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ORIGINAL PAPER

Calcium Signaling Involvement in Cadmium-Induced Astrocyte Cytotoxicity and Cell Death Through Activation of MAPK and PI3K/Akt Signaling Pathways

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Abstract Cadmium (Cd), a highly ubiquitous toxic heavy metal, can contaminate the environment, including agricultural soil, water and air, via industrial runoff and other sources of pollution. Cd accumulated in the body via direct exposure or through the food chain results in neurodegeneration and many other diseases. Previous studies on its toxicity in the central nervous system (CNS) focused mainly on neurons. To obtain a more comprehensive understanding of Cd toxicity for the CNS, we investigated how astrocytes respond to acute and chronic Cd exposure and its toxic molecular mechanisms. When primary cultures of cerebral cortical astrocytes incubated with 1–300 μM $CdCl_2$, morphological changes, LDH release and cell death were observed in a time and dose-dependent manner. Further studies demonstrated that acute and chronic Cd treatment phosphorylated JNK, p38 and Akt to different degrees, while ERK1/2 was only phosphorylated under low doses of Cd (10 μM) exposure. Inhibition of JNK and PI3K/Akt, but not of p38, could partially protect astrocyte from cytotoxicity in chronic and acute Cd exposure. Moreover, Cd also induced a strong calcium signal, while BAPTA, a specific intracellular calcium (Ca^{2+}) chelator, prevented Cd-induced intracellular increase of calcium levels in astrocytes; inhibited the Cd-induced activation of ERK1/2, JNK, p38 and Akt; and also significantly reduced astrocyte cell death. All of these results suggested that the Cd–Ca^{2+}–MAPK and PI3K/Akt signaling pathways were involved in Cd-induced toxicity in astrocytes. This toxicity involvement indicates that these pathways may be exploited as a target for the prevention of Cd-induced neurodegenerative diseases.

Keywords Astrocytes · Cadmium · Cytotoxicity · MAPK · PI3K/Akt

Abbreviations

Cd	Cadmium
Ca^{2+}	Calcium
CNS	Central nervous system
AD	Alzheimer's disease
PD	Parkinson's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood–brain barrier
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3 kinase
p-ERK1/2	Phosphorylated extracellular regulated kinase

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p-JNK	Phosphorylated c-Jun N-terminal kinase
p-p38	Phosphorylated p38
p-Akt	Phosphorylated protein kinase B
FBS	Fetal bovine serum

Introduction

Cadmium (Cd), an extremely toxic heavy metal that is widely distributed in the environment, is commonly found in industrial workplaces, contaminated food and cigarette smoke. Cd in biological systems is present as the Cd^{2+} ion, which structurally resembles calcium (Ca^{2+}) and has a long biological half-life, mainly due to its low rate of excretion from the body [1]. Thus, prolonged exposure to Cd, either through direct contact or ingestion, will cause toxic effects due to its accumulation in a variety of tissues, including the central nervous system (CNS), kidney and liver [2]. Cd may induce Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders [3–5]. In addition, clinical data have shown that Cd contributes to neurological illnesses, such as olfactory dysfunction; learning disabilities and hyperactivity in children; and neurobehavioral defects in attention, psychomotor speed, and memory in workers [2, 6]. A 20-year epidemiological investigation in China revealed that Cd pollution is extremely serious and has created a severe threat to the health of local residents [5, 7].

Growing evidence from in vivo studies indicates that Cd is able to enter the CNS via changing the permeability and disrupting the integrity of the blood–brain barrier (BBB). Such Cd-induced disruption is a potentially important cause of brain dysfunction including behavioral disorders [8, 9]. Astrocytes are the most numerous glial cells in CNS and are the key components of BBB. Therefore, astrocytes are the first brain cells to encounter Cd after it crosses the BBB [10]. Astrocytes ought to respond to Cd by changing its normal physiological functions. Studies have increasingly demonstrated that astrocytes could respond to various stimuli and insults to secrete cytokines that modulate the survival of neurons in the brain and implicate the chronic neuro-inflammation that mediates AD neurodegeneration [11]. Meanwhile, astrocytes are also known to be essential in maintaining the extracellular microenvironment, scavenging free radical and metabolic toxins and producing neurotrophic factors and/or growth factors [12]. A toxic impact on these functions ought to affect its normal interaction with neurons and neuronal survival. Neurons appear to be more susceptible than astrocytes to toxicity, injury and cell death. However, toxins entering the brain would first disturb astrocytic functions before affecting neurons, thereby contributing to secondary damage and exacerbating the primary prejudicial response of neurons to the toxin.

Previous research on Cd toxicity in CNS has mainly focused on neurons. To obtain a more comprehensive understanding of Cd toxicity to the CNS, we have investigated the effects of acute and chronic Cd toxicity and its toxic molecular mechanisms in primary cultures of cerebral cortical astrocytes.

Materials and Methods

Primary Cultures of Cerebral Cortical Astrocytes

Primary cultures of astrocytes were prepared from cerebral cortices of newborn ICR mice as reported previously [13, 14]. In brief, cerebral cortices from newborn ICR mice (Department of Laboratory Animal Science, Peking University Health Science Center) freed of meninges and olfactory bulbs were cut into small cubes (<1 mm^3) in Dulbecco's Modified Eagle's Medium (DMEM). The vortex disrupted the tissue for 90 s and the resulting suspension was passed through two sterile nylon Nitex sieves of pore sizes 70 and 10 μm (Spectrum Laboratories, Inc., USA). The mixture with 10 % (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) was distributed into 35 mm culture dishes (Corning Incorporated, USA) at 4×10^5 cell/mL containing 2 mL DMEM. All cultures were incubated in a Heraus CO_2 incubator (Thermo Fisher Scientific, USA) at 37 °C with 5 % CO_2 (v/v). The culture medium was changed twice per week and 7 % (v/v) FBS was used after 2 weeks. Confluent and mature cultures used for experiments were at least 4 weeks old.

Cd and Inhibitor Treatments

The stock solutions of $CdCl_2$ (100 mM; Sigma-Aldrich, USA) were diluted with the medium and sterilized by filtration (0.22 μm; Millipore, USA). Astrocytes were incubated with different concentrations of $CdCl_2$ for designated times, representing chronic and acute Cd neurotoxicity. To avoid the changes in astrocytes due to lack of serum in experiments that required more than 1 day of incubation, some cultures were incubated in DMEM with FBS. In the time-course study, cells were incubated with 1, 2, or 5 μM $CdCl_2$ for 0–5 days in DMEM supplemented with/without 7 % FBS; or 10 or 20 μM $CdCl_2$ for 0–12 h in a serum free medium, representing chronic Cd neurotoxicity. Untreated control cells were incubated with the serum-free medium without $CdCl_2$. Acute Cd poisoning was treated similarly, but cells were incubated with 100 or 300 μM $CdCl_2$ for 0.5–2 h. For inhibitor treatments, cultures were pre-treated

with a 40 μM U0126 (MEK/ERK signal pathway inhibitor; Promega, USA); a 40 μM SP600125 (JNK inhibitor; Sigma-Aldrich, USA); a 40 μM SB203580 (p38 inhibitor; Sigma-Aldrich, USA); and a 40 μM LY294002 (PI3K/Akt inhibitor; Sigma-Aldrich, USA), or 20 μM BAPTA-AM (calcium chelator; EMD Millipore, USA). Pre-treatments were 30 min in a serum-free medium before Cd treatment. During Cd incubation, the inhibitor remained in the serum-free incubation medium.

Observation and Assessment of Cell Morphology

Astrocyte morphology was assessed with a phase contrast microscope at various time intervals of incubation with Cd. After the exposure to different concentrations of Cd at designated times, an inverted Microscope (Leica Microsystems, Germany) under phase contrast mode was used to trace the morphological changes of Cd-treated astrocytes.

Western Blot Analysis

The total protein of Cd-treated astrocytes was extracted and Western blot analysis was performed according to a previously published procedure [15, 16]. Briefly, the cultures were washed with ice-cold PBS three times Proteins were extracted in 100 μL of RIPA buffer [20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1 % NP-40, 1 % SDS, 0.5 % sodium deoxycholate, 1 mM PMSF, and protease inhibitor cocktail, including phosphatase inhibitors (Roche, Sweden)] for each dish. The cell lysate was centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was collected. Protein concentration was determined with a Lowry Protein Assay. Equal amounts of proteins from cell lysate was denatured in a protein loading buffer for 10 min at 100 °C and separated on a 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Proteins were then transferred to PVDF membranes. Blocking was performed by incubation in TBST [20 mM Tris-buffered 150 mM saline (pH 7.5) with 0.1 % Tween 20] containing 5 % BSA for 1 h at room temperature. Primary antibodies against p-ERK1/2 (1:2000, Santa Cruz Biotechnology, Inc., USA), p-JNK (1:2000, Santa Cruz Biotechnology, Inc., USA), p-p38 (1:1000, Cell Signaling Technology, Inc., USA), p-Akt (Ser 473) (1:1000, Cell Signaling Technology, Inc., USA) and GAPDH (1:2000, Cell Signaling Technology, Inc., USA) were diluted in TBST containing 5 % BSA and incubated overnight at 4 °C. The membranes were washed with TBST every 10 min for three times, then incubated in HRP-conjugated secondary antibodies (1:2000, Jackson ImmunoResearch Laboratories, Inc., USA) for 1 h at room temperature. After washing, the signals were detected by chemiluminescence with ECL solution (Santa Cruz Biotechnology, Inc., USA).

