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Inhibition of Cadmium-Induced Oxidative Injury in Rat Primary Astrocytes by the Addition of Antioxidants and the Reduction of Intracellular Calcium

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Abstract Exposure of the brain to cadmium ions (Cd^{2+}) is believed to lead to neurological disorders of the central nervous system (CNS). In this study, we tested the hypothesis that astrocytes, the major CNS-supporting cells, are resistant to Cd^{2+}-induced injury compared with cortical neurons and microglia (CNS macrophages). However, treatment with $CdCl_2$ for 24 h at concentrations higher than 20 μM substantially induced astrocytic cytotoxicity, which also resulted from long-term exposure to 5 μM of $CdCl_2$. Intracellular calcium levels were found to rapidly increase after the addition of $CdCl_2$ into astrocytes, which led to a rise in reactive oxygen species (ROS) and to mitochondrial impairment. In accordance, preexposure to the extracellular calcium chelator EGTA effectively reduced ROS production and increased survival of Cd^{2+}-treated astrocytes. Adenovirus-mediated transfer of superoxide dismutase (SOD) or glutathione peroxidase (GPx) genes increased survival of Cd^{2+}-exposed astrocytes. In addition, increased ROS generation and astrocytic cell death due to Cd^{2+} exposure was inhibited when astrocytes were treated with the polyphenolic compound ellagic acid (EA). Taken together, Cd^{2+}-induced astrocytic cell death resulted from disrupted calcium homeostasis and an increase in ROS. Moreover, our findings demonstrate that enhancement of the activity of intracellular antioxidant enzymes and supplementation with a phenolic compound, a natural antioxidant, improves survival of Cd^{2+}-primed astrocytes. This information provides a useful approach for treating Cd^{2+}-induced CNS neurological disorders. J. Cell. Biochem. 103: 825–834, 2008.

Key words: glia; cadmium; oxidative stress; calcium; free radical

Glia play an important role in neuronal survival and transmission; therefore, they are functionally considered to be neuronal support cells. Astrocytes, the most abundant glial cells in the central nervous system (CNS), serve several functions. They provide energy substrates to neurons, maintain ionic and neurotransmitter homeostasis, regulate synaptic transmission, and stimulate neurogenesis [Ransom et al., 2003]. In terms of their structure, these cells extend processes called footplates or end-feet, which surround the vascular surfaces of the brain and pia tissues to form a perivascular glial limiting membrane and a pial-glial limiting membrane [Voutsinos-Porche et al., 2003]. Their cell bodies are juxtaposed between neurons and the capillary endothelium to form the blood–brain barrier. Given these cytoarchitectural features, astrocytes are the first cells of the brain parenchyma to encounter foreign molecules crossing the blood–brain barrier [Tiffany-Castiglioni et al., 1989]. When the CNS is injured, astrocytes become reactivated, they produce proinflammatory mediators, and they form physical barrier that hinders axonal regeneration and tissue repair [Silver and

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Miller, 2004]. Compelling evidence has emerged to indicate that astrocytes may support neuronal survival and tissue repair after CNS injury by producing neurotrophic factors and/or growth factors, by serving as free radical scavengers, and by removing excess extracellular glutamate [Ransom et al., 2003].

Cadmium ion (Cd^{2+}), as used in various industrial activities, is a carcinogen and induces tumors in many human organs [Waalkes, 2000]. In vivo and in vitro studies have indicated that acute or chronic exposure to Cd^{2+} causes cell necrosis and/or apoptosis. Cd^{2+} induces oxidative stress by disrupting intracellular Ca^{2+} homeostasis and by causing the accumulation of reactive oxygen species (ROS) [Bagchi et al., 1995; Hassoun and Stohs, 1996; Beyersmann and Hechtenberg, 1997; Shaikh et al., 1999; Szuster-Ciesielska et al., 2000; Shen et al., 2001; Lemarie et al., 2004]. Numerous studies provide evidence that Cd^{2+}-induced neuronal toxicity and damage to the brain tissue is due to ROS-triggered oxidative stress resulting from inhibition of endogenous antioxidant enzymes and depletion of glutathione [Acan and Tezcan, 1995; Kumar et al., 1996; Almazan et al., 2000; Watjen et al., 2002; Lopez et al., 2003; Watjen and Beyersmann, 2004; Im et al., 2006]. Oxidative stress in association with ROS-induced mitochondrial membrane dysfunction mediates Cd^{2+}-related apoptosis of cultured cortical neurons [Lopez et al., 2003, 2006]. Recent studies have shown that thiol-containing compounds, such as glutathione, cysteine, and *N*-acetylcysteine, can completely block Cd^{2+}-induced death of astrocytes [Im et al., 2006]. However, caspase-3 inhibitor and familiar antioxidants (e.g., vitamin E, vitamin C, caffeic acid) fail to suppress the effect of Cd^{2+} on astrocytes [Im et al., 2006]. Although the findings point to glutathione depletion as the basis for Cd^{2+}-induced astrocytic death, whether ROS, caspase activation, and intracellular calcium (Ca^{2+}) homeostasis are involved in Cd^{2+}-induced astrocytic death remains to be determined.

Herein, we demonstrate that both Ca^{2+}- and ROS-dependent mechanisms are involved in Cd^{2+}-induced astrocytic death. Our data also show that the antioxidant EA, gene transfer of SOD and GPx, or extracellular/intracellular calcium chelators (EGTA/BAPTA-AM) prevents astrocytes from Cd^{2+}-induced oxidative insult.

