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CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death

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Abstract

Cadmium (Cd), a toxic environmental contaminant, induces neurodegenerative diseases. Recently we have shown that Cd elevates intracellular free calcium ion ($[Ca^{2+}]_i$) level, leading to neuronal apoptosis partly by activating mitogen-activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) pathways. However, the underlying mechanism remains to be elucidated. Here we show that the effects of Cd elevated $[Ca^{2+}]_i$ on MAPK and mTOR network as well as neuronal cell death are through stimulating phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII). This is supported by the findings that chelating intracellular Ca^{2+} with BAPTA/AM or preventing Cd-induced $[Ca^{2+}]_i$ elevation using 2-aminoethoxydiphenyl borate (2-APB) blocked Cd activation of CaMKII. Inhibiting CaMKII with KN93 or silencing CaMKII attenuated Cd activation of MAPK/mTOR pathways and cell death. Furthermore, inhibitors of mTOR (rapamycin), JNK (SP600125) and Erk1/2 (U0126), but not of p38 (PD169316), prevented Cd-induced neuronal cell death in part through inhibition of $[Ca^{2+}]_i$ elevation and CaMKII phosphorylation. The results indicate that Cd activates MAPK/mTOR network triggering neuronal cell death, by stimulating CaMKII. Our findings underscore a central role of CaMKII in the neurotoxicology of Cd, and suggest that manipulation of intracellular Ca^{2+} level or CaMKII activity may be exploited for prevention of Cd-induced neurodegenerative disorders.

Keywords

cadmium; apoptosis; calcium ion; calcium/calmodulin-dependent protein kinase II; mitogen-activated protein kinase; mammalian target of rapamycin

Introduction

Cadmium (Cd) pollution in the environment may be accumulated in human organs through direct exposure or food chain, resulting in many diseases such as pulmonary edema, respiratory tract irritation, renal dysfunction, anemia, osteoporosis, and cancer in humans

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(Satarug *et al.*, 2000; Kim *et al.*, 2005; Lau *et al.*, 2006; Chen *et al.*, 2008a). Cd also severely affects the function of the nervous system (Lopez *et al.*, 2003), with symptoms including headache and vertigo, olfactory dysfunction, parkinsonian-like symptoms, slowing of visuomotor functioning, peripheral neuropathy, decreased equilibrium, decreased ability to concentrate, and learning disabilities (Pihl and Parkes, 1977; Kim *et al.*, 2005; Monroe and Halvorsen, 2006). A growing number of clinical investigations have pointed to Cd intoxication as a possible etiological factor of neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease and Huntington's disease (Okuda *et al.*, 1997; Johnson, 2001; Panayi *et al.*, 2002). However, the exact mechanism(s) through which Cd elicits its neurotoxic effects is still unresolved.

Multiple studies have shown that the mitogen-activated protein kinases (MAPKs) signaling pathways, which comprise a highly conserved cascade of serine/threonine (Ser/Thr) kinases connecting cell surface receptors to regulatory targets in response to various stimuli, play a critical role in neuronal apoptosis (Kyriakis and Avruch, 2001; Pearson *et al.*, 2001; Li *et al.*, 2004). There exist at least three distinct groups of MAPKs, including extracellular signal-regulated protein kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK, in mammalian cells. Erk1/2 can be activated by growth factors, and is involved in cellular proliferation, differentiation and development, whereas JNK and p38 signaling cascades can be activated by environmental stress and inflammatory cytokines, and have been shown to promote neuronal cell death (Rockwell *et al.*, 2004). Mammalian target of rapamycin (mTOR), a Ser/Thr kinase, lies downstream of protein kinase B (Akt/PKB) (Kim *et al.*, 2000; Huang and Houghton, 2003), and senses mitogenic stimuli, nutrient conditions (Fang *et al.*, 2001; Hara *et al.*, 2002; Kim *et al.*, 2002) and ATP (Dennis *et al.*, 2001), regulating cell proliferation, growth and survival (Bjornsti and Houghton, 2004). Akt may positively regulate mTOR, leading to increased phosphorylation of ribosomal p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), the two best characterized downstream effector molecules of mTOR (Bjornsti and Houghton, 2004). We have demonstrated that all three MAPK members can be activated by Cd in neuronal (PC12 and SH-SY5Y) cells, and identified that Cd-induced neuronal apoptosis is partially associated with activation of JNK, Erk1/2, but not by p38 signaling (Chen *et al.* 2008b). Especially, we have pinpointed that Cd induces apoptosis of neuronal cells in part by activation of Akt/mTOR signaling pathway (Chen *et al.*, 2008b). However, it is still not well understood how Cd activates MAPKs and mTOR signaling pathways in the neuronal cells.

Other studies has implicated that Cd-induced cell apoptosis involves a sustained elevation of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Shen *et al.*, 2001; Li *et al.*, 2003; Lemarie *et al.*, 2004; Liu *et al.*, 2007; Biagioli *et al.*, 2008; Wang *et al.*, 2008). Prolonged change in calcium distribution in cells triggers various cascades that lead to apoptosis (Hajnoczky *et al.*, 2003). Recently we have shown that Cd-induced $[\text{Ca}^{2+}]_i$ elevation activates MAPK and mTOR pathways, thereby leading to apoptosis of neuronal cells (Xu *et al.*, 2011). However, the underlying mechanism remains elusive. Calcium/calmodulin-dependent protein kinase II (CaMKII), a Ser/Thr specific protein kinase, is a general integrator of Ca^{2+} signaling (Colbran and Brown, 2004; Liu and Templeton, 2007). CaMKII is activated in the presence of Ca^{2+} and calmodulin (CaM), which leads to autophosphorylation, generating a Ca^{2+} /CaM-independent form of the enzyme (Schworer *et al.*, 1986). In addition, CaMKII transduces signals to MAPKs involved in cell proliferation or apoptosis (Wright *et al.*, 1997; Yamanaka *et al.*, 2007; Kim *et al.*, 2008a; Kim *et al.*, 2008b; Lin *et al.*, 2008; Liu and Templeton, 2008; Olofsson *et al.*, 2008). This prompted us to study whether Cd activates MAPK and mTOR pathways triggering neuronal apoptosis, through CaMKII activation by elevated $[\text{Ca}^{2+}]_i$.