Measurement of LDH Release

Astrocytes in cultures were treated as described above in 35 mm dishes with Cd at different concentrations for designated times. Fifty microliter of incubation media from each dish were transferred to a new 96-well plate and measured for LDH content, according to the procedure of the test kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, USA) and a previously published procedure [17].

Annexin V-FITC/PI/Hoechst Staining and Cell Apoptosis Detection

Astrocytes in cultures after treatment as described above in 35 mm dishes with Cd performed Annexin V-FITC/PI/Hoechst staining according to the procedure of the test kit (Annexin V-FITC Apoptosis Detection Kit, Biosea, China). Briefly, the cultures were washed twice with ice-cold PBS; and then 10 μL Annexin V-FITC and 10 μL of PI in 1 mL Annexin V binding buffer were added to each culture and incubated for 15 min. After re-washing with PBS, the cultures were fixed with 4 % PFA for 20 min at room temperature. 2 μg/mL of Hoechst 33342 (Sigma-Aldrich, USA) in PBS was added to each culture and incubated for 10 min. After re-washing with PBS, the cultures were observed under fluorescent microscopy (Leica Microsystems, Germany).

Calcium Imaging

Calcium imaging was performed with Fluo-3-AM according to a previously published procedure [16]. Before imaging, primary cultures of astrocytes were washed once with HEPES-buffered saline (HBS; 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 2 mM $CaCl_2$, 25 mM HEPES, pH 7.2) and then incubated in HBS containing 5 μM Fluo-3 AM (Biotium, USA) and 0.02 % pluronic acid for 20 min at 37 °C. Cells were observed with an Olympus IX71 microscope (Olympus, Japan).

Statistical Analysis

Data from at least three independent experiments was expressed in the form of mean ± SEM. All analyzes were performed using Prism 5.0 statistical software (Graph Pad Software, USA). Significance tests were performed using one-way ANOVA followed by Newman–Keuls post hoc tests. Differences with $P < 0.05$ were considered as statistically significant.

Results

Cd Altered Morphology and Reduced Viability of Astrocytes in a Time and Dose-Dependent Manner

We initially evaluated Cd cytotoxicity on primary cultures of cerebral cortical astrocytes by phase-contrast microscopy. Time lapse recording was used to follow morphological changes in astrocytes under chronic and acute Cd-treatments. Micrographs of primary cultures of astrocytes treated with increasing dosages of Cd for designated times were captured with phase contrast microscopy. In Fig. 1 (simulating chronic exposure in the absence of FBS), we found that astrocytes under 1 μM Cd began to contract and lose its cellular integrity on Day 2. Severe cell death was observed on Day 4 and 5. With Cd at 2 μM, astrocytes began to loosen the culture integrity on Day 1, and serious cell retraction and death were observed on Day 2. Cultures under 5 μM Cd showed an earlier integrity changes than

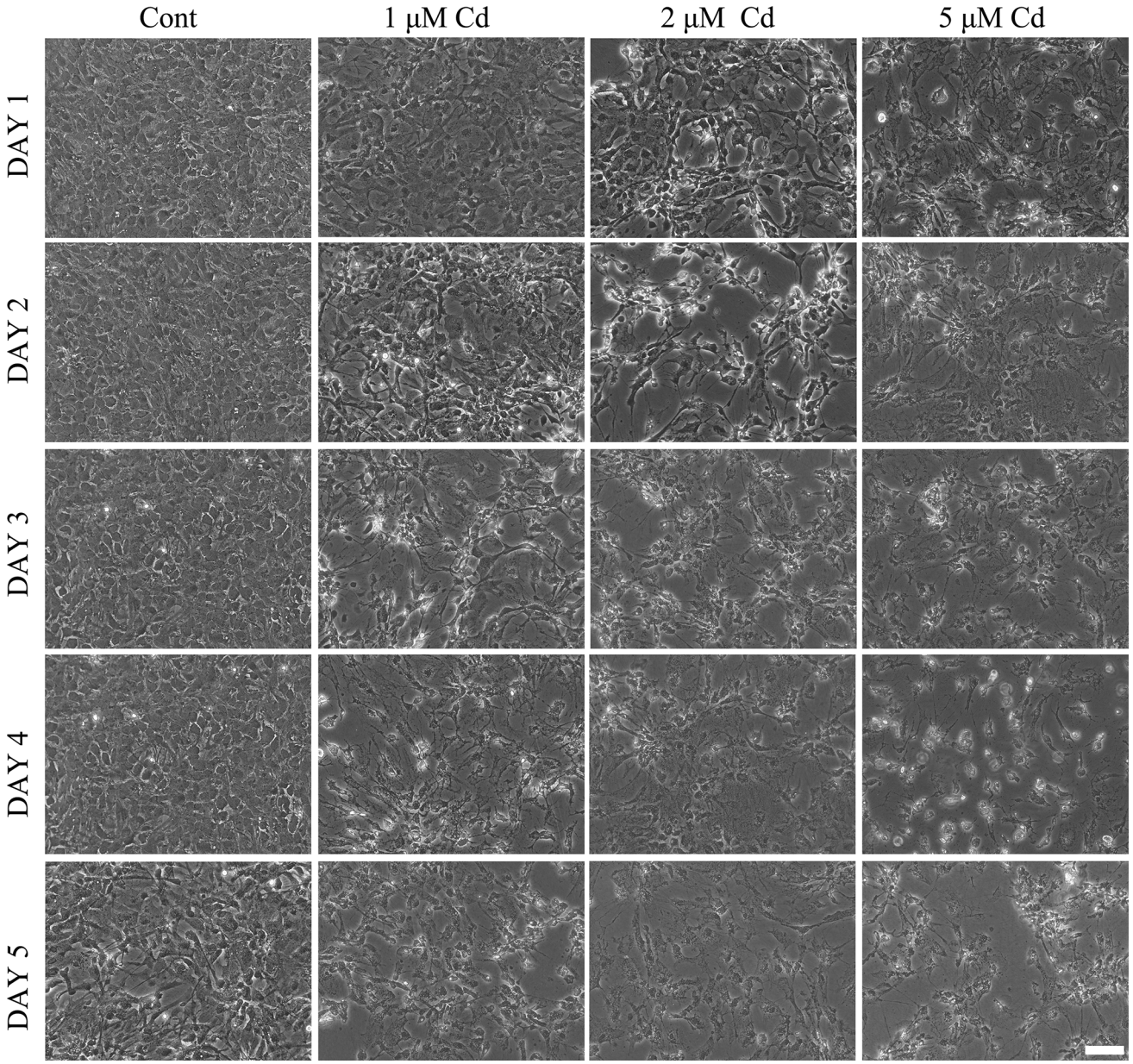

Fig. 1 Phase-contrast microscopy showed low level Cd-induced astrocytes cellular morphological changes and cell death in a time and dose-dependent manner. Phase contrast micrographs of primary cultures of astrocytes in the absence of FBS after low dosages of Cd (0, 1, 2, 5 μM) treatment at day 1, 2, 3, 4 and 5, respectively. Pictures were representative data from at least 3 independent experiments. *Bar* = 50 μm

