Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 69 (2005) 133–146
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Activation of protein kinase Cδ by proteolytic cleavage contributes to manganese-induced apoptosis in dopaminergic cells: protective role of Bcl-2

Masashi Kitazawa[a], Vellareddy Anantharam[a], Yongjie Yang[a], Yoko Hirata[b], Arthi Kanthasamy[a], Anumantha G. Kanthasamy[a,*]

[a]*Parkinson Disorders Research Laboratory, Department of Biomedical Sciences, Iowa State University, 2062 Vetmed, Ames, IA 50011-1250, USA*
[b]*Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu 501-1193, Japan*

Received 7 May 2004; accepted 24 August 2004

Abstract

Chronic inorganic manganese exposure causes selective toxicity to the nigrostriatal dopaminergic system, resulting in a Parkinsonian-like neurological condition known as Manganism. Apoptosis has been shown to occur in manganese-induced neurotoxicity; however, the down-stream cellular target of caspase-3 that contributes to DNA fragmentation is not established. Herein, we demonstrate that proteolytic activation of protein kinase Cδ (PKCδ) by caspase-3 plays a critical role in manganese-induced apoptotic cell death. Treatment of PC12 cells with manganese caused a sequential activation of mitochondrial-dependent pro-apoptotic events, including mitochondrial membrane depolarization, cytochrome c release, caspase-3 activation, and DNA fragmentation. Overexpression of Bcl-2 in PC12 cells remarkably attenuated each of these events, indicating that the mitochondrial-dependent apoptotic cascade contributes to manganese-induced apoptosis. Furthermore, PKCδ was proteolytically cleaved by caspase-3, causing a persistent activation of the kinase. The manganese-induced proteolytic cleavage of PKCδ was significantly blocked by Bcl-2-overexpression. Administration of active recombinant PKCδ induced DNA fragmentation in PC12 cells, suggesting a pro-apoptotic role of PKCδ. Furthermore, expression of catalytically inactive mutant PKCδ^{K376R} via a lentiviral gene delivery system effectively attenuated manganese-induced apoptosis. Together, these results suggest that the mitochondrial-dependent caspase cascade mediates apoptosis via proteolytic activation of PKCδ in manganese-induced neurotoxicity.

Keywords: Mitochondria; Oxidative stress; PKC; Gene delivery; Manganese; Parkinson's disease

1. Introduction

Chronic exposure to high levels of manganese is known to cause neurological symptoms similar to idiopathic Parkinson's disease (PD) in both humans and laboratory animals [1–5]. Manganese exposure is associated with many occupations including mining, automotive mechanics, dry cell battery manufacturing, welding, and agricultural application of manganese containing pesticides [6]. Additionally, the manganese containing organic compound methylcyclopentadienyl manganese tricarbonyl (MMT) is used as a gasoline additive in many countries, and inorganic manganese is emitted upon combustion [7,8]. Consequently, environmental exposure to manganese is likely to increase, creating concerns regarding the health risks associated with chronic manganese exposure [9–11]. Manganese induces behavioral changes and a variety of cellular changes including GSH depletion, dopamine depletion, GABA release, increased oxidative stress, and impairment of the energy metabolism and anti-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immuno-sorbent assay; $MnCl_2$, manganese chloride; PC12, rat pheochromocytoma cells; PKC, protein kinase C; ROS, reactive oxygen species; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

* Corresponding author. Tel.: +1 515 294 2516; fax: +1 515 294 2315.
E-mail address: akanthas@iastate.edu (A.G. Kanthasamy).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.08.035

oxidant systems [2,12–15]. Apoptosis is recognized as a major cell death process in neurodegenerative disorders including Parkinson's disease [16,17]. Manganese induces apoptosis in human B cells [18] and in rat pheochromocytoma (PC12) cells [19,20]. In addition, manganese enhances oxidative stress-mediated L-DOPA toxicity, indicating that manganese is a potent dopaminergic neurotoxicant [21]. The mitochondrion is an important cellular target in manganese-induced apoptosis in dopaminergic cells [22–24]. The Bcl-2 protein family has a central regulatory role in mitochondrial-dependent apoptosis [25]. Overexpression of Bcl-2 proteins attenuates apoptosis induced by various chemicals [18,26,27], demonstrating the important anti-apoptotic function of Bcl-2 proteins.

Although apoptosis has been shown to be important in manganese-induced neurotoxicity, the down-stream targets of activated caspase-3 in manganese-induced apoptosis that result in DNA fragmentation are not well established. In the present study, we examined the sequential activation of apoptotic signaling molecules in PC12 cells, an in vitro model that has been used widely to elucidate the effects of neurotoxic agents on dopaminergic cells [28–30]. Herein, we demonstrate that proteolytic cleavage of protein kinase Cδ (PKCδ) by caspase-3 is a critical event in manganese-induced apoptosis.

2. Materials and methods

2.1. Materials

Manganese chloride, mouse monoclonal β-actin antibody, propidium iodide, and human recombinant active PKCδ protein were purchased from Sigma Chemical Co. Phorbol-12-myristate-13-acetate (TPA) was purchased from Calbiochem. The caspase-3 substrate Ac-DEVD-AMC was purchased from Bachem Biosciences Inc. The caspase-9 substrate Ac-LEHD-AMC and the caspase-3 inhibitor Z-DEVD-FMK were purchased from Alexis Biochemicals. The broad-spectrum caspase inhibitor Z-VAD-FMK was purchased from Enzyme System. Acridine orange and Cy3-conjugated goat anti-mouse secondary antibody was purchased from Molecular Probes. Rabbit polyclonal anti-PKCδ antibody and mouse monoclonal Bcl-2 antibody were purchased from Santa Cruz Biotechnology Inc. The anti-V5 mouse antibody was purchased from Invitrogen Inc. The ECL Western blotting analysis kit was purchased from Amersham Pharmacia Biotech Inc. The cytochrome c ELISA kit was obtained from MBL International Corp. The Cell Death Detection ELISA Plus Assay Kit was purchased from Roche Molecular Biochemicals. The TUNEL kit was purchased from Upstate Biotechnology. BioPORTER protein delivery reagent was purchased from Gene Therapy Systems. Dulbecco's modified Eagle medium (DMEM) was purchased from Mediatech Inc. Heat-inactivated horse serum and fetal bovine serum were purchased from Invitrogen. Other routine laboratory chemicals were obtained from Fisher Scientific. The Bcl-2 transfected PC12 cells and vector transfected PC12 cells were generous gifts from Drs. Yutaka Eguchi and Yoshihide Tsujimoto at Osaka University (Osaka, Japan), respectively. The PKCδ^{K376R} construct was a kind gift from Dr. Reyland at the University of Colorado (Boulder, CO).

2.2. Cell culture

Vector and Bcl-2-overexpressing PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 7% heat-inactivated horse serum and 4% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. PKCδ^{K376R} and LacZ expressing PC12 cells were grown in RPMI-1640 medium containing 10% heat-inactivated horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 50 units penicillin, and 50 μg/ml streptomycin [30,31].

2.3. Cytotoxicity assay

Vector and Bcl-2-overexpressing PC12 cells ($\sim 1 \times 10^5$ cells/well) were subcultured separately in 24-well tissue culture plates for 24 h and treated with 200–1000 μM $MnCl_2$ for 72 h. Cell death was determined by trypan blue exclusion method with an Improved Nebauer type Hemacytometer. The cell viability was normalized as percent of control.

2.4. Cytochrome c detection assay

Manganese-induced cytochrome c release was measured using a cytochrome c ELISA kit as described previously [30,31]. Briefly, vector and Bcl-2-overexpressing PC12 cells ($\sim 5 \times 10^6$ cells) were exposed to 1 mM $MnCl_2$ for up to 32 h. After treatments, cytoplasmic fractions were collected and levels of cytosolic cytochrome C were analyzed by ELISA as described previously [31]. The optical density of each well was measured at 450 nm using a microplate reader. The concentration of cytochrome c was calibrated from a standard curve based on reference standards.

2.5. Mitochondrial membrane potential detection assay

Mitochondrial membrane potential ($\Delta\Psi_m$) depolarization was assessed using a Becton Dickinson FACScan flow cytometer [32]. Vector and Bcl-2-overexpressing PC12 cells ($\sim 1 \times 10^6$ cells) were exposed to 1 mM $MnCl_2$ for up to 40 h, and then 40 nM 3,3′-dihexaoxacarbocyanine iodide ($DiOC_6$) was added for 15 min. The cells were then washed once and resuspended with phosphate buffered

saline (PBS), and $\Delta\Psi_m$ was measured by flow cytometry with excitation at 484 nm and emission at 501 nm.

2.6. Caspase-3 and caspase-8 activity assay

Vector and Bcl-2-overexpressing PC12 cells (~(1–2) × 10^5 cells/well) were subcultured in a 24-well tissue culture plate for 24 h and treated with (1 mM) $MnCl_2$ for 0–48 h. Cells were washed once with PBS (pH 7.4) and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin. Cells were then incubated at 37 °C for 20 min to allow complete lysis. Lysates were quickly centrifuged at 10,000 × *g*, and cell-free supernatants were collected. Caspase-3 activity was measured using the caspase-3 specific fluorescent substrate Ac-DEVD-AMC, as previously described [31,33]. Formation of 7-amino-4-methylcoumarine (AMC) resulting from caspase substrate cleavage was measured by using fluorescent plate reader with excitation at 380 nm (slit width 10 nm) and emission at 460 nm (slit width 20 nm). Caspase-3 activity was expressed as fluorescence unit (FU) per mg protein per hour.

2.7. Isolation of cytosolic fractions

Vector and Bcl-2-overexpressing PC12 cells (~1 × 10^7 cells) were exposed to (1 mM) $MnCl_2$ at 37 °C for the indicated periods. Cells were washed once with ice-cold PBS and resuspended in homogenization buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium fluoride, and 50 μM sodium orthovanadate. Cells were then sonicated for 10 s and centrifuged at 1,00,000 × *g* for 60 min at 4 °C to produce the supernatant cytosolic fractions. To collect membrane fractions, pellets were dissolved in homogenization buffer containing 1% Triton X-100 to form suspensions and then sonicated and centrifuged at 10,000 × *g* for 30 min. The protein concentration of each cytosolic fraction was determined using Bradford protein assay dye reagent. Cytosolic fraction samples were mixed with 2× gel loading buffer containing 10% SDS and 200 mM DTT and boiled for 5 min prior to loading [30,31].

