Original Article

Cadmium Activates Reactive Oxygen Species-dependent AKT/mTOR and Mitochondrial Apoptotic Pathways in Neuronal Cells*

YUAN Yan[1,2,†], WANG Yi[1,2,†], HU Fei Fei[1,2], JIANG Chen Yang[1,2], ZHANG Ya Jing[1,2], YANG Jin Long[1,2], ZHAO Shi Wen[1,2], GU Jian Hong[1,2], LIU Xue Zhong[1,2], BIAN Jian Chun[1,2], and LIU Zong Ping[1,2,#]

1. College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, Jiangsu, China; 2. Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, Jiangsu, China

Abstract

Objective To examine the role of Cd-induced reactive oxygen species (ROS) generation in the apoptosis of neuronal cells.

Methods Neuronal cells (primary rat cerebral cortical neurons and PC12 cells) were incubated with or without Cd post-pretreatment with rapamycin (Rap) or N-acetyl-L-cysteine (NAC). Cell viability was determined by MTT assay, apoptosis was examined using flow cytometry and fluorescence microscopy, and the activation of phosphoinositide 3'-kinase/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and mitochondrial apoptotic pathways were measured by western blotting or immunofluorescence assays.

Results Cd-induced activation of Akt/mTOR signaling, including Akt, mTOR, p70 S6 kinase (p70 S6K), and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). Rap, an mTOR inhibitor and NAC, a ROS scavenger, blocked Cd-induced activation of Akt/mTOR signaling and apoptosis of neuronal cells. Furthermore, NAC blocked the decrease of B-cell lymphoma 2/Bcl-2 associated X protein (Bcl-2/Bax) ratio, release of cytochrome c, cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), and nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease G (Endo G).

Conclusion Cd-induced ROS generation activates Akt/mTOR and mitochondrial pathways, leading to apoptosis of neuronal cells. Our findings suggest that mTOR inhibitors or antioxidants have potential for preventing Cd-induced neurodegenerative diseases.

Key words: Cadmium; Apoptosis; AKT/mTOR pathway; Mitochondrial apoptotic pathway; Primary rat cerebral cortical neurons; PC12 cells

Biomed Environ Sci, 2016; 29(2): 117-126 *doi: 10.3967/bes2016.013* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

*This work was supported by the National Natural Science Foundation of China (No. 31101866 and 31302058); a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), China Postdoctoral Science Foundation funded project (2015M581874); and Jiangsu Planned Projects for Postdoctoral Research Funds (1501072A).

†These authors contributed equally to this work.

#Correspondence should be addressed to Dr LIU Zong Ping, Tel: 86-514-87991448, Fax: 86-514-87972218, E-mail: liuzongping@yzu.edu.cn

Biographical notes of the first authors: YUAN Yan, female, born in 1979, Associate Professor and PhD, majoring in neurotoxicology caused by cadmium; WANG Yi, female, born in 1979, lectorate and PhD, majoring in neurotoxicology caused by cadmium.

INTRODUCTION

Cd is a metal frequently used in various industrial activities such as battery and alloy manufacturing, and it is a ubiquitous environmental contaminant present in tobacco smoke and food[1-2]. It is a toxic metal capable of damaging several organs, including the brain[3-4]. Previous studies have shown that Cd-induced neurotoxicity is a result of reactive oxygen species (ROS) generation, which leads to oxidative stress[5-8]. Under pathological conditions, excessive amounts of Cd-induced ROS can activate related signaling pathways, resulting in apoptosis of neuronal cells[6,9].

The mammalian target of rapamycin (mTOR), a 289-kD Ser/Thr kinase, lies downstream of protein kinase B (Akt/PKB) and regulates cell proliferation, growth, and survival[10]. Activation of Akt may positively regulate mTOR, leading to increased phosphorylation of p70 S6 kinase (p70 S6K) and eukaryotic initiation factor 4E binding protein 1(4E-BP1), the two best-characterized downstream effector molecules of mTOR[10-11]. Cd-induced ROS generation has been reported to be related to activation of the mTOR pathway in neuronal model cells such as rat pheochromocytoma (PC12) and human neuroblastoma (SH-SY5Y) cell lines[7]. This prompted us to examine whether Cd activates mTOR by inducing ROS in primary neurons.

Mitochondria have been shown to play a central role in apoptosis[12]. Mitochondrial damage results in the release of pro-apoptotic proteins such as cytochrome c (Cyt C) and apoptosis-inducing factor (AIF), which trigger caspase-dependent or caspase-independent cell death[13]. Recently, we demonstrated that Cd-induced apoptosis is partially associated with the activation of caspase-dependent and caspase-independent mitochondrial signaling pathways in primary neurons and PC12 cells[14-15]. Excess generation of ROS might contribute to mitochondrial damage and cause cell death by triggering endogenous apoptotic cascade reactions[16]. However, little is known about the role of ROS in Cd-mediated activation of mitochondrial signaling pathways in neuronal cells.

Here, we show that Cd induces ROS generation, which is correlated with activation of the Akt/mTOR pathway, that in turns leads to neuronal apoptosis. We also show that Cd-induced generation of ROS activates caspase-dependent and caspase-independent mitochondrial signaling pathways, which leads to neuronal apoptosis.

MATERIALS AND METHODS

Reagents

NEUROBASAL™ Medium and B27 Supplement were purchased from Invitrogen (Grand Island, NY, USA). Fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM)-F12 (1:1), cadmium acetate ($CdAc_2$), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Poly-D-lysine (PDL), Hoechst 33258, rapamycin (Rap), and penicillin/streptomycin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). RPMI 1640 medium and horse serum were supplied by Life Technologies (Grand Island, NY, USA). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from BD Biosciences (San Diego, CA, USA). Antibodies against Akt, phospho-Akt (Thr308), mTOR, phospho-mTOR (Ser2448), p70 S6K, phospho-p70 S6K (Thr389), phospho-4E-BP1 (Thr37/46), cleaved caspase-3, cleaved poly (ADP-ribose) polymerase (PARP), cytochrome c (Cyt C), B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), β-actin, and cyclooxygenase (COX)-IV were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against apoptosis-inducing factor (AIF) and endonuclease G (Endo G) were obtained from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tetramethylrhodamine (TRITC)-conjugated AffiniPure goat anti-rabbit IgG and FITC-conjugated AffiniPure goat anti-rabbit IgG were obtained from Bioworld Technology (Minneapolis, MN, USA). Enhanced chemiluminescence solution was obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents were of analytical grade.