MATERIALS AND METHODS

Medium, B-27 serum supplement and antibiotics used in this study were purchased from Invitrogen (Carlsbad, CA). Cell cultureware and petri dishes were obtained from BD Biosciences (San Jose, CA). Fetal bovine serum (FBS) and calf serum (CS) were the products of Hyclone Laboratories (Logan, UT). Poly-D-lysine (PDL) and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase (LDH) assay kit and Caspase-3 activity assay kit were from Roche Diagnostics (Indianapolis, IN) and Promega (Madison, WI), respectively. Ca^{2+}-sensitive dye Fluo-4-AM, JC-1, and CM-H_2DCFDA were the products from Molecular Probes (Eugene, OR). SOD-525 kit was from R&D System (Minneapolis, MN). The specific inhibitor for caspase-3, Z-DEVD-fmk, was purchased from Tocris Bioscience (Ellisville, MO) and BioVision (Mountain View, CA). The polyphenolic compound EA was the product from Sigma.

Preparation of Primary Rat Cortical Neurons

Cortical neuronal cultures were prepared described as follows. Cerebral cortices were removed from embryonic day 17–18 rat brains. Animal care was provided in accordance with the *Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals proved by* Institutional Animal Care and Use Committee in National Cheng Kung University. The tissues were dissected mechanically in the Dulbecco's Modified Eagle's Medium (DMEM) containing 0.0025% trypsin/EDTA, and then passed through a 70 μm pore nylon mesh. After centrifugation, cells were seeded at the density of 1×10^5 cells/well onto a 24-well plates coated with 0.0025% PDL in 0.1 M boric acid buffer, and then cultured in DMEM/F-12 containing 10% heat-inactivated horse serum for 2 h at 37°C, 5% CO_2. The medium was then changed with Neurobasal medium containing 1 × B-27 serum supplement every 2 days, and the cultures were maintained in Neurobasal medium plus B-27 for 9–10 days.

Preparation of Primary Rat Microglia and Astrocytes

Primary rat astrocytic culture was prepared as described below. Cerebral cortices from neonatal Sprague–Dawley rat brains (P1–2) were

removed and carefully dissected. The tissue was dissociated in DMEM containing 0.0025% trypsin/EDTA and passed through a 70 μm pore nylon mesh. After centrifugation, the cell pellet was resuspended in DMEM/F12 medium containing 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. The cells (10^7 cells/flask) were then plated onto PDL-coated 75T tissue culture flasks. The medium was renewed every 2–3 days. Eight days later, microglia were collected using the shake-off method [McCarthy and de Vellis, 1980], and replated onto a 96-well plate at the density of 2×10^4 cells/well, which was used to examine the effect of $CdCl_2$ on microglial cell viability/toxicity. There were approximately 90–92% astrocytes on the flask after shake-off procedure, which were verified using immunostaining for the specific astrocytic marker GFAP. Less than 5–10% of cells were B4 isolectin$^+$ microglia in astrocyte culture.

MTT Cell Viability Assay

MTT reacts with mitochondrial dehydrogenase in viable cells to generate a formazan product. Thus, measurement of the level of the formazan product by a colorimetric method is correlated with the relative number of cells. To study the effect of $CdCl_2$ on the cell viability of astrocytes, microglia, and neurons, cells were treated with $CdCl_2$ (0–50 μM) in serum-free DMEM medium (DMEM) for distinct time courses, followed by MTT assay. For long-term treatment, astrocytes were cultured in DMEM medium with 1 or 5 μM of $CdCl_2$ for 5 days or for 8 days. The medium was replenished every 2–3 days. MTT assay was performed by the addition of MTT solution (5 mg/ml) to each well, and 4 h later the culture was incubated with SDS (10% in 0.01 N HCl) was added to each well, and the culture was then incubated overnight at 37°C. MTT absorbance was measured using an ELISA reader at 595 nm. The data are presented as the percentage versus control which is expressed as 100% cell viability.

LDH Assay

Cell cytotoxicity analysis by measuring LDH activity in the culture medium was performed to validate MTT cell viability at 24 h post-treatment with $CdCl_2$. Astrocytes were treated with different concentration (1–100 μM) of $CdCl_2$ in DMEM for 24 h. The supernatant was collected, centrifuged at 1,500 rpm for 10 min to remove cell debris, and subsequently subjected to LDH assay following the procedure provided by the vendor (Roche Diagnostics). The data are presented as relative LDH activity (fold of control).

Caspase-3 Activity Assay

Astrocytes were replated into 100 mm petri dishes at the density of 5×10^6 cells/dish for 3 days, and then treated with $CdCl_2$ (10, 20, 30, and 50 μM) in DMEM for 2 h. These cells were collected, lysed in the lysis buffer provided by the vendor (Promega), and centrifuged at 10,000 rpm for 2 min. Total protein in cell lysate was measured using Bio-Rad DC kit (Bio-Rad, Hercules, CA). The cell lysate (50 μg) was incubated with a caspase-3 substrate Ac-DEVD-pNA for 2 h, and then subjected to the assay of caspase-3 activity. Upon the cleavage of the caspase-3, free pNA with yellow color can be released. Thus, caspase-3 activity was measured by monitoring the intensity of yellow color by an ELISA reader at 405 nm. The data are presented as the percentage versus control (100% activity).