2.8. Western blotting

Proteins in cytosolic fractions were resolved by 10–12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membrane [30,31]. Non-specific binding sites were blocked by 5% non-fat milk blocking solution. The membrane were then treated with anti-PKCδ (1:2000 dilution) antibody, followed by secondary horseradish peroxidase-conjugated anti-rabbit (1:2000 dilution) antibody. Antibody-bound proteins were detected by an enhanced chemiluminescence (ECL) system [30,31]. To confirm equal protein loading in each lane, antibodies were stripped from membranes with stripping buffer and reprobed with β-actin (1:5000 dilution).

2.9. Protein kinase Cδ activity assay

PKCδ enzymatic activity was measured using an immunoprecipitation kinase assay as previously described [31,34]. Vector and Bcl-2-overexpressing PC12 cells were exposed to 1 mM $MnCl_2$ for 36 h, and cytosolic fractions were collected. After the immunoprecipitation with PKCδ, 25 μl of samples were incubated with 20 μl of reaction buffer containing 0.4 mg of histone H1 and 5 μCi of [γ-^{32}P]ATP (4500 Ci/mM) for 10 min at 30 °C. SDS gel loading buffer (2×) was added to terminate the reaction, the samples were boiled for 5 min and the products were separated on a 12.5% SDS–PAGE gel. The H1 phosphorylated bands were detected using a Personal Molecular Imager (FX model, Bio-Rad Laboratories) and quantified using Quantity One 4.2.0 software (Bio-Rad Laboratories).

2.10. In situ apoptotic labeling

Acridine orange and propidium iodide double staining was performed to assess DNA damage in apoptotic cells [35]. Vector and Bcl-2-overexpressing PC12 cells were grown on cover slips coated with type I rat tail collagen (6 μg/cm^2) for 24 h at 37 °C, and 1 mM $MnCl_2$ was added for the indicated periods. After the exposures, cells were washed once with PBS and stained with 5 μM acridine orange and 5 μM propidium iodide for 10 min or 10 μM Hoechst 33342 for 5 min at room temperature in the dark. The cells were washed once with PBS and mounted in a perfusion chamber with HBSS buffer and observed under a Nikon DiaPhot microscope (Nikon Inc., Melville, NY) with the excitation wavelength at 360, 488, or 540 nm for Hoechst 33342, acridine orange, or propidium iodide, respectively. Fluorescent images were captured with a SPOT digital camera.

2.11. DNA fragmentation assay

DNA fragmentation was measured using a Cell Death Detection ELISA Plus Assay Kit, as described previously [31,34]. Vector and Bcl-2-overexpressing PC12 cells (~1 × 10^6 cells) were subcultured in six-well culture plates for 24 h and exposed to 1 mM $MnCl_2$ with or without Z-DEVD-FMK for the indicated periods at 37 °C. The number of apoptotic PC12 cells was determined exactly as described by the manufacturer, and DNA fragmentation was expressed as a percentage of control.

2.12. In vitro delivery of catalytically active PKCδ

Vector expressing PC12 cells (~(1–2) × 10^5 cells/well) were subcultured in 24-well tissue culture plates for 24 h.

Human recombinant catalytically active PKCδ protein was delivered into cells using BioPORTER reagent, strictly following the manufacturer's protocol. Briefly, cells were incubated for 4 h with 5 ng of recombinant PKCδ or BioPORTER reagent in serum-free DMEM. Cells were counted and equal numbers of cells were analyzed by the DNA fragmentation assay as described above. The efficiency of the protein delivery system was also determined by delivering 5 ng of FITC-labeled goat IgG (supplied with the kit) into vector PC12 cells. Cells were washed three times with PBS and observed under fluorescent microscope at emission 488 nm. Fluorescent-positive cells were counted in five randomly selected regions and the efficiency was determined to be approximately 70%.

2.13. Lentiviral-mediated transfection of $PKC\delta^{K376R}$ gene in PC12 cells

The ViraPower™ Lentiviral Expression System (Invitrogen) was employed to introduce a catalytically inactive $PKC\delta^{K376R}$ (lysine-to-arginine mutation at position 376) into PC12 cells (herein referred to as PKCδ-DN). PKCδ-DN was subcloned into the kit-supplied expression vector plenti6/V5-D-TOPO (herein named plenti/PKCδ-DN) by PCR. Primers were: forward, 5′caccatggcacccttcctgctc3′ and reverse, 5′aatgtccaggaattgctcaaac3′. Standard cloning procedures were used and to produce lentiviral containing plenti6/PKCδ-DN, the plenti6/PKCδ-DN construct as well as supporting plasmids (supplied with the kit) were transfected into human 293FT cells with the use of lipofectamine 2000 as described in the kit's instructions. The lentivirus in the medium was collected by centrifuging at $1500 \times g$ for 15 min, 48–72 h post-transfection. Lentivirus containing plenti/lacZ was also produced to serve as a vector control. Lentivirus containing plenti/PKCδ-DN and polybrene (6 μg/ml) were added into cultured PC12 cells (5×10^4) for 24 h, and then the cultured medium was replaced with fresh medium. After 24 h, 1 mM $MnCl_2$ was added to PC12 cells and incubated for an additional 24 h. PKCδ-DN or lacZ expressing PC12 cells were identified by immunostaining of the C-terminal V5 epitope on expressed protein.

2.14. In situ TUNEL staining

TUNEL staining and immunostaining of the V5 epitope were performed sequentially. After $MnCl_2$ treatment, vector PC12 cells were collected by Cytospin (Thermo Electron) and stained for TUNEL following the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde for 15 min and permeabolized with 0.2% Triton-X containing 0.2% BSA in PBS for another 15 min. Cells were then incubated in TdT end-labeling cocktail containing Biotin-dUTP, TdT buffer, and TdT for 60 min at RT, and reaction was stopped by immersing cells in 1× TB buffer. After the wash, PC12 cells were incubated in blocking reagent containing avidin-FITC for 30 min at RT and washed with 1× PBS. The V5 epitope immunostaining was performed by incubating PC12 cells with anti-V5 mouse antibody (1:200) for 60 min at RT and then Cy3-conjugated goat anti-mouse secondary antibody (1:400) for 45 min and washed with 1× PBS. Finally, nuclei were counterstained with Hoechst 33342 for 3 min. Cells were washed with 1× PBS and mounted on slides. The cells were observed under Nikon fluorescence microscopy (Model TE-2000U) and pictures were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). About 200 PKCδ-DN or lacZ expressing PC12 cells were examined and both TUNEL and V5-positive cells were quantitatively analyzed and plotted in Prism software.

2.15. Data analysis and statistics

Data from caspase activity, cytochrome c release, and DNA fragmentation assays were first analyzed using one-way ANOVA. Dunnett's post-test or Bonfferoni's multiple comparison test was then performed to compare treated samples, and $p < 0.05$ was considered significant.

3. Results

3.1. Dose-dependent manganese-induced cytotoxicity in vector PC12 cells and Bcl-2-overexpressing PC12 cells

A dose–response cytotoxicity study was performed to determine the optimal manganese concentrations for additional mechanistic studies in vector control PC12 cells and Bcl-2-overexpressing PC12 cells. Exposure to various concentrations of manganese (0.2–1 mM) over 72 h caused a dose-dependent cytotoxicity in vector PC12 cells (Fig. 1). Manganese-induced cytotoxicity was significantly suppressed in Bcl-2-overexpressing PC12 cells ($p < 0.01$) as compared to the vector expressing PC12 cells. A 72-h exposure to 1 mM manganese produced approximately 50% cell death in the Bcl-2-overexpressing PC12 cells and 75% cell death in the vector PC12 cells. However, exposure to 1 mM manganese for 36 h resulted in 29% cell death in vector cells but no significant cell death in Bcl-2-overexpressing cells (data not shown). Therefore, we used 1 mM manganese in subsequent experiments to evaluate the cellular mechanism of apoptosis in dopaminergic cells.

3.2. Manganese affects mitochondrial function and releases pro-apoptotic cytochrome c

Manganese reportedly accumulates in mitochondria and inhibits mitochondrial complex I and/or II activity [24,36,37]. We examined the effect of manganese on mitochondrial functions and found that the mitochondrial membrane potential ($\Delta\Psi_m$) was depolarized in a

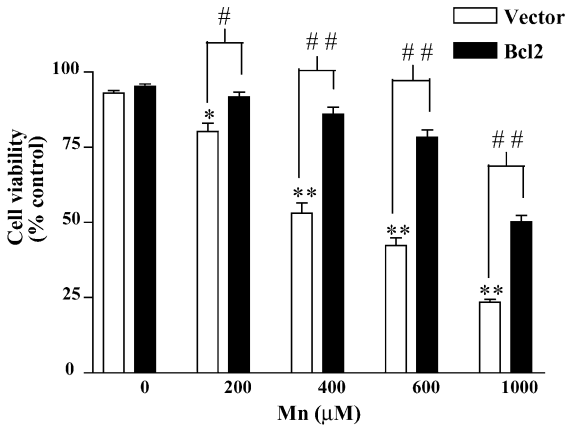

Fig. 1. Manganese-induced cytotoxicity in PC12 cells. Vector PC12 cells and Bcl-2-overexpressing PC12 cells were treated with 200, 400, 600, and 1000 μM $MnCl_2$ for 72 h, and cytotoxicity was determined using the trypan blue exclusion technique by counting live (clear) and dead (stained) cells in three to five randomly selected fields. Each bar represents mean ± S.E.M. for $n = 6$. Asterisks ($^{*}p < 0.05$ or $^{**}p < 0.01$) indicate significant difference between control and manganese treatment in vector cells and pound signs ($^{\#}p < 0.05$ or $^{\#\#}p < 0.01$) indicate significant difference between manganese-treated vector and Bcl-2-overexpressing cells.