Ethics Statement

Fetal Sprague-Dawley rats at 18-19 d of gestational age were obtained from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The Animal Care and Use Committee of Yangzhou University approved all experiments and procedures conducted in the animals [approval ID: SYXK (Su) 2007-0005].

Cell Culture

The rat pheochromocytoma (PC12) cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and was used for no more than 10 passages. PC12 cells were cultured in antibiotic-free RPMI 1640 medium supplemented with 10% horse serum and 5% FCS. Cells were maintained in a humid incubator (37 °C, 5% CO_2).

Primary rat cerebral cortical neurons were cultured from fetal Sprague-Dawley rats at 18-19 d of gestational age, as described previously[17]. The cells were used for experiments after 6 d of culture.

Cell Viability Assay

PC12 Cells were seeded at a density of 2×10^4 cells/well in 96-well plates precoated with PDL. Cells were treated with 10 μmol/L $CdAc_2$ for 24 h following pre-incubation with or without NAC (100 μmol/L) for 1 h, with six replicates of each treatment. At the designated time points, cell viability was measured by the MTT assay, which is based on the conversion of the tetrazolium salt to the colored product, formazan. In brief, 20 μL MTT solution (0.5 g/L in PBS) was added into each well of the 96-well plates (containing 100 μL medium and cells) 4 h before the end of incubation. The supernatant was then discarded, and 150 μL DMSO was added to dissolve the formazan. The absorbance was measured at 570/630 nm by the microplate reader (Sunrise, Austria).

Apoptosis Assay

PC12 cells were cultured in six-well plates precoated with PDL at a density of 5×10^5 cells/well. After 24 h of Rap (200 nm) pre-incubation, cells were co-treated with or without $CdAc_2$ (10 μmol/L) for 24 h, followed by performing an apoptosis assay using the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining kit according to the manufacturer's instructions. The percentage of apoptotic cells was quantified by a BD-FACS Aria flow cytometer (Becton-Dickinson, San Jose, CA, USA). The apoptosis percentage was summed up from primary apoptosis (Annexin V^+/PI^-) and late apoptosis (Annexin V^+/PI^+).

Hoechst 33258 Staining

Primary rat cerebral cortical neurons and PC12 cells were cultured in six-well plates (precoated with PDL) at a density of 5×10^5 cells/well. After 24 h of Rap (200 nmol/L) or 1 h of NAC (100 μmol/L) pre-incubation, cells were co-treated with or without $CdAc_2$ (10 μmol/L) for 24 h. An apoptosis assay was then performed using the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining kit according to the manufacturer's instructions.

Apoptotic morphological changes in the nuclear chromatin of primary rat cerebral cortical neurons and PC12 cells were examined using Hoechst 33258 staining as previously described[18].

Subcellular Fractionation

After treatment, primary rat cerebral cortical neurons or PC12 cells were briefly washed twice with cold phosphate-buffered saline (PBS). To obtain cytosolic and mitochondrial protein extracts, cells were subfractionated in homogenization buffer. The cytosolic and mitochondrial fractions were isolated separately by centrifugation as described[19]. The resulting pellet was composed of the mitochondrial fraction and the supernatant was composed of the cytosolic fraction.

Western Blotting Analysis

Primary rat cerebral cortical neurons or PC12 cells were treated with or without 10 μmol/L Cd for 24 h following 1 h of NAC (100 μmol/L) pre-incubation or 24 h of Rap (200 nmol/L) pre-incubation. After treatment, western blotting analysis was performed as described previously[14-15]. Equal amounts of protein were separated on 10%-12% SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking in 5% fat-free milk for 2 h, the membranes were incubated overnight at 4 °C with antibodies against cleaved caspase-3, cleaved PARP, cytochrome c, COX-IV, Akt, phospho-Akt (Thr308), mTOR, phospho-mTOR (Ser2448), p70 S6K, phospho-p70 S6K (Thr389), phospho-4E-BP1 (Thr37/46) (1:1000 dilution) or Bcl-2, Bax, and β-actin (1:2000 dilution). Detection was performed with the appropriate HRP-conjugated secondary antibodies (1:5000 dilution) and an enhanced chemiluminescence reagent. All assays were performed in duplicate.

Immunofluorescence Assays

PC12 cells were seeded at a density of 1×10^6 cells/well in six-well plates. Cells were treated with or without 10 μmol/L Cd for 24 h following 1 h pre-incubation with or without NAC (100 μmol/L); the treatments were performed in triplicate. After washing in PBS, cells were fixed on coverslips with 4%

paraformaldehyde. Then, the cells were permeabilized with 0.5% Triton X-100 and blocked with 5% BSA. Cells were incubated overnight with rabbit anti-AIF (1:100 dilution) or anti-EndoG (1:100 dilution) antibody in blocking solution at 4 °C, rinsed in blocking solution, and then stained with goat anti-rabbit IgG-FITC (1:200 dilution) for 1 h at room temperature. Cells were co-stained with Hoechst 33258 to visualize the nuclear morphology. Samples were examined under a fluorescence microscope to analyze AIF or EndoG translocation into the nucleus.

Statistical Analysis

Values were expressed as the mean±standard deviation (SD), and significance was assessed by the Student's *t*-test. Results were considered highly significant at $P<0.01$.

RESULTS

Cd Induces Activation of AKT/mTOR Signaling

Primary rat cerebral cortical neurons and PC12 cells were treated with different concentrations of $CdAc_2$ (0-20 μmol/L) for 24 h, or with 10 μmol/L $CdAc_2$ for 0-24 h. Activation of Akt/mTOR signaling was detected by western blotting (Figure 1). As shown in Figure 1A, treatment with 0-20 μmol/L Cd for 24 h resulted in a concentration-dependent increase in the phosphorylation of Akt, as well as mTOR and its downstream effector molecules (p70 S6K and 4E-BP1) in primary rat cerebral cortical neurons and PC12 cells. Furthermore, Cd induced the activation of Akt/mTOR signaling within 24 h (Figure 1B). Cd did not obviously alter total protein levels (Figure 1). These findings clearly indicate that Cd activates Akt/mTOR signaling pathways in neuronal cells.

