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Dopaminergic Neurotoxicant 6-OHDA Induces Oxidative Damage through Proteolytic Activation of PKCδ in Cell Culture and Animal Models of Parkinson's Disease

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Abstract

The neurotoxicant 6-hydroxydopamine (6-OHDA) is used to investigate the cellular and molecular mechanisms underlying selective degeneration of dopaminergic neurons in Parkinson's disease (PD). Oxidative stress and caspase activation contribute to the 6-OHDA-induced apoptotic cell death of dopaminergic neurons. In the present study, we sought to systematically characterize the key downstream signaling molecule involved in 6-OHDA-induced dopaminergic degeneration in cell culture and animal models of PD. Treatment of mesencephalic dopaminergic neuronal N27 cells with 6-OHDA (100 μM) for 24h significantly reduced mitochondrial activity and increased cytosolic cytochrome c, followed by sequential activation of caspase-9 and caspase-3. Co-treatment with the free radical scavenger MnTBAP (10 μM) significantly attenuated 6-OHDA-induced caspase activities. Interestingly, 6-OHDA induced proteolytic cleavage and activation of protein kinase C delta (PKCδ) was completely suppressed by treatment with a caspase-3-specific inhibitor, Z-DEVD-FMK (50 μM). Furthermore, expression of caspase-3 cleavage site-resistant mutant PKCδ^{D327A} and kinase dead PKCδ^{K376R} or siRNA-mediated knockdown of PKCδ protected against 6-OHDA-induced neuronal cell death, suggesting that caspase-3-dependent PKCδ promotes oxidative stress-induced dopaminergic degeneration. Suppression of PKCδ expression by siRNA also effectively protected N27 cells from 6-OHDA-induced apoptotic cell death. PKCδ cleavage was also observed in the substantia nigra of 6-OHDA-injected C57 black mice but not in control animals. Viral-mediated delivery of PKCδ^{D327A} protein protected against 6-OHDA-induced PKCδ activation in mouse substantia nigra. Collectively, these results strongly suggest that proteolytic activation of PKCδ is a key downstream event in dopaminergic degeneration, and these results may have important translational value for development of novel treatment strategies for PD.

Keywords

Oxidative Stress; 6-OHDA; PKC delta; Apoptosis; animal model; Parkinson's disease

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Conflict of interest statement
All authors declare that there is no conflict of interests.

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting more than 1% of the population over the age of 60. The significant loss of dopaminergic neurons (>70%) in the substantia nigra pars compacta is a hallmark of PD (Burke and Kholodilov, 1998; Orth and Tabrizi, 2003). Metabolic or neurotoxic insults often cause oxidative stress-mediated neuronal apoptosis and thereby contribute to the pathogenesis and progression of several forms of neurodegenerative diseases, including PD. Oxidative stress results from the imbalance of pro-oxidant/antioxidant homeostasis, leading to the generation of toxic reactive oxygen species (ROS). Oxidative stress often causes extensive damage to lipids, proteins, and DNA, resulting in cell death by a variety of different mechanisms including activation or inactivation of various apoptotic cell signaling molecules. Typically, unregulated ROS generation results in calcium dysregulation that leads to excitotoxic cell death or to mitochondrial dysfunction, resulting in activation of the apoptotic caspase cascade (Henchcliffe and Beal, 2008; Zhou *et al.*, 2008; Naoi *et al.*, 2009; Tsang and Chung, 2009; Cannon and Greenamyre, 2010; Kanthasamy *et al.*, 2010).

MPTP, 6-OHDA and rotenone are neurotoxins commonly used to investigate the pathogenesis and progression of PD in cellular and animal models (Moore *et al.*, 2005; Andrabi *et al.*, 2008; Henchcliffe and Beal, 2008; Zhou *et al.*, 2008; Lee *et al.*, 2009; Naoi *et al.*, 2009; Shin *et al.*, 2009; Tsang and Chung, 2009; Beal, 2010; Cannon and Greenamyre, 2010; Horowitz and Greenamyre, 2010; Kanthasamy *et al.*, 2010; Gao *et al.*, 2011). Results from MPTP and 6-OHDA studies suggest that oxidative stress-mediated apoptotic cell death is a major contributing factor to the selective degeneration of dopaminergic neurons in the PD model. 6-OHDA has been shown to impair mitochondrial dysfunction and caspase activation in a variety of cell lines and primary neuronal cultures (Choi *et al.*, 1999; Dodel *et al.*, 1999; Elkon *et al.*, 2004; Jordan *et al.*, 2004; Liu *et al.*, 2007; Puttonen *et al.*, 2008; Kim *et al.*, 2010; Kupershmidt *et al.*, 2010; Yoon *et al.*, 2010). However, the role of cell signaling molecules downstream of caspases in the degeneration of dopaminergic neurons in PD is yet to be identified.

Recently, we identified the novel PKC isoform protein kinase Cδ (PKCδ) is highly expressed in nigral dopaminergic neurons (Zhang *et al.*, 2007b) and the kinase is a prominent endogenous substrate for caspase-3, as well as a key player in oxidative stress-induced neuronal apoptosis (Kanthasamy *et al.*, 2003). The PKCδ is proteolytically cleaved by caspase-3 to yield a 38 kDa regulatory fragment and a 41 kDa persistently active catalytic fragment (Greenberg *et al.*, 1998; Brodie and Blumberg, 2003). In the present study, we examined the role of PKCδ in 6-OHDA-induced dopaminergic cell death by using loss of PKCδ function dominant negative mutants and RNAi-mediated knockdown of PKCδ expression. Both cell culture and animal models were used to delineate the PKCδ signaling in 6-OHDA-triggered oxidative damage. We demonstrated that proteolytic cleavage of PKCδ by caspase-3 is a critical event in 6-OHDA-induced dopaminergic degeneration in cell culture and animal models of PD.

Materials and Methods

Materials

ATP, Protein-A-Sepharose, 6-hydroxydopamine, and β-actin mouse monoclonal antibody were obtained from Sigma, Z-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-DEVD-FMK), Acetyl-Leu-Glu-His-Asp-7-amino-4-fluorcoumarin (Ac-LEHD-AFC) and acetyl-Asp-Glu-Val-Asp-7-amino-4-fluorcoumarin (Ac-DEVD-AFC), were obtained from MP Biochemicals (Irvine, CA). Anti-PKCδ antibody (rabbit polyclonal) was purchased from Santa Cruz

Biotechnology (Santa Cruz, CA), and the ECL chemiluminescence kit was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Sytox green nucleic acid fluorescence stain was purchased from Molecular Probes (Eugene, OR). Cell Death Detection ELISA Plus assay kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). [^{32}P-]ATP was purchased from Perkin Elmer (Downers Grove, IL). Bradford protein assay kit was purchased from Bio-Rad (Hercules, CA). RPMI-1640 medium, horse serum, fetal bovine serum, L-glutamine, penicillin, and streptomycin were obtained from Invitrogen (Gaithersburg, MD). Plasmids for pPKCδ^{K376R}-green fluorescent protein (GFP) and pEGFP-N1 were kind gifts from Dr. Stuart Yuspa (National Cancer Institute, Bethesda, MD). PKCδ^{D327A}-GFP (PKCδ-CRM) construct was a kind gift from Dr. Mary Reyland at the University of Colorado (Boulder, CO).

Cell culture

The immortalized rat mesencephalic dopaminergic neuronal cell line (N27) was developed from the ventral mesencephalon, a region of the brain that is directly affected in PD (Zhou *et al.*, 2000). N27 cells represent a homogenous population of tyrosine hydroxylase-positive (TH^+) neurons with functional characteristics including dopamine synthesis and cellular signaling pathway (Zhang *et al.*, 2007b, Jin *et al.*, 2011a, Jin *et al.*, 2011b). We and others have extensively used this cell model for studying neurodegenerative mechanisms in PD (Kaul *et al.*, 2003; Kitazawa *et al.*, 2003; Anantharam *et al.*, 2004; Latchoumycandane *et al.*, 2005; Kanthasamy *et al.*, 2010). The cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U penicillin and 50 μg/ml streptomycin (Kaul *et al.*, 2003; Anantharam *et al.*, 2004). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. N27 cells of passage 2–4 were used for most of the experiments.

Primary mesencephalic neuronal culture

Primary mesencephalic neuronal cultures were prepared from the ventral mesencephalon from embryonic day 15–17 Sprague Dawley rats as described previously (Yang *et al.*, 2004; Afeseh Ngwa *et al.*, 2009; Song *et al.* 2010). Briefly, ventral mesencephalic brain regions were dissected out of the rat embryos and the tissues were maintained on ice-cold, calcium-free EBSS supplemented with gentamycin (50 mg/ml) and penicillin/streptomycin (200 U), and then dissociated in EBSS solution containing trypsin (0.25%) for 15 min. The dissociated cells were plated at equal density (0.5×10^6 cells) in 30 mm-diameter tissue culture wells precoated with poly-L-lysine (1 mg/ml). The primary cultures were maintained in a chemically defined, serum-free media consisting of neurobasal medium fortified with B-27 supplements, L-glutamine (500 μM), penicillin (100 U), and streptomycin (100 μg/ml) (Life Technologies). The cells were maintained in a CO_2 incubator at 5% CO_2 and 37°C for 24 h, and then treated with cytosine arabinoside (10 μM) for 24 h to inhibit glial cell proliferation. Half of the culture medium was replaced every 2 days. Approximately 6–7 day-old cultures were used for experiments.