Materials and Methods

Materials

Cadmium chloride (Sigma, St. Louis, MO, USA) was dissolved in sterile distilled water to prepare the stock solutions (0–120 μ M), filtered through a 0.22 μ m pore size membrane, aliquoted, and stored at room temperature. Rapamycin (ALEXIS, San Diego, CA, USA) was dissolved in DMSO to prepare a 100 μ g/ml stock solution and was stored at -20°C . Fluo-3/AM was from Fluka (Buchs, SG, Switzerland). Dulbecco's Modified Eagle Medium (DMEM), 0.05% Trypsin-EDTA, NEUROBASAL™ Media, and B27 Supplement were purchased from Invitrogen (Grand Island, NY, USA), whereas horse serum and fetal bovine serum (FBS) were supplied by Minhai Corp. (Lanzhou, China). Enhanced chemiluminescence solution was from Millipore (Billerica, MA, USA). 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) and 2-aminoethoxydiphenyl borane (2-APB) were purchased from Calbiochem (San Diego, CA, USA). KN93 was from ALEXIS, whereas SP600125, U0126, and PD169316 were from Sigma. The following antibodies were used: CaMKII, phospho-CaMKII (Thr286), phospho-Akt (Ser473), phospho-S6K1 (Thr389), phospho-Erk1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-4E-BP1 (Thr70), 4E-BP1 (all from Cell Signaling Technology, Beverly, MA, USA), Akt, S6K1, Erk2, JNK1, phospho-JNK (Thr183/Tyr185), c-Jun, phospho-c-Jun (Ser63), p38 (all from 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). Poly-D-lysine (PDL) and 4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Other chemicals were purchased from local commercial sources and were of analytical grade quality.

Cell culture

Rat pheochromocytoma (PC12) cell line was from American Type Culture Collection (ATCC) (Manassas, VA, USA). PC12 cells were used for no more than ten passages, and cultured in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 . Primary cortical neurons were obtained from fetal mice at 16–18 days of gestation and cultured as described (Chen *et al.*, 2010).

Lentiviral shRNA cloning, production, and infection

To generate lentiviral shRNA to CaMKII, oligonucleotides containing the target sequences were synthesized, annealed and inserted into FSIPPW lentiviral vector via the EcoRI/BamHI restriction enzyme site, as described previously (Liu *et al.*, 2008). Oligonucleotides used were: sense: 5'-AATCCCCCTGATTGAAGCCATCAACATGCAAGAGATGTTGATGGCTTCAATCAGTTTTTG-3', anti-sense: 5'-GATCCAAAACTGATTGAAGCCATCAACATCTCTTGCATGTTGATGGCTTCAATCAGGGG-3'. Lentiviral shRNA targeting GFP (control) was described (Liu *et al.*, 2008). To produce lentivirus with CaMKII shRNA, above constructs were transfected to 293TD cells, then the lentivirus-containing supernatant was used to infect targeting cells following the procedure described (Chen *et al.*, 2011).

Cell viability assay and morphological analysis

Primary neurons, PC12 cells, or PC12 cells infected with lentiviral shRNA to CaMKII or GFP (control), respectively, were seeded at a density of 1×10^4 cells/well in a flat-bottomed 96-well plate, pre-coated with PDL (0.2 μ g/ml). Next day, cells were treated with 0–120 μ M Cd for 24 h, with 20 μ M Cd for 0–24 h, or with/without 10 and 20 μ M Cd for indicated time

following pre-incubation with/without 100 μM 2-APB or 10 μM KN93 for 1 h with five replicates of each treatment. Subsequently, each well was added 0.01 ml (5 mg/ml) of MTT reagent and incubated for 4 h. After the incubation, the incubation precipitates were dissolved with 0.1 ml of SDS. Cell viability was determined by measuring the optical density (OD) at 570 nm using an ELx800 Microplate Reader (Bio-Tek Instruments, Inc. Winooski, Vermont, USA). In addition, the cells were also seeded at a density of 5×10^5 cells/well in a PDL-coated six-well plate. Next day, cells were exposed to Cd (10 and 20 μM) following pre-incubation with/without 20 μM BAPTA/AM, 2-APB, or KN93 for 1 h. After incubation for 24 h, the images were taken with a Nikon Eclipse TE2000-U inverted phase-contrast microscope (Nikon, Japan) (200 $\times$) equipped with a digital camera.

Flow cytometry for $[\text{Ca}^{2+}]_i$ staining

PC12 cells were seeded in 60-mm dishes, pre-coated with PDL, at a density of 1×10^6 cells/dish in the complete growth medium. Next day, cells were exposed to Cd (0–20 μM) for 4 h and 24 h with triplicate of each treatment, followed by harvesting cells, washing 3 times with PBS. Subsequently, cell suspensions (100 μl) for $[\text{Ca}^{2+}]_i$ staining were loaded with 5 μM Fluo-3/AM for 30 min at 37°C in the dark, and then washed once with PBS to remove the extracellular Fluo-3/AM. Fluo-3/AM was replaced by PBS as a negative control. All samples were analyzed to detect the status of $[\text{Ca}^{2+}]_i$ under a fluorescence-activated cell sorter (FACS) Vantage SE flow cytometer (Becton Dickinson, California, USA) using the CellQuest software.

Analysis for $[\text{Ca}^{2+}]_i$ fluorescence intensity

PC12 cells were seeded at a density of 5×10^5 cells/well in the complete growth medium in a PDL-coated six-well plate. Next day, cells were treated with/without Cd (10 and 20 μM) for 4 h and 24 h following pre-incubation with/without rapamycin (Rap, 0.2 $\mu\text{g/ml}$) for 48 h, or with/without SP600125 (20 μM), U0126 (5 μM), PD169316 (20 μM), BAPTA/AM (20 μM), or 2-APB (100 μM) for 1 h, followed by harvesting cells and washing with PBS. Subsequently, cell suspensions (100 μl) for $[\text{Ca}^{2+}]_i$ analysis were loaded with Fluo-3/AM as described above. Afterwards, $[\text{Ca}^{2+}]_i$ fluorescent intensity was detected as described (Chen *et al.*, 2011).

Western blot analysis

Western blotting was performed as described (Chen *et al.*, 2011).

Statistical analysis

Results were expressed as mean values $\pm$ standard error (mean $\pm$ S.E.). Statistical analysis was performed by Student's *t*-test (STATISTICA, Statsoft Inc, Tulsa, OK). A level of *P* < 0.05 was considered to be significant.

