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Effects of Cadmium on Structure and Enzymatic Activity of Cu,Zn-SOD and Oxidative Status in Neural Cells

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Abstract Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder disease. Ten percent of the ALS patients are congenital (familial ALS), and the other 90% are sporadic ALS (SALS). It has been shown that mutations found in the Cu,Zn-SOD cause 20% of the familial ALS due to its low enzyme activity. We hypothesized that heavy metals may interfere the structure of Cu,Zn-SOD protein to suppress its activity in some of the SALS. In this study, we expressed and characterized the recombinant human Cu,Zn-SOD under various concentrations of Cu^{2+}, Zn^{2+}, and Cd^{2+}. By atomic absorption spectrophotometry, we demonstrated that adding of cadmium significantly increased the content of cadmium ion, but reduced its Zn^{2+} content and enzyme activity of the Cu,Zn-SOD protein. The data of circular dichroism spectra demonstrated that the secondary structure of Cu,Zn-SOD/Cd is different from Cu,Zn-SOD, but close to apo-SOD. In addition to the effect of cadmium on Cu,Zn-SOD, cadmium was also shown to induce neural cell apoptosis. To further investigate the mechanism of neural cell apoptosis induced by cadmium, we used proteomics to analyze the altered protein expressions in neural cells treated with cadmium. The altered proteins include cellular structural proteins, stress-related and chaperone proteins, proteins involved in reactive oxygen species (ROS), enzyme proteins, and proteins that mediated cell death and survival signaling. Taken together, in this paper, we demonstrate that cadmium decreases the content of Zn^{2+}, changes the conformation of Cu,Zn-SOD protein to decrease its enzyme activity, and causes oxidative stress-induced neural cell apoptosis. J. Cell. Biochem. 98: 577–589, 2006.

Key words: amylotrophic lateral sclerosis (ALS); Cu,Zn-SOD; cadmium; proteomics; oxidative stress; altered protein profile

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that leads to the selective loss of motor neurons. Ten percent of the ALS patients are congenital cases called familial ALS (FALS) which is caused by mutations of the Cu,Zn-superoxide dismutase (Cu,Zn-SOD) gene [Majoor-Krakauer et al., 2003]. The other 90% of ALS cases which have unknown causes are called sporadic ALS (SALS). Both FALS and SALS have similar clinical features and share some common pathological mechanism [Mulder et al., 1986; Piao et al., 2003].

Decreasing of Cu,Zn-SOD activity was reported to be correlated with ALS occurrence. There are two hypotheses which theorize the relationship between Cu,Zn-SOD and ALS. One is the misfolding of mutant protein, the other is the intracellular aggregation of Cu,Zn-SOD. The mutant protein may alter its catalytic mechanism and allow the production of oxidants such as peroxynitrite [Estevez et al., 1999] and hydrogen peroxide [Yim et al., 1996]. These reactive nitrogen and oxygen species accumulated in cells to result in damages to proteins, nucleic acids, and lipids, and finally caused cell toxicity. In support this hypothesis, the studies by de la Torre et al.

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[1996] and Ravikumar et al. [2000] revealed that the enzyme activities of superoxide dismutase, catalase and other enzymes which involved in free radical scavenging are decreased in patients with the neuropsychiatric disorder and Parkinson's disease. In addition, Cu,Zn-SOD inclusion bodies have also been shown to be a common pathological finding in motor neuron and neighboring astrocytes of ALS patients [Watanabe et al., 2001]. Together with these observations suggest that the misfolding and aggregation of Cu,Zn-SOD may result in cell cytotoxicity [Caughey and Lansbury, 2003].

Cu,Zn-SOD is a dimeric enzyme that dismutates superoxide to molecular oxygen and hydrogen speroxide. The dismutation process of Cu,Zn-SOD protein is in a two-step catalytic cycle, which involves the alternate reduction and oxidation of an essential copper in the active site. A structural zinc atom is closely coordinated with the copper through a shared bridging histidine and limits the copper's redox activity in Cu,Zn-SOD with endogenous antioxidants [Hart et al., 1999]. The wild-type SOD mostly folds into a soluble form protein, and it would be readily treated with copper to yield fully active SOD. The zinc-deficient SOD could activate apoptosis of motor neurons by promoting the formation of peroxynitrite [Yim et al., 1996; Estevez et al., 1999].

Cadmium and zinc constitute group IIB of the periodic table and share certain similar biological responses. Cadmium is known as a potent toxic metal in industrial and food contamination, and it is harmful to human because it accumulates in liver, kidney, and other tissues. It has been reported that cadmium exposure causes ALS by reducing the superoxide dismutase activity [Bar-sela et al., 2001]. However, the mechanism of cadmium decrease the activity of Cu,Zn-SOD protein is not clear. We hypothesize that cadmium may cause the misfolding of Cu,Zn-SOD protein to alter its catalytic mechanism. In this study, we constructed, expressed, and purified human Cu,Zn-SOD in *E. coli* culture system to investigate role of cadmium on Cu,Zn SOD protein activity. Our results demonstrated that cadmium decreases the Cu,Zn-SOD activity by changing the protein conformations. Furthermore, we demonstrate that cadmium treatment strongly induces the expression of metallothionein and decreased the Zn^{2+} content in the Cu,Zn-SOD protein.

Cadmium also induces different biochemical changes, which are typically associated with apoptosis [Eneman et al., 2000]. Apoptosis is particularly important in the development of the central nervous system, where neurons compete for a limited amount of survival factors nine neurotropic factors. If the embryos are exposed to cadmium before neurulation, the most dramatic malformation, an opening in the anterior neural pore (exencephaly) is observed. If the exposure occurs after the closure of the neural tube, there is a shift to rib and upper limb defects [Ferm, 1971; Nakashima et al., 1988]. In this study, we also demonstrate that cadmium induces apoptosis in the neural N2A cells. We further applied a two-dimensional electrophoresis-based proteomic approach to study cellular protein alterations associated with cadmium-induced apoptosis. Proteomic studies indicate that cadmium-induced cellular oxidation is related to its cytotoxicity. Decreasing SOD activity in cadmium-treated cells could also be caused by reactive oxygen species (ROS)-induced protein conformational change.

