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Celastrol Attenuates Cadmium-Induced Neuronal Apoptosis via Inhibiting Ca^{2+}-CaMKII-Dependent Akt/mTOR Pathway

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Abstract

Cadmium (Cd), an environmental and industrial pollutant, affects the nervous system and consequential neurodegenerative disorders. Recently, we have shown that celastrol prevents Cd-induced neuronal cell death partially by suppressing Akt/mTOR pathway. However, the underlying mechanism remains to be elucidated. Here, we show that celastrol attenuated Cd-elevated intracellular-free calcium ($[Ca^{2+}]_i$) level and apoptosis in neuronal cells. Celastrol prevented Cd-induced neuronal apoptosis by inhibiting Akt-mediated mTOR pathway, as inhibition of Akt with Akt inhibitor X or ectopic expression of dominant negative Akt reinforced celastrol's prevention of Cd-induced phosphorylation of S6K1/4E-BP1 and cell apoptosis. Furthermore, chelating intracellular Ca^{2+} with BAPTA/AM or preventing $[Ca^{2+}]_i$ elevation using EGTA potentiated celastrol's repression of Cd-induced $[Ca^{2+}]_i$ elevation and consequential activation of Akt/mTOR pathway and cell apoptosis. Moreover, celastrol blocked Cd-elicited phosphorylation of CaMKII, and pretreatment with BAPTA/AM or EGTA enhanced celastrol's suppression of Cd-increased phosphorylation of CaMKII in neuronal cells, implying that celastrol hinders $[Ca^{2+}]_i$-mediated

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CaMKII phosphorylation. Inhibiting CaMKII with KN93 or silencing CaMKII attenuated Cd activation of Akt/mTOR pathway and cell apoptosis, and this was strengthened by celastrol. Taken together, these data demonstrate that celastrol attenuates Cd-induced neuronal apoptosis via inhibiting Ca^{2+}-CaMKII-dependent Akt/mTOR pathway. Our findings underscore that celastrol may act as a neuroprotective agent for the prevention of Cd-induced neurodegenerative disorders.

Cadmium (Cd), a common toxic environmental and industrial pollutant, can easily traverse the blood–brain barrier and accumulate in the brain leading to high neurotoxicity (Wang and Du, 2013; Xu et al., 2016). Epidemiological and clinical data have documented that Cd exposure results in a variety of neurological symptoms, including severe headaches and vertigo, visuomotor dysfunction, peripheral neuropathy, decreased equilibrium, neurobehavioral defects in attention, learning disabilities and hyperactivity, and olfactory dysfunction (Pihl and Parkes, 1977; Marlowe et al., 1985; Bar-Sela et al., 2001; Chen et al., 2011; Xu et al., 2016). Multiple studies have pointed to Cd as a potential risk factor in the development of neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Okuda et al., 1997; Bar-Sela et al., 2001; Johnson, 2001; Panayi et al., 2002; Chen et al., 2011). Thus, it is of great importance to unveil the underlying mechanisms of Cd-induced neurotoxicity and to find a novel therapeutic target and strategy to prevent the neurotoxicity of Cd.

It is well known that protein kinase B (Akt/PKB) plays a critical role in the regulation of neuronal cell survival (Dudek et al., 1997). Mammalian target of rapamycin (mTOR) activity modulates differentiation, growth, and survival of neurons, which is vital for synaptic plasticity, learning and memory formation, and food uptake in adult brain (Jaworski and Sheng, 2006; Swiech et al., 2008). Activated Akt, as a main upstream mediator of mTOR, positively regulates mTOR, leading to increased phosphorylation of ribosomal p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (Bjornsti and Houghton, 2004; Laplante and Sabatini, 2012; Cornu et al., 2013). So the phosphorylation level of S6K1 and 4E-BP1 is widely used as a surrogate of mTOR activity. Celastrol, a triterpene that is extracted from the roots of *Tripterygium wilfordi* (thunder god vine) plant, is a potent compound with antioxidant, anti-apoptotic, anti-inflammatory, anti-carcinogenic, and anti-obesity properties (Tao et al., 2001; Salminen et al., 2010; Kannaiyan et al., 2011; Li et al., 2015; Liu et al., 2015). Of note, celastrol also has neuroprotective benefit in the models of neurodegenerative disorders, such as PD, AD, and ALS (Allison et al., 2001; Cleren et al., 2005; Kiaei et al., 2005). Recent studies have shown that celastrol suppresses the growth of various cancer cells by inhibiting Akt and/or mTOR signaling (Pang et al., 2010; Kannaiyan et al., 2011; Ma et al., 2014; Sha et al., 2015). Our group has demonstrated that Cd induces neuronal cell death in part by activation of Akt/mTOR signaling pathway (Chen et al., 2008), and found that celastrol prevents Cd from activation of Akt/mTOR pathway and apoptosis in neuronal cells (Chen et al., 2014). However, how celastrol attenuates Cd-induced activation of Akt/mTOR signaling and neuronal apoptosis is largely unknown.

Calcium ion (Ca^{2+}), as an intracellular second messenger, modulates a variety of physiological responses of neurons to neurotransmitters and neurotrophic factors, including cell survival responses (Neher and Sakaba, 2008; Surmeier et al., 2010; Xu et al., 2011). A series of studies have shown that disturbances in cellular Ca^{2+} homeostasis result in synaptic dysfunction, impaired plasticity, and neuronal degeneration (Gibbons et al., 1993; Mattson, 2007; Toescu and Verkhratsky, 2007; Marambaud et al., 2009). Calcium/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitously expressed multifunctional Ser/Thr kinase activated by Ca^{2+}/calmodulin (CaM) complex (Schworer et al., 1986; Colbran and Brown, 2004). It has been reported that CaMKII functions through Ca^{2+} signaling to regulate the survival and apoptosis of neuronal cells (Yamanaka et al., 2007; Kim et al., 2008; Song et al., 2010; Chen et al., 2011). Our recent studies have disclosed that Cd-induced elevation of intracellular-free Ca^{2+} ($[Ca^{2+}]_i$) activates CaMKII-dependent Akt/mTOR pathway leading to neuronal cell death (Chen et al., 2011; Xu et al., 2011). This prompted us to study whether celastrol inhibits Cd activation of Akt/mTOR pathway triggering neuronal apoptosis by preventing Cd from activating $[Ca^{2+}]_i$-CaMKII. In the present study, we show that celastrol's prevention of Cd-activated Akt/mTOR pathway contributing to neuronal apoptosis was Ca^{2+}-dependent. Further investigation found that celastrol hindered $[Ca^{2+}]_i$-mediated CaMKII phosphorylation, thereby preventing Cd from activation of Akt/mTOR pathway and neuronal apoptosis. Our findings underscore that celastrol may act as a neuroprotective agent for the prevention of Cd-induced neurodegenerative disorders.

Materials and Methods

Materials

Cadmium chloride, 4′,6-diamidino-2-phenylindole (DAPI), poly-D-lysine (PDL), ethylene glycol tetra-acetic acid (EGTA), and protease inhibitor cocktail were purchased from Sigma (St Louis, MO). 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA/AM) was from Calbiochem (San Diego, CA). KN93 was acquired from ALEXIS Biochemicals Corporation (San Diego, CA). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit was from Promega (Madison, WI). Enhanced chemiluminescence solution was from Millipore (Billerica, MA). Dulbecco's modified Eagle's medium (DMEM), 0.05% Trypsin-EDTA, NEUROBASALTM Media, B27 Supplement, and Fluo-3/AM were purchased from Invitrogen (Grand Island, NY). Horse serum and fetal bovine serum (FBS) were obtained from Hyclone (Logan, UT). The following antibodies were used: phospho-CaMKII (Thr286), phospho-Akt (Ser473), phospho-S6K1 (Thr389), phospho-4E-BP1 (Thr70), 4E-BP1, cleaved-caspase-3, PARP (Cell Signaling Technology, Beverly, MA); Akt, S6K1, CaMKII (Santa Cruz Biotechnology, Santa Cruz, CA); β-tubulin, phospho-Akt (Thr308), and HA (Sigma); Goat anti-rabbit IgG-horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP (Pierce, Rockford, IL). Other chemicals were purchased from local commercial sources and were of analytical grade.

Cell culture

Rat pheochromocytoma (PC12) cells (American Type Culture Collection, Manassas, VA), seeded in a six-well plate (5×10^5 cells/well) or 96-well plate (1×10^4 cells/well) pre-coated with 0.2 μg/ml PDL, were grown in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS. Cells were maintained in a humidified incubator of 5% CO_2 at 37°C. Primary murine neurons, isolated from fetal mouse cerebral cortexes of 16–18 days of gestation in female ICR mice (being pregnant), were seeded in a six-well plate (5×10^5 cells/well) or 96-well plate (1×10^4 cells/well) coated with 10 μg/ml PDL for experiments after 6 days of culture as described (Chen et al., 2010, 2014). All procedures used in this study were approved by 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.

Recombinant adenoviral constructs and infection of cells

Recombinant adenovirus encoding HA-tagged dominant negative Akt (Ad-dn-Akt, T308A/S473A) was generously provided from Dr. Kenneth Walsh (Boston University, Boston, MA), and the control virus expressing the green fluorescent protein (GFP) (Ad-GFP) was described previously (Liu et al., 2010). For experiments, PC12 cells were grown in the growth medium and infected with the individual adenovirus for 24 h at 5 of multiplicity of infection (MOI = 5). Subsequently, cells were used for experiments. Ad-GFP served as a control. Expression of HA-tagged dn-Akt was determined by Western blotting with antibodies to HA.