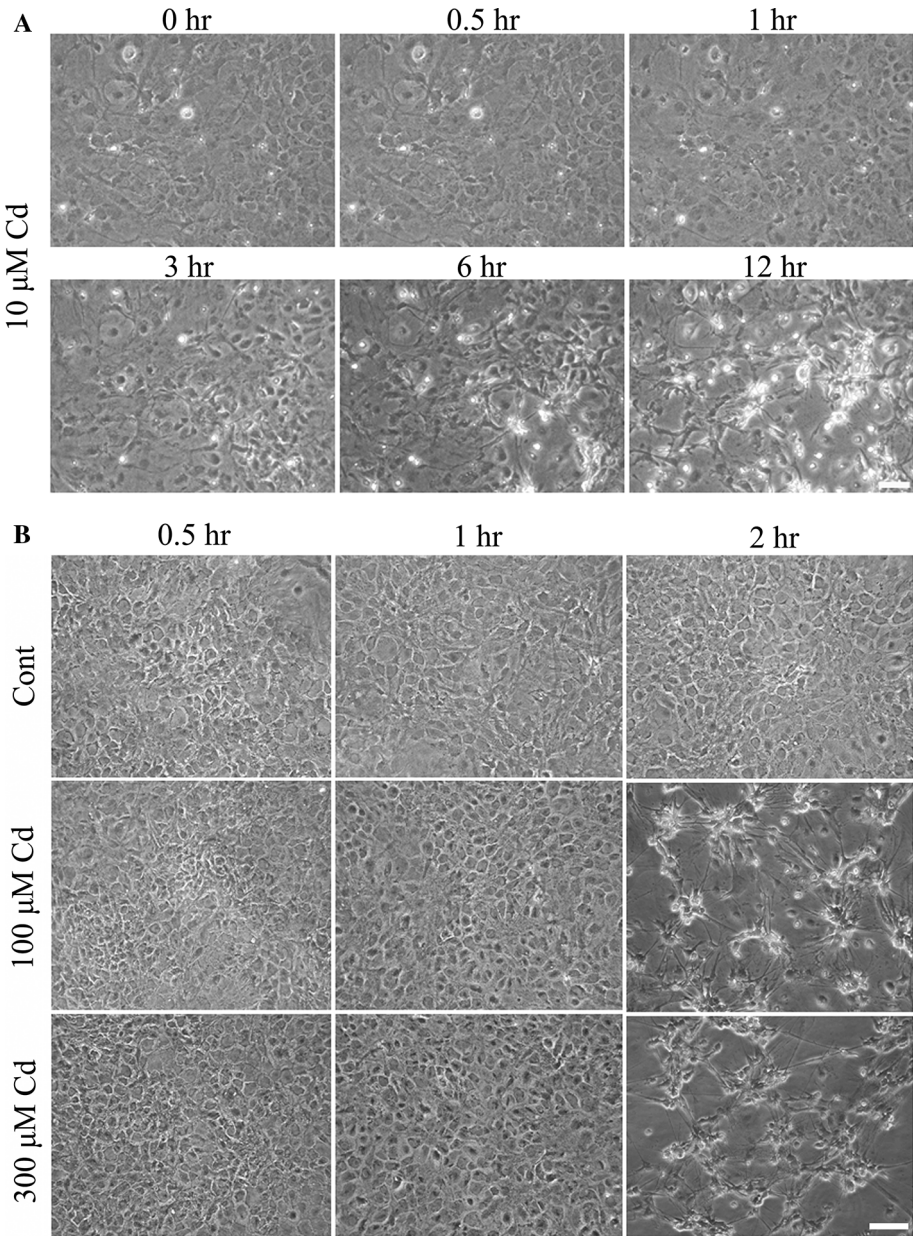

Fig. 2 Phase-contrast microscopy showed high level Cd-induced astrocytes cellular morphological changes and cell death in a time and dose-dependent manner. Phase contrast micrographs of primary cultures of astrocytes after 10 μM Cd treatment at 0, 0.5, 1, 3, 6, and 12 h (**a**); and 100 and 300 μM Cd treatment at 0.5, 1 and 2 h (**b**). *Bar* = 50 μm

2 μM in Day 1, while integrity changes under 10 μM occurred in 12 h (Fig. 2a). Chronic Cd incubation might last for more than 24 h, therefore, we compared cultures with/without FBS under low Cd chronic exposure. It showed that low Cd exposure induced a much slower cellular morphological change in the presence of FBS than in its absence. This data suggested that FBS may bind to Cd and reduce the Cd availability to the cells. In another hand, this reduction of availability might play a protective role to the cells (Fig S1). In Fig. 2b (simulating acute exposure, in the absence of FBS), astrocytes under 100 or 300 μM of Cd, showed cell processes retraction, loosening of integrity and death within 2 h of exposure. Based on these morphological observations, we found that the Cd cytotoxicity to astrocytes was dose and time dependent.

Apoptotic morphological changes induced by Cd in cerebral cortical astrocytes were assessed by Annexin V-FITC/PI/Hoechst staining (Fig. 3a). After exposure to 10 μM Cd for 6 h, the cells with Annexin V-positive (green), PI-negative, and Hoechst (blue) staining showed nuclear morphological changes typical of early stage apoptosis. That is, there were condensed nuclear chromatin and fragmented nuclei characterized by a scattered, drop-like structure, with the nuclei of apoptotic cells smaller than the nuclei of intact cells. However, the majority of cells had normal nuclei which were unaltered, and the

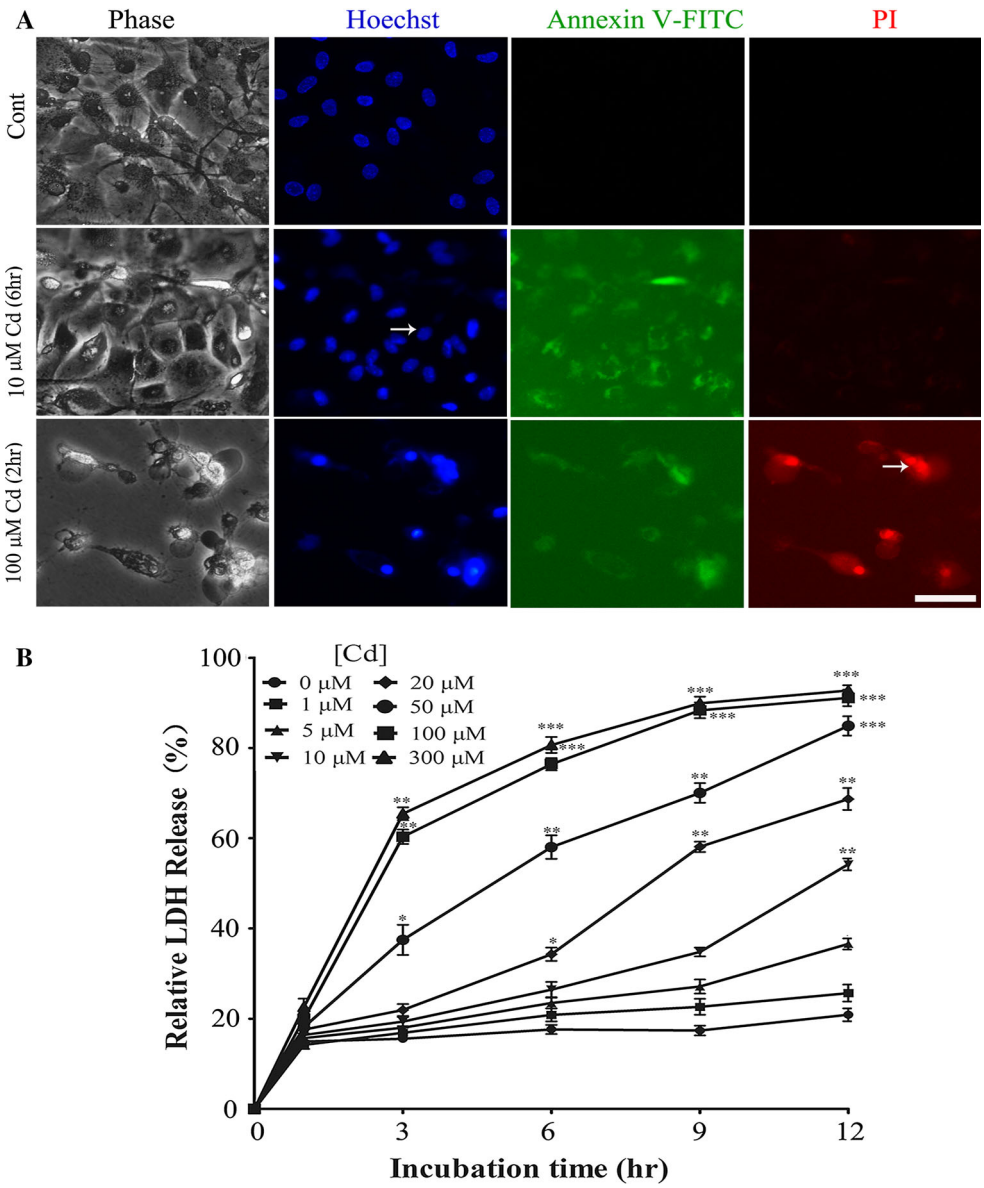

Fig. 3 Cd induced astrocytic apoptosis/necrosis and LDH release in a time and dose dependent manner. **a** Cd-induced apoptosis and necrosis in a culture of astrocytes treated with 10 μM M Cd (*up*) for 6 h or 100 μM M Cd (*down*) for 2 h by Annexin V-FITC/PI/Hoechst staining (*arrows*, apoptotic or necrotic cells) under a fluorescence microscope. *Bar* = 50 μm. **b** LDH release from primary cultures of astrocytes after different concentrations of Cd (0, 1, 5, 10, 20, 50, 100 or 300 μM) treatment at 0, 1, 3, 6, 9 and 12 h. *vs. 0, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 4$ (number of experiments correspond to experiments performed in distinct preparations)

chromatin appeared to spread uniformly throughout the entire nucleus. There were only a small number of early stage apoptotic cells after exposure to 10 μM Cd for 6 h.