Cd-Induced Neuronal Cell Apoptosis is Associated with Activation of Akt/mTOR Signaling

To ascertain whether Cd-induced activation of mTOR signaling is related to neuronal apoptosis, primary rat cerebral cortical neurons and PC12 cells were pre-treated with Rap (200 nmol/L), an inhibitor of mTOR, for 24 h, and then exposed to Cd (10 μmol/L) for 24 h. This was followed by Hoechst 33258 staining (primary rat cerebral cortical neurons and PC12 cells) or flow cytometry (PC12 cells). Rap alone did not appear to alter nuclear morphology or apoptosis rate. However, Rap markedly prevented Cd-induced increase of apoptosis rate and the appearance of nuclear morphological changes typical of apoptosis [i.e., condensed nuclear chromatin and fragmented nuclei characterized by a scattered, drop-like structure (Figure 2A-2C)]. Furthermore, using western blotting, we confirmed that the Cd-activated phosphorylation of Akt, mTOR, p70 S6K, and 4E-BP1 was severely blocked by Rap (Figure 2D). Therefore, our data clearly indicated that Cd may at least partially induce apoptosis of the neuronal cells through activation of the mTOR signaling pathway.

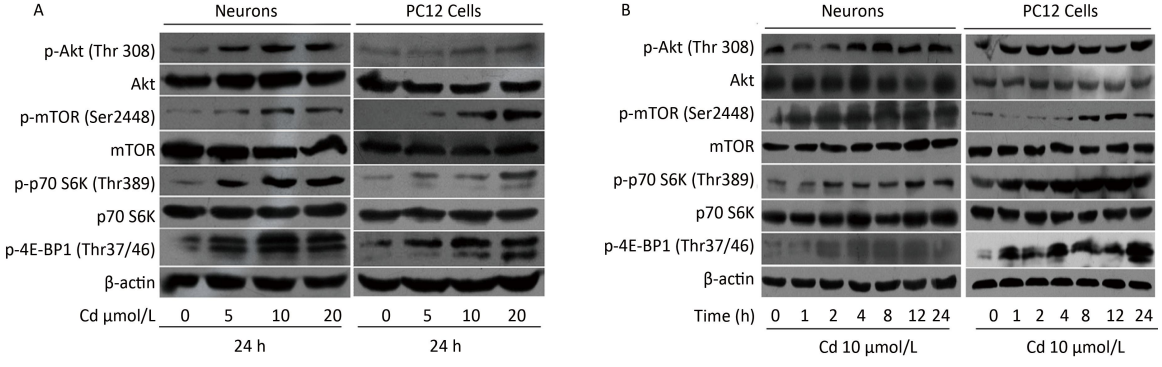

Figure 1. Cd activated Akt/mTOR signaling pathways in neuronal cells. Primary rat cerebral cortical neurons and PC12 cells treated with Cd (0-20 μmol/L) for 24 h (A), or with Cd (10 μmol/L) for 0-24 h (B) were harvested and total lysates were subjected to western blotting analysis using antibodies against the indicated proteins. (A and B) Cd induced activation of Akt, mTOR, p70 S6K, and 4E-BP1 in primary rat cerebral cortical neurons and PC12 cells. β-actin was used as a loading control. Similar results were observed in at least three independent experiments.

Cd-Induced Generation of ROS Activates Akt/mTOR Apoptotic Pathway in Neuronal Cells

Recently, we have demonstrated that Cd-induced apoptosis is associated with induction of ROS generation in primary neurons[20]. The antioxidant NAC, an ROS scavenger, has been shown to block Cd-induced ROS production and neuronal apoptosis in primary neurons and PC12 and SH-SY5Y cells[6,21]. To confirm that Cd-induced neuronal apoptosis is indeed due to its induction of ROS generation, primary rat cerebral cortical neurons and PC12 cells were pretreated with NAC (100 μmol/L) for 1 h, and then co-treated with or without Cd (10 μmol/L) for 24 h. Apoptotic morphological analysis revealed that NAC partially abolished Cd-induced nuclear morphological changes typical of apoptosis (Figure 3A and 3B). Furthermore, MTT assay results (Figure 3C) further demonstrated that NAC can partially suppress Cd-induced loss of cell viability in PC12 cells. These results suggest that Cd-induced neuronal apoptosis may be associated with its induction of ROS.