Animal studies

Six- to 8-week-old 26/C57/Bl mice weighing 25–30 g were housed in standard conditions: constant temperature (22 ± 1°C), humidity (relative, 30%), and a 12-h light/dark cycle. Mice were allowed free access to food and water. Use of the animals and protocol procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. C57 black mice were injected stereotaxically with 4 μl of 1 μg/l 6-OHDA (in 0.2% ascorbic acid) into the right substantia nigra (SN) and saline into the left SN at 10 angles of the following coordinates from the bregma: AP: −3.2; ML: 2.0; and DV: −4.7 as described previously (Filipov *et al.*, 2002). The mice were sacrificed 7 days later; left and right striatum and SN were removed and processed for Western blot and

immunoprecipitation kinase assays. For PKCδ-CRM lentiviral studies in animals, C57 black mice were injected stereotaxically with lenti-PKCδ-CRM (2 μl viral particles (5×10^9 gc/mL) at a rate of 0.2 μl/min using a microinjection pump into the right SN and lenti-LacZ into the left SN. After 6 days of lentiviral injection, the mice were re-injected with 4μg 6-OHDA. The mice were sacrificed 7-days later; left and right striatum and SN were removed and subjected to neurochemical, biochemical, and immunohistochemical analyses including western blot and immunoprecipitation kinase assays.

Assessment of cell viability

Cell viability was determined by trypan blue exclusion method and Sytox assay after exposing N27 cells to 100 μM 6-OHDA (Latchoumycandane *et al.*, 2005). Sytox assay was determined by using a cell-impermeable nucleic acid fluorescence-based dye (Sytox), which enters into dead cells and intercalates with DNA to produce green fluorescence (Jin *et al.*, 2011a). Sytox-stained cells were then observed using a Nikon TE 200 fluorescence microscope, and images were captured with a Spot RT camera.

Determination of mitochondrial activity

The N27 cells were incubated with 100 μM 6-OHDA for 12–24 h, and mitochondrial activity was determined by using MTT (3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyl tetrazolium bromide) assay, which is based on the formation of formazan from tetrazolium inside the active mitochondria (Latchoumycandane *et al.*, 2005). Briefly, N27 cells were plated in 24-well plates and treated with 6-OHDA. After treatment, the cells were washed once with PBS and then incubated in serum-free medium containing 0.25 mg/ml MTT for 2 h at 37°C. Formation of formazan was measured at 570 nm with reference wavelength at 630 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

Determination of cytosolic cytochrome c

The cytosolic cytochrome c levels were measured in cytosolic fractions obtained from control and 6-OHDA-treated N27 cells using an ELISA Kit as described previously (Anantharam *et al.*, 2002). Briefly, N27 cells (~5×10^6 cells) were exposed to 100 μM 6-OHDA for 3 and 6 h. After treatment, cytosolic fractions were obtained from lysed cells and the level of cytosolic cytochrome c was quantified by ELISA assay using a SpectraMax Gemini XS microplate reader.

Determination of caspase-9 and -3 activities

The activities of caspase-9 and -3 were measured using caspase-9- and -3-specific fluorescence substrates, Ac-LEHD-AFC and Ac-DEVD-AFC, respectively as described previously (Kaul *et al.*, 2003). Briefly, cells ($1–2 \times 10^5$ cells/well) were subcultured in 24-well tissue culture plates and treated with 100 μM 6-OHDA for 3, 6, 12 and 24 h. Formation of 7-amino-4-methylcoumarine (AMC), resulting from caspase substrate cleavage, was measured using a SpectraMax Gemini XS microplate reader with excitation at 400 nm (slit width 10 nm) and emission at 505 nm (slit width 20 nm). Enzyme activities are expressed as fluorescence units/mg protein/h.

Determination of proteolytic activation of PKCδ

N27 cells (~1×10^7 cells) were exposed to 100 μM 6-OHDA for 12 and 24 h and cell lysates were prepared as described previously (Kaul *et al.*, 2003). N27 cells were washed with phosphate buffered saline (PBS) and resuspended in homogenization buffer containing 25 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF and 4 μg/ml each of aprotonin and leupeptin. The cells were sonicated for 15 sec under ice-cold

conditions, and then the samples from control and treated cells were centrifuged at 16,000 × *g* for 60 min at 4 °C. The supernatants were collected as cell lysates. Mouse brain nigral lysates from 6-OHDA treated animals were prepared as described previously (Zhang et al., 2007). Cell and mouse brain lysates containing equal protein were mixed with 2x gel loading buffer containing 10% SDS and 200 mM DTT, and placed in a boiling water bath for 5 min. Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane (Bio-Rad Laboratories). The non-specific binding sites were blocked with 5% non-fat dry milk blocking solution (Amersham Pharmacia Biotech), and the membrane was then treated with anti-PKCδ (1:2000 dilution) antibody (Santa Cruz) or tyrosine hydroxylase antibody (1:2000) followed by secondary HRP-conjugated anti-rabbit or anti-mouse (1:2000) antibody. Antibody-bound proteins were detected by an enhanced chemiluminescence (ECL) system using a Kodak Imager (Kodak Image Station 2000R, Eastman Kodak Company, New Haven, CT). To confirm equal protein in each lane, membranes were reprobed with -actin (1:5000).

Determination of PKCδ activity

PKCδ enzymatic activity was measured using immunoprecipitation as described previously (Reyland *et al.*, 1999; Anantharam *et al.*, 2002). The proteins were immunoprecipitated from cell and mouse brain lysates overnight at 4 °C using anti-PKCδ antibody. After immunoprecipitation, 25μl of samples containing PKCδ bound to Sepharose A beads were incubated with 25μl of reaction buffer containing 0.4 mg histone H1 and 5 μCi of [γ-^{32}P] ATP (4,500 Ci/mM) for 10 min at 30 °C. The reaction was terminated after mixing with SDS gel loading buffer (2x) and boiling for 5 min. The samples were separated on a 15% SDS-PAGE, and histone-phosphorylated bands were detected using Phosphoimager (Personal Molecular Imager FX, Bio-Rad Laboratories, Hercules, CA) and quantified using Quantity One 4.2.0 Software (Bio-Rad Laboratories).

Lentiviral-mediated transduction of caspase resistant PKCδ^{D327A} and kinase inactive PKCδ^{K376R} mutant proteins

We used the ViraPower Lentiviral Expression System (Invitrogen) to stably transduce a caspase-resistant mutant of PKCδ (PKCδ^{D327A}, lysine to arginine mutation at position 327) into N27 cells or primary mesencephalic neurons. Initially, the cDNA coding for PKCδ^{D327A} (herein referred to as plenti6/PKCδ-CRM) was transferred from pEGFP-N1 vector to plenti6/V5-D-TOPO expression vector using standard cloning procedures. The PCR primers were: forward, 5′CACCATGGCACCCTTCCTGCTC3′ and reverse, 5′AATGTCCAGGAATTGCTCAAAC 3′. The cloning and preparation of lentivirus coding for pLenti6PKCδ^{K376R}–DN have been described previously (Kitazawa *et al.*, 2005). Standard expression procedures were used to produce lentiviruses. Briefly, the plenti6/PKCδ-CRM constructs were transfected into human 293FT cells using Lipofectamine 2000 transfection reagent as per the manufacturer's protocol (Invitrogen). The lentivirus in the medium was collected by centrifuging at 1500 × *g* for 15 min 48–72 h post-transfection. Lentivirus containing plenti/LacZ was also produced to serve as a vector control. Lentiviruses containing either plenti/PKCδ^{D327A}-CRM or plenti/PKCδ^{K376R}-DN and polybrene (6 μg/ml) were added into cultured N27 cells. Stable cell lines were established by selection in 10 μg/ml Blasticidin 48 h after transfection. Colonies were isolated, then replated and grown to confluence in T75 flasks. Subsequently, the stable cell lines were maintained in 5 μg/ml blasticidin. PKCδ-CRM-V5-, PKCδ-DN-V5- or LacZ-V5-expressing N27 cells were identified by immunostaining of the V5 epitope present in the C-terminus.

PKCδ siRNA transfections

PKCδ-siRNA was prepared by an *in vitro* transcription method as described previously (Yang *et al.*, 2004). Transfection of N27 cells was carried out using a commercially

available TKO transfection reagent (Mirus Corporation, Madison, WI). Briefly, diluted TKO reagent was mixed with either 25 nM PKCδ siRNA duplex or 25 nM non-specific siRNA to form a lipid-siRNA complex; this complex was then added to the N27 cells (50–70% confluent). After 24 h, N27 cells were treated with 100 μM 6-OHDA for an additional 24 h, then used for DNA fragmentation assay. The siRNA-transfected cells were viewed under a Nikon TE 200 fluorescence microscope and images were captured with a Spot RT camera.