Lentiviral shRNA cloning, production, and infection

Lentiviral shRNAs to CaMKII and GFP (as control) were generated and used as described (Chen et al., 2011). For use, monolayer PC12 cells, when grown to about 70% confluence, were infected with above lentivirus-containing supernatant in the presence of 8 μg/ml polybrene and exposed to 2 μg/ml puromycin after 24 h of infection. In 5 days, cells were used for experiments.

Assay for cell viability

PC12 cells and primary neurons, or PC12 cells infected with Addn-Akt or Ad-GFP, respectively, seeded in a 96-well plate (1×10^4 cells/well) pre-coated with PDL (0.2 μg/ml for PC12 cells; 10 μg/ml for primary neurons), were exposed to Cd (10 and/or 20 μM) for 24 h following pretreatment with/without celastrol (1 μM) for 1 h with five replicates of each treatment. Subsequently, cell viability, after incubation with MTS reagent (One Solution reagent) (20 μl/well) for 3 h, was monitored by the optical density (OD) at 490 nm using a Victor X3 Light Plate Reader (PerkinElmer, Waltham, MA).

DAPI staining

PC12 cells and primary neurons, or PC12 cells infected with lentiviral shRNA to CaMKII or GFP, or PC12 cells infected with Ad-dn-Akt or Ad-GFP, respectively, seeded at a density of 5×10^5 cells/well in a six-well plate containing a PDL-coated glass coverslip per well, were pretreated with/without celastrol (1 μM) for 1 h, or pretreated with/without Akt

inhibitor X (20 μM), BAPTA/AM (20 μM), EGTA (100 μM), or KN93 (10 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 and/or 20 μM) for 24 h. Afterwards, the apoptotic cells with fragmented and condensed nuclei were determined using DAPI staining as described (Chen et al., 2008). Finally, photographs were captured under a fluorescent microscope (Leica DMi8, Wetzlar, Germany) equipped with a digital camera.

Analysis for $[Ca^{2+}]_i$ imaging

PC12 cells and primary neurons, seeded at a density of 5×10^5 cells/well in a six-well plate containing a PDL-coated glass coverslip per well, were pretreated with/without celastrol (1 μM) for 1 h, or pretreated with/without BAPTA/AM (20 μM) or EGTA (100 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 and/or 20 μM) for 24 h. The cells were then loaded with 5 μM Fluo-3/AM for 40 min. Finally, all stained coverslips were rinsed twice with PBS, followed by imaging under a fluorescence microscope. Integral optical density (IOD) for quantitative analysis of the fluorescence intensity was measured by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Newburyport, MA).

Western blot analysis

PC12 cells and primary neurons, or PC12 cells infected with lentiviral shRNA to CaMKII or GFP, or PC12 cells infected with Ad-dn-Akt or Ad-GFP, respectively, after treatments, Western blotting was performed, and the blots for detected protein were semi-quantified using NIH Image J software (National Institutes of Health, Bethesda, MD) as described previously (Chen et al., 2010, 2014).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). 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

Celastrol prevents Cd-induced neuronal apoptosis by attenuating Cd-elevated $[Ca^{2+}]_i$

Recently, we have shown that Cd induces apoptosis of PC12, SH-SY5Y cells, and primary neurons by induction of $[Ca^{2+}]_i$ elevation (Xu et al., 2011), and celastrol prevents Cd-evoked neuronal apoptosis (Chen et al., 2014). This prompted us to study whether celastrol prevents Cd-induced apoptosis by attenuating Cd-elevated $[Ca^{2+}]_i$ in the neuronal cells. To this end, PC12 cells and primary neurons were pretreated with/without celastrol (1 μM) for 1 h and then exposed to Cd (10 and 20 μM) for 24 h, followed by imaging $[Ca^{2+}]_i$ by a calcium indicator dye, Fluo-3/AM. Imaged and quantified results revealed that treatment with Cd for 24 h evoked strong $[Ca^{2+}]_i$ fluorescence (in green) in the cells, which was potently repressed by celastrol pretreatment (Fig. 1A and B). Consistently, pretreatment with celastrol also conferred high resistance to Cd-decreased cell viability of PC12 cells and primary neurons

(Fig. 1C), implying that celastrol attenuates Cd-induced cell death by preventing Cd from elevating $[Ca^{2+}]_i$ in neuronal cells.

Using DAPI staining, we found that pretreatment with celastrol obviously reduced the percentage of cells with fragmented nuclei (arrows) in PC12 cells and primary neurons in response to Cd, compared with the control (Fig. 2A and B). In addition, using Western blot analysis, we also observed that treatment with Cd for 8 h resulted in robust cleavages of caspase-3 and PARP in the cells, which were dramatically blocked by celastrol (Fig. 2C). Collectively, our results support the notion that celastrol attenuates Cd-elevated $[Ca^{2+}]_i$, thereby preventing Cd-induced apoptosis in neuronal cells.

Celastrol prevents Cd-induced neuronal apoptosis by blocking Akt-mediated activation of mTOR pathway

Akt is a major regulator of neuronal cell survival (Dudek et al., 1997). Our recent studies have documented that celastrol may inhibit Cd-induced activation of Akt/mTOR signaling pathway and apoptosis in neuronal cells (Chen et al., 2014). In line with the above findings, here we also observed that pretreatment with celastrol (1 μM) for 1 h markedly blocked the phosphorylation of Akt (Ser473 and Thr308), S6K1 (Thr389), and 4E-BP1 (Thr70) in PC12 and primary neurons induced by 8-h exposure with Cd (10 and 20 μM), as detected by Western blot analysis (Fig. 3A and B). As Akt lies upstream of mTOR (Laplante and Sabatini, 2012; Cornu et al., 2013), we next sought to determine whether celastrol prevents Cd from activation of mTOR-dependent neuronal apoptosis through blocking activation of Akt. For this, PC12 cells and primary neurons were pre-incubated with/without Akt inhibitor X alone, or in combination with celastrol. We found that pretreatment with Akt inhibitor X (20 μM) or celastrol (1 μM) alone substantially suppressed Cd-induced p-Akt, p-S6K1, p-4E-BP1, and cleaved-caspase-3 in the cells (Fig. 3C and D). Especially, co-treatment with celastrol/Akt inhibitor X exhibited a stronger inhibitory effect on Cd-induced events (Fig. 3C and D). Consistently, the combination of celastrol with Akt inhibition X also showed more potent attenuation of Cd-elicited apoptosis than celastrol or Akt inhibitor X alone, as evidenced by the decreased percentage of cells with fragmented nuclei (Fig. 3E).

To confirm the above findings, PC12 cells, infected with recombinant adenoviruses-expressing HA-tagged dominant negative Akt (Ad-dn-Akt) and a control virus-encoding GFP alone (Ad-GFP), respectively, were exposed to Cd (10 μM) for 8 h or 24 h post-pretreatment with/without celastrol (1 μM) for 1 h. As expected, a high level of HA-tagged dn-Akt was seen in Ad-dn-Akt-infected cells, but not in Ad-GFP-infected cells (as control) (Fig. 4A). Of importance, we showed that ectopic expression of dn-Akt profoundly attenuated Cd-induced p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 (Fig. 4A and B), cell viability reduction (Fig. 4C), and cell apoptosis (Fig. 4D), and potentiated celastrol's inhibition of Cd-induced events (Fig. 4A–D). Taken together, our results indicate celastrol prevents Cd-induced neuronal apoptosis by inhibiting Akt-mediated activation of mTOR pathway.

Celastrol blocks Cd-induced activation of Akt/mTOR pathway and neuronal apoptosis via preventing Cd from elevating $[Ca^{2+}]_i$

Having observed that celastrol's attenuation of Cd-elevated $[Ca^{2+}]_i$ links to its prevention of neuronal apoptosis (Figs. 1 and 2), and celastrol prevents Cd-induced neuronal apoptosis by inhibiting Akt-mediated activation of mTOR pathway (Fig. 3), next, we asked whether celastrol inhibition of Cd-elevated $[Ca^{2+}]_i$ plays a pivotal role in repressing Cd-induced activation of Akt/mTOR signaling and apoptosis in neuronal cells. To this end, PC12 cells and primary neurons were pretreated with/without BAPTA/AM (20 μM), an intracellular Ca^{2+} chelator, for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 μM) for 8 h or 24 h. We found that the combination of celastrol with BAPTA/AM attenuated Cd-triggered $[Ca^{2+}]_i$ elevation (Fig. 5A) and the phosphorylation of Akt, S6K1, and 4E-BP1 (Fig. 5B and C) more potently than celastrol or BAPTA/AM alone in the cells. The inhibitory effect of BAPTA/AM on Cd-induced cleavage of caspase-3 was also remarkably potentiated by co-treatment with celastrol (Fig. 5B and C). In line with this, BAPTA/AM obviously prevented Cd-induced nuclear fragmentation and condensation in PC12 cells and primary neurons, and this effect was strengthened by celastrol as well (Fig. 5D). The results suggest that celastrol's prevention of Cd-activated Akt/mTOR pathway and neuronal apoptosis was Ca^{2+}-dependent.

To further determine how celastrol attenuation of Cd-elevated $[Ca^{2+}]_i$ contributes to its suppression of Akt/mTOR signaling pathway and neuronal apoptosis, we extended our studies using EGTA (100 μM), an extracellular Ca^{2+} chelator. The results showed that inhibition of extracellular Ca^{2+} influx by EGTA (Fig. 6A) profoundly prevented Cd from activating the phosphorylation of Akt, S6K1, and 4E-BP1 (Fig. 6B and C), and partially blocked the cleavage of caspase-3 and apoptosis in PC12 cells and primary neurons in response to Cd (Fig. 6B–D). Of importance, co-treatment with celastrol/EGTA caused a more effective inhibition of Cd-elicited events (Fig. 6A–D). These findings indicate that celastrol's interference with Cd-induced extracellular Ca^{2+} influx links to its prevention of Akt/mTOR signaling pathway and neuronal apoptosis. Collectively, our data strongly support the notion that celastrol blocks Cd-induced neuronal apoptosis via preventing Cd elevation of $[Ca^{2+}]_i$, thus inhibiting Akt/mTOR pathway.