MATERIALS AND METHODS

Materials and Reagents

A human Cu,Zn-SOD cDNA fragment was isolated using the polymerase chain reaction (PCR) technique from the human liver cDNA library and a polyclonal antibody raised against human Cu,Zn-SOD, which was produced in our laboratory. RNAzol reagent was purchased from Tel-Test, Inc., Superscript II RT, oligonucleotides were purchased from Invitrogen Life technologies Co. (Groningen, The Netherlands), and HiFi-DNA polymerase was purchased from Yeastern Biotech Co., Ltd. (Taiwan). Restriction endonucleases, T4 DNA ligase, RQ1 RNase-Free DnaseI, Rnasin, pGEM-T easy vector, and Isopropyl-L-D-thiogalactoside (IPTG) were purchased from Promega Co. (Madison, WI). Pfu polymerase and *Escherichia coli* strain JM109 (DE3) were obtained from Stratagene (La Jolla, CA). Plasmid pQE30 and Ni^{2+}-nitrilotriacetic acid Sepharose superflow were purchased from Qiagen (Valencia, CA). Anti-metallothionein antibody was generated against a polymer of rat liver MT-II. Horseradish peroxidase (HRP) conjugated anti-rabbit IgG was purchased from Jackson ImmunoResearch Lab (West Grove, Pennsylvania). Nickel bead column and chemiluminescence detection ECL plus

were from Amersham Pharmacia Biotech. (Buckinghamshire, UK). All chemicals were of reagent grade.

Expression of Cu,Zn-SOD Protein in the *E. coli* System

Total RNA was isolated from human liver using the RNAzol reagent in accordance with the instructions from the manufacture. The conversion of cDNA from mRNA was performed as described previously [Chou et al., 2005].

The full-length cDNA covering the complete open reading frame (ORF) of human Cu, Zn-SOD was amplified with a Cu,Zn-SOD sense primer, Cu,Zn-SOD-F: 5′-ATG GCG ACG AAG GCC GTG TGC GTG CTG-3′, and a Cu,Zn-SOD anti-sense primer Cu,Zn-SOD-R: 5′-TTA TTG GGC GAT CCC AAT TAC ACC ACA-3′. PCR amplification was performed in a 50 μl reaction mixture containing 2 μl first strand cDNA, 0.5 μg of primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 units of HiFi-DNA polymerase. The PCR amplification program is 94°C for 3 min; 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and the final extension at 72°C for 15 min. The PCR products were ligated into pGEM-T easy vector and subjected to sequence analysis.

The expression plasmid, pQE-SOD1, was constructed by inserting the full-length human Cu,Zn-SOD cDNA into pQE30 at the *Sph*I and *Pst*I sites, which allows the generation of the Cu,Zn-SOD protein with in-framed His-tag at the N-terminal. The Cu,Zn-SOD protein was expressed in *E. coli* JM109 induced by 1 mM IPTG (isopropyl-s-D-thiogalactopyranoside) at 37°C for 4 h. We harvested all the cells for purification of Cu,Zn-SOD protein. In briefly, cells were resuspended in PBS and sonicated three times at 10-s intervals on an ice bath to disrupt the cells. We harvested the supernatant after centrifugation at 13,000 rpm for 5 min, and then collected the suspension to interact with Ni^{2+}-nitrilotriacetic acid Sepharose metal affinity resins at 4°C overnight with mild agitation. The resins were centrifuged and washed twice with 10 mM Tris-HCl buffer containing 1 M NaCl and then eluted with 50–200 mM imidazole solution according to the manufacturer's instructions.

Cu,Zn-SOD Activity

SOD activity was measured by the inhibition of pyrogallol auto-oxidation [Marklund and Marklund, 1974]. The purified apo-SOD, Cu,Zn-SOD, or Cu,Zn-SOD with 500 nM cadmium protein (Cu,Zn-SOD/Cd) was dissolved in an assay buffer containing 50 mmol/L Tris (pH 8.2) and 1 mmol/L diethyltriamine pentoacetic acid (DTPA). Protein concentration in the supernatant was determined by Bradford assay. Pyrogallol (Sigma, MO) auto-oxidation was measured as the rate of change of the absorbance at 420 nm over 3 min of 200 μmol/L pyrogallol, diluted from 0.5 M HCl stock into assay buffer. SOD activity was expressed as units per milligram (U/mg) of protein.

Atomic Absorption Spectrophotometer Analysis

The Cu,Zn-SOD protein was expressed under treatment of 0.2 mM equal amount of Cu^{2+} and Zn^{2+} and increasing concentrations of cadmium (0–500 nM) in *E. coli*. The expressed protein was then purified, dissolved in TBS buffer at pH 7.4, and the concentration of metal ion (Cd^{2+}, Zn^{2+}, and Cu^{2+}) in protein was determined by atomic absorption analysis (Graphite Furnace Atomic Absorption Spectrophotometer, GFAAS, Z-5000, Hitach). The wavelength was 324.8 nm for Cu^{2+}, 213.9 nm for Zn^{2+}, and 228.8 nm for Cd^{2+}.

Measurement of CD Spectra

The concentration of expressed SOD, Cu, Zn-SOD, or Cu,Zn-SOD/Cd for CD analysis was determined by Bradford assay and dissolved in potassium phosphate buffer at pH 7.4 in a concentration of 2.5 μg/μl. The CD spectra were measured by a Jasco J-715 spectropolarimeter and the SELCON 3 software was used to analyze the data. The mean residue ellipticity was estimated from the mean residue weight, which was calculated from the primary structure.

Treatment of N2A Neuroblastoma Cells With Cadmium and Analysis of Cell Cycle by Flow Cytometry

Mouse N2A neuroblastoma cells (obtained from ATCC, Manassas, VA) were cultivated for 24 h in serum-free medium and then either left untreated or treated with 10 μM of cadmium chloride for 24 h. At the end of experiment, cells were harvested, resuspended in PBS solution, stained by propidium iodide, and cell-cycle

distribution was analyzed with a FACStar Plus flow cytometer as described [Lau et al., 2004]. For each sample, 1×10^6 cells were analyzed, providing a solid statistical basis for the determination of the percentage of cells in each cell-cycle phase using the Lysis II software program.

