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Research Article

Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway

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Abstract. The cytotoxicity of cadmium (Cd) induced autophagy and apoptosis in MES-13 cells was determined by flow cytometry. Autophagy was also assessed by formation of autophagosomes and processing of LC3. Pharmacological inhibition of autophagy resulted in increased of cell viability, suggesting autophagy plays a role in cell death in Cd-treated mesangial cells. Cd also induced a rapid elevation in cytosolic calcium ($[Ca^{2+}]_i$), and modulation of $[Ca^{2+}]_i$ via treatment with IP_3R inhibitor or knockdown of calcineurin resulted in a change in the proportion of cell death, suggesting that the release of calcium from the ER plays a crucial role in Cd-induced cell death. Inhibition of Cd-induced ERK activation by PD 98059 suppressed Cd-induced autophagy, and BAPTA-AM eliminated activation of ERK. BAPTA-AM also inhibited Cd-induced mitochondrial depolarization and activation of caspases. These findings demonstrated that Cd induces both autophagy and apoptosis through elevation of $[Ca^{2+}]_i$, followed by Ca^{2+}-ERK and Ca^{2+}-mitochondria-caspase signaling pathways.

Keywords. Autophagy, apoptosis, cadmium, calcium, calcineurin, ERK, mitochondria.

Introduction

Cell death is commonly subdivided into three categories: apoptosis (type I), autophagic cell death (type II), and necrosis (type III) [1]. Apoptosis – typical cell death with morphological and biochemical characteristics such as mitochondrial depolarization, chromatin condensation, and nucleosomal ladder formation – is mediated by the activation of caspase and other factors released from mitochondria. The molecular mechanism of apoptosis has been extensively studied, but the role and mechanism of autophagy are still obscure. Autophagy, an evolutionarily conserved

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mechanism for sequestering and degrading long-lived cellular proteins and damaged organelles, is important in normal development and as a response to changing environmental stimuli. Its alteration may result in cancer development, bacterial and viral infections, neurodegenerative disorders, and cardiovascular diseases [2, 3]. The formation of double membrane vesicles, called autophagosomes, is the main characteristic of autophagy, which requires the two ubiquitin-like conjugation systems (Atg-Atg12 and Atg8 ligation) and activation of class III phosphatidylinositol-3-kinase [4, 5]. Knowledge of the regulation of autophagy is increasing, and many signaling pathways – including the target of rapamycin (TOR), phosphatidylinositol-3-kinase-I (PI3K-I)/protein kinase B (PKB), GTPase, calcium, and mitogen-activated protein kinases (MAPKs) – all play important roles in it [6, 7].

The role of autophagy in cell death is controversial. Autophagy reportedly plays a protective role in allowing cells to survive during nutrient deprivation, and cells undergo apoptosis when autophagy is inhibited [8, 9]. However, the morphological features of autophagy have also been observed in dying cells in which caspases are suppressed or insufficiently activated [10, 11]. A more recent report demonstrated that oxidative stress-induced autophagic cell death is independent of apoptosis [12], indicating that autophagy and apoptosis may interact or occur independently of each other. Therefore, the interplay between autophagy and apoptosis is unclear and the scenario of autophagy in cell fate is controversial.

Calcium is a universal messenger regulating many physiological and pathological functions, such as secretion, contraction, metabolism, gene transcription, and cell death [13, 14]. Temporally and spatially organized elevation of calcium in the cytosol, mitochondria, and nucleus is one of the most commonly recognized intracellular signals [15]. However, prolonged changes in calcium distribution in cells can trigger various cascades that lead to cell death. Endoplasmic reticulum (ER) is the major calcium storage unit in the cell. A recent report demonstrated that accumulation of calcium in the mitochondria and apoptosis induced by ER stress can be reduced by pretreatment with an inhibitor of the calcium channel of the ER (inositol-1,4,5-trisphosphate receptor, IP_3 R) [16], implicating the release of calcium from the ER as possibly leading to cell death. Moreover, it was reported that the release of calcium from ER can be inhibited by dephosphorylation of IP_3R through activation of calcineurin, a calcium-dependent serine/threonine phosphatase [17], suggesting that calcineurin may be a protective factor in calcium-mediated cell death. Furthermore, recent evidence has shown that cells undergo disruption of calcium homeostasis and BAX/BAK-mediated apoptosis after photodynamic therapy, but undergo autophagic cell death if BAX/BAK are knocked out [18]. Therefore, variations in the concentration of calcium in cells seem to play an important role in regulating apoptosis and autophagy.

Cadmium, an environmental pollutant with high cytotoxicity, accumulates in the liver and kidney after exposure through routes such as oral ingestion or inhalation. Previous publications have shown that environmental Cd exposure results in renal dysfunction [19–21] and an increased urinary excretion of low molecular weight proteins [21], suggesting glomeruli and mesangial cells may be significant targets of Cd-related renal dysfunction. It has been proven that Cd-induced nephrotoxicity may be due to contraction of mesangial cells in response to Cd [22]. Recently, Liu and Templeton [23] demonstrated that Cd-induced apoptosis in mesangial cells occurs through a calcium-dependent pathway. Furthermore, cell death induced by Cd occurs through a caspase-dependent pathway accompanied by the release of cytochrome *c* [24, 25] or through a caspase-independent cell death accompanied by reactive oxygen species (ROS) bursts and translocation of the apoptosis-inducing factor (AIF) [26, 27]. However, the role of autophagy in this Cd-mediated cell death is unclear.

In the present study, we investigate the cytotoxicity of Cd toward MES-13 mesangial cells. We have shown that Cd induces an increase of $[Ca^{2+}]_i$ followed by extracellular signal-regulated kinase (ERK) activation and mitochondrial depolarization, and then triggers autophagy and apoptosis. Our results provide molecular evidence to demonstrate a novel finding that Cd induces two types of cell death, including calcium-ERK-mediated autophagy and calcium-mitochondria-caspase-mediated apoptosis.

Materials and Methods

Cell culture, treatment, and chemicals. Mesangial cells (MES-13) obtained from American Tissue Culture Collection (ATCC, CRL-1927, Manassas, VA) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) and F-12 (3:1) complex medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (pH 7.4) in a humidified atmosphere containing 5% CO_2. Mesangial cells (at 50% confluence) were treated with 6 μM of $CdCl_2$ for the indicated time periods. All experiments in this study were done in complete medium unless otherwise indicated. DMEM, FBS, penicillin, and strepto-

mycin were purchased from HyClone (Logan, UT). Cadmium chloride, bovine serum albumin (BSA), acridine orange, 1,2-bis(2-amino-phenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA-AM), 3-methyladenine (3-MA), 5,5',6,6',-tetrachloro-1,1',3,3',-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), Fluo-3 AM, PD 98059, U0126, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical (St. Louis, MO). 2-aminoethoxydiphenyl borate (2-APB) was from Calbiochem (San Diego, CA). Rabbit monoclonal anti-p-p42/44 ERK (clone E-8), rabbit polyclonal anti-p42/44 ERK, anti-PARP, and anti-caspase 3 and 9 were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-microtubule-associated protein 1 light chain 3 (LC3) was from MBL International (Nagoya, Japan). Mouse monoclonal anti-GAPDH was from Chemicon International (Temecula, CA). Annexin-V-FITC reagent and n-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were supplied by Biovision (Mountain View, CA). The secondary antibodies, including horseradish peroxidase (HRP)-conjugated goat anti-mouse and -rabbit immunoglobulin G (IgG), were from Pierce (Rockford, IL) and Jackson ImmunoResearch Laboratories (West Pine, PA), respectively. The polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). The Protein Assay Dye Reagent was from Bio-Rad Laboratories (Hercules, CA).