Results

Cd induced CaMKII phosphorylation in neuronal cells

Activation of CaMKII is pro-apoptotic or anti-apoptotic depending on cell types or experimental conditions (Wright *et al.*, 1997; Fladmark *et al.*, 2002; Liu and Templeton, 2007; Rokhlin *et al.*, 2007; Vila-Petroff *et al.*, 2007). To determine the role of CaMKII activity in Cd-induced neuronal apoptosis, PC12 cells and primary neurons, respectively, were exposed to 0–20 μM Cd for 12 h, or to 20 μM Cd for different time (0–12 h). We found that exposure of neuronal cells to Cd resulted in phospho-CaMKII increase in a time- and concentration-dependent manner (Fig. 1a and b), and a dramatically increased phosphorylation of CaMKII was seen at 12 h of exposure to 10 and 20 μM Cd (Fig. 1a) and

at 8–12 h post exposure to 20 μM Cd (Fig. 1*b*), respectively. Furthermore, Cd-elevated phospho-CaMKII level was consistent with decreased cell viability (Fig. 1*c* and *d*) or increased apoptosis in PC12 cells and/or primary neurons (Chen *et al.*, 2008*b*), suggesting that Cd-induced neuronal apoptosis might be associated with its induction of CaMKII phosphorylation

Cd-induced phosphorylation of CaMKII was attributed to $[\text{Ca}^{2+}]_i$ elevation in neuronal cells

CaMKII is a general integrator of Ca^{2+} signaling (Liu and Templeton, 2007), which is activated in the presence of Ca^{2+} and calmodulin, resulting in autophosphorylation and generation of a Ca^{2+} /CaM-independent form of the enzyme (Schworer *et al.*, 1986). Our recent studies have demonstrated that Cd-induced neuronal apoptosis is associated with its induction of $[\text{Ca}^{2+}]_i$ elevation, and unveiled that Cd-elevated $[\text{Ca}^{2+}]_i$ activates MAPK/mTOR pathways and apoptosis in neuronal cells through calcium-binding protein CaM (Xu *et al.*, 2011). Therefore, we proposed that Cd induction of $[\text{Ca}^{2+}]_i$ elevation may activate MAPK/mTOR pathways and neuronal apoptosis by induction of CaMKII phosphorylation. To test the hypothesis, first of all, we examined whether Cd-elevated $[\text{Ca}^{2+}]_i$ really activates CaMKII in neuronal cells. Consistent with our previous findings (Xu *et al.*, 2011), treatment with Cd for 4 h and 24 h resulted in a concentration-dependent increase of $[\text{Ca}^{2+}]_i$ at concentrations of 0–20 μM in PC12 cells (Fig. 2*a* and *b*). Pre-treatment for 1 h with 20 μM BAPTA/AM, an intracellular Ca^{2+} chelator, significantly attenuated $[\text{Ca}^{2+}]_i$ elevation induced by Cd (10 and 20 μM) exposure for 4 h and 24 h in PC12 cells (Fig. 2*c*) or primary neurons (data not shown). Interestingly, chelating intracellular Ca^{2+} with BAPTA/AM obviously inhibited Cd-induced CaMKII phosphorylation in PC12 cells and primary neurons (Fig. 2*d*).

2-APB, at concentrations of ≥ 50 μM , functions not only as a membrane-permeable inhibitor of inositol 1,4,5-trisphosphate (IP_3) receptors on endoplasmic reticulum (ER), but also as an inhibitor of extracellular Ca^{2+} influx through the Ca^{2+} release activated Ca^{2+} (CRAC) channels on plasma membrane (Prakriya and Lewis, 2001; Bilmen *et al.*, 2002; Braun *et al.*, 2003). Next, 2-APB was employed to prevent Cd-induced $[\text{Ca}^{2+}]_i$ elevation. As expected, pretreatment with 2-APB (100 μM) markedly attenuated Cd-induced $[\text{Ca}^{2+}]_i$ elevation (Fig. 3*a*). Like BAPTA/AM, 2-APB also partially blocked Cd activation of CaMKII in PC12 cells or primary neurons (Fig. 3*b*). Taken together, the results indicate Cd induction of $[\text{Ca}^{2+}]_i$ elevation stimulates CaMKII phosphorylation.

Cd elicited CaMKII phosphorylation leading to activation of MAPK and mTOR pathways and neuronal apoptosis

To unravel whether Cd induction of CaMKII phosphorylation is correlated with its activation of MAPK and mTOR signaling pathways as well as apoptosis in neuronal cells, PC12 cells were exposed to Cd (10 and 20 μM) for 12 h after pretreatment with CaMKII inhibitor KN93 (10 μM) for 1 h. We found that Cd-induced phosphorylation of CaMKII was obviously attenuated by KN93 in PC12 cells or primary neurons (Fig. 4*a*). Morphological analysis (Fig. 4*b*) revealed that KN93 itself did not alter cell shape. Cd alone (10 and 20 μM) induced cell roundup and shrinkage. However, KN93 partially prevented Cd-induced morphological change. Results from the MTT assay (Fig. 4*c*) further demonstrated that KN93 significantly suppressed Cd-induced loss of cell viability in PC12 cells and primary neurons. Of importance, KN93 partially blocked Cd-induced phosphorylation of Erk1/2, JNK, and p38 MAPK in PC12 cells and primary neurons (Fig. 4*d*). Cd-activated phosphorylation of Akt, S6K and 4E-BP1 in PC12 cells and primary neurons were also markedly reduced by KN93 (Fig. 4*e*).

To further substantiate the role of CaMKII in Cd activation of MAPK/mTOR pathways and neuronal cell death, expression of CaMKII α was silenced by RNA interference. As shown in Fig. 5a, lentiviral shRNA to CaMKII α , but not to GFP, downregulated CaMKII expression by ~90% in PC12 cells. Downregulation of CaMKII in part prevented Cd-induced cell death in PC12 cells (Fig. 5b and c). Furthermore, silencing CaMKII obviously attenuated Cd-induced phosphorylation of CaMKII (Fig. 5d). Consistently, downregulation of CaMKII conferred partial resistance to Cd activation of MAPKs and mTOR signaling pathways (Fig. 5d and e). The results indicate that Cd activates MAPK/mTOR signaling pathways and neuronal apoptosis by inducing CaMKII phosphorylation.