2D-Gel Electrophoresis, Image Analysis and MS Ppeptide Fingerprinting

2D-gel electrophoresis (2DE) was carried out with Amersham Biosystems Ettan™ IPGphor™ Isoelectric Focusing System and Ettan™ Dalt*six* Tank (18 cm) units, in accordance with a protocol previously described [He et al., 2003]. Protein samples (150 μg) were extracted from N2A cells treated with 10 μM $CdCl_2$, and untreated cells were used as a control. Triplicate electrophoreses were performed to ensure reproducibility. All gels were visualized by silver staining [He et al., 2003]. Image acquisition and analysis were performed with an Image Scanner (Amersham) and Image Master 2D Elite software (Amersham). Comparisons were made between gel images of protein profiles obtained from cells treated with cadmium and images from untreated cells. Altered protein spots that changed consistently and significantly (>twofold difference) were selected for analysis with MALDI-TOF mass spectrometry. These protein spots were cut off in small pieces and subjected to in-gel trypsin digestion overnight. Peptide mass spectra were recorded and previously described parameters for spectral acquisition were used [He et al., 2003]. Protein identification with 25 ppm or lower mass error and MOWSE scores over 300 were obtained in most analyses during database matching using MS-Fit (http://prospector.ucsf.edu/). Duplicate or triplicate runs were made to ensure the accuracy of analyses.

SDS–PAGE and Western Blot Analysis

The cells were lysed and protein extraction was performed. SDS–PAGE and Western blot analysis were carried out as described previously [Lau et al., 2004]. The samples were separated on 10 % SDS gel and electrophoretically transferred to PVDF membrane (Amersham). The membranes were blotted with 5% skim milk, washed, and probed with primary antibodies against SOD, VDAC1, peroxiredoxin 1, thioredoxin, HSPgp96, PDI, and β-actin, respectively. After washing, the membranes were incubated with corresponding secondary antibodies and visualized by enhanced chemiluminescence (ECL) in accordance with the manufacturer's recommendations (Amersham).

Statistical Analysis

All experiments were repeated at least three times. The data are expressed as mean ± SD. Difference in means was assessed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparisons test.

RESULTS

Expression and Characterization of Cu,Zn-SOD Protein in *E coli* System

To identify the relationship between the structure and enzyme activity of Cu,Zn-SOD protein, we constructed the gene of human Cu,Zn-SOD in pQE20 and expressed the protein in *E. coli* culture system. *E. coli* was treated with equal amounts of Cu^{2+} and Zn^{2+} (0–2 mM) and the recombinant Cu, Zn-SOD protein was expressed by IPTG (1 mM) induction. As shown in Figure 1A, the expressed Cu,Zn-SOD protein showed the best enzyme activity at 0.5 mM Cu^{2+} and Zn^{2+} concentration. The human Cu,Zn-SOD protein is a major protein expressed in *E. coli* under such a culture condition, and it was identified as a 23 kDa monomer and few dimmer protein bands by SDS–PAGE and Western blot analysis (Fig. 1B). The expression of Cu,Zn-SOD protein was not affected at the cadmium concentration ≤500 nM (Fig. 1B, lanes 2–6 and lanes 2′–6′). The growth rate of *E. coli* cells was significantly suppressed by cadmium at the concentration >500 nM (Fig. 1C). The enzyme activities of expressed Cu,Zn-SOD protein affected by cadmium were determined by pyrogallol auto-oxidation analysis. The effect of cadmium on suppression of the enzyme activity of Cu,Zn-SOD protein was shown in a dose-dependent manner (Fig. 1D).

Effect of Cadmium on the Complexion Ability of SOD Protein With Zn^{2+} and Cu^{2+}

In order to identify how cadmium suppressed the enzyme activity of Cu,Zn-SOD protein, we tested the ability of cadmium to complete or replace the metal ion binding of the Cu,Zn-SOD protein. The Cu,Zn-SOD was purified by a Nickel-bead column, and was displayed as a

