Involvement of Oxidized Peroxiredoxin-3 in Cadmium- and Ceramide-induced Apoptosis of Human Neuroblastoma Cells

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Cadmium (Cd) is an environmental pollutant that produces numerous toxic effects in humans. Previous reports have demonstrated that Cd induces neurodegeneration and other diseases. To study the mechanism of Cd-induced neurodegeneration, SH-SY5Y cells were exposed to Cd. Cd-induced ceramide production was observed in the cells in a time- and dose-dependent manner. Ceramide is a well known lipid second messenger. Previous reports have shown that accumulation of ceramide, triggered by oxidative stress or anticancer drugs, induces apoptosis. To identify proteins involved in cellular responses to Cd or ceramide, we performed comparative proteome analysis using high-resolution two-dimensional gel electrophoresis (2-DE). Four different proteins were identified by combination of mass spectrometry and peptide fingerprinting. Among them, the level of oxidized peroxiredoxin-3 (Prx-3) simultaneously increased in Cd- and ceramide-exposed cells. Furthermore, we observed Cd- or ceramide-induced generation of reactive oxygen species (ROS), leading to SH-SY5Y cell apoptosis. Pretreatment with Trolox helped scavenge Cd- or ceramide-induced ROS and prevented apoptosis, suggesting that Cd- or ceramide-induced cell death can be attributed to ROS production. Here, we elucidate the link between Cd-induced apoptosis and ROS scavenger system in SH-SY5Y cells.

Key words —— cadmium, ceramide, peroxiredoxin-3, reactive oxygen species

INTRODUCTION

Cadmium (Cd), a heavy metal widely used in industry, is known to cause occupational and environmental hazards.[1)] The toxicity of Cd as an industrial chemical, a food contaminant, and one of the major components of cigarette smoke is well established.[2, 3)] In mammals, Cd exerts multiple toxic effects including nephrotoxicity, hepatotoxicity, immunotoxicity, and neurotoxicity. It has been classified as a human carcinogen by the International Agency for Research on Cancer. In addition, Cd has been reported to induce apoptosis, both *in vivo* and *in vitro,* of many tissues including respiratory organs,[4)] testis,[5–7)] kidney,[8, 9)] liver,[10)] and immune cells.[11, 12)] The nervous system is also one of the targets of Cd toxicity.[13)] Of the hazards associated with exposure to Cd, central nervous system disorders have been reported in the case of Itai-itai disease and in various clinical studies on children and workers exposed to Cd.[14)] However, the mechanism of toxicity on neurons has not been fully understood.

In various cell types, intracellular calcium levels were found to rapidly increase after the addition of Cd[15)] which led to a rise in reactive oxygen species (ROS)[16)] and to mitochondrial impairment.[17)] Cd interferes with antioxidant defense mechanisms and stimulates the production of ROS, which may act as signaling molecules in the induction of gene expression and apoptosis.[18)] Also, it had been reported that Cd-induced apoptosis is mediated by the caspase-dependent pathway.[19, 20)]

Ceramide is known to be a sphingolipid-derived second messenger that plays a role in stress response, cell growth, differentiation, and cell death.[21)] In general, ceramide is produced via the

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hydrolysis of sphingomyelin as a result of sphingomyelinase (SMase) activation, or *de novo* synthesis beginning with serine and palmitic acid. Stress stimuli such as tumor necrosis factor (TNF)-α, Fas ligand, oxidative stress, growth factor withdrawal, anticancer drugs, and UV are known to elevate endogenous levels of ceramides.[22] Several lines of evidence suggest that ROS and cellular redox potential, which is mainly regulated by cellular glutathione, are tightly linked to the regulation of SMase activation.[23] On the other hand, C_2-ceramide, a cell-permeable ceramide, induced the increase in ROS in mitochondria and caused apoptosis of human retinal pigmented epithelial cells. This treatment also induced the activation of caspase-3 and the increase in mitochondrial membrane permeability transition.[24]

Mitochondria are the major source of cellular ROS, accounting for up to 90% of the total production.[25] Intracellular H_2O_2 is removed mostly by catalase, glutathione peroxidase, and peroxiredoxin (Prx). In mammals, six distinct Prx family members have been identified.[26–28] Prx are a ubiquitous family of thiol peroxidases that protect cells from peroxides and have a putative role in redox signaling.[29] Among them, Prx-3 is mitochondrial antioxidant protein and member of the Prx family that can scavenge H_2O_2. On reaction with H_2O_2, the redox-sensitive cysteine (Cys) residue of each subunit of the Prx homodimer is oxidized to cysteine-sulfenic acid (Cys-SOH), which then reacts with a neighboring reduced cysteine (Cys-SH) of the other subunit to form an intermolecular disulfide. This disulfide is reduced specifically by thioredoxin, not by glutathione or glutaredoxin.[30]

Previous reports have demonstrated that both Cd and ceramide induce apoptosis via ROS production. However, the mechanism by which apoptosis is effected has not been fully elucidated. In the present study, ceramide level in the Cd-exposed cells were found to be elevated and the changes in the quantitative levels of proteome in Cd- and C_6-ceramide-exposed cells were analyzed by high-resolution two-dimensional gel electrophoresis (2-DE). Results of the proteomic analysis revealed that levels of oxidized Prx-3 were significantly elevated by both Cd and C_6-ceramide, and not by other heavy metals such as mercury and lead. However, levels of another ROS scavenger protein, thioredoxin-2 (Trx-2), were significantly decreased in Cd-treated cells. The present study suggests that Cd may induce apoptosis through the up-regulation of ROS generation as well as the down-regulation of the ROS scavenging system.