Mitochondrial Membrane Potential Assay

The mitochondrial membrane potential (MMP) of astrocytes was measured by flow cytometry with the lipophilic cationic probe JC-1. Astrocytes (1×10^6 cells) were treated with 30 and 50 μM of $CdCl_2$ for 6 h. The cells were then incubated with 10 μM of JC-1 for 30 min at 37°C, and trypsinized using 0.0025% trypsin/EDTA. The cells are centrifuged, resuspended in a total volume of 1 ml, and analyzed using FACScan flow cytometry (BD Biosciences). Two excitation wavelengths, 527 nm (green) and 590 nm (red) are used to detect the JC-1 monomer form and the JC-1 aggregate form, respectively. The red fluorescence is predominantly detected in healthy cells with high MMP, while it is decreased in damaged mitochondria. Alternatively, after astrocytes were exposed to 30 μM of $CdCl_2$ for 6 h, the cells were incubated with 10 μM of JC-1 for 30 min at 37°C, and then fixed in 4% paraformylaldehydre for 15 min. JC-1 staining in astrocytes was visualized under an epifluorescence microscope equipped with a cooling digital imaging system.

Calcium Imaging

Astrocytes were preincubated with 5 μM of Ca^{2+}-sensitive dye Fluo-4-AM for 20 min under

the dark, and then washed twice with Hank's balanced salt solution (HBSS) containing 20 mM HEPES. The culture was mounted on Olympus IX71 Fluorescence inverted microscope equipped with 40× oil immersion lens (NA 1.35), perfused with HEPES-buffered HBSS for 30 s, and then with HEPES-buffered HBSS containing 30 μM of $CdCl_2$ for 5 min. Time-lapse images were recorded at the rate of 1 s/frame by using a cooled CCD camera with TILL Photonics Imaging System Software (IMAGO-QE; TILL Photonics GmbH, CA). The Ca^{2+}-sensitive dye Fluo-4-AM was excited by the 488 nm line of a Xenon short arc lamp and the emission wavelength was 530 nm. Regions (approximately 25 cells/region) of interest (ROIs) were selected, and the normalized fluorescence changes F/F_0. F is the fluorescence intensity at every time scale and F_0 is the averaged fluorescence intensity at the first 30 s of HEPES-buffered HBSS perfusion.

Measurement of ROS

Astrocytes were replated onto a 96-well plate at the density of 5×10^4 cells/well for 3 days. The cultures were loaded at 37°C for 1 h in DMEM containing 10 μM of CM-H_2DCFDA that is converted from nonfluorescence DCF to the highly fluorescence DCF through oxidation by intracellular ROS. The cultures were then followed by treatment with the different concentration of $CdCl_2$ for 3 h. The fluorescence was measured or in an automatic microtiter reader at the excitation wavelength 485 nm and the emission wavelength 535 nm (PerkinElmer HT7000). The percentage of intracellular ROS levels was determined by normalizing the fluorescence intensity of the treated culture to control.

SOD Enzymatic Activity Assay

Astrocytes were replated into 100 mm petri dish at the density of 5×10^6 cells/dish for 3 days, and then treated with 30 μM of $CdCl_2$ with or without 30 μM of antioxidant ellagic acid (EA) in DMEM for 3 h. Astrocytes were incubated in lysis buffer (0.5% NP-40, 1 mM EDTA in PBS), and cell lysate was then centrifuged at 12,000 rpm for 10 min. The supernatant was analyzed for total SOD activity (Cu/Zn-SOD and Mn-SOD) using the BIOXYTECH SOD-525 kit. The method was based on a SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c] fluorine in an aqueous alkaline solution that yields a chromophore with maximum absorbance at 525 nm. Total protein was measured using Bio-Rad DC kit (Bio-Rad). SOD activity was indicated as units per milligram of protein. The data are expressed as the percentage versus control (100% activity).

Infection of Recombinant Adenovirus Encoding Human Superoxide Dismutase (hSOD), Catalase (hCAT), and Glutathione Peroxidase (hGPx)

Recombinant adenoviral vectors with human SOD, CAT, and GPx genes were provided by Dr. Song-Kun Shyue, Institute of Biomedical Sciences, Academia Sinica, Taiwan. Human Cu, Zn-SOD, GPx, or CAT cDNA containing the entire coding sequence was subcloned into the adenovirus shuttle plasmid vector, which contains a promoter of the human phosphoglycerate kinase (PGK) and a polyadenylation signal of bovine growth hormone [Lin et al., 2004, 2005]. For infection, astrocytes were seeded onto a 24-well plate at the density of 1×10^5 cells/well, and then infected with various adenoviruses at approximately 10–20 MOI for 24 h. The culture medium was replaced with fresh DMEM medium containing 10% CS for 48 h, and then the cultures were treated with $CdCl_2$ for 24 h. MTT assay was performed to evaluate the effect of these recombinant adenoviral vectors on astrocytic cell survival.

Statistical Analysis

Data are expressed as mean ± SEM. Each experiment was repeated at least three times. Statistical significance of differences between the two groups of data (P-value <0.05) was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison (Minitab, State College, PA).