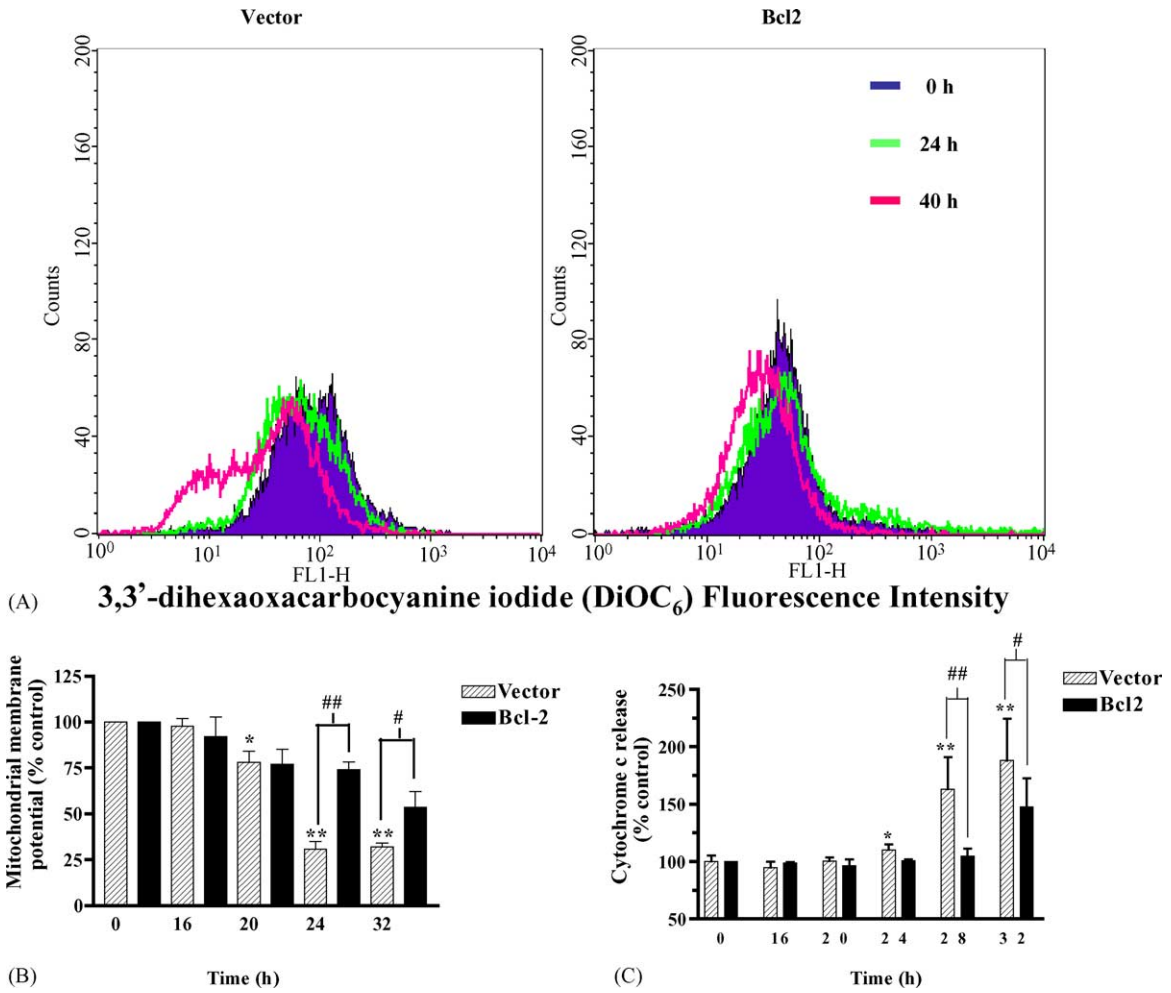

Fig. 2. Effect of manganese on mitochondrial function and cytochrome c release. Vector PC12 and Bcl-2-overexpressing PC12 cells were exposed to 1 mM $MnCl_2$ for 24–40 h. At the end of the exposure time, mitochondrial membrane potential ($\Delta\Psi_m$) was measured by flow cytometry using $DiOC_6$ (40 nM) as described in the Section 2. (A) The histogram shows the shift in the mitochondrial membrane potential ($\Delta\Psi_m$) at 0, 24, and 40 h. (B) The graph represents the reduction in $\Delta\Psi_m$ as percent of control at 16, 20, 24, and 32 h time points. (C) Release of cytochrome c into cytosol was detected using a cytochrome c ELISA kit as described in the Section 2. Each bar in B and C represents mean ± S.E.M. for $n = 6$. Asterisks ($^{*}p < 0.05$ or $^{**}p < 0.01$) indicate a significant difference between control and manganese treatment in vector cells, and pound signs ($^{\#}p < 0.05$ or $^{\#\#}p < 0.01$) indicate a significant difference between manganese-treated vector and Bcl-2-overexpressing cells.

time-dependent manner following 1 mM manganese treatment in vector PC12 cells (Fig. 2A). The statistically significant changes ($p < 0.05$) in depolarization of $\Delta\Psi_m$ were detected as early as 20 h and continued in a time-dependent manner up to 32 h (Fig. 2B). Bcl-2-overexpression effectively suppressed and delayed the manganese-induced depolarization of $\Delta\Psi_m$.

Mitochondrial insults often result in release of cytochrome c into the cytoplasm to initiate the apoptotic caspase cascade. Manganese caused significant ($p < 0.05$) release of cytochrome c in the cytosol starting at 24-h post-exposure (Fig. 2C), the time point just following the significant $\Delta\Psi_m$ depolarization. However, cytosolic cytochrome c levels were not significantly increased in Bcl-2-overexpressing PC12 cells up to 32 h (Fig. 2C), indicating that Bcl-2-overexpression protects against manganese toxicity at the mitochondrial level.

3.3. Manganese induces the caspase-3 cascade in PC12 cells

Since cytochrome c release is known to initiate the caspase cascade following binding to apoptosis activating factor-1 (Apaf-1) [38–41], the next series of experiments was designed to examine whether or not manganese exposure induces the effector caspase-3 in dopaminergic cells. Exposure of vector PC12 cells to manganese (1 mM) time-dependence increased caspase-3 activity 1.3- to 4.3-fold above basal levels at 24–48-h post-exposure (Fig. 3A). Thus, the caspase-dependent cell death process was initiated between 24 and 28 h after manganese exposure, concomitant with depolarization of $\Delta\Psi_m$ and cytochrome c release. We also found that the manganese-induced caspase-3 activation between 28 and 48 h was significantly suppressed ($p < 0.05$) in cells overexpressing Bcl-2. To determine if caspase-3 activation was mediated by initiator caspase-9, we evaluated the effect of the caspase-9 specific inhibitor Z-LEHD-FMK. Pre-treatment with Z-LEHD-FMK (50 or 100 μM) inhibited manganese-induced caspase activity in a dose-dependent manner, indicating that manganese-induced caspase-3 activation is mainly dependent on caspase-9 (Fig. 3B). Caspase-8, another initiator caspase linked to receptor-mediated apoptosis, was unaltered up to 32 h of manganese exposure (Fig. 3C), suggesting that the mitochondrial-dependent caspase cascade is predominantly activated during manganese treatment.

3.4. Manganese-induced proteolytic cleavage of PKCδ

We recently showed that PKCδ is an important substrate for caspase-3, which proteolytically cleaves and activates the kinase to promote apoptosis in dopaminergic cells [30,31]. We monitored PKCδ proteolytic cleavage every 4 h for 48 h following manganese (1 mM) treatment in both vector PC12 and Bcl-2-overexpressing PC12 cells. Native PKCδ (74 kDa) was proteolytically cleaved into a catalytically active fragment (41 kDa), which started to appear at 20 h and became more significant at later time points in vector PC12 cells (Fig. 4A). PKCδ cleavage increased in a time-dependent manner up to 48 h, and the time course of PKCδ cleavage paralleled caspase-3 activation. Lower concentrations of manganese ranging 100–200 μM also induced caspase-3 activation and PKCδ cleavage when the treatment period was increased to 96 h (data not shown). In Bcl-2-overexpressing PC12 cells, no

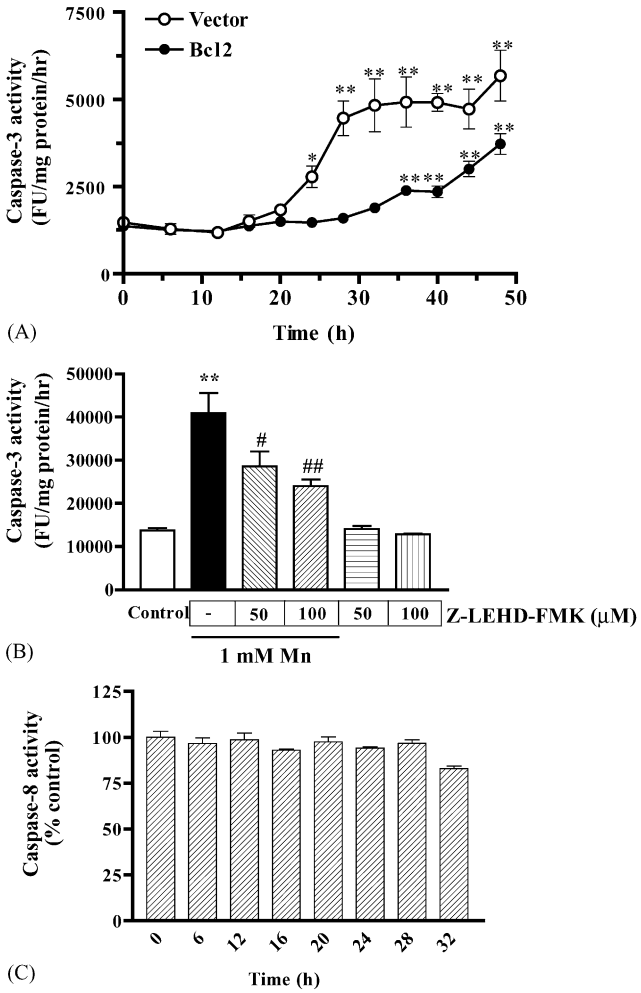

Fig. 3. Manganese-induced caspase-3 and caspase-8 activity in vector PC12 and Bcl-2-overexpressing PC12 cells. Vector PC12 and Bcl-2-overexpressing PC12 cells were treated with 1 mM $MnCl_2$ for 0–48 h in the presence or absence of 50–100 μM Z-LEHD-FMK, a caspase-9 inhibitor. Cytosolic supernatants were collected and caspase activities were measured by incubating the supernatant with 50 μM Ac-DEVD-AMC (fluorogenic caspase-3 substrate) or 50 μM Ac-IETD-AMC (fluorogenic caspase-8 substrate) for 1 h at 37 °C, and expressed as fluorescent unit (FU) per mg protein per hour or as percent control. (A) Time course of caspase-3 activity in vector and Bcl-2-expressing PC12 cells. Each point represents mean ± S.E.M. for $n = 6$–9. (B) Caspase-3 activity in the presence of caspase-9 inhibitor, Z-LEHD-FMK in vector PC12 cells. Each bar represents mean ± S.E.M. for $n = 6$–9. (C) Time course of caspase-8 activity in vector PC12 cells. Each bar represents mean ± S.E.M. for $n = 6$. Asterisks ($^{*}p < 0.05$ or $^{**}p < 0.01$) indicate significant difference compared with control cells and pound sign ($^{\#}p < 0.05$ or $^{\#\#}p < 0.01$) indicates significant difference compared with manganese-treated cells.