Measurement of cell viability. Cell viability was measured by the MTT assay, which is based on the conversion of the tetrazolium salt to the colored product, formazan. In brief, 10 μl MTT solution (5.5 mg/ml in PBS) was added into each well of the 96-well plate (containing 100 μl medium and cells) 4 h before the end of incubation. The supernatant was then discarded, and 100 μl DMSO was added to dissolve the formazan. The absorbance was measured at 550 nm using a Thermo Varioskan Flash Reader (Thermo Electron Corporation, France).

Measurement of apoptosis. Apoptosis was analyzed by detecting phosphatidylserine externalization using flow cytometry with a two-color analysis of FITC-labeled annexin V/PI double-staining according to a previous publication [26]. In brief, the trypsinized adhered cells and the suspended cells in medium were collected in HEPES buffer containing 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Subsequently, cells were stained with annexin V (1 μg/ml)/PI (0.2 ng/ml) for 15 min and were then analyzed by flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA). The percentage of apoptosis was summed up from primary apoptosis (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+).

Measurement of acidic vesicular organelles (AVOs). The percentage of autophagic cell death was analyzed using flow cytometry with acridine orange dyeing according to published procedures [28, 29]. After Cd treatment, cells were stained with acridine orange (1 μg/ml) for a period of 20 min. The trypsinized adhered cells and the suspended cells in medium were collected in phenol red-free growth medium (GIBCO). The fluorescence emission of green (510~530 nm) and red (650 nm) from 1 X 10^4 cells was measured with a flow cytometer using CellQuest software. The percentage of autophagy was summed up from upper-left and upper-right quadrants.

Measurement of the mitochondrial membrane potential (ΔΨm). JC-1, a lipophilic cationic fluorescence dye with the ability to enter mitochondria was used as a measure of ΔΨm according to our previous report [30]. After treatment, cells were incubated with JC-1 (5 μg/ml) dissolved in DMSO for 30 min at 37°C. The trypsinized adhered cells and the suspended cells in medium were then collected in 0.5 ml PBS and analyzed on a flow cytometer. The percentage of cells with depolarized mitochondria was the sum of the upper-right and down-right quadrants.

Measurement of intracellular calcium. After treatment with or without Cd, the mesangial cells were harvested and incubated with 500 nM Fluo-3 AM dye for a total of 30 min at 37°C and then immediately analyzed on a flow cytometer using FL-1 as a detector. The relative intracellular calcium concentrations were calculated from the ratio of the geographic mean values of the FL-1 peak generated from Cd-treated cells over each respective control as indicated in the figure legend.

Electron microscopy. To morphologically observe the induction of autophagy in Cd-treated mesangial cells, we performed an ultrastructural analysis according to published procedures [28]. After being treated with 6 μM Cd for 24 h, cells were washed twice with PBS and fixed with ice-cold glutaraldehyde (3% in 0.1 M cacodylate buffer, pH 7.4) for 30 min. Cells were post-fixed in osmium tetroxide and embedded in Epon, before being cut and stained with uranyl acetate/lead citrate (Fluka, Chemie AG, Buchs, Switzerland) and viewed with a Hitachi H600 electron microscope (Hitachi Instrument, Tokyo, Japan).

Western blot analysis. Cells were scraped and lysed with 50 μl of lysis buffer containing 25 mM HEPES,

1.5% Triton X-100, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 0.1 mM sodium deoxycholate, and a protease inhibitor cocktail (Roche, Boehringer Mannheim, Germany) [31]. After that, sampling buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, and 140 mM β-mercaptoethanol) was added to each lysate, and subsequently boiled for 7 min and centrifuged at 15,000 *g* for 5 min. The protein amount was determined using Bio-Rad Protein Assay Dye Reagent. Proteins electrotransferred onto PVDF membranes were immunoblotted with anti-p-p42/44 ERK (1:500 dilution), anti-p42/44 ERK (1:4,000 dilution), anti-LC3 (1:1,000 dilution), anti-caspase 3 (1:1,000 dilution) and anti-caspase 9 (1:1,000 dilution), anti-PARP (1: 500), or anti-GAPDH (1:10,000 dilution) antibodies. Detection was performed with appropriate HRP-conjugated secondary antibodies (1:20,000 dilution) and enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). The band density was quantified with Gel-Pro Analyzer densitometry software (Media Cybernetics).

Knockdown of calcineurin. According to the manufacturer's instructions, mesangial cells were transfected with 20 nM siRNA targeted against calcineurin (5'-AACCUCGUGUGGAU AUCUUdTdT-3; 5'-AACAAGAUCCGAGCAAUAGdTdT-3') [32] or control siRNA (Cat. No. 12935–200, from Invitrogen) using lipofectamine^{TM} RNAimax reagent (Invitrogen, San Diego, CA). After transfection, the medium was refreshed. Transfected cells were incubated for 48 h, followed by treatment with Cd and then analyzed by flow cytometry with acridine orange and annexin V/PI double-staining.

Statistical analysis. Values were expressed as the mean ± standard deviation (SD) and were calculated either by Student's *t*-test (for two groups) or one-way ANOVA followed by Duncan's multiple-range test (for three or more groups). A value of $p<0.05$ was considered statistically significant.