RESULTS

Toxicity of Cd^{2+} to Neurons, Microglia, and Astrocytes

When cortical neurons were treated by $CdCl_2$ at the concentration range of 1–5 μM in DMEM for 24 h, 50% reduction in neuronal cell viability was observed (Fig. 1A). We also found that exposure to $CdCl_2$ at 0.5–1.0 μM for 24 h resulted in approximately 50% microglial cell death (Fig. 1B). However, treatment with 1–10 μM of $CdCl_2$ in DMEM did not affect cellular viability of astrocytes (Figs. 2 and 3). These

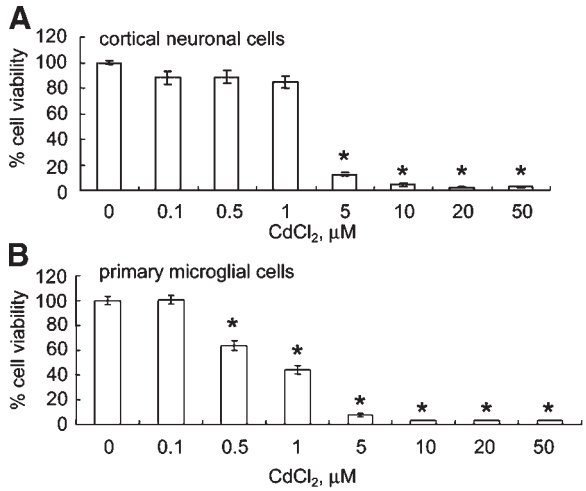

Fig. 1. Cell viability of cortical neurons or microglia at 24 h post-$CdCl_2$ exposures. Cell viability of cortical neurons (**A**) and microglia (**B**) was determined by MTT assay. Data are mean values (±SEM) of all independent experiments ($n \geq 3$). *Significance is relative to untreated control ($P < 0.05$).

results indicated that microglia and neurons were less resistant to Cd^{2+} insult than astrocytes were.

Morphologic examination indicated that treatment with $CdCl_2$ at 20, 30, 50, or 100 μM in DMEM resulted in shrinkage of astrocytic cell bodies (Fig. 2, arrows) and in extraction of their processes (Fig. 2, arrowheads). Moreover, $CdCl_2$ reduced astrocytic cell viability in a dose- and time-dependent manner (Fig. 3A). Further confirming this result was the finding that $CdCl_2$ increased levels of LDH released from astrocytes in a dose-dependent manner at 24 h (Fig. 3B). Moreover, repeated treatment with 5 μM of $CdCl_2$ in DMEM for 8 d substantially induced astrocytic cell death (Fig. 3C), demonstrating that long-term exposure to the low dose of Cd^{2+} could cause astrocytic cell death.

Involvement of a Caspase-3 Independent Pathway in Cd^{2+}-Induced Astrocytic Cell Death

When astrocytes were exposed to various concentrations of $CdCl_2$ for 2 h, caspase-3 activity did not markedly increase (Fig. 4A). The addition of Z-DVAD.fmk to astrocytes exposed to 30 μM of $CdCl_2$ failed to protect them from Cd^{2+}-induced death (Fig. 4B). The results demonstrated regulation of Cd^{2+}-induced astrocytic cell death by means of a caspase-3 independent pathway.

Attenuation of Mitochondrial Membrane Potential in Cd^{2+}-Treated Astrocytes

MMP loss was observed among astrocytes treated with $CdCl_2$ at 30 μM (7.8%) and 50 μM (17.7%) for 6 h compared with untreated astrocytes (2.4%; Fig. 5A, R2 region). JC-1 staining indicated a well-organized distribution of strong, punctate JC-1 aggregates in astrocytes without $CdCl_2$ treatment (Fig. 5B). A scattering of JC-1 aggregates with diffuse fluorescence (Fig. 5B) was found in astrocytes exposed to 30 μM of $CdCl_2$ for 6 h.

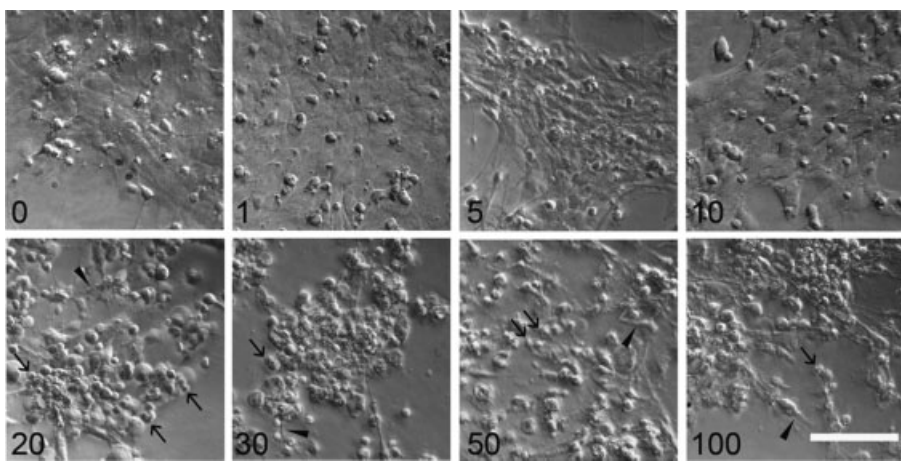

Fig. 2. Morphological examination of astrocytes after $CdCl_2$ treatment for 24 h. Astrocytes were treated with $CdCl_2$ for 24 h at the indicated concentrations. The morphological examination showed the shrinkage of astrocytic cell bodies (arrows) and the extraction of astrocytic processes (arrowheads) after exposure to $CdCl_2$ at the concentrations of greater than 20 μM. Scale bar, 100 μm.