Celastrol prevents Cd-induced neuronal apoptosis by inhibiting Ca^{2+}-CaMKII-dependent Akt/mTOR signaling pathway

CaMKII is a general integrator of Ca^{2+} signaling (Colbran and Brown, 2004; Liu and Templeton, 2007). Recently, we have demonstrated that Cd-elevated $[Ca^{2+}]_i$ activates CaMKII-dependent Akt/mTOR pathway leading to neuronal cell death (Chen et al., 2011). Thus, we reasoned that celastrol prevents Cd-induced neuronal apoptosis by inhibiting Ca^{2+}-CaMKII-dependent Akt/mTOR signaling pathway. To this end, firstly, PC12 cells and primary neurons were pretreated with/without celastrol (1 μM) for 1 h and then exposed to Cd (10 and 20 μM) for 8 h, followed by Western blot analysis. As shown in Figure 7A and B, pretreatment with celastrol dramatically reversed Cd-increased phosphorylation of CaMKII. This was in agreement with the observation that there existed a significant inhibitory effect of celastrol on Cd-induced cell viability reduction (Fig. 1C) or apoptosis (Fig. 2A–C) in PC12 cells and primary neurons. The results suggest that celastrol

may protect against neuronal apoptosis through preventing Cd from triggering CaMKII phosphorylation.

Next, we studied whether celastrol inhibition of Cd-induced CaMKII phosphorylation is dependent on the level of $[Ca^{2+}]_i$. To answer this question, PC12 cells and primary neurons were pretreated with/without BAPTA/AM (20 μM) or EGTA (100 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10) for 8 h. As predicted, chelating $[Ca^{2+}]_i$ with BAPTA/AM or preventing $[Ca^{2+}]_i$ elevation using EGTA obviously enhanced celastrol's inhibition of Cd-induced CaMKII phosphorylation in the cells (Fig. 7C and D), indicating that celastrol prevents Cd-elevated $[Ca^{2+}]_I$, resulting in inhibition of CaMKII phosphorylation.

To elucidate whether celastrol inhibition of CaMKII phosphorylation is associated with preventing activation of Akt/mTOR pathway and apoptosis in neuronal cells exposed to Cd, PC12 cells and primary neurons were pretreated with/without CaMKII inhibitor KN93 (10 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10) for 8 h or 24 h. As demonstrated in Figure 8A and B, KN93 powerfully reinforced the inhibitory effects of celastrol on Cd-elicited p-CaMKII, p-Akt, p-S6K1, and p-4E-BP1 in the cells. Also, co-treatment with celastrol/KN93 rescued cells from Cd-induced cleaved-caspase-3 and apoptosis more potently than celastrol or KN93 alone (Fig. 8A–C). These results support the idea that celastrol blocks Cd-induced activation Akt/mTOR pathway and apoptosis in part by inhibiting CaMKII in neuronal cells.

To further corroborate the role of CaMKII in celastrol blockage of Cd-activated Akt/mTOR pathway and neuronal apoptosis, PC12 cells, infected with lentiviral shRNA to CaMKII or GFP, were exposed to Cd (10 μM) for 8 h or 24 h following pretreatment with/without celastrol (1 μM) for 1 h. As shown in Figure 8D, CaMKII expression was downregulated by ~90% in shRNA CaMKII-infected cells compared to shRNA GFP-infected cells. Silencing CaMKII obviously strengthened celastrol suppression of Cd-induced phosphorylation of CaMKII, Akt, S6K1, and 4E-BP1 (Fig. 8D and E). Consistently, downregulation of CaMKII also reinforced the inhibitory effects of celastrol on Cd-induced cleavage of caspase-3 (Fig. 8D and E) and apoptosis (Fig. 8F) in the cells. Taken together, these data verify that celastrol protects against Cd-induced neuronal apoptosis by inhibiting Ca^{2+}-CaMKII-dependent Akt/mTOR signaling pathway.

Discussion

Accumulated evidence has demonstrated the association of Cd exposure with a variety of neurological symptoms (Pihl and Parkes, 1977; Marlowe et al., 1985; Bar-Sela et al., 2001; Chen et al., 2011; Xu et al., 2016), and underlined the intoxication of Cd as a possible etiological factor of neurodegenerative diseases, such as PD, AD, HD, and ALS (Okuda et al., 1997; Bar-Sela et al., 2001; Johnson, 2001; Panayi et al., 2002; Chen et al., 2011). A series of studies from our group have shown that Cd activates Akt/mTOR pathway contributing to neuronal cell death (Chen et al., 2008), and pinpointed that Cd elevation of $[Ca^{2+}]_i$ activates CaMKII-dependent Akt/mTOR pathway leading to neuronal cell death (Chen et al., 2011; Xu et al., 2011), indicating the importance of Ca^{2+} signaling in activating

Akt/mTOR-mediated apoptosis in neuronal cells in response to Cd (Chen et al., 2008, 2011; Xu et al., 2011). Celastrol, a plant-derived triterpene, has been used as a clinical therapeutic agent due to its lack of toxicity (Cleren et al., 2005; Pang et al., 2010). It also possesses neuroprotective benefit in the models of neurodegenerative disorders, such as PD, AD, and ALS (Allison et al., 2001; Cleren et al., 2005; Kiaei et al., 2005). Recently, we have demonstrated that celastrol prevents Cd-induced neuronal cell death partially by suppressing Akt/mTOR pathway. Herein, we provide evidence that celastrol efficiently prevented Cd-induced $[Ca^{2+}]_i$ elevation, thereby suppressing activation of CaMKII in neuronal cells. To our knowledge, this study is the first to demonstrate that the neuroprotective effect of celastrol is associated with its attenuating Cd-induced Ca^{2+}/CaMKII-dependent activation of Akt/mTOR pathway.

Considering that disturbances in cellular Ca^{2+} homeostasis have been highlighted in various neuropathological conditions, such as synaptic dysfunction, impaired plasticity, and neuronal degeneration, including Cd-induced neurotoxicity (Gibbons et al., 1993; Mattson, 2007; Toescu and Verkhratsky, 2007; Marambaud et al., 2009; Chen et al., 2011; Xu et al., 2011), we firstly tested whether celastrol affects Cd-induced $[Ca^{2+}]_i$ elevation in neuronal cells. As expected, celastrol indeed attenuated Cd-elevated $[Ca^{2+}]_i$ contributing to apoptosis in PC12 cells and primary neurons (Figs. 1 and 2). In this study, the fluorescent intensity of $[Ca^{2+}]_i$ is evaluated by imaging with a Ca^{2+} indicator dye, Fluo-3/AM, based on multiple data of Fluo-3/AM as a suitable tool to record the effect of Cd on $[Ca^{2+}]_i$ in various living cells (Marchi et al., 2000; Wang et al., 2008, 2009; Chen et al., 2011). Athough Fluo-3/AM could be responsive to intracellular free Cd^{2+} in Cd-exposed neuronal cells, as shown by the data that Cd^{2+} may enter neuronal cells through Ca^{2+} channels (Taki, 2013), other studies have documented that Cd^{2+} can produce the strongest probe signal rise in free solution and the lowest fluorescence increase in cells, underlining that interference of Cd^{2+} with Fluo-3/AM does not affect Ca^{2+} measurements in living cells (Marchi et al., 2000). Alterations of Fluo-3/AM produced by Cd^{2+} in free solution become negligible in the cellular environment, and the responses of the probe within cells are consistent with expected Cd^{2+} effects on cell Ca^{2+}, rather than with the artifacts derived from metal/probe interactions (Marchi et al., 2000). Therefore, the use of Fluo-3/AM for fluorescent Ca^{2+} probe and digital imaging can be considered a suitable technique for studies concerning Cd^{2+} effects on the intracellular Ca^{2+}. However, it is worthy to explore whether changes in $[Ca^{2+}]_i$ levels, by taking advantage of multicolor imaging techniques with appropriate fluorescent indicators, could be simultaneously monitored with Cd^{2+} levels. This may reveal the mechanistic details of Cd-induced toxicity more substantially (Taki, 2013). More studies are needed, by designing the molecules that can distinguish the behaviors between Cd^{2+} and Ca^{2+} based on their distinct fluorescence features.

It is well known that mTOR functions as at least two complexes, mTORC1 and mTORC2 (Laplante and Sabatini, 2012; Cornu et al., 2013). mTORC1 regulates phosphorylation of S6K1 and 4E-BP1, and mTORC2 regulates phosphorylation of Akt (Laplante and Sabatini, 2012; Cornu et al., 2013). In the current study, we observed that celastrol significantly blocked Cd-induced phosphorylation of Akt (Ser473 and Thr308), S6K1 (Thr389), and 4E-BP1 (Thr70) in PC12 and primary neurons (Fig. 3A), suggesting that celastrol inhibits both mTORC1 and mTORC2 in neuronal cells in response to Cd. We have recently shown

that Cd results in neuronal apoptosis in part through activation of mTOR signaling pathway (Chen et al., 2008, 2014), and unveiled that both mTORC1-mediated S6K1/4E-BP1 and mTORC2-mediated Akt pathways contribute to Cd-induced cell death (Xu et al., 2015). In this study, we also revealed that pharmacological inhibition of Akt with Akt inhibitor X reinforced celastrol's prevention of Cd-induced phosphorylation of Akt (Ser473 and Thr308), S6K1 (Thr389), and 4E-BP1 (Thr70), as well as cell apoptosis (Fig. 3). This is further supported by the results that overexpression of dn-Akt significantly attenuated Cd-induced events, and this was potentiated by celastrol (Fig. 4). It is noteworthy that the effect for co-treatment with celastrol/Akt inhibitor X is additive in protecting cells against Cd-induced apoptosis. This may be attributed to two reasons: (i) neither celastrol nor Akt inhibitor X was used at their full effects and concentrations could be higher; (ii) celastrol and Akt inhibitor X have different targets, therefore enhancing their common effect. Collectively, these findings support the idea that celastrol interferes with Cd-induced activation of Akt/mTOR-dependent neuronal apoptosis, and imply that celastrol may exert its protection against Cd-induced neuronal cell damage by inhibiting both mTORC1 and mTORC2 pathways.