Meanwhile, after 100 μM Cd for 2 h incubation, we could see many cells with Annexin V-positive (green), PI-positive staining (red), and also fragmented and smaller nuclei. These results suggested that the main consequence of a high dose of Cd was to rapidly induce secondary necrosis or late stage apoptosis.

All of these morphological observations were consistent with the results, when LDH is released into the medium from astrocytes after similar Cd treatments (Fig. 3b). In primary cultures of astrocytes, Cd at doses of 1, 5 and 10 μM did not induce any significant increase in LDH level in the medium up to 9 h of incubation. It was at 12 h incubation, and 10 μM that Cd began to induce a significant increase of LDH release. As for 20 μM Cd, LDH release was significantly higher than the control at 6 h. As for 50, 100, and 300 μM Cd at 3 h incubation, LDH releases reached 37.48, 60.34 and 65.45 %, respectively. The release continued to increase with the time of Cd incubation. Again, this LDH data also suggested a time and dose-dependent Cd cytotoxicity on astrocytes.

Changes of p-ERK1/2, p-JNK, p-p38 and p-Akt with Chronic Cd Treatments

Cytotoxicity-induced cell death from heavy metal exposure often involves the activation of the MAPK (ERK1/2, JNK and p38) and PI3K/Akt signaling pathways [18, 19]. We

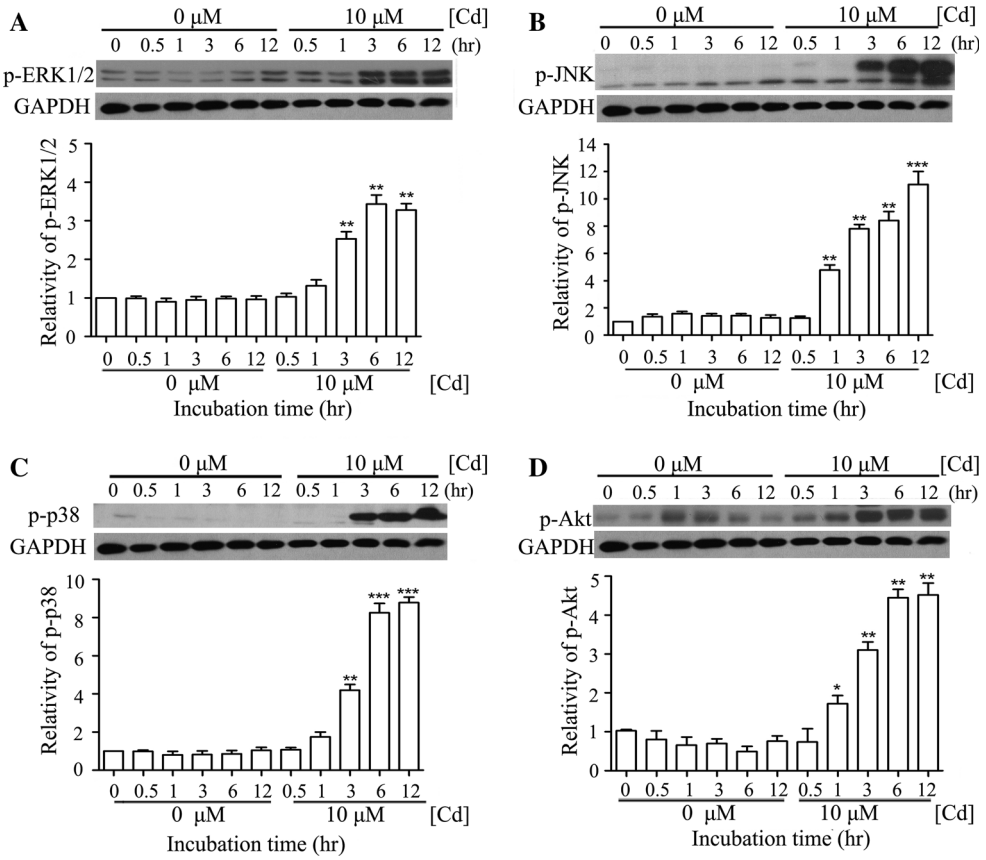

Fig. 4 Activation of ERK1/2, JNK, p38 and Akt in astrocytes exposed to low doses of Cd. Representative Western blots (*up*) and statistical results (*down*) revealed activation of ERK1/2 (**a**), JNK (**b**), p38 (**c**) and Akt (**d**) treated with/without 10 μM Cd at 0, 0.5, 1, 3, 6 and 12 h in astrocytes in serum free DMEM. GAPDH was detected as an internal control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, $n = 4$

firstly studied whether the chronic Cd treatment affects MAPK and PI3K/Akt activation by measuring their phosphorylation. At 10 μM Cd treatment, the p-ERK1/2 level was elevated significantly at 3 h, while it reached a 3.5 fold increase at 12 h (Fig. 4a). The p-JNK levels elevated earlier than p-ERK1/2, and were already significant at 1 h of Cd treatment (Fig. 4b), and the level continued to increase significantly in a time-dependent manner and reached a tenfold increase within the 12 h incubation. The p-p38 began to rise at 3 h Cd treatment, and its levels continued to increase in the 12 h incubation period (Fig. 4c). The p-Akt level began to significantly increase after 1 h of Cd treatment and continued to rise during the 12 h incubation period (Fig. 4d). Through comparative analysis, we can see the p-JNK and p-Akt levels increased at the early time of 1 h, and p-JNK and p-p38 levels elevated to much higher levels at 12 h incubation with 10 μM Cd treatment. These data suggested that JNK was the most sensitive signal molecule among the four signaling pathways.

Changes of p-ERK1/2, p-JNK, p-p38 and p-Akt with Acute Cd Treatments

We continued to examine whether the acute Cd treatment affects MAPK and PI3K/Akt activation. Under acute Cd treatment, there was no significant increase in p-ERK1/2 at 100 or 300 μM Cd for up to 2 h, as compared to no Cd treatment (Fig. 5a). In contrast, p-JNK was already significantly increased at 0.25 h, reaching an optimal level at 1 h, and remained at this level at 2 h (Fig. 5b). p-p38 appeared to elevate with statistical significance at 0.25 h at 300 μM but not at the 100 μM Cd treatment. At 0.5 h, p-p38 significantly increased under both Cd concentrations tested. It continued to increase significantly within the 2 h incubation time under both concentrations (Fig. 5c). As for p-Akt, it was also up-regulated at 0.25 h, significantly at 300 but not at 100 μM Cd treatment. Its levels peaked at 0.5 and 1 h at both concentrations; however, its levels were decreased at 2 h (Fig. 5d). The p-JNK, p-p38 and p-Akt levels were all increased at the early time of 0.25 h, and the p-p38 level was elevated to a higher level at 2 h incubation with 300 μM Cd. These results suggested that p38 was the most sensitive signal molecule among the four signaling pathways.

Inhibition of JNK and PI3K/Akt Activation Partially Attenuated Cd-Induced Astrocyte Death

To determine the role of MAPK and PI3K/Akt signaling pathways in Cd-induced astrocytic cytotoxicity, different