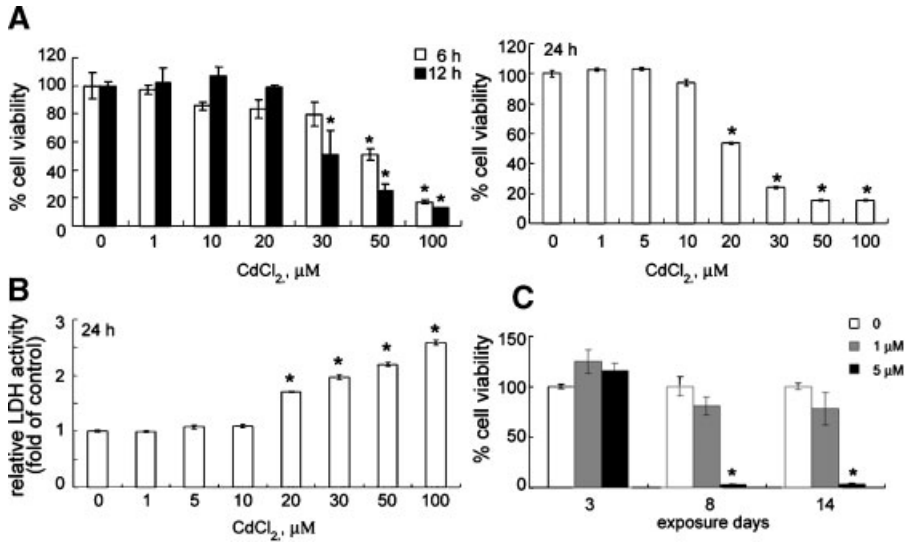

Fig. 3. Cd^{2+}-induced reduction in astrocytic cell viability. **A**: Astrocytic viability was analyzed by MTT assay after treatment with $CdCl_2$ at various concentrations for 6, 12, and 24 h. **B**: Astrocytic toxicity was analyzed by LDH assay after treatment with $CdCl_2$ at the indicated concentrations for 24 h. **C**: Astrocytic viability was examined by MTT assay after long-term treatment with $CdCl_2$ (1 and 5 μM) for 3, 8, and 14 days. Data are mean values (±SEM) of all independent experiments ($n \geq 3$). *Significance is relative to untreated control ($P < 0.05$).

Cd^{2+}-Induced Oxidative Stress and Disruption of Intracellular Calcium Homeostasis

Treatment with $CdCl_2$ for 3 h considerably increased ROS generation in astrocytes (Fig. 6A). Intracellular Ca^{2+} levels markedly increased after the addition of 30 μM of $CdCl_2$ into the astrocytic culture (Fig. 6C).

The application of EGTA to the astrocytes effectively blocked the Cd^{2+}-induced increase in ROS levels (Fig. 7A). Pretreatment with EGTA for 5 min reduced Cd^{2+}-induced astrocytic death in a dose-dependent way (Fig. 7B). In addition, pretreatment of the astrocytes with 10 μM of BAPTA-AM for 15 min effectively attenuated Cd^{2+}-induced death (Fig. 7C). Thus, Cd^{2+}-induced astrocytic cell death involved a Ca^{2+}- and ROS-dependent mechanism in association with mitochondrial dysfunction.

Delivery of Antioxidant Enzyme Genes and Reduction of Cd^{2+}-Induced Cell Death With EA

We next asked whether the antioxidant enzyme gene delivery (SOD, CAT, or GPx) via adenovirus can prevent astrocytes from Cd^{2+}-

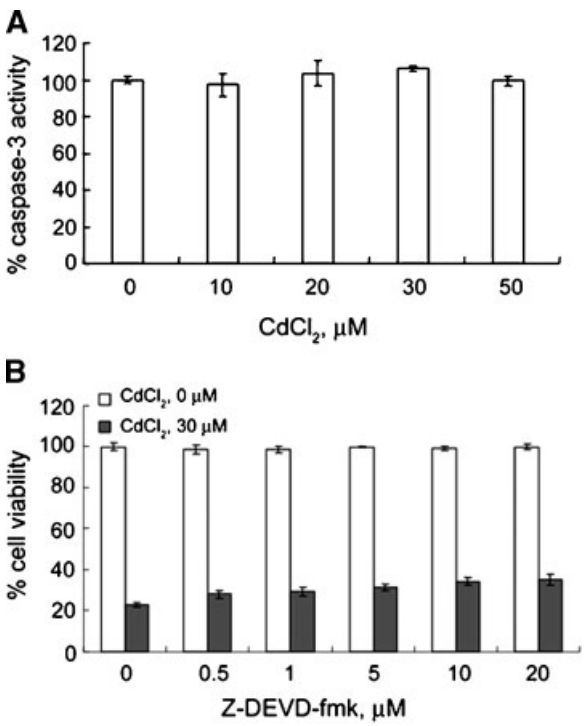

Fig. 4. Caspase-3 independent effect on Cd^{2+}-induced astrocytic cell death. **A**: Caspase-3 activity assay of astrocytes that were treated for 2 h with $CdCl_2$ at the indicated concentrations. **B**: Astrocytic viability was examined by MTT assay at 24 h after treatment with 30 μM of $CdCl_2$ in the presence of a caspase-3 inhibitor Z-DEVD-fmk at the concentrations indicated as above. Data are mean values (±SEM) of all independent experiments ($n = 3$). Data are mean values (±SEM) of at least three independent experiments.

