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N-acetyl-L-cysteine protects against cadmium-induced neuronal apoptosis by inhibiting ROS-dependent activation of Akt/mTOR pathway in mouse brain

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Abstract

Aims—This study explores the neuroprotective effects and mechanisms of N-acetyl-L-cysteine (NAC) in mice exposed to cadmium (Cd).

Methods—NAC (150 mg/kg) was intraperitoneally administered to mice exposed to Cd (10–50 mg/L) in drinking water for 6 weeks. The changes of cell damage and death, reactive oxygen species (ROS), antioxidant enzymes, as well as Akt/mammalian target of rapamycin (mTOR) signaling pathway in brain neurons were assessed. To verify the role of mTOR activation in Cd-induced neurotoxicity, mice also received a subacute regimen of intraperitoneally administered Cd (1 mg/kg) with/without rapamycin (7.5 mg/kg) for 11 days.

Results—Chronic exposure of mice to Cd induced brain damage or neuronal cell death, due to ROS induction. Co-administration of NAC significantly reduced Cd levels in the plasma and brain of the animals. NAC prevented Cd-induced ROS and significantly attenuated Cd-induced brain damage or neuronal cell death. The protective effect of NAC was mediated, at least partially, by elevating the activities of Cu/Zn-superoxide dismutase, catalase and glutathione peroxidase, as well as the level of glutathione in the brain. Furthermore, Cd-induced activation of Akt/mTOR pathway in the brain was also inhibited by NAC. Rapamycin *in vitro* and *in vivo* protected against Cd-induced neurotoxicity.

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Conclusions—NAC protects against Cd-induced neuronal apoptosis in mouse brain partially by inhibiting ROS-dependent activation of Akt/mTOR pathway. The findings highlight that NAC may be exploited for prevention and treatment of Cd-induced neurodegenerative diseases.

Keywords

N-acetyl-L-cysteine; cadmium; neuronal apoptosis; mammalian target of rapamycin; reactive oxygen species

Introduction

Cadmium, a toxic transition metal, is mainly released from cigarette smoking, smelting and refining of metals, and burning of chemical fuels and municipal wastes, resulting in pollution of air, water, and soil. In addition to occupational exposure, the food chain and smoking are the two main sources of accumulation of Cd in human organs [1, 2]. As the half-life of Cd in human body is about 15–20 years, evidently, the content of Cd in human body is likely to increase in the future and might lead to a higher incidence of Cd-related diseases including carcinogenesis, immunodepression neurodegeneration [3–5]. It has been described that Cd contributes to the dysfunction of the nervous system such as learning disabilities and hyperactivity in children [6, 7], olfactory dysfunction, and neurobehavioural defects in attention, psychomotor speed, and memory in workers [8–10]. Growing evidence implicates that Cd neurotoxicity is a possible aetiological factor in neurodegenerative diseases [10–12]. However, the exact mechanism(s) by which Cd elicits its neurotoxic effects on brain is still not clear.

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, has been widely recognized as a central controller for cell proliferation/growth and survival [13]. The mTOR signaling pathway is composed of protein kinase B (Akt/PKB) as the main upstream mediator and two best characterized downstream effector molecules, ribosomal p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), and activated Akt may positively regulate mTOR, leading to increased phosphorylation of S6K1 and 4E-BP1 [13]. Studies have shown that mTOR activity regulates survival, differentiation and development of neurons, which is crucial for synaptic plasticity, learning and memory formation, and food uptake in adult brain [14, 15]. Recent studies have revealed that mTOR activity is modified in various pathological states of the nervous system, including brain tumours, tuberous sclerosis, cortical dysplasia and neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [14]. Recently we have demonstrated that Cd activates mTOR signaling pathway in PC12, SH-SY5Y cells and murine primary neurons, leading to neuronal apoptosis [16, 17]. However, whether and how exposure to Cd *in vivo* contributes to neurotoxicity via activation of mTOR signaling is largely unknown.

Extensive studies have shown that oxidative stress, e.g., reactive oxygen species (ROS), is a prominent feature of many neurodegenerative disorders including AD, PD, and amyotrophic lateral sclerosis [18–20]. Under pathological conditions, excessive or sustained ROS induced by Cd or other stimuli directly oxidize lipids, proteins, and nucleic acids, which lead to

damage of the basic cell structures and result in cellular dysfunction and cell death [21, 22]. Clinical data have shown that ROS level is elevated in the brains of patients suffering from AD or HD [23]. Increasing evidence suggests that the excessive production of ROS in the brain, and the imbalance between oxidative stress and antioxidant defenses is related to Cd exposure [24, 25].

Antioxidant drugs are becoming increasingly popular in the prevention of oxidative stress-related neurodegenerative disorders and hold promise as potential therapeutic agents [19, 24]. N-acetyl-L-cysteine (NAC), a thiol-containing compound, has been shown to act as an antioxidant by raising intracellular level of cysteine and restoring the pool of intracellular glutathione (GSH), or by directly scavenging ROS [24, 26]. NAC has the capacity to inhibit both acute brain injuries and chronic neurodegenerative disorders such as trauma, hypoxic-ischaemic brain injury and AD [26-28]. Goncalves et al have recently demonstrated that NAC may ameliorate Cd-induced neurotoxicity and improve the memory and learning processes of Cd-intoxicated rats [24]. In our recent studies, we observed that Cd induces the generation of ROS, which activates mTOR pathway leading to apoptosis in PC12 and SH-SY5Y cells, and NAC potently prevents the events [11, 29]. However, it is unknown whether supplementation of NAC is an effective means of preventing Cd-induced brain damage or neuronal cell death *in vivo*.

Here, we show that chronic exposure of mice to Cd induced brain damage or neuronal death, which was closely associated with induction of ROS and activation of Akt/mTOR pathway in the brain. Co-administration of NAC prevented Cd-induced neurotoxicity, at least in part by decreasing the level of Cd, increasing the activities of Cu/Zn-superoxide dismutase (Cu/Zn-SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as elevating the level of GSH, in the brain.

Materials and Methods

Chemicals

Cadmium chloride, xanthine, xanthine oxidase, cytochrome c, bovine serum albumin (BSA), superoxide dismutase (SOD), poly-D-lysine (PDL), N-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 2',7'-dichlorofluorescein (DCF), 3,3'-diaminobenzidine tetrachloride (DAB), 4',6-diamidino-2-phenylindole (DAPI), and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). Rapamycin was from ALEXIS (San Diego, CA, USA). Enhanced chemiluminescence solution was from Millipore (Billerica, MA, USA), whereas normal goat serum from Chemicon International Inc (Temecula, CA, USA). The following antibodies were used: phospho-Akt (Ser473), phospho-S6K1 (Thr389), phospho-4E-BP1 (Thr70), 4E-BP1 (Cell Signaling Technology, Beverly, MA, USA), Akt, S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -tubulin (Sigma), goat anti-rabbit IgG-horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP (Pierce, Rockford, IL, USA). Other chemicals were purchased from local commercial sources and were of analytical grade.