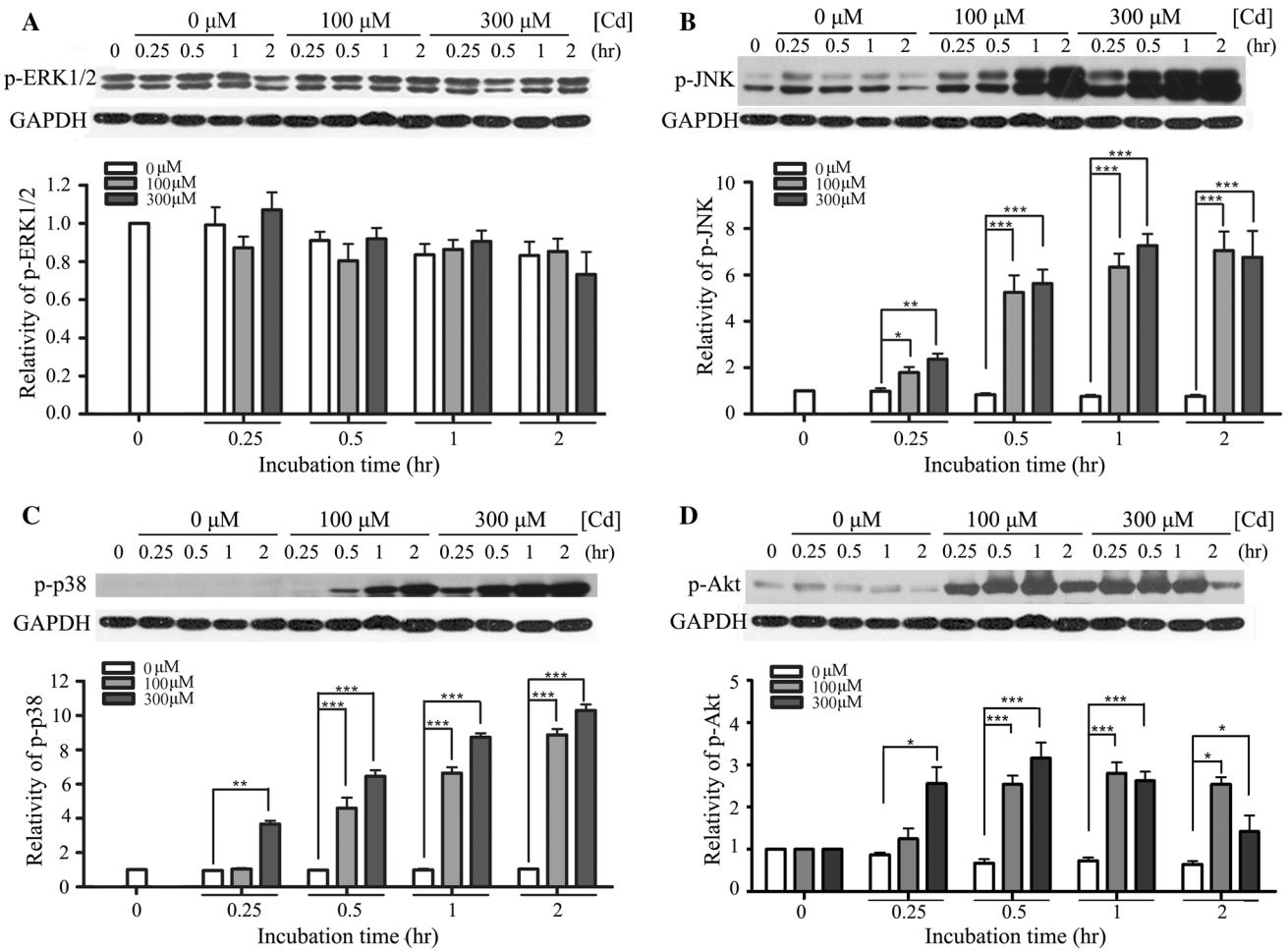

Fig. 5 Activation of JNK, p38 and Akt in astrocytes exposed to high doses of Cd. **a** representative Western blots (*up*) and statistical results (*down*) revealed that ERK1/2 was not activated at any time measured. Representative Western blots (*up*) and statistical results (*down*) revealed activation of JNK (**b**), p38 (**c**), and Akt (**d**) treated with/without 100 or 300 μM Cd at 0, 0.25, 0.5, 1 and 2 h in astrocytes in serum free DMEM. GAPDH was detected as an internal control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, $n = 4$

inhibitors of the MAPK and PI3K/Akt signaling pathways were utilized. There was a significant increase of LDH release in astrocytes exposed to 10 μM Cd for 12 h, but no significant change at 5 μM. So we chose 10 μM as a more suitable concentration to determine the role of the different signaling pathways in cell damage. We pre-treated cultures with 40 μM U0126, SP600125, SB203580 or LY294002 for 30 min in serum-free media before Cd treatments. After that, we measured the LDH release into culture mediums following 12 h of 10 μM Cd treatment. The results indicated that SP600125, U0126 and LY294002 could reduce the LDH release to different degrees, with the most significant reduction produced by SP600125 (from 52.86 to 32.45 %) at 12 h. U0126 (to 40.02 %) and LY294002 (to 36.84 %) also significantly attenuated LDH release. Interestingly, there was no significant difference after blocking p38 with SB203580 (Fig. 6a). We also found that when inhibitor incubation took place alone for 12 h, there was no significant increase of LDH release, which indicated that the inhibitors used had no significant toxicity to astrocytes (Fig S2). Furthermore, statistical analysis indicted that the combined pre-treatment with SP600125 and LY294002 (to 24.68 %) reduced more LDH release than when only one of them was used (Fig. 6a).

We performed similar measurements with 100 μM Cd treatment. Among the 4 inhibitors, only SP600125 (52.36–31.56 %) and LY294002 (52.36–37.56 %) reduced LDH release at 2 h, while U0126 or SB203580 did not induce any significant reduction in LDH release at this time point. These results indicated that only SP600125 and LY294002 could reduce Cd-induced astrocyte death. In addition, we found that the combined pre-treatment with SP600125 and LY294002 also reduced more LDH release (to 25.12 %) than when only one was used. These data suggested that Cd-induced activation of JNK and Akt from different signaling pathways may have an additive effect

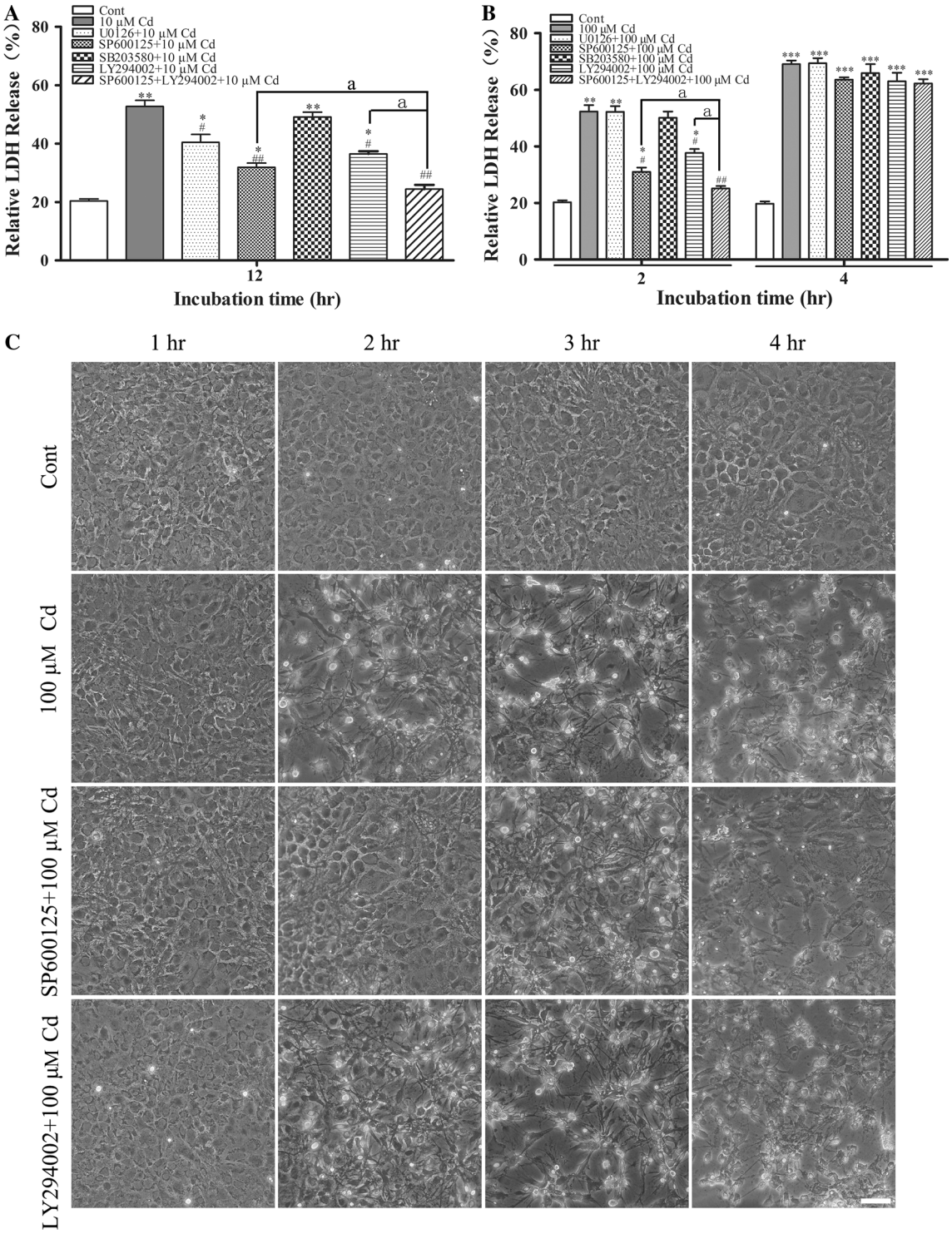

Fig. 6 SP600125 (JNK inhibitor) and LY294002 (PI3K/Akt inhibitor) partly reduced Cd-induced astrocyte death. LDH release measurements of astrocytes after U0126, SP600125, SB203580 or LY294002 pre-incubation for 30 min, before **a** 10 μM Cd treatment at 6 and 12 h, or **b** 100 μM Cd treatment at 1, 2 and 4 h. *vs. Cont, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $^{\#}$vs. Cd, $^{\#}P < 0.01$, $^{\#\#}P < 0.001$, ^{a}vs. SP600125 + LY294002 + Cd, $^{a}P < 0.01$, $n = 4$. **c** Phase contrast micrographs of primary cultures of astrocytes pre-incubation with 40 μM SP600125 or 40 μM LY294002 for 30 min, before subsequent 100 μM Cd treatment for 1, 2, 3 and 4 h. *Bar* = 50 μm

on astrocyte death. At 4 h of 100 μM Cd treatment, none of the four inhibitors exerted detectable reductions in Cd-induced LDH release (Fig. 6b).

