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Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis☆

Sun Don Kim[a,d], Chang Kyu Moon[c], Su-Yong Eun[a,b], Pan Dong Ryu[d], Sangmee Ahn Jo[a,b,*]

[a] Department of Biomedical Sciences, National Institute of Health, Seoul 122-701, Republic of Korea
[b] Biomedical Brain Research Center, National Institute of Health, Seoul 122-701, Republic of Korea
[c] College of Pharmacy, Seoul National University, Seoul 122-701, Republic of Korea
[d] College of Veterinary Medicine, Seoul National University, Seoul 122-701, Republic of Korea

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Abstract

Cd induces oxidative stress and apoptosis in various cells by activating mitogen-activated protein kinases (MAPKs), but the precise signaling components of the MAPK cascade and their role in neuronal apoptosis are still unclear. Here, we report that Cd treatment of SH-SY5Y cells caused apoptosis through sequential phosphorylation of the apoptosis signal regulating kinase 1, MAPK kinase 4, c-Jun N-terminal kinase (JNK), and c-Jun as determined by overexpression of dominant negative (DN) constructs of these genes or using a specific JNK inhibitor SP600125. Both Cd-induced JNK and c-Jun phosphorylation and apoptosis were inhibited dramatically by *N*-acetyl-L-cysteine, a free radical scavenger. In addition, caspase inhibitors, zDEVD and zVAD, reduced apoptosis but not JNK and c-Jun phosphorylation induced by Cd, while overexpression of DN JNK1 inhibited caspase-3 activity. Taken together, our data suggested that the JNK/c-Jun signaling cascade plays a crucial role in Cd-induced neuronal cell apoptosis and provides a molecular linkage between oxidative stress and neuronal apoptosis.

Keywords: Cadmium; Oxidative stress; MAP kinases; c-Jun; Caspase-3; Apoptosis; Signaling

Cadmium (Cd) is a ubiquitous environmental toxic heavy metal that causes pulmonary edema, respiratory tract irritation, renal dysfunction, anemia, osteoporosis, and cancer in humans. In addition, workers exposed to Cd showed olfactory dysfunction and neurobehavioral defects in attention, psychomotor speed, and memory. Cd generates reactive oxygen species (ROS) in various cells [1] and reacts readily with thiol groups of proteins [2], thereby disrupting the structure of cellular proteins. They also induce lipid peroxidation [3]. Cd is an effective inducer of a number of stress proteins including heme oxygenase-1, heat shock protein 70, and metallothionein [4–6], and transcription factors such as c-Jun, c-Myc, and c-Fos [7,8].

One of the most relevant signaling pathways involved in both the stress response and apoptosis is the mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases. To date, three MAP kinase cascades that converge on extracellular signal-regulated protein kinase (ERK1/2), c-Jun N-terminal kinases

☆ *Abbreviations:* Cd, cadmium; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, p38 MAP kinase; MKK4/7, MAP kinase kinase 4/7; ASK-1, apoptosis signal regulating kinase; DN, dominant negative.

* Corresponding author. Fax: +822 388 0924.
E-mail address: sangmeeaj@nih.go.kr (S.A. Jo).

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(JNKs), and p38 MAP kinase (p38) have been extensively characterized. These MAPKs are regulated by the distinct stimuli [9,10]. ERK is predominantly activated by growth factors or mitogens leading to cell differentiation, growth, and survival. On the other hand, JNK and p38 are preferentially activated by oxidative stress and cytokines resulting in inflammation and apoptosis. One apoptotic pathway that appears to be particularly important in neuronal cell apoptosis includes JNKs. Accumulated evidences indicate that JNK activation plays critical roles in many paradigms of neuronal cell death as diverse as MPTP, β-amyloid exposure, low potassium, trophic factor withdrawal, oxidative stress, and UV irradiation [11–14]. c-Jun, a well-known downstream target of activated JNK, also plays an obligatory role in cell death. Neuronal c-Jun levels are elevated in response to trophic factor withdrawal, cytotoxic insults, and cadmium treatment [7,15]. Although extensive knowledge about the JNK/c-Jun pathway in apoptosis was documented, it is not clearly understood how c-Jun activation is involved in neuronal cell death signaling, except its role in inhibition of cytochrome *c* release from the mitochondria [16].

Several upstream molecules of JNK have also been identified. JNK can be activated by MAPK kinases (MAPKKs) including mitogen-activated protein kinase kinase 4 and 7 (MKK4 and MKK7). MAPKKs in turn can be activated by several MAPKK kinases (MAPKKKs) including the MEKK group (MEKK1 through 4), the mixed-lineage protein kinase group (MLK1 through 3, DLK, and LZK), the apoptosis signal regulating kinase group (ASK1 and 2), TAK1, and TPL2 [17]. Of the MAPKKK molecules, ASK1 is ubiquitously expressed and activates the MKK4/7-JNK and MKK3/6-p38 signaling cascades [18]. Overexpression of ASK1 induced apoptosis or dominant negative ASK1 (ASK1-DN) protected it [19], suggesting that ASK1 is a pivotal component in stress or cytokine-induced apoptosis.