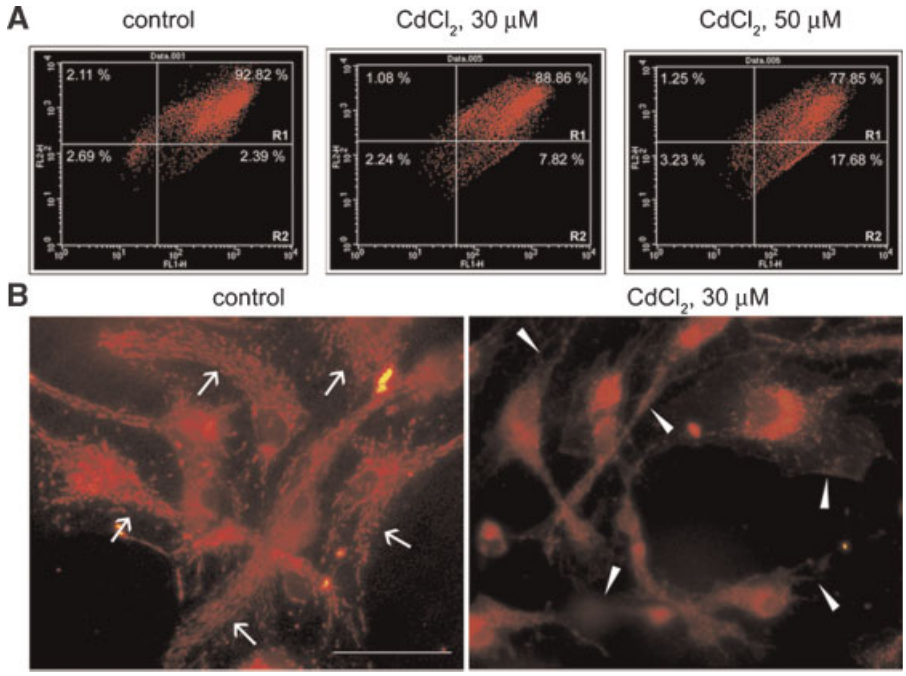

Fig. 5. Effect of $CdCl_2$ on astrocytic mitochondrial impairment. **A**: Astrocytic mitochondrial membrane potential (MMP) alteration was examined by flow cytometric analysis using the fluorescent cationic dye JC-1 after treatment with 30 and 50 μM of $CdCl_2$ for 6 h. **B**: Higher magnification images show that the strong punctate red fluorescence intensity (arrows) of aggregated JC-1 complexes was detected in control astrocyte cultures, whereas a decrease observed in astrocytes treated with 30 μM of $CdCl_2$ (arrowheads) for 6 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

induced cell death. The expression of SOD and CAT in infected cells has been previously verified [Lin et al., 2004, 2005], and GPx expression was validated by examining GPx mRNA levels in infected cells using RT-PCR (data not shown). Gene transfer for SOD and GPx substantially increased the survival of Cd^{2+}-treated astrocytes (Fig. 8A), whereas CAT gene therapy did not reduced Cd^{2+}-induced astrocytic toxicity.

The natural antioxidant compound EA reduced Cd^{2+}-induced toxicity in astrocytes (Fig. 8B). Moreover, the addition of EA to Cd^{2+}-treated astrocytes considerably suppressed ROS generation (Fig. 8C). Although we observed a tendency for $CdCl_2$ to reduce SOD activity in astrocytes, this inhibition was not biostatistically different from results in the control culture (Fig. 8D). In addition, EA did not substantially increase SOD activity in Cd^{2+}-treated astrocytes compared with the EA-treated control (Fig. 8D), indicating that EA-induced astrocytic cell survival was mainly due to its effect in the blockage of Cd^{2+}-induced ROS generation.

DISCUSSION

Cd^{2+}-induced toxicity of astrocytes reportedly results from the depletion of glutathione [Im et al., 2006]. We demonstrate here that Cd^{2+}-induced astrocytic cell death was via the caspase-3-independent, but ROS-dependent pathway. This finding is similar to observations in neuronal and nonneuronal cells [Hassoun and Stohs, 1996; Shaikh et al., 1999; Lemarie et al., 2004; Shih et al., 2005; Im et al., 2006]. Further experiments by intracellular calcium measurement and pharmacological approach indicate that Cd^{2+} resulted in astrocytic death possibly through Ca^{2+}/ROS-dependent signaling pathway. Elevation of antioxidant enzyme SOD and GPx levels, as well as treatment with the potent antioxidant EA, could attenuate Cd^{2+}-induced astrocytic cell death.