Many studies on the effect of Cd on cytosolic Ca^{2+} level, using an intracellular Ca^{2+} chelator, BAPTA/AM, have demonstrated that Cd disrupts $[Ca^{2+}]_i$ homeostasis, leading to apoptosis in a variety of cells, such as skin epidermal cells (Son et al., 2010), hepatic cells (Lemarie et al., 2004; Xie et al., 2010), mesangial cells (Wang et al., 2008), renal tubular cells (Yeh et al., 2009), thyroid cancer cells (Liu et al., 2007), thymocytes (Shen et al., 2001), murine macrophages (Kim and Sharma, 2004), and neuronal cells (PC12, SH-SY5Y, primary neurons) (Chen et al., 2011; Xu et al., 2011). EGTA, an extracellular Ca^{2+} chelator, which renders the inaccessibility of extracellular Ca^{2+} to the cells, has been shown to have substantial inhibitory effects on Cd-induced $[Ca^{2+}]_i$ elevation, growth arrest, mitochodrial activity, and apoptosis in various cells (Kim and Sharma, 2004; Yang et al., 2008; Wang et al., 2009; Xie et al., 2010; Xu et al., 2011). Based on these data, in this study, we employed BAPTA/AM and EGTA as pharmacological chelators for drawing and evaluating the role of Ca^{2+} in celastrol's attenuation of Cd-induced neuronal apoptosis. We demonstrated that chelating intracellular Ca^{2+} with BAPTA/AM or preventing $[Ca^{2+}]_i$ elevation using EGTA potentiated celastrol's repression of Cd-induced $[Ca^{2+}]_i$ elevation and consequential activation of Akt/mTOR pathway and cell apoptosis. Taken together, these findings support that celastrol counteracts Cd neurotoxicity by blocking Cd-induced $[Ca^{2+}]_i$ elevation, thereby preventing activation of Akt/ mTOR signaling pathway.

CaMKII, a ubiquitously expressed multifunctional Ser/Thr kinase, has been reported to regulate the survival and apoptosis of neuronal cells through Ca^{2+} signaling (Yamanaka et al., 2007; Kim et al., 2008; Song et al., 2010; Chen et al., 2011). Since CaMKII acts as a general integrator of Ca^{2+} signaling, we speculated that celastrol likely prevents activation of Akt/mTOR pathway and neuronal apoptosis by inhibiting Ca^{2+}-dependent CaMKII phosphorylation. Indeed, in this study, we showed that celastrol blocked Cd-elicited phosphorylation of CaMKII (Fig. 7A and B), and pretreatment with BAPTA/AM, or EGTA enhanced celastrol's suppression of Cd-increased phosphorylation of CaMKII in neuronal cells (Fig. 7C and D), suggesting that celastrol hinders Cd elevation of $[Ca^{2+}]_i$-mediated CaMKII phosphorylation. To unveil whether CaMKII activity is essential for

celastrol's inhibition of Cd-activated Akt/mTOR signaling and apoptosis in neuronal cells, KN93, a specific inhibitor of CaMKII (Choi et al., 2006), was employed. We found that KN93 potently enhanced the inhibitory effects of celastrol on Akt, S6K1, and 4E-BP1 phosphorylation, as well as cleaved-caspase-3 in PC12 cells and primary neurons induced by Cd (Fig. 8A and B), and effectively strengthened celastrol's rescue of cells from apoptosis (Fig. 8C). Similar results were observed in the cells treated with lentiviral shRNA to CaMKII (Fig. 8D–F). These results strongly support that celastrol has beneficial roles in preventing Cd-elevated $[Ca^{2+}]_i$-dependent CaMKII phosphorylation, thereby inhibiting activation of Akt/mTOR signaling pathway and neuronal apoptosis.

A new question that arises from this work is how celastrol suppresses Cd-elevated $[Ca^{2+}]_i$-dependent CaMKII-Akt/mTOR pathway and neuronal apoptosis. Many studies have reported that the interconnection between cellular Ca^{2+} and free radicals, such as reactive oxygen species (ROS), alters the structures and functions of cellular proteins, and also activates or inhibits related signaling pathways, leading to neuronal apoptosis (Miller et al., 2009; Circu and Aw, 2010; Niizuma et al., 2010; Franklin, 2011; Cheng et al., 2012). Especially, abnormally high $[Ca^{2+}]_i$ level evokes ROS overproduction, and thus activates stress cascades, resulting in apoptosis (Gunter and Sheu, 2009; Cheng et al., 2012). In turn, excess ROS can also promote Ca^{2+} overload and sensitize the bioactivity of Ca^{2+} (Kim et al., 2006; Cheng et al., 2012). It is well known that celastrol belongs to triterpene family with antioxidant property and oxidative stability (Salminen et al., 2010; Jaquet et al., 2011). During our research, we also observed that when celastrol-pretreated PC12 cells and primary neurons were exposed to Cd for 24 h, cellular ROS overproduction was significantly reduced compared to the vehicle-pretreated cells (data not shown). In addition, of note, mitochondria play a crucial role in cellular Ca^{2+} and redox homeostasis and apoptosis induction (Koopman et al., 2010; Cheng et al., 2012). Cd triggers the high ROS levels in the mitochondria of PC12 cells, anterior pituitary cells, cortical neurons, and brain (Wang et al., 2004; Lopez et al., 2006; Poliandri et al., 2006). In our experiments, we also noticed that celastrol attenuated mitochondrial ROS-dependent apoptosis in neuronal cells in response to Cd (data not shown). Thus, we tentatively conclude that celastrol may modulate the crosstalk between Ca^{2+} signaling and mitochondrial ROS, thereby suppressing Cd activation of Akt/mTOR signaling pathway leading to apoptosis in neuronal cells in response to Cd. Undoubtedly, more studies are needed to address this issue.

In conclusion, we have demonstrated that celastrol prevented Cd-activated Akt and consequential mTOR pathway contributing to neuronal apoptosis in a Ca^{2+}-dependent manner. Further, we found that celastrol hindered Cd elevation of $[Ca^{2+}]_i$-mediated CaMKII phosphorylation, thereby preventing Cd from activation of Akt/mTOR pathway and neuronal apoptosis (Fig. 9). Our findings underscore that celastrol may act as a neuroprotective agent for the prevention of Cd-activated Ca^{2+}-CaMKII-Akt-mTOR pathway associated with neurodegenerative disorders.

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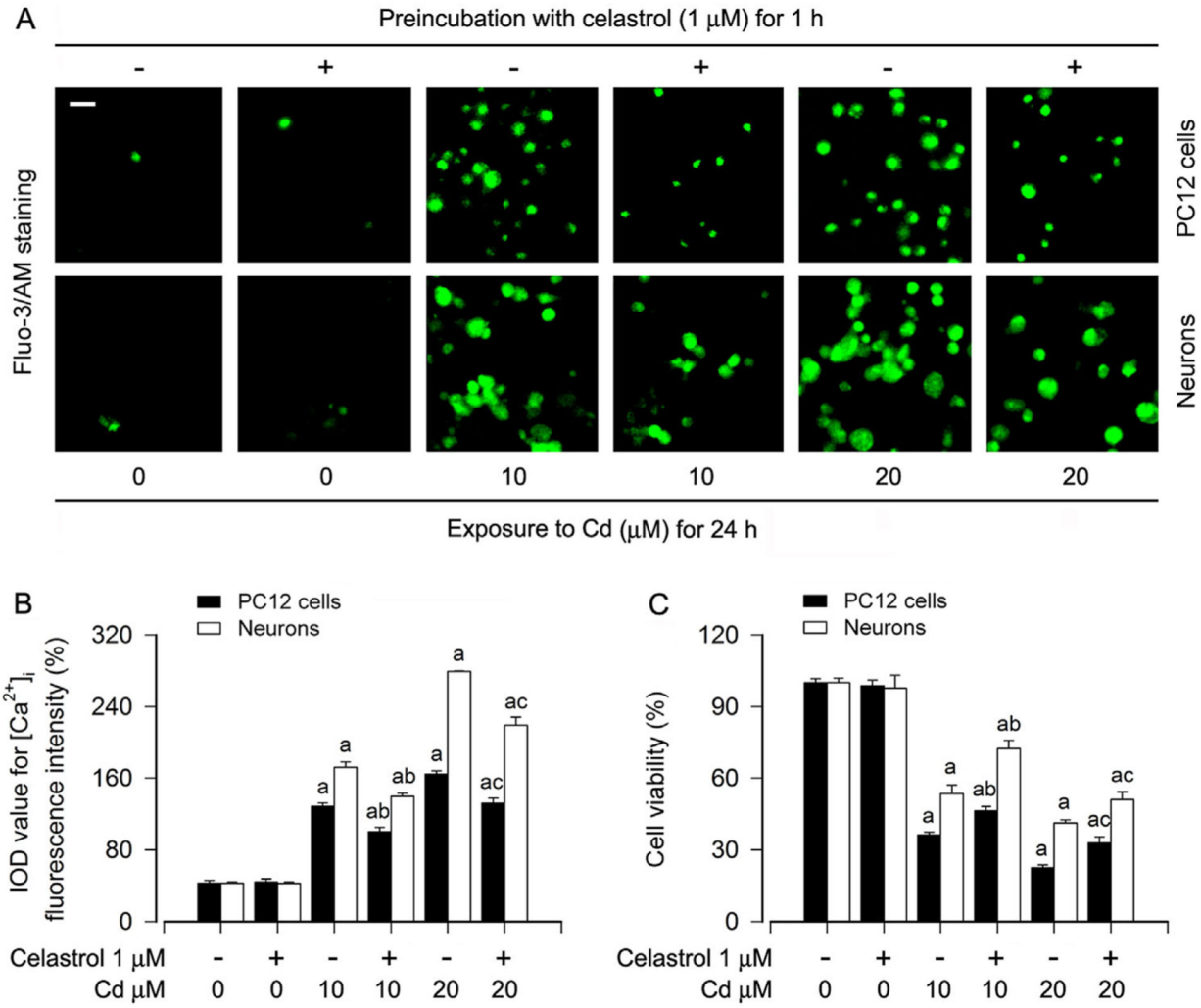

Fig. 1.