Determination of DNA fragmentation by ELISA

Extent of DNA fragmentation was measured using Cell Death Detection ELISA Plus Assay Kits, as per manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). This is a highly sensitive assay for the detection of early apoptotic events that measures the amount of histone-associated low molecular weight DNA in the cytoplasm of cells. Briefly, N27 cells were exposed to 100 μM 6-OHDA with or without 50 μM Z-DEVD-FMK (caspase-3-specific inhibitor) for 24 h. The fragmented DNA was quantified colorimetrically using a SpectraMax microplate reader (Anantharam *et al.*, 2002).

Statistical analysis

All the data were analyzed using Prism 4.0 Software (GraphPad, San Diego, CA). Data were first analyzed using one-way ANOVA. Then Dunnett's post hoc test was performed to compare the differences between control and treated samples. P-values <0.05 were considered significant.

Results

6-OHDA induces neuronal cell death

Quantification of cell viability or cell death is an important parameter in assessing neurotoxin-induced cytotoxic cell death. The dopaminergic neuronal cells derived from mesencephalic regions are more vulnerable to neurotoxin- and oxidative-stress-induced cell death. In the present study, cell viability was determined by trypan blue exclusion method and cell death was measured by Sytox green cell death assay. N27 cells treated with 100 μM 6-OHDA showed decreased cell viability (50% reduction) at 24 h as compared to control cells (Fig. 1A). Sytox green dye enters the cells via damaged plasma membrane, intercalates with DNA and emits green fluorescence. After 24 h treatment with 100 μM 6-OHDA, Sytox green-positive cells were viewed under a Nikon TE 200 fluorescence microscope, and images were captured with a Spot RT camera. The Sytox assay revealed 50% cell death as compared to control groups (Fig. 1B), confirming the results obtained with trypan blue exclusion assay. Together, these results suggest that exposure to 100 μM 6-OHDA for 24 h causes significant cell death in dopaminergic neuronal cells.

6-OHDA alters mitochondrial activity and release of cytochrome c

It is well known that mitochondria play a significant role in apoptosis. In response to a variety of stress stimuli, mitochondrial proteins, such as cytochrome c, are released into the cytosol, to further activate the downstream apoptotic caspase cascade (Susin *et al.*, 1999). In the present study, mitochondrial activity was assessed by the intake and conversion of tetrazolium salts into formazan by intact mitochondria inside the cell. 6-OHDA treatment significantly decreased the mitochondrial activity as compared to control cells in a time-dependent manner (Fig. 2A). Furthermore, 6-OHDA also induced the release of cytochrome c from the mitochondria into the cytosol, as determined by ELISA. Cytochrome c release was observed as early as 3 h after 6-OHDA treatment, and continued to increase steadily up to 6 h (Fig. 2B). Exposure to 100 μM 6-OHDA for 3 and 6 h increased cytosolic cytochrome

c levels by 80% and 200%, respectively, as compared with corresponding control cells, suggesting that mitochondrial dysfunction contributes to 6-OHDA-induced cell death.

6-OHDA activates caspase-3 and -9 in a time-dependent manner

Cytochrome c released from mitochondria is known to activate multiple caspases that play an important role in the execution of apoptosis in neuronal and non-neuronal cells. Exposure to 100 μM 6-OHDA induced increases in caspase-9 and -3 enzyme activities up to 12 h in a time-dependent manner, as compared to control (Fig. 3). The activity of caspase-9 increased by 75% and 65% at 6 h and 12 h, respectively, as compared to untreated controls (Fig. 3A). The activity of caspase-3 increased by 150% and 200% at 6 h and 12 h, respectively (Fig. 3B). These data indicate that caspase-9 activity peaks at 6 h or earlier and precedes caspase-3 activity, which peaks at 12 h or later. Co-treatment with 10 μM MnTBAP, a SOD mimetic, significantly inhibited 6-OHDA-induced increases in caspase-3 activity when compared with 6-OHDA-treated cells alone (Fig. 3C). MnTBAP inhibited caspase-3 activity by 71% and 74% at 6 h and12 h, respectively, when compared to 6-OHDA treated cells. This suggests that free radicals are critically involved in 6-OHDA-induced caspase-3 activation.

Caspase-3 mediates proteolytic activation of PKCδ in 6-OHDA-treated neuronal cells

In the present study, exposure to 100 μM 6-OHDA for 12 and 24 h resulted in concomitant increases in the magnitude of 41 kDa catalytic and 38 kDa regulatory PKCδ fragments (Fig. 4A), as compared to control, in a time-dependent manner. Co-treatment with 50 μM Z-DEVD-FMK, a caspase-3 specific inhibitor, almost completely blocked the 6-OHDA-induced PKCδ cleavage at both time points of 12 h and 24 h, suggesting that PKCδ cleavage is mediated via caspase-3 (Fig. 4A).

A dramatic increase in PKCδ enzymatic activity, as determined by immunoprecipitation kinase assays, suggested that an increase in the PKCδ kinase activity parallels the proteolytic cleavage products (Fig. 4B). A 24 h treatment with 100 μM 6-OHDA induced a ten-fold increase in PKCδ kinase activity. The kinase activity assay was performed in the absence of lipids to determine the activity contributed solely by the proteolytically-cleaved PKCδ catalytic fragment. The increase in PKCδ kinase activity was almost completely blocked in cells co-treated with 50 μM Z-DEVD-FMK, suggesting that caspase-3 mediates 6-OHDA-induced activation of PKCδ.

Suppression of 6-OHDA-induced DNA fragmentation by caspase-3 inhibitor or free radical scavenger

Chromatin condensation and DNA fragmentation are key markers of apoptosis and have been shown to increase in dopaminergic neuronal cells upon treatment with dopaminergic toxins (Anantharam *et al.*, 2002; Kitazawa *et al.*, 2003). DNA fragmentation was measured quantitatively by ELISA (Roche Molecular Biochemicals), as previously described (Kitazawa *et al.*, 2002). Exposure to 100 μM 6-OHDA significantly increased DNA fragmentation by 70% at 24 h, as compared to control (Fig. 5). However, co-treatment with 50 μM Z-DEVD-FMK or 10 μM MnTBAP significantly blocked 6-OHDA-induced DNA fragmentation by 95% and 92%, respectively (Fig. 5), suggesting that both generation of free radicals and activation of caspase-3 are essential for 6-OHDA-induced apoptosis.

Caspase cleavage-resistant mutant PKCδ^{D327A}-CRM protects against 6-OHDA-induced PKCδ cleavage, apoptotic cell death and TH neuronal cell loss

To determine the effect of caspase-3 on 6-OHDA-induced cleavage of PKCδ, N27 cells were engineered to stably express caspase cleavage-resistant PKCδ mutant (PKCδ^{D327A}-

CRM) and then challenged with 6-OHDA. Figure 6A shows N27 cells stably expressing PKCδ-CRM-V5 fusion protein immunostained with V5 antibody. Exposure to 100 μM 6-OHDA failed to induce proteolytic cleavage of PKCδ in PKCδ-CRM transfected cells for up to 24 h as compared to 6-OHDA-treated naïve N27 cells (Fig. 4A). In a similar fashion, treatment with 6-OHDA also failed to induce a significant increase in DNA fragmentation in N27 cells stably expressing PKCδ-CRM compared to LacZ control N27 cells (Fig. 6B).

To determine whether PKCδ-CRM can also protect against 6-OHDA-induced dopaminergic cell death in primary cultures, rat mesencephalic dopaminergic primary neurons were transiently transfected with plasmids PKCδ^{D327A}-CRM-V5 and Lac-V5 using a nucleofector device. The transfection efficiency was determined to be greater than 80% in both PKCδ-CRM and LacZ-transfected primary neurons as determined by V5-immunostaining. Twenty-four h after transfection, the primary neurons were treated with 30 μM 6-OHDA for 3 h. After treatment, the primary neurons were fixed and immunostained for tyrosine hydroxylase (TH). Immunostaining experiments revealed that 6-OHDA induced a significant loss in the number of TH$^+$ neurons in LacZ-transfected primary neurons as compared to untreated LacZ-transfected primary neurons (Fig. 7B). However, 6-OHDA failed to induce a significant loss in the number of TH$^+$ neurons in PKCδ-CRM-transfected primary neurons as compared to untreated PKCδ-CRM-transfected primary neurons (Fig. 7B). LacZ vector transfected primary neurons were affected by 6-OHDA while PKCδ-CRM-transfected primary neurons remained intact (Fig. 7A), indicating PKCδ-CRM offered significant protection against apoptotic cell death and TH$^+$ neuronal cell loss.

