure. Taken together, it is thought that secondary products such as p-quinone and H$_2$O$_2$ play an important role in the maximum activation of caspases induced by 6-OHDA. All quinones are redox cycling agents that generate ROS [35]. On the other hand, partially substituted quinones such as p-quinone also function as arylating agents that react with nucleophiles such as the reduced sulphydryl groups in GSH and cysteine, or the cysteinyl residues in proteins to form covalently linked quinone-thiol adducts [35,36]. Arylating quinones have unique biological properties such as high cytotoxicity that are not commonly shared by nonarylating quinones and arylated thiol adducts [35,37]. Although the precise mechanism of p-quinone-mediated cytotoxicity has not been fully elucidated, it appears that adduct formation between p-quinone and the cellular components largely contributes to H$_2$O$_2$-independent 6-OHDA cytotoxicity.

It has been demonstrated that the caspase cascades can be activated and amplified after the mitochondrial release of cytochrome c into the cytosol via the formation of a protein complex comprising cytochrome c and the apoptotic protease-activating factor-1 (Apaf-1)—the apoptosome—in the presence of dATP or ATP [24,38]. It is also known that H$_2$O$_2$ can induce the release of cytochrome c, and we observed the presence of cytosolic cytochrome c under conditions of oxidative stress induced by H$_2$O$_2$. With regard to the effect of catalase addition and the washing procedure (Fig. 7), it was thought that the H$_2$O$_2$ generated from 6-OHDA acted as a major player in the release of cytochrome c induced by 6-OHDA under the conditions employed.

![Fig. 9. Proposed pathways for 6-OHDA-induced PC12 cell death. Based on our findings in this work, H$_2$O$_2$-dependent and -independent action is involved in 6-OHDA-induced PC12 cell death. Added 6-OHDA is rapidly oxidized and generates both H$_2$O$_2$ and p-quinone. The generated H$_2$O$_2$ induces Prx oxidation, cytochrome c release, and caspase-3 activation, which are inhibited by catalase. On the other hand, the generated p-quinone might induce caspase-3 activation via both caspase-8 and caspase-12 activation in a mitochondria-independent manner.](image-url)
The results shown in Figs. 6 and 7 suggest the involvement of a cytochrome c-independent caspase activation pathway in 6-OHDA-induced cytotoxicity. As shown in Fig. 8, not only a caspase-9 inhibitor but also a caspase-8 inhibitor exhibited a significant protective effect against 6-OHDA-induced caspase-3 activation (Fig. 8A). Using MN9D cells and primary cultures of mesencephalic neurons treated with 6-OHDA, it has been demonstrated that a caspase-8-mediated apoptotic pathway is activated [9]. It has been reported that caspase-8-mediated cleavage of Bid promotes the mitochondrial release of cytochrome c into the cytosol [39]. It is also known that caspase-8 can directly cleave and activate caspase-3 [40]. Considering the results presented in this study, it appears that the latter action of caspase-8 is evident in PC12 cells treated with 6-OHDA.

Interestingly, a calpain inhibitor also exhibited an inhibitory effect on the caspase-3 activation induced by 6-OHDA (Fig. 8B). Calpain is a Ca2+-responsive protease associated with the cleavage of caspase-12, a key signal involved in endoplasmic reticulum (ER) stress-induced apoptosis [41]. Previous studies have reported on the involvement of ER stress in 6-OHDA-induced neurotoxicity [42,43]. There is considerable evidence indicating the involvement of ER stress in the pathogenesis of PD. For example, the loss of parkin, the second gene with disruption of protein folding [45]. On the other hand, it has been suggested that a calpain/caspase-12 pathway is involved in the activation of caspase-3 that is induced by 6-OHDA. This pathway has been postulated to be activated following the disruption of protein folding [45]. On the other hand, it has been recently reported that ER stress is a cellular mechanism for arylating quinone toxicity [46]. Further studies are necessary for the elucidation of the mechanism of ER stress induced by 6-OHDA, particularly the effect of p-quinone on the state of protein folding.

In conclusion, the present study clearly demonstrates that the H2O2 generated from 6-OHDA induces Prx oxidation, cytochrome c release, and caspase activation, while the generated secondary products, including p-quinone, are involved in caspase-3 activation in a cytochrome c-independent manner (Fig. 9). The present results have important implications in the development of antioxidant therapies for PD.

Acknowledgment

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (18790081).

References