Effects of administered celastrol in PC12 cells and primary neurons on Cd-elevated $[Ca^{2+}]_i$ and cell viability reduction. PC12 cells and primary neurons were pretreated with celastrol (1 μM) for 1 h, and then exposed to Cd (10 and 20 μM) for 24 h. (A) $[Ca^{2+}]_i$ imaging was detected using an intracellular Ca^{2+} indicator dye Fluo-3/AM, showing that Cd-evoked strong $[Ca^{2+}]_i$ fluorescence (in green) was potently repressed by celastrol in the cells. Scale bar: 20 μm. (B) Quantitative analysis of $[Ca^{2+}]_i$ fluorescence intensity was measured by Image-Pro Plus 6.0 software. (C) Cell viability was assayed using MTS reagents, showing that celastrol markedly attenuated Cd-induced cell viability reduction in PC12 cells and primary neurons. Results are presented as mean ± SEM (n = 5). $^{a}P < 0.05$, difference with control group; $^{b}P < 0.05$, difference with 10 μM Cd group; $^{c}P < 0.05$, difference with 20 μM Cd group.

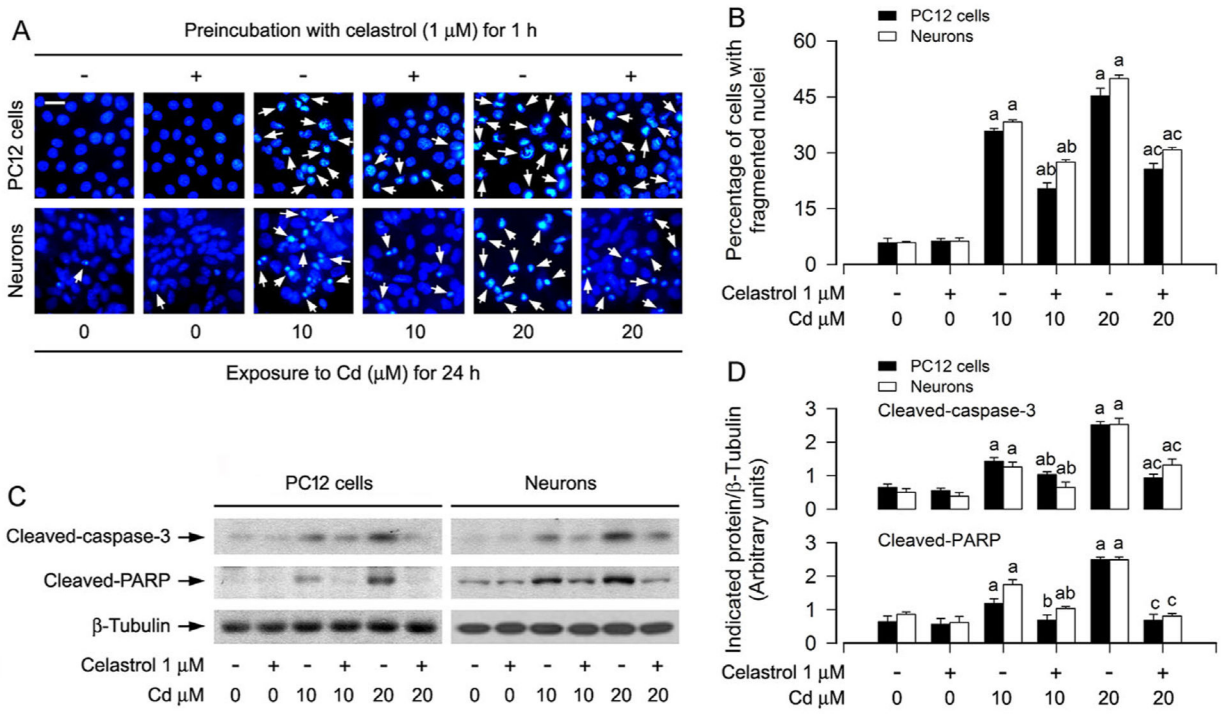

Fig. 2.

Inhibitory effect of administered celastrol in PC12 cells and primary neurons on cell apoptosis in response to Cd. PC12 cells and primary neurons were pretreated with celastrol (1 μM) for 1 h, and then exposed to Cd (10 and 20 μM) for 8 h (for Western blotting) or 24 h (for DAPI staining). (A) Apoptotic cells were evaluated by nuclear fragmentation and condensation (arrows) using DAPI staining. Scale bar: 20 μm. (B) The percentages of cells with fragmented nuclei were quantified, showing that celastrol obviously relieved Cd-induced apoptosis in PC12 cells and primary neurons. (C) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (D) The blots for cleaved-caspase-3 and cleaved-PARP were semi-quantified. Results are presented as mean ± SEM (n = 3–5). $^{a}P < 0.05$, difference with control group; $^{b}P < 0.05$, difference with 10 μM Cd group; $^{c}P < 0.05$, difference with 20 μM Cd group.

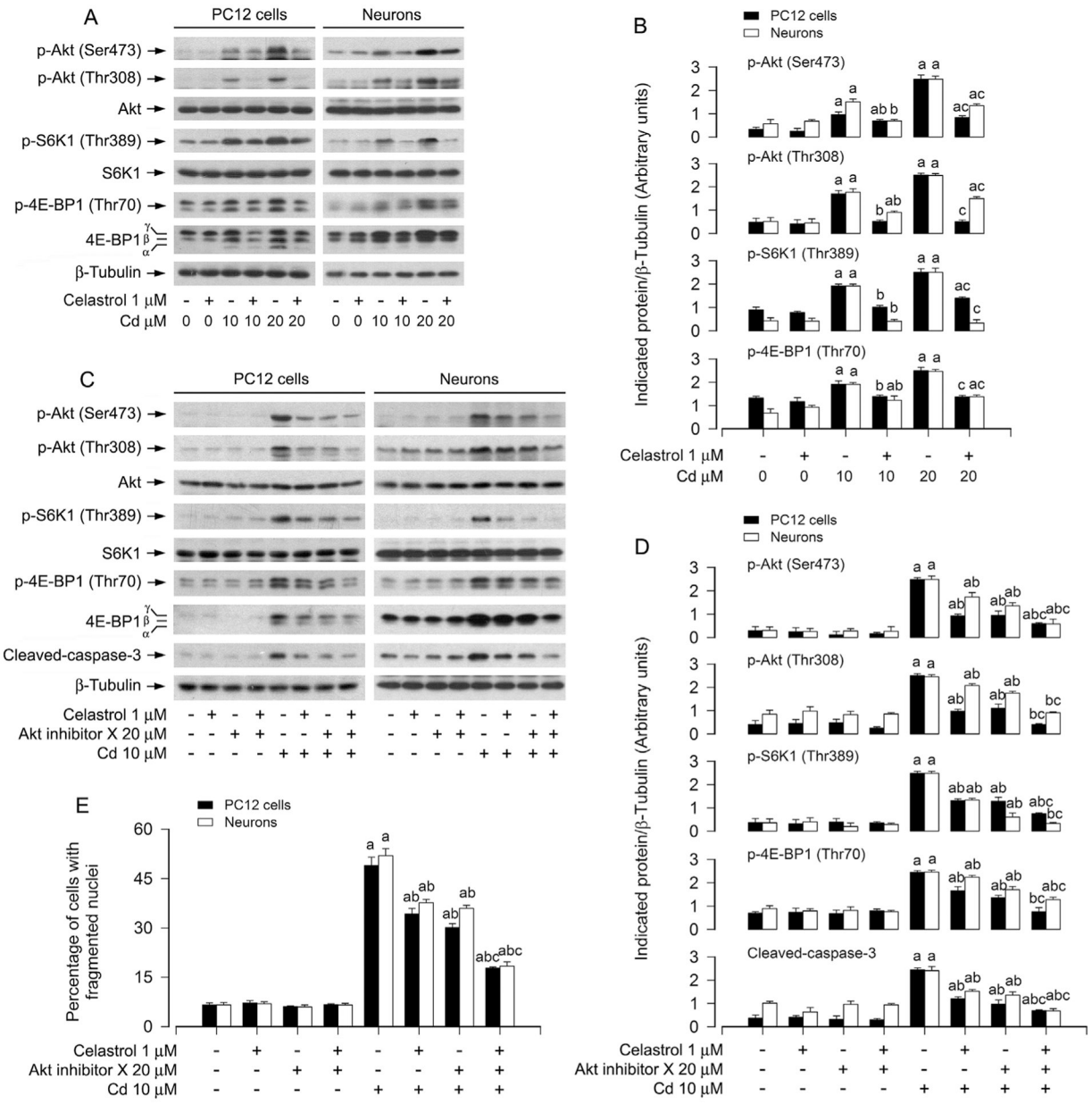

Fig. 3.