Furthermore, we confirmed the effects of SP600125 and LY294002 on delaying astrocyte death under 100 μM Cd treatments by examining the morphological changes of cells in culture for up to 4 h. Both SP600125 and LY294002 could prevent the loosening of culture integrity caused by 100 μM. But we also noticed that there was an obvious better maintenance of integrity in SP600125 than LY294002 treated cultures, while there was no difference between groups at 4 h (Fig. 6c). The number of dead cells in the SP600125 group appeared to be lower, while LY294002 had no effect at 3 h, as compared to 100 μM Cd exposure at 3 h (Fig. 6c). Taken together, these data suggested that both SP600125 and LY294002 reduced Cd-induced astrocyte death, which suggested that JNK and Akt were involved in Cd-induced astrocytic cytotoxicity.

Cd Induced Calcium Signaling While BAPTA Inhibited Cd-Induced Activation of JNK and Akt and Protected Astrocytes from Death

Studies have shown that Cd disrupts intracellular free calcium homeostasis, leading to neural death [20]. We investigated the changes in calcium levels in astrocytes under low and high-level Cd treatments. Firstly, there was no significant increase of fluo-3 fluorescent in a Ca^{2+}-free medium under 100 μM Cd exposures in astrocytes (Fig S3). There was an increase in calcium levels from 15 to 60 min under 10 μM or 100 μM Cd exposures, with a much stronger signal in cultures with 100 μM Cd treatment (Fig. 7a). The relative intensity of calcium signaling rose to 2.8 fold under 10 μM Cd incubation after 60 min incubation, while it rapidly increased to 5.5 fold at 100 μM Cd exposure (Fig. 7b). These results suggest that the degree and speed of increase in calcium levels is much higher and faster at 100 μM Cd. Western blots demonstrated that BAPTA-AM treatment could block the elevation of p-ERK1/2 induced by 10 μM Cd, but BAPTA-AM had no effect on p-ERK1/2 levels with or without 100 μM Cd treatment. The activations of JNK and p38 were blocked after 30 min of BAPTA-AM pre-incubation before the treatment by 10 μM Cd for 6 h or 100 μM Cd for 1 h. Furthermore, we have analyzed the two isoforms of p-JNK separately, and found that the effect of BAPTA on p-JNK2 is more obvious than the effect on p-JNK1. p-Akt increase was also inhibited in the 100 μM Cd treatment group and was inhibited to a much lower degree of 10 μM Cd treatment (Fig. 7c and Fig S4). These results indicated that ERK1/2, JNK, p38, and Akt activation might be partly dependent on the increase of the intracellular calcium signal by Cd treatment in astrocytes.

After blocking of the calcium increases by BAPTA, we measured the LDH release under 10 μM Cd treatment at 12 h as an index of cell damage. Data revealed that BAPTA could decrease the LDH release from 52.86 to 23.86 %. This reduction was equivalent to the combined effect of SP600125 and LY2940002 (from 52.86 to 24.68 %) at 12 h Cd incubation (Fig. 7d). Under 2 h 100 μM Cd treatment, BAPTA also exerted a similar reduction effect on LDH release (from 52.36 to 24.08 %). This reduction was a combined effect of SP600125 and LY294002 (52.36 to 25.12 %). However, BAPTA effects vanished at 4 h of 100 μM Cd treatment (Fig. 7e).

In addition, phase-contrast microscopy showed that pre-incubation with BAPTA-AM for 30 min in serum-free media before Cd treatment significantly reduced Cd-induced astrocyte death (Fig. 8). This reduction was almost to the same degree as the combined effect of SP600125 and LY294002 treatments. All of these results supported the ability of the BAPTA inhibited calcium signal to prevent Cd-induced activation of the MAPK and PI3K/Akt signaling pathways, and protected astrocyte from death.

Discussion

Cd, a highly toxic heavy metal, is classified as a human carcinogen by the International Agency for Research on Cancer, and can affect cell proliferation, differentiation, apoptosis and other cellular activities [6, 21]. Accumulating evidence implicates Cd as severely affecting the function of the CNS, and thereby contributing to neurodegenerative diseases and neurological disorders [22, 23]. In vivo and in vitro studies have also indicated that acute or chronic exposure to Cd causes death in neurons, including apoptosis and necrosis [23, 24]. It's well known that astrocytes perform a very important role in the pathology and physiology of CNS function, including a protective role in neuronal survival and tissue repair after CNS injury via cleaning up metabolic toxins and free radicals [12, 25]. Previous study mainly focused on the Cd toxicity to neurons, now it's very necessary to investigate the astrocytic response to Cd in addition to the neuronal response, so as to get a comprehensive understanding of the CNS response to a toxin. More importantly, the underlying mechanism remains to be determined. The Cd toxicity in astrocytes was previously investigated [26], but this study was mainly focus on addressing the signaling mechanisms involved. In our study, we found that Cd induced astrocyte cytotoxicity in a time and dose dependent manner is associated not only with activation of MAPK cascade, but also with activation of the PI3K/Akt signaling pathway.

We initially observed, in detail, dose and time dependent Cd-induced morphological changes and LDH release

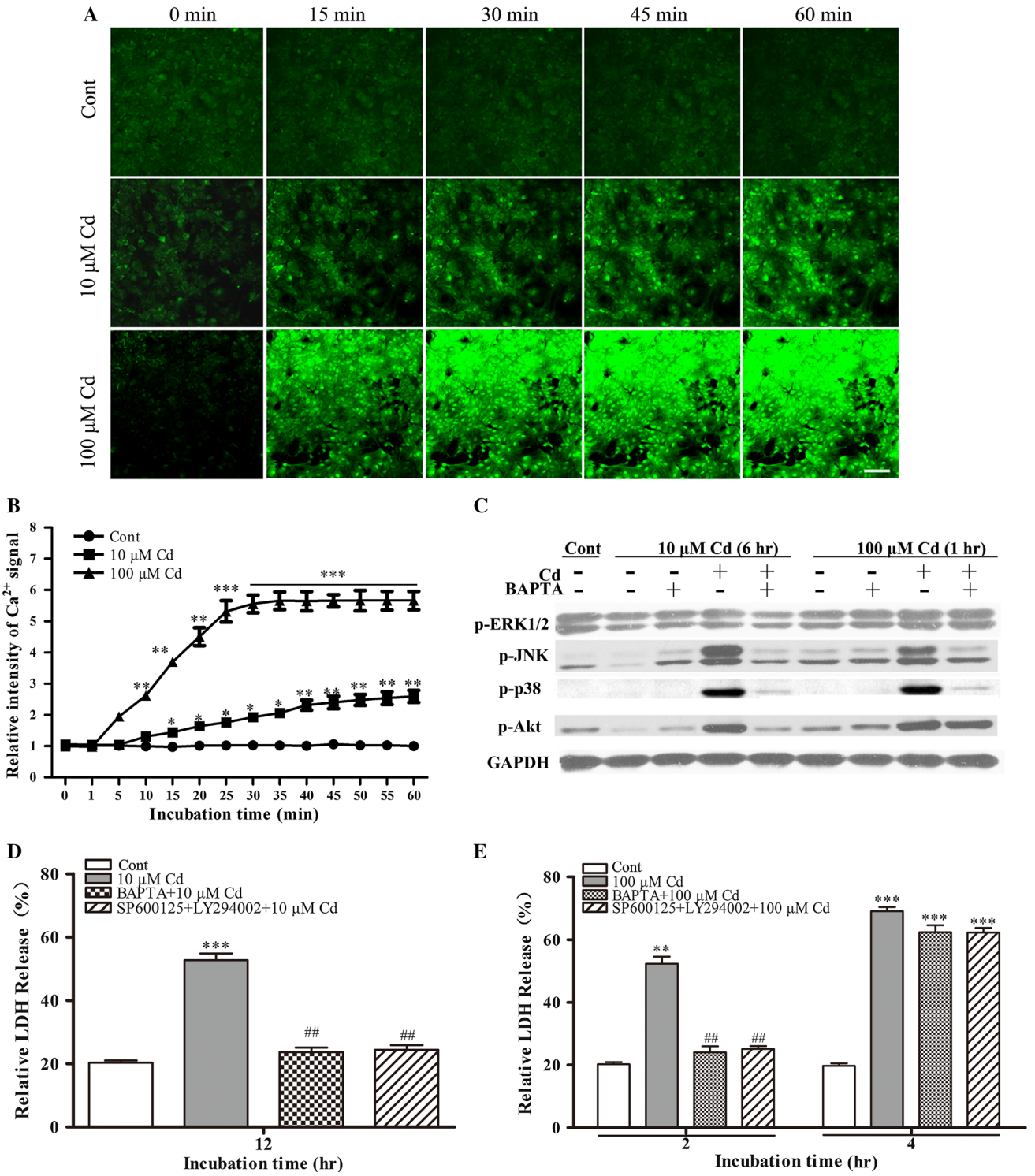

Fig. 7 BAPTA inhibited Cd-induced activation of MAPK and PI3K/Akt and prevented astrocyte from Cd-induced death. **a** Time lapse recordings of calcium levels during Cd treatment (10 or 100 μM) in primary cultures of astrocytes with the calcium indicator Fluo-3 at time points of 0, 15, 30, 45, and 60 min, *bar* = 80 μm. **b** Relative intensity of calcium signal under Cd exposure (10 or 100 μM) at different time points. **c** Western blots to detect the effect of BAPTA-AM pre-incubation on activation of ERK1/2, JNK, p38 and Akt with (+)/without (−) Cd treatment (10 μM Cd for 6 h and 100 μM Cd for 1 h) in astrocytes Cont: normal control without treatment. GAPDH was measured as an internal control. **d**, **e** LDH release measurements of astrocytes after 20 μM BAPTA or 40 μM SP600125 + 40 μM LY294002 pre-incubation for 30 min before **c** 10 μM Cd treatment at 6 and 12 h, or **d** 100 μM Cd treatment at 1, 2, and 4 h. *vs. Cont, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}$vs. Cd, $^{\#\#}P < 0.01$, $n = 4$