Results

Cd triggers autophagy and apoptosis in mesangial cells. Apoptosis and autophagy are, respectively, considered to be type I and II cell death. To investigate the types of cell death induced by Cd, acridine orange and annexin V/PI double-staining were employed with a flow cytometer using mesangial cells exposed to various concentrations of Cd. As revealed in Figure 1(A, upper panel of C), we observed that the percentage of AVO was increased to 50.5% at a concentration of 12 μM Cd. Nevertheless, if mesangial cells were treated with 24 μM of Cd, the ratio of AVO was decreased as the total apoptosis was increased to 35.3% and 67.8%, respectively (Fig. 1A, B, upper panel of C). Here, using MTT assay, we also observed cell viability was decreased as dose- and time-dependent manners after treatment with Cd (lower panel of Figure 1C, D). These results suggested that Cd-induced cytotoxicity in mesangial cells might be exerted through autophagy and apoptosis. Furthermore, autophagy was the predominant type of cell death in the mesangial cells exposed to less than 12 μM Cd. In this study, we also observed that mesangial cells underwent autophagy after treatment with serum starvation for 28 h, but without a corresponding increase in apoptosis (Fig. 1A, B, upper panel of C). Therefore, autophagy and apoptosis do not always occur simultaneously in mesangial cells. To observe the effects of exposure time on cell death, mesangial cells were treated with 6 μM Cd for various time periods as indicated in upper panel of Figure 1D. After treatment with Cd, the percentage of AVO and phosphatidylserine externalization increased by 39.2% and 18.2%, respectively, at 28 h. Next, we tested whether treatment with Cd caused processing of full-length LC3-I to LC3-II, a hallmark of autophagy. To do this we used immunoblot analysis to detect the extracted lysates from mesangial cells treated with or without Cd for 8, 16, 24, and 28 h. As shown in Figure 1E, we observed that the levels of LC3-II proteins increased after treatment with Cd. Furthermore, to confirm the occurrence of autophagy, we examined the formation of autophagosomes after exposure to Cd for 24 h. As revealed by transmission electron microscopy, the untreated mesangial cells exhibited large nuclei, surrounded by cytoplasm with healthy-looking mitochondria (Fig. 1F). After treatment with Cd for 24 h, we found that the cytoplasm was full of autophagosomes containing visible cytoplasmic contents (Fig. 1F, as indicated by arrows). To further ascertain the role of autophagy in Cd-induced cytotoxicity, 3-MA, an autophagy inhibitor, was employed to detect its effect on Cd-induced cell death. As revealed in Figure 2A, the percentage of autophagy was decreased from 36.3 to 21.3%. There was, however, no significant effect on apoptosis by 3-MA (Fig. 2A), indicating that autophagy does not serve as a protective scenario for apoptosis in Cd-induced cell death. Furthermore, using a broad caspase spectrum inhibitor, Z-VAD-fmk, we sought to test the role of the caspase cascade in Cd-induced apoptosis. We observed that after 28 h of Cd exposure, the percentage of apoptosis was effectively reduced by Z-VAD-fmk, but there was only a minor effect ($p>0.05$) on autophagy (Fig. 2B). Using MTT assay to monitor the effects of 3-MA on cell viability, we

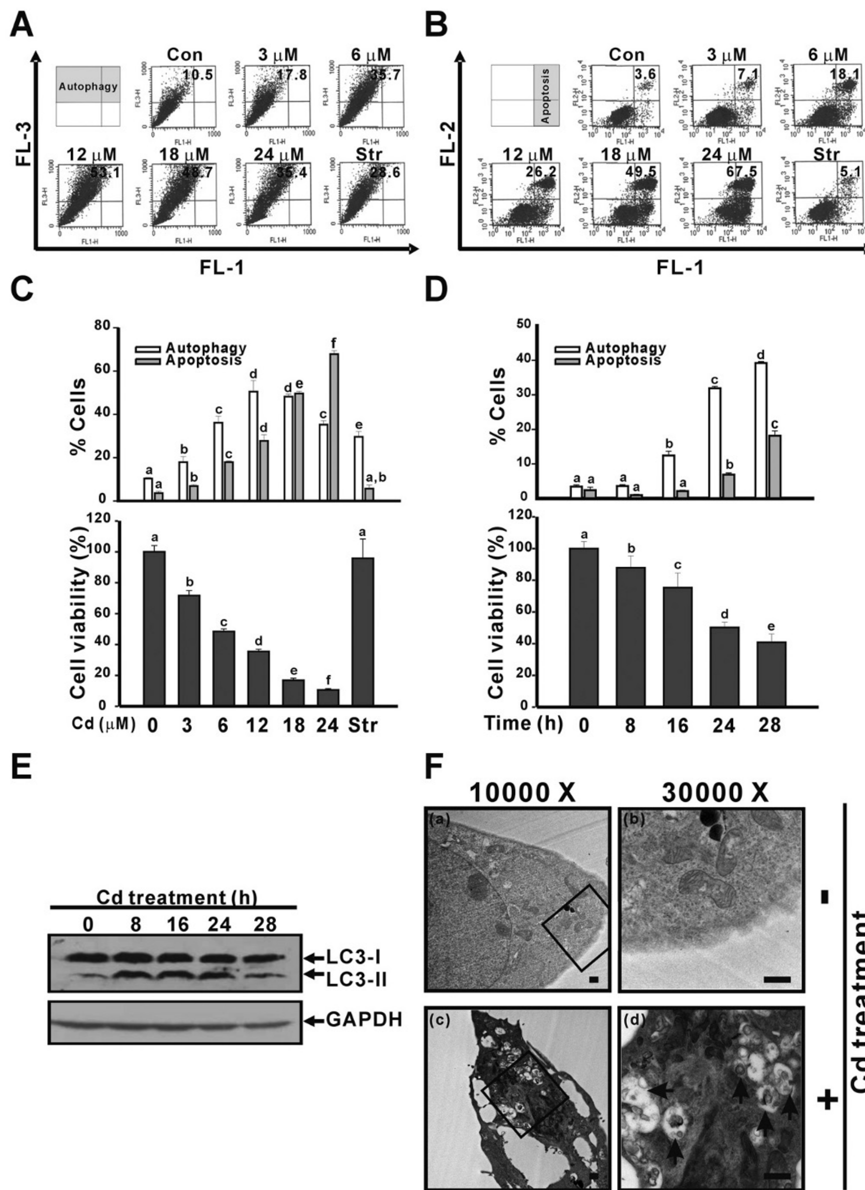

Figure 1. Autophagy and apoptosis are induced by Cd in mesangial cells. Dose response of Cd-induced autophagy and apoptosis. Cells were treated with indicated concentration of Cd for 28 h and then autophagy and apoptosis were analyzed on a flow cytometer using acridine orange (*A*) and annexin V/PI staining methods (*B*), respectively. Data presented in panel A and B are representative of three independent experiments, and their statistical results are presented in the upper panel of *C*. Con, control. Str, cells were starved with 0.1 % serum for 28 h to induce autophagy, which serves as a positive control for autophagy. For the upper panel of *D*, cells were treated with 6 μM Cd for the indicated periods of time and then analyzed by acridine orange staining and annexin V/PI double-staining using flow cytometry for autophagy and apoptosis, respectively. The cell viability of dose (lower panel of *C*) and time-course (lower panel of *D*) after treatment with Cd were determined by the MTT assay (n=6). (*E*) Cells were incubated with Cd for the indicated times. Cell lysates were analyzed using Western blotting with anti-LC3 and -GAPDH antibodies. GAPDH was used as an internal control to normalize the amount of proteins applied in each lane. (*F*) Mesangial cells treated with or without Cd for 24 h were analyzed on a transmission electron microscope. Panel b and d are enlargements of the enclosed area in panel a and c, respectively. Autophagosomes containing cytoplasmic contents were indicated by arrows. Bars, 500 nm. Significant difference compared with the respective control was evaluated using one-way ANOVA followed by Duncan's multiple-range test. Columns not sharing the same superscript differ significantly ($p<0.05$).