Cd^{2+} has been reported to trigger caspase-3 activation to induce the apoptosis of several cell types, such as human lymphoma U937 cells [Li et al., 2000], murine macrophages [Kim and Sharma, 2006], and cortical neurons [Lopez

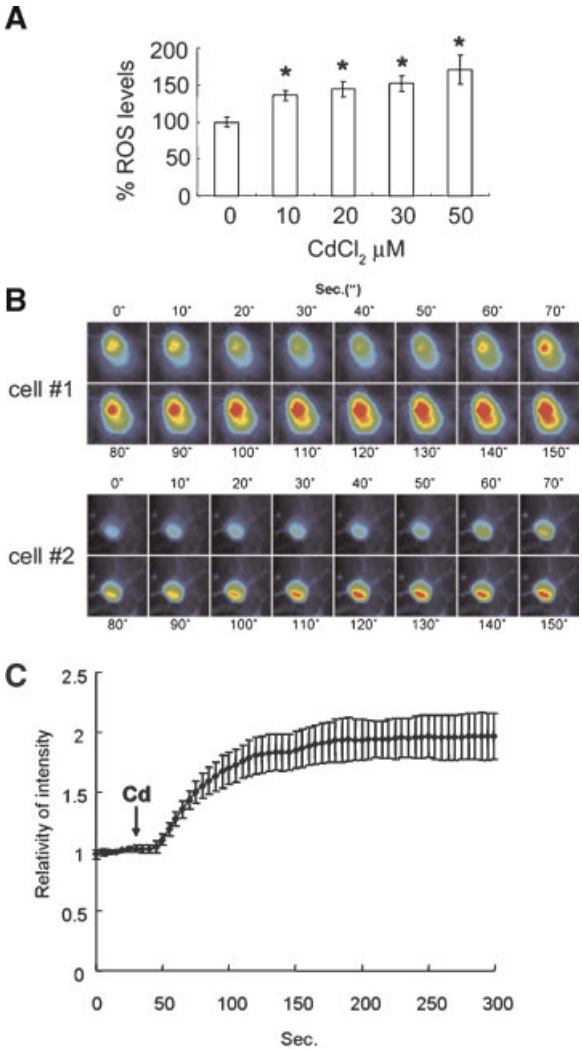

Fig. 6. A rise of ROS and intracellular calcium levels in astrocytes by $CdCl_2$ treatment. **A**: An increase in ROS generation was detected in astrocytes after exposure to $CdCl_2$ at the indicated concentrations for 3 h. Data are mean values (±SEM) of three independent experiments. *Significance is relative to untreated control ($P < 0.05$). The real-time Ca^{2+} image of Fluo-4-AM preloaded atrocytes was recorded at every 10-s time interval after perfusion with 30 μM of $CdCl_2$ in HEPES-HBSS as described in Materials and Methods Section. **B**: A series of fluorescence imaging of two astrocytes indicated as cell #1 and cell #2 was shown. **C**: The quantification of the fluorescence intensity indicated a rapid rise of intracellular Ca^{2+} after the addition of 30 μM of $CdCl_2$ to Fluo-4-AM-loaded astrocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

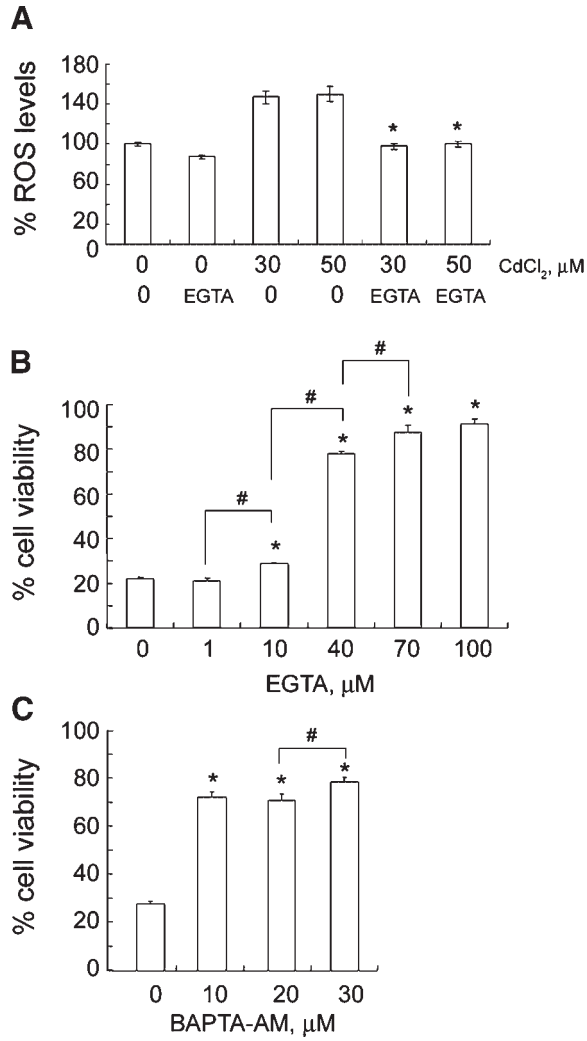

Fig. 7. Reduction of Cd^{2+}-induced astrocytic death by EGTA and BAPTA-AM. **A**: ROS detection was performed at 3 h after treatment with $CdCl_2$ (30 and 50 μM) in the presence of extracellular calcium chelator EGTA (40 μM). **B**: Astrocytic viability was examined by MTT assay at 24 h after treatment with $CdCl_2$ (30 μM) in the presence of EGTA at the indicated concentrations. **C**: Astrocytic viability was analyzed by MTT assay at 24 h after treatment with $CdCl_2$ (30 μM) in the presence of intracellular calcium chelator BAPTA-AM at the indicated concentrations. Data are mean values (±SEM) of three independent experiments. *Significance is relative to untreated control ($P < 0.05$). Significance is relative to cd^{2+}-treated group ($P < 0.05$).

et al., 2003]. However, contrary findings have indicated that Cd^{2+}-induced cell death could involve a caspase-3-independent pathway [Ishido et al., 1999; Harstad and Klaassen, 2002; Shih et al., 2005; Im et al., 2006]. Similar to these latter observations, our findings indicate neither an increase in caspase-3 activity nor reduction in cell death with the caspase-3 inhibitor among Cd^{2+}-treated astrocytes. Apparently, Cd^{2+} effect on astrocytic cell death is caspase-3 independent.