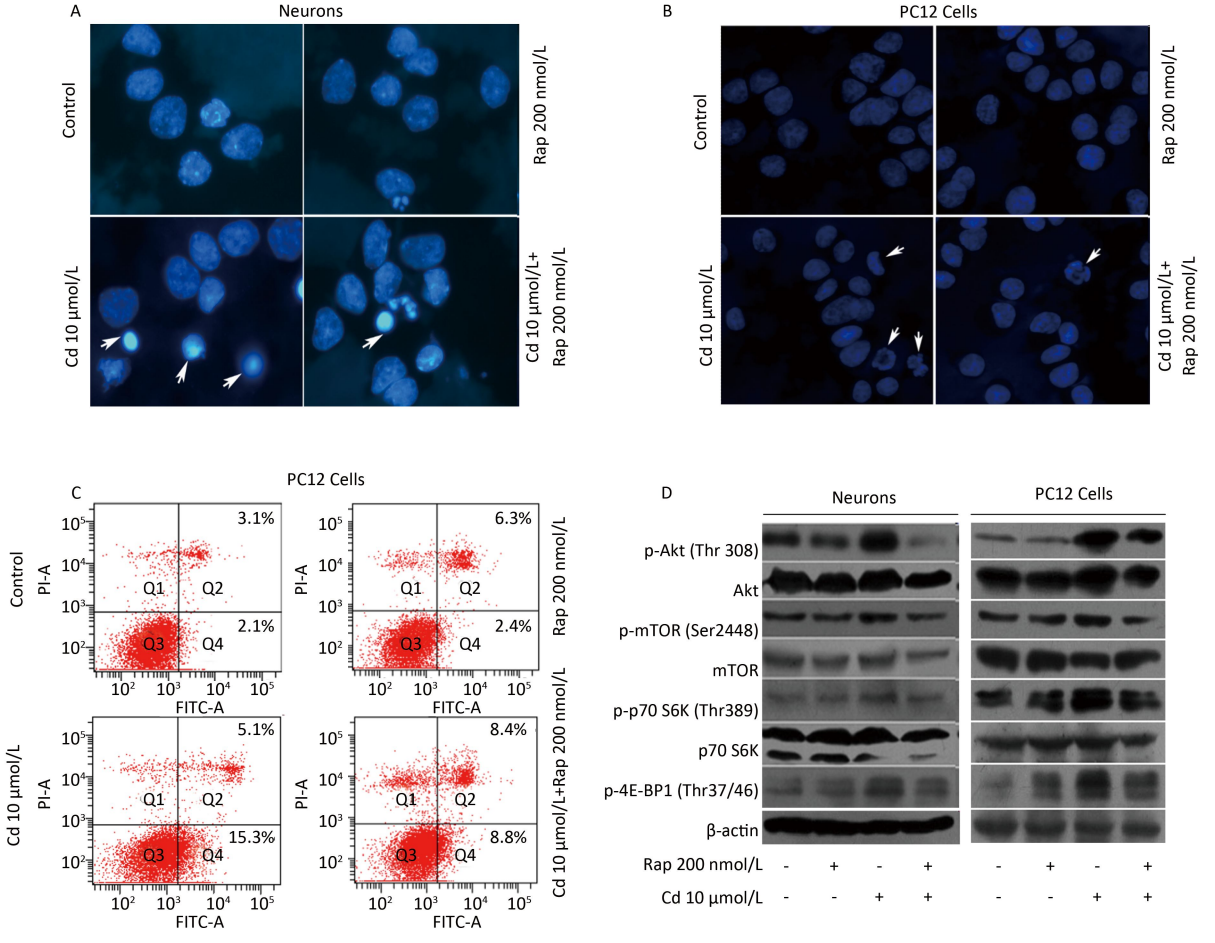

Figure 2. Inhibition of mTOR by rapamycin (Rap) attenuated Cd-activated mTOR signaling pathways as well as neuronal apoptosis. Primary rat cerebral cortical neurons and/or PC12 cells were pretreated with or without Rap (200 nm) for 24 h and then exposed to Cd (10 μmol/L) for 24 h. (A and B) Rap blocked Cd-induced apoptotic morphological changes in primary rat cerebral cortical neurons and PC12 cells. Morphology of apoptosis was visualized by Hoechst 33258 staining under a fluorescence microscope. The arrows indicate apoptotic neuronal cells showing nuclear condensation. The original magnification is 1000×. All experiments were performed twice. (C) Rap partially rescued cells from Cd-induced apoptosis. Following Annexin V-FITC and PI double staining, apoptosis rate of PC12 cells was analyzed by flow cytometry. A representative experimental result is shown. (D) Rap blocked Cd-induced phosphorylation of Akt, mTOR, p70 S6K, and 4E-BP1 in primary rat cerebral cortical neurons and PC12 cells. The cell lysates were subjected to western blotting using indicated antibodies. β-actin was used as a loading control. Similar results were observed in at least three independent experiments.

To ascertain whether phosphorylation of Akt and mTOR and its downstream effector molecules (p70 S6K and 4E-BP1) is attributable to the Cd-induced generation of ROS, primary rat cerebral cortical neurons and PC12 cells were pretreated with NAC (100 μmol/L) for 1 h and then co-treated with or without Cd (10 μmol/L) for 24 h. Treatment was followed by a western blot analysis. Our results showed that Cd-induced phosphorylation of Akt, mTOR, p70 S6K, and 4E-BP1 was almost completely blocked by NAC (Figure 3D). Collectively, these findings indicate that Cd activates the Akt/mTOR signaling pathway by induction of ROS in neuronal cells.

Cd-Induced Generation of ROS Activates Mitochondrial Apoptotic Pathway in Neuronal Cells

Recently, we have demonstrated that activation of the caspase-dependent and caspase-independent mitochondrial apoptotic pathways partially contribute to Cd-induced apoptosis of neuronal cells (primary rat