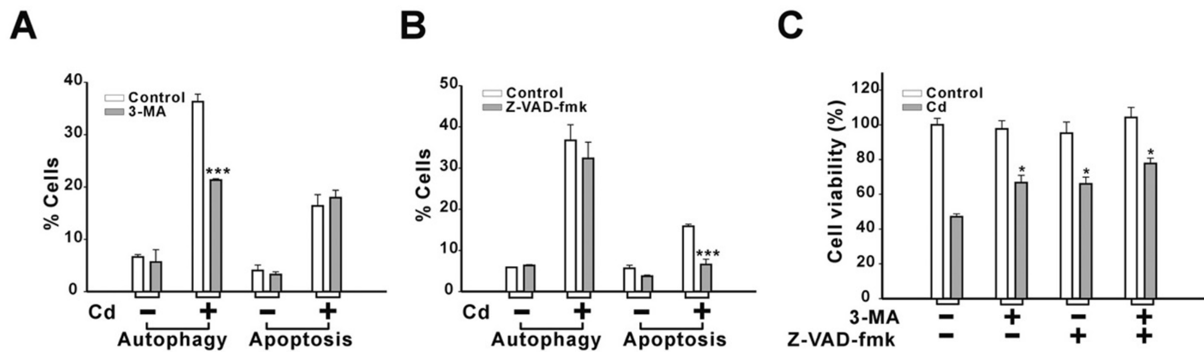

Figure 2. Suppression of autophagy increases cell viability. (*A*) Cells were pretreated with 2 mM 3-MA, an inhibitor of autophagy, for 1 h, followed by treatment with Cd for another 28 h to determine autophagy and apoptosis. ***$p<0.001$ *vs* the respective control, student's *t*-test. (*B*) Cd-induced apoptosis occurs through a caspase-dependent pathway. Cells were pretreated with 100 μM Z-VAD-fmk, a pan-inhibitor of caspase, for 1 h, followed by treatment with Cd for another 28 h to determine the extent of autophagy and apoptosis. ***$p<0.001$ *vs* the respective control; student's *t*-test. (*C*) Mesangial cells were pretreated with 3-MA or Z-VAD-fmk for 1 h, followed by treatment with Cd for 28 h to determine cell viability using MTT assay. *$p<0.05$, significantly different compared with the group of Cd treatment only by one-way ANOVA followed by Duncan's multiple-range test, n=4.

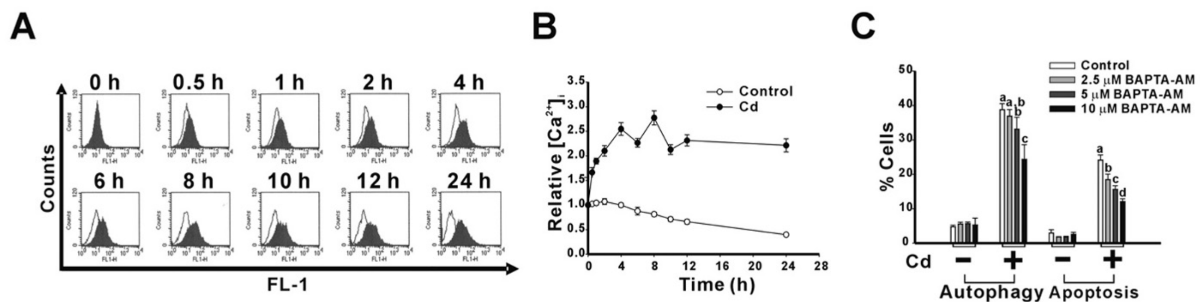

Figure 3. Cd-induced autophagy and apoptosis arise through calcium-mediated signaling. Cells were treated with Cd for the indicated time periods and incubated with 0.5 μM Fluo-3 AM dye for a total of 30 min before analysis by flow cytometry. Data presented in panel *A* is a representative of three independent experiments, and their statistical results are presented in panel *B*. The cytosolic calcium was elevated significantly ($p < 0.001$, *vs* the control, student's *t*-test) after treatment with Cd from 0.5 to 24 h. (*C*) Cd-induced apoptosis and autophagy were suppressed by cytosolic calcium chelator. Cells were pretreated with various concentrations of BAPTA-AM, a chelator of cytosolic calcium, for 1 h, followed by treatment with Cd for another 28 h to determine autophagy and apoptosis. Significant difference compared with the respective control was evaluated using one-way ANOVA followed by Duncan's multiple-range test. Columns not sharing the same superscript differ significantly ($p<0.05$).

observed that the suppression of cell viability resulting from treatment with Cd was reversed from 47.2 % to 66.7 % (Fig. 2C), suggesting that Cd induces an autophagic cell death. The recovery of cell viability was also observed after treatment with Z-VAD-fmk (Fig. 2C). Furthermore, co-treatment with Z-VAD-fmk and 3-MA has mild additive effects on cell viability (Fig. 2C). Collectively, Cd induces two types of cell death in mesangial cells, including both autophagy and apoptosis.

Cd induces calcium-dependent cell death. Variations in cytosolic calcium concentration have been demonstrated to participate in Cd-induced apoptosis in different cell lines [30, 33–36], but the role of calcium in autophagy is still unclear. To confirm the variation in calcium after treatment with Cd in mesangial cells, $[Ca^{2+}]_i$ was measured over time using flow cytometry with a calcium indicator dye, Fluo-3 AM. In Figure 3A, B, $[Ca^{2+}]_i$ was elevated after treatment with Cd for 30 min, climbing 2.8 fold after 8 h. An 8 h-time point was used in the following experiments to detect the effect of indicated treatment on calcium since $[Ca^{2+}]_i$ was elevated to the maximal level at this time point. Next, to evaluate the effects of variations in cytoplasmic calcium on Cd-induced cell death, we detected the percentages of apoptosis and autophagy after exposure to Cd with or without various concentrations of BAPTA-AM, a chelator of cytosolic calcium. As shown in Figure 3C, pretreatment with 10 μM BAPTA-AM before exposure to Cd resulted in markedly reduced percentages of autophagy and apoptosis, from 38.8 % and 24.1 % to 24.3 % and 12.1 %, respectively, suggesting