Cell culture

Primary murine neurons were isolated from mice as described [30]. Isolated cells were seeded at a density of 5×10^5 in a 6-well plate containing a glass coverslip per well or at a density of 2×10^6 cells/well in a 6-well plate coated with 10 $\mu\text{g/ml}$ PDL in NEUROBASAL™ Media (Invitrogen) supplemented with 2% B27 Supplement (Invitrogen), 2 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 $\mu\text{g/ml}$ insulin (Sigma), and 40 $\mu\text{g/ml}$ of gentamicin (Invitrogen), and grown in a humid incubator (37°C, 5% CO₂). Fresh medium was replaced every 3 days. The cells were used for experiments after 6 days of culture.

Assay for cell DAPI staining, and morphology

Isolated murine primary neurons were treated with/without Cd (10 and 20 μM) for 24 h following pre-incubation with/without rapamycin (0.2 $\mu\text{g/ml}$) for 48 h with triplicates of each treatment. Subsequently, cells were fixed with 4% paraformaldehyde prepared in PBS for 2 h at 4°C. The cells were washed three times with PBS, and then stained with DAPI (4 $\mu\text{g/ml}$ in deionized water) for 30 min at room temperature in the dark. Following a brief washing with PBS, slides were mounted in glycerol/PBS (1:1, v/v) containing 2.5% 1,4-diazabicyclo-(2,2,2)octane. Photographs were taken with a fluorescence microscopy (Nikon 80i, Japan) equipped with digital camera. Cells with condensed nuclei were scored to be apoptotic. In addition, for cell morphological analysis, after treatment for 24 h, images were taken with a Nikon Eclipse TE2000-U inverted phase-contrast microscope (Nikon, Japan) (200 $\times$) equipped with digital camera.

Animals and administration with Cd and NAC or with Cd and rapamycin

One hundred twenty male ICR mice, weighing 25-30 g, were obtained from the Laboratory Animal Center, Nanjing Medical University (Nanjing, China). All animals were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee, and were in compliance with the guidelines set forth by the Guide for the Care and Use of Laboratory Animals. The mice were housed at room temperature (20-25°C), relative humidity of 60%, subjected to a 12 h-light/dark cycle under conventional barrier protection, and supplied with water and feed *ad libitum*. After acclimatization to these conditions for 1 week, the mice were carried out for two series of experiments.

For experiments of treatment with Cd $\pm$ NAC, 80 mice were randomly divided into 8 groups (10 mice/group), which included normal control group, NAC treatment group, three Cd treatment groups, and three Cd/NAC treatment groups. The control group and the NAC treatment group received normal distilled water as drinking water, but the mice in the NAC treatment group were also intraperitoneally injected with NAC solution, which was dissolved in the physiological saline, at a dose of 150 mg/kg body weight every other day. The three Cd-treated groups were only given drinking water containing 10, 25, 50 mg/L of Cd, respectively. The three Cd/NAC treatment groups received drinking water containing 10, 25, 50 mg/L of Cd, respectively, plus each having intraperitoneal injection of NAC solution (150 mg/kg body weight) once every other day. The experiment lasted for 6 weeks. At the end of the experiment, all mice were anesthetized to collect blood samples by enucleating eyeballs. Blood samples were collected into test tubes containing heparin

sodium (1:500), and immediately centrifuged at 3000 rpm for 10 min at 4°C. The obtained plasma was stored at -20°C for analysis. Finally, all animals were sacrificed by cervical dislocation, and brain tissues were immediately removed, and fixed in 4% paraformaldehyde or stored at -80°C for further analysis.

For experiments of treatment with Cd ± rapamycin, rapamycin was dissolved in 0.2 ml of 100% ethanol and then diluted 100-fold with 40% propyleneglycol to obtain a final concentration of 0.75 mg/ml [31]. Forty mice were divided into 4 groups (10 mice/group), including Cd/rapamycin, Cd/vehicle, saline/rapamycin, and saline/vehicle groups. A subacute Cd regimen (1 mg/kg), with some modifications as described [32], was used. In brief, animals from each group received one intraperitoneal injection of Cd solution daily (1 mg/kg in saline) for 5 days and were sacrificed at the indicated time points after the last Cd injection. Rapamycin (7.5 mg/kg) [31] or vehicle was administered intraperitoneally daily, starting 2 days before the first Cd/saline injection and continuing for 4 days after the last Cd/saline injection. Rapamycin/vehicle was administered 30 min before each Cd/saline injection. Four days after the last Cd injection, all animals were sacrificed and brain tissues were immediately collected as described above.

Measurement of Cd level in plasma and brain tissues

To determine the level of Cd in plasma, 0.25 ml of plasma sample from each animal was digested by 1 ml of pure HNO₃ in an acid-washed tube for 4 h at room temperature. Subsequently, 0.5 ml of 30% H₂O₂ was added into the tube, followed by incubation in a water bath at 100°C overnight in order to digest the residual fat. The sample was adjusted to 2 ml by adding 2% HNO₃ for analysis.

To determine the level of Cd in brain tissue, approximately 0.1 g of brain sample was weighed, transferred into an acid-washed beaker, and digested in 1.5 ml of pure HNO₃ for 4 h and further with 0.75 ml of 30% H₂O₂ for 1 h in order to digest the residual fat. Next, the beaker was incubated on a low-temperature electric hot plate (at 120-150°C) until the digestion were completed, as indicated by being free of particulates and color in the solution, and the liquid was completely evaporated. The sample was finally adjusted to 3 ml by adding 2% HNO₃ for analysis.

The levels of Cd in the plasma and brain samples were determined using inductively coupled plasma-quadrupole mass spectrometer (ICP-QMS) ELAN 9000 (Perkin-Elmer Corp., Norwalk, CT, USA). The operating parameters for ICP-QMS are shown in Table 1. Recovery was established using Cd standard with quantification of ¹¹²Cd and ¹¹⁴Cd isotopes. The limit of detection for Cd was 3 pg/ml. The ICP-QMS was optimized using ¹¹⁵In isotope standards. Data are expressed as µg/L and ng/g for plasma and brain tissue, respectively. All reagents were of analytical grade, and ultrapure de-ionized water (18 MΩcm⁻¹) from a Milli-Q analytical reagent-grade water purification system (Millipore) was used throughout. Glassware was washed in pure HNO₃ and rinsed with the de-ionized water.