The addition of Cd^{2+} robustly to increased intracellular Ca^{2+} levels in astrocytes, an observation consistent with results showing

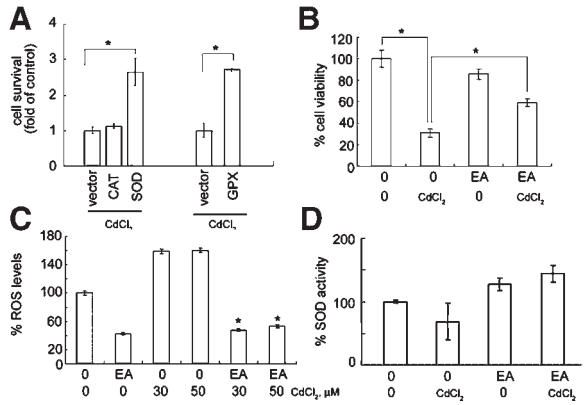

Fig. 8. Effect of antioxidant ellagic acid and antioxidant enzymes on the survival of Cd^{2+}-treated astrocytes. **A**: Astrocytic survival was examined by MTT assay after astrocytes were infected with adenoviral-hSOD, adenoviral-hCAT and adenoviral-hGPX for 48 h, and then treatment with $CdCl_2$ (20 μM) for another 24 h. Data are mean values (±SEM) of three sister cultures, and the experiments were repeated twice with similar results. *Significance is relative to Cd^{2+}-untreated control vector groups ($P < 0.05$). **B**: Astrocytic viability was analyzed at 24 h after treatment with $CdCl_2$ (30 μM) and 30 μM of ellagic acid (EA). Data are mean values (±SEM) of all independent experiments ($n \geq 3$). *$P < 0.05$. **C**: ROS detection was performed at 3 h after treatment with $CdCl_2$ (30 and 50 μM) in the presence or absence of EA (30 μM). Data are mean values (±SEM) of all independent experiments ($n \geq 3$). *Significance is relative to Cd^{2+}-treated groups ($P < 0.05$). **D**: SOD activity was analyzed after treatment with $CdCl_2$ (30 μM) in the presence or absence of EA (30 μM) for 3 h. Data are mean values (±SEM) of three independent experiments.

alteration of Ca^{2+} homeostasis due to Cd^{2+} in human hepatocarcinoma Hep3B cells [Lemarie et al., 2004]. Similar to the findings that disrupted Ca^{2+} impairs mitochondrial function and subsequently causes apoptosis of Hep3B cells [Lemarie et al., 2004], loss of mitochondrial membrane potential occurs in Cd^{2+}-treated astrocytes.

Data suggest that ROS-induced necrosis and/or apoptosis is likely to be a central mechanism of Cd^{2+}-induced neuronal cell death [Lopez et al., 2003, 2006]. We have found that treatment with Cd^{2+} increased intracellular ROS levels at 3 h, with MMP loss in a dose-dependent pattern at 6 h. Our further finding that EGTA suppressed intracellular ROS generation at 3 h and increased the viability of Cd^{2+}-treated astrocytes at 24 h suggest that disrupted Ca^{2+} homeostasis in astrocytes by Cd^{2+} lead to cell death from ROS generation and MMP reduction.

Reduced glutathione levels have been demonstrated in neurons [Lopez et al., 2006] and astrocytes by Cd^{2+} [Im et al., 2006]. In contrast to the finding that Cd^{2+} increased neuronal CAT and SOD activity at concentrations of 50–100 μM [Lopez et al., 2006], our results have shown insignificant change in the activity of these enzymes in astrocytes treated with 30 μM of $CdCl_2$. Yet, the transfer of SOD and GPx genes can increase the survival of Cd^{2+}-treated astrocytes.

The plant phenol EA has an antioxidant property and chemopreventive activity [Smith and Gupta, 1996; Dorai and Aggarwal, 2004], and it acts as a potent chelating compound to suppress nickel-induced oxidative stress in the liver and kidney [Ahmed et al., 1999]. In addition, EA inhibits nitric oxide production in C6 astrocytic cells [Soliman and Mazzio, 1998]. Consistent with its effect on these cell types, EA is a potent antioxidant that attenuated Cd^{2+}-induced ROS generation, as observed in our study. However, our results indicate that EA had no effect on SOD activity in Cd^{2+}-treated astrocytes. Taken together, our results show that the increase of antioxidant defense system by treating with EA or by enhancing the activity of SOD and GPx prevented astrocytes from Cd^{2+} insult.

In conclusion, our data show that astrocytes, compared with microglia and neurons, were resistant to Cd^{2+}-induced injury. Nevertheless, treatment with high concentrations of Cd^{2+} or long-term exposure to low concentrations of Cd^{2+} could result in astrocytic cell death. The mechanism of Cd^{2+}-mediated apoptosis of astrocytes was Ca^{2+}/ROS dependent. This mechanism could be blocked with the Ca^{2+} chelator EGTA, antioxidant enzymes (SOD and GPx), and with natural antioxidant phenol compounds such as EA.

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