MATERIAL AND METHODS

Materials —— Cadmium chloride ($CdCl_2$), lead nitrate ($PbNO_3$), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl)tetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), C_6-ceramide, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), and CHAPS were purchased from Sigma (St. Louis, MO, U.S.A.). [^{3}H]Palmitic acid was purchased from Amersham Corp. (Piscataway, NJ, U.S.A.). Methyl mercuric chloride (CH_3HgCl, 98%) was purchased from Tokyokasei Co. (Tokyo, Japan). All equipment for isoelectric focusing (IEF) and SDS-PAGE were from Amersham Pharmacia Biotech (Uppsala, Sweden). Silica gel 60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). Lactate dehydrogenase enzyme assay kit (Cytotox 96, Non-radioactive cytotoxicity assay kit) and Trolox were obtained from Promega (Madison, WI, U.S.A.). Antibodies to Prx-3, Prx-3-SO_3, and Trx-2 were purchased from Labfrontier Co. (Seoul, South Korea). All other chemicals were of the highest purity available from commercial sources.

Cell Culture —— SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Biowhittaker, Walkersville, MD, U.S.A.) with 10% fetal bovine serum, 100 U/ml each of penicillin and streptomycin (Gibco, Grand Island, NY, U.S.A.). Cultures were maintained in an incubator at 37°C in an atmosphere containing 5% CO_2. Cells were detached from the plates by 0.25% trypsin containing 1 mM EDTA. Cells were exposed to Cd or ceramide by incubating in DMEM/F12 containing 2% fetal bovine serum.

DNA Fragmentation —— Control and Cd-treated cells were cultured in growth medium for 13 hr at 37°C. Cells (1×10^7) were collected, washed with phosphate buffered saline (PBS), and gently lysed at 4°C in buffer containing 5 mM Tris-HCl pH 7.4, 20 mM EDTA, and 0.5% Triton X-100. Samples were centrifuged at $12000 \times g$ for 20 min to separate the intact chromatin from DNA fragments. DNA was extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1, v/v/v) to the extracted DNA was added 100 μg/ml RNase A, followed by incuba-

tion at 37°C for 30 min. The enzyme reaction was stopped with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v). The DNA fragments were then precipitated with 70% ethanol in the presence of 0.3 M sodium acetate, pH 5.2. After washing with 70% ethanol, DNA pellet was dissolved in tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA samples were analyzed by electrophoresis on 1.8% agarose gel containing ethidium bromide.

Lactate Dehydrogenase (LDH) Release and MTT Reduction Assay —— For LDH release assay, control and Cd-treated cells were cultured in 24 well plates for 13 hr at 37°C. LDH activity was determined in 50-μl aliquots of culture medium with the Cyto Tox 96 assay kit from Promega according to the manufacturer's instructions. The absorbance of each sample was measured at 490–492 nm. LDH levels are measured in both the culture supernatant and the cell lysate. The percentage LDH release is calculated by dividing the level of LDH in the medium by the total amount of LDH in the medium and cell lysate. LDH activity was expressed as percent of control. Alternatively, cell viability was determined by MTT assay. At the end of Cd or ceramide treatment procedure, MTT stock solution was added to each culture well and the cells were incubated at 37°C for 2 hr. After the medium was removed carefully, the precipitated formazan was dissolved with dimethyl sulfoxide. Cell viability was determined by measuring absorbance at 570 nm using a Genius Pro EIA plate reader (Tecan Group Ltd., Maennedorf, Switzerland).

DAPI Staining —— Apoptotic cells with condensed or fragmented nuclei were visualized by staining with DAPI. An aliquot of 5×10^5 cells per well of SH-SY5Y cells had been treated with Cd or ceramide at indicated concentration for 13 hr before the cells were isolated by centrifugation for DAPI staining. Cells were fixed with methanol/acetic acid (3 : 1, v/v) for 30 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml of DAPI at 37°C for 15 min. The cells were then washed with PBS and examined by fluorescence microscope (Nikon, Tokyo, Japan).