Although several cellular stresses have been shown to activate p38, JNK, and ERK in neuronal cells [15,20,21], it is unclear which MAPK cascade leads to apoptosis induced by Cd in neuronal cells. Furthermore, upstream and downstream effector molecules of the death-associated MAPK pathway are not understood. Here, we report that ROS produced by Cd plays an important role in apoptosis in SH-SY5Y cells and the apoptosis occurs through activation of the ASK1/MKK4/JNK/c-Jun cascade and caspase-3.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco-BRL (Gland Island, NY). The MAPK inhibitors, SB203580, U0126, and SP600125 and the caspase inhibitors, z-Val-Ala-Asp-CH_2F (zVAD-fmk) and z-ASP (OCH_3)-Glu (OCH_3)-Val-Asp-CH_2F (zDEVD-fmk), were obtained from Calbiochem–Novabiochem (San Diego, CA). Anti-JNK, p38, ERK, c-Jun, phospho-JNK (Thr-183/Tyr-185), phospho-ERK (Thr-202/Tyr-204), phospho-c-Jun (Ser-63), and caspase-3 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-specific p38 (Thr-180/Tyr-182) antibody was obtained from Pharmingen (San Diego, CA). Cadmium chloride, *N*-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Nonidet-P40 (NP-40), and other chemicals were obtained from Sigma (St. Louis, MO).

Cell cultures. SH-SY5Y human neuroblastoma cells (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. Cells were maintained at 37 °C in a humid atmosphere containing 95% air/5% CO_2.

Transfection. Plasmids containing dominant forms of JNK1 (Thr180-Pro-Tyr182 replaced with Ala-Pro-Phe; DN JNK1) and MKK7 (K149A; MKK7A; DN MKK7) were kindly provided by Dr. J. Han (Scripps Research Institute, USA). Plasmids containing wild forms of MKK7 and MKK4, and dominant negative form of MKK4 (K129R; MKK4-KR; DN MKK4) were kindly provided by Dr. E. Choi (Korea University, Korea). Plasmids containing wild and dominant forms of ASK1 (K709M; DN ASK1) were kindly provided by Dr. H. Ichijo (Tokyo Medical and Dental University, Japan). Plasmid containing dominant form of c-Jun (c-JunΔ169; DN c-Jun) was kindly provided by Dr. J. Ham (University College London, UK). For transient transfection, SH-SY5Y cells were plated in a washed glass coverslip or 60 mm dish 1 day before transfection and transfected with plasmids (1 μg for coverslip or 2 μg for 60 mm dish) using lipofectAMINE Plus reagent (Gibco-BRL, Gland Island, NY) according to the manufacturer's recommendation. After 3 h the transfection medium was replaced with fresh culture media and the cells were incubated for another 16–20 h prior to treatment with Cd. For stable transfection, cells were plated at 5×10^5 cells/60 mm dish and transfected with the construct (DN MKK4) using lipofectAMINE Plus reagent and the transfected cells were selected in the presence of G418 (800 μg/ml).

DAPI staining. SH-SY5Y cells transiently transfected with expression vectors encoding the indicated genes and pEGFP (Clontech laboratories, Palo Alto, CA) were established. For inhibitor experiments, cells were treated with an appropriate concentration of Cd for the indicated time following preincubation with each inhibitor for 30 min. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) prepared in PBS for 30 min at room temperature. Following treatment with RNase (1 mg/ml), cells were treated with 4′6-diamidino-2-phenylindole (DAPI, 10 μg/ml) for 30 min. Apoptotic cells with condensed or fragmented nuclei were visualized under a fluorescence microscope. Approximately 100–200 cells/well were assessed. The percentage of apoptotic cell was determined as a ratio of GFP-expressing cells with apoptotic nuclei divided by the total number of GFP-expressing cells.

MTT assay. Cell viability was assessed by a MTT assay described previously in [22]. Briefly, SH-SY5Y cells plated at a density of 2.5×10^4 in 96-well microtiter plates were cultured overnight, treated with Cd for the indicated time, rinsed with phosphate-buffered saline (PBS), pH 7.2, and incubated with MTT solution (0.5 mg/ml) for 4 h. The reagent was reduced by living cells to form insoluble blue formazan products. The cells were then washed, solubilized with 100 μl solution containing 10% sodium dodecyl sulfate (SDS) and 0.01 N HCl, and quantified by measuring absorbance at 540 nm.

TUNEL staining. In order to detect apoptotic cells, TUNEL staining was carried out using an In situ Cell death Detection Kit (Roche, Indianapolis, IN). Cells on coverslips were fixed with 4% PFA prepared in PBS for 30 min, rinsed with PBS, and permeabilized with 0.1% Triton X-100 for 2 min. Aliquots (50 μl) of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluo-

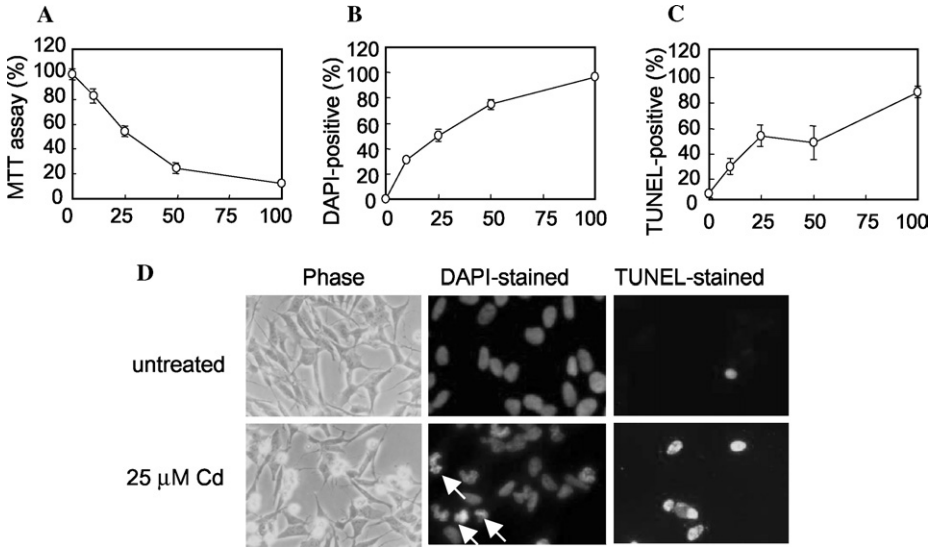

Fig. 1. Cd induces apoptosis of SH-SY5Y neuronal cells. SH-SY5Y cells grown in the growth medium were treated with varying concentrations (0–100 μM) of Cd for 12 h, and cell death was assessed by the MTT assay (A), DAPI staining (B), and TUNEL staining (C) as described in the Materials and methods. Data represent the average ± SD from two to four independent experiments. (D) Phase-contrast microscopic images showed the damaged cells which are more rounded in shape and smaller in size after Cd-treatment. DAPI-positive apoptotic cells showing fragmented or condensed nuclei (arrows) and TUNEL-positive cells (arrows) were also observed in Cd-treated cells.