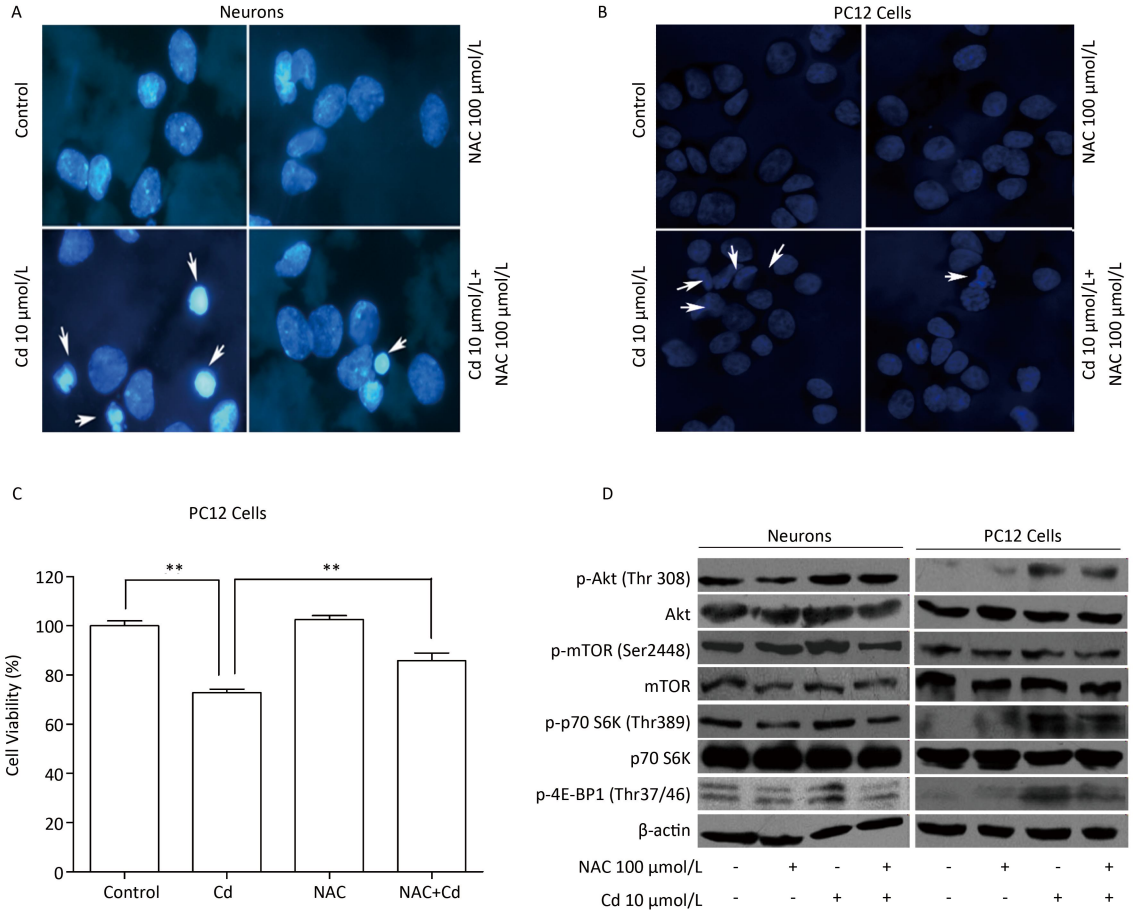

Figure 3. Cd-induced generation of ROS activates Akt/mTOR apoptotic signaling pathway in neuronal cells. Primary rat cerebral cortical neurons and PC12 cells, pretreated with or without NAC (100 μmol/L) for 1 h, were exposed to Cd (10 μmol/L) for 24 h. (A and B) NAC blocked Cd-induced apoptotic morphological changes in primary rat cerebral cortical neurons and PC12 cells. Morphology of apoptosis was visualized by Hoechst 33258 staining under a fluorescence microscope. The arrows indicate apoptotic neuronal cells showing nuclear condensation. The original magnification is 1000×. All experiments were performed twice. (C) NAC partially rescued cells from Cd-induced cell death. Cell viability of PC12 was evaluated by MTT assay. Results are presented as mean±SD (n=6). $^{**}P$<0.01 compared with indicated groups. (D) NAC partially blocked Cd-induced phosphorylation of Akt, mTOR, p70 S6K, and 4E-BP1 in primary rat cerebral cortical neurons and PC12 cells. The cell lysates were subjected to western blotting using indicated antibodies. β-actin was used as a loading control. Similar results were observed in at least three independent experiments.

cerebral cortical neurons and PC12 cells)[14,17]. However, whether Cd-induced ROS production and the consequent apoptosis of neuronal cells occur through activation of the caspase-dependent or the caspase-independent pathway remain to be clarified. Therefore, PC12 cells were pretreated with or without NAC (100 μmol/L) for 1 h, followed by exposure to Cd (10 μmol/L) for 24 h. We found that pretreatment with NAC inhibited Cd-induced up-regulation of Bax and down-regulation Bcl-2 levels and Bcl-2: Bax ratio (Figure 4A and 4B). In addition, the Cd-induced release of Cyt C from the mitochondria into the cytosol was attenuated by NAC (Figure 4C and 4D). Activation of caspase-3 and PARP was also blocked by NAC (Figure 4E and 4F). The role of ROS in the release of AIF and Endo G was further examined. As shown in Figure 5A and 5B, immunofluorescence staining of PC12 cells pretreated with NAC prior to Cd exposure revealed a punctuate mitochondrial pattern of AIF and Endo G, similar to that observed in untreated cells. Collectively, these findings support the notion that the Cd-induced generation of ROS activates the caspase-dependent and caspase-independent mitochondrial pathways, triggering apoptosis of neuronal cells.