Inhibitors of mTOR, JNK and Erk1/2, but not p38, partially inhibited Cd-induced [Ca²⁺]_i elevation and CaMKII phosphorylation, preventing neuronal cell death

Recently we have demonstrated that rapamycin, a specific inhibitor of mTOR (Huang and Houghton, 2003), may exert a beneficial effect against Cd-induced neuronal cell death by blocking mTOR-mediated phosphorylation of S6K1 and 4E-BP1 (Chen *et al.*, 2008b). We have also shown that inhibitors of JNK and Erk1/2 partially prevent Cd-induced neuronal apoptosis by inhibiting phosphorylation of JNK and Erk1/2, respectively (Chen *et al.*, 2008b). Cd induced [Ca²⁺]_i elevation activates MAPKs and mTOR network (Xu *et al.*, 2011). To investigate whether MAPKs and mTOR pathways reversely regulate Cd-induced intracellular Ca²⁺ elevation and CaMKII phosphorylation, PC12 cells and/or primary neurons were exposed to Cd (10 and 20 μ M) for 4 h and 24 h, or 12 h after pretreatment with rapamycin, JNK inhibitor SP600125, MEK1/2 (upstream of Erk1/2) inhibitor U0126, or p38 inhibitor PD169316 for indicated time, respectively. As shown in Fig. 6a, pretreatment with rapamycin obviously reduced Cd-induced [Ca²⁺]_i elevation in PC12 cells. Consistently, we found that rapamycin dramatically blocked activation of mTOR-mediated S6K1 and 4E-BP1 with a concomitant reduction of CaMKII phosphorylation in Cd-exposed PC12 cells and primary neurons (Fig. 6b). Similar effects of 20 μ M SP600125 (Fig. 6c and d) and 5 μ M U0126 (Fig. 6e and f), but not 20 μ M PD169316 (Fig. 6g and h), on Cd-induced [Ca²⁺]_i elevation and CaMKII activation were seen in PC12 cells and/or primary neurons. The findings suggest that inhibitors of mTOR, JNK and Erk1/2 may prevent Cd-induced neuronal cell death in part through inhibition of [Ca²⁺]_i elevation and CaMKII phosphorylation.

Discussion

Calcium ion (Ca²⁺), as a second messenger, mediates a variety of physiological responses of neurons to neurotransmitters and neurotrophic factors (Cheng *et al.*, 2003). However, deregulated cellular Ca²⁺ homeostasis contributes to neuronal cell death, which is implicated in neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (Gibbons *et al.*, 1993; Kawahara and Kuroda, 2000; Mattson, 2007; Marambaud *et al.*, 2009). Cd, a highly toxic heavy metal, occurs frequently in the polluted environment and has a long biological half-life (15–20 years) in humans (Jin *et al.*, 1998). Its targets of toxicity including liver, lung, kidney, testis, bone, etc., resulting in many diseases such as pulmonary edema, respiratory tract irritation, renal dysfunction, anemia, osteoporosis, and cancer in humans (Satarug *et al.*, 2000; Kim *et al.*, 2005; Lau *et al.*, 2006; Chen *et al.*, 2008a). Cd also severely affects the function of the nervous system by its induction of neuronal apoptosis, and thereby contributes to neurodegenerative diseases (Lopez *et al.*, 2003). We previously have demonstrated that Cd induces apoptosis of neuronal (PC12 and SH-SY5Y) cells via activation of MAPK and mTOR signaling network (Chen *et al.*, 2008b). Recently our further studies revealed that Cd elevated [Ca²⁺]_i level, leading to neuronal apoptosis in part through activation of MAPKs and mTOR pathways (Xu *et al.*, 2011). Here, we provide evidence that the effects of Cd-elevated [Ca²⁺]_i on MAPKs and mTOR network as well as

neuronal cell death are through stimulating phosphorylation of CaMKII. The data indicate that CaMKII, as a key signaling protein, plays a bridging role between Cd induction of $[Ca^{2+}]_i$ elevation and activation of MAPK and mTOR network, leading to neuronal cell death.

CaMKII, a multifunctional Ser/Thr kinase ubiquitously expressed in all neuronal compartments, is prominent among the Ca^{2+} -sensitive processes (Colbran and Brown, 2004). It is activated upon binding of Ca^{2+} -containing CaM, which undergoes autophosphorylation, and then takes on autonomous activity independent of calmodulin binding (Hudmon and Schulman, 2002). Based on the unique regulatory properties of CaMKII and our recent findings that Cd induces $[Ca^{2+}]_i$ elevation with a concomitant increase of CaM function contributing to neuronal apoptosis, we speculated that CaMKII is an 'interpreter' of Cd induction of Ca^{2+} signaling, leading to apoptosis of neuronal cells. In the present study, we observed that exposure of neuronal cells (PC12 cells and primary neurons) to Cd resulted in phospho-CaMKII increase in a time- and concentration-dependent manner, which was consistent with decreased cell viability (Fig. 1c and d) or increased apoptosis of PC12 cells (Chen *et al.*, 2008b). Chelation of intracellular Ca^{2+} with BAPTA/AM blocked Cd activation of CaMKII. We have recently noticed that BAPTA/AM attenuated Cd activation of MAPK and mTOR signaling pathways as well as cell death in PC12 cells or primary neurons (Xu *et al.*, 2011). These findings suggest that Cd-elevated $[Ca^{2+}]_i$ activates CaMKII, which might be associated with activation of MAPK and mTOR signaling pathways, leading to neuronal apoptosis.

Many cell death stimuli are known to alter the concentration of Ca^{2+} in the cytosol and the storage of Ca^{2+} in the intracellular organelles (Baffy *et al.*, 1993; Bian *et al.*, 1997). $[Ca^{2+}]_i$ increase is usually attribute to Ca^{2+} mobilization from intracellular stores and/or Ca^{2+} entry from the extracellular space (Jan *et al.*, 2001). The ER is one of the major calcium storage units in cells, and blockers of the ER calcium channel, such as IP_3 receptors, can effectively prevent Ca^{2+} release induced by various stimuli including Cd (Wang *et al.*, 2007; Wang *et al.*, 2008). Influx of Ca^{2+} from the extracellular environment, following internal Ca^{2+} store depletion, provides the elevated and sustained $[Ca^{2+}]_i$ levels, which is associated with the activation of the CRAC channels on the plasma membrane (Prakriya and Lewis, 2001; Lewis *et al.*, 2008). Studies have demonstrated that 2-APB ($\geq 50 \mu M$) inhibits both IP_3 receptors and CRAC channels, blocking ER Ca^{2+} release and extracellular Ca^{2+} influx (Prakriya and Lewis, 2001; Bilmen *et al.*, 2002; Braun *et al.*, 2003). Therefore, 2-APB (100 μM) was employed to prevent Cd-induced $[Ca^{2+}]_i$ elevation. As expected, 2-APB did block Cd-induced $[Ca^{2+}]_i$ elevation. Importantly, like BAPTA/AM, 2-APB remarkably attenuated Cd-induced phosphorylation of CaMKII. Furthermore, we found that Cd-activated MAPK/mTOR pathways were partially inhibited by 2-APB as well (data not shown). This is in line with our recent findings that pretreatment with ethylene glycol tetra-acetic acid (EGTA), an extracellular Ca^{2+} chelator, which renders the inaccessibility of extracellular Ca^{2+} to the cells, dramatically prevented Cd-induced $[Ca^{2+}]_i$ elevation and activation of MAPK and mTOR pathways, as well as cell death (Xu *et al.*, 2011). Taken together, our results suggest that Cd may induce ER Ca^{2+} release via activation of IP_3 receptors on the ER and extracellular Ca^{2+} influx by activation of CRAC channels on plasma membrane, resulting in $[Ca^{2+}]_i$ elevation contributing to CaMKII phosphorylation.