Assay of malondialdehyde (MDA) and GSH levels

Brain tissue was homogenized with 1:10 (w/v) PBS on ice. The homogenate was centrifuged at 3000 rpm for 15 min at 4°C, and the supernatant was collected. Protein concentration in

the supernatant was determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL, USA). The concentrations of malondialdehyde (MDA), as a maker of lipid peroxidation, and GSH in the samples were measured and expressed as nM/mg protein using the MDA and GSH assay kits (Jiancheng Institute of Biotechnology, Nanjing, China), according to the manufacturer's protocols.

Assay of Cu/Zn-SOD, CAT and GPx activities

Cu/Zn-SOD activity was assayed as described by McCord and Fridowich [33]. Solution A was obtained by mixing with 100 ml of 50 mM PBS (pH 7.4) containing 0.1 mM EDTA and 2 μ M cytochrome c prepared in 10 ml of 0.001 N NaOH solution containing 5 μ M xanthine. Solution B contained 0.2 U xanthine oxidase/ml and 0.1 mM EDTA. Brain tissue supernatant (50 μ l) was mixed with 2.9 ml of solution A, and the reaction was started by adding 50 μ l of solution B. The absorbance was measured at 550 nm using a Synergy™ 2 Multi-function Microplate Reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA). A blank control was run by replacing the supernatant with 50 μ l of ultra pure water. The activity of Cu/Zn-SOD was expressed as U/mg protein with reference to the activity of a standard curve of bovine Cu/Zn-SOD under the same conditions.

CAT activity of brain tissue supernatant was determined according to the method of Aebi [34]. In short, 0.65 ml of 50 mM PBS (pH 7.0) and a 50 μ l sample were added to a quartz cuvette, and the reaction was started by adding 0.3 ml of 30 mM H₂O₂. The decomposition of H₂O₂ was monitored at 240 nm at 25°C. CAT activity was calculated as nM H₂O₂ consumed/min/mg protein and data are expressed as U/mg protein.

GPx activity was measured as described by Lu et al [35]. In brief, tert-Butylhydroperoxide was used as a substrate. The assay examined the enzymatic reduction of H₂O₂ by GPx through consumption of GSH that is restored from oxidized glutathione GSSG in a coupled enzymatic reaction by GR. GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance due to NADPH consumption was measured at 340 nm. GPx activity was calculated using the molar extinction coefficient of 6.22 mM⁻¹ cm⁻¹. One unit of GPx was defined as the amount of enzyme that catalyzed the oxidation of 1.0 μ M of NADPH to NADP⁺ per min at 25 °C.

Assay of ROS

For brain tissues, ROS was measured based on the oxidation of DCFH-DA to DCF, as described previously [36]. Briefly, the brain homogenate was diluted 1:20 (vol/vol) with ice-cold Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.0 mM CaCl₂, 10 mM D-glucose, and 5 mM HEPES, pH 7.4) to obtain a concentration of 10 mg tissue/ml. The reaction mixture (1 ml) containing Locke's buffer (pH 7.4), 0.2 ml homogenate and 10 μ l of DCFH-DA (5 mM) was incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After further incubation for 30 min, Fluorescent intensity for the conversion of DCFH-DA to fluorescent product DCF was recorded by excitation at 485 nm and emission at 535 nm using a Synergy™ 2 Multi-function Microplate Reader. Background fluorescence (conversion of DCFH-DA in the absence of homogenate) was corrected by the inclusion of

parallel blanks. ROS formation was quantified from a DCF-standard curve and data are expressed as pM DCF formed/min/mg protein.

Analysis of transmission electron microscopy

To evaluate the changes in the ultrastructure of brain neurons between different experimental groups treated with or without Cd in the presence or absence of NAC, cerebral cortex from each group was promptly fixed with 4% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C for 3 h. A secondary fixation employed 1% osmium tetroxide in the same buffer for 2 h post overnight at 4°C by washing in PBS, followed by dehydration in a series of acetone (30, 50, 70, 80, 90 and 100%) and embedding in Epon 812. The specimens for electron microscopic observation were selected from 1 µm semi-thin sectional samples stained via 1% toluidine blue (pH 7.8). Ultra-thin sections (60 nm) were cut on an ultramicrotome (Leica Co., Wetzlar, Germany), collected on copper grids, and stained with 4% uranyl acetate and lead citrate. Neuronal ultrastructure was observed and photographed by transmission electron microscope (TEM) (Hitachi H-600-II, Hitachi Ltd., Tokyo, Japan).

Light microscopy

To detect the changes in the histology of brain neurons between different experimental groups treated with or without Cd in the presence or absence of rapamycin, brain tissues of two mice from each group, respectively, were fixed with 4% paraformaldehyde prepared in 0.1 M PBS (pH 7.4) at 4°C for 24 h. The samples were then dehydrated in increasing concentrations of ethanol (70, 85, 95 and 100%), embedded in paraffin and sectioned serially at 5 µm. After coronal sections were assembled on glass slides and stained with haematoxylin and eosin (H&E), the cerebral cortex and hippocampal subfields were viewed and photographed under a Nikon light microscope equipped with a digital camera (Eclipse 50iTa, Japan).

TUNEL staining

Neuronal apoptosis was determined by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining. The standard protocol for stained specimens was performed according to the manufacture's instructions of *In Situ* Cell Death Detection Kit® (Roche, Mannheim, Germany). Briefly, paraffin-embedded brain tissues were sectioned, followed by deparaffinization in xylene and rehydration in decreasing stages of ethanol (absolute, 95%, 90%, 80%, 70%, diluted in double distilled water). Each section was incubated with proteinase K working solution (20 µg/ml in 10 mM Tris/HCl, pH 7.4) for 15 min at room temperature and washed twice with PBS, followed by addition of TUNEL reaction mixture (TdT enzyme solution and labeling solution) to the samples and incubation for 1 h in a dark and humidified incubator at 37°C. After TUNEL labeling reaction, all stained specimens were rinsed three times with PBS and mounted with coverslips containing a mounting medium. Finally, the samples were analyzed by fluorescence microscopy (Nikon 80i, Japan) equipped with digital camera. For quantitative analysis of the fluorescence staining, the integral optical density (IOD) was measured by Image-Pro Plus 6.0 software (Media Cybernetics Inc., Newburyport, MA,

USA). For analysis, plaque areas were excluded and IOD in 0.01 mm² area (four areas per slide, three slides from each brain sample) was estimated at a consistent position per section.