rescent dUTP were added to the samples and incubated at 37 °C for 60 min in a dark, humidified condition. After washing with PBS, the coverslips were observed under a fluorescence microscope.

Western blot analysis. Cells were lysed in cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholic acid, and 0.1% SDS) containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin), and cellular debris was removed by centrifugation at 14,000*g* for 20 min at 4 °C. Aliquot (40 μg) of total protein fraction was separated using 12.5% SDS–PAGE and the proteins were transferred onto a nitrocellulose filter membrane (Amersham Bioscience, Germany). After blocking the membrane with 5% fat-free milk in TBST buffer (25 mM Tris–HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, and 0.2% Tween 20) for 1 h, the membrane was incubated with appropriate primary and secondary antibodies for 1 h each, and developed using an enhanced chemiluminescence kit (Amersham Bioscience, Germany). All the antibodies (anti-JNK, ERK, p38, c-Jun, phospho-JNK, phospho-ERK, phospho-p38, phospho-c-Jun, and caspase-3 antibodies) were used at 1:1000 dilution. For reprobing, membranes were stripped at 50 °C for 30 min in 62.5 mM Tris–HCl, pH 6.8, containing 100 mM β-mercaptoethanol and 2% (w/v) SDS.

Statistical analyses. Data are expressed as means ± SD. Data were evaluated with one-way analysis of variance (ANOVA) followed by Student's *t* test. Statistically significant differences were reported as $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. Data with values of $p < 0.05$ were generally accepted as statistically significant.

Results

Cd induced apoptosis of neuronal cells through phosphorylation of JNK

To assess the toxic effects of Cd on human neuronal cell, SH-SY5Y cells were treated with Cd for 12 h at various concentrations (0–100 μM) and assayed for cell viability using the MTT assay, DAPI staining, and TUNEL staining. Loss of viability occurred in a dose-dependent manner in response to Cd treatment (Fig. 1A). At 25 μM Cd, the cell viability reduced to about 54.1 ± 3.4% of the untreated control cells. Phase-contrast microscopic observation showed the damaged cells which were more rounded in shape and smaller in size (Fig. 1D). To confirm whether the loss of viability was attributable to apoptosis, we performed nuclear staining with a fluorescence dye DAPI (Figs. 1B and D). Nuclear fragmentation and condensation were hardly observed in the untreated cells while approximately 50 ± 4.0% cells treated with 25 μM Cd showed the fragmentation and condensation of nuclei as apoptotic bodies (arrows). In addition, the cleaved DNAs were labeled using TUNEL staining and the number of TUNEL-positive cells increased in a dose-dependent manner (Figs. 1C and D). Thus, apoptotic cell death seemed to be the major cause involved in Cd-induced reduction of cell viability.

Cd has been reported to activate the MAP kinases, ERK, p38, and JNK, but the activated kinase by Cd is likely to be different depending on cell types and the dose tested [23]. Thus, we examined changes of phosphorylation of these kinase the following Cd treatment in SH-SY5Y cells by Western blot analysis using each phospho-specific antibody. As shown in Fig. 2A, 25 μM Cd treatment caused phosphorylation of both JNK and p38; activation of JNK and p38 appeared to be maximum at 6 h after Cd treatment, was sustained upto at 12 h, and disappeared at 24 h. We hardly detected activation earlier than 3 h (data not shown). Phosphorylation of JNK and p38 was dose-dependent at the concentration of 10–100 μM Cd (Fig. 2B). In contrast, ERK was highly phosphorylated in the untreated cells and was unchanged in response to Cd treatment.