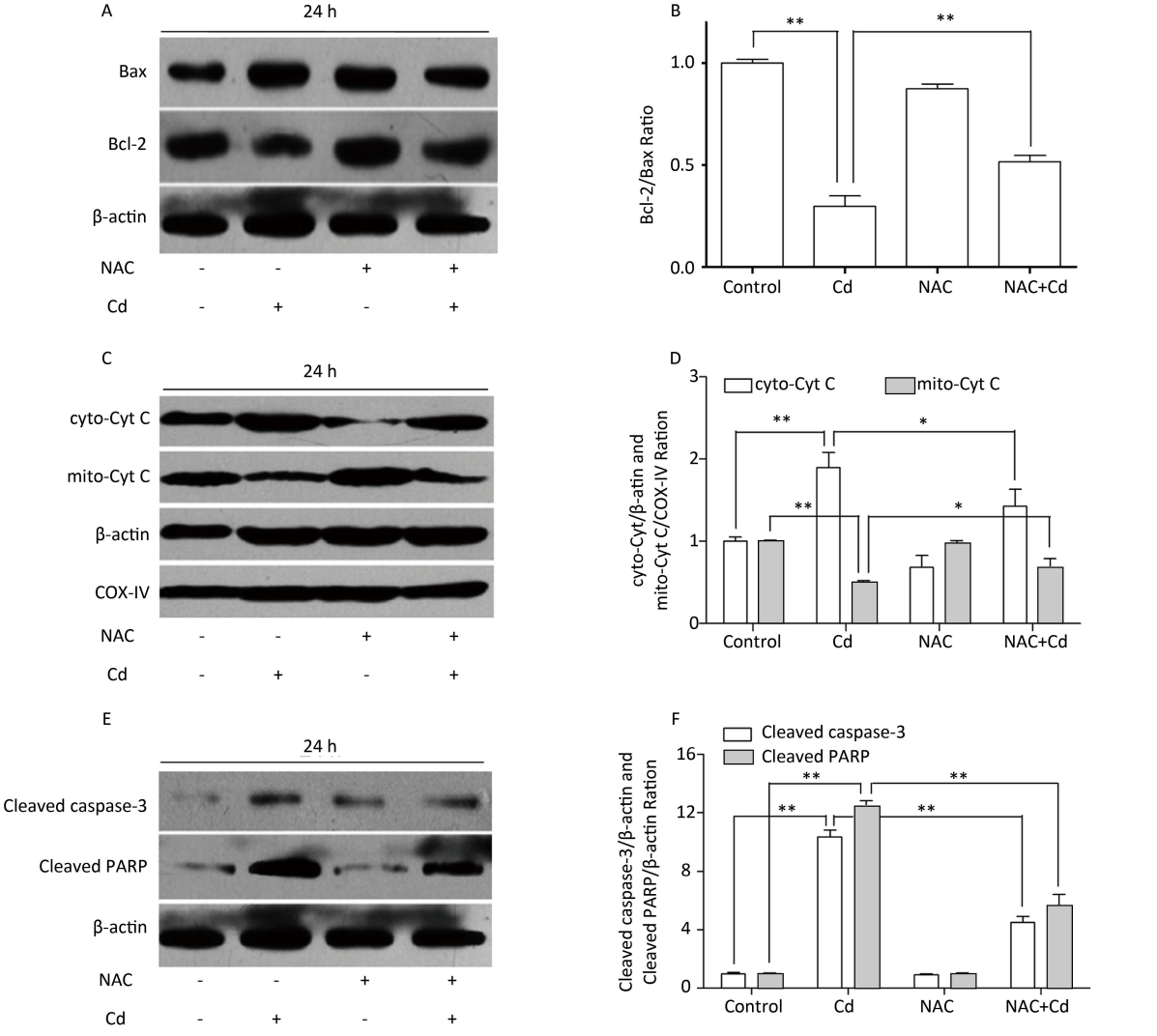

Figure 4. Cd-induced generation of ROS activates caspase-dependent mitochondrial apoptotic signaling pathway in neuronal cells. PC12 cells, pretreated with or without NAC (100 μmol/L) for 1 h, were exposed to Cd (10 μmol/L) for 24 h. (A-F) NAC blocks Cd-induced down-regulation of Bcl-2/Bax ratio, release of Cyt C, cleavages of caspase-3 and PARP. The cell lysates were subjected to western blotting using indicated antibodies (A, C, E). COX-IV and β-actin were used as internal controls of mitochondrial and cytosolic subfraction, respectively. Statistical results are presented in (B, D, F). Mean values were derived from three independent experiments. $^{**}P<0.01$ compared with indicated groups.

DISCUSSION

Oxidative stress is a key risk factor for undesirable biological reactions and functional cell damage and has been implicated in various disease processes, including neurodegenerative disease[22]. Studies have shown that oxidative stress is a possible mechanism of Cd toxicity[23-24]. Cd induces excessive amounts of ROS, creating damage from oxidative stress that results in many pathophysiological processes and the development of disease[25]. ROS, which are secondary mediators, participate in various physiological responses including proliferation, differentiation, and apoptosis[26-27]. Cd is a well-known inducer of ROS generation in cells[23,28]. Chen et al.[6] found that ROS generation was induced by Cd in a time- and dose-dependent manner in PC12 and SH-SY5Y cells. In our previous studies, we also detected ROS generation induced by Cd in primary neurons[20]. It has been documented that Cd induces production of ROS, which can destroy DNA, proteins, and lipid function, and activate signaling pathways that cause cell death[6,9,28-29]. Thus, oxidative stress has a critical function in Cd-induced toxicity.

Akt/mTOR signaling has been widely recognized as a central and positive controller of cell growth, proliferation, and survival[10,30]. mTOR activity is modified in various pathological states of the nervous system, including brain tumors and neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease[31]. Studies have shown that under different stress conditions, the consequences of activation of the Akt/mTOR pathway in cells may be completely different. Induction of neuronal cell death by hydrogen peroxide is partly associated with the inhibition of the mTOR signaling pathway[32]. On the other hand, the activation of mTOR signaling contributes to Cd-induced apoptosis in PC12 and SH-SY5Y cells[33]. In the present study, our results indicate that Cd induced the activation of Akt/mTOR signaling including Akt, mTOR, p70 S6K, and 4E-BP1 (Figure 1). Rap, an mTOR inhibitor, blocked Cd-induced activation of Akt/mTOR signaling and partially prevented Cd-induced apoptosis of neuronal cells (Figure 2), which suggests that activation of the Akt/mTOR signaling pathway may contribute to Cd-induced apoptosis of neuronal cells. Inhibition of mTOR by Rap may have a beneficial effect against Cd-induced neuronal apoptosis.

Activation of mTOR signaling by ROS has been previously described[7,34]. Chen et al.[7] demonstrated that Cd induces ROS generation, resulting in activation of Akt/mTOR in PC12 and SH-SY5Y cells. This prompted us to investigate whether Cd activates Akt/mTOR signaling through induction of ROS in primary neurons. Here, we present evidence that Cd induces ROS-dependent activation of Akt/mTOR signaling in primary rat cerebral cortical neurons and PC12 cells. This is strongly supported by the finding that NAC, a ROS scavenger, blocked Cd-induced phosphorylation of Akt, mTOR, p70 S6K, and 4E-BP1 and prevented apoptosis in neuronal cells (Figure 3). Collectively, our findings suggest that Cd-induced generation of ROS activates the Akt/mTOR pathway, leading to apoptosis of neuronal cells.

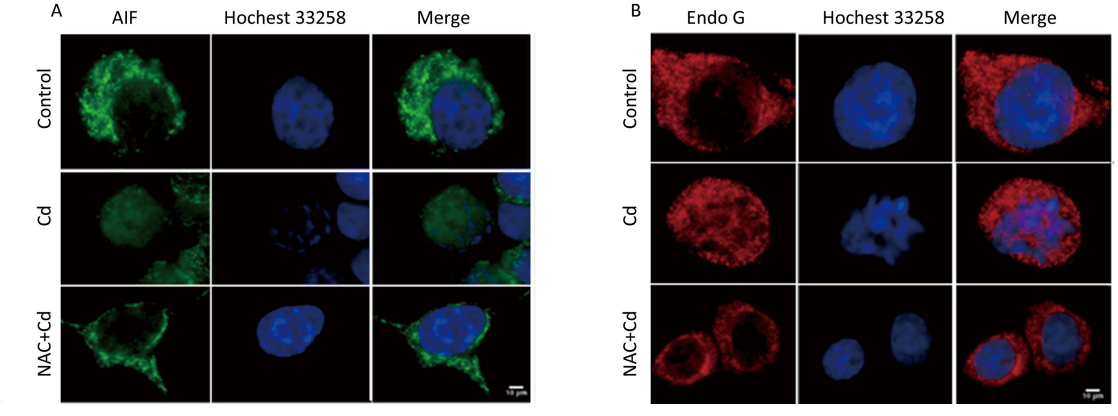

Figure 5. Cd-induced generation of ROS activates caspase-independent mitochondrial apoptotic signaling pathway in neuronal cells. PC12 cells, pretreated with or without NAC (100 μmol/L) for 1 h, were exposed to Cd (10 μmol/L) for 24 h. (A and B) NAC attenuates Cd-induced AIF and EndoG nuclear translocation. After fixation and permeabilization, immunostaining was performed with antibodies against AIF or Endo G, cells were simultaneously stained with Hoechst 33258 to detect the nuclei and subsequently viewed under fluorescence microscopy. The original magnification is 1000×. The experiments were repeated at least three times, with similar results. Bar=10 μm.