To demonstrate that CaMKII is essential for Cd activation of MAPK and mTOR pathways as well as neuronal cell death, pharmacological inhibition or genetic manipulation of CaMKII activity was utilized. Pretreatment with KN93, a specific inhibitor of CaMKII (Choi *et al.*, 2006), blocked Cd-induced CaMKII phosphorylation, and partially prevented Cd-induced death in PC12 cells and primary neurons (Fig. 4a–c). Consistently, Cd activation of MAPK and mTOR pathways was obviously blocked by KN93 in PC12 cells

and primary neurons as well (Fig. 4d and e). Furthermore, downregulation of CaMKII by RNA interference also attenuated Cd activation of MAPK and mTOR pathways as well as neuronal cell death. These findings strongly suggest that Cd activates MAPK and mTOR signaling pathways by CaMKII phosphorylation, leading to neuronal cell death.

Recently we have shown that a selective mTOR inhibitor, rapamycin, may dramatically rescue Cd-induced neuronal cell death by blocking mTOR-mediated phosphorylation of S6K1 and 4E-BP1 (Chen *et al.*, 2008b). Consistently, in the study, we noted that rapamycin was able to attenuate Cd-induced $[Ca^{2+}]_i$ elevation and CaMKII phosphorylation in PC12 cells and/or primary neurons. In addition, we also found that SP600125 (a specific JNK inhibitor) and U0126 (a specific inhibitor of MEK1/2, upstream kinases of Erk1/2), but not PD169136 (a specific p38 inhibitor), could prevent Cd-induced $[Ca^{2+}]_i$ elevation and CaMKII activation in PC12 cells and/or primary neurons. The results are consistent with our previous findings that inhibition of JNK or Erk1/2 by specific inhibitors or RNA interference partially prevented Cd-induced neuronal apoptosis (Chen *et al.*, 2008b). Our data suggest that JNK, Erk1/2 and mTOR are involved in controlling the cellular Ca^{2+} homeostasis, and their inhibitors prevent neuronal cell death, not only by directly inhibiting the activities of the kinases, but also by inhibiting Cd induction of $[Ca^{2+}]_i$ elevation, which, in turn, results in less activation of CaMKII-MAPK/mTOR signaling network.

Four isoforms of p38 ($-\alpha$, $-\beta$, $-\gamma$, and $-\delta$) have been identified and especially, various isoforms of p38 have unique cellular functions (Wang *et al.*, 1998; Enslen *et al.*, 2000; Zhang *et al.*, 2011). In this study, an antibody to phospho-p38 (Thr180/Tyr182) (Cat.# 9215, Cell Signaling) was used, which cannot differentiate isoforms of p38- α , $-\beta$, $-\gamma$, and $-\delta$. Therefore, currently we do not know what isoforms of p38 MAPK is activated by Cd-activated CaMKII. As activation of p38 MAPK is not involved in Cd-induced neuronal cell death (Chen *et al.*, 2008b), we did not further study what isoforms of p38 was activated.

In summary, we have shown that Cd-elevated $[Ca^{2+}]_i$ activates CaMKII in neuronal cells, which elicits MAPK and mTOR network, leading to neuronal cell death. Our findings implicate an important role of CaMKII in Cd neurotoxicology, and suggest that manipulation of intracellular Ca^{2+} level or CaMKII activity may be exploited for prevention of Cd-induced neurodegenerative disorders.

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Abbreviations

2-APB	2-aminoethoxydiphenyl borate
4E-BP1	eukaryotic initiation factor 4E binding protein 1
Akt	protein kinase B (PKB)
BAPTA/AM	1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester
$[Ca^{2+}]_i$	intracellular free calcium ion

Cd	cadmium
CaMKII	Ca ²⁺ /Calmodulin-dependent protein kinase II
CRAC channels	Ca ²⁺ release activated Ca ²⁺ channels
DMEM	Dulbecco's Modified Eagle's Medium
ER	endoplasmic reticulum
Erk1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
IP₃	inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
PDL	poly-D-lysine
PI3K	phosphatidylinositol 3'-kinase
S6K1	S6 kinase 1
Ser/Thr	serine/threonine
SDS	sodium dodecyl sulfate

References

- Ang ES, Zhang P, Steer JH, Tan JW, Yip K, Zheng MH, Joyce DA, Xu J. Calcium/calmodulin-dependent kinase activity is required for efficient induction of osteoclast differentiation and bone resorption by receptor activator of nuclear factor kappa B ligand (RANKL). *J Cell Physiol.* 2007; 212:787–795. [PubMed: 17477372]
- Baffy G, Miyashita T, Williamson JR, Reed JC. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J Biol Chem.* 1993; 268:6511–6519. [PubMed: 8454620]
- Biagioli M, Pifferi S, Ragghianti M, Bucci S, Rizzuto R, Pinton P. Endoplasmic reticulum stress and alteration in calcium homeostasis are involved in cadmium-induced apoptosis. *Cell Calcium.* 2008; 43:184–195. [PubMed: 17588656]
- Bian X, Hughes FM Jr, Huang Y, Cidlowski JA, Putney JW Jr. Roles of cytoplasmic Ca²⁺ and intracellular Ca²⁺ stores in induction and suppression of apoptosis in S49 cells. *Am J Physiol.* 1997; 272:C1241–1249. [PubMed: 9142849]
- Bilmen JG, Wootton LL, Godfrey RE, Smart OS, Michelangeli F. Inhibition of SERCA Ca²⁺ pumps by 2-aminoethoxydiphenyl borate (2-APB). 2-APB reduces both Ca²⁺ binding and phosphoryl transfer from ATP, by interfering with the pathway leading to the Ca²⁺-binding sites. *Eur J Biochem.* 2002; 269:3678–3687. [PubMed: 12153564]
- Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer.* 2004; 4:335–348. [PubMed: 15122205]
- Braun FJ, Aziz O, Putney JW Jr. 2-aminoethoxydiphenyl borane activates a novel calcium-permeable cation channel. *Mol Pharmacol.* 2003; 63:1304–1311. [PubMed: 12761340]