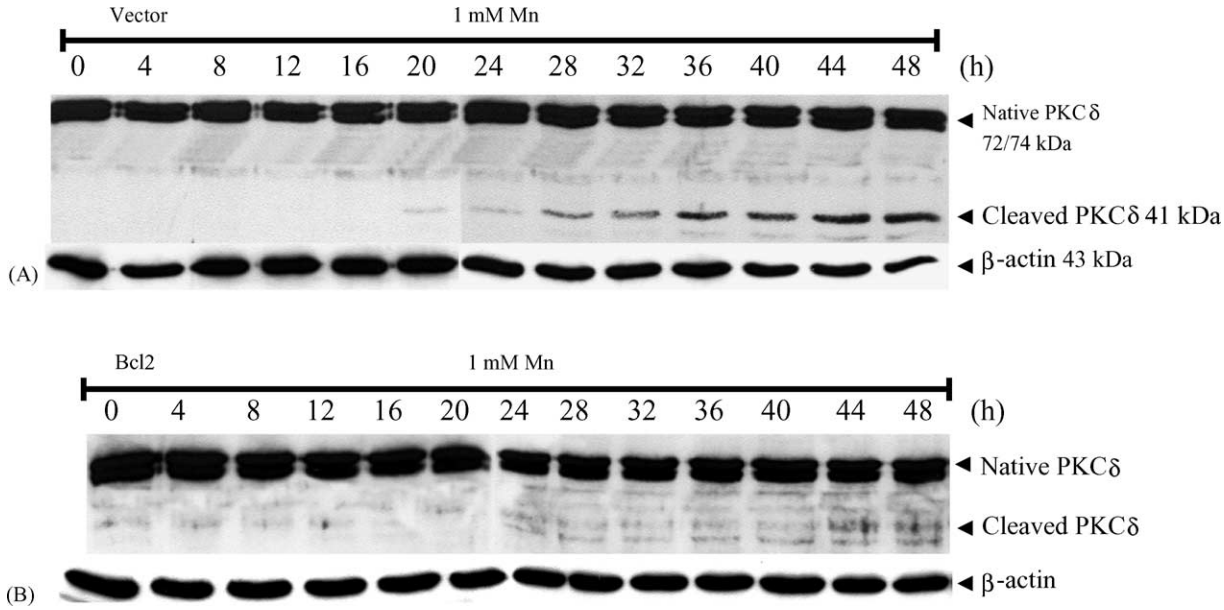

Fig. 4. Manganese induces proteolytic cleavage of PKCδ in PC12 cells. (A) Vector PC12 or (B) Bcl-2-overexpressing PC12 cells were treated with 1 mM $MnCl_2$ for 0–48 h. Cytosolic fractions were collected, and equal amounts of proteins were resolved on 10% SDS–PAGE followed by polyclonal PKCδ antibody. Native PKCδ appears at 72–74 kDa and catalytically active cleaved PKCδ appears at around 41 kDa. Equal protein loading was confirmed by reprobing with β-actin.

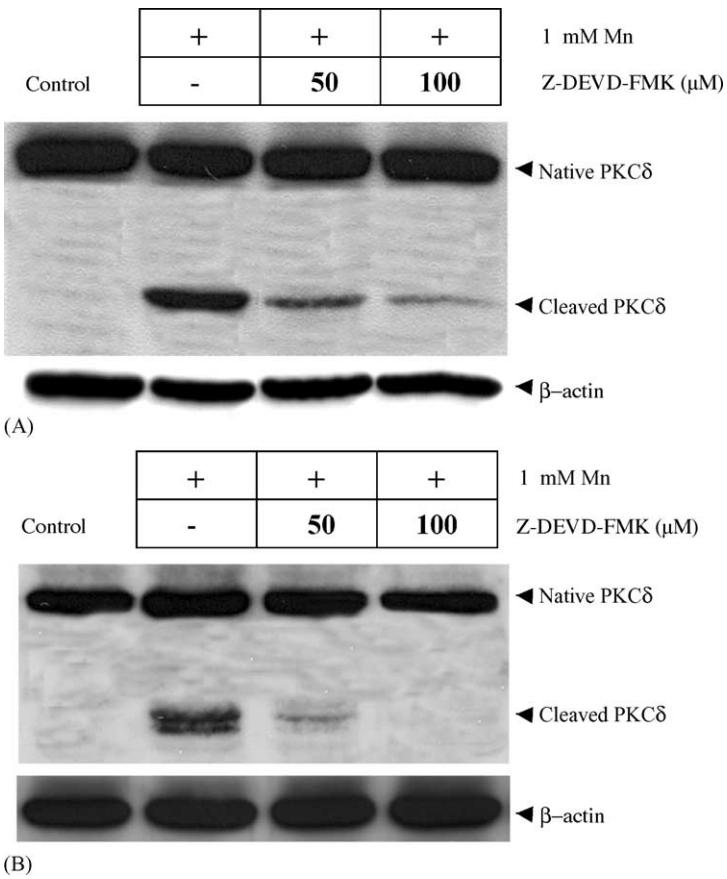

Fig. 5. Effect of caspase inhibitors on Manganese induces proteolytic cleavage of PKCδ Vector PC12 cells were treated with 1 mM $MnCl_2$ with or without (A) 50–100 μM Z-VAD-FMK, a caspase inhibitor and (B) 50–100 μM Z-DEVD-FMK, a caspase-3 specific inhibitor. Caspase inhibitors were added for 30 min prior to the treatment with manganese for 36 h. Cytosolic fractions were isolated, and proteins were resolved on 10% SDS–PAGE. Native PKCδ (72–74 kDa) and cleaved PKCδ (41 kDa) were detected. Equal protein loading was confirmed by reprobing with β-actin.

well-defined bands corresponding to the proteolytically cleaved product of PKCδ were detected during manganese exposure (Fig. 4B), indicating that Bcl-2 can suppress the caspase-3-mediated apoptotic cell death process.

To confirm that manganese-induced proteolytic cleavage of PKCδ was caspase-3 mediated, we separately pre-treated vector PC12 cells for 1 h with the pan-caspase inhibitor Z-VAD-FMK or the caspase-3 specific inhibitor Z-DEVD-FMK, and then treated the cells with 1 mM manganese for 36 h. Pre-treatment with 50–100 μM Z-VAD-FMK (Fig. 5A) or 50–100 μM Z-DEVD-FMK (Fig. 5B) significantly inhibited manganese-induced proteolytic cleavage of PKCδ in a dose-dependent manner, suggesting that the manganese-induced proteolytic activation of PKCδ was caspase-3-dependent. Manganese treatment did not induce translocation of PKCδ to the membrane, a common mechanism of PKCδ activation, in either vector PC12 or Bcl-2-overexpressing PC12 cells (data not shown). Furthermore, neither a decreased total Bcl-2 protein level nor cleaved product of Bcl-2 was observed during manganese treatment.

3.5. Manganese increases PKCδ kinase activity via proteolytic cleavage

To further examine whether manganese-induced proteolytic cleavage of PKCδ also increased PKCδ kinase activity in the cells, we measured PKCδ enzyme activity using a ^{32}P radiolabeled histone phosphorylation assay following immunoprecipitation of the kinase. In vector PC12 cells, PKCδ kinase activity increased 2–3-fold over basal levels after a 36-h exposure to 1 mM manganese (Fig. 6). On the other hand, in Bcl-2-overexpressing PC12 cells, there was no increase in PKCδ activity at 36 h.

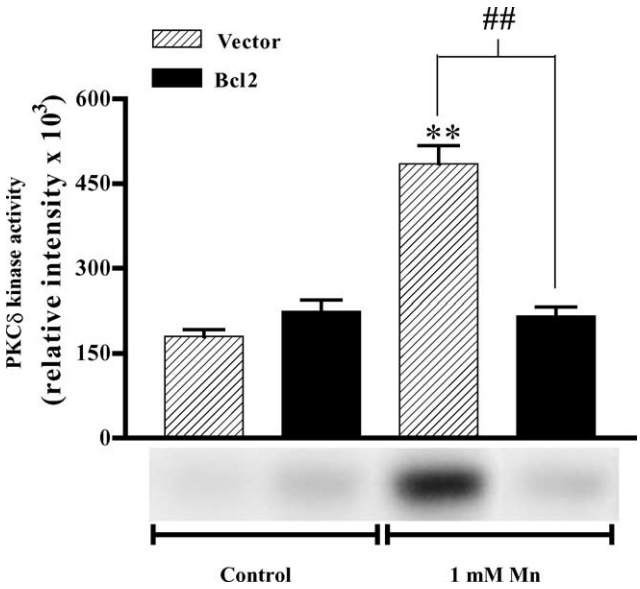

Fig. 6. Manganese-induced PKCδ activity. Vector PC12 and Bcl-2-overexpressing PC12 cells were exposed to 1 mM MnCl2 for 36 h, and cytosolic fractions were collected. Then, samples were immunoprecipitated with PKCδ, and kinase activity was measured as described in the Section 2. Each bar represents mean ± S.E.M. for $n = 6$. Asterisks ($^{**}p < 0.01$) indicate a significant difference between control and manganese treatment in vector cells, and pound signs ($^{\#\#}p < 0.01$) indicate a significant difference between manganese-treated vector and Bcl-2-overexpressing cells.