Loss-of-function-PKCδ^{K376R}-DN mutant rescues neuronal cells against 6-OHDA-induced apoptotic cell death

To determine whether enzyme activity of cleaved PKCδ also is critical for 6-OHDA-induced cell death, we engineered N27 cells to stably express loss-of-function kinase dead PKCδ mutant (PKCδ^{K376R}-DN) in which lysine at position 376 in the catalytic site is mutated to arginine, rendering the kinase inactive. We then challenged the cells with 6-OHDA. Figure 8A shows N27 cells stably expressing PKCδ-DN-V5 fusion protein immunostained with V5 antibody. Exposure to 100 μM 6-OHDA resulted in a 75% increase in DNA fragmentation in N27 cells stably expressing LacZ vector as compared to untreated LacZ-expressing cells. However, 6-OHDA treatment failed to induce a significant increase in DNA fragmentation in N27 cells stably expressing PKCδ-DN compared to untreated PKCδ-DN expressing cells (Fig. 8B). These results strongly suggest that PKCδ kinase activity is essential for 6-OHDA-induced apoptotic cell death in dopaminergic neuronal clonal cells.

RNAi-mediated knockdown of PKCδ rescues neuronal cells against 6-OHDA-induced DNA fragmentation

To further substantiate the functional role of PKCδ in 6-OHDA-induced neuronal cell death, we examined the effect of siRNA targeted to PKCδ on 6-OHDA-induced DNA fragmentation. We recently developed siRNAs targeted against the coding region of PKCδ that specifically suppress PKCδ expression without producing any cytotoxic effect in dopaminergic cells (Yang *et al.*, 2004). PKCδ-siRNA dramatically suppressed PKCδ protein expression, as demonstrated by secondary staining with Alexa 488 in Cy3-labeled PKCδ-siRNA (siRNA PKCδ-4) transfected N27 cells, compared to non-specific siRNA (siRNA-NS) transfected N27 cells (Fig. 9A). SiRNA transfection studies revealed that exposure to 100 μM 6-OHDA for 24 h resulted in a significant increase in DNA fragmentation in non-specific siRNA transfected N27 cells, whereas 6-OHDA failed to induce a significant increase in DNA fragmentation in PKCδ siRNA-transfected cells (Fig. 9B), thus confirming the pivotal role of PKCδ in 6-OHDA-induced dopaminergic cell death.

Proteolytic cleavage of PKCδ in the substantia nigra of 6-OHDA injected animals

To determine if 6-OHDA-induced proteolytic activation of PKCδ observed in N27 cells (Fig. 4) also occurs *in vivo*, we examined the proteolytic activation of PKCδ in an animal PD model. Adult C57 black male mice were stereotaxically injected with 4 ug 6-OHDA in the right substantia nigra (SN), and saline was injected into the left SN as described in the Methods section. After 7 days animals were sacrificed and then nigral tissues were dissected out using a tissue punch. Western blot analysis of nigral tissue lysate revealed the proteolytic cleavage of native PKCδ (72–74 kDa) into a PKCδ catalytic fragment (41 kDa) and a 38 kDa regulatory fragment in the 6-OHDA-injected side (Fig. 10A). No cleavage was observed in saline-injected substantia nigra. Tyrosine hydroxylase (TH) immunoblot of each sample was used as a marker of nigral tissue, and β-actin bands represented equal protein loading. These results indicate that the 6-OHDA-induced PKCδ proteolytic cleavage is not limited to cell culture models, but also occurs in mouse nigral tissues following 6-OHDA treatment. The proteolytic cleavage of PKCδ was also followed by a dramatic increase in the PKCδ enzymatic activity, as determined by immunoprecipitation kinase assays. This suggests that an increase in the PKCδ kinase activity parallels the proteolytic cleavage products observed in 6-OHDA-injected substantia nigra as compared to saline-injected substantia nigra (Fig. 10B). The kinase activity assay was performed in the absence of lipids to determine the activity contributed solely by the proteolytically cleaved PKCδ catalytic fragment. These data suggest that proteolytic cleavage of PKCδ may play a critical role in the 6-OHDA-induced dopaminergic cell death observed in animal models of PD.

Caspase cleavage-resistant mutant PKCδ^{D327A}-CRM protects against 6-OHDA-induced PKCδ cleavage and activation in mouse substantia nigra

Since PKCδ^{D327A}-CRM protected against 6-OHDA-induced proteolytic activation in N27 cells, we next determined whether PKCδ^{D327A}-CRM protects against 6-OHDA-induced proteolytic activation of PKCδ in mouse brain substantia nigra. Adult C57 black male mice were stereotaxically injected with lentivirus coding for PKCδ^{D327A}-CRM in the left SN and LacZ in the right SN 6 days prior to injection of 4 μg 6-OHDA in the right SN and saline into the left SN. Six days later, 12 animals were sacrificed, nigral tissues were dissected out, and Western blot and kinase assays were performed. Western blot analysis of nigral tissue lysate revealed the proteolytic cleavage of native PKCδ in the LacZ and 6-OHDA-injected side (Fig. 11A), whereas proteolytic cleavage of PKCδ was significantly blocked in the PKCδ^{D37A}-CRM- and 6-OHDA-injected side. β-actin bands represented equal protein loading. These results indicate that the caspase-cleavage resistant PKCδ^{D37A}-CRM significantly prevented 6-OHDA-induced PKCδ proteolytic cleavage in a dominant-negative manner in mouse nigral tissues similar to that observed in cell cultures studies. The proteolytic cleavage of PKCδ was also followed by a dramatic increase in the PKCδ enzymatic activity in the LacZ-injected side, whereas PKCδ enzymatic activity was significantly blocked in the PKCδ^{D37A}-CRM-injected side following 6-OHDA treatment, as assessed by immunoprecipitation kinase assays (Fig. 11B). This suggests that PKCδ^{D37A}-CRM also attenuates 6-OHDA-induced increases in PKCδ kinase activity in mouse substantia nigra similar to that observed in N27 cells.

Discussion

We reported that PKCδ is an oxidative stress sensitive kinase activated by pro-oxidants such as hydrogen peroxide or neurotoxic agents such as manganese and pesticides in neuronal culture models (Kaul *et al.*, 2003; Kitazawa *et al.*, 2004; Yang *et al.*, 2004; Latchoumycandane *et al.*, 2005). In this report, we extended our previous studies to investigate the role of proteolytically activated PKCδ in mediating dopaminergic cell death in cell culture and animal PD models using 6-OHDA. The 6-OHDA-treatment induced

apoptosis in mesencephalic dopaminergic neuronal cells (N27) via free radical generation, mitochondrial dysfunction, cytochrome c release, activation of caspase-9 and caspase-3, proteolytic activation of PKCδ and DNA fragmentation. PKCδ siRNA and loss-of-function PKCδ mutants ($PKC\delta^{D327A}$ and $PKC\delta^{K376R}$) all rescued 6-OHDA-induced cell death and TH^+ neuronal cell loss in both N27 cells and in primary mesencephalic neurons. Loss-of-function $PKC\delta^{D327A}$ mutant also rescued 6-OHDA-induced proteolytic activation in the mouse brain SN.

Oxidative stress results from an imbalance between ROS generation, antioxidant levels and/or the depletion of enzymatic or non-enzymatic scavengers of ROS. The neurotoxin 6-OHDA, a hydroxylated analogue of dopamine, enters the cells via the dopamine transporter. Inside the cell, Fe^{2+} and H_2O_2 enhance the formation of 6-OHDA by non-enzymatic hydroxylation of dopamine (Linert *et al.*, 1996). It has been reported that alterations in mitochondrial membrane permeability result in the release of cytochrome c into the cytosol (Fiskum *et al.*, 2003; Polster and Fiskum, 2004). In this study, N27 cells exposed to 6-OHDA induced cytotoxic cell death in a time- and dose-dependent manner. Based on cytotoxicity experiments, as well as a review of literature, we used 100 μM 6-OHDA for all subsequent studies (Choi *et al.*, 2000). In this study, we show that treatment with 100 μM 6-OHDA induces altered mitochondrial activity, as determined by the MTT assay. These alterations in mitochondrial activity were followed by an increase in cytosolic cytochrome c levels. Once cytochrome c is released from the mitochondria, it forms a complex with apoptosis protease- activating factor-1 (Apaf-1) and activates caspase-9, which in turn activates caspase-3 (Zhou and Tang, 2002; Yuyama *et al.*, 2003; Polster and Fiskum, 2004). We demonstrated that co-treatment with MnTBAP almost completely blocked 6-OHDA-induced increases in DNA fragmentation, suggesting that ROS serves as an initiator of 6-OHDA-induced apoptotic cell death.

We found that exposure of N27 cells to 6-OHDA resulted in increases in both caspase-9 and caspase-3 activities, indicating that caspases play a role in 6-OHDA-induced dopaminergic cell death. Co-treatment with 10 μM MnTBAP almost completely blocked 6-OHDA-induced increases in caspase-3 activity, indicating that ROS mediates caspase-3 activation. These results are in agreement with those from recent studies by our lab and from others in which both caspase-9 and caspase-3 activation was observed in neuronal cultures after MPP^+(1-methyl-4-phenylpyridinium) treatment (Kaul *et al.*, 2003; Yang *et al.*, 2004). Our results are also in agreement with previous studies, which have reported oxidative stress, alterations in mitochondrial function, cytochrome c release, and caspase-9 and caspase-3 activation during 6-OHDA-induced cell death in the PC12 pheochromocytoma cell line (Elkon *et al.*, 2004), SH-SY5Y human neuroblastoma cells (Jordan *et al.*, 2004), MN9D murine dopaminergic cells (Choi *et al.*, 1999) and cerebellar granule cells (Dodel *et al.*, 1999).