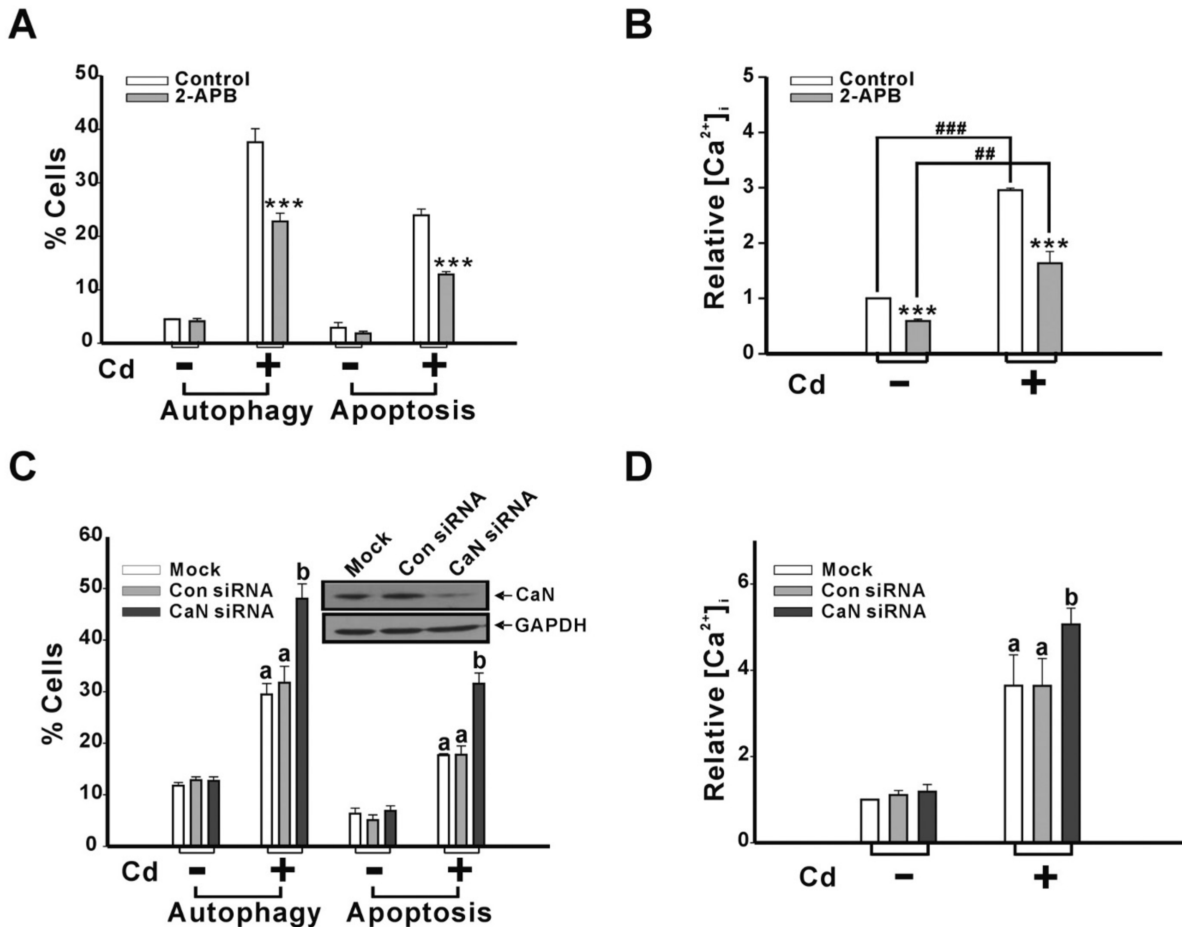

Figure 4. Calcium released from ER through IP_3R channels is a major cause of Cd-induced autophagy and apoptosis. (*A*) Cells were pretreated with 60 μM 2-APB, an inhibitor of IP_3R, for 1 h, followed by treatment with Cd for 28 h to determine autophagy and apoptosis. (*B*) Cd-induced elevation of cytosolic calcium was diminished by 2-APB. Cells were pretreated with 2-APB for 1 h, followed by treatment with Cd for another 8 h. The variation of cytosolic calcium was analyzed by Fluo-3 AM staining using flow cytometry. ***$p<0.001$ *vs* the respective control; ##$p<0.01$, ###$p<0.001$ *vs* the indicated group, student's *t*-test. (*C*) Promotion of Cd-induced cell death by silencing the calcineurin gene. Cells were transfected with control or calcineurin siRNA for 48 h, followed by treatment with Cd for 28 h to determine autophagy and apoptosis. The inset of panel *C* indicated a parallel immunoblot assay using anti-calcineurin and -GAPDH antibodies to monitor the efficiency of siRNA. GAPDH was used as an internal control to normalize the amount of proteins applied in each lane. (*D*) The elevation of cytosolic calcium induced by Cd was potentiated by knockdown of calcineurin. Cells with or without knockdown of calcineurin were treated with Cd for 8 h, and then analyzed by Fluo-3 AM staining using flow cytometry. Con, control; CaN, calcineurin. Significant difference with the respective control was evaluated using one-way ANOVA followed by Duncan's multiple-range test. Columns not sharing the same superscript differ significantly ($p<0.05$).

that cytosolic calcium may play a pivotal role in Cd-induced cell death.

The ER is one of the major calcium storage units in cells, and many reports have demonstrated that blockers of the ER calcium channel can effectively inhibit apoptosis induced by various stimuli, suggesting that the release of calcium from the ER may lead to apoptosis. Therefore, we employed 2-APB, a blocker of the ER calcium channel (IP_3R), to explore its effects on Cd-induced cell death. As shown in Figure 4A, we observed that 2-APB was able to suppress Cd-induced autophagy and apoptosis; additionally, elevation of $[Ca^{2+}]_i$ induced by Cd was suppressed by 2-APB from 3.0 to 1.6 fold after treatment with Cd for 8 h (Fig. 4B). As demonstrated in the literature, dephosphorylation of IP_3R by calcineurin decreases the release of calcium from the ER [37, 38]. Here, using siRNA to knockdown the expression of calcineurin (see inset of Fig. 4C), Cd-induced autophagy and apoptosis increased from 29.5 % and 17.7 % to 48.1 % and 31.5 %, respectively (Fig. 4C). Furthermore, using flow cytometry with Fluo-3 AM staining, we also detected that $[Ca^{2+}]_i$ had increased from 3.6 to 5.1 folds after treatment with Cd for 8 h (Fig. 4D). Taken together, these results demonstrated that cell death induced by Cd in mesangial cells is linked to the release of calcium from the ER.