Preventive effects of administered celastrol and/or Akt inhibitor X in PC12 cells and primary neurons on Cd-induced activation of Akt/mTOR pathway and cell apoptosis. PC12 cells and primary neurons were pretreated with/without celastrol (1 μM) for 1 h, or pretreated with/without Akt inhibitor X (20 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 and/or 20 μM) for 8 h (for Western blotting) or 24 h (for DAPI staining). (A and C) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (B and D) The blots for p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. (E) Apoptotic cells were evaluated by nuclear fragmentation and condensation using DAPI staining. Results are presented as mean

± SEM (n = 3–5). [a]$P < 0.05$, difference with control group; [b]$P < 0.05$, difference with 10 μM Cd group; [c]$P < 0.05$, difference with 20 μM Cd group; [d]$P < 0.05$, difference with Cd/Akt inhibitor X group or Cd/Celastrol group.

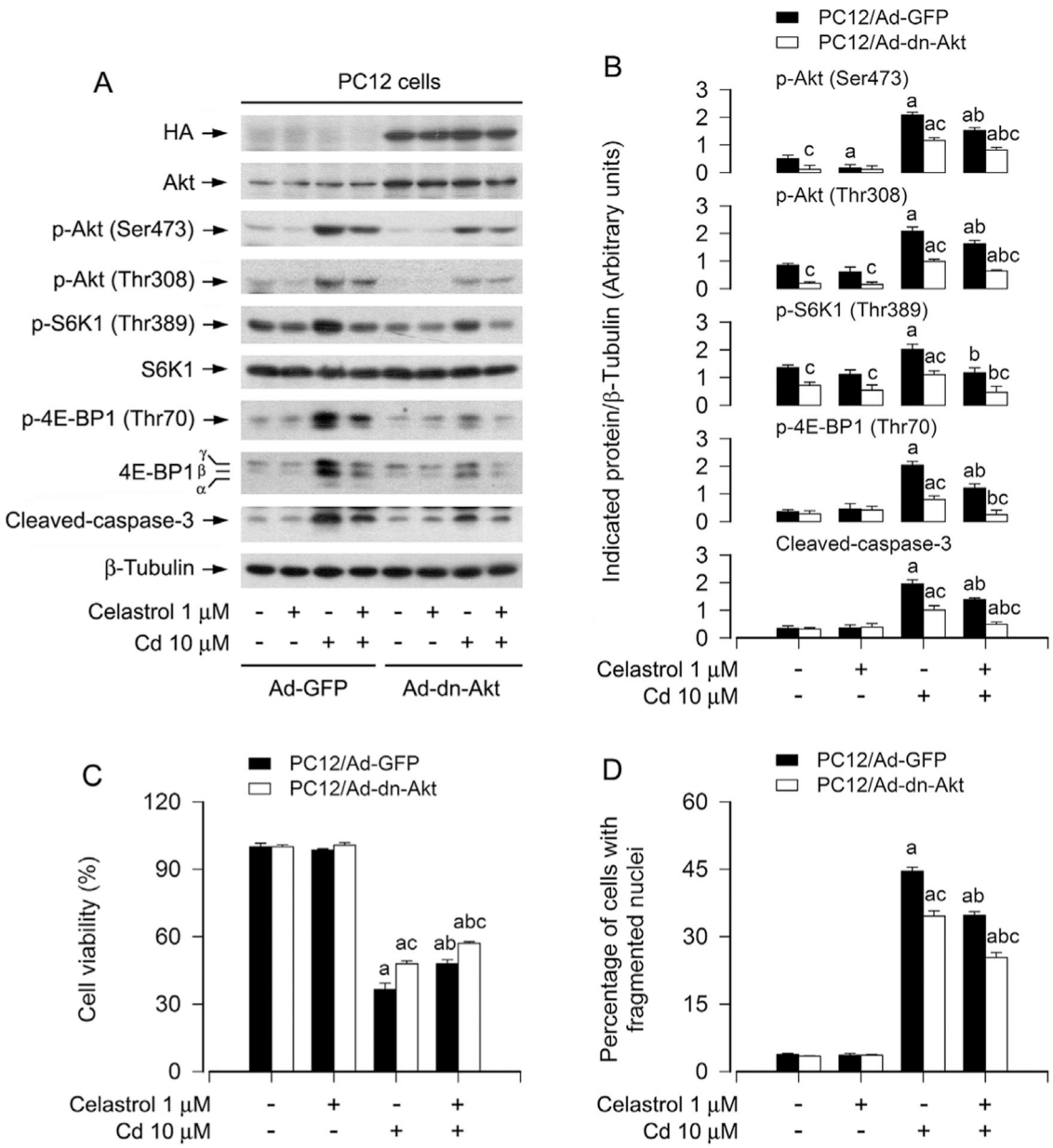

Fig. 4.

Effects of ectopic expression of dominant negative Akt in PC12 cells on celastrol's prevention from Cd-induced activation of mTOR pathway and apoptotic cell death. PC12 cells, infected with recombinant adenovirus expressing dominant negative (dn) Akt (Ad-dn-Akt) or GFP (Ad-GFP) (as control), were pretreated with/without celastrol (1 μM) for 1 h and then exposed to Cd (10 μM) for 8 h (for Western blotting) or 24 h (for cell viability assay and DAPI staining). (A) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (B) The blots for p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. (C) Cell viability was detected by measuring OD at 490 nm using MTS reagents. (D) Apoptotic cells were evaluated by

nuclear fragmentation and condensation using DAPI staining. Results are presented as mean ± SEM (n = 3–5). [a]$P < 0.05$, difference with control group; [b]$P < 0.05$, difference with 10 μM Cd group; [c]$P < 0.05$, Ad-dn-Akt group versus Ad-GFP group.

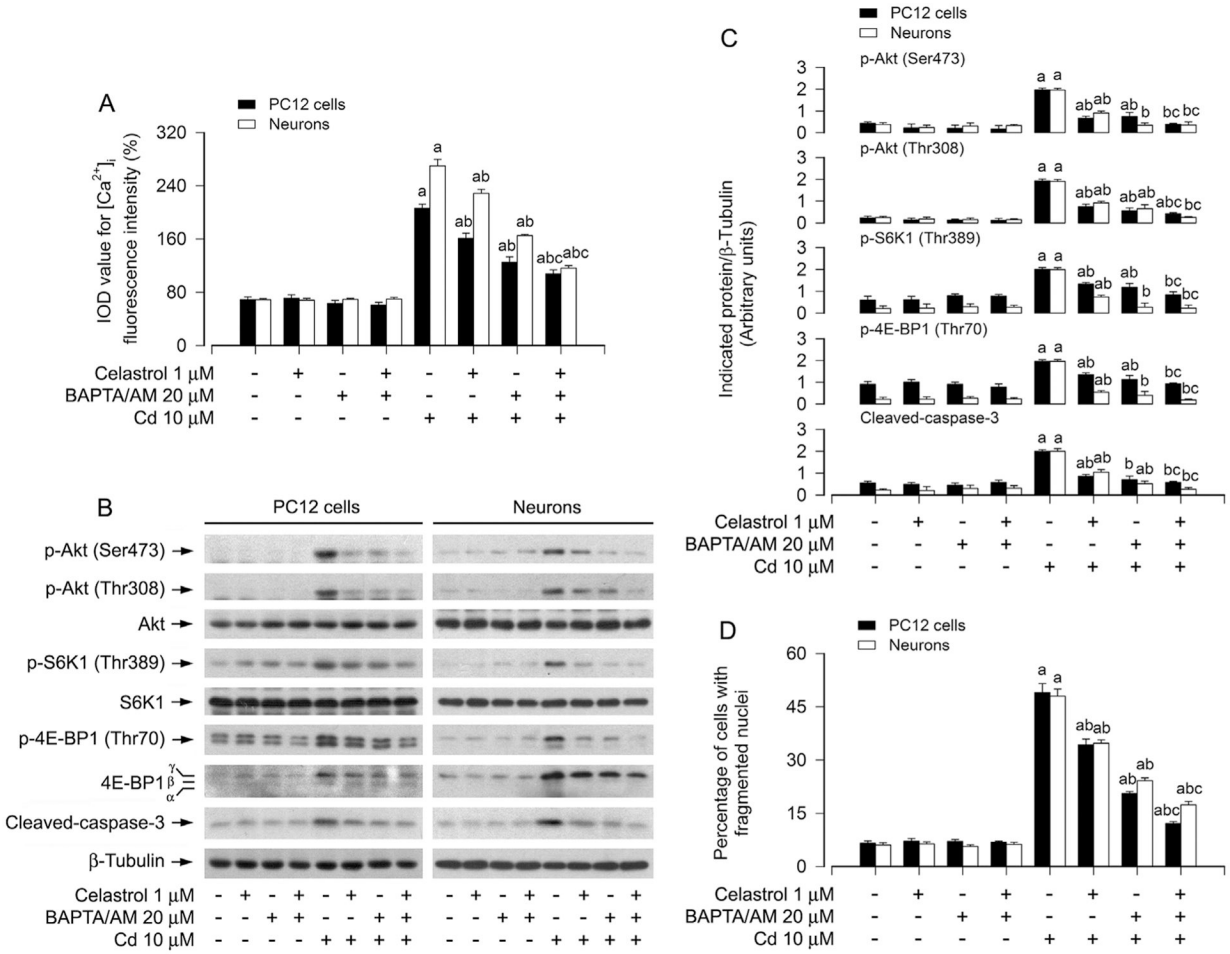

Fig. 5.