Detection of Ceramide —— 2×10^5 Cells were plated in 6 well culture dishes and labeled with [^{3}H]palmitic acid (1 μCi/ml) for 24 hr. After the exposure to Cd for 13 hr, the medium was aspirated, the cell monolayers were rinsed twice with ice-cold PBS, and 0.8 ml of ice-cold methanol containing 0.5N-HCl was added. Cells were scraped free of the substratum using a plastic scraper, and lipids were extracted using chloroform. The lipids were incubated in 0.1 N-methanolic potassium hydroxide (KOH) at 37°C for 1 hr to hydrolyze the phospholipids. The resulting lipids were evaporated to dryness using nitrogen stream. The lipids were dissolved in chloroform/methanol (1 : 1, v/v) and spotted on TLC silica gel plates. The plates were developed in chloroform/methanol/acetic acid/H_2O (85 : 4.5 : 5 : 0.5, v/v/v/v). The labeled ceramide was identified by comparison with commercial lipid standards for ceramide (BioMol, Plymouth Meeting, PA, U.S.A.), which were run on the same plate. After drying, the lipids were visualized by iodine vapor staining. The radioactive spots corresponding to ceramide were scraped off the plate and mixed with 2.5 ml of a scintillation solution (Insta gel-XF, Packard Instrument Co., Meriden, CT, U.S.A.). Radioactivity was determined using a β-scintillation counter (Tri-carb 1600 TR, Packard Instrument Co.).

Proteomics Analysis —— SH-SY5Y cells were lysed in 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, and 0.002% bromphenol blue. After incubation with DNase/RNase/Mg^{2+} solution at 37°C for 2 hr, DTT (100 mM) and 0.5% IPG buffer were added. IEF was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences, Uppsala, Sweden) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3500 V during 3 hr for sample entry followed by constant 3500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 × 24 cm, 10–16%). SDS-PAGE was performed using Hoefer DALT 2D system (Amersham Biosciences) following manufacturer's instruction. 2D gels were run at 20°C for 1700 Vh. And then 2D gels were silver stained but fixing and sensitization step with glutaraldehyde was omitted.

Quantitative analysis of digitized images was carried out using the PDQuest (version 7.0, BioRad, Hercules, CA, U.S.A.) software according to the protocols provided by the manufacturer. Protein spots were enzymatically digested in-gel and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain,

dried to remove solvent and then rehydrated with trypsin (8–10 ng/μl) and incubated 10 hr at 37°C. The proteolytic reaction was terminated by addition of 5 μl 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extract of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C_{18}ZipTips (Millipore, Bedford, MA, U.S.A.), and peptides eluted in 1–5 μl of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 μl of mixture spotted onto a target plate.

Matrix-assisted Laser Desorption/ionization Time-of-flight (MALDI-TOF) Analysis and Database Search —— Protein analysis were performed using an Ettan MALDI-TOF (Amersham Biosciences). Peptides were evaporated with a N2 laser at 337 nm, and using a delayed extraction approach. They were accelerated with 20 kV injection pulse for time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe) was used for protein identification by peptide mass fingerprinting.

Analysis of Intracellular ROS Production —— Before treatment with $CdCl_2$ and C_6-ceramide, cells were incubated in culture medium enriched with 25 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) for 1 hr. At the indicated times, the cells were scraped off. Cell suspension was centrifuged at $1000 \times g$ for 5 min at 4°C. After the cells were washed in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 1.8 mM $CaCl_2$, 5 mM glucose), they were resuspended in 0.25 ml HEPES buffer. The cells were sonicated for 30 sec at 4°C. The formation of dichlorofluorescin (DCF) was determined fluorometrically using a FL600 fluorescence spectrophotometer (Bio Tek, Winooski, VT, U.S.A.) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were calculated as fluorescence intensity/mg of protein, represented as % of control.

Western Blot Analysis —— SH-SY5Y cells (1×10^7) were incubated with 10 μM $CdCl_2$ and 20 μM C_6-ceramide. After 13 hr, the cells were scraped and lysed by sonication in buffer A (25 mM Tris, pH 7.4, 1 mM EDTA, 10 mM mercaptoethanol, and protease inhibitor cocktail). The samples were mixed with an aliquot of Laemmli's buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10 mM mercaptoethanol, and 0.002% bromophenol blue). After boiling for 10 min, the samples were cooled at room temperature. The samples were separated in 15% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked for 2 hr at room temperature in 5% non-fat milk in tris buffered saline (TBS) (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 2.7 mM KCl). After blocking, the membrane was incubated for 6 hr with the primary antibody at 4°C. After three washes with TBS/0.1% Tween 20, membranes were incubated with alkaline phosphatase-conjugated secondary antibody at 1 : 2000 dilution for 2 hr. Detection was performed by a pre-formulated substrate kit for use with NBT/BCIP (1-Step™ NBT/BCIP, Pierce, Rockford, IL, U.S.A.).

Statistical Analysis —— The data are presented as means ± S.D. Statistical significance was assessed with one-way analysis of variance (ANOVA) and in all cases the criterion for significance was $p < 0.05$.

RESULTS

Cd-induced Dose- and Time-dependent Ceramide Production

We examined the effect of Cd on the production of endogenous ceramide in SH-SY5Y neuroblastoma cells. After labeling with [^{3}H]palmitic acid for 12 hr, cells were treated with 20 μM $CdCl_2$ for the indicated times. As shown in Fig. 1, Cd increased ceramide production in a time- (Fig. 1A) and dose-dependent (Fig. 1B) manner; 1.6- and 3.0-fold by 10 and 20 μM Cd, respectively, and 2.2-fold at 10 hr.