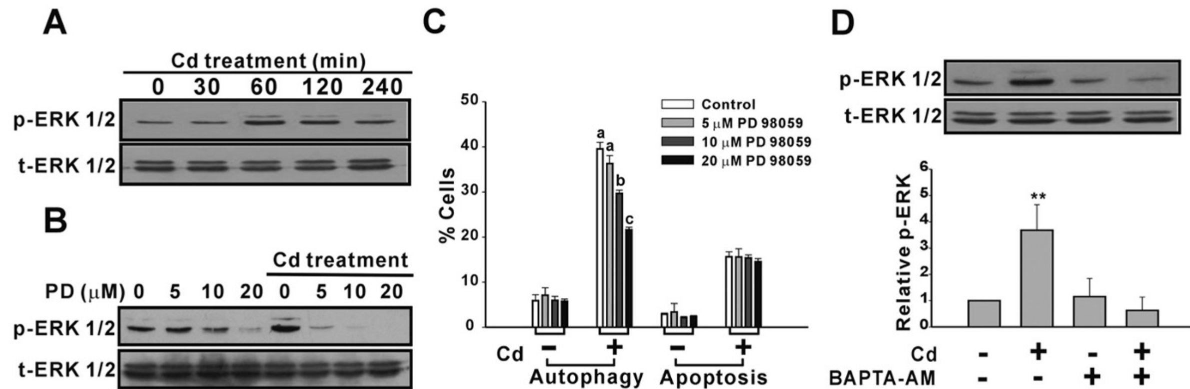

Figure 5. Cd-induced autophagy is mediated through calcium-ERK signaling. (*A*) Time course of activation of ERK during Cd-induced cell death. Cells were treated with Cd for the indicated periods of time and then analyzed by Western blotting with anti-p-ERK and -t-ERK antibodies. t-ERK was used as an internal control to normalize the amount of proteins in each lane. (*B*) Activation of ERK was inhibited by treatment with inhibitor of MEK 1/2. Cells pretreated with PD 98059 for 1 h, followed by treatment with Cd for 1 h. Cell lysates were analyzed by Western blotting with anti-p-ERK and -t-ERK antibodies. t-ERK was used as an internal control to normalize the amount of proteins in each lane. (*C*) Cells were pretreated with various concentrations of PD 98059 for 1 h, followed by treatment with Cd for another 28 h to determine the extent of autophagy and apoptosis. Significant difference with the respective control was evaluated using one-way ANOVA followed by Duncan's multiple-range test. Columns not sharing the same superscript differ significantly ($p<0.05$). (*D*) Activation of ERK induced by Cd occurs through calcium signaling. Cells were pretreated with 10 μM BAPTA-AM for 1 h, followed by treatment with Cd for 60 min. Cell lysates were analyzed by Western blotting with anti-p-ERK and -t-ERK antibodies. t-ERK was used as an internal control to normalize the amount of proteins in each lane. $^{**}p<0.01$, significantly different compared with the control, student's *t*-test.

Cd-induced autophagy is mediated by Ca^{2+}-ERK signaling. The activation of MAPKs, such as ERK, JNK and p38, is involved in the regulation of autophagy [39, 40]. It was reported that Cd-induced ERK activation in mesangial cells is mediated by MKK-7 [41, 42]. Therefore, we proposed that Cd-activated ERK may be involved in regulating autophagy. To confirm this possibility, we sought to determine whether ERK contributes to this response. Using an immunoblot assay, we detected that ERK was activated after treatment with Cd within 1 h, and reached the maximal level at 1 h-time point (Fig. 5A). As revealed in Fig. 5B, we observed that the activation of ERK was reduced by pretreatment with a specific MEK 1/2 inhibitor, PD 98059. Furthermore, the PD 98059 (Fig. 5C) and U0126 (Suppl. Fig. 1) were able to suppress the Cd-induced autophagy as revealed by flow cytometry with acridine orange staining, suggesting the activation of ERK is involved in Cd-induced autophagy. However, the proportion of apoptosis was not significantly reduced by PD 98059 (Fig. 5C), and there was only a minor effect on Cd-induced apoptosis in cells pretreated with 10 μM U0126 (Suppl. Fig. 1), indicating that Cd-induced activation of ERK plays a major role in autophagy, but has only a minor effect on apoptosis Furthermore, it has been reported that disturbances of cytosolic calcium can modulate the activation of ERK in fibroblasts and neuron cells through Ras-dependent and -independent signaling [43]. Following this line of reasoning, we tried to link ERK activation with the elevation of $[Ca^{2+}]_i$. As shown in Fig. 5D, ERK activation was reduced by BAPTA-AM (10 μM). Taken together, our results suggest that Cd-induced activation of ERK is mediated by an increase of $[Ca^{2+}]_i$, which resulted in an induction of autophagy.

Cd-induced apoptosis occurs through Ca^{2+}-mitochondria signaling. Our previous report demonstrated that Cd may exert its toxicity through calcium signaling accompanied by depolarization of mitochondria [30]. To investigate the effects of Cd on the mitochondria in mesangial cells, using JC-1 as an indicator of mitochondrial membrane potential ($\Delta\Psi$m), we observed that the ratio of cells with depolarized mitochondria increased from 2.3 % to 20.0 % after treatment with Cd for 28 h (Fig. 6A, B). However, reduction of cytosolic calcium by BAPTA-AM or 2-APB subsequently decreased the percentage of cells harboring damaged mitochondria from 20.0 % to 11.0 % or 14.1 %, respectively. Since activation of caspase 9 and 3 is a downstream event that follows the collapse of mitochondria, we used immunoblots to detect that the activation of caspase 9 and 3 and the cleaved form of PARP had increased after treatment with Cd (Fig. 6C). Furthermore, the activation of caspases and cleavage of PARP were suppressed by BAPTA-AM (Fig. 6D), suggesting calcium-mediated mitochondria-caspase activation is involved in Cd-induced apoptosis. Collectively, our results showed that Cd-induced apoptosis occurred through a calcium-mitochondria signaling pathway.