Immunohistochemistry

After sections received deparaffinization and rehydration treatments, antigen retrieval was performed in 0.01M citrate buffer (pH 6.0) and endogenous peroxidase activity in the sectioned brain tissues was blocked with 3% H₂O₂ in methanol for 1 h. Following each step of the procedure, several washings in 0.1 M PBS (pH 7.4) were performed. Tissue sections were blocked in 3% normal goat serum (diluted in PBS containing 1% BSA) for 1 h at room temperature, and then incubated at 4°C overnight with the primary antibody to phospho-4E-BP1 (Thr70) (1:100, diluted in PBS containing 1% BSA). Subsequently, biotinylated goat anti-rabbit IgG secondary antibody (1:100, diluted in PBS containing 1% BSA) was applied for 90 min at room temperature, followed by incubation for 1 h with a streptavidin-biotin-horseradish peroxidase complex (SABC) (Boster Bio-engineering Limited Company, Wuhan, China). Horseradish peroxidase was reacted with 0.025% DAB in PBS containing 0.01% H₂O₂ for 3 min to yield a permanent deposit, and counterstained with Mayor's haematoxylin. Finally, stained sections were rinsed in distilled water, dehydrated in ethanol, cleared in xylene and mounted with coverslips. The specificity of the staining was evaluated by omitting the primary antibody. The images of stained sections were taken with a Nikon Eclipse Ti-S microscopy equipped with a digital camera.

Western blot analysis

Brain tissues homogenized in 3 ml of ice-cold RIPA buffer [50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1% Triton X-100; 10 mM NaF; 1 mM Na₃VO₄; protease inhibitor cocktail (1:1000)]. For *in vitro* murine primary neurons, after treatment, cells were briefly washed with cold PBS, and then on ice, lysed in RIPA buffer. Homogenates or lysates were sonicated for 10 s and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as a standard (Pierce). Equivalent amounts of protein were separated on 7.5-12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using enhanced chemiluminescence reagent (Millipore). To check the amount of protein loaded, the immunoblots were treated with stripping solution (62.5 mM Tris buffer, pH 6.7, containing 2% SDS and 100 mM β-mercaptoethanol) for 30 min at 50°C and incubated with mouse monoclonal anti-β-tubulin antibody (Sigma) followed by horseradish peroxidase-coupled goat anti-mouse IgG (Pierce).

Statistical analysis

Results were expressed as means and standard error of the mean (mean ± SEM). The Student's *t*-test for non-paired replicates was used to identify statistically significant differences between treatment means. Group variability and interaction were compared

using either one-way or two-way ANOVA followed by Bonferroni's post-tests to compare replicate means. Significance was accepted at $P < 0.05$.

Results

Administration of NAC reduces Cd levels in the plasma and brain of mice exposed to Cd

Cd severely affects the function of the nervous system [4], with symptoms including headache and vertigo, olfactory dysfunction, and parkinsonian-like symptoms [6, 7]. To investigate the toxicity of Cd in brain, and the protection of NAC against the neurotoxicity of Cd *in vivo*, eighty male ICR mice were randomly divided into 8 groups (10 mice/group) and exposed to drinking water containing different concentrations (0, 10, 25, 50 mg/L) of Cd for 6 weeks, with or without intraperitoneal injection of NAC (150 mg/kg body weight) once every other day. At the end of the experiments, plasma and brain samples from all animals were collected. By ICP-QMS analysis, we found that Cd levels in the brain tissues of the normal control and NAC alone-treated mice were approximately 15 and 17 ng/g wet weight, respectively. Exposure of the animals to drinking water containing 10, 25 and 50 mg/L of Cd increased Cd levels in the brain tissues by approximately 2, 3.6 and 4-fold, respectively, compared to that in the normal control (Fig. 1A). Interestingly, co-administration of NAC significantly reduced Cd levels in the brain of the mice exposed to Cd (10-50 mg/L) (Fig. 1A). Similar tendency was also seen in the plasma samples (Fig. 1B). The results suggest that administration of NAC can effectively reduce Cd levels in the plasma and brain of mice exposed to Cd.

Ultrastructure of the brain tissues from mice exposed to Cd

To elucidate how Cd causes brain damage in mice and how NAC prevents this event, we examined the ultrastructure of the cortex neurons by transmission electron microscopy. In the neurons from the normal control or NAC alone-treated samples, normal cytoplasm, organelles and nucleus were visualized. In particular, there existed abundance of mitochondrion with structural integrity and finely characteristic cristae, tubular sacs of rough endoplasmic reticulum (ER), ribosome, and elements of Golgi apparatus in the perinuclear region (Fig. 2A- and B-arrow inserted picture for magnification). The large round nucleus contained homogeneously dispersed chromatin and one or two electron dense nucleoli (Fig. 2A and B). However, in the neurons from Cd-treated samples, there were some mitochondria with distinct vacuolation, irregular swelling and cristae disruption, the vacuolated ER with dilated cisterns, the increase in the number of lysosomes or autolysosomes, as indicated by asterisks and arrows, as well as the inserted picture for higher magnification (Fig. 2C and E). Also, Cd induced chromatin condensation into small clumps abutting the nuclear envelope, revealing some typical structural changes of apoptosis (Fig. 2C and E). Of importance, NAC dramatically prevented Cd-induced morphological changes in neurons, showing a profile similar to that in the normal control (Fig. 2D and F, arrows and the inserted picture for higher magnification). The findings clearly indicate that NAC can efficiently protect brain neurons from Cd-induced severe damages.

NAC prevents ROS induction and neuronal apoptosis in the brain of Cd-exposed mice

Studies have consistently shown that the toxicity of Cd is related to its induction of ROS in various types of cells [37, 38]. Cd-induced ROS causes neuronal apoptosis *in vitro* [3, 39], but whether this occurs *in vivo* remains to be defined. Here we found that exposure of mice to drinking water containing 10-50 mg/L of Cd for 6 weeks resulted in a significant increase in ROS level in the brain of the animals (Fig. 3A). Administration of NAC (150 mg/kg) did not significantly alter the ROS level in the brain, but significantly attenuated Cd-induced ROS (Fig. 3A). Of note, NAC was able to restore the ROS to the basal level in all Cd-treated groups, compared to the normal control group (Fig. 3A). In addition, neuronal apoptosis in cerebral cortex and hippocampus CA1 area was evaluated using TUNEL staining. We found that the number of TUNEL-positive cells significantly increased in cerebral cortex and hippocampus of Cd-exposed mice, and this effect was Cd dose-dependent (Fig. 3B and C). In contrast, administration of NAC dramatically decreased the number of TUNEL-positive cells in the cerebral cortex and hippocampus of the mice exposed to Cd (Fig. 3B and C). The findings clearly indicate that chronic exposure of mice to Cd induces ROS, leading to neuronal cell death in cerebral cortex and hippocampus of the animals, which can be potentially prevented by co-administration of NAC.