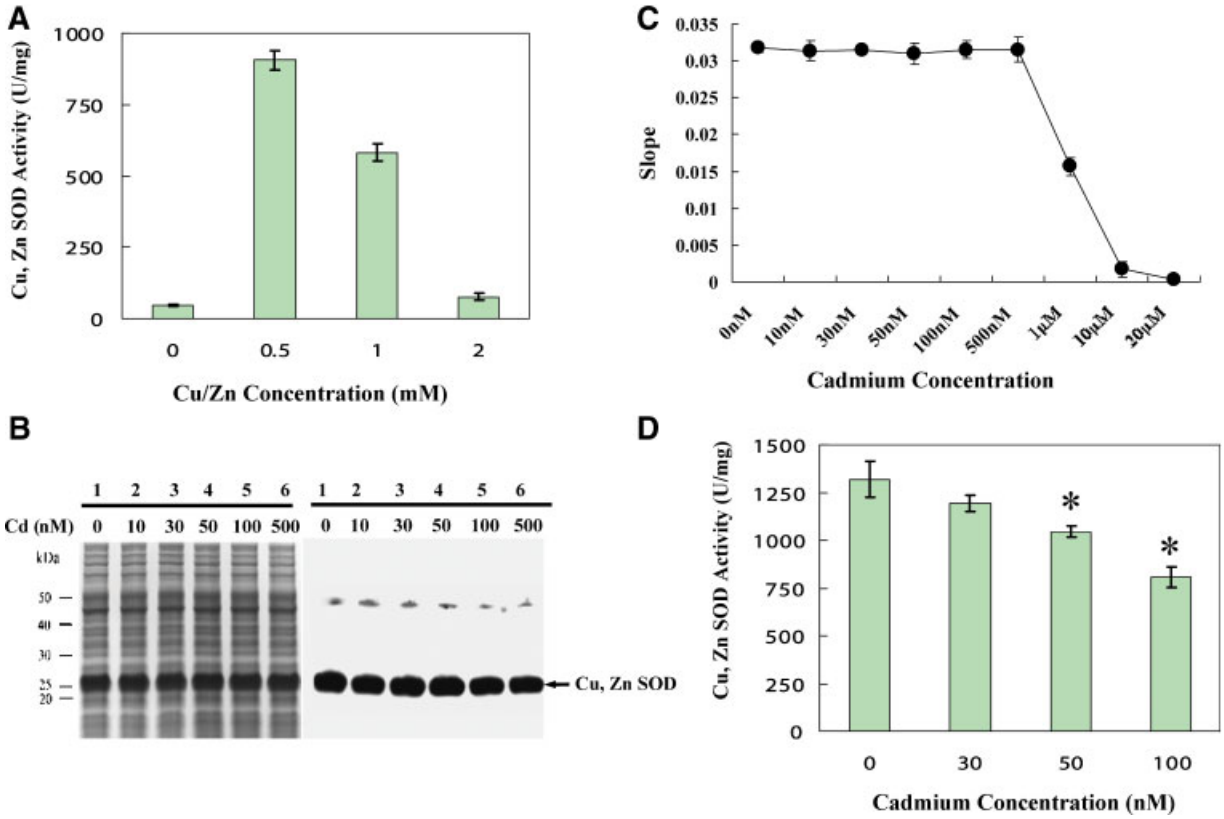


Fig. 1. Expression and characterization of Cu,Zn-SOD protein in *E coli* system. The effect of equal amount of Cu^{2+} and Zn^{2+} (0, 0.5, 1, and 2 mM) on SOD activity was shown in (**A**). The expression and identification of Cu,Zn-SOD in *E. coli* treated with 0-500 nM cadmium was shown in (**B**). Relative growth rate of *E coli* treated with increasing concentration of cadmium was detected by OD_{600} analysis (**C**). The effect of increasing concentration of cadmium on the enzyme activity of Cu, Zn-SOD protein was shown in (**D**). Shown in D is the average of three independent experiment assays and SD. $^*P < 0.05$ by one-way ANOVA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

single protein band on SDS–PAGE (Fig. 2A). The concentrations of metal ions (Cd^{2+}, Zn^{2+}, and Cu^{2+}) in the purified Cu,Zn-SOD proteins under various culture conditions were then determined by atomic absorption spectrophotometry. As shown in Figrue 2B and C, adding increasing concentrations of cadmium to the culture medium correspondingly increased the content of cadmium ions, but decreased the content of zinc ions in purified Cu,Zn-SOD protein. The Cd^{2+} content increased about fivefold (from 10 to 80 nmol/mg) (Fig. 2B), but the content of Zn^{2+} decreased greatly to 1/7 (from 7 to 1 μmol/mg) (Fig. 2C) when 500 nM cadmium was added. Copper ion content in Cu,Zn-SOD protein did not change under the same culture conditions (Fig. 2D).

Effect of Cadmium on the Conformation of the Cu,Zn-SOD Protein

The effect of cadmium on the secondary structure of the expressed-Cu,Zn-SOD protein was analyzed by circular dichroism (CD) spectroscopy (Fig. 3). Based on the CD spectra of protein conformation [Chen et al., 1974; Chang et al., 1978; Perczel et al., 1992], the expressed-SOD protein exhibits a minimum molecular ellipticity at 208–210 nm, which is a character of α-helical conformation. The protein is not completely unordered since it did not exhibit a strong negative band near 197 nm. Furthermore, the spectra of 217 nm bear some resemblance of the β-form conformation, suggesting that Cu,Zn-SOD protein contains some order structure besides the helix, which is likely a mixture of β-form, β-turn, and unordered form. The conformation of purified Cu,Zn-SOD is quite different from the apo-SOD protein (Fig. 3, dot-dash line vs. dotted line), this observation is in coincident with the difference of enzyme activities between these two proteins (Fig. 1D). This observation indicates the important role of Cu^{2+} and Zn^{2+} in the structure and function of SOD protein. Furthermore, the

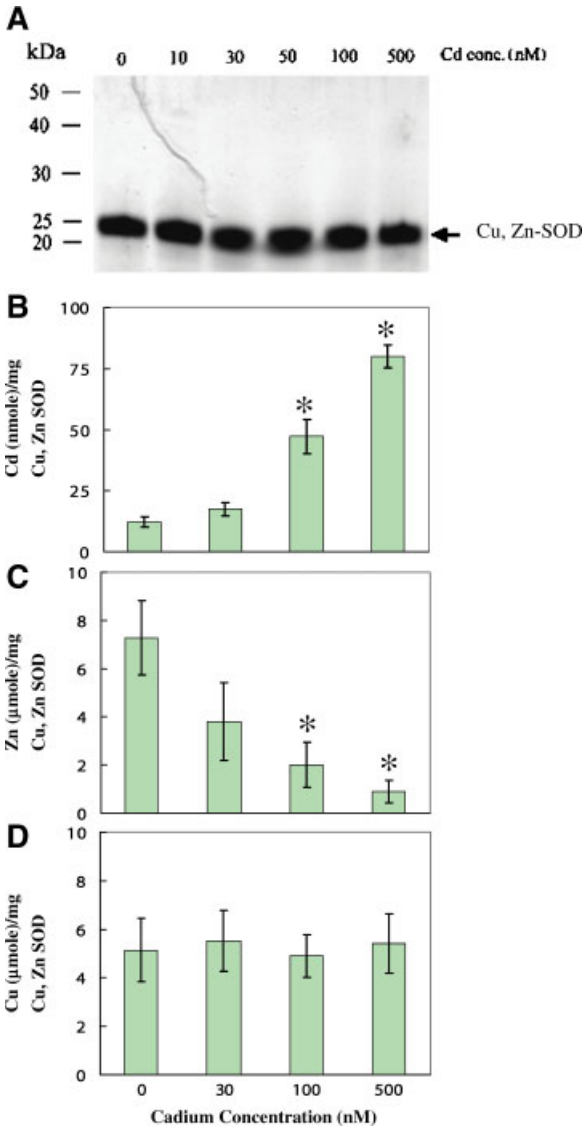


Fig. 2. Effect of cadmium on the ability of SOD to complex with metal ion. The Cu,Zn-SOD protein was expressed under treatment of equal amount of 0.2 mM Cu^{2+} and Zn^{2+} and/or increasing concentration of cadmium in *E. coli*. The purification and identification of the Cu,Zn-SOD protein under various treatments are shown in (**A**). The Cu,Zn-SOD protein under various treatments was collected, and dissolved in TBS buffer at pH 7.4. The concentration of each metal ion in Cu,Zn-SOD protein was then determined by atomic absorption analysis Cd^{2+} (**B**), Zn^{2+} (**C**), and Cu^{2+} (**D**). Shown in B, C, and D are the average of three independent experiment assays and SD. $^*P < 0.05$ by one-way ANOVA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

conformation of Cu,Zn-SOD/Cd is different from Cu,Zn-SOD with increasing α-helix structure and decreasing random coil structure (solid line vs. dot-dash line). Together with the previous results, these results support the fact that cadmium may cause the lost of zinc ions in the Cu,Zn-SOD protein to change the conformation and then suppress its enzyme activity.