Events that occur downstream of caspase-3 activation are still unclear. PKCδ has been reported to be an endogenous substrate for caspase-3 (Reyland *et al.*, 1999; Anantharam *et al.*, 2002; Kaul *et al.*, 2003; Kitazawa *et al.*, 2003), and activated caspase-3 cleaves PKCδ, yielding a 38 kDa regulatory fragment and a 41 kDa persistently active catalytic fragment (Greenberg *et al.*, 1998; Brodie and Blumberg, 2003). Recently, we established that caspase-3-mediated proteolytic activation of PKCδ is an important downstream event in apoptotic cell death of dopaminergic neurons. In this study, we demonstrate that 6-OHDA induces caspase-3-dependent proteolytic cleavage of PKCδ, and causes increases in PKCδ kinase activity in N27 cells in a time-dependent manner. This suggests that 6-OHDA-treated N27 cells have higher levels of persistently active catalytic PKCδ fragments. Furthermore, we demonstrated that N27 cells and primary mesencephalic neurons engineered to express PKC-CRM or catalytically inactive $PKC\delta^{K376R}$ protein (PKCδ-DN) were both resistant to

6-OHDA-induced cell death. The protective effect of PKCδ-siRNA further confirmed the proapoptotic function of the kinase. These *in vitro* results strongly implicate PKCδ in oxidative damage of dopaminergic neurons. Furthermore, 6-OHDA also induced proteolytic cleavage and activation in mouse substantia nigra, suggesting that PKCδ may mediate 6-OHDA-induced neurodegeneration in animals. Loss-of-function $PKC\delta^{D327A}$ mutant also significantly attenuated 6-OHDA-induced proteolytic activation in mouse brain SN, providing *in vivo* evidence for our hypothesis. While we were completing these studies, another research group reported that PKCδ is activated in adrenal chromaffin cells (Hanrott *et al.*, 2006) and in a rat model (Hanrott *et al.*, 2008). However, our systematic investigation using dominant negative mutants, CRM mutants and siRNA provides clear evidence for proapoptotic function of PKCδ in dopaminergic neurodegeneration.

The role of PKCδ in nigral neurons is beginning to emerge. We recently reported that PKCδ is highly expressed in nigral dopaminergic neurons (Zhang *et al.*, 2007b). In addition, we demonstrated that PKCδ also negatively regulates tyrosine hydroxylase activity and dopamine synthesis by enhancing protein phosphatase-2A activity in dopaminergic neurons (Zhang *et al.*, 2007b). Recently, we have also showed that PKCδ contributes manganese-induced tyrosine hydroxylase activity (Zhang *et al.*, 2011). Previously, we demonstrated -synuclein interacts with proapoptotic proteins PKCδ and BAD to protect dopaminergic neuronal cells against MPP^+-induced apoptotic cell death (Kaul *et al.*, 2005a). We also showed that tyrosine phosphorylation regulates the proteolytic activation of PKCδ in dopaminergic neuronal cells (Kaul *et al.*, 2005b). Furthermore, we reported that α-synuclein negatively regulates protein kinase Cδ expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyl-transferase activity (Jin *et al.*, 2011b). Very recently, we have implicated transcriptional regulation of PKCδ in oxidative stress-induced neuronal cell death (Jin *et al.*, 2011b). With respect to environmental neurotoxic chemical exposure and pathogenesis of PD, we showed that exposure to the transition metal manganese, pesticides and proteasome inhibitors can trigger a PKCδ-dependent apoptotic cascade in dopaminergic neuronal cells. Collectively, our findings demonstrate that PKCδ plays a critical role in mediating nigral dopaminergic neurodegeneration during neurotoxic insults. These results also suggest that PKC may serve as a potential therapeutic target for development of novel neuroprotective strategies for treatment of PD. In this regard, we extended our mechanistic findings into translational research, in which we showed that the pharmacological inhibitor rottlerin protected against MPTP-induced motor deficits, dopamine depletion and TH neuronal cell loss in an animal model of PD (Zhang *et al.*, 2007a).

In summary, the present study demonstrates that the oxidative stressor 6-OHDA activates the PKCδ-dependent apoptotic pathway in both cell culture and animal models of PD. The use of loss-of-function PKCδ mutants, as well as selective targeting of the proapoptotic kinase PKCδ by siRNA, clearly establishes PKCδ as a key downstream mediator of 6-OHDA-induced apoptosis. These findings indicate that further exploration of PKCδ signaling in the nigral dopaminergic system may have a significant therapeutic implication in the treatment of PD related neurological disorders.

Acknowledgments

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Abbreviations

6-OHDA	6-hydroxydopamine
PD	Parkinson's disease
SN	substantia nigra
PKCδ	protein kinase C delta
ROS	reactive oxygen species
TH	tyrosine hydroxylase
MTT	3-(4,5-Dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
AMC	7-amino-4-methylcoumarine
Z-DEVD-FMK	Z-Asp-Glu-Val-Asp-fluoromethyl ketone
Ac-LEHD-AFC	Acetyl-Leu-Glu-His-Asp-7-amino-4-fluorcoumarin
Ac-DEVD-AFC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-fluorcoumarin

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Highlights

- Dopaminergic neurotoxicant 6-hydroxydopamine (6-OHDA) activates the mitochondrial-apoptotic signaling pathway.
- Proteolytic activation of PKCδ is a key downstream event of 6-OHDA-induced neurotoxicity.
- Expression of cleavage resistant PKCδ^{D327A}, kinase dead PKCδ^{K376R} or PKCδ siRNA protects against 6-OHDA-induced dopaminergic cell death.
- Viral-mediated delivery of PKCδ^{D327A} attenuates 6-OHDA induced PKCδ proteolytic activation in mouse substantia nigra.
- The study strongly suggests that PKCδ proteolytic activation is a key downstream event in dopaminergic degeneration.

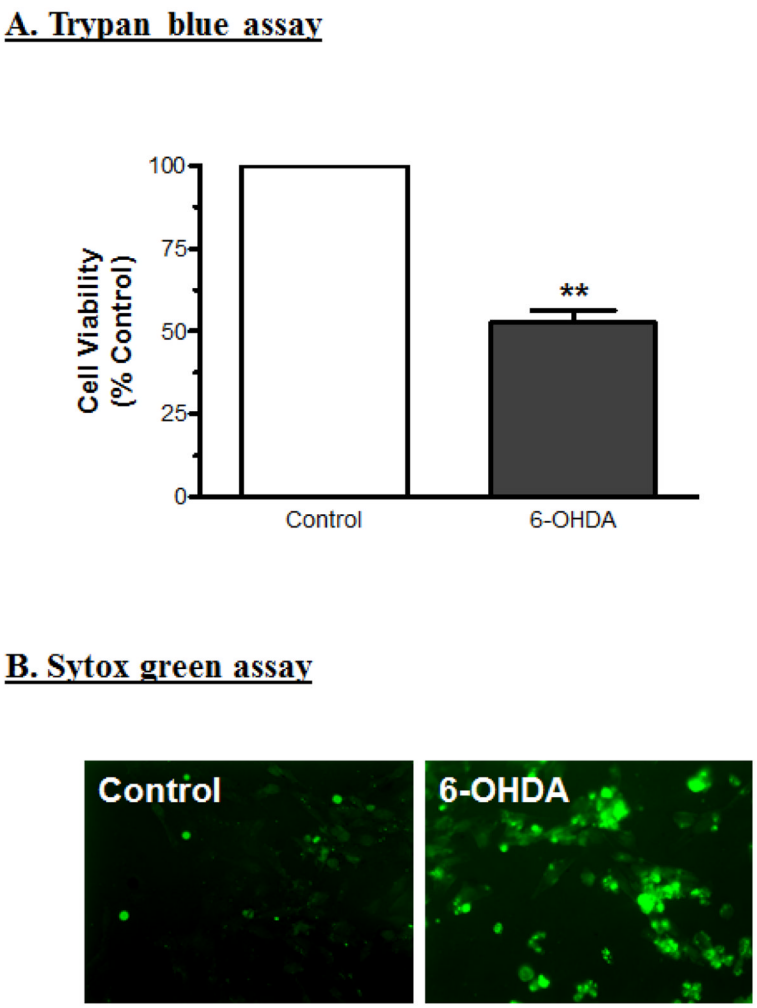

Fig. 1.

Dopaminergic neuronal cytotoxicity induced by the neurotoxin 6-OHDA. (A) Cell viability was measured by the trypan blue exclusion method after exposure to 100 μM 6-OHDA for 24 h. The data represent mean ± SEM for at least two separate experiments in triplicate. ** indicates a significant difference between the means at $p<0.01$. (B) Cell death was also assessed qualitatively using Sytox green nucleic acid stain. N27 cells were exposed to 100 μM 6-OHDA for 24 h with 1 μM Sytox green, and then observed under fluorescence microscopy with excitation of 485 nm and emission of 538 nm. The Sytox green-positive cells represent dead cells after exposure to 6-OHDA.