NAC inhibits Cd induction of ROS by intervening MDA, GSH and antioxidant enzymes in the brain of mice

ROS level is tightly balanced in cells by the rate of production/clearance of ROS, catalyzed through oxidant and antioxidant enzymes [40, 41]. Cu/Zn-SOD is a primary enzyme that catalyzes superoxide anions ($O_2^{\bullet-}$) into peroxides, which are converted into water by CAT and GPx [41]. The amount of MDA is an indicator of oxidative stress status of a cell or tissue. GSH plays an important role in maintaining the intracellular redox balance and protecting the brain [21, 42]. To understand how NAC protects brain from Cd-induced oxidative damage, we investigated whether NAC affects the levels of MDA and GSH, as well as the activities of antioxidant enzymes, including Cu/Zn-SOD, CAT and GPx. As shown in Fig. 4, exposure of mice to drinking water containing 10-50 mg/L of Cd for 6 weeks significantly increased MDA levels, but significantly decreased GSH levels in the brain, compared to the control. Treatment with NAC alone decreased MDA level and increased GSH level in the brain slightly, but not significantly. However, co-administration of NAC very potently inhibited Cd-elevated MDA nearly to the control level, and restored Cd-decreased GSH close to the basal level in the brain (Fig. 4A and B). Similarly, exposure of mice to drinking water containing 10-50 mg/L of Cd for 6 weeks significantly decreased the activities of Cu/Zn-SOD, CAT and GPx dose-dependently in the brain, which were nearly rescued to the control level by NAC (Fig. 5A-C). These results imply that NAC may prevent ROS production by intervening MDA, GSH and antioxidant enzymes in the brain of Cd-exposed mice.

NAC prevents neuronal apoptosis by inhibiting ROS-activated Akt/mTOR pathway in the brain of Cd-exposed mice

Recently, we have demonstrated that Cd activates Akt/mTOR signaling pathway in PC12 and SH-SY5Y cells and primary neurons, leading to caspase-dependent apoptosis [16, 17].

We have also identified that Cd activation of Akt/mTOR pathway is due to induction of ROS in PC12 cells [11]. To explore the molecular mechanism by which Cd induces neuronal cell death *in vivo*, we tested whether Cd induction of ROS activates Akt/mTOR pathway, in the brain of Cd-exposed mice. By Western blot analysis, we found that exposure of mice to Cd-containing drinking water for 6 weeks resulted in a strong phosphorylation of Akt, S6K and 4E-BP1 in the brain, which was profoundly attenuated by co-administration of NAC (150 kg/kg) (Fig.6A and B). By immunohistochemistry, we also observed an intense staining of phospho-4E-BP1 in the cerebral cortex and hippocampus from Cd-exposed mice, which was abolished by NAC as well (Fig. 6C). In addition, Cd induced robust activation of caspase-3 in the brain, as detected by increased cleavage of caspase-3, and NAC drastically attenuated this event (Fig. 6D and E), which was in line with increased apoptosis as detected by TUNEL staining (Fig.3B and C).

To gain more insights into the event that NAC protects against Cd-induced neuronal apoptosis by inhibiting Akt/mTOR pathway *in vivo*, we extended our studies using rapamycin, a selective inhibitor of mTOR. Pretreatment of the primary neurons with rapamycin (0.2 µg/ml) for 48 h, mimicking the effect of NAC, dramatically inhibited Cd-induced phosphorylation of Akt, S6K1 and 4E-BP1 (Fig.7A and B), and obviously attenuated Cd-induced neuronal cell death (Fig.7C-E).

Based on the above *in vitro* findings, we next examined the effects of rapamycin *in vivo* on the subacute Cd model in which higher dose of Cd (1 mg/kg) are administered to mice intraperitoneally daily for 5 days, resulting in maximal induction of apoptotic profiles in the cerebral cortex and hippocampus 4 days after the last injection, as observed by us previously (data not shown). Rapamycin (7.5 mg/kg) [31] was administered to mice intraperitoneally once per day, 2 days before Cd treatment, which was the highest dose that could be used without major toxicity (peripheral effects) when administered along with Cd. Animals then received five Cd injections. Rapamycin or vehicle was administered 30 min before Cd. Mice were killed 4 days after the last Cd injection. Western blot analysis showed that administration of rapamycin *in vivo* fully blocked Cd-induced phosphorylation of Akt, S6K, and 4E-BP1 in the brain (Fig. 8A and B). Cd activation of caspase-3 was strikingly repressed as well (Fig. 8C and D). By H&E staining, the histology of the cerebral cortex and hippocampus was detected under a light microscope. Compared with the control, treatment with rapamycin alone did not apparently alter the histological structure of the cerebral cortex or hippocampus. However, treatment with Cd caused brain damage. As shown in Fig.8E, the stratified structure in the cortex was obviously disorganized, and the cellular distribution across the cortex exhibited blurred compartmental boundaries in the Cd-treated mice, compared to the normal control or rapamycin alone-treated counterparts. Furthermore, the distinctive cortical plate appeared much less compact, and the subplate region was poorly defined in Cd-treated mice. In addition, an infracted and abnormal appearance of histology was seen in the hippocampus of Cd-treated group. Of note, rapamycin remarkably prevented Cd-induced structural abnormality in the cortex and hippocampus (Fig. 8E). Consistently, rapamycin potently attenuated the number of TUNEL-positive cells in the cerebral cortex and hippocampus of Cd-treated mice (Fig. 8F). Recently, our studies have shown that rapamycin *in vitro* prevented Cd-induced death in PC12 cells by suppressing Cd-induced

ROS [11]. Of interest, rapamycin *in vivo* also inhibited Cd-induced ROS in the brain tissues (Fig. 8G). Taken together, the results strongly support that NAC protects against Cd-induced neuronal apoptosis in mouse brain by inhibiting ROS-activated Akt/mTOR pathway.