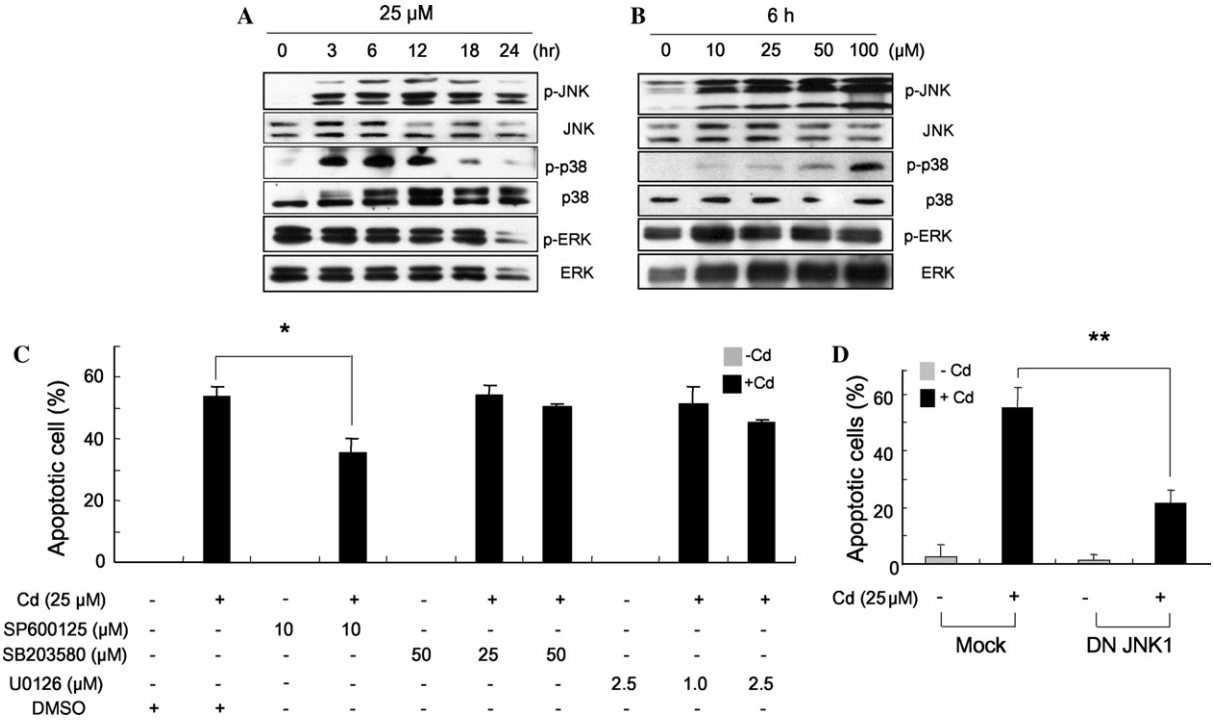

Fig. 2. Phosphorylation of JNK mediates Cd-induced neuronal cell apoptosis. (A,B) SH-SY5Y cells were treated with 25 μM Cd for the indicated time or at varying concentrations for 6 h. Then, the total protein was extracted and separated by SDS–PAGE, and the Western blot analysis was performed using appropriate antibodies. (C) Cells were treated with Cd (25 μM, 12 h) following pretreatment with a JNK inhibitor SP600125, a p38 inhibitor SB203580 or an ERK inhibitor U0126 for 30 min, and the apoptotic cells were counted by the DAPI staining. (D) Cells transiently transfected with DN JNK1 or mock vector together with pEGFP were treated with 25 μM Cd for 12 h, and the apoptotic cells were counted following the DAPI staining. Cells showing condensed or fragmented nuclei were counted under a fluorescence microscope. Data represent the average ± SD of the percent apoptotic cells relative to the total GFP-positive cells (100%) from at least three independent experiments. *$p < 0.05$ and **$p < 0.01$.

As JNK and p38 were activated during Cd-induced apoptosis of SH-SY5Y cells, we examined whether inhibition of these kinases would result in inhibition of apoptosis. Thus, cells were treated with 25 μM Cd for 12 h after pretreatment with a JNK inhibitor SP600125 (10 μM), a p38 inhibitor SB203580 (25, 50 μM), or an ERK inhibitor U0126 (1.0, 2.5 μM) for 30 min, and then apoptotic cells were counted by DAPI staining (Fig. 2C). Pretreatment with SP600125 significantly reduced the number of Cd-induced apoptotic cells to 35.6 ± 4.6% from 49.0 ± 1.8% of Cd-treated cells (Fig. 2C). In contrast, SB203580 and U0126 did not blocked significantly apoptosis induced by Cd. Role of JNK in neuronal cell apoptosis was further confirmed by cotransfection study using dominant negative construct of JNK1 (DN JNK1). Cells transiently cotransfected with DN JNK1 and pEGFP were treated with 25 μM Cd for 12 h, and the apoptotic cells were counted after DAPI staining. Fig. 2D showed that DN JNK1 construct significantly reduced Cd-induced apoptosis by 60.6% compared to mock construct (55.1 ± 7.4%, mock; 21.7 ± 4.3%, DN JNK1). Taken together, these results suggest that activation of JNK, but not p38 and ERK, seemed to be linked to Cd-induced apoptosis of SH-SY5Y cells.

Phosphorylation of c-Jun is involved in Cd-induced neuronal cell apoptosis

Although several publications have demonstrated that activation of the JNK and c-Jun pathway leads to neuronal cell death induced by trophic factor withdrawal [16] and ischemia [24], the role of c-Jun expression and activation by other extracellular stimuli such as Cd intoxication has not been studied. Thus, we examined by Western blot analysis both the expression and phosphorylation of c-Jun by using anti-c-Jun and phospho- (Ser-63) c-Jun antibodies, respectively. As shown in Fig. 3A, Cd increased both the c-Jun protein level and phosphorylation of c-Jun in a concentration- and time-dependent manner. The Cd-induced c-Jun phosphorylation was dependent on the activation of JNK since pretreatment of cells with a JNK inhibitor SP600125 (10 μM) for 30 min (Fig. 3B) or overexpression of DN JNK1 (Fig. 3C) significantly decreased c-Jun phosphorylation induced by Cd treatment. In addition, when the cells transiently cotransfected with a plasmid expressing dominant negative form of c-Jun (c-JunΔ169; DN c-Jun) and pEGFP were treated with 25 μM Cd for 12 h, DN c-Jun blocked Cd-induced apoptosis by 52.9 ± % (53.5 ± 3.9%, mock; 22.9 ± 6.0%, mutant). Thus, c-Jun phosphorylation