Cd- and Ceramide-induced Cell Death

It was previously reported that Cd- or ceramide-induced DNA fragmentation, a biochemical characteristic of apoptotic cells.[11,13] Cd- and C_6-ceramide induced cell death in SH-SY5Y cells as determined by MTT assay. Cd- (Fig. 2A) and C_6-ceramide-induced (Fig. 2B) cell death increased in a dose-dependent manner. To characterize the cell death induced by Cd- or C_6-ceramide, we examined the nuclear morphology of the cells using the fluorescent DNA-binding agent, DAPI. As shown in Fig. 3A, Cd- and C_6-ceramide-treated cells clearly exhibited condensed fragmented nuclei, an indication of apoptotic cell death. To fur-

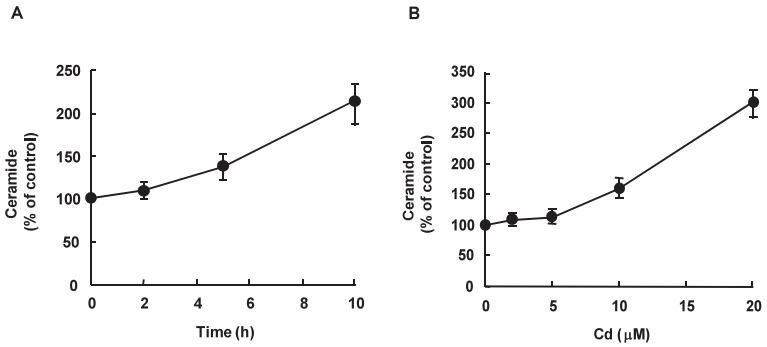

Fig. 1. Cd-increased Ceramide Production in a Dose- and Time-dependent Manner in SH-SY5Y Cells

SH-SY5Y neuroblastoma cells (2×10^5) were labeled with [^{3}H]palmitic acid (1 μCi/ml) in DMEM/F12 containing 2% FBS medium for 12 hr and treated with 20 μM $CdCl_2$ for the indicated times (A) and various concentrations for 13 hr (B). Lipids were extracted and resolved by thin layer chromatography (TLC). Lipid spots were scraped for the measurement of radioactivity by β-liquid scintillation counting. Data are means ± S.D. from three independent experiments. Each value represents the percent of each [^{3}H]-labeled lipid relative to the control at each time point.

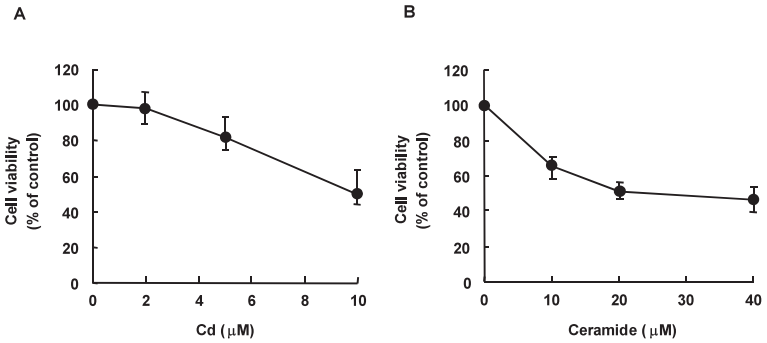

Fig. 2. Cd- and C_6-ceramide Induced Cell Death

SH-SY5Y neuroblastoma cells (5×10^5) were treated with the indicated concentrations of $CdCl_2$ (A) and C_6-ceramide (B) for 13 hr. Cell viability was determined by MTT assay as described in the MATERIALS AND METHODS. The data represent mean ± S.D. from three independent experiments.

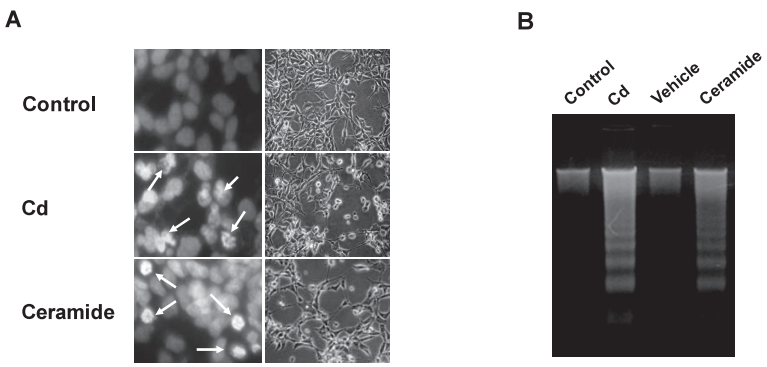

Fig. 3. Cd- and C_6-ceramide Induced Apoptosis

(A) SH-SY5Y neuroblastoma cells (5×10^5) were treated with 10 μM $CdCl_2$ or 20 μM C_6-ceramide for 13 hr and then stained with DAPI. Apoptotic cells were identified by observation with fluorescence microscope. (B) SH-SY5Y neuroblastoma cells were treated with 10 μM $CdCl_2$ or 20 μM C_6-ceramide for 13 hr. DNA was prepared as described in MATERIALS AND METHODS, and DNA fragments were analyzed by 1.8% agarose gel electrophoresis. The data is representative of three independent experiments.

ther confirm and evaluate the induction of apoptosis, we studied DNA fragmentation. Cells treated with Cd and ceramide showed considerable DNA fragmentation (Fig. 3B).