Discussion

Cd, an environmental toxin, adversely affects biological systems in various ways [24]. In humans, clinical and epidemiological data show that Cd exerts its toxic effects not only on the kidneys, liver and testis but also on the central nervous system (CNS) [24, 43]. Cd can penetrate the blood-brain barrier and accumulate in the brain contributing to the development of CNS damage [25, 44]. Cd toxicity leads to brain cellular dysfunction, lethal cerebral oedema and parkinsonism [10, 24]. Studies have shown that low Cd dosage can initiate apoptotic cell death in distinct brain regions via oxidative stress or excessive amounts of ROS generation, which is thought to play an important role in human neurodegenerative diseases [18-20, 24, 45]. Therefore, it is of great importance to find a novel therapeutic target and strategy to control the oxidative damage of Cd on brain in individuals with Cd-induced neurodegenerative diseases. The free radical scavenger or antioxidant NAC has been shown to directly reduce ROS level [24]. Recently, we have demonstrated that Cd-induced ROS activates the mTOR pathway leading to apoptosis in PC12 and SH-SY5Y cells, and NAC potentially blocks these events [11]. However, whether this can be recapitulated in the brain remains to be defined. Here, we provide evidence that Cd-induced neuronal apoptosis was closely associated with ROS induction and activation of Akt/mTOR pathway in the brain of mice. NAC protected against Cd-induced apoptosis of neurons in the brain through inhibiting ROS induction. Further, we found that NAC attenuated Cd-induced ROS by intervening MDA, GSH and antioxidant enzymes, including Cu/Zn-SOD, CAT and GPx, in the brain of mice.

Occupational exposure, dietary consumption and cigarette smoking are the main sources of Cd contamination in humans [1, 2]. Cd has a long biological half-life mainly due to its low rate of excretion from the body. Thus, prolonged exposure to Cd will cause toxic effects due to its accumulation over time in a variety of tissues [46]. A growing number of clinical investigations have pointed to Cd intoxication involved in brain neurons contributing to brain damage and neuronal cell apoptosis, as a possible aetiological factor of neurodegenerative diseases [10-12, 46, 47]. To imitate the status of human “environmental” and “occupational” exposure to Cd, Brzóska *et al* have worked out a rat model using 5 mg/L and 50 mg/L Cd in drinking water for 6-24 weeks accordingly, respectively [48, 49]. In addition, administration of Cd (1 mg/kg) by intramuscular injection once daily for 4 months (long-term exposure) affects total antioxidant status in adult rat brain [50, 51]. For a subchronic mouse model of exposure to Cd, animals receiving intraperitoneal injection of Cd (3 mg/kg) 5 days per week for 2 weeks or subcutaneous injection of Cd (10 µM/kg) 5 times per week for 4 weeks is also documented [52-54]. Based on the data from others and our preliminary experiments, in the current study, mice were exposed to drinking water containing 10-50 mg/L of Cd for 6 weeks, and co-administration of NAC was given by intraperitoneal injection at a dose of 150 mg/kg to elucidate neuroprotective effects and mechanisms of administered NAC in mice chronically exposed to Cd.

In this study, we found that chronic exposure of mice to drinking water containing 10-50 mg/L Cd for 6 weeks resulted in a significant Cd increase in the plasma and brain. When mice were exposed to Cd-containing water, a dose-dependent increase of Cd level occurred in the brain or plasma. Interestingly, in this study we observed that co-administration of NAC negated this effect, and was able to restore Cd close to normal control levels in the plasma and brain of mice exposed to Cd-containing drinking water. This is consistent with the evidence observed with various chelating agents e.g. thiol reagents, dithiothreitol or L-cysteine resulting in a decrease of tissue Cd concentration [50]. Many data have shown that NAC is a precursor of cysteine and GSH. NAC pretreatment before Cd exposure protected pig kidney epithelial cells (LLC-PK1) from Cd-induced cytotoxicity, not only by increasing the intra- and extracellular concentration of GSH, but also by extracellular reactions, lowering the uptake of Cd by the cells [55]. Also, α -lipoic acid, an antioxidant, can chelate several divalent cations, e.g. Mn^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , and Pb^{2+} [27, 56, 57]. Jalilehvand et al have demonstrated that NAC may chelate Cd^{2+} to form a complex in aqueous solution [58]. Whether NAC reduces Cd levels in the plasma and brain of Cd-exposed mice by a similar mechanism remains to be determined.

The mitochondrion is not only the energy factory but also the main generator of ROS for cells. Under physiological conditions, ROS derived from the mitochondria are important for a multitude of cell signaling processes; however, under pathological conditions, excessive ROS are formed and reduce mitochondrial biogenesis [59-61]. In turn, mitochondrial dysfunction further stimulates ROS generation and reduces mitochondrial respiration [62]. In cells, ROS homeostasis is tightly controlled by the ROS-generating and -eliminating systems [40]. The endogenous antioxidant enzymes such as SOD, CAT, and GPx play crucial roles in the elimination of ROS [40, 59]. SOD decomposes $O_2^{\bullet-}$ and produces H_2O_2 , which can be further catalyzed to water by CAT and GPx in the cells [41]. If the balance between ROS production and elimination is broken, the antioxidant enzymes may be exhausted [41]. Cd is a well-known inducer of ROS generation in cells [63]. In the previous studies, we also detected ROS generation induced by Cd in a time- and dose-dependent manner in PC12 and SH-SY5Y cells [11]. Here, we found that chronic treatment with 10-50 mg/L Cd-containing drinking water for 6 weeks dramatically impaired mitochondrial function, and lowered the activities of these antioxidant enzymes. All of these changes resulted in elevation of ROS level in the brain. As lipid peroxidation induced by ROS may also contribute to the neurotoxicity in mouse brain, we tested whether Cd affects the level of MDA, a product of lipid peroxidation. In Cd-exposed mice, elevated MDA content was observed in the brain, compared to normal control mice. In line with the above findings, we also observed that the number of TUNEL-positive cells significantly increased in cerebral cortex and hippocampus of Cd-exposed mice, suggesting that Cd induces ROS by inhibiting the activities of antioxidant enzymes leading to apoptosis of neuronal cells. Of interest, NAC significantly rescued the activities of all these enzymes, almost completely reduced ROS and MDA to the control levels, and potently decreased the number of TUNEL-positive cells in the brain of Cd-exposed mice. Our results strongly support the notion that oxidative stress-mediated apoptosis may play a central role in Cd-induced neurotoxicity.

A question that arises from this work is how Cd reduces the activities of SOD, CAT and GPx. It has been reported that Cd has a high affinity for sulfhydryl (-SH) groups [64-66].