Recent studies have shown that Cd can induce apoptosis by causing mitochondrial dysfunction and activating caspase cascade signals[16,35]. Mitochondria-dependent apoptosis is an active process of cell death mediated by at least two different pathways. A major pathway that has been established involves the activation of caspases and is mediated by sequential mitochondrial release of Cyt C, downstream activation of the initiator caspase-9 and executor caspase-3, and final cleavage of specific substrates. A second pathway that has been identified involves nuclear translocation of mitochondrial proteins such as Endo G and AIF, which can induce DNA fragmentation independently of caspase recruitment[36]. Studies have shown that Cd may trigger cell death by caspase-dependent and/or caspase-independent apoptotic mechanisms, depending on cell types[37-39]. Recently, we have demonstrated that Cd-induced neuronal (primary rat cerebral cortical neurons and PC12 cells) apoptosis was partially associated with the activation of caspase-dependent and caspase-independent mitochondrial signaling pathways by decreasing the Bcl-2: Bax ratio, activating caspase-9/3 and PARP, releasing Cyt C, and inducing nuclear translocation of AIF and Endo G[14-15]. Furthermore, the ERK- and JNK-mediated mitochondrial apoptotic pathways play an important role in Cd-induced neuronal apoptosis[14-15]. Studies have shown that excessive generation of ROS may contribute to the activation of mitochondrial signaling pathway[16,23,40]. However, little is known about the role of ROS in Cd-mediated activation of mitochondrial signaling pathways in neuronal cells. The Bcl-2 family, which includes a balance of pro-apoptotic (eg, Bax, and Bak) and anti-apoptotic (eg, Bcl-2, and Bcl-xL) members, has been demonstrated to induce the release of mitochondria death effectors (such as Cyt C) and the onset of mitochondrial dysfunction that subsequently regulates apoptotic pathways[41-42]. It has been reported that ROS regulates the expression of Bcl-2 and Bax[40]. To determine whether ROS regulates Bcl-2 signaling in neuronal cells after Cd treatment, we detected the expression levels of Bcl-2 and Bax. As shown in Figure 4A and 4B, NAC significantly suppressed Cd-induced down-regulation of Bcl-2: Bax ratio in PC12 cells. In addition, NAC attenuated the Cd-induced release of Cyt C (Figure 4C and 4D), cleavage of PARP, and subsequent activation of caspase-3 (Figure 4E and 4F). Therefore, these results show that ROS mediates the caspase-dependent mitochondrial signaling pathway in Cd-induced neuronal apoptosis. AIF and Endo G are two caspase-independent mitochondrial proteins[36]. Our data showed that NAC blocked Cd-induced nuclear translocation of AIF and Endo G (Figure 5). These results show that ROS also mediates the caspase-independent mitochondrial signaling pathway in Cd-induced neuronal apoptosis. Taken together, these findings indicate that the ROS-mediated mitochondrial pathway may partially account for Cd-induced apoptosis in neuronal cells.

In summary, here we show that Cd-induced generation of ROS activates the Akt/mTOR apoptotic pathway and leads to the apoptosis of neuronal cells. To our knowledge, this is first study to demonstrate that the ROS-mediated caspase-dependent and caspase-independent mitochondrial apoptotic pathways play an important role in Cd-induced neuronal apoptosis. Our findings suggest that antioxidants (eg, NAC) or inhibitors of mTOR may be exploited for the prevention of Cd-induced neurodegenerative disease.

Received: September 16, 2015;
Accepted: January 26, 2016

REFERENCES

1. Cai Q, Long ML, Zhu M, et al. Food chain transfer of cadmium and lead to cattle in a lead-zinc smelter in Guizhou, China. Environ Pollut, 2009; 157, 3078-82.
2. Jarup L, Akesson A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharm, 2009; 238, 201-8.
3. Lopez E, Figueroa S, Oset-Gasque MJ, et al. Apoptosis and necrosis: two distinct events induced by cadmium in cortical neurons in culture. Brit J Pharmacol, 2003; 138, 901-11.
4. Mendez-Armenta M, Rios C. Cadmium neurotoxicity. Environ Toxicol Phar, 2007; 23, 350-8.
5. Kanter M, Unsal C, Aktas C, et al. Neuroprotective effect of quercetin against oxidative damage and neuronal apoptosis caused by cadmium in hippocampus. Toxicol Ind Health, 2013; Nov 5.
6. Chen L, Liu L, Huang S. Cadmium activates the mitogen-activated protein kinase (MAPK) pathway via induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5. Free Radical Bio Med, 2008; 45, 1035-44.
7. Chen L, Xu B, Liu L, et al. Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death. Free Radical Bio Med, 2011; 50, 624-32.
8. Unsal C, Kanter M, Aktas C, et al. Role of quercetin in cadmium-induced oxidative stress, neuronal damage, and apoptosis in rats. Toxicol Ind Health, 2015; Dec 31, 1106-51.
9. Kim SD, Moon CK, Eun SY, et al. Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. Biochem Bioph Res Co, 2005; 328, 326-34.
10. Bai X, Jiang Y. Key factors in mTOR regulation. Cell Mol Life Sci, 2010; 67, 239-53.
11. Polak P, Hall MN. mTOR and the control of whole body metabolism. Curr Opin Cell Biol, 2009; 21, 209-18.