- 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 Radic Biol Med*. 2008a; 45:1035–1044. [PubMed: 18703135]
- Chen L, Liu L, Luo Y, Huang S. MAPK and mTOR pathways are involved in cadmium-induced neuronal apoptosis. *J Neurochem*. 2008b; 105:251–261. [PubMed: 18021293]
- Chen L, Xu B, Liu L, Luo Y, Yin J, Zhou H, Chen W, Shen T, Han X, Huang S. Hydrogen peroxide inhibits mTOR signaling by activation of AMPK α leading to apoptosis of neuronal cells. *Lab Invest*. 2010; 90:762–773. [PubMed: 20142804]
- Chen L, Xu B, Liu L, Luo Y, Zhou H, Chen W, Shen T, Han X, Kontos CD, Huang S. Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death. *Free Radic Biol Med*. 2011; 50:624–632. [PubMed: 21195169]
- Cheng A, Wang S, Yang D, Xiao R, Mattson MP. Calmodulin mediates brain-derived neurotrophic factor cell survival signaling upstream of Akt kinase in embryonic neocortical neurons. *J Biol Chem*. 2003; 278:7591–7599. [PubMed: 12488453]
- Choi SS, Seo YJ, Shim EJ, Kwon MS, Lee JY, Ham YO, Suh HW. Involvement of phosphorylated Ca²⁺/calmodulin-dependent protein kinase II and phosphorylated extracellular signal-regulated protein in the mouse formalin pain model. *Brain Res*. 2006; 1108:28–38. [PubMed: 16863646]
- Colbran RJ, Brown AM. Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. *Curr Opin Neurobiol*. 2004; 14:318–327. [PubMed: 15194112]
- Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G. Mammalian TOR: A Homeostatic ATP Sensor. *Science*. 2001; 294:1102–1105. [PubMed: 11691993]
- Enslen H, Branch DM, Davis RJ. Molecular determinants that mediate selective activation of p38 MAP kinase isoforms. *EMBO J*. 2000; 19:1301–1311. [PubMed: 10716930]
- Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic Acid-Mediated Mitogenic Activation of mTOR Signaling. *Science*. 2001; 294:1942–1945. [PubMed: 11729323]
- Fladmark KE, Brustugun OT, Mellgren G, Krakstad C, Boe R, Vintermyr OK, Schulman H, Doskeland SO. Ca²⁺/calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. *J Biol Chem*. 2002; 277:2804–2811. [PubMed: 11713251]
- Gibbons SJ, Brorson JR, Bleakman D, Chard PS, Miller RJ. Calcium influx and neurodegeneration. *Ann N Y Acad Sci*. 1993; 679:22–33. [PubMed: 8390144]
- Hajnoczky G, Davies E, Madesh M. Calcium signaling and apoptosis. *Biochem Biophys Res Commun*. 2003; 304:445–454. [PubMed: 12729578]
- Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, KY. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell Mol Life Sci*. 2002; 110:177–189.
- Huang S, Houghton PJ. Targeting mTOR signaling for cancer therapy. *Curr Opin Pharmacol*. 2003; 3:371–377. [PubMed: 12901945]
- Hudmon A, Schulman H. Neuronal Ca²⁺/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu Rev Biochem*. 2002; 71:473–510. [PubMed: 12045104]
- Jan CR, Cheng JS, Roan CJ, Lee KC, Chen WC, Chou KJ, Tang KY, Wang JL. Effect of diethylstilbestrol (DES) on intracellular Ca²⁺ levels in renal tubular cells. *Steroids*. 2001; 66:505–510. [PubMed: 11182139]
- Jin T, Lu J, Nordberg M. Toxicokinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. *Neurotoxicology*. 1998; 19:529–535. [PubMed: 9745907]
- Johnson S. Gradual micronutrient accumulation and depletion in Alzheimer's disease. *Med Hypotheses*. 2001; 56:595–597. [PubMed: 11399105]
- Kawahara M, Kuroda Y. Molecular mechanism of neurodegeneration induced by Alzheimer's beta-amyloid protein: channel formation and disruption of calcium homeostasis. *Brain Res Bull*. 2000; 53:389–397. [PubMed: 11136994]
- Kim BW, Choi M, Kim YS, Park H, Lee HR, Yun CO, Kim EJ, Choi JS, Kim S, Rhim H, Kaang BK, Son H. Vascular endothelial growth factor (VEGF) signaling regulates hippocampal neurons by elevation of intracellular calcium and activation of calcium/calmodulin protein kinase II and mammalian target of rapamycin. *Cell Signal*. 2008a; 20:714–725. [PubMed: 18221855]