Many SH groups exist in the active sites of SOD, CAT, and GPx, which are associated with their antioxidant activities [65, 66]. Likely, Cd may form complexes with SH groups of these enzymes, leading to a decrease of the enzymatic activities. Our results support the proposal that the depletion of intracellular SH groups by Cd is the prerequisite for ROS induction as well as disruption of intracellular organelles [65, 67]. It is known that SH-containing nutrients or amino acids play an important role in detoxification and protection of cells and cellular components against oxidative stress [24, 68, 69]. As NAC is an excellent source of SH groups, we deduce that NAC may provide enough SH groups for cells, which may competitively prevent Cd from interaction with the SH groups in the antioxidant enzymes. Clearly, more studies are needed to address this issue.

mTOR has been widely recognized as a central controller for cell proliferation, growth and survival [13]. In adult brain, mTOR is crucial for synaptic plasticity, learning and memory formation, and brain control of food uptake [14, 15]. Recent studies have shown that mTOR activity is modified in various pathological states of the nervous system, including brain tumours, tuberous sclerosis, and neurodegenerative disorders such as AD, PD, and HD [14]. For example, mTOR is excessively activated in the case of tuberous sclerosis, neurofibromatosis I or AD brain, but inactivated in PD and HD [14]. Because Cd potentially induces apoptosis of neuronal cells [3, 4], we originally speculated that Cd might suppress mTOR signaling. However, to our surprise, exposure of PC12 and SH-SY5Y cells to Cd induced robust phosphorylation of mTOR and its downstream effector molecules, such as S6K1 and 4E-BP1, and inhibition of mTOR by rapamycin prevented Cd-induced apoptosis [17], indicating that Cd causes neuronal apoptosis by activation of the mTOR pathway. Recently we have further demonstrated that Cd induced apoptosis of PC12 and SH-SY5Y cells by ROS-mediated mTOR activation [11]. In the present study, using Western blotting, we validated that exposure of mice to Cd-containing drinking water resulted in strong phosphorylation of Akt, S6K and 4E-BP1 in the brain. By immunohistochemical analysis, we also observed a very high level of phospho-4E-BP1 expression in the cerebral cortex and hippocampus of Cd-exposed mice. Importantly, NAC obviously reversed Cd-induced activation of Akt/mTOR pathway in the mouse brain. Furthermore, consistent with our previous findings in PC12 and SH-SY5Y cells [11], we also found that pretreatment with NAC (5 mM) for 1 h was able to prevent Cd (10-20 μ M, 24 h)-induced cell death in the murine primary neurons (data not shown). To corroborate the above findings, rapamycin, a selective inhibitor of mTOR, was utilized. Pretreatment of the primary neurons with rapamycin for 48 h, mimicking the effect of NAC, dramatically inhibited Cd-induced phosphorylation of Akt, S6K1 and 4E-BP1, and obviously attenuated Cd-induced neuronal cell death. Further, we extended the experiments for rapamycin *in vivo*. We observed that administration of rapamycin *in vivo* also blocked Cd-induced phosphorylation of Akt, S6K, and 4E-BP1, as well as activation of caspase-3 in the brain (Fig. 8A-D). Concurrently, rapamycin potentially attenuated Cd-induced brain damage and neuronal cell death, as evidenced by the histology and the number of TUNEL-positive cells in the cerebral cortex and hippocampus. Furthermore, rapamycin *in vivo* also inhibited Cd-induced ROS in the brain tissues (Fig. 8G), which is in line with our previous observations that rapamycin *in vitro* protects against Cd-induced neuronal cell death by suppressing Cd-induced ROS via down-regulating expression of a ROS generating enzyme (NOX2) and its regulatory

proteins [11]. Collectively, our findings indicate that Cd induction of ROS activates Akt/mTOR pathway, leading to neuronal apoptosis not only *in vitro*, but also *in vivo*, and NAC protects from Cd-induced neuronal apoptosis in mouse brain in part by inhibiting ROS-activated Akt/mTOR signaling pathway.

In conclusion, here we have shown that chronic exposure of mice to Cd-containing drinking water induced brain damage or neuronal cell death by induction of ROS, which could be remarkably attenuated by co-administration of NAC. Mechanistically, NAC reduced Cd level, increased Cu/Zn-SOD, CAT and GPx activities, and elevated GSH level, thereby reducing ROS and MDA levels and blocking ROS-activation of Akt/mTOR signaling pathway in the brain. Our findings suggest that inhibitors of mTOR, SH-containing nutrients/antioxidants, and especially NAC may be exploited, for prevention and treatment of Cd-induced neurodegenerative diseases.

Acknowledgments

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Fig. 1.

NAC reduces Cd levels in the plasma and brain of Cd-exposed mice. (A, B) A significant dose-dependent increase of Cd level was detected, by ICP-QMS, in the plasma (A) and brain (B) in mice exposed to Cd-containing drinking water for 6 weeks. Co-treatment with NAC greatly restored Cd close to the normal control level in the plasma (A) and brain (B) of Cd-exposed mice. Each value is presented as mean $\pm$ SEM, n=5. * P < 0.05 vs control group.

Fig. 2.

NAC prevents Cd-induced neuronal cell injuries in the brain neurons of mice. Ultra-thin cerebral cortex sections were stained with uranyl acetate and lead citrate, followed by examining the ultrastructure of neurons under a transmission electron microscope. Typical ultrastructural micrographs for the cerebral cortex neurons of mice exposed to drinking water containing 0, 10 and 25 mg/L of Cd for 6 weeks, together co-treatment with or without NAC (150 mg/kg), are shown. (A and B) Normal control group (0 mg/L Cd) and NAC alone treatment group, showing normal appearance of cytoplasm, organelles and nucleus in neurons. (C and E) 10 and 25 mg/L Cd treatment groups, respectively, showing some mitochondria with distinct vacuolation, irregular swelling and cristae disruption, the vacuolated ER with dilated cisterns, the increase in the number of lysosomes or autolysosomes as indicated by asterisks and by arrows or the inserted picture for magnification. (D and F) 10 and 25 mg/L Cd/NAC treatment groups. NAC dramatically prevented Cd-induced morphological changes in neurons, showing a profile similar to that in the normal control. Scale Bar: 50 nm.

Fig. 3.

NAC prevents Cd induction of ROS and neuronal cell death in the brain of mice. (A) Exposure of mice to drinking water containing of 10-50 mg/L of Cd for 6 weeks resulted in a significant increase of ROS level in the brain, which was almost completely blocked by co-treatment with NAC (150 mg/kg). ROS level was measured based on the oxidation of DCFH-DA to DCF using a microplate reader. (B) Neuronal apoptosis in cerebral cortex and hippocampus CA1 sections was evaluated by in situ detection of fragmented DNA using TUNEL staining. The apoptotic cells were stained in red. In cerebral cortex and hippocampus CA1 sections, the upper panels show a general view of apoptotic cells, and the lower panels show the magnifications of the fields selected in the upper panels. Scale bar: 50 μ m. (C) Analysis of the number of TUNEL-positive cells in cerebral cortex and hippocampal CA1 sections, showing that NAC potently decreased the number of TUNEL-positive cells induced by Cd-containing drinking water. Each value is presented as mean $\pm$ SEM, n=5. * P < 0.05, ** P < 0.01 vs control group; ### P < 0.01 vs Cd treatment group.

Fig. 4.