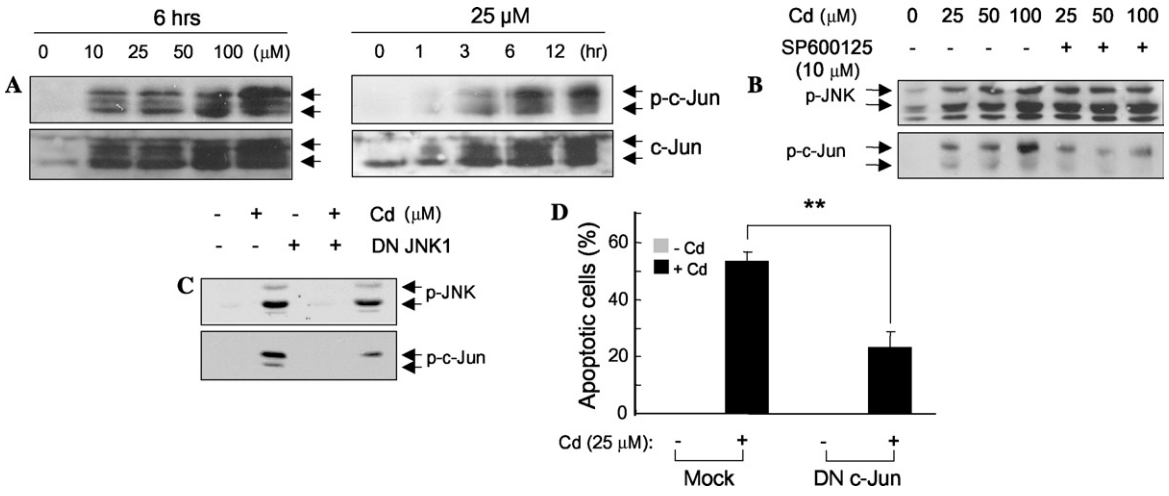

Fig. 3. C-Jun is involved in Cd-induced neuronal cell apoptosis. (A) Expression and phosphorylation of c-Jun induced by Cd was measured by Western blot analysis. SH-SY5Y cells were treated with 25 μM Cd for the indicated times or at the varying concentrations for 6 h. Then, the total protein fraction was extracted and subjected to SDS–PAGE and Western blot analysis using anti-c-Jun or phospho-c-Jun antibody. (B) Cells were pretreated with 10 μM SP600125 for 30 min and then with 25 μM Cd for 12 h. The total proteins were extracted and separated by SDS–PAGE and the Western blot analysis was performed using anti-phospho-JNK or phospho-c-Jun antibody. (C) Cells were transiently transfected with dominant negative JNK1. At 24 h after transfection, the cells were treated with 25 μM Cd for 12 h. The total proteins were extracted and separated by SDS–PAGE, and the Western blot analysis was performed using anti-phospho-JNK or phospho-c-Jun antibody. (D) Cells were transiently transfected with a dominant negative construct of c-Jun (DN c-Jun) or a mock vector together with pEGFP as a transfection control. At 24 h after transfection, the cells were treated with 25 μM Cd for 12 h, fixed with 4% PFA, and stained with 10 μg/ml DAPI. Data represent the average ± SD of the percent apoptotic cells relative to the total GFP-positive cells (100%) from three independent experiments. $^{**}p < 0.01$.

which is dependent on JNK is likely to contribute to Cd-induced neuronal cell death in SH-SY5Y cells.

MKK4, but not MKK7, mediates Cd-induced neuronal cell apoptosis

Both MKK4 and MKK7 are known as upstream kinases of JNK [25,26]. To examine the role of these two MKKs in Cd-induced cell death and the effect on Cd-induced JNK activation, SH-SY5Y cells transiently transfected with wild, dominant negative constructs of MKK4 (K129R; DN MKK4) or MKK7 (K149A; DN MKK7) were treated with 25 μM Cd for 12 h. A pEGFP vector was cotransfected to select transfected cells. Overexpression of wild MKK4 alone caused significant increase in apoptosis (9.45 ± 2.7%, mock; 22.2 ± 3.1%, wild MKK4). In addition, overexpression of DN MKK4 significantly inhibited Cd-induced apoptosis by 51.1% (58.5 ± 2.7%, mock; 28.6 ± 1.3%, DN MKK4), but DN MKK7 (64.0 ± 2.1%) caused no significant change (Fig. 4A). As shown in Fig. 4B, DN MKK4 also suppressed phosphorylation of both JNK and c-Jun. These results suggest that MKK4 but not MKK7 serves as an upstream kinase of JNK during Cd-induced neuronal cell apoptosis.

ASK1 mediates Cd-induced neuronal cell apoptosis

ASK1 was identified as a MKK kinase which in turn activates the JNK or p38 signaling cascade [18]. Previ-

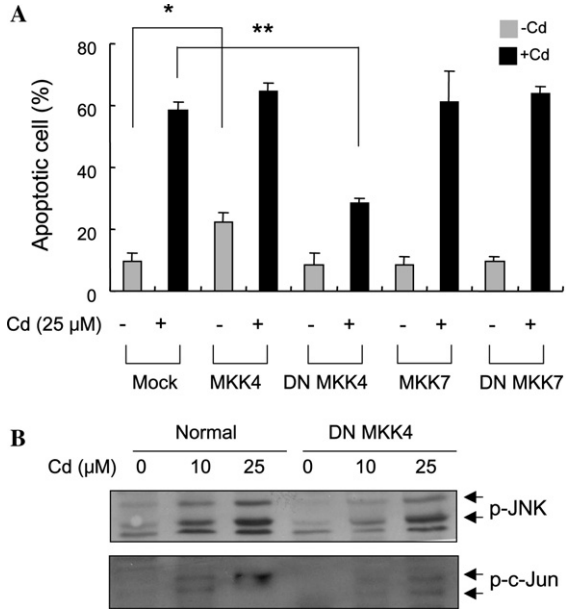

Fig. 4. MKK4, but not MKK7, mediates Cd-induced neuronal cell apoptosis. (A) SH-SY5Y cells transiently transfected with a mock, wild, or dominant negative construct of MKK4 (DN MKK4; K129R) and MKK7 (DN MKK7; K149A) were treated with 25 μM Cd for 12 h, and analyzed for apoptotic cell death by the DAPI staining. Data represent the average ± SD of the percent apoptotic cells relative to the total GFP-positive cells (100%) from two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) Cells stably transfected with DN MKK4 were treated with 10 or 25 μM Cd for 12 h. The total proteins were extracted and separated by SDS–PAGE, and the Western blot analysis was performed using anti-phospho-JNK or phospho-c-Jun antibody.