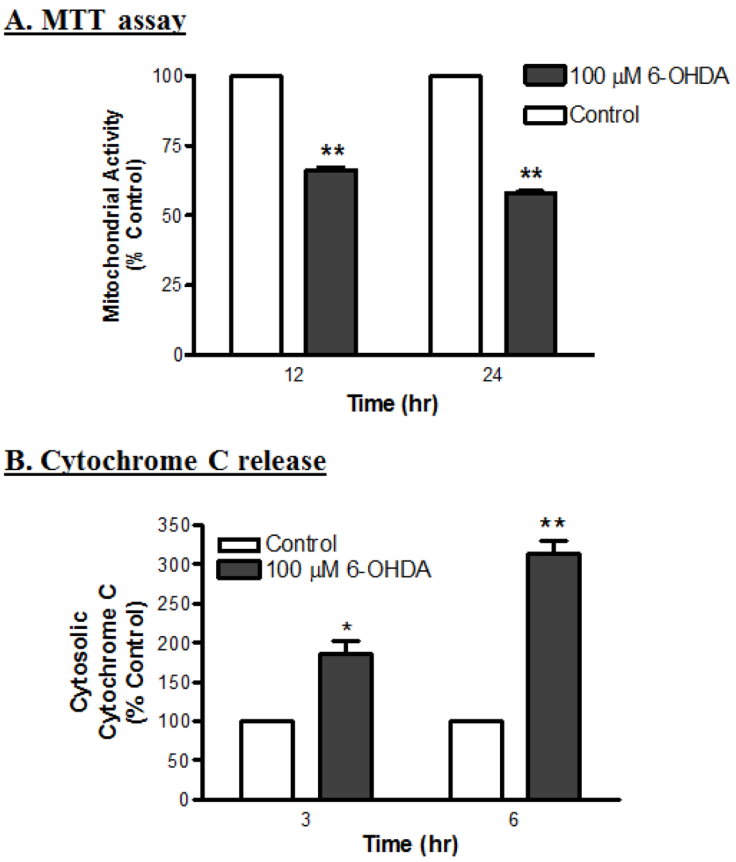

Fig. 2.

6-OHDA impairs mitochondrial activity and induces cytochrome c release in dopaminergic neurons. (A) Relative mitochondrial activity was determined using MTT; the assay is based on the formation of formazan from tetrazolium inside the active mitochondria. The mitochondrial activity decreased significantly after exposure to 6-OHDA for 12 and 24 h (**p<0.01; n=6). (B) Cytosolic cytochrome c release was determined using an ELISA kit; the levels in the cytosolic fraction increased significantly after 3 and 6 h treatments with 6-OHDA (*p<0.05; **p<0.01; n=8).

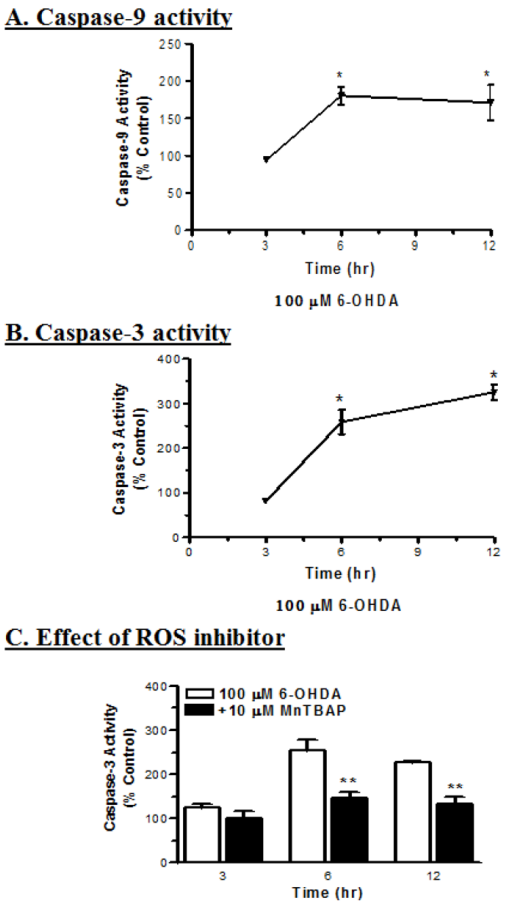

Fig. 3.

6-OHDA induces ROS-mediated activation of caspases in dopaminergic neuronal cells. The activities of caspase-9 (A) and caspase-3 (B) were determined using fluorogenic substrates, Ac-LEHD-AMC and Ac-DEVD-AMC, respectively, with excitation of 380 nm and emission of 460 nm. Significant increases in the activities of caspase-9 (n=6; *p<0.05) and caspase-3 (n=6; *p<0.05) were observed after exposure to 6-OHDA for 6 and 12 h. (C) Co-treatment with 10 μM MnTBAP (free radical scavenger) protected against 6-OHDA-induced changes in caspase-3 activities, with significant differences between time points (n=6; **p<0.01).

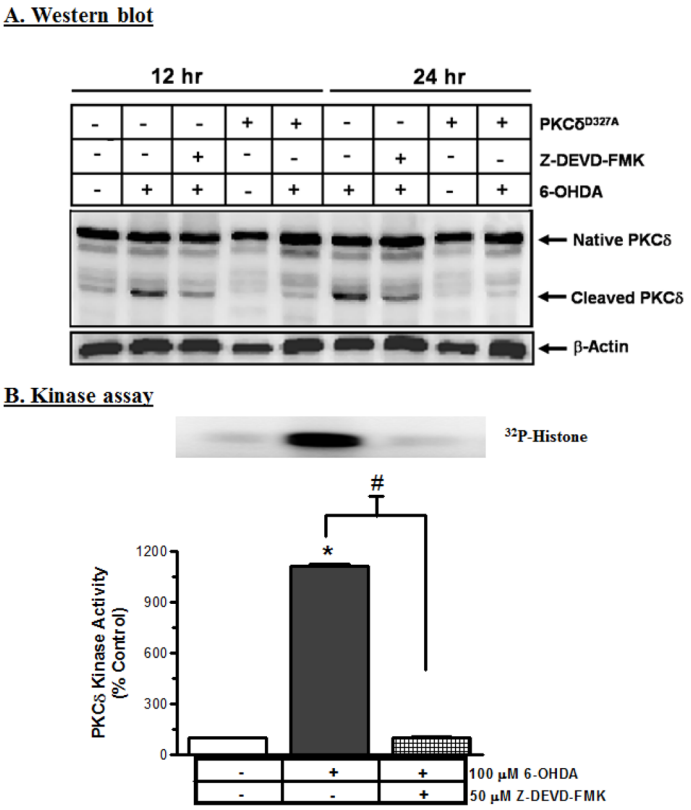

Fig. 4.

6-OHDA induces caspase-3-mediated proteolytic activation of PKCδ. (A) Proteolytic activation of PKCδ was determined in N27 cells and the cells overexpressing cleavage-resistant PKCδ^{D327A} protein after exposure to 6-OHDA with or without Z-DEVD-FMK (caspase-3 inhibitor) by Western blot. The results show that 6-OHDA induces caspase-3-mediated PKCδ cleavage; the induction was blocked by the caspase-3 inhibitor and by overexpression of PKCδ cleavage resistant protein (CRM). (B) PKCδ kinase activity was determined by immunoprecipitation followed by [^{32}P]-phosphorylation, and then scanned with a phosphoimager. The data were analyzed using Quantity One Software. The results indicate that 6-OHDA induces PKCδ kinase activity (*$p<0.05$); that activity was completely inhibited by Z-DEVD-FMK.

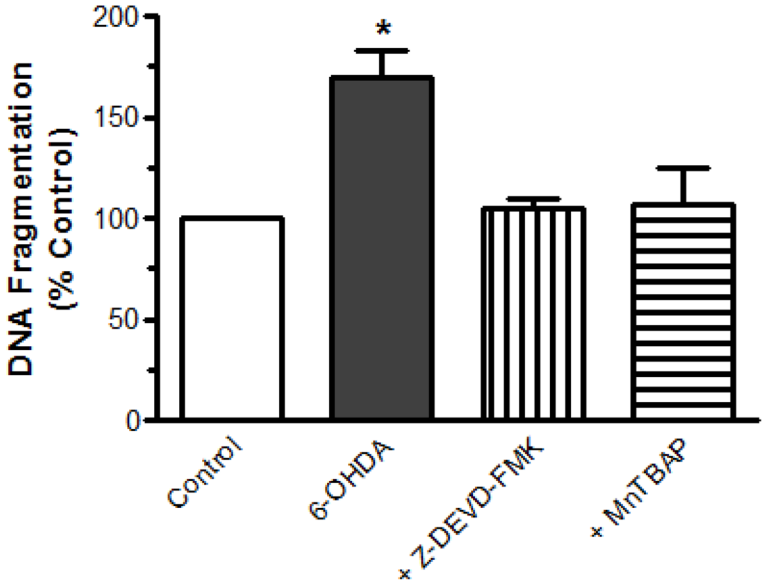

Fig. 5.