Cadmium Induced the Expression of Metallothionein

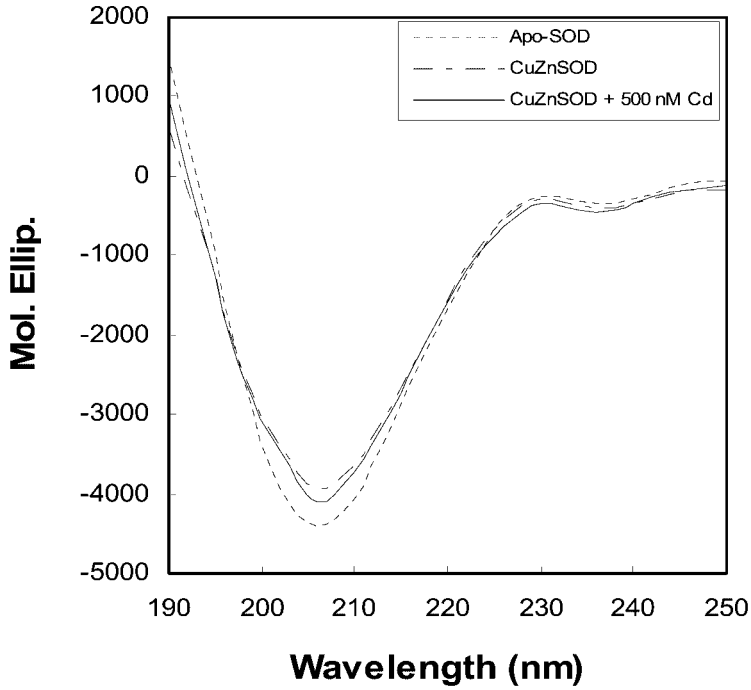
Cadmium is known as a strong inducer of metallothionein (MT) expression [Price-Haughey et al., 1986]. MT has been shown the high capacity to bind zinc ions in mammalian cells [Van Campenhout et al., 2004]. We demonstrated that cadmium significantly induce MT expression in *E. coli* by Western blot analysis (Fig. 4). Since the zinc ions are believed to stabilize the three-dimensional structure of SOD protein and to confer the enzyme activity of SOD, we suggest that high level of apo-MT induced by cadmium may sequester zinc from the Cu,Zn-SOD enzyme to decrease the SOD activity.

Cadmium Induces Apoptosis in Neural Cells

It has been reported that neural tube undergo apoptosis and degeneration in cadmium-treated embryos [Chan and Cheng, 2003]. In this study, the effects of cadmium on neural cell death were further investigated by flow cytometric analysis. The percentage of apoptotic cells in mouse N2A neuroblastoma cells after cadmium treatment was dramatically higher than that of the control cells (Fig. 5). These results confirmed our previous findings that cadmium induces cell apoptosis [Eneman et al., 2000; Shih et al., 2005].

Protein Alterations in the Cells Treated With Cadmium

To further investigate the mechanism of neural cell apoptosis induced by cadmium, protein profiles of control and cadmium-treated N2A cells were studied by comparative proteomic analysis. Representative 2-D gel images for control- and cadmium-treated N2A cells are shown in Figure 6A and B, respectively. Figure 6 shows image overviews for a typical master gel of a N2A cell treated with or without cadmium for 24 h. Over 1,000 protein spots were clearly separated on the gels. Circles highlight major areas where significant and consistent alterations of protein expression were identified. These altered proteins were distributed evenly throughout the entire gel, indicating that multiple clusters of proteins are involved in the process of cadmium-induced apoptosis.



Program SELCON3	α- helix	β- sheet	Turn	Random
SOD	11.6 %	54.2 %	19.8 %	14.7 %
Cu,Zn-SOD	6.2 %	49.1 %	20.8 %	22.9 %
Cu,Zn-SOD+Cd	7.9 %	49.4 %	21 %	21.1 %

Fig. 3. Effect of cadmium on the secondary conformation of Cu,Zn-SOD protein. The Cu,Zn-SOD protein was dissolved in 5 mM TBS buffer at pH 7.4 and showed as the dash line, apo-SOD protein was shown as the dotted line, and Cu,Zn-SOD/Cd was shown as the dot-dash line.

Table I shows protein ID obtained through peptide mass fingerprinting, together with proportionate differences of expression and major functions of the identified altered proteins through peptide fingerprinting matching. These proteins can be classified into several categories based on their major biological functions, including cellular structural proteins, stress-related and chaperone proteins, proteins involved in ROS, enzyme proteins, and translation factors, proteins that mediate cell death and survival signaling. Table II summarizes the parameters obtained in database matching for protein identification, including NCBI database access number, sequence coverage, and experimental mass and p*I*.

Table I illustrates the upregulation of stress-related chaperones and redox-related proteins, including heat shock protein gp96 precursor (HSP gp96), peroxiredoxin 1 and 6, and thioredoxin was also observed in the cells treated with cadmium. Table I also displays the signal transduction proteins altered in the cells treated with cadmium. The expressions of oncoprotein 18 (Op18) and cyclophilin A (Cyp A) were elevated by cadmium. Calcyclin binding protein (CacyBP), Rho GDP dissociation inhibitor alpha (GDI), and Ras-related nuclear protein were regulated in the same manner by cadmium. Ion-related signal transduction protein, such as voltage-dependent anion channel 1 (VDAC1), was upregulated under cadmium treatment.