Analysis and Identification of Proteome

To identify proteins that show enhanced expression on exposure to Cd or those that mediate Cd-induced cell death, proteomic analysis was per-

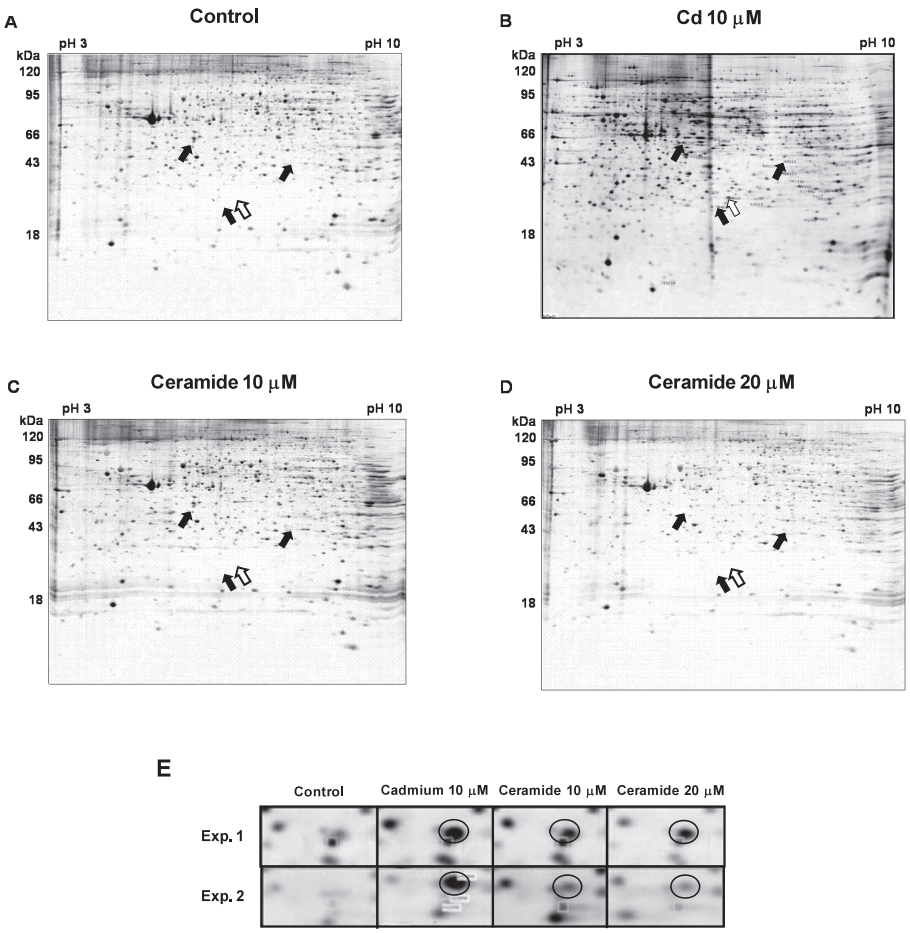

Fig. 4. Comparative Analysis of 2-DE from Cd- and Ceramide-treated SH-SY5Y Cells

(A–D) Cd- and ceramide-treated SH-SY5Y neuroblastoma cells were examined by 2-DE. Proteins (200 μg) were focused in the first dimension. Second dimension was performed using a 10–16% SDS-PAGE. Gels were detected by silver staining. A protein level in Cd- and ceramide-treated cells was compared to that in control cells. Simultaneously expressed proteins in Cd- and C_6-ceramide-treated cells are indicated with black arrows and Prx-3 were indicated with white arrows. (E) The spots were identified from the database and were subjected to MALDI-TOF analysis. The elevated protein was confirmed as Prx-3. The data is representative of three independent experiments.

formed. Whole cell lysates obtained from cells treated with Cd- or C_6-ceramide for 13 hr were analyzed by 2-DE. As shown in Fig. 4, proteins were resolved. The fifteen spots were differentially expressed between control and treated samples. Among them, we found four spots that simultaneously showed increased expression in Cd- and C_6-ceramide-treated cells. We suggested that these spots may be candidate proteins and analyzed the indicated spots by MALDI-TOF analysis. The four proteins were identified as B-TFIID transcription factor-associated, 170kDa (Mot1 homolog, S. cerevisiae) (BTAF1) RNA polymerase 2, ferritin light chain, Prx-3, phosphatidylinositol transfer protein. Among them, Prx-3 was initially selected for further characterization. Previous reports have demonstrated that both Cd and ceramide induce apoptosis via ROS production. On reacting with H_2O_2, the redox-sensitive Cys residue of Prx-3 is oxidized to Cys-SOH.[30] In the present study, we detected a protein spot with an apparent molecular mass of 32 kDa and a isoelectric point (pI) of 5.9, which was speculated to be oxidized Prx-3 because its pI value was lower than the theoretical pI of Prx-3. As shown in Fig. 4E, enlarged 2-DE images shown that Cd- and C_6-ceramide significantly enhanced Prx-3 expression.