- Kim D, Sarbassov D, Ali S, King J, Latek R, Erdjument-Bromage H, Tempst P, Sabatini D. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*. 2002; 110:163–175. [PubMed: 12150925]
- Kim S, Jung Y, Kim D, Koh H, Chung J. Extracellular zinc activates p70 S6 kinase through the phosphatidylinositol 3-kinase signaling pathway. *J Biol Chem*. 2000; 275:25979–25984. [PubMed: 10851233]
- Kim SD, Moon CK, Eun SY, Ryu PD, Jo SA. Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. *Biochem Biophys Res Commun*. 2005; 328:326–334. [PubMed: 15670787]
- Kim SM, Park JG, Baek WK, Suh MH, Lee H, Yoo SK, Jung KH, Suh SI, Jang BC. Cadmium specifically induces MKP-1 expression via the glutathione depletion-mediated p38 MAPK activation in C6 glioma cells. *Neurosci Lett*. 2008b; 440:289–293. [PubMed: 18573614]
- Kyriakis JM, Avruch J. Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation. *Physiol Rev*. 2001; 81:807–869. [PubMed: 11274345]
- Lau A, Zhang J, Chiu J. Acquired tolerance in cadmium-adapted lung epithelial cells: roles of the c-Jun N-terminal kinase signaling pathway and basal level of metallothionein. *Toxicol Appl Pharmacol*. 2006; 215:1–8. [PubMed: 16516943]
- Lemarie A, Lagadic-Gossman D, Morzadec C, Allain N, Fardel O, Vernhet L. Cadmium induces caspase-independent apoptosis in liver Hep3B cells: role for calcium in signaling oxidative stress-related impairment of mitochondria and relocation of endonuclease G and apoptosis-inducing factor. *Free Radic Biol Med*. 2004; 36:1517–1531. [PubMed: 15182854]
- Lewis TL, Brundage KM, Brundage RA, Barnett JB. 3,4-Dichloropropionanilide (DCPA) inhibits T-cell activation by altering the intracellular calcium concentration following store depletion. *Toxicol Sci*. 2008; 103:97–107. [PubMed: 18281253]
- Li M, Xia T, Jiang CS, Li LJ, Fu JL, Zhou ZC. Cadmium directly induced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis. *Toxicology*. 2003; 194:19–33. [PubMed: 14636693]
- Li Z, Arnaud L, Rockwell P, Figueiredo-Pereira ME. A single amino acid substitution in a proteasome subunit triggers aggregation of ubiquitinated proteins in stressed neuronal cells. *J Neurochem*. 2004; 90:19–28. [PubMed: 15198663]
- Lin CH, Chen PS, Gean PW. Glutamate preconditioning prevents neuronal death induced by combined oxygen-glucose deprivation in cultured cortical neurons. *Eur J Pharmacol*. 2008; 589:85–93. [PubMed: 18589412]
- Liu L, Chen L, Chung J, Huang S. Rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins. *Oncogene*. 2008; 27:4998–5010. [PubMed: 18504440]
- Liu Y, Templeton DM. Cadmium activates CaMK-II and initiates CaMK-II-dependent apoptosis in mesangial cells. *FEBS Lett*. 2007; 581:1481–1486. [PubMed: 17367784]
- Liu Y, Templeton DM. Initiation of caspase-independent death in mouse mesangial cells by Cd²⁺: involvement of p38 kinase and CaMK-II. *J Cell Physiol*. 2008; 217:307–318. [PubMed: 18506790]
- Liu ZM, Chen GG, Vlantis AC, Tse GM, Shum CK, van Hasselt CA. Calcium-mediated activation of PI3K and p53 leads to apoptosis in thyroid carcinoma cells. *Cell Mol Life Sci*. 2007; 64:1428–1436. [PubMed: 17514353]
- Lopez E, Figueroa S, Oset-Gasque M, MPG. Apoptosis and necrosis: two distinct events induced by cadmium in cortical neurons in culture. *Br J Pharmacol*. 2003; 138:901–911. [PubMed: 12642392]
- Marambaud P, Dreses-Werringloer U, Vingtdeux V. Calcium signaling in neurodegeneration. *Mol Neurodegener*. 2009; 4:20. [PubMed: 19419557]
- Mattson MP. Calcium and neurodegeneration. *Aging Cell*. 2007; 6:337–350. [PubMed: 17328689]
- Monroe RK, Halvorsen SW. Cadmium blocks receptor-mediated Jak/STAT signaling in neurons by oxidative stress. *Free Radic Biol Med*. 2006; 41:493–502. [PubMed: 16843830]
- Okuda B, Iwamoto Y, Tachibana H, Sugita M. Parkinsonism after acute cadmium poisoning. *Clin Neurol Neurosurg*. 1997; 99:263–265. [PubMed: 9491302]
- Olofsson MH, Havelka AM, Brnjic S, Shoshan MC, Linder S. Charting calcium-regulated apoptosis pathways using chemical biology: role of calmodulin kinase II. *BMC Chem Biol*. 2008; 8:2.

- Panayi AE, Spyrou NM, Iversen BS, White MA, Part P. Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry. *J Neurol Sci.* 2002; 195:1–10. [PubMed: 11867068]
- Pearson G, Robinson F, Beers Gibson T, Xu B-e, Karandikar M, Berman K, Cobb MH. Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. *Endocr Rev.* 2001; 22:153–183. [PubMed: 11294822]
- Pihl RO, Parkes M. Hair element content in learning disabled children. *Science.* 1977; 198:204–206. [PubMed: 905825]
- Prakriya M, Lewis RS. Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethylidiphenyl borate (2-APB) occurs independently of IP(3) receptors. *J Physiol.* 2001; 536:3–19. [PubMed: 11579153]
- Rockwell P, Martinez J, Papa L, Gomes E. Redox regulates COX-2 upregulation and cell death in the neuronal response to cadmium. *Cell Signal.* 2004; 16:343–353. [PubMed: 14687664]
- Rokhlin OW, Taghiyev AF, Bayer KU, Bumcrot D, Koteliansk VE, Glover RA, Cohen MB. Calcium/calmodulin-dependent kinase II plays an important role in prostate cancer cell survival. *Cancer Boil Ther.* 2007; 6:732–742.
- Satarug S, Baker J, Reilly P, Esumi H, Moore M. Evidence for a synergistic interaction between cadmium and endotoxin toxicity and for nitric oxide and cadmium displacement of metals in the kidney. *Nitric Oxide.* 2000; 4:431–440. [PubMed: 10944428]
- Schworer CM, Colbran RJ, Soderling TR. Reversible generation of a Ca^{2+} -independent form of Ca^{2+} (calmodulin)-dependent protein kinase II by an autophosphorylation mechanism. *J Biol Chem.* 1986; 261:8581–8584. [PubMed: 3722161]
- Shen HM, Dong SY, Ong CN. Critical role of calcium overloading in cadmium-induced apoptosis in mouse thymocytes. *Toxicology and applied pharmacology.* 2001; 171:12–19. [PubMed: 11181107]
- Vila-Petroff M, Salas MA, Said M, Valverde CA, Sapia L, Portiansky E, Hajjar RJ, Kranias EG, Mundina-Weilenmann C, Mattiazzi A. CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury. *Cardiovasc Res.* 2007; 73:689–698. [PubMed: 17217936]
- Wang SH, Shih YL, Ko WC, Wei YH, Shih CM. Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway. *Cell Mol Life Sci.* 2008; 65:3640–3652. [PubMed: 18850067]
- Wang SS, Chen L, Xia SK. Cadmium is acutely toxic for murine hepatocytes: effects on intracellular free Ca^{2+} homeostasis. *Physiol Res.* 2007; 56:193–201. [PubMed: 16555952]
- Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J, Chien KR. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogenactivated protein kinase family. *J Biol Chem.* 1998; 273:2161–2168. [PubMed: 9442057]
- Wright SC, Schellenberger U, Ji L, Wang H, Larrick JW. Calmodulin-dependent protein kinase II mediates signal transduction in apoptosis. *FASEB J.* 1997; 11:843–849. [PubMed: 9285482]
- Xu B, Chen S, Luo Y, Chen Z, Liu L, Zhou H, Chen W, Shen T, Han X, Chen L, Huang S. Calcium signaling is involved in cadmium-induced neuronal apoptosis via induction of reactive oxygen species and activation of MAPK/mTOR network. *PloS ONE.* 2011; 6:e19052. [PubMed: 21544200]
- Yamanaka A, Hiragami Y, Maeda N, Toku S, Kawahara M, Naito Y, Yamamoto H. Involvement of CaM kinase II in gonadotropin-releasing hormone-induced activation of MAP kinase in cultured hypothalamic neurons. *Arch Biochem Biophys.* 2007; 466:234–241. [PubMed: 17706588]
- Zhang J, Harrison JS, Studzinski GP. Isoforms of p38MAPK gamma and delta contribute to differentiation of human AML cells induced by 1,25-dihydroxyvitamin D3. *Exp Cell Res.* 2011; 317:117–130. [PubMed: 20804750]

Fig. 1.