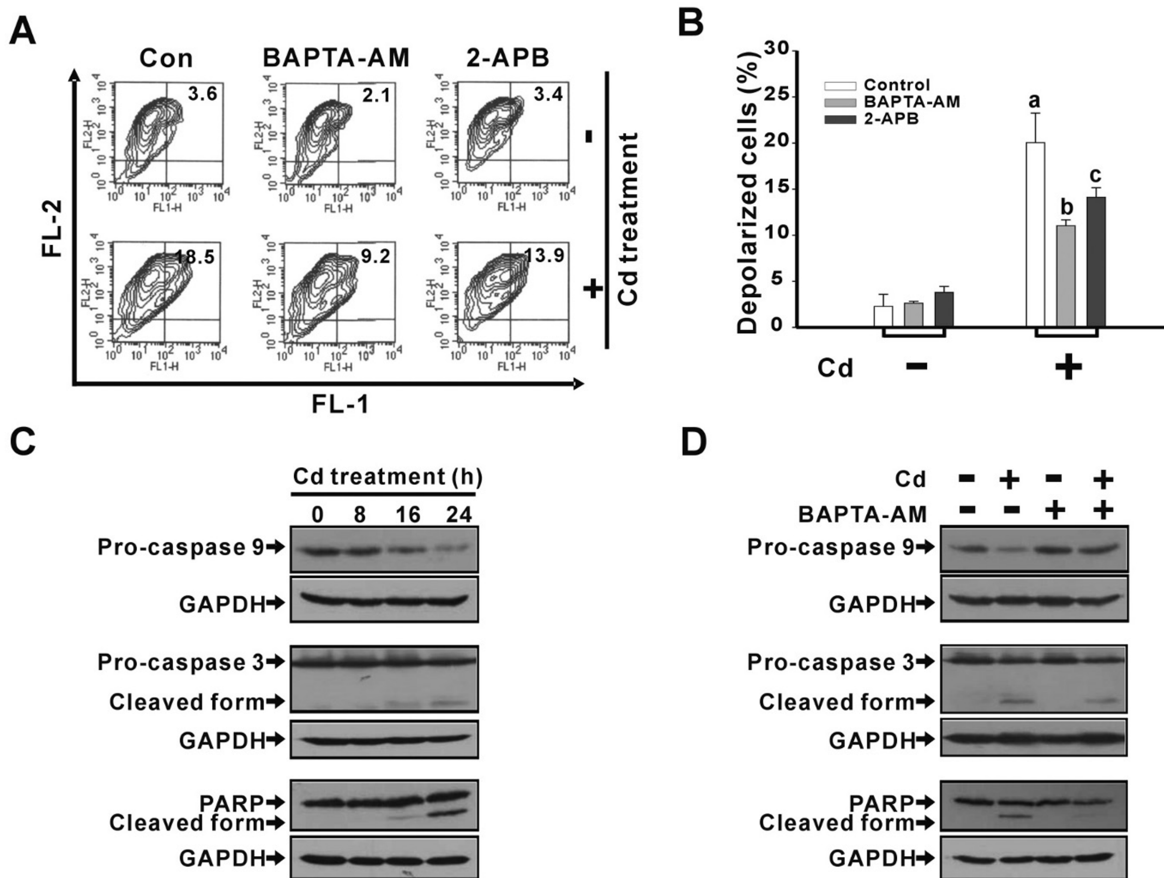

Figure 6. The apoptotic effect of cadmium occurs through calcium-mitochondria signaling. (*A*) Cells were pretreated with BAPTA-AM (10 μM), or 2-APB (60 μM) for 1 h, followed by treatment with Cd for another 28 h. The mitochondrial membrane potential was analyzed by JC-1 staining using flow cytometry. Three independent experiments were performed, and the statistical results are presented in panel *B*. Significant difference with the respective control was evaluated using one-way ANOVA followed by Duncan's multiple-range test. Columns not sharing the same superscript differ significantly ($p<0.05$). (*C*) Activation of the caspase cascade during Cd-induced cell death. Cells were treated with Cd for the indicated periods of time and then analyzed by Western blotting with anti-caspase 9 or anti-caspase 3, or anti-PARP antibodies. GAPDH was used as an internal control to normalize the amount of proteins in each lane. (*D*) Activation of caspases induced by Cd occurs through calcium signaling. Cells were pretreated with BAPTA-AM for 1 h, followed by treatment with Cd for 28 h. Cells lysates were analyzed by Western blotting with anti-caspase 9 or anti-caspase 3, or anti-PARP antibodies. GAPDH was used as an internal control to normalize the amount of proteins in each lane.

Discussion

Renal dysfunctions induced by Cd are associated with alterations of intrarenal hemodynamics and glomerular filtration, and glomeruli and their contractile mesangial cells have been identified as targets of Cd exposure [21]. In this study, we have shown that Cd-induced autophagy and apoptosis are mediated through a calcium signaling pathway. We know that Ca^{2+}-ERK was involved in the regulation of Cd-induced autophagy because MEK1/2 inhibitor (PD 98059 and U0126) reduced Cd-induced autophagy. Moreover, depolarization of mitochondria and the activation of caspase 9 and 3 were also detected after treatment with Cd. BAPTA-AM was able to reduce mitochondrial collapse, caspase activation, and the cleavage of PARP. Collectively, our results demonstrate that Cd induces two types of cell death which includes Ca^{2+}-ERK-mediated autophagy and Ca^{2+}-mitochondria-caspase-mediated apoptosis (Fig. 7).

That autophagy serves as a type of cell death is supported by past studies demonstrating autophagosome accumulation and LC3-II protein increase in dying cells or an elevation of cell viability resulting from autophagy suppression [44, 45]. However, emerging data are showing that the cytotoxicity of Cd, a toxic metal which mainly accumulates in the kidneys, occurs through calcium-mediated apoptosis, but not autophagy, in various cell types such as macrophages, liver cells, human lymphoma cells, kidney proximal tubule cells, and mtDNA-depleted cells [30, 33–36]. In this study, we have shown that Cd

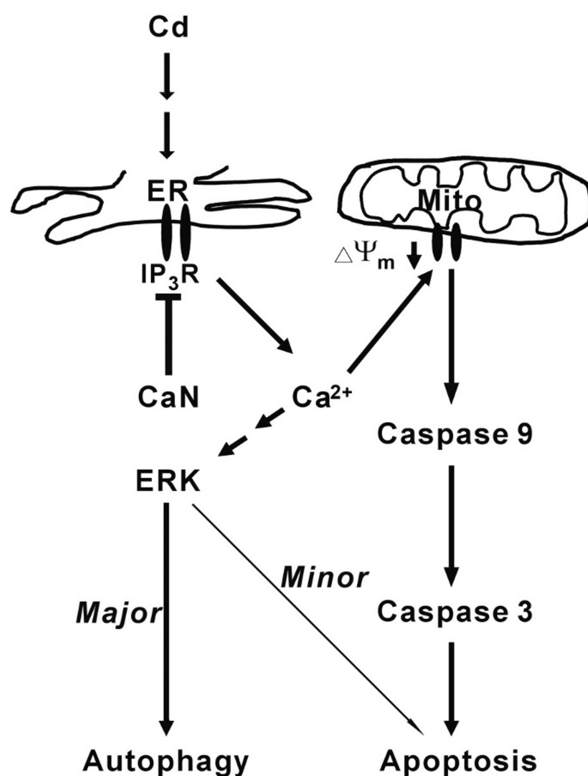

Figure 7. A proposed model of Cd toxicity toward mesangial cells. The cytotoxicity of Cd is initiated through release of calcium from IP_3R channel of the ER, and then leads to ERK activation and mitochondrial depolarization. The calcium-mediated ERK activation plays major and minor roles in Cd-induced autophagy and apoptosis, respectively. CaN, calcineurin; ER, endoplasmic reticulum; IP_3R, inositol-1,4,5-triphosphate receptor; Mito, mitochondria; $\Delta\Psi m$, mitochondrial membrane potential.

induced two types of cell death since a standard autophagy inhibitor, 3-MA, offered protection against Cd-induced cell death. However, the cross-interaction between apoptosis and autophagy needs further investigation. It is worth noting that, in this study, treatment with 3-MA, an autophagy inhibitor, or Z-VAD-fmk, an apoptosis inhibitor, did not affect the percentage of apoptosis or autophagy, respectively (Fig. 2A, B). Additionally, as revealed in Figure 2C, the suppression of autophagy and apoptosis by cotreatment with 3-MA and Z-VAD-fmk did not completely recover the decreased cell viability by Cd treatment. This might, at least in part, contribute by other mechanisms, such as Cd toxicity on the DNA repairing systems [46] and/or activation of P53, followed by cell cycle arrest and then induction of cell death [47]. Consistent with our results, Chen et al [12] demonstrated that suppression of oxidative stress-induced autophagic cell death by treatment with 3-MA or Atgs siRNA does not affect the percentage of apoptosis. Furthermore, Z-VAD-fmk also had no effect on oxidative stress-induced autophagy, according to flow cytometry with acridine orange staining [12], all of which suggested that interaction between autophagy and apoptosis may not be necessary for cell death in some systems.