Cd- and C_6-ceramide Induced ROS Production

As Prx-3 is known to be a mitochondrial antioxidant protein, we measured the production of ROS in Cd- and C_6-ceramide-treated cells. Figure 5 shows the changes in DCF fluorescence intensity in Cd- and C_6-ceramide-treated cells. Treatment with

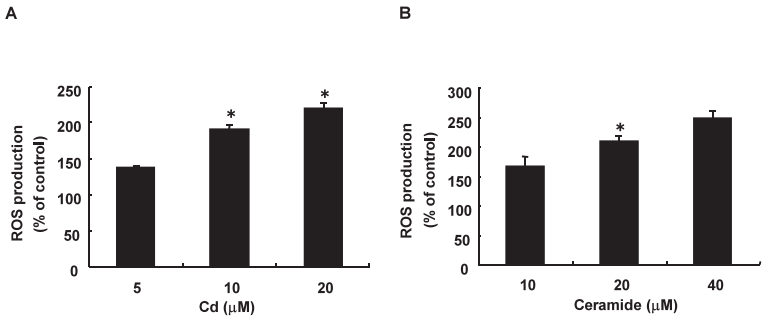

Fig. 5. Cd- and C_6-ceramide Induced ROS Production in SH-SY5Y Cells

SH-SY5Y neuroblastoma cells (5×10^5) were pretreated with 25 μM DCFH-DA for 1 hr, then Cd (A) and ceramide (B) were added to the culture medium for 13 hr. The formation of DCF was determined fluorometrically using a microplate fluorescence reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were represented as fluorescence intensity/mg of protein. The data represent mean ± S.D. from three independent experiments. $^*p < 0.05$.

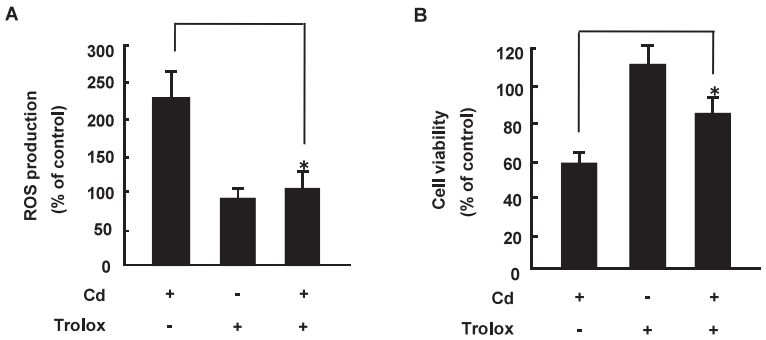

Fig. 6. Trolox Prevented Cd-induced ROS Production and Cell Death in SH-SY5Y Cells

SH-SY5Y neuroblastoma cells (5×10^5) were pretreated with trolox (300 μM) for 1 hr in serum-deprived medium. After treatment with 25 μM DCFH-DA for 1 hr, then 10 μM $CdCl_2$ was added to the culture medium for 13 hr. The formation of DCF was determined using a fluorescence reader. Results are represented as fluorescence intensity/mg of protein (A). And, cell viability was measured by MTT assay (B). The data represent mean ± S.D. from three independent experiments. $^*p < 0.05$.

10 μM Cd and 20 μM C_6-ceramide enhanced ROS production by approximately 2-fold. Pretreatment with trolox, an antioxidant, completely inhibited the Cd-induced ROS production (Fig. 6A), whereas trolox partially inhibited cell viability (Fig. 6B).

Immunological Analysis of Prx-3

First, to immunologically detect Prx-3 protein, the proteome of the lysates from Cd-treated and control cells were resolved in a 2-DE gel. As shown in Fig. 7A–B, the spot of Prx-3 was detected as an acidic satellite spot in Cd-treated cells, and not in control cells. This spot is supposed to be the oxidized form of Prx-3; termed Prx-3-SO_3, because the pI is 5.9 and much lower than the pI of Prx-3 which is 7.2. Thus, we further confirmed that the detected Prx-3 is an oxidized form via immunoblot analysis using anti-Prx-3-SO_3 antibody. Fig. 7C shows that the antibody reacted with the oxidized form of Prx-3 only in Cd- and C_6-ceramide-treated cells.

Cd Exposure Increased Prx-3-SO_3

It is known that three (Cd, lead, mercury) heavy metals induced apoptosis in various cells through a common mechanism. Similar to Cd, lead and mercury are known to induce apoptosis by the increase in Ca^{2+} or ROS and thus damaged neuronal cells.[31] To determine whether the up-regulation of Prx-3-SO_3 is specific to Cd among heavy metals, the cells were exposed to mercury, lead, and Cd at the indicated concentrations for 13 hr. These metals induced LDH release in a dose-dependent manner although different in sensitivity to the amount (Fig. 8A–C). To detect oxidized Prx-3, cells were exposed to 10 μM Cd, 4 μM mercury, and 100 μM lead for 13 hr and cell death was observed. The cells were lysed and subjected to immunoblot analysis using anti-Prx-3-SO_3 antibody. As shown in Fig. 8D, levels of the oxidized form of Prx-3 were found to be increased only in cells treated with Cd, and not those treated with mercury or lead.