NAC decreases MDA and increases GSH level in the brain of Cd-exposed mice. (A) Comparison of MDA levels in the brain between all groups. (B) Comparison of GSH levels in the brain between all groups. Each value is presented as mean $\pm$ SEM, n=5. * P < 0.05, ** P < 0.01 vs control group; # P < 0.05, ## P < 0.01 vs Cd treatment group.

Fig. 5.

NAC restores Cu/Zn-SOD, CAT and GPx activities in the brain of Cd-exposed mice. (A) Comparison of Cu/Zn-SOD activities in the brain between all groups. (B) Comparison of CAT activities in the brain between all groups. (C) Comparison of GPx activities in the brain between all groups. Each value is presented as mean $\pm$ SEM, $n=5$. * $P < 0.05$ vs control group; # $P < 0.05$ vs Cd treatment group.

Fig. 6.

NAC inhibits activation of Akt/mTOR signaling and caspase-3 in the brain of Cd-exposed mice. Brain tissues in all groups were homogenized and supernatants were collected. Equivalent amounts of protein were subjected to Western blotting using indicated antibodies. β -tubulin was used as a loading control. Similar results were observed in at least three independent experiments. (A) Representative immunoblots for p-Akt (Ser473), total Akt, p-S6K1 (Thr389), total S6K1, p-4EBP1 (Thr70), total 4EBP1, and β -tubulin in mouse brain tissues. (B) Blots for p-Akt, p-S6K1, p-4EBP1 were semi-quantified using NIH image J. Relative density is expressed as the ratio (p-Akt/ β -tubulin; p-S6K1/ β -tubulin; p-4EBP1/ β -tubulin). (C) Phospho-4EBP1 in the cerebral cortex and hippocampus was determined using immunohistochemistry, showing that Cd induced a dramatic level of p-4EBP1 (Thr70) expression in the cerebral cortex and hippocampus of mice, which was abolished by NAC. (D) Representative immunoblot for cleaved-caspase-3 in mouse brain tissues. (E) Blot for cleaved-caspase-3 was semi-quantified using NIH image J. Relative density is expressed as the ratio cleaved-caspase-3/ β -tubulin. Each value is presented as mean $\pm$ SEM, $n=3$. ^a $P < 0.05$, ^b $P < 0.01$ vs control group; ^c $P < 0.05$ vs 10 mg/L Cd treatment group; ^d $P < 0.05$ vs 25 mg/L Cd treatment group.

Fig. 7.

Rapamycin inhibits Cd-induced activation of Akt/mTOR signaling and neuronal cell death in the murine primary neurons. Primary neurons were pretreated with/without rapamycin (Rap, 0.2 μg/ml) for 48 h, and then exposed to Cd (10 and/or 20 μM) for 4 h or 24 h. The cell lysates were subjected to Western blotting using indicated antibodies. β-tubulin was used as a loading control. Similar results were observed in at least three independent experiments. The manifestations for cell death were evaluated using DAPI staining and cell morphological analysis. (A) Representative immunoblots for p-Akt (Ser473), total Akt, p-S6K1 (Thr389), total S6K1, p-4EBP1 (Thr70), total 4E-BP1, and β-tubulin in all groups. (B) Blots for p-Akt, p-S6K1, p-4E-BP1 were semi-quantified using NIH image J. Relative density is expressed as the ratio (p-Akt/β-tubulin; p-S6K1/β-tubulin; p-4E-BP1/β-tubulin). (C) Pretreatment with rapamycin obviously attenuated Cd inhibition of cell viability. (D) Four typical fluorescent micrographs for apoptotic cells with nuclear condensation and fragmentation (arrows) are shown. Scale bar: 10 μm. (E) Cd significantly increased percentage of cells with condensed and fragmented nuclei, which was potently attenuated by pretreatment with rapamycin. All values are presented as mean ± SEM, n=3. ^a*P* < 0.05, ^b*P* < 0.01 vs control group; ^c*P* < 0.01 vs 10 μM Cd group; ^d*P* < 0.01 vs 20 μM Cd group.

Fig. 8.

Rapamycin attenuated brain damage and cell death in the subacute Cd-treated mice by inhibiting activation of Akt/mTOR signaling and caspase-3. A subacute regimen of intraperitoneally administered Cd (1 mg/kg) ± rapamycin (7.5 mg/kg) for 11 days in mice was subjected. Brain tissues from all groups were homogenized and supernatants were collected. Equivalent amounts of protein were subjected to Western blotting using indicated antibodies. Semi-thin cerebral cortex and hippocampus sections were stained with H&E and histologically assessed using a light microscope. Neuronal apoptosis in cerebral cortex and hippocampus CA1 sections was evaluated by *in situ* detection of fragmented DNA using TUNEL staining. ROS level was measured based on the oxidation of DCFH-DA to DCF using a microplate reader. (A and C) Representative immunoblots for p-Akt (Ser473), total Akt, p-S6K1 (Thr389), total S6K1, p-4EBP1 (Thr70), total 4E-BP1, pro-caspase-3, cleaved-caspase-3, and β-tubulin in mouse brain tissues. (B and D) Blots for p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. Relative density is expressed as the ratio (p-Akt/β-tubulin; p-S6K1/β-tubulin; p-4E-BP1/β-tubulin; cleaved-caspase-3/β-tubulin). (E) Four typical light micrographs for the cerebral cortex and hippocampus of mice exposed to Cd, together co-treatment with or without rapamycin, are shown. Note: Cd induced obvious perturbation of anatomic structure in cerebral cortex and distinct abnormality of histological appearance in hippocampus, which were obviously prevented by rapamycin. CP, cortical plate; CP1-3, upper, middle, and lower tiers of the cortical plate; IZ, intermediate zone; MZ, marginal zone; SP, subplate; VZ/SVZ, ventricular/subventricular zones. (F) Statistical analysis of the number of TUNEL-positive cells in cerebral cortex and hippocampal CA1 sections, showing that rapamycin potently attenuated the number of TUNEL-positive cells induced by Cd. (G) Rapamycin inhibited Cd-induced ROS in the brain tissues. Each value is

presented as mean $\pm$ SEM, n=3-5. ^a*P* <0.05, ^b*P* <0.01 vs control group; ^c*P* <0.05 vs 1 mg/kg Cd treatment group.

Table 1

ICP-QMS operating conditions and measurement parameters

RF Power	1100 W
Nebulizer gas flow	0.94 L/min
Dwell time	25 ms
Speed of peristaltic pump	26 rpm
Scan mode	Peak hopping
Reading	50 sweeps/1 reading/2 replicates
Detector	Pulse
Sampler/Skimmer cones	Nickel
Spray chamber	Ryton® Double-pass Scott-type spray chamber
Nebulizer	Gem-tip Cross-Flow pneumatic nebulizer