Western blot analysis was used to confirm the altered expressions of proteins in response to the treatment with cadmium. Figure 7 shows the Western blot results of VDAC1,

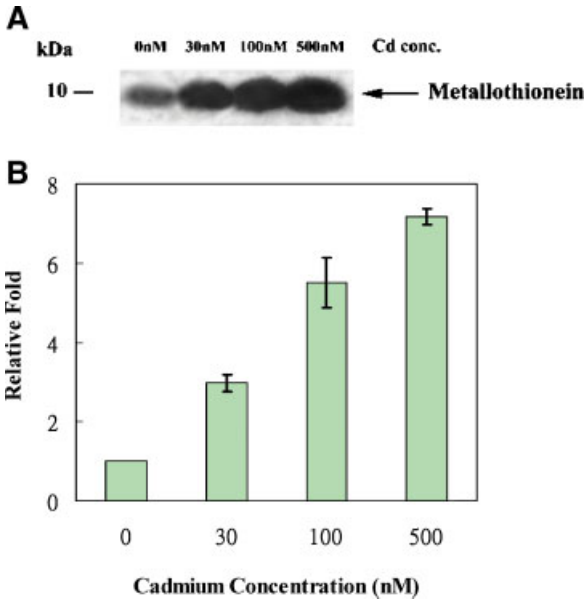


Fig. 4. Effect of cadmium on metallothionein protein expression in *E. coli*. The expression of metallothionein protein in *E coli* treated with increasing concentration of cadmium (0, 30, 100, and 500 nM) was detected by Western blot using rabbit anti-metallothionein polyclonal antibody (**A**). The relative fold of cadmium-induced metallothionein protein expression was analyzed and shown in (**B**). Shown in B are the average of three independent experiment assays and SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

peroxiredoxin 1, thioredoxin, HSPgp96, and PDI. The altered expression ratio of these proteins matched well with the ratio detected in 2DE.

DISCUSSION

Cadmium is a potent toxic metal which is very harmful to the environment and humans due to its tendency of in vivo accumulation in tissues. The toxicity of cadmium as an industrial pollutant, a food contaminant and in cigarettee smoke has been well established [Morselt, 1991]. Cadmium would accumulate in cells and be toxic in many organs, such as kidney, liver, lung, testis, brain, bone, blood system, etc [WHO, 1992]. However, the molecular mechanism responsible for the toxic effects of cadmium is far from being fully understood.

Superoxide dimutatse, such as Cu,Zn-SOD, is an important enzyme for scavenging-free radicals in cells. Without correct protein folding, this enzyme will lose its activity and be correlated with many diseases like ALS. Metal ions in Cu,Zn-SOD protein contribute the electrostatic stability to maintain their dimensional construction. Cu^{2+} is believed to localize at the bottom of a deep channel as the active site of Cu,Zn-SOD while the superoxide anion is electrostatically driven, and Zn^{2+} would move in close coordination with the Cu^{2+} through a share bridge histidine to contribute a catalytic structure [Tainer et al., 1983; Fisher et al., 1991]. Without the zinc involved in this structure, the molecules would not fold properly and performed actively.

In our results, we have demonstrated that Cu,Zn-SOD activity is strongly inhibited by cadmium. In supporting our observation, it has also been demonstrated that cadmium can replace Zn^{2+} to reduce superoxide dismutase activity [Bauer et al., 1980; Kofod et al., 1991]. Cadmium and zinc constitute group IIB of the periodic table and share certain similar biologi-

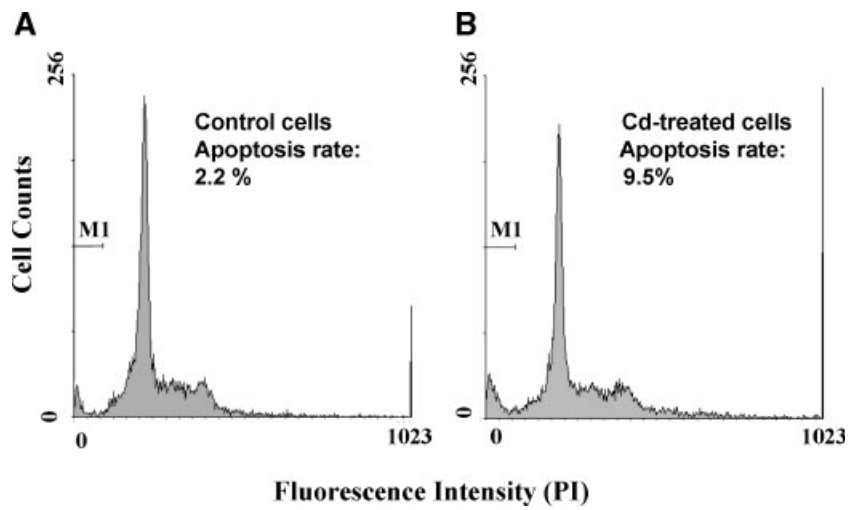


Fig. 5. Analysis of cadmium-induced neural N2A cell apoptosis by flow cytometry. N2A cells untreated (**A**) or treated (**B**) with 10 μM cadmium for 24 h were collected and the apoptosis rate of the cells were analyzed.