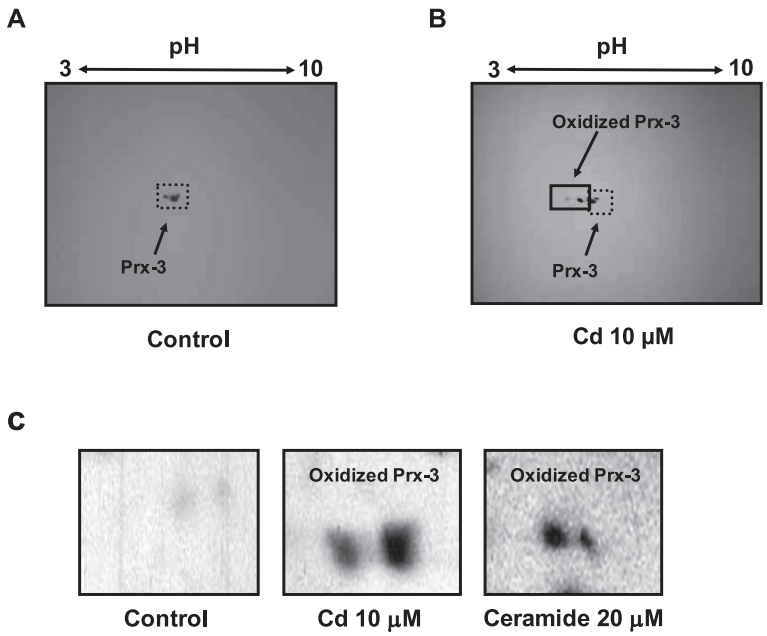

Fig. 7. Cd-increased Oxidized Prx-3 Levels in SH-SY5Y Cells

(A–B) SH-SY5Y neuroblastoma cells were exposed to 10 μM $CdCl_2$ for 13 hr. SH-SY5Y cells were disrupted and cell lysates examined by 2-DE. The gels were transferred to a nitrocellulose membrane. The blot was probed with a mouse monoclonal Prx-3 antibody and detected using NBT/BCIP kit. (A, control cells; B, Cd-treated cells). (C) SH-SY5Y neuroblastoma cells were exposed to $CdCl_2$ and ceramide for 13 hr. SH-SY5Y cells were disrupted and examined by 2-DE. The gel was transferred to a nitrocellulose membrane. The blot was probed with a rabbit polyclonal Prx-3-SO_3 antibody and detected using NBT/BCIP kit. The data are representative of three independent experiments.

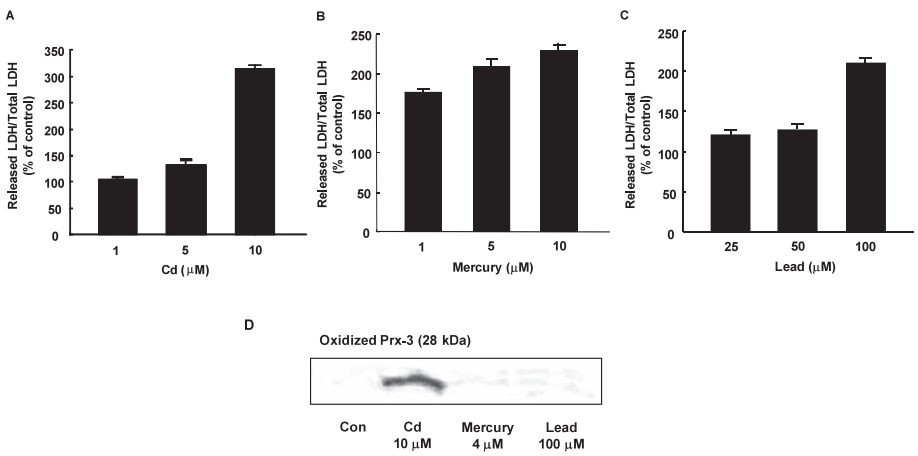

Fig. 8. Cd, Mercury, and Lead Induced cell Death and Expression of Oxidized Prx-3 in SH-SY5Y Cell

(A–C) SH-SY5Y neuroblastoma cells were treated with indicated concentrations of Cd, mercury, and lead for 13 hr. Cytotoxicity was determined by LDH assay as described in the MATERIAL AND METHOD. The percentage LDH release is calculated by dividing the level of LDH in the medium by the total amount of LDH in the medium and cell lysate. LDH activity was expressed as percent of control. The data represent mean ± S.D. from three independent experiments. (D) SH-SY5Y neuroblastoma cells were exposed to Cd, mercury, and lead for 13 hr. Cell lysate were used for western blotting. Each sample was separated using a 15% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was probed with a rabbit polyclonal Prx-3-SO_3 antibody and detected using NBT/BCIP kit. The data are representative of three independent experiments.