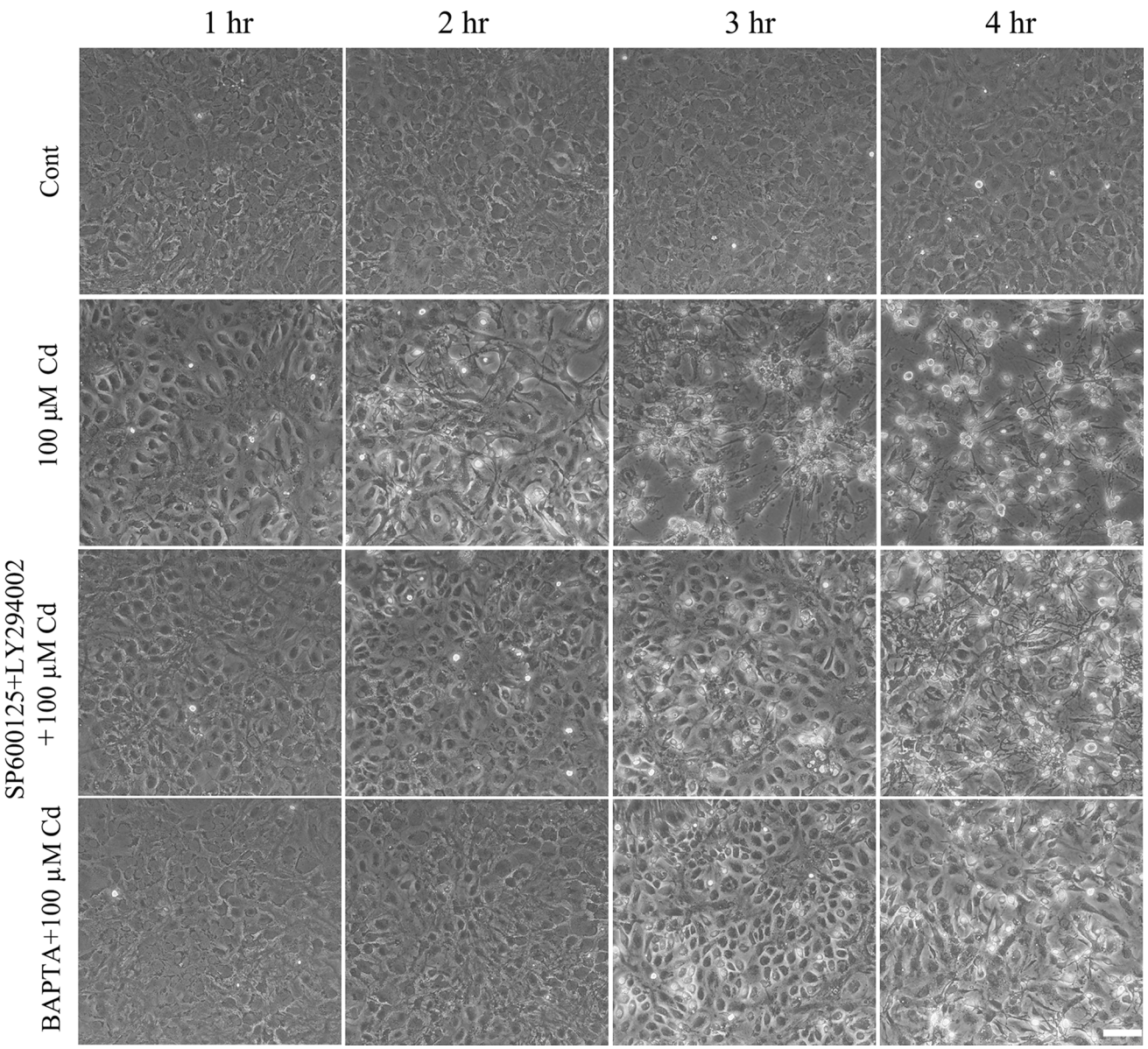

Fig. 8 Phase-contrast microscopy showed BAPTA delayed Cd-induced astrocyte death. Phase contrast micrographs of primary cultures of astrocytes after 100 μM Cd treatment, a combination of 40 μM SP600125 and LY294002 pre-incubation for 30 min before subsequent 100 μM Cd treatment, and 20 μM BAPTA-AM pre-incubation for 30 min before subsequent 100 μM Cd treatment for 1, 2, 3 and 4 h. *Bar* = 50 μm

in primary cultures of astrocytes under both chronic and acute Cd exposure. Obviously, astrocytes are more resistant than neurons to heavy metal induced cytotoxicity, but Cd can still induce astrocyte cytotoxicity and cell death after 10 μM Cd exposure for 12 h or 100 μM Cd exposure for 2 h in a time and dose-dependent manner [15, 20]. Compared to other studies on Cd-induced neuronal death, our results show that neurons are less resistant and more sensitive to Cd insults than astrocytes [26, 27]. When cortical neurons were treated by Cd at the concentration range of 1–5 μM in DMEM for 24 h, a 50 % reduction in neuronal cell viability was observed [18, 26, 27]. However, treatment with up to 10 μM of Cd in DMEM for 24 h can affect cellular viability of astrocytes to 50 %. Neurons were, in terms of survival, clearly less resistant to Cd insult. But on the other hand our results showed that cortical astrocytes underwent apoptosis when exposed to 1–10 μM of Cd in the absence of serum for 24 h. However, higher Cd concentrations mainly induced necrosis quickly. These results suggest that Cd induces either apoptosis or necrosis in a primary culture of astrocytes, depending on the Cd concentration. This dual mechanism induced by Cd is not

surprising because classical apoptosis and necrosis represent only the extreme ends of a broad range of possible morphological and biochemical deaths and these two death types can occur simultaneously in cell cultures exposed to the same stimulus [23].

There has been lots of research performed on Cd-induced neuronal toxicity and the mechanisms involved are rather clear. It has been reported that there are many different possible mechanisms of Cd cytotoxicity and Cd-induced cell death in neurons. Such mechanisms include reactive oxygen species (ROS) production, calcium homeostasis disorders, downstream signaling pathways, mitochondrial apoptosis, the interaction of Cd with other metals such as cobalt (Cu), zinc (Zn), arsenic (As), and lead (Pb) [2, 11, 28].

Multiple studies have demonstrated that Cd cytotoxicity may trigger cell death by selective activation of ERK1/2, JNK, p38, and/or Akt in various types of cell, including PC12 cells, SH-SY5H cells, cortical neurons and endothelial cells [18–20, 29, 30]. Moreover, cell death depends on the cell types and the concentration of Cd tested [31]. Increasing evidence indicates that Cd-induced neuron and astrocyte toxicity is due to induction of ROS, leading to oxidative stress [26, 32, 33]. Previous study has reported on mechanisms of Cd-induced neuronal toxicity, and demonstrated that Cd activates the MAPK and mTOR signaling pathways by induction of ROS generation. This sequence not only activates the upstream kinases of ERK1/2 and JNK, but also the Akt/mTOR pathway, leading to apoptosis of neuronal cells [32–34]. During Cd-induced neurons cytotoxicity, Cd induces oxidative stress by disrupting intracellular calcium homeostasis and by causing the accumulation of ROS in neurons [24, 26]. Cd-induced toxicity in astrocytes, is now known to involve ROS from oxidative stress, and ROS may be one of the early signal changes in Cd-induced astrocytes cytotoxicity as neurons [26, 35]. However, the signal pathway involved in the depletion of glutathione and calcium homeostasis disorders are unknown [26, 35]. Moreover, most of the previous studies were performed in cell lines, such as PC12, SH-SY5Y, C6 [18, 36]. Studies using a primary culture of neurons and astrocytes for Cd toxicity are rather rare.