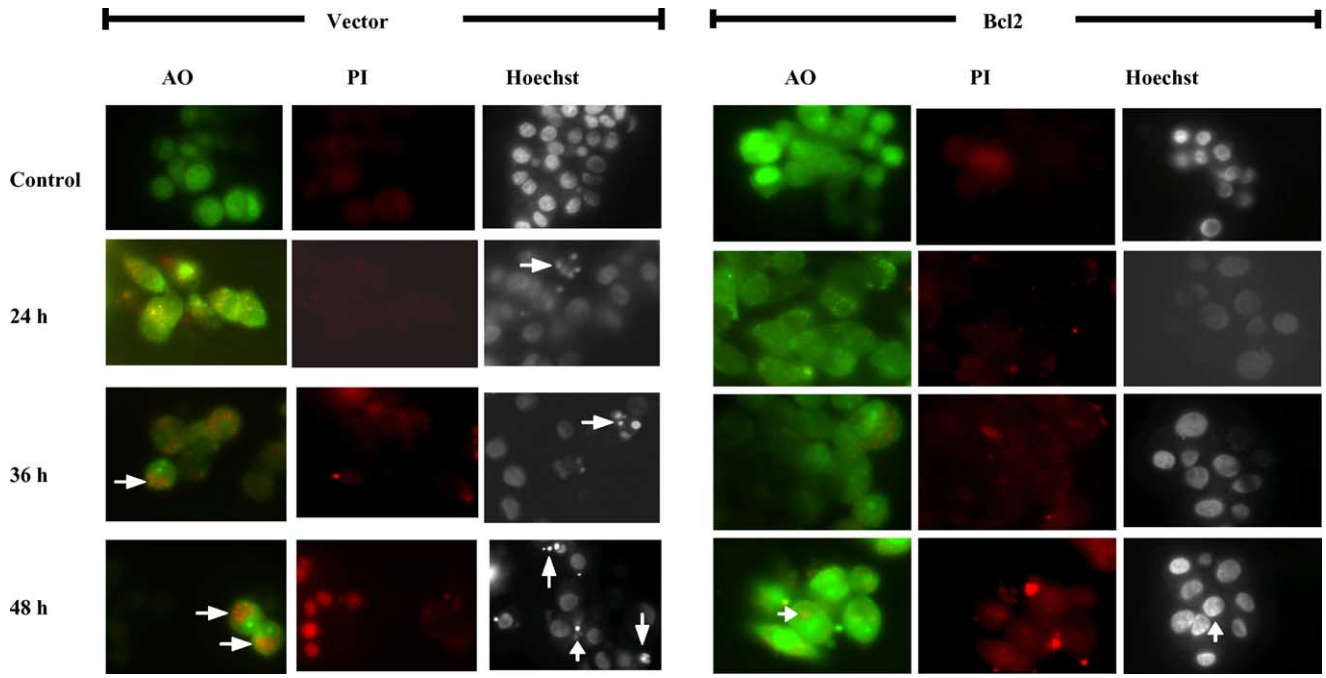

Fig. 7. Manganese-induced apoptosis in PC12 cells: in situ study. Qualitative measurement of apoptosis using the acridine orange (AO), propidium iodide (PI), and Hoechst 33342 (Hoechst) dyes. Cells were plated on collagen-coated cover slips and exposed to 1 mM $MnCl_2$ for 24–48 h. Cells were then stained with 5 μg/ml AO + 5 μg/ml PI or 10 μg/ml Hoechst and observed under the fluorescent microscope. Arrows indicate cells undergoing apoptosis.

These results, combined with data from the proteolytic cleavage assays (Figs. 4 and 5), indicate that manganese-induced proteolytic cleavage results in increased PKCδ kinase activity.

3.6. Manganese-induced DNA fragmentation in PC12 cells

We examined manganese-induced apoptotic cell death using acridine orange/propidium iodide double in situ staining or Hoechst 33342 nuclear staining. Both acridine orange and Hoechst 33342 are used to detect chromatin integrity; an index of apoptosis [35], and propidium iodide detects cells undergoing necrosis or the late stage of apoptosis. Apoptotic bodies and chromatin condensation started to appear at 24 h and increased with time up to 48 h in 1 mM manganese-treated vector PC12 cells (Fig. 7). None of the manganese-treated cells stained for propidium iodide, suggesting that necrosis is not occurring during the treatment period (Fig. 7). The membrane integrity was normal in Bcl-2-overexpressing PC12 cells, and very few of the cells were apoptotic during the entire 48 h of manganese exposure.

To further confirm the results obtained by in situ fluorometric detection of apoptosis and to assess the involvement of caspases and PKCδ in mediation of apoptosis, a quantitative DNA fragmentation assay was performed. We confirmed the effect of Bcl-2 on manganese-induced DNA fragmentation using an ELISA assay. DNA fragmentation was detected in vector PC12 cells as early as 24 h after exposure to 1 mM manganese, and it increased time-dependently by 8-, 15-, and 21-fold at 24, 36, and 48 h, respectively (Fig. 8A). DNA fragmentation was dramatically attenuated at all time points in manganese-treated Bcl-2-overexpressing PC12 cells, indicating that Bcl-2 can protect dopaminergic cells from apoptosis during manganese exposure.

We next examined the effect of caspase inhibitors on DNA fragmentation during manganese treatment. In Fig. 8B, manganese-induced DNA fragmentation was significantly ($p < 0.01$) suppressed by pre-treatment with 100 μM Z-VAD-FMK, a potent caspase inhibitor, at 36-h post-exposure in vector PC12 cells. In addition, 100 μM Z-DEVD-FMK, a caspase-3 specific inhibitor, also significantly ($p < 0.01$) attenuated manganese-induced DNA fragmentation at 36 h, although not as effectively as Z-VAD-FMK. The caspase inhibitors alone did not significantly alter basal DNA fragmentation levels during the entire experimental period (data not shown).

3.7. Pro-apoptotic role of PKCδ in dopaminergic cells: studies on PKC down-regulation, recombinant PKCδ and PKCδ dominant-negative mutant

In the next series of experiments, we determined whether or not proteolytic activation of PKCδ contributes

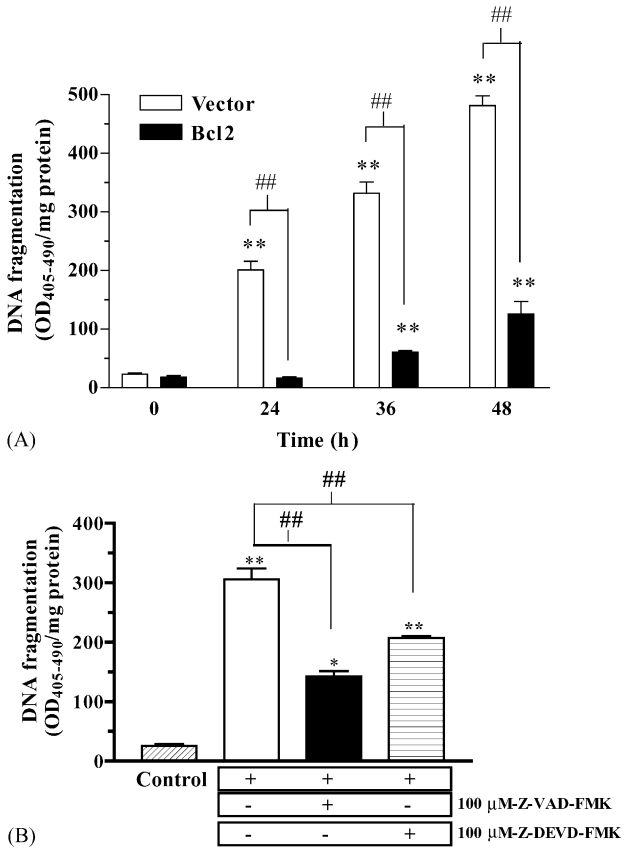

Fig. 8. Manganese-induced DNA fragmentation in PC12 cells. (A) Apoptotic cell death was determined by measuring DNA fragmentation. Cells were treated with 1 mM $MnCl_2$ for 24–48 h, and the level of DNA fragmentation was analyzed using a DNA ELISA kit. Each bar represents mean ± S.E.M. ($n = 6$–9). (B) Vector PC12 cells were exposed to 1 mM $MnCl_2$ with or without 100 μM Z-VAD-FMK, a pan-caspase inhibitor, or 100 μM Z-DEVD-FMK, a caspase-3 specific inhibitor, for 36 h. DNA fragmentation was determined using a DNA ELISA assay kit. Each bar represents mean ± S.E.M. ($n = 6$–9). Asterisks ($^{*}p < 0.05$ or $^{**}p < 0.01$) indicate a significant difference between control and manganese treatment and pound signs ($^{(\#\#}p < 0.01$) indicate a significant difference between manganese-treated vector and Bcl-2-overexpressing cells or between manganese- and inhibitor-treated cells.

to manganese-induced apoptosis in dopaminergic cells. Chronic treatment with low-dose TPA down-regulates PKCs [42,43]. Vector PC12 cells were treated with 0.2 μM TPA for 24 h to down-regulate PKC proteins, and cells were then exposed to manganese (1 mM) for 36 h. As shown in Fig. 9A, 24 h of TPA treatment resulted in a prolonged down-regulation of PKCδ for more than 72 h. Other PKC isoforms, except for the atypical PKCζ, were also down-regulated in the same manner (data not shown). We found that these PKC-deficient dopaminergic cells are significantly ($p < 0.01$) more resistant to manganese-induced DNA fragmentation as compared to control cells (Fig. 9B). Although, TPA down-regulates most of the PKC isoforms, this initial experiment in PKC-deficient cells provides support that PKCs are important in manganese-induced apoptosis.