Immunological Analysis of Trx-2

Prx-3 is known to contain a mitochondrial localization sequence and is found exclusively in mitochondria. It uses mitochondrial Trx-2 as an electron donor for its peroxidase activity.[32] We examined whether Trx-2 is associated with oxidized Prx-3 upregulation in Cd-treated cells. Immunoblot analysis revealed that the protein level of Trx-2 was decreased in Cd-treated cells (Fig. 9).

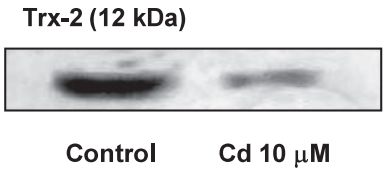

Fig. 9. Cd Decreased Trx-2 in SH-SY5Y Cells

SH-SY5Y neuroblastoma cells were exposed to 10 μM $CdCl_2$ for 13 hr. Cell lysates were used for western blotting. Each sample was separated using a 15% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was probed with a rabbit polyclonal Trx-2 antibody and detected using NBT/BCIP kit. The data are representative of four independent experiments.

DISCUSSION

Cd is an important polluting agent that is widely used in industry. It is known that both the acute or chronic exposure to Cd is associated with damage at different levels.[33–35] Cd exerts multiple toxic effects including neurotoxicity, nephrotoxicity, hepatotoxicity, and immunotoxicity. The toxicity of Cd is thought to be due to induction of apoptosis.[36] Many reports have discussed the mechanism of Cd-induced apoptosis. In various cell types, Cd interferes with antioxidant defense systems and activates the production of ROS.

Lipid-derived pro-apoptotic ceramide has emerged as an important intracellular signaling molecule that mediates diverse cellular effects; of which, apoptosis has attracted significant interest.[37, 38] Many stress stimuli result in the elevation of ceramide levels and apoptotic cells. Although the biochemical mechanism by which ceramide triggers apoptosis is not fully understood, there are considerable lines of evidence suggesting that it is the key mediator of this response.

Our results demonstrate that Cd induces apoptosis with a significant effect at 10 μM after 13 hr of incubation (Figs. 2, 3). We employed the proteomics approach to identify proteins involved in the cellular adaptive response to Cd toxicity. To examine the change in protein expression level after exposure to Cd and a cell-permeable ceramide, proteome obtained from Cd- and C_6-ceramide-treated cells was analyzed by 2-DE. The expression levels of several proteins in Cd- and C_6-ceramide-treated cells were different from those in untreated cells. In many reports, Cd exposure stimulated the expression of various genes that encode defense and repair proteins, oncogenes, and signal transduction proteins such as heat shock protein, c-fos, c-myc, c-jun, and metallothionein.[39–41] Comparative analysis of proteome profiles between the normal culture and Cd-exposed cells, allowed the identification of four proteins whose levels were altered in the Cd- and C_6-ceramide-treated cells. One of the identified proteins is Prx-3.

Prx-3 is an antioxidant enzyme in mitochondria that could be a neuroprotective protein—lower Prx-3 expression level in the hippocampus might be correlated with higher sensitivity of hippocampal neurons to excitotoxic damage.[42–45] Prx-3 is a critical regulator of mitochondrial H_2O_2, which itself promotes apoptosis in cooperation with other mediators of apoptotic signaling. Our results show that Cd- and C_6-ceramide increase the level of oxidized Prx-3 (Fig. 7), suggesting that Cd- and C_6-ceramide may induce apoptosis by lowering the capacity of the reducing system of cells. On the other hand, mercury and lead did not increase the level of oxidized Prx-3 (Fig. 8), suggesting that Cd may up-regulate Prx-3-SO_3, but not Prx-3, through the generation of ceramide. We have demonstrated the protective function of Prx-3 and thus propose that mitochondrial protection through scavenging of mitochondrial ROS might represent a strategy for clinically useful neuroprotective therapy.

Prx-3, which contains a mitochondrial localization sequence, is found exclusively in the mitochondrion, and uses mitochondrial Trx-2 as the electron donor for its peroxidase activity.[32] Interestingly, our results showed that the exposure to Cd decreased the protein level of Trx-2 (Fig. 9). Specific sub-mitochondrial localization to the inner mitochondrial membrane and high resistance to oxidation suggest that Trx-2 provides a primary line of defense against ROS, which is generated during mitochondrial respiratory chain functioning.[46] In addition, Trx-2 has documented anti-apoptotic action, in particular, based on the prevention of cytochrome c release from the mitochondria.

In summary, ceramide, a pro-apoptotic mediator capable of generating ROS, was produced by the exposure of human neuroblastoma cells to Cd. Proteomic analysis showed that the level of oxidized Prx-3 was increased by Cd- and C_6-ceramide, whereas Trx-2 level was decreased by Cd. This effect may be specific to Cd among three (Cd, lead, mercury) heavy metals. Together, our results suggest that the Cd-induced production of ceramide may cause apoptosis and increase the cellular level of ROS through the up-regulation of Prx-3-SO_3 and the down-regulation of Trx-2.

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