Effects of chelating $[Ca^{2+}]_i$ with BAPTA/AM in PC 12 cells and primary neurons on celastrol's inhibition of Cd-induced activation of Akt/mTOR pathway and cell apoptosis. PC12 cells and primary neurons were pretreated with/without BAPTA/AM (20 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 μM) for 8 h (for Western blotting) or 24 h (for $[Ca^{2+}]_i$ imaging, DAPI staining). (A) $[Ca^{2+}]_i$ levels were imaged and quantified using an intracellular Ca^{2+} indicator dye Fluo-3/AM. (B) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (C) The blots for p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. (D) The percentages of apoptotic cells with fragmented nuclei were quantified by DAPI staining. Results are presented as mean ± SEM (n = 3–5). $^{a}P < 0.05$, difference with control group; $^{b}P < 0.05$, difference with 10 μM Cd group; $^{c}P < 0.05$, difference with Cd/BAPTA/AM group or Cd/Celastrol group.

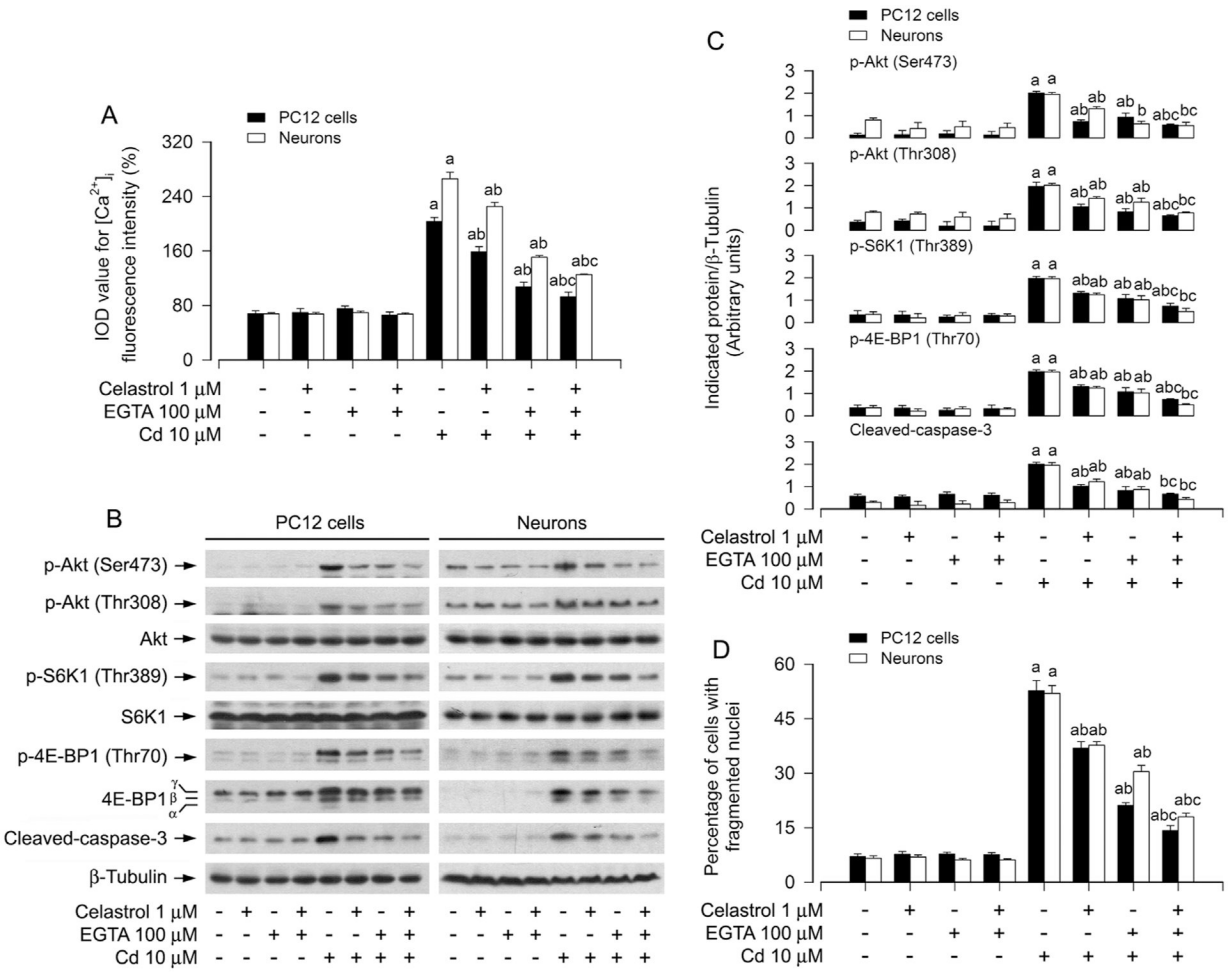

Fig. 6.

Effects of preventing $[Ca^{2+}]_i$ elevation using EGTA in PC 12 cells and primary neurons on celastrol's blockage of Cd-induced activation of Akt/mTOR pathway and cell apoptosis. PC12 cells and primary neurons were pretreated with/without EGTA (100 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 μM) for 8 h (for Western blotting) or 24 h (for $[Ca^{2+}]_i$ imaging, DAPI staining). (A) $[Ca^{2+}]_i$ levels were imaged and quantified using an intracellular Ca^{2+} indicator dye Fluo-3/AM. (B) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (C) The blots for p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. (D) The percentages of apoptotic cells with fragmented nuclei were quantified by DAPI staining. Results are presented as mean ± SEM (n = 3–5). $^{a}P < 0.05$, difference with control group; $^{b}P < 0.05$, difference with 10 μM Cd group; $^{c}P < 0.05$, difference with Cd/EGTA group or Cd/Celastrol group.

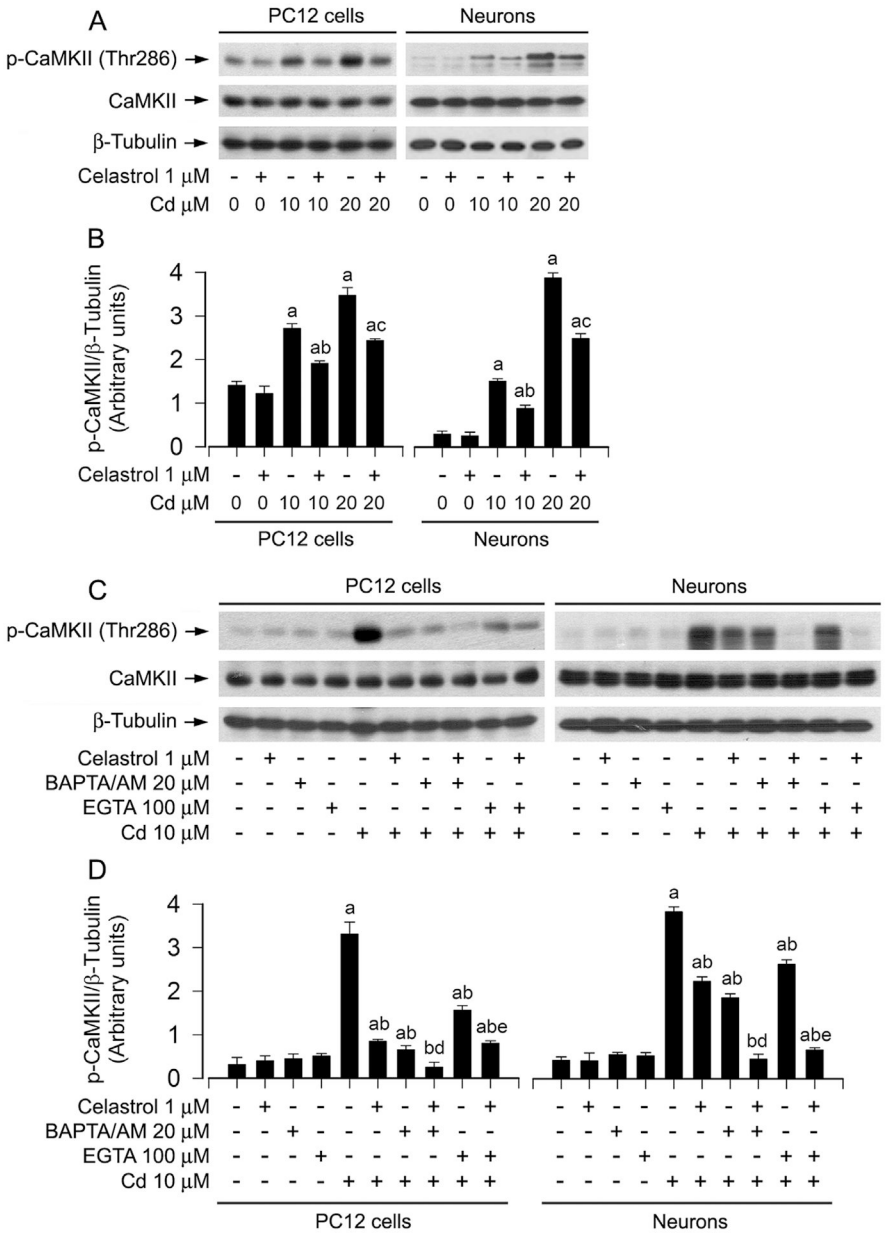

Fig. 7.