ROS and caspase-3 mediate 6-OHDA-induced DNA fragmentation. The cells were cultured and treated with 100 μM 6-OHDA for 24 h in the presence or absence of a caspase-3 inhibitor, 50 μM Z-DEVD-FMK, or a free radical scavenger, 10 μM MnTBAP. DNA fragmentation was quantified using ELISA. The data are represented as the mean ± SEM of six individual measurements. *$p<0.05$ indicates significant differences between 6-OHDA-treated cells and control and inhibitor- or scavenger-treated cells.

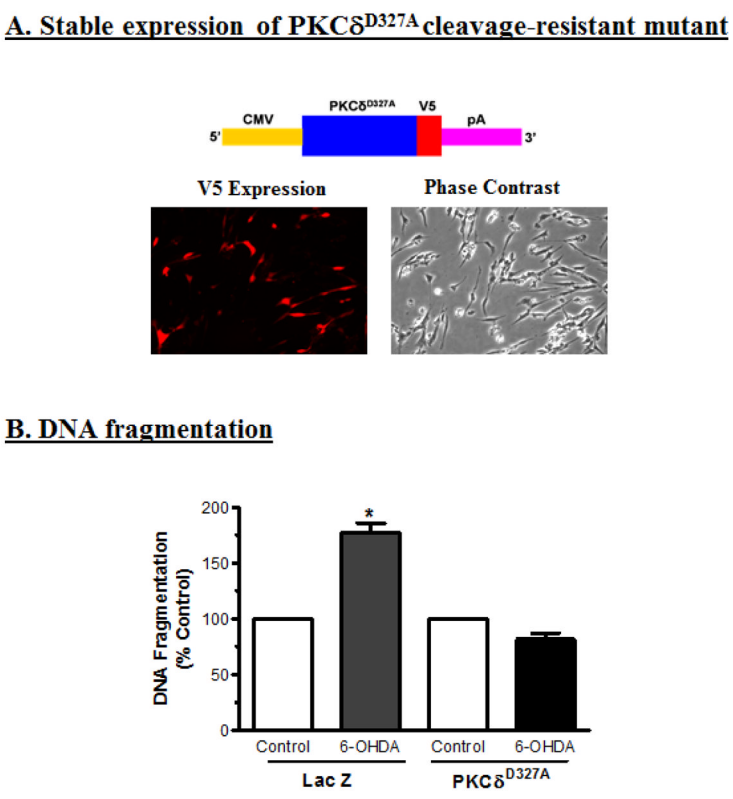

Fig. 6.

Overexpression of cleavage-resistant PKCδ^{D327A} mutant (PKCδ-CRM) blocks 6-OHDA-induced apoptosis. (A) Schematic of plasmid constructs: PKCδ-CRMV5 construct encodes cleavage resistant mutant of PKCδ-CRM with V5 tag, transcript mRNA is transcribed under the 5′ human cytomegalovirus (CMV) immediate early promoter, and the mRNA is stabilized with the 3′ SV40 mRNA polyadenylation signal (pA). Fluorescent images showing the stable expression of V5 tag in N27 cells expressing the PKCδ-CRM-V5 tag (200X magnification). (B) PKCδ-CRM blocks 6-OHDA-induced DNA fragmentation. The N27 cells stably expressing vector (LacZ) or PKCδ-CRM-V5 protein were treated with 100 μM 6-OHDA for 24 h, then DNA fragmentation was measured by ELISA. The data represent mean ± SEM of six individual measurements. The asterisk (*$p<0.05$) indicates a significant difference between the 100 μM 6-OHDA-treated PKCδ-CRM-V5 mutant cells and the 100 μM 6-OHDA-treated LacZ vector control cells.

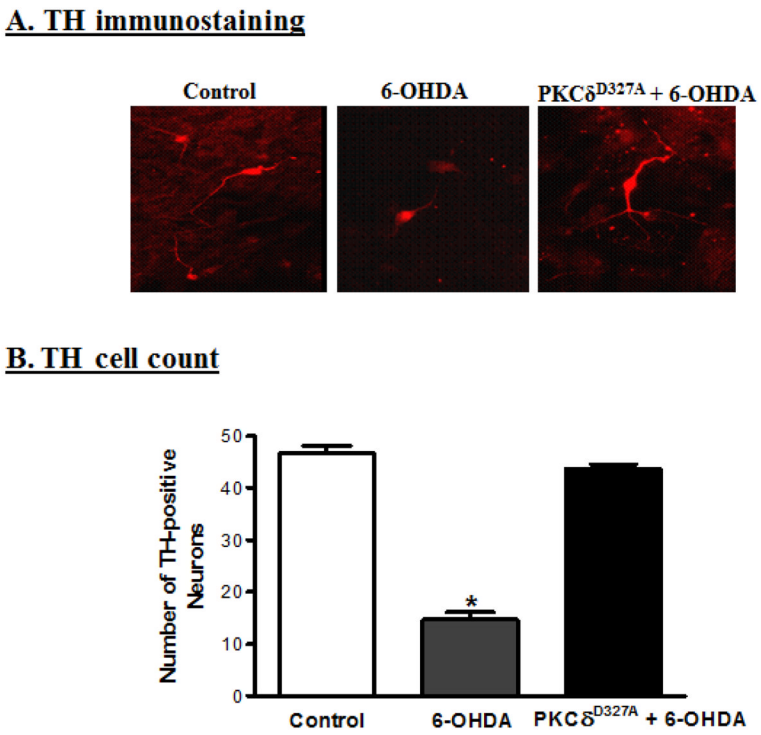

Fig. 7.

Expression of PKCδ-CRM blocks 6-OHDA-induced primary dopaminergic neuronal cell death. (A) Primary dopaminergic neuronal cells from E16 embryos were collected and transfected with PKCδ-CRM. The primary neurons were exposed to 30 μM 6-OHDA for 3 h and then stained for tyrosine hydroxylase. PKCδ-CRM-expressing primary neurons were protected from 6-OHDA-induced cell death as compared to 6-OHDA-treated primary neurons. (B) The TH-positive neurons were counted by fluorescence microscopy using Metamorph Software. PKCδ-CRM-expressing primary dopaminergic neurons blocked 90% of cell death, compared to non-transfected cells treated with 6-OHDA (*$p<0.05$).

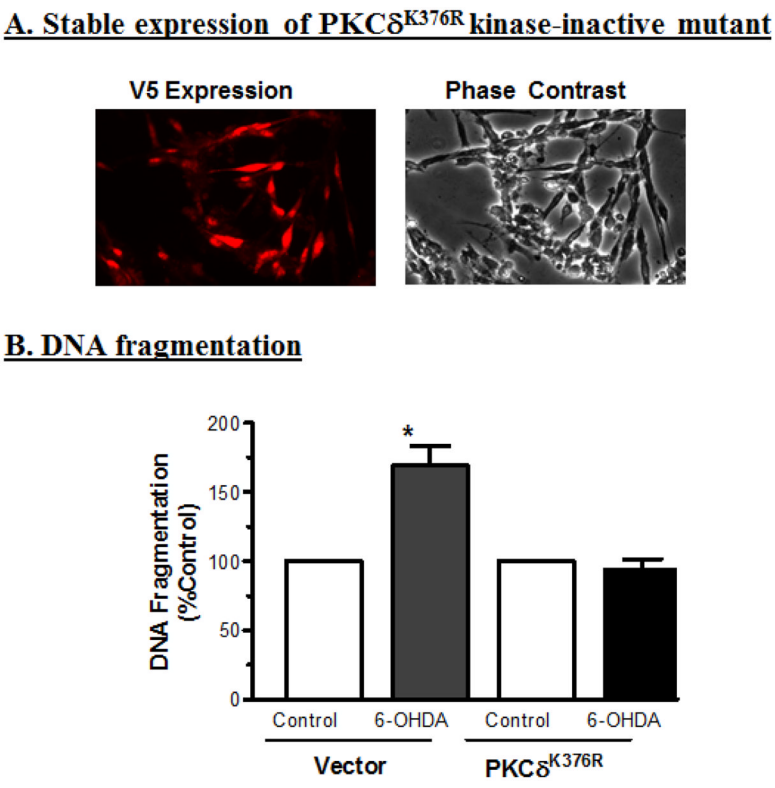

Fig. 8.

Overexpression of catalytically inactive PKCδ^{K376R} protein (PKCδ-DN) attenuates 6-OHDA-induced apoptosis. (A) Fluorescent images showing the stable expression of PKCδ-DN-V5 (left panel), and phase contrast image (right panel) in N27 cells. (B) DNA fragmentation assay shows significant protection against 6-OHDA in the cells overexpressing catalytically inactive kinase dead PKCδ-DN mutant as compared to wild-type (LacZ) cells. *$p<0.05$ indicates a significant difference between the 100 μM 6-OHDA-treated PKCδ-DN mutant cells and the 100 μM 6-OHDA-treated LacZ vector control cells.