ous studies demonstrated that overexpression of wild-type or constitutively active ASK1 induced apoptosis while kinase-inactive mutant of ASK1 inhibited apoptosis induced by various stresses including TNF and anandamide [27,28]. To examine the possible role of ASK1 as an upstream MAP kinase in regulating JNK and c-Jun activation, and Cd-induced apoptosis, cells transiently transfected with a dominant negative construct of ASK1 (K709M; DN ASK1) were treated with 25 μM Cd for 12 h. As shown in Fig. 5A, both JNK and c-Jun phosphorylation induced by Cd treatment reduced significantly by transient transfection with DN ASK1. Overexpression of wild ASK1 construct alone induced significant increase (15.1 ± 3.0%) of apoptosis compared with mock construct and enhanced apoptosis further following Cd treatment (59.9 ± 3.0%), confirming that ASK1 functions as an apoptosis-inducer (Fig. 5B). Consistent with this observation, overexpression of DN ASK1 blocked efficiently apoptosis caused by Cd (47.3 ± 3.4%, mock; 20.3 ± 4.8%, mutant). These data suggest that ASK1 mediates Cd-induced apoptosis by possibly phosphorylating downstream activators, JNK and c-Jun.

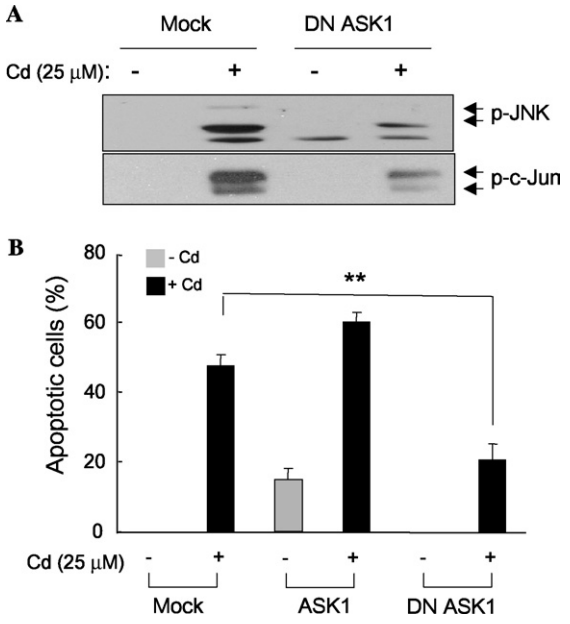

Fig. 5. ASK1 mediates Cd-induced neuronal cell apoptosis. (A) SH-SY5Y cells transiently transfected with a mock or dominant negative construct of ASK1 were treated with 25 μM Cd for 12 h. Then, the cells were extracted and separated by SDS–PAGE, and the Western blot analysis was performed with anti-phospho-JNK or phospho-c-Jun antibody. (B) Cells were transiently transfected with a mock, wild or dominant negative construct of ASK1 together with pEGFP. At 24 h after transfection, the cells were treated with 25 μM Cd for 12 h, fixed with 4% PFA, and stained with 10 μg/ml DAPI. Data represent the average ± SD of the percent apoptotic cells relative to the total GFP-positive cells (100%) from three independent experiments. $^{**}p < 0.01$.

NAC blocks Cd-induced JNK and c-Jun phosphorylation as well as neuronal cell apoptosis

Cd has been shown to induce oxidative stress [1,2]. To investigate a possible relationship between the JNK pathway and oxidative stress induced by Cd, we examined the effect of NAC, a free radical scavenger, on Cd-induced JNK and c-Jun phosphorylation and cell death. As shown in Fig. 6, NAC at the 1 and 5 mM effectively inhibited Cd-induced cell death, reducing cell death from 48.9 ± 1.8% (untreated control) to 25.6 ± 0.1% and 7.6 ± 1.6%, respectively (Fig. 6A). NAC also blocked significantly the Cd-induced JNK and c-Jun phosphorylation in a dose-dependent manner (Fig. 6B). These results suggested that ROS generated by Cd plays an important role in inducing neuronal cell death probably by regulating the activity of JNK and c-Jun.

Cd-induced neuronal cell apoptosis is dependent on caspase activation which is controlled by JNK/c-Jun phosphorylation

It is known that apoptosis requires a sequential activation of many caspases. Furthermore, previous data showed that caspases are involved in MAPK-mediated apoptosis caused by stress-inducing agents. Thus, we examined a possible association between caspases and the JNK/c-Jun signaling cascade during Cd-mediated apoptosis. Preincubation of cells for 2 h with a broad