Cd induced CaMKII phosphorylation in neuronal cells. (a) PC12 cells or primary neurons were treated with 0–20 μM Cd for 12 h, or (b) with 20 μM Cd for 0–12 h. Total 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. Cd induced phosphorylation of CaMKII in a concentration-dependent (a) and time-dependent (b) manner. (c and d) Cell viability of PC12 cells or primary neurons treated with different concentration of Cd for 24 h (c), or treated with 20 μM Cd for various time (d) was evaluated using MTT assay. Results are presented as mean ± SE; $n=4-6$. ** $P<0.01$ difference with control group.

Fig. 2.

Cd induced CaMKII phosphorylation via induction of [Ca²⁺]_i elevation. (a) Representative data show increasing [Ca²⁺]_i fluorescent intensity (shift to right) at 4 and 24 h post treatment of PC12 cells with Cd (0–20 μ M) using a fluorescence-activated cell sorter (FACS), with a fluorescent probe, Fluo-3/AM. (b) Quantitative analysis of FACS assay (a) shows changes of [Ca²⁺]_i fluorescence intensity in Cd-treated PC12 cells. (c) PC12 was pretreated with/without 20 μ M BAPTA/AM for 1 h, and then exposed to Cd (10 and 20 μ M) for 4 h and 24 h. [Ca²⁺]_i fluorescent intensity was assayed by Fluo-3/AM using a microplate reader, showing that BAPTA/AM strongly suppressed Cd-induced [Ca²⁺]_i elevation in the cells. (d) PC12 cells or primary neurons treated with/without Cd (10 and 20 μ M) for 12 h post pretreatment with BAPTA/AM for 1 h were harvested. 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. Results are presented as mean $\pm$ SE; $n=3-5$. ^b $P<0.01$, difference with control group; ^c $P<0.01$, difference with 10 μ M Cd group; ^d $P<0.01$, difference with 20 μ M Cd group.

Fig. 3.

Prevention of $[Ca^{2+}]_i$ elevation by 2-APB attenuated Cd-induced CaMKII phosphorylation. PC12 cells or primary neurons were pretreated with/without 2-APB (100 μ M) for 1 h, and then exposed to Cd (10 and 20 μ M) for indicated time. (a) $[Ca^{2+}]_i$ fluorescent intensity was assayed by Fluo-3/AM using a microplate reader, showing that 2-APB markedly attenuated Cd-induced $[Ca^{2+}]_i$ elevation in the cells. (b) Cd induction of CaMKII phosphorylation was inhibited by 2-APB, as detected by Western blotting using indicated antibodies. β -tubulin was used as a loading control. Similar results were observed in at least three independent experiments. Results are presented as mean $\pm$ SE; $n=5$. ^b $P<0.01$, difference with control group; ^c $P<0.01$, difference with 10 μ M Cd group; ^d $P<0.01$, difference with 20 μ M Cd group.

Fig. 4. Inhibition of CaMKII by KN93 attenuated Cd activation of MAPK and mTOR signaling pathways as well as neuronal cell death. PC12 cells and/or primary neurons were pretreated with/without KN93 (10 μM) for 1 h, and then exposed to Cd (10 and 20 μM) for indicated time. (a) KN93 obviously blocked Cd-induced CaMKII phosphorylation in PC12 cells and primary neurons. (b) Morphology of PC12 cells was visualized under a Nikon Eclipse TE2000-U inverted phase-contrast microscope (200×) equipped with digital camera. KN93 partially rescued cells from Cd-induced apoptosis. (c) Cell viability for PC12 cells and primary neurons was evaluated by MTT assay. Results are presented as mean ± SE; *n*=5. ^b*P*<0.01, difference with control group; ^c*P*<0.01, difference with 10 μM Cd

group; ^d $P < 0.01$, difference with 20 μM Cd group. (d and e) Cd-induced phosphorylation of JNK, Erk1/2, and p38, as well as Akt and mTOR-mediated S6K1 and 4E-BP1 was inhibited in part by KN93 in PC12 cells and primary neurons. 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.

Fig. 5. Downregulation of CaMKII prevented Cd activation of MAPK and mTOR signaling pathways as well as neuronal cell death. (a) Downregulation of CaMKII (by ~ 90%) by lentiviral shRNA to CaMKII and GFP (as control) in PC12 cells, as detected by Western blotting with antibodies to CaMKII. (b) Morphology of lentiviral shRNA-infected cells, treated with/without Cd (10 and 20 μM) for 24 h, was visualized under a Nikon Eclipse TE2000-U inverted phase-contrast microscope (200×) equipped with digital camera. (c) Cell viability was evaluated using MTT assay. Results are presented as mean ± SE; $n=8$. ** $P<0.01$, difference with control group; ## $P<0.01$, CaMKII shRNA group vs GFP shRNA group. (d and e) lentiviral shRNA-infected cells were exposed to Cd (10 and 20 μM) for 12 h, followed by Western blotting with indicated antibodies, showing that downregulation of CaMKII conferred partial resistance to Cd activation of MAPKs and

mTOR signaling pathways. β -tubulin was used as a loading control. Similar results were observed in at least three independent experiments.

Fig. 6.

Inhibitors of mTOR, JNK and Erk1/2, but not of p38, partially inhibited Cd-induced $[Ca^{2+}]_i$ elevation and CaMKII phosphorylation, preventing neuronal apoptosis. PC12 cells or primary neurons were exposed to Cd (10 and 20 μ M) for indicated time after pretreatment with 0.2 mg/ml mTOR inhibitor rapamycin (Rap) for 48 h or with 20 μ M JNK inhibitor SP600125, 5 μ M MEK1/2 (upstream of Erk1/2) inhibitor U0126, or 20 μ M p38 inhibitor PD169136 for 30 min, respectively. (a) Rap obviously reduced Cd-induced $[Ca^{2+}]_i$ elevation. (b) Rap blocked activation of mTOR-mediated S6K1 and 4E-BP1 with a concomitant reduction of CaMKII phosphorylation in Cd-exposed PC12 cells. SP600125 (c and d) and U0126 (e and f), but not PD169136 (g and h), partially prevented Cd-induced $[Ca^{2+}]_i$ elevation and CaMKII phosphorylation. $[Ca^{2+}]_i$ fluorescent intensity was evaluated

by a fluorescent probe, Fluo-3/AM, using a microplate reader. Western blot analysis was performed using indicated antibodies. The blots were probed for β -tubulin as a loading control. Similar results were observed in at least three independent experiments. Results are presented as mean $\pm$ SE; $n=5$. ^b $P<0.01$, difference with control group; ^c $P<0.01$, difference with 10 μ M Cd group; ^d $P<0.01$, difference with 20 μ M Cd group.