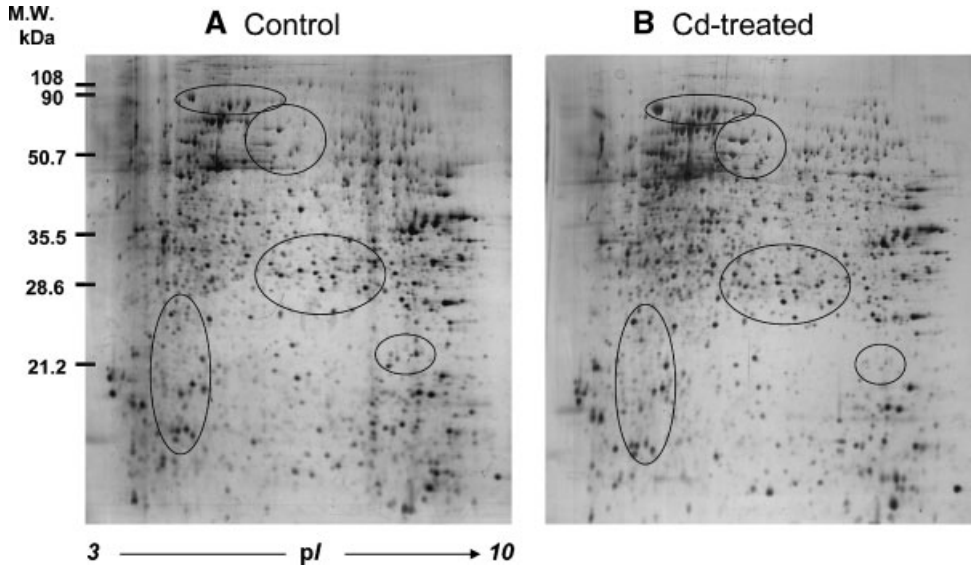


Fig. 6. Analysis of the cadmium-induced protein expression in neural N2A cell by two-dimensional electrophoresis. Representative of 2-D gel images for control (**A**) and cadmium-treated N2A cells (**B**) for 24 h. Areas where significant and consistent alterations of protein expression identified are circled.

cal responses, but cadmium has larger atomic radium than zinc. Under a higher cadmium concentration, cadmium, as the larger radium ions, may act as a steric hindrance to interfere with the incorporation of zinc to SOD protein. This happens in many enzymatic reactions. Alternatively, a biological mechanism could be turned on by adding cadmium to decrease the zinc level in the culture medium, which interfere the incorporation of zinc into protein molecule. In addition, studies by Casalino et al. [2002] showed that adding of Zn^{2+} did not restore Cu,Zn-SOD activity. This observation further supports our result of a disproportional increase of cadmium and decrease of Zn^{2+} content in expressed-Cu,Zn-SOD protein while Cd^{2+} was added in culture medium (Fig. 2B,C). Therefore, we suggest that Cd-induced the lost of Zn^{2+} maybe via a competitive inhibition mechanism or an alternative biological pathway in the protein instead of chemical substitution.

Several protective agents, including metallothionein and glutathione, play an important role in detoxification processes [Brugnera et al., 1994; Cherian, 1994; Heyes and McLellan, 1999]. Studies by Van Campenhout et al. [2004] reveal that cadmium could induce the synthesis of metallothionein. It has been demonstrated that metallothionein owns the Zn^{2+} binding capacity [Van Campenhout et al., 2004], thus the high expression of metallothionein could probably cause zinc deficiency temporarily. Since Zn^{2+} has been shown to stabilize

TABLE I. Protein Alterations in N2A Cell Under Treatment of 10 μM $CdCl_2$ for 24 h

Protein ID	Folds changed	Major functions
Heat shock protein gp96 precursor (HSPgp96)	+4.1	Stress-related, chaperone
Peroxiredoxin 1	+2.9	Chaperone, and redox activities
Peroxiredoxin 6	+2.7	Chaperone, and redox activities
Thioredoxin	+4.5	Redox activities
Protein disulfide isomerase (PDI)	−3.8	Translation factor, chaperone
Oncoprotein 18 (Op18)	+3.4	Cell proliferation or differentiation
Cyclophilin A (Cyp A)	+1.4	Cell proliferation or differentiation
Calcyclin binding protein, Siah-interacting protein (CacyBP)	−2.4	Cell proliferation or differentiation
Rho GDP dissociation inhibitor alpha (GDI)	+3.1	Cell proliferation or differentiation
Porin isoform 1, voltage-dependent anion channel 1 (VDAC1)	+5.7	Cell proliferation or differentiation

TABLE II. Results of MALDI-TOF Mass Spectra and Database Searching for Protein Identification

Protein ID	ACC# (NCBI)	Sequence coverage	Experimental mass (kDa)/pI
Heat shock protein gp96 precursor (HSPgp96)	15010550	17.0%	90195/4.7
Peroxiredoxin 1	4505591	39.0%	22111/8.3
Peroxiredoxin 6	1718024	50.0%	25035/6.0
Thioredoxin	135773	45.0%	11738/4.8
Protein disulfide isomerase (PDI)	7437388	26.0%	56783/6.0
Oncoprotein 18 (Op18)	5031851	42.0%	17303/5.8
Cyclophilin A (Cyp A)	1334908	34.0%	23207/5.0
Calcyclin binding protein, Siah-interacting protein (CacyBP)	5454052	23.0%	27774/4.7
Rho GDP dissociation inhibitor alpha (GDI)	1351015	22.0%	24423/7.0
Porin isoform 1, voltage-dependent anion channel 1 (VDAC1)	13899241	12.0%	17068/6.6
Stratifin	5454052	23.0%	27774/4.7

the three-dimensional structures of SOD protein, the decrease of zinc content in cellular would interfere with the post-translational modification during the folding process of the Cu,Zn-SOD protein. Therefore, the depletion of zinc ion by Cd-induced metallothionein may be the most possible cause to change the protein secondary conformation of the active site and further alter its functional activity.

In this experiment, we successfully established an enzymologic system to monitor the conformational change of SOD protein caused by heavy metal contamination. We found that cadmium significantly depleted zinc content in the Cu,Zn-SOD protein to change the functional protein conformation and reduce its enzyme activity. In addition, this effect is correlated with its strong induction of metallothionein expression. The lost of Cu,Zn-SOD activity due to an improper folding or misfolding which has similar effect as the mutations found in the Cu,Zn-SOD is considered as an important cause of SALS. In addition, cadmium is a potent neuron cell poison known to cause oxidative stress [Stohs and Bagchi, 1995]. Our previous results also showed that cadmium decreased intracellular glutathione concentrations [Shukla et al., 2000]. Oxidative stress is one of the mechanisms that contribute to structural changes or misfolding of proteins such as SOD. Lipid peroxidation has been considered as the primary mechanism for cadmium toxicity [Muller, 1986; Yiin et al., 1990; Manca et al., 1999]. It has been proposed that the enhancement of lipid peroxidation by cadmium in rats is a consequence of a decrease in superoxide dismutase and catalase activity [Hussain et al., 1987; Shukla et al., 1989; Stajn et al., 1997]. Substantial evidence has accumulated showing that oxidative stress may play an important role in neurodegeneration [Coyle and Puttfarcken, 1993; Cohen and Wemer, 1994; Williams, 1995].