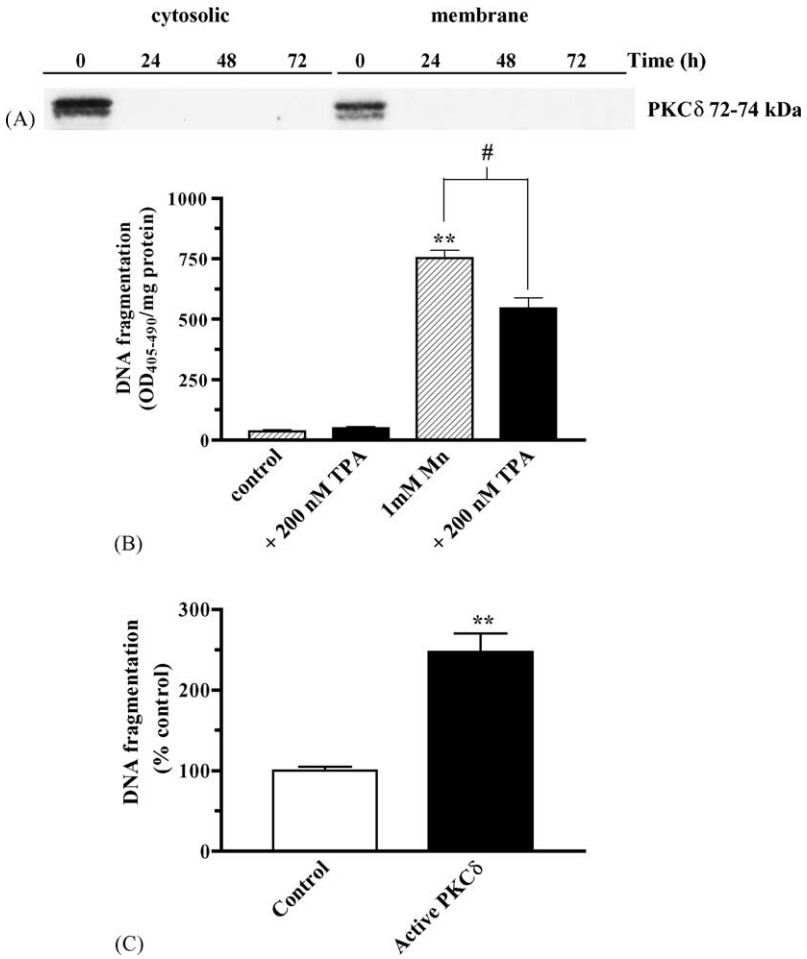

Fig. 9. Catalytically active PKCδ protein plays a critical role in execution of DNA fragmentation. (A) Vector PC12 cells were treated with 0.2 μM TPA for 24–72 h and down-regulation of PKCδ was determined by Western blot. (B) PKC down-regulated vector PC12 cells were treated with 1 mM $MnCl_2$ for 36 h, and DNA fragmentation was quantified by ELISA assay. Each bar represents mean ± S.E.M. ($n = 6$). Asterisks ($^{**}p < 0.01$) indicate a significant difference between control and manganese treatment, and pound signs ($^{\#}p < 0.05$) indicate a significant difference between normal vector cells and PKC down-regulated vector cells following manganese treatment. (C) Vector PC12 cells were treated with 10 μl BioPORTER reagent +5 ng catalytically active PKCδ protein in serum-free DMEM for 4 h to successfully deliver active PKCδ protein into the cells. For comparison, 5 ng of heat-inactivated PKCδ protein (15 min in boiling water) or solvent (PBS) was delivered by BioPORTER reagent. Following the 4 h treatment, equal numbers of cells were collected and processed to determine DNA fragmentation using a DNA ELISA assay kit. DNA fragmentation was expressed as percent control (10 μl BioPORTER reagent + PBS). Each bar represents mean ± S.E.M. ($n = 6$). Asterisks ($^{**}p < 0.01$) indicate a significant difference between control and catalytically active PKCδ protein-treated cells.

To determine the specific role of PKCδ in apoptosis in dopaminergic cells, we delivered the active recombinant PKCδ protein into cells and then monitored DNA fragmentation. We recently demonstrated, along with others, that various biologically active proteins, including enzymes, could be delivered into cells using a new lipid-mediated protein delivery system known as BioPORTER [31,44]. Human recombinant PKCδ was administered (5 ng) to cells by the BioPORTER system. The delivery effectiveness of BioPORTER was determined using FITC-labeled antibody, and treated cells were observed under a fluorescence microscope [30]. In our previous study, we confirmed the biological activity of the recombinant PKCδ administered to cells [45]. DNA fragmentation was increased by 2.5-fold in the active recombinant PKCδ delivered cells compared to the BioPORTER reagent-treated (control) cells (Fig. 9C). Thus, catalytically active PKCδ plays an important role in the execution of apoptosis.

To further confirm the pro-apoptotic role of PKCδ in manganese-induced cell death, a dominant-negative mutant of PKCδ (PKCδ-DN, with a lysine-to-arginine mutation at position 376 which renders the kinase inactive) was introduced into PC12 cells by a lentiviral-mediated gene delivery system. Cells were then treated with 1 mM $MnCl_2$ for 24 h. LacZ was separately introduced into PC12 cells and treated with $MnCl_2$ to serve as a vector control. PKCδ-DN or LacZ expressing PC12 cells were identified by immunostaining of the V5 epitope. Apoptotic PC12 cells were identified by TUNEL-positive staining and Hoechst 33342 staining. PKCδ-DN expressing PC12 cells (about 30% V5-positive) showed intact nuclear morphology

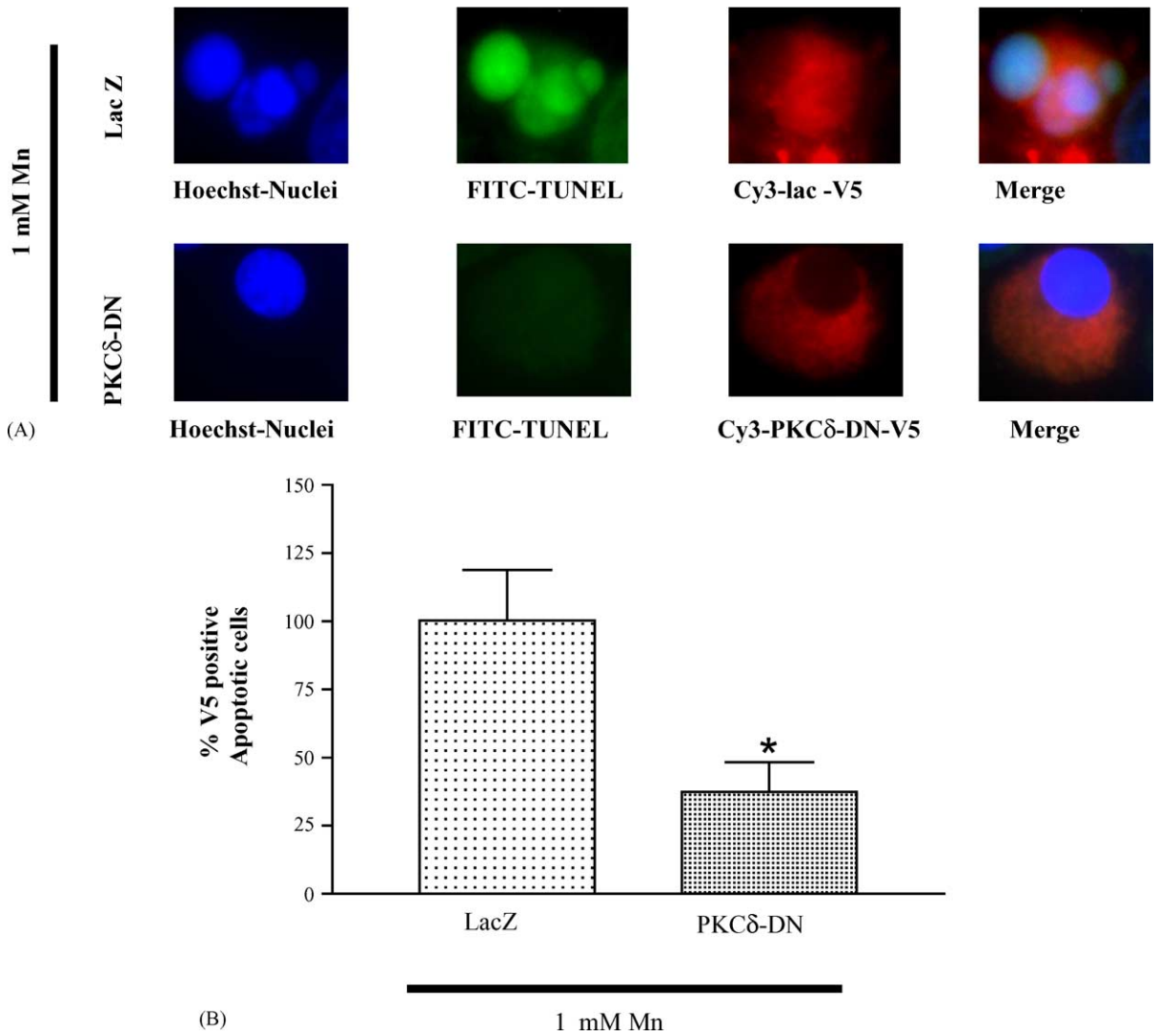

Fig. 10. Overexpression of dominant-negative mutant PKCδ^{K376R} (PKCδ-DN) by lentiviral mediated transfection attenuated manganese-induced apoptosis in PC12 cells. PC12 cells were transfected with LacZ or PKCδ-DN using a V5-tagged lentiviral gene delivery system, and then cells were exposed to 1 mM MnCl2 for 24 h. (A) PKCδ-DN or LacZ expressing PC12 cells were identified by immunostaining of the V5 epitope (red). Apoptotic PC12 cells were identified by both TUNEL staining (green) and nuclear morphology changes revealed by Hoechst staining (blue). (B) The percentage of V5 apoptotic cells in PKCδ-DN or LacZ expressing PC12 cells after manganese exposure. Each bar represents mean ± S.E.M. ($n = 4$). Asterisk ($^{*}p < 0.05$) indicates a significant difference between LacZ and PKCδ-DN expressing cells.

and no TUNEL signal after manganese treatment (Fig. 10A). However, strong TUNEL staining and nucleic condensation into apoptotic bodies, as revealed by Hoechst 33342 staining, were observed in LacZ expressing PC12 cells. The TUNEL staining signal overlaid very well with Hoechst 33342 staining, confirming that the TUNEL signal was not induced by non-specific binding. The quantitative analysis of apoptosis after manganese exposure in PKCδ-DN or LacZ expressing PC12 cells is summarized in Fig. 10B. The percentage of apoptotic PC12 cells was about 2.5-fold higher in LacZ expressing PC12 cells than in PKCδ-DN expressing PC12 cells. This result strongly indicates that PKCδ-DN significantly attenuated apoptosis in PC12 cells induced by manganese treatment, demonstrating therefore, that PKCδ promotes manganese-induced apoptosis in PC12 cells.