Effect of administered celastrol and/or BAPTA/AM or EGTA in PC12 cells and primary neurons on phosphorylation of CaMKII induced by Cd. PC12 cells and primary neurons were pretreated with/without celastrol (1 μM) for 1 h, or pretreated with/without BAPTA/AM (20 μM) or EGTA (100 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 and/or 20 μM) for 8 h. (A and C) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (B and D) The blots for p-CaMKII were semi-quantified. Results are presented as mean ± SEM (n = 3). [a]$P < 0.05$, difference with control group; [b]$P < 0.05$, difference with 10 μM Cd group;

[c]$P < 0.05$, difference with 20 μM Cd group; [d]$P < 0.05$, difference with Cd/BAPTA/AM group; [e]$P < 0.05$, difference with Cd/EGTA group.

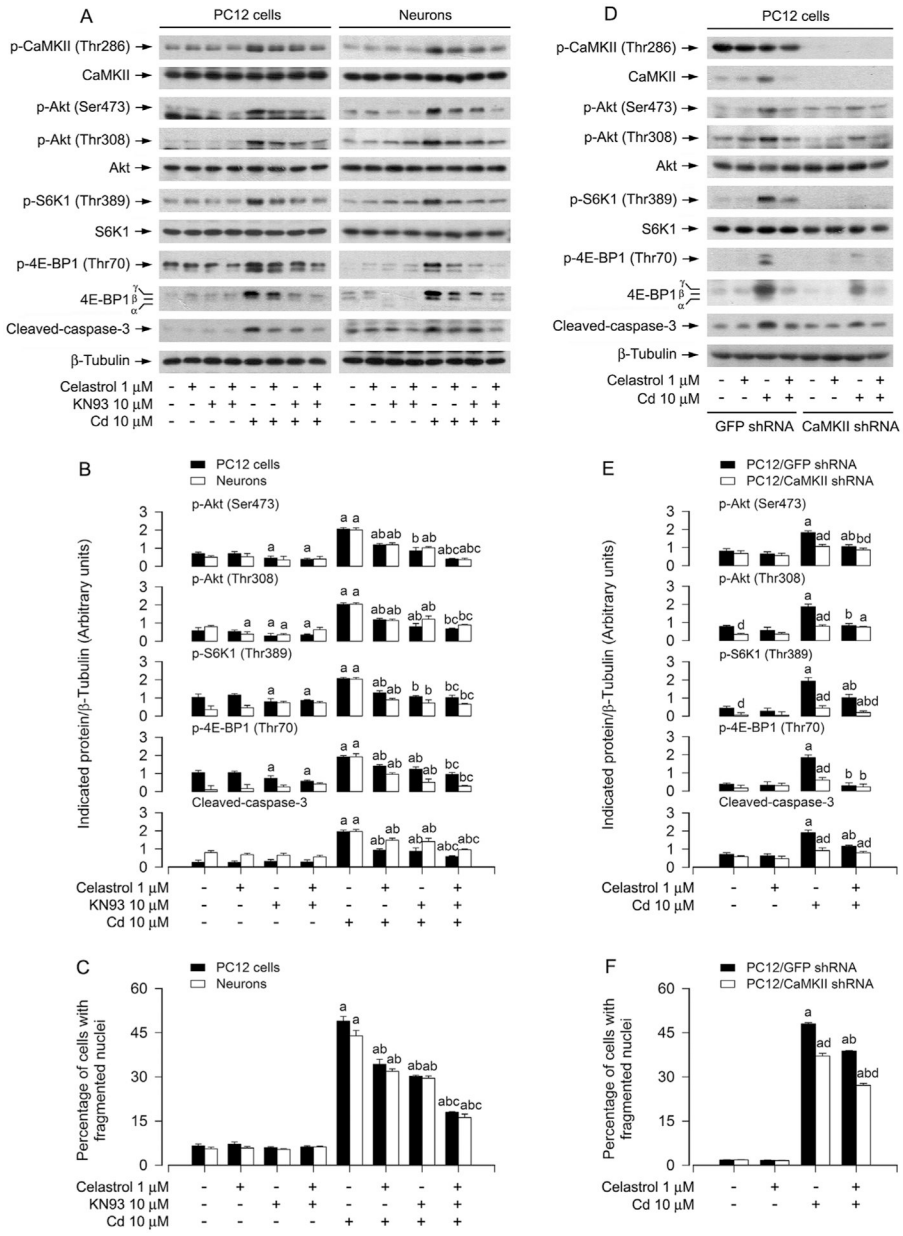

Fig. 8.

Effects of inhibition of CaMKII by KN93 or downregulation of CaMKII in PC12 cells and/or primary neurons on celastrol's prevention from Cd-induced activation of Akt/mTOR pathway and apoptotic cell death. PC12 cells and primary neurons, or PC12 cells infected with lentiviral shRNA to CaMKII or GFP (as control), respectively, were pretreated with/without celastrol (1 μM) for 1 h, or pretreated with/without KN93 (10 μM) for 1 h and then celastrol (1 μM) for 1 h, followed by exposure to Cd (10 μM) for 8 h (for Western blotting) or 24 h (for DAPI staining). (A and D) Cell lysates were subjected to Western blot analysis using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (B and E) The blots for p-CaMKII, p-Akt, p-S6K1, p-4E-BP1, cleaved-caspase-3 were semi-quantified. (C and F)

The percentages of apoptotic cells with fragmented nuclei were quantified by DAPI staining. Results are presented as mean ± SEM (n = 3–5). [a]$P < 0.05$, difference with control group; [b]$P < 0.05$, difference with 10 μM Cd group; [c]$P < 0.05$, difference with Cd/KN93 group or Cd/Celastrol group; [d]$P < 0.05$, CaMKII shRNA group versus GFP shRNA group.

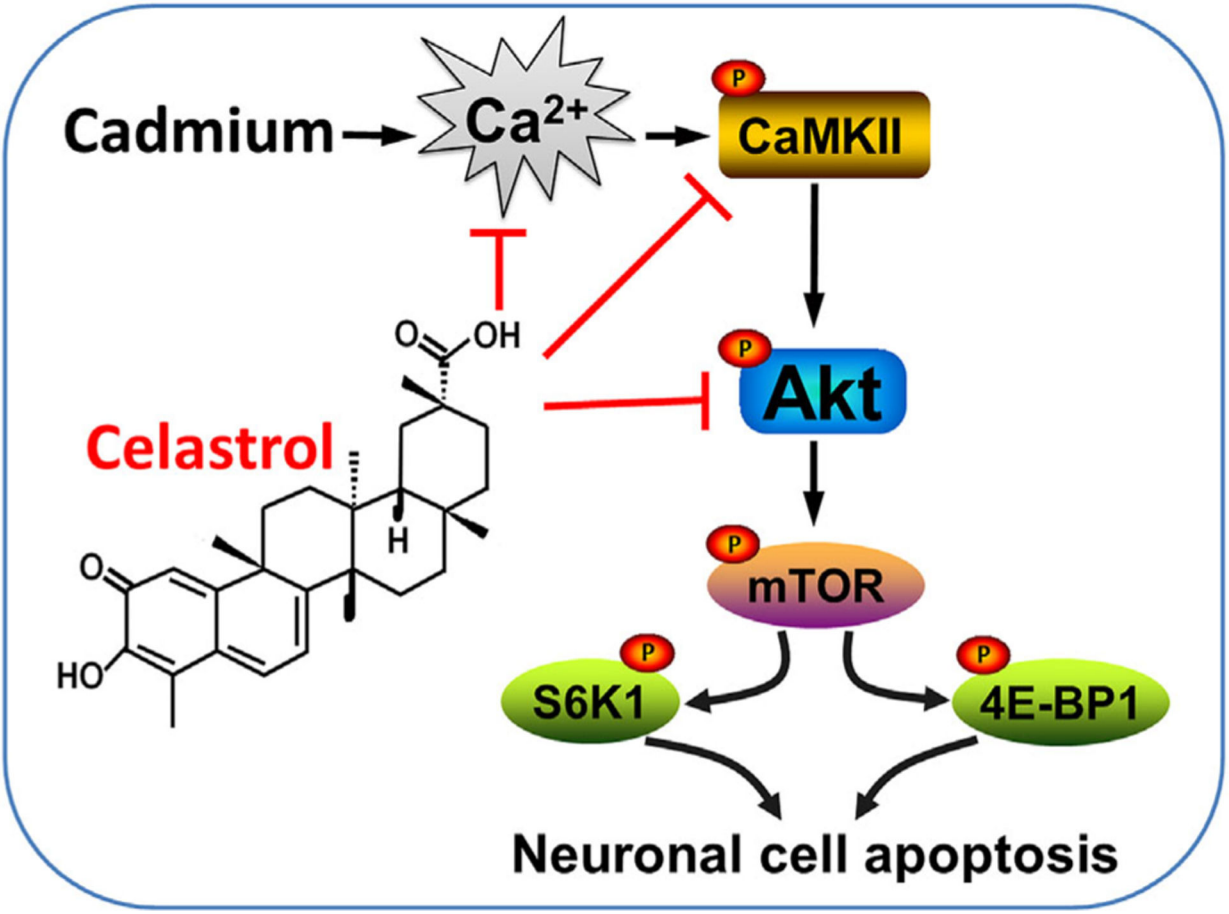

Fig. 9.

Schematic model of the preventive effect of celastrol on Cd-evoked neuronal apoptosis. Celastrol prevents Cd-induced elevation of $[Ca^{2+}]_i$, which results in inhibition of CaMKII phosphorylation. This leads to suppression of CaMKII-dependent Akt/mTOR pathway, thereby preventing Cd-induced apoptosis in neuronal cells.