Much of our new understanding of astrocytes has been derived from studies conducted with primary cultures of astrocytes, which have been an invaluable tool for studying roles of astrocytes in physiological and pathological conditions [37]. Despite their greater Cd-tolerance, astrocytes are very sensitive to Cd treatment, and the functional changes thereby induced may also contribute to neuronal cell death under cytotoxicity. Here our study was focused on the signal transduction pathways and confirmed that Cd-induced intracellular calcium increase led to MAPK and PI3K/Akt activation in a primary culture of astrocytes.

In this study, we detected the activation of MAPK (ERK1/2, JNK and p38) and PI3K-Akt/protein kinase B (PKB) signaling pathways by Western blot. We found that JNK, p38 and Akt were all activated to different degrees under low and high levels of Cd treatment at various times. We also found that ERK1/2 was only activated at low dose exposure to Cd. JNK is preferentially activated by oxidative stress and cytokines resulting in inflammation and apoptosis [38], and Akt plays a crucial role in regulating cell growth, differentiation, and survival/death [39]. Furthermore, we found that both JNK and Akt were activated to a faster and higher degree when under acute and chronic Cd exposure. This finding suggested that they may play a more important role in Cd-induced astrocyte death. Surprisingly, under high-level Cd exposure, there was no significant activation of ERK1/2, which is predominantly activated by growth factors or mitogens, leading to cell differentiation, growth, and survival. One possible reason was that the cytotoxicity and damage under 100/300 μM Cd treatment were too severe and mainly induced necrosis, which may differ from the effect of chronic Cd exposure. All three MAPK members (including ERK1/2, JNK and p38) and PI3K/Akt were activated in neurons and astrocytes exposed to Cd at low Cd concentration of 10 μM. In addition, activation of MAPK and PI3K/Akt takes place at 2 h after 10–20 μM Cd incubation in neurons [18, 20], while the time point is 1 h in astrocytes. This finding suggested astrocytes may be more sensitive to MAPK and PI3K/Ak signal pathways under Cd exposure than neurons.

Activation of MAPK and/or PI3K/Akt pathways may promote cell survival or cell death, depending on the stimuli [20, 40]. By using different specific inhibitors for MAPK and PI3K/Akt, then, we observed the effect of these two signal pathways on Cd-induced astrocytes cytotoxicity. We thereby found that SP600125 and LY294002 partially blocked Cd-induced astrocyte cytotoxicity at high and low Cd exposure. This finding in turn suggests that activation of JNK and Akt were an upstream signal of cell death. Inhibiting ERK1/2 with U0126 had a much weaker effect on reduced Cd-induced death only at low Cd levels, but not at high Cd exposure, and inhibiting p38 with SB203580 had no effect at all.

Cd rapidly activated ERK1/2, JNK, p38 and mTOR, and inhibition of ERK1/2, JNK, and mTOR—but not p38—partially protected the cells from Cd-induced neuron apoptosis [18, 20, 24]. Yang et al. [26] found that the addition of antioxidants inhibited Cd-induced oxidative injury in primary rat cultures of astrocytes. Some reports indicate that a calcium signaling pathway mediates sustained activation of MAPK and mTOR/PI3K in primary rat cerebral cortical neuron via a mitochondrial apoptotic pathway [24, 41, 42]. Chen et al. [43] have found that celastrol protects against Cd-induced apoptotic cell death

in neuronal cells via inhibiting activation of JNK and the Akt/mTOR network. All of these findings indicate that exposure to Cd activates MAPK and PI3K/Akt in both neurons and astrocytes and that there's no significant difference between the activation and effect in neurons and in astrocytes. Moreover, inhibition of JNK and Akt activation partly blocks Cd-induced neuronal and astrocytic death [33].

Calcium is a ubiquitous intracellular ion that acts as a signaling mediator in numerous cellular processes including cell proliferation, differentiation, and survival/death, and is similar in many respects to Cd [1]. Intracellular calcium homeostasis is crucial in maintaining the normal function of the cell, in that variations in the concentration of calcium in cells can determine cell survival or death [31]. Recent evidence strongly supports the theory that Ca^{2+} dysregulation is involved in many diseases, such as AD [44]. Due to Cd^{2+} structurally resembling Ca^{2+}, it can disrupt intracellular free calcium homeostasis, leading to apoptosis in murine neurons [18]. Moreover, a number of studies have demonstrated that Cd interacts with the functions of many Ca^{2+}-dependent regulatory proteins such as MAPK, protein kinase C (PKC), and phospholipase C, thus interfering with calcium homeostasis [24, 41, 45].

Previous researches have shown that astrocytes propagate intercellular calcium waves over long distances in response to stimulation, and, similar to neurons, release gliotransmitters in a Ca^{2+}-dependent manner [16]. However, little is known about the role of Ca^{2+} signaling in Cd-mediated activation of MAPK/mTOR pathways and apoptosis in astrocytes. Marchi et al. [46] have demonstrated that a Ca^{2+} probe (fluo-2-AM and fluo-3-AM) is a suitable tool to study the effects of heavy metals (Cd^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+}) on Ca^{2+} in living cells. However, previous studies have suggested that Fluo-3 fluorescence increases upon binding of Cd [47]. In order to rule out this possibility, another experiment has been performed using a Ca^{2+}-free medium, and we found that there was no significant increase of fluo-3 fluorescent. Here we observed there was a significant increase of Ca^{2+} with fluo-3 as a Ca^{2+} probe in astrocytes under Cd treatment at low or high Cd exposure at the beginning time of 30 and 15 min, respectively. Chelating free Ca^{2+} with BAPTA, which has no chelating effect on Cd, has been widely used as a specific intracellular Ca^{2+} chelator to study the effects of Cd-induced Ca^{2+} levels in PC12 cells, SH-SY5Y cells, and primary cultures of neurons [20, 24].

We observed that chelating free Ca^{2+} with BAPTA reduced Cd-induced astrocyte death at high and low Cd doses exposure. In addition, BAPTA blocked Cd-induced JNK, p38 and Akt activation at 6 h at low doses and also ERK1/2, JNK, p38 and Akt activation at 1 h at high doses. In primary cultures of cortical neurons, Cd elevates intracellular free Ca^{2+} levels, leading to neuronal apoptosis partly by activating MAPK and mTOR pathways, while a central role in the neurotoxicology of Cd is played by CaMKII pivot [24, 41]. These results suggested that increased levels of Ca^{2+} stimulated the up-regulation of MAPK and PI3K/Akt under low and high Cd exposure, which also occurs in primary cultures of cerebral cortical astrocytes as neurons exposed to Cd.

In our previous published studies, our lab has performed a lot of investigation regarding the response of astrocytes and neurons under different injury conditions. Injury conditions covered included traumatic injury, ischemic injury, and excitotoxic injury, and we have also elucidated the possible mechanisms and signaling pathways involved [13, 15–17]. We have found that MAPK and PI3K/Akt were activated to different degrees at different time points in our ischemia and scratch models. Furthermore, we found that ischemia activates the JNK/c-Jun/AP-1 pathway to up-regulate 14-3-3gamma, enabling it to play an important protective role in astrocytes under ischemia. We also found that traumatic scratch injury triggers calcium influx to activate the JNK/c-Jun/AP-1 pathway and switch on GFAP expression in traumatic-induced astrogliosis [15, 16]. These findings are consistent with a report from another laboratory indicating that reactive astrogliosis in the rat glioma 9L cell line was up-regulated by combined mechanical and chemical injuries, which suggested that activation of MAPK signal pathways may participate in Cd-induced astrogliosis [48]. All of these results indicated that astrocytes play different roles in responding to toxicity, injury and damage stimulation, which may affect their normal interaction with neurons in the CNS.

In summary, we have found that Cd induced significant cytotoxicity and cell death in primary cultures of astrocytes. Morphological changes and cell death were observed in a time and dose-dependent manner at both low and high levels of Cd exposure. MAPK and PI3K/Akt were activated to different levels under Cd treatment. Moreover, JNK and Akt, but not p38, may be two important signaling pathways that participate in Cd cytotoxicity in astrocytes. Furthermore, the activation of MAPK and PI3K/Akt depended on increased levels of intracellular Ca^{2+}, while chelating intracellular Ca^{2+} with BAPTA blocked the activation of MAPK and PI3K/Akt signal pathways. Moreover, chelating intracellular Ca^{2+} also reduced Cd-induced astrocytes death via the inhibition of JNK and Akt activation. Taken together, our findings support the notion that the regulation of Cd-induced Ca^{2+} homeostasis may be a good strategy for prevention of Cd-related diseases in the CNS. Most important of all, we have shown that astrocytes are very responsive to heavy metal toxicity, due to their particular role and function in BBB, homeostasis, and interaction with neurons. Data collected from astrocytes

and from neurons would be indispensable for us to obtain a more comprehensive understanding of the CNS response to toxins.

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Compliance with Ethical Standards

Conflict of interest All authors have no conflict of interest.

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