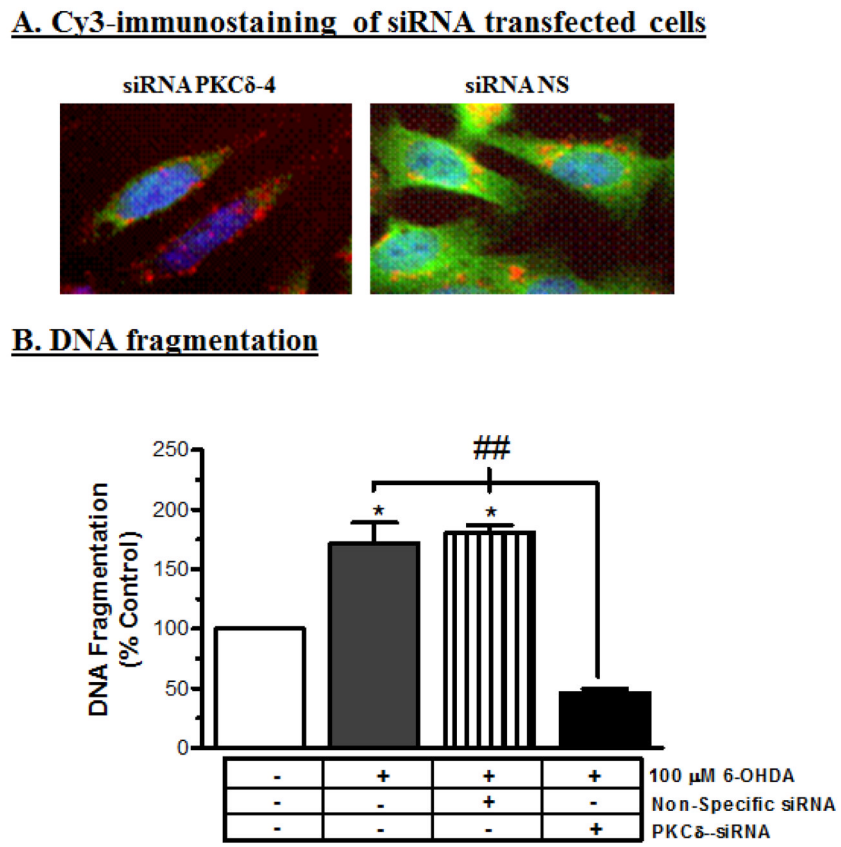

Fig. 9.

Suppression of PKCδ by siRNA protects N27 cells from 6-OHDA-induced apoptotic cell death. (A) PKCδ siRNA suppresses PKCδ protein expression. N27 cells were transfected with cy3-labeled PKCδ siRNA (25 nM) or cy3-labeled non-specific (NS) siRNA; PKCδ expression was observed following immunostaining using anti-PKCδ antibody and Alexa 488 secondary antibody. The nuclei were visualized by Hoechst 33342 counterstaining. Merged images show expression of PKCδ (green), cy3-labeled siRNA or siRNA–NS 9 (red) and nuclear staining (blue). The cells were observed under a fluorescence microscope. (B) PKCδ siRNA attenuates 6-OHDA-induced DNA fragmentation. The N27 cells were transfected with siRNA-δ-4 (25 nM) or non-specific siRNA for 24 h; cells were then treated with 100 μM 6-OHDA for an additional 24 h, and then DNA fragmentation was quantified. The data represent the mean ± SEM from six individual measurements. * indicates differences between 6-OHDA-treated non-transfected and NS siRNA cells and the untreated control cells ($p<0.05$). ## indicates the differences between 6-OHDA-treated siRNA cells and 6-OHDA-treated non-transfected and NS siRNA cells ($p<0.01$).

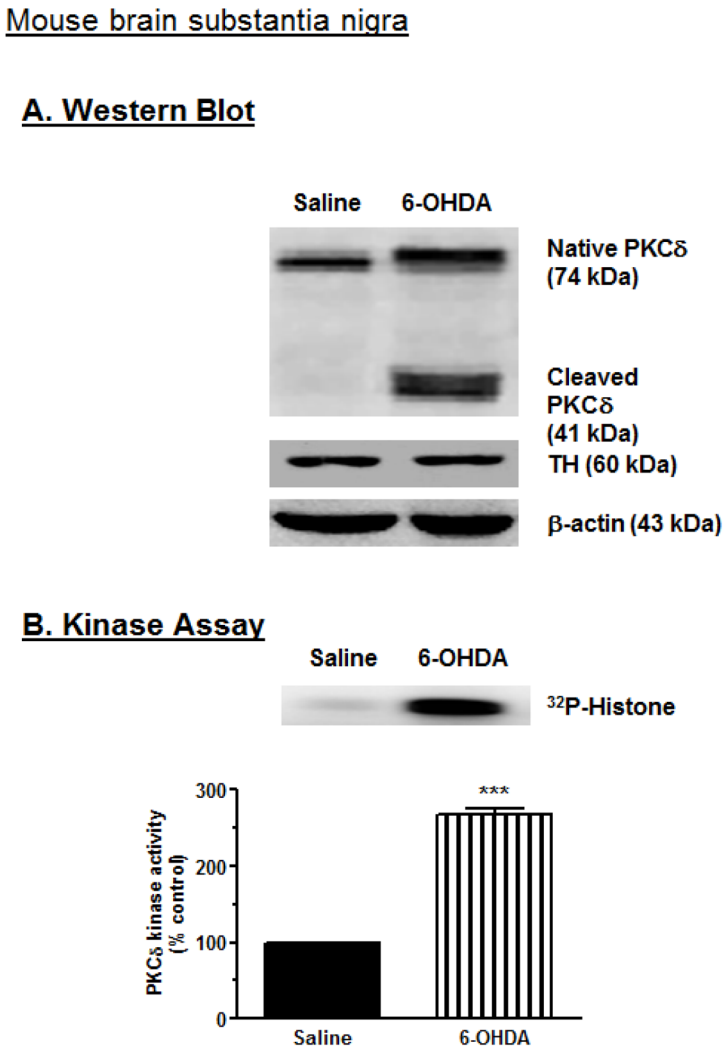

Fig. 10.

6-OHDA induces proteolytic activation of PKCδ in mouse substantia nigra. (A) Mice were stereotaxically injected with 6-OHDA, and proteolytic cleavage of PKCδ in mouse substantia nigra was analyzed using Western blot. The results showed that 6-OHDA induces PKCδ cleavage. (B) PKCδ enzyme activity was determined by immunoprecipitation kinase assay with [^{32}P]-phosphorylation, and then scanned using a phosphoimager. The densitometric analysis of the [^{32}P]-phosphorylated 32 kDa histone band is shown. The data represent mean ± SEM from 3 independent images. *** indicates a significant difference between the 10 μM 6-OHDA-injected and saline-injected substantia nigra ($p<0.001$).

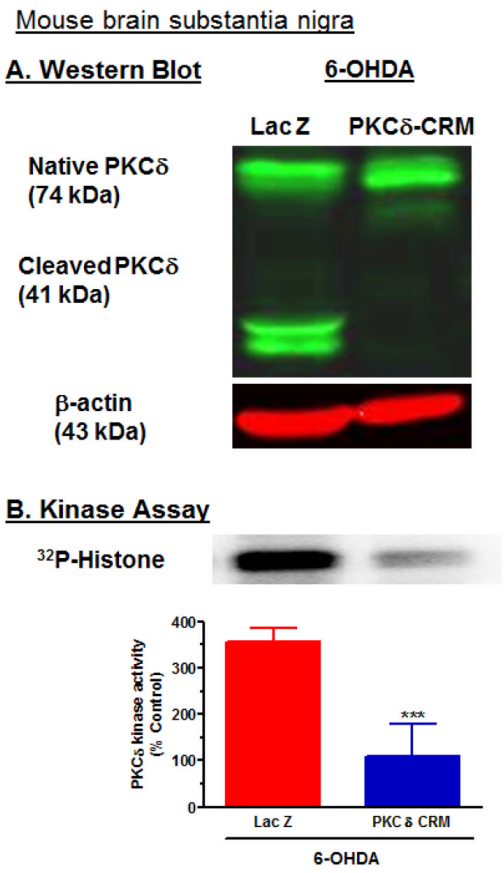

Fig. 11.

Caspase-3 cleavage-resistant PKCδ^{D327A} CRM mutant prevents 6-OHDA-induced proteolytic activation of PKCδ in mouse substantia nigra. (A) Mice were stereotaxically injected with either LacZ or PKCδ^{D327A} CRM and 6-OHDA, and proteolytic cleavage of PKCδ in mouse SN was measured, as described in the Methods. SN samples were analyzed using Western blot and immunoprecipitation kinase assays. The results showed that 6-OHDA induces PKCδ cleavage. (B) PKCδ enzyme activity was determined by immunoprecipitation kinase assay with [^{32}P]-phosphorylation, and then scanned using a phosphoimager. The densitometric analysis of the [^{32}P]-phosphorylated 32 kDa histone band is shown. The data represent mean ± SEM from 3 independent images. *** indicates a significant difference between the 10 μM 6-OHDA and LacZ-injected and 6-OHDA and PKCδ^{D327A} CRM-injected SN ($p<0.001$).