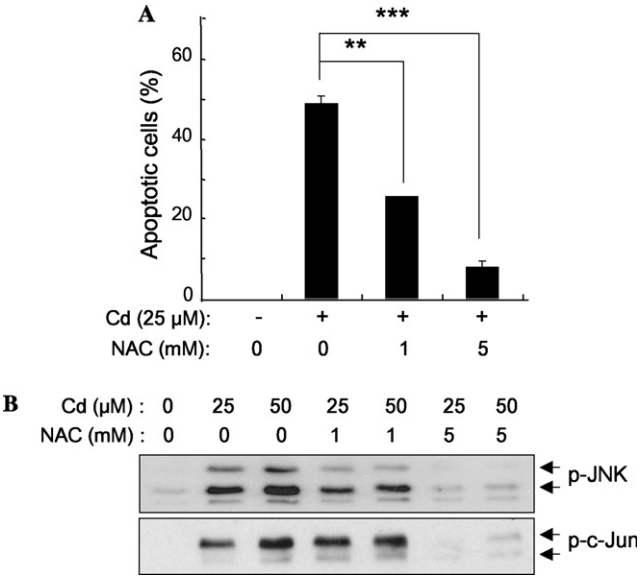

Fig. 6. NAC blocks Cd-induced phosphorylation of JNK and c-Jun as well as neuronal cell apoptosis. SH-SY5Y cells were pretreated with 1 or 5 mM *N*-acetyl-L-cysteine (NAC) for 1 h and then treated with 25 or 50 μM Cd for 12 h. Apoptosis was assayed by the DAPI staining (A) and phosphorylation of JNK and c-Jun was analyzed by the Western blot analysis (B). Data represent the average ± SD of the percent apoptotic cells relative to the total GFP-positive cells (100%) from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$.

spectrum caspase inhibitor, zVAD-fmk (100 μM) and a caspase-3-specific inhibitor, zDEVD-fmk (100 μM), significantly suppressed cell death induced by Cd (25 μM, for 12 h) by 31.9% and 37.9%, respectively (52.0 ± 4.9%, Cd-treated; 35.4 ± 1.8%, zVAD; and 32.3 ± 0.7%, zDEVD). Higher dose (200 μM) of zDEVD rescued cells further by 60.9%. This result suggested that caspases including caspase-3 are likely to be involved in apoptosis induced by Cd in SH-SY5Y cells. Pretreatment with caspase inhibitors did not block phosphorylation of JNK and c-Jun (Fig. 7B). In contrast, Cd induced caspase-3 activation as assessed by cleavage of procaspase-3, and transient transfection with DN JNK1 inhibited Cd-induced caspase-3 activation. These findings suggest that Cd-induced apoptosis is dependent on caspase activation, and JNK and c-Jun lie upstream of caspase protease in a cascade.

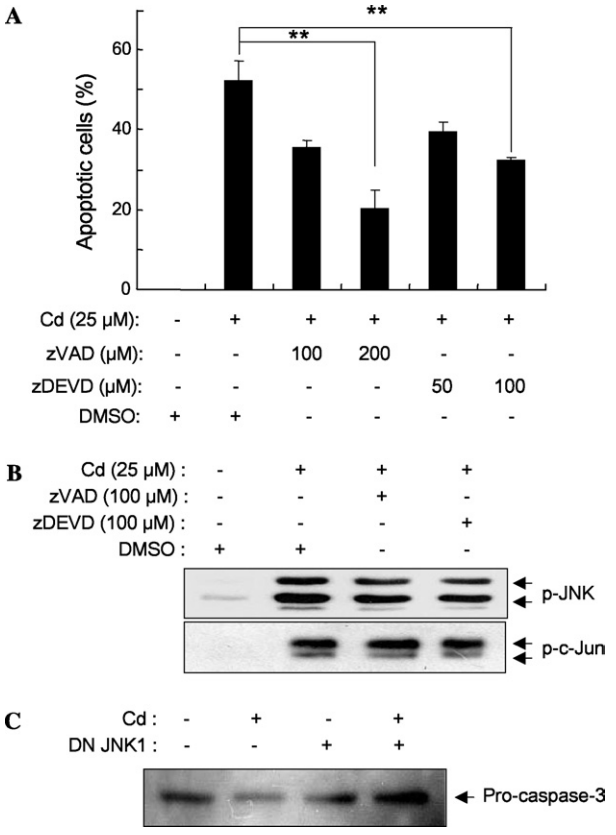

Fig. 7. Cd-induced caspase-dependent apoptosis and caspase activation is downstream of JNK phosphorylation.SH-SY5Y cells were pretreated with a broad spectrum caspase inhibitor, zVAD-fmk (100 μM), or a caspase-3-specific inhibitor, zDEVD-fmk (100 μM), for 2 h and then incubated with 25 μM Cd for 12 h. Apoptotic cell death and phosphorylation of JNK and c-Jun were assayed by the DAPI staining (A) and Western blot analysis (B), respectively. Data represent the average ± SD from three independent experiments. $^{**}p < 0.01$. (C) SH-SY5Y cells transiently transfected with DN JNK1 were treated with 25 μM Cd for 12 h and then subjected to Western blot analysis using anti-procaspase-3 antibody. Reduced band intensity refers caspase activation.

Discussion

Cd-induced apoptosis was described previously in various cells including neuronal cell [5,15,23,29] but its underlying molecular mechanism was not clearly elucidated. In the present study, we used SH-SY5Y cell, a human neuroblastoma cell line, in order to examine the intracellular signaling pathways involved in Cd-induced neuronal cell apoptosis. We found that Cd induced apoptosis as determined by TUNEL assay and DAPI staining, and that the apoptosis occurs through activation of the ASK-1/MKK4/JNK/c-Jun signaling pathway and their downstream molecule, caspase-3.