Alterations in cellular oxidative status have been shown to play an important role in inducing apoptotic cell death [Green and Reed, 1998; Raha and Robinson, 2001; Armstrong et al., 2002]. High levels of ROS induce a state of oxidative stress in cells, which may damage cellular DNA, proteins, and lipids, and result in cell-cycle arrest, cellular senescence, and cell death [Martindale and Holbrook, 2002]. Cells are equipped with multiple antioxidant defense mechanisms for survival [Nakamura et al., 1997; Mates et al., 1999]. Cadmium-induced alterations of the proteins involved in defending oxidative stress by upregulating the expressions of thioredoxin, peroxiredoxin 1, and peroxiredoxin 6 (Table I and Fig. 7). Our results (Fig. 7) also showed that thioredoxin was highly upregulated in cadmium-treated N2A cells, which suggests that cadmium may induce oxidative stress in neural cells. Peroxiredoxins (Prxs) are a group of recently characterized thiol-containing proteins with efficient antioxidant capacity, capable of antagonizing hydrogen peroxide in living cells. The peroxiredoxins also influence a variety of cellular processes that are sensitive to ROS and play a role in signal transduction and gene expression related to alterations in cellular ROS levels [Lim et al., 1998]. Our results further suggest that cadmium may induce ROS, which may subsequently alter mitochondria membrane potential and ultimately induce mitochondria-mediated apoptosis.

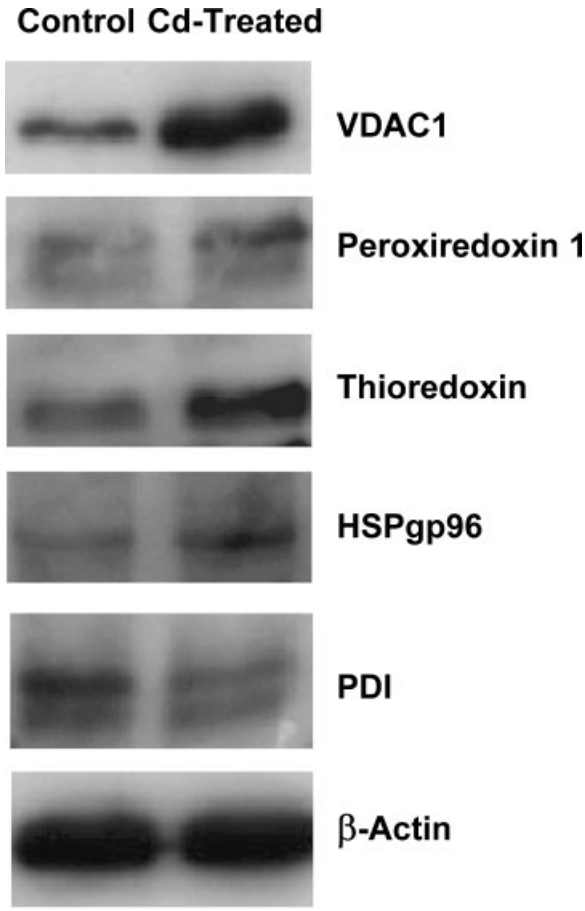


Fig. 7. Western blot analysis of the altered proteins in cadmium-treated N2A cells. N2A cells untreated (Control) or treated (Cd-Treated) with 10 μM cadmium for 24 h were collected. The soluble fractions of all cell lysates were resolved by 10 % SDS–PAGE and immunodetected by Western blotting using specific antibodies including VDAC1, peroxiredoxin 1, Thioredoxin, HSPgp96, PDI, and β-actin as indicated. Monoclonal anti-β-actin antibody was used to monitor the loading difference. The data are representative of three-independent experiments.

Mitochondria are mediated by VDAC1. VDAC1 is a mitochondrial outer membrane channel protein, which plays a role in the permeability transition [Bernardi et al., 1994; Zoratti and Szabo, 1995]. VDAC1 also plays an essential role in Bax/Bak-induced apoptotic mitochondrial changes in the process of apoptosis in mammalian cells [Shimizu et al., 1999; Shimizu et al., 2000; Madesh and Hajnoczky, 2001]. In this process, the pro-apoptotic proteins Bax and Bak bind to VDAC1 and enhance its permeability so that cytochrome *c* passes through the channel and releases to cytoplasm, and then induces the process of apoptosis [Shimizu et al., 1999, 2000; Madesh and Hajnoczky, 2001]. Our data on VDAC1 upregulation suggest that cadmium may induce cell death via the mitochondria-mediated apoptosis pathway.

Protein disulfide-isomerase (PDI) was downregulated in N2A cells treated with cadmium (Table I). PDI is a multifunctional protein mainly located in the endoplasmic reticulum (ER). During protein folding in the ER, PDI catalyzes thiol/disulfide exchange, including disulfide bond formation and rearrangement reactions [Lyles and Gilbert, 1991]. The thiol/disulfide centers of the two thioredoxin-like domains function as two independent active sites. These two sites could lead to increased PDI expression in neurons, and result in both attenuation of the loss of cell viability in vitro and reduction of the number of DNA-fragmented cells in the rat hippocampal CA1 subregion in vivo [Lyles and Gilbert, 1991]. Recent reports demonstrate that upregulated PDI may exert a protective effect against apoptotic cell death [Tanaka et al., 2000; Ko et al., 2002]. The observed downregulated expression of PDI in our study indicates that cadmium triggers neural cells towards apoptosis. Calcyclin binding protein (CacyBP), a novel protein target of calcyclin involved in calcium signaling pathways in neuronal tissue was upregulated in proliferating and differentiating cells [Nowotny et al., 2000]. Downregulation of CacyBP expression by cadmium-treatment (Table I) further suggests that cadmium may induce cell apoptosis through affecting the processes of neural cell proliferation and differentiation.

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