12.Hockenbery DM, Giedt CD, O'Neill JW, et al. Mitochondria and apoptosis: new therapeutic targets. Adv Cancer Res, 2002; 85, 203-42.
13.Polster BM, Fiskum G. Mitochondrial mechanisms of neural cell apoptosis. J Neurochem, 2004; 90, 1281-9.
14.Jiang C, Yuan Y, Hu F, et al. Cadmium induces PC12 cells apoptosis via an extracellular signal-regulated kinase and c-Jun N-terminal kinase-mediated mitochondrial apoptotic pathway. Biol Trace Elem Res, 2014; 158, 249-58.
15.Yuan Y, Jiang C, Hu F, et al. The role of mitogen-activated protein kinase in cadmium-induced primary rat cerebral cortical neurons apoptosis via a mitochondrial apoptotic pathway. J Trace Elem Med Biol, 2015; 29, 275-83.
16.Gobe G, Crane D. Mitochondria, reactive oxygen species and cadmium toxicity in the kidney. Toxicol Lett, 2010; 198, 49-55.
17.Yuan Y, Jiang CY, Xu H, et al. Cadmium-induced apoptosis in primary rat cerebral cortical neurons culture is mediated by a calcium signaling pathway. PloS One, 2013; 8, e64330.
18.Zhang Q, Xu X, Yuan Y, et al. IPS-1 plays a dual function to directly induce apoptosis in murine melanoma cells by inactivated Sendai virus. Int J Cancer, 2014; 134, 224-34.
19.Jayanthi S, Deng X, Noailles PA, et al. Methamphetamine induces neuronal apoptosis via cross-talks between endoplasmic reticulum and mitochondria-dependent death cascades. Faseb J, 2004; 18, 238-51.
20.Yan Y, Bian JC, Zhong LX, et al. Oxidative stress and apoptotic changes of rat cerebral cortical neurons exposed to cadmium in vitro. Biomed Environ Sci, 2012; 25, 172-81.
21.Yuan Y, Liu XZ, Bian JC, et al. Cadmium-induced lipid peroxidation and protective effect of N-acetyl cysteine in cerebral cortical neurons of rats. Chin J vet Sci, 2011; 31, 391-3. (In Chinese)
22.Pitocco D, Zaccardi F, Di Stasio E, et al. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud, 2010; 7, 15-25.
23.Chang KC, Hsu CC, Liu SH, et al. Cadmium induces apoptosis in pancreatic β-cells through a mitochondria-dependent pathway: the role of oxidative stress-mediated c-jun N-terminal kinase activation. PLoS One, 2013; 8, e54374.
24.Nazimabashir, Manoharan V, Miltonprabu S. Cadmium induced cardiac oxidative stress in rats and its attenuation by GSP through the activation of Nrf2 signaling pathway. Chem Biol Interact, 2015; 242, 179-93.
25.Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology, 2011; 283, 65-7.
26.Morgan MJ, Liu ZG. Reactive oxygen species in TNFalpha-induced signaling and cell death. Mol Cells, 2010; 30, 1-12.
27.Schippers JH, Nguyen HM, Lu D, et al. ROS homeostasis during development: an evolutionary conserved strategy. Cell Mol Life Sci, 2012; 69, 3245-57.
28.Khojastehfar A, Aghaei M, Gharagozloo M, et al. Cadmium induces reactive oxygen species-dependent apoptosis in MCF-7 human breast cancer cell line. Toxicol Mech Method, 2015; 25, 48-55.
29.Bagchi D, Joshi SS, Bagchi M, et al. Cadmium- and chromium-induced oxidative stress, DNA damage, and apoptotic cell death in cultured human chronic myelogenous leukemic K562 cells, promyelocytic leukemic HL-60 cells, and normal human peripheral blood mononuclear cells. J Biochem Mol Toxic, 2000; 14, 33-41.
30.Don AS, Tsang CK, Kazdoba TM, et al. Targeting mTOR as a novel therapeutic strategy for traumatic CNS injuries. Drug Discov Today, 2012; 17, 861-8.
31.Swiech L, Perycz M, Malik A, et al. Role of mTOR in physiology and pathology of the nervous system. Biochim Biophys Acta, 2008; 1784, 116-32.
32.Chen L, Xu B, Liu L, et al. Hydrogen peroxide inhibits mTOR signaling by activation of AMPKa leading to apoptosis of neuronal cells. Lab Invest, 2010; 90, 762-73.
33.Chen L, Liu L, Luo Y, et al. MAPK and mTOR pathways are involved in cadmium-induced neuronal apoptosis. J Neurochem, 2008; 105, 251-61.
34.Zhang L, Wang H, Xu J, et al. Inhibition of cathepsin S induces autophagy and apoptosis in human glioblastoma cell lines through ROS-mediated PI3K/AKT/mTOR/p70S6K and JNK signaling pathways. Toxicol Lett, 2014; 228, 248-59.
35.Ben P, Zhang Z, Xuan C, et al. Protective effect of L-theanine on cadmium-induced apoptosis in PC12 cells by inhibting the mitochondria-mediated pathway. Neurochem Res, 2015; 40, 1661-70
36.van Loo G, Saelens X, van Gurp M, et al. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ, 2002; 9, 1031-42.
37.Oh SH, Lim SC. A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicol Appl Pharm, 2006; 212, 212-23.
38.Pathak N, Mitra S, Khandelwal S. Cadmium induces thymocyte apoptosis via caspase-dependent and caspase-independent pathways. J Biochem Mol Toxic, 2013; 27, 193-203.
39.Shih YL, Lin CJ, Hsu SW, et al. Cadmium toxicity toward caspase-independent apoptosis through the mitochondria-calcium pathway in mtDNA-depleted cells. Ann NY Acad Sci, 2005; 1042, 497-505.
40.Deng S, Tang S, Zhang S, et al. Furazolidone induces apoptosis through activating reactive oxygen species-dependent mitochondrial signaling pathway and suppressing PI3K/Akt signaling pathway in HepG2 cells. Food Chem Toxicol, 2015; 75, 173-86.
41.Leibowitz B, Yu J. Mitochondrial signaling in cell death via the Bcl-2 family. Cancer Biol Ther, 2010; 9, 417-22.
42.Czabotar PE, Lessene G, Strasser A, et al. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol, 2014; 15, 49-63.