4. Discussion

In the present study, we systematically characterized the cell death mechanisms associated with manganese toxicity, and demonstrated that (i) manganese activates the mitochondrial-dependent caspase cascade; (ii) the mitochondrial anti-apoptotic protein Bcl-2 negatively modulates the manganese-induced apoptotic cascade; and (iii) caspase-3-dependent proteolytic activation of PKCδ is a critical down-stream effector of manganese-induced apoptosis. Importantly, to our knowledge, this is the first report establishing PKCδ as a key biochemical target in manganese-induced apoptotic cell death.

Manganese primarily targets the nigrostriatal system, including the globus pallidus and substantia nigra [4,5,46,47]. Manganese has been reported to accumulate

in mitochondria and inhibit mitochondrial complex I and II activities [36,48,49]. Reactive oxygen species (ROS) are generated following inhibition of the mitochondrial electron transport chain [50]. In a recent study, manganese increased ROS generation in isolated mitochondrial fractions from mouse brains [51]. Also, we demonstrated that ROS plays a causal role in apoptotic cell death following exposure to the organic manganese compound methylcyclopentadienyl manganese tricarbonyl (MMT) [31]. ROS, in a dopamine rich environment, contributes to oxidative stress by forming dopamine derived quinone radicals [52,53]. Therefore, the increased susceptibility of the nigral system to manganese-induced neurotoxic insult can be attributed to the enhanced sensitivity of nigrostriatal neurons to oxidative stress. Although, we did not measure ROS generation in the present study, the observed mitochondrial membrane depolarization indicates the possible involvement of oxidative insult in manganese-induced neurotoxicity.

Mitochondrial membrane depolarization results in the rapid release of the pro-apoptotic factor cytochrome c into the cytoplasm to initiate the caspase cascade, an early and critical step in the apoptotic pathway [38,41,54–58]. Our results are consistent with other recent reports of caspase-3 activation observed during manganese toxicity in other in vitro models [20,59,60]. However, Oubrahim et al. [60] reported that manganese-induced caspase-3 activation and apoptosis in HeLa cells (derived from human epithelial adenocarcinoma) were not mediated by mitochondria because manganese did not alter the mitochondrial membrane potential ($\Delta\Psi_m$). Mitochondria from cancer cells appear to be resistant to manganese toxicity. Another study by Roth et al. [20] concluded that manganese-induced apoptosis is a caspase-independent process because the general caspase inhibitor Z-VAD-FMK failed to attenuate manganese-induced cell death in PC12 cells at 24 h. Since the investigators did not measure DNA fragmentation directly, the functional role of caspase-3 in the apoptotic process is difficult to ascertain. Also, they examined the effect of Z-VAD-FMK on manganese toxicity only up to 24 h, and apoptosis could have occurred at a later time point. Our data show that caspase-3 activation starts to rise at 24 h and peaks around 36 h, whereas maximal DNA fragmentation occurs around 36–48 h after manganese treatment. Thus, the manganese-induced cell death process appears to be cell type specific and time-dependent. Our data clearly demonstrate that chronic manganese exposure targets mitochondria and activates caspase-dependent apoptosis in dopaminergic cells, which are known to be selectively affected in manganese-induced neurotoxicity. Furthermore, attenuation of manganese-induced depolarization of $\Delta\Psi_m$, cytochrome c release, caspase-3 activation, and apoptosis by the mitochondrial anti-apoptotic protein Bcl-2 indicates that mitochondria may serve as an initial regulator of manganese-induced apoptosis in dopaminergic cells. Previous studies have shown caspase-dependent degradation of Bcl-2 during the apoptotic process [61,62], however, we observed no Bcl-2 degradation during manganese treatment. In addition, recent evidence indicates that manganese may affect the endoplasmic reticulum (ER) and induce the caspase-12-dependent apoptotic cascade [59,63], targeting multiple organelles and promoting cell death.

Caspase-dependent apoptosis has been reported in dopaminergic degeneration resulting from exposure to various dopaminergic neurotoxins, including MPP^+ [30,31,45,64,65] as well as in brains of PD patients [66]. However, the key down-stream events that contribute to DNA fragmentation are not well characterized. One significant finding of the current study is the identification of PKCδ proteolytic activation induced by manganese exposure. PKCδ belongs to a novel isoform family of PKCs and is activated in a calcium-independent manner. Traditionally, PKCs have been considered to have anti-apoptotic functions, but PKCδ is emerging as a key pro-apoptotic factor. Lipid-dependent translocation and proteolytic activation are two major activation mechanisms of PKCδ [34,67–69]. Also, activation of PKCδ by tyrosine phosphorylation has been reported [70]. Recently, we demonstrated that PKCδ proteolytic activation mediates apoptotic cell death in dopaminergic cells following exposure to dieldrin and MPP^+ [30,45]. In those studies as well as in the present study, PKCδ was not activated by translocation, indicating that proteolytic activation is the primary mode of activation in dopaminergic cells. Induction of DNA fragmentation by delivery of the catalytically active PKCδ protein and attenuation of manganese-induced DNA fragmentation by pharmacological and genetic modulation of PKCδ (kinase-inactive mutant) clearly show that PKCδ is a key down-stream substrate of caspase-3. The cellular substrates of PKCδ that specifically mediate apoptotic cell death have not yet been defined. However, several signaling molecules such as DNA protein kinase (DNA-PK), MAP-kinase, scrambalase, and NF-kappa transcription factor have been proposed to function as down-stream effectors of PKCδ [71–74]. Previously, Hirata et al. [19] reported activation of the JNK pathway by phosphorylation of a serine–threonine kinase in Manganese-treated PC12 cells. PKCδ-dependent phosphorylation of JNK has been implicated in apoptosis [72].

In conclusion, manganese treatment in dopaminergic cells induces caspase-3-dependent proteolytic activation of PKCδ, and the active PKCδ contributes to apoptotic cell death. Identification of critical cellular targets of PKCδ important in apoptosis following manganese treatment may provide insight into neurodegenerative processes associated with manganese exposure.

Acknowledgements

This study is supported by National Institute of Health (NIH) grants ES10586 and NS45133.

References

[1] Graumann R, Paris I, Martinez-Alvarado P, Rumanque P, Perez-Pastene C, Cardenas SP, et al. Oxidation of dopamine to aminochrome as a mechanism for neurodegeneration of dopaminergic systems in Parkinson's disease. Possible neuroprotective role of DT-diaphorase. Pol J Pharmacol 2002;54:573–9.

[2] Aschner M. Manganese neurotoxicity and oxidative damage. In: Connor JR, editor. Metals and oxidative damage in neurological disorders. New York: Plenum; 1997. p. 77–93.

[3] Erikson KM, Aschner M. Manganese neurotoxicity and glutamate–GABA interaction. Neurochem Int 2003;43:475–80.

[4] McKinney AM, Filice RW, Teksam M, Casey S, Truwit C, Clark HB, et al. Diffusion abnormalities of the globi pallidi in manganese neurotoxicity. Neuroradiology 2004;46:291–5.

[5] Pal PK, Samii A, Calne DB. Manganese neurotoxicity: a review of clinical features, imaging and pathology. Neurotoxicology 1999;20: 227–38.

[6] Gerber GB, Leonard A, Hantson P. Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. Crit Rev Oncol Hematol 2002;42:25–34.

[7] Aschner M. Manganese: brain transport and emerging research needs. Environ Health Perspect 2000;108(Suppl 3):429–32.

[8] Zayed J, Gerin M, Loranger S, Sierra P, Begin D, Kennedy G. Occupational and environmental exposure of garage workers and taxi drivers to airborne manganese arising from the use of methylcyclopentadienyl manganese tricarbonyl in unleaded gasoline. Am Ind Hyg Assoc J 1994;55:53–8.

[9] Zayed J, Vyskocil A, Kennedy G. Environmental contamination and human exposure to manganese—contribution of methylcyclopentadienyl manganese tricarbonyl in unleaded gasoline. Int Arch Occup Environ Health 1999;72:7–13.

[10] Lynam DR, Roos JW, Pfeifer GD, Fort BF, Pullin TG. Environmental effects and exposures to manganese from use of methylcyclopentadienyl manganese tricarbonyl (MMT) in gasoline. Neurotoxicology 1999;20:145–50.

[11] Kaiser J. Manganese: a high-octane dispute. Science 2003;300: 926–8.

[12] Calabresi P, Ammassari-Teule M, Gubellini P, Sancesario G, Morello M, Centonze D, et al. A synaptic mechanism underlying the behavioral abnormalities induced by manganese intoxication. Neurobiol Dis 2001;8:419–32.

[13] Brouillet EP, Shinobu L, McGarvey U, Hochberg F, Beal MF. Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism. Exp Neurol 1993;120:89–94.

[14] Mustafa SJ, Chandra SV. Levels of 5-hydroxytryptamine, dopamine and norepinephrine in whole brain of rabbits in chronic manganese toxicity. J Neurochem 1971;18:931–3.

[15] Defazio G, Soleo L, Zefferino R, Livrea P. Manganese toxicity in serumless dissociated mesencephalic and striatal primary culture. Brain Res Bull 1996;40:257–62.