The transition metals frequently cause the elevation of $[Ca^{2+}]_i$ which leads to a cytotoxicity effect [48]. Our results also demonstrated that elevation of $[Ca^{2+}]_i$ plays a crucial role not only in the apoptosis but also in the autophagy induced by Cd. Consistent with our results, vitamin D and its pharmacological analog (EB1089), ATP, ionomycin, and photodynamic therapy are known to induce calcium-mediated autophagy [18, 49], suggesting that disturbance of calcium plays an important role in the induction of autophagy. More importantly, herein we report for the first time that Cd exhibited its cytotoxicity *via* autophagy through Ca^{2+}-ERK signaling. In addition, a recent report demonstrated that elevation of $[Ca^{2+}]_i$ in Cd-exposed hepatocytes occurs through extracellular calcium entry and release of calcium from intracellular stores [50]. In this study, we observed that inhibition of IP_3R by 2-APB to abolish calcium release from the ER significantly reduced Cd-induced autophagy and apoptosis. Furthermore, a knockdown of calcineurin caused mesangial cells to be sensitized to Cd by way of enhancing cytoplasmic calcium from the ER, suggesting that release of calcium through IP_3R may be one of the major factors behind Cd-induced cell death. It was reported that Cd could induce an increase of IP_3, a ligand of IP_3R, within 5 min after treatment with Cd [51], suggesting that Cd could activate the IP_3R through increase of intracellular IP_3, resulted in release of calcium from ER. Consistent with our results, inhibition of calcineurin resulted in augmentation of the PKC-dependent phosphorylation status in IP_3R- and IP_3-induced Ca^{2+} release [37, 38], suggesting that calcineurin appears to be a major regulator of the Ca^{2+} conductance of IP_3R. However, it was reported that calcineurin could play a protective role in the acute ischemia or taxol-induced apoptosis through dephosphorylation of nuclear factor of activated T cells (NF-AT) and Bcl-2 [52, 53]. Therefore, we could not exclude the possibility that another mechanism is involved in the calcineurin-mediated protective effect in Cd-induced cell death. In this study, we use BAPTA-AM to chelate intracellular calcium to demonstrate that calcium plays a crucial role in Cd-induced cell death. However, it was reported that 5F-BAPTA is able to bind to Cd, which may decrease its cytotoxicity [54]. In this study, it is conceivable that calcium plays a positive regulator in Cd-induced cell death, since suppressing the function of the ER calcium channel, IP_3R, through an IP_3R inhibitor decreases the $[Ca^{2+}]_i$ and then

diminishes the apoptogenic and autophagic activities of Cd. Contrary to our results, knockdown or blockage of IP_3R strongly stimulates autophagy [55]. Additionally, autophagy is inhibited by agents with the ability to release cytosolic calcium from intracellular stores such as thapsigargin, 2,5-di-(tert-buty)-1,4- benzohydroquinone (tBuBHQ), and A23187 [56]. Therefore, the role of calcium release through IP_3R in regulating cell death is still controversial and merits further elucidation.

The molecular mechanism of calcium-mediated autophagy is unclear. Recently, it was reported that Bax/Bak double-knockout MEF cells are prevented from undergoing apoptosis but do undergo autophagic cell death resulting from disruption of cytosolic calcium after treatment with photodynamic therapy [18]. Additionally, calmodulin-dependent kinase-beta is involved in inducting autophagy after treatment with calcium-mobilizing agents [49]. In this study, we demonstrated that the calcium-mediated activation of ERK plays a major role in regulating Cd-induced autophagy. Consistent with our results, it was reported that Cd leads to ERK activation through the MKK7 pathway [41, 42], but the role of ERK in Cd-induced cell death was not mentioned. Furthermore, a recent report regarding Cd-induced apoptosis in thyroid carcinoma cells demonstrated that pretreatment with BAPTA-AM decreased the phosphorylation of ERK induced by Cd, suggesting Cd-mediated ERK activation occurs through calcium signaling [47]. ERK has been considered a survival signaling pathway that is activated after treatment with various stimuli [57–59]. Recently, however, increasing evidence has indicated that ERK plays a central role in promoting apoptosis and non-apoptotic cell death [60, 61]. Oxidative stress induced by treatment with 1-methyl-4-phenylpyridinium (MPP^+) or overexpression of the neurotrophin receptor tropomyosin-related kinase A (TrKA) induced autophagy in ERK-dependent signaling [62–64], suggesting that activation of ERK may be a common event in the induction of autophagy. However, it has been reported that oridonin-induced autophagy is accompanied by downregulation of the phosphorylation of ERK, and pretreatment with 3-MA, an inhibitor of autophagy, results in a reduction of autophagy and upregulation of ERK phosphorylation [65]. Therefore, the role of ERK in autophagy is controversial. Additionally, it is known that the inhibition of cisplatin-activated ERK can prevent the release of cytochrome *c* and activation of caspase 3 [66], implying that ERK may be involved in cisplatin-induced apoptosis. In this study, we observed that the inhibition of ERK had mild effect on phosphatidylserine externalization (Suppl. Fig. 1). Furthermore, a recent report showed that inhibition of ERK by PD 98059 had no effect on the Cd-induced Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II)-mediated apoptosis in mesangial cells [23], all of which suggesting that the activation of ERK may not play a major role in Cd-induced apoptosis. One of the possible explanations is that responses can vary with the cell line and treatment involved. However, the detailed mechanism of ERK in regulating Cd-induced autophagy is unclear and is worth to further elucidate.

The connection between mitochondrial depolarization and apoptosis is well established. Our previous report proved that Cd-induced caspase-independent apoptosis is accompanied by a depolarization of mitochondria through calcium-mediated signaling [30]. In this study, we showed that Cd induced caspase-dependent apoptosis, accompanied by the depolarization of mitochondria, and BAPTA-AM prevented the activation of caspase 3 and collapse of mitochondria, suggesting that calcium-mitochondria-caspase is the major apoptosis signal in Cd-treated mesangial cells. Mitochondria are considered to be one of the calcium storehouses and buffering organelles of cells. The accumulation of calcium in mitochondria occurs through the mitochondrial calcium uniporter. Our earlier publication demonstrated that Cd-induced apoptosis in mtDNA-depleted cells is blocked by mitochondrial calcium uniporter inhibitor [30]. Furthermore, it was published that Cd entering mitochondria through the mitochondrial calcium uniporter resulted in mitochondrial swelling and release of cytochrome *c* and AIF [67, 68], suggesting that the calcium channel of mitochondria may play a pivotal role in Cd-induced apoptosis. On the other hand, mitochondrial depolarization has been demonstrated to be a cause, while inducing autophagy [69, 70]. In this study, 2-APB and BAPTA-AM were able to prevent the collapse of mitochondria and autophagy from Cd toxicity, suggesting mitochondrial depolarization caused by the elevation of $[Ca^{2+}]_i$ may play a role in Cd-induced autophagy. Nevertheless, direct evidence is required to support this hypothesis.

To the best of our knowledge, this is the first report to demonstrate that Cd induces two types of cell death, which includes both apoptosis and autophagy, and both types are mediated by calcium signaling. Furthermore, our study elucidated that calcium-mediated autophagy and apoptosis occur through the ERK-dependent and mitochondria-caspase signaling pathways, respectively.

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-008-8383-9) and is accessible for authorized users.

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