Three distinct MAP kinases, ERK, p38, and JNK, have been characterized and reported to be involved in apoptosis in many different paradigms of cellular toxicity. Although MAP kinases play an important role in apoptosis, the role they play in Cd-induced apoptosis is not clear and seems to be different depending on cell types and environmental conditions. It has been reported that JNK is involved in apoptosis of lung carcinoma cells [23] and murine macrophage cells [30] while it is not associated with apoptosis in HT4 neuronal cells [15] in which p38 plays a major role. Thus, the JNK function in apoptosis was controversial. From this study, we found that Cd activated JNK and inhibition of JNK activity by a JNK inhibitor, SP600125, or overexpression of DN MKK4, a direct upstream kinase of JNK, reversed Cd-induced apoptosis. In contrast, SB 203580, a p38 kinase-specific inhibitor, and U0126, an ERK-specific inhibitor, had no effect on Cd-induced apoptosis. These results suggested that JNK mediates apoptosis induced by Cd. Although stress stimuli usually activated p38 and this activation caused apoptosis in some cellular damage models, it seemed to be irrelevant in others. p38 activation was required for apoptosis induced by Cd, trophic factor withdrawal, and ischemia [29,31,32] while in contrast, inability of SB203580 to prevent apoptosis induced by UV and *S*-nitrosoglutathione [33,34] proves the weak correlation of p38 with apoptosis. Similar contradictory results were obtained in neuronal cell. In a recent study with HT4 cell, inhibition of p38, but not JNK, blocked cell death induced by Cd [15], while in our study with the SH-SY5Y cell, JNK inhibition, but not p38, did block Cd-induced apoptosis (Fig. 2). Such a discrepancy suggests the existence of marked differences in regulation of the stress responses depending on cell type, albeit it is difficult to explain about them at the moment. Whether JNK or p38 is implicated with apoptosis could also depend on cellular insult in a cell type. One recent paper suggested that mechanistically different forms of glutamate- (caspase-independent) and trophic factor withdrawal (caspase-dependent)-induced cell death required p38 and JNK activation, respectively [35], suggesting close association of JNK with caspase-dependent apoptosis. The involve-

ment of JNK in Cd-induced apoptosis was further confirmed by the activation of c-Jun, a well-known downstream activator of JNK.

In addition to a variety of growth factors, cytokines, and mitogens, ERKs are also known to be activated by cytotoxic insults. Cellular ERK activation either inhibits or causes no effect on apoptosis in some cells [23,29], but enhances apoptosis in other cells [20,36]. Even in the same cell, role of ERK activation is likely to be different depending on cellular insults. In SH-SY5Y neuronal cells, for example, hydrogen peroxide induced apoptosis which was inhibited by pretreatment with an ERK inhibitor [20], while Cd caused no changes in ERK activation in our study, suggesting that different cytotoxic insults are likely to be linked to different signaling molecules.

Both MKK4 and MKK7 are activators of JNK while MKK7 is specific to JNK [26]. In brain, MKK4 are much more highly expressed than MKK7 [26]. Although expression of MKK4 and MKK7 in SH-SY5Y cells has not been examined, overexpression of DN MKK4 blocked significantly both JNK and c-Jun phosphorylation and apoptosis while that of DN MKK7 did not (Fig. 4). Thus, Cd-induced neurotoxicity occurs at least in part through activation of MKK4 and is likely to be independent of MKK7 in neuronal cells. Such specificity in MAPKK activation by various stimuli has been reported in other cases. Tumor necrosis factor-α and low potassium activated MKK7 more than MKK4 while anisomycin and UV activated MKK4 more than MKK7 [13,37].

Among MAPKKKs, ASK1 is known to be activated in response to various cytotoxic stresses including ROS and induce both JNK and p38 activation and apoptosis [27]. Expectedly, the results demonstrated that DN ASK1 not only rescued the cadmium-induced apoptosis (Fig. 5) but also reduced JNK and c-Jun phosphorylation induced by Cd. Thus, ASK1 seems to be a key molecule in Cd-induced apoptosis, albeit contribution of other MAPKKKs should be studied elsewhere. Overall, ASK1/MKK4/JNK and c-Jun play a critical role in signaling pathways involved in Cd-induced neuronal apoptosis in human neuronal cells.

The mechanism of Cd toxicity involves the generation of oxidative stress. Cadmium generates hydrogen peroxide [30] and free radical scavenger or antioxidant such as NAC and TROLOX clearly reduced Cd-induced toxicity [38]. NAC almost completely abolished Cd-induced JNK and c-Jun activation as well as apoptosis shown in the present study (Fig. 6), suggesting that free radicals are involved in its effect and are the major mediator for the Cd-induced cell death. In addition, the present study demonstrated that generation of ROS occurs upstream of JNK and c-Jun activation since NAC abolished phosphorylation of these signaling molecules.

One well-known response involved in apoptosis is the caspase activation. As seen in Fig. 7 Cd activates caspase-3 and a caspase-3 inhibitor, DEVD, blocked apoptosis induced by Cd, suggesting that Cd caused caspase-dependent apoptosis in SH-SY5Y neuronal cells. In contrast, Cd is likely to induce caspase-independent apoptosis in other neuronal and Hep3B liver cells which is associated with nuclear translocation of endonuclease G and apoptosis-inducing factor [15,39]. Prior studies placed JNK/c-Jun activation upstream of mitochondrial cytochrome *c* release which leads to caspase activation [40]; the observations that the caspase inhibitors did not block JNK/c-Jun activation and that DN JNK did caspase activation are consistent with such findings.

In summary, the present study identified JNK as a key molecule involved in Cd-induced neurotoxicity and demonstrated the detailed MAPK signaling pathway in which sequential activation of ASK1/MEKK/JNK and c-Jun plays an important role. ROS generated by Cd treatment seemed to serve as an upstream regulator of these kinase cascade while caspase served as a downstream regulator. Role of JNK and p38 in Cd-induced apoptosis is likely to be different due to cell lines and environment.

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