[16] Honig LS, Rosenberg RN. Apoptosis and neurologic disease. Am J Med 2000;108:317–30.

[17] Sathasivam S, Ince PG, Shaw PJ. Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. Neuropathol Appl Neurobiol 2001;27:257–74.

[18] Schrantz N, Blanchard DA, Mitenne F, Auffredou MT, Vazquez A, Leca G. Manganese induces apoptosis of human B cells: caspase-dependent cell death blocked by bcl-2. Cell Death Differ 1999;6: 445–53.

[19] Hirata Y, Adachi K, Kiuchi K. Activation of JNK pathway and induction of apoptosis by manganese in PC12 cells. J Neurochem 1998;71:1607–15.

[20] Roth JA, Feng L, Walowitz J, Browne RW. Manganese-induced rat pheochromocytoma (PC12) cell death is independent of caspase activation. J Neurosci Res 2000;61:162–71.

[21] Migheli R, Godani C, Sciola L, Delogu MR, Serra PA, Zangani D, et al. Enhancing effect of manganese on L-DOPA-induced apoptosis in PC12 cells: role of oxidative stress. J Neurochem 1999;73:1155–63.

[22] Gavin CE, Gunter KK, Gunter TE. Manganese and calcium transport in mitochondria: implications for manganese toxicity. Neurotoxicology 1999;20:445–53.

[23] Hirata Y, Kiuchi K, Nagatsu T. Manganese mimics the action of 1-methyl-4-phenylpyridinium ion, a dopaminergic neurotoxin, in rat striatal tissue slices. Neurosci Lett 2001;311:53–6.

[24] Malecki EA. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. Brain Res Bull 2001;55:225–8.

[25] Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells 1998;3:697–707.

[26] Fabisiak JP, Kagan VE, Ritov VB, Johnson DE, Lazo JS. Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. Am J Physiol 1997;272:C675–84.

[27] Yang L, Matthews RT, Schulz JB, Klockgether T, Liao AW, Martinou JC, et al. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. J Neurosci 1998;18: 8145–52.

[28] Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. Free Radic Biol Med 2001;31: 1473–85.

[29] Shafer TJ, Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. Neurotoxicology 1991;12:473–92.

[30] Kitazawa M, Anantharam V, Kanthasamy AG. Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. Neuroscience 2003;119: 945–64.

[31] Anantharam V, Kitazawa M, Wagner J, Kaul S, Kanthasamy AG. Caspase-3-dependent proteolytic cleavage of protein kinase Cdelta is essential for oxidative stress-mediated dopaminergic cell death after exposure to methylcyclopentadienyl manganese tricarbonyl. J Neurosci 2002;22:1738–51.

[32] Hishita T, Tada-Oikawa S, Tohyama K, Miura Y, Nishihara T, Tohyama Y, et al. Caspase-3 activation by lysosomal enzymes in cytochrome c-independent apoptosis in myelodysplastic syndrome-derived cell line P39. Cancer Res 2001;61:2878–84.

[33] Yoshimura S, Banno Y, Nakashima S, Takenaka K, Sakai H, Nishimura Y, et al. Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. Inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. J Biol Chem 1998;273:6921–7.

[34] Reyland ME, Anderson SM, Matassa AA, Barzen KA, Quissell DO. Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. J Biol Chem 1999;274:19115–23.

[35] Pulliam L, Stubblebine M, Hyun W. Quantification of neurotoxicity and identification of cellular subsets in a three-dimensional brain model. Cytometry 1998;32:66–9.

[36] Galvani P, Fumagalli P, Santagostino A. Vulnerability of mitochondrial complex I in PC12 cells exposed to manganese. Eur J Pharmacol 1995;293:377–83.

[37] Zheng W, Ren S, Graziano JH. Manganese inhibits mitochondrial aconitase: a mechanism of manganese neurotoxicity. Brain Res 1998;799:334–42.

[38] Jellinger KA. Parkinson disease with old-age onset. Arch Neurol 2003;60:1814–5.

[39] Jordan J, Cena V, Prehn JH. Mitochondrial control of neuron death and its role in neurodegenerative disorders. J Physiol Biochem 2003;59:129–41.

[40] Fiskum G, Starkov A, Polster BM, Chinopoulos C. Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease. Ann NY Acad Sci 2003;991:111–9.

[41] Earnshaw WC. Apoptosis. A cellular poison cupboard. Nature 1999;397:. 387, 389.
[42] Kikkawa U, Kishimoto A, Nishizuka Y. The protein kinase C family: heterogeneity and its implications. Annu Rev Biochem 1989;58: 31–44.
[43] Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, et al. Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem 1989; 264:4088–92.
[44] Boyle DL, Carman P, Takemoto L. Translocation of macromolecules into whole rat lenses in culture. Mol Vis 2002;8:226–34.
[45] Kaul S, Kanthasamy A, Kitazawa M, Anantharam V, Kanthasamy AG. Caspase-3 dependent proteolytic activation of protein kinase C delta mediates and regulates 1-methyl-4-phenylpyridinium (MPP+)-induced apoptotic cell death in dopaminergic cells: relevance to oxidative stress in dopaminergic degeneration. Eur J Neurosci 2003;18:1387–401.
[46] Diaz-Veliz G, Mora S, Gomez P, Dossi MT, Montiel J, Arriagada C, et al. Behavioral effects of manganese injected in the rat substantia nigra are potentiated by dicumarol, a DT-diaphorase inhibitor. Pharmacol Biochem Behav 2004;77:245–51.
[47] Spadoni F, Stefani A, Morello M, Lavaroni F, Giacomini P, Sancesario G. Selective vulnerability of pallidal neurons in the early phases of manganese intoxication. Exp Brain Res 2000;135:544–51.
[48] Gavin CE, Gunter KK, Gunter TE. Mn2+ sequestration by mitochondria and inhibition of oxidative phosphorylation. Toxicol Appl Pharmacol 1992;115:1–5.
[49] Maynard LS, Cotzias GC. Partition of manganese among organs and intracellular organelles in the rat. J Biol Chem 1955;214:489–95.
[50] Kushnareva Y, Murphy AN, Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD(P)+ oxidation-reduction state. Biochem J 2002;368:545–53.
[51] HaMai D, Campbell A, Bondy SC. Modulation of oxidative events by multivalent manganese complexes in brain tissue. Free Radic Biol Med 2001;31:763–8.
[52] Graham DG. Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. Neurotoxicology 1984;5:83–95.
[53] Stokes AH, Hastings TG, Vrana KE. Cytotoxic and genotoxic potential of dopamine. J Neurosci Res 1999;55:659–65.
[54] Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22.
[55] Hashimoto M, Rockenstein E, Crews L, Masliah E. Role of protein aggregation in mitochondrial dysfunction and neurodegeneration in Alzheimer's and Parkinson's diseases. Neuromolecular Med 2003; 4:21–36.
[56] Iverson SL, Orrenius S. The cardiolipin–cytochrome c interaction and the mitochondrial regulation of apoptosis. Arch Biochem Biophys 2004;423:37–46.
[57] Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Klose J, et al. Mitochondrial dysfunction and oxidative damage in Parkin-deficient mice. J Biol Chem 2004;279:18614–22.
[58] Rego AC, Oliveira CR. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. Neurochem Res 2003; 28:1563–74.
[59] Chun HS, Lee H, Son JH. Manganese induces endoplasmic reticulum (ER) stress and activates multiple caspases in nigral dopaminergic neuronal cells, SN4741. Neurosci Lett 2001;316:5–8.
[60] Oubrahim H, Stadtman ER, Chock PB. Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells. Proc Natl Acad Sci USA 2001;98:9505–10.
[61] Liang Y, Nylander KD, Yan C, Schor NF. Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2. Mol Pharmacol 2002;61:142–9.
[62] Liang Y, Yan C, Nylander KD, Schor NF. Early events in Bcl-2-enhanced apoptosis. Apoptosis 2003;8:609–16.
[63] Oubrahim H, Chock PB, Stadtman ER. Manganese(II) induces apoptotic cell death in NIH3T3 cells via a caspase-12-dependent pathway. J Biol Chem 2002;277:20135–8.
[64] Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH. Dopaminergic cell death induced by MPP(+), oxidant and specific neurotoxicants shares the common molecular mechanism. J Neurochem 2001;76:1010–21.
[65] Turmel H, Hartmann A, Parain K, Douhou A, Srinivasan A, Agid Y, et al. Caspase-3 activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Mov Disord 2001;16:185–9.
[66] Hartmann A, Hunot S, Michel PP, Muriel MP, Vyas S, Faucheux BA, et al. Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proc Natl Acad Sci USA 2000;97:2875–80.
[67] Pongracz J, Webb P, Wang K, Deacon E, Lunn OJ, Lord JM. Spontaneous neutrophil apoptosis involves caspase 3-mediated activation of protein kinase C-delta. J Biol Chem 1999;274:37329–34.
[68] Li L, Lorenzo PS, Bogi K, Blumberg PM, Yuspa SH. Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. Mol Cell Biol 1999;19: 8547–58.
[69] Majumder PK, Pandey P, Sun X, Cheng K, Datta R, Saxena S, et al. Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome c release and apoptosis. J Biol Chem 2000; 275:21793–6.
[70] Kikkawa U, Matsuzaki H, Yamamoto T. Protein kinase Cdelta (PKCdelta): activation mechanisms and functions. J Biochem Tokyo 2002;132:831–9.
[71] Bharti A, Kraeft SK, Gounder M, Pandey P, Jin S, Yuan ZM, et al. Inactivation of DNA-dependent protein kinase by protein kinase Cdelta: implications for apoptosis. Mol Cell Biol 1998;18: 6719–28.
[72] Chen WC, Chen CC. Signal transduction of arginine vasopressin-induced arachidonic acid release in H9c2 cardiac myoblasts: role of Ca2+ and the protein kinase C-dependent activation of p42 mitogen-activated protein kinase. Endocrinology 1999;140:1639–48.
[73] Vancurova I, Miskolci V, Davidson D. NF-kappa B activation in tumor necrosis factor alpha-stimulated neutrophils is mediated by protein kinase Cdelta. Correlation to nuclear Ikappa Balpha. J Biol Chem 2001;276:19746–52.
[74] Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, et al. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem 2000; 275:23065–73.