

# Cadmium Toxicity toward Autophagy through ROS-Activated GSK-3 $\beta$ in Mesangial Cells

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We previously demonstrated that cadmium (Cd) is able to induce autophagic cell death through a calcium-extracellular signal-regulated kinase pathway. Here, the object of this study is to investigate the role of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) in the induction of autophagy. After treatment with Cd, MES-13 mesangial cells were determined to have undergone autophagy based on the formation of acidic vesicular organelles and autophagosomes as well as on the processing of microtubule-associated protein 1 light chain 3, using flow cytometry with acridine orange staining, electron microscopy, and immunoblot, respectively. Use of the GSK-3 $\beta$  inhibitor SB 216763 or the small interfering RNA technique to knockdown the expression of GSK-3 $\beta$  resulted in a decrease of Cd-induced autophagy. In contrast, overexpression of GSK-3 $\beta$  by transient transfection potentiated Cd toxicity toward the mesangial cells, suggesting that GSK-3 $\beta$  plays a crucial role in regulating Cd-induced autophagy. Moreover, a decrease of the phosphorylated level at Ser9 of GSK-3 $\beta$  was observed by immunoblot after treatment with Cd, indicating GSK-3 $\beta$  was activated by Cd. This phenomenon was reversed by the reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC), demonstrated that ROS might activate GSK-3 $\beta$ . In fact, intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was 2.6-fold elevated after 3 h of exposure to Cd. Both Cd-induced ROS bursts and autophagy were reduced by NAC and vitamin E. In summary, this study demonstrated that, in MES-13 mesangial cells, Cd-induced autophagy was mediated through the ROS-GSK-3 $\beta$  signaling pathway.

**Key Words:** cadmium; autophagy; GSK-3 $\beta$ ; ROS; mesangial cells.

Cadmium, a heavy metal, is an environmental pollutant with high cytotoxicity. Epidemiological information on occupationally exposed populations in highly contaminated areas has established

that an overload of Cd produces adverse health effects like pulmonary disease, carcinogenicity, and hepato- and nephrotoxicity. Animal study demonstrated that the major accumulation organs after Cd exposure are kidneys and liver (Friberg *et al.*, 1974). Long-term exposure of Cd may lead to renal dysfunction (Jarup, 2002) and increase urinary excretion of low molecular weight proteins (Jarup, 2002), suggesting glomeruli and mesangial cells may be involved in Cd-induced renal dysfunction. Contraction of mesangial cells induced by Cd resulted in nephrotoxicity (Hirano *et al.*, 2005). Recently, Liu and Templeton (2007) demonstrated that Cd-induced a calcium-mediated apoptosis in mesangial cells. Cd interferes with cell proliferation and development and is implicated in either apoptosis or necrosis, depending on the exposure conditions and the model used (Galan *et al.*, 2001; Ishido *et al.*, 2002; Shih *et al.*, 2003).

Autophagy, an evolutionarily conserved process for degrading and recycling of long-lived cellular proteins and damaged organelles, is characterized by double-membraned vacuoles (autophagosomes) sequestered in the cytoplasmic fraction (Hippert *et al.*, 2006). Autophagy is important to normal development, which is an adapting mechanism to respond to changing environmental stimuli (Hippert *et al.*, 2006), and its dysregulation may lead to cancer, neurodegenerative disorders, and cardiovascular diseases (Shintani and Klionsky, 2004). The regulation of autophagy is a very complex process which includes many signaling pathways, such as the target of rapamycin, phosphatidylinositol 3-kinase-I/protein kinase B, guanosine triphosphate phosphohydrolase, calcium, and mitogen-activated protein kinase pathways (Gozuacik and Kimchi, 2004; Yang *et al.*, 2005). Though the molecular mechanism of apoptosis is well documented, that of autophagy is remains unclear, and its role in cell death is controversial. It was reported that autophagy serves as a protective mechanism against cell death during nutrient deprivation, and cells undergo apoptotic cell death when autophagy is inhibited (Boya *et al.*, 2005; Lum *et al.*, 2005). However, autophagy has also been observed as a cell death no matter caspase is activated or not (Gozuacik and Kimchi, 2004; Shimizu *et al.*, 2004; Yu *et al.*, 2004). Our previous report has

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demonstrated that Cd not only induces apoptosis, but also triggers a calcium-extracellular signal-regulated kinase (ERK) mediated autophagic cell death in mesangial cells (Wang *et al.*, 2008). However, the calcium chelator (1,2-bis-[2-aminophenoxy]-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester) or ERK inhibitor could not completely reduce Cd-induced autophagy (Wang *et al.*, 2008), suggesting that some other signaling pathways may be involved in Cd-induced autophagy and warrant for further investigation.

Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a serine/threonine kinase, is regulated through multiple mechanisms, including the phosphorylation of GSK-3 $\beta$  itself, intracellular location, and formation of a protein complex (Frame and Cohen, 2001; Jope and Johnson, 2004). The activation of GSK-3 $\beta$  kinase is through dephosphorylation at Ser9 or phosphorylation at Tyr216 (Forde and Dale, 2007). GSK-3 $\beta$  is involved in many diseases, such as bipolar mood disease (Gould *et al.*, 2004), schizophrenia (Emamian *et al.*, 2004), Alzheimer's disease (Bhat and Budd, 2002), diabetes mellitus (Wagman *et al.*, 2004; Woodgett, 2003), and cancer (Mazor *et al.*, 2004; Ougolkov *et al.*, 2005). It has been reported that overexpression of GSK-3 $\beta$  induces type I cell death (apoptosis) in neuron cells, but inhibition of GSK-3 $\beta$  can abolish the apoptotic response (Pap and Cooper, 1998). In addition, Hoefflich *et al.* (2000) observed that loss of GSK-3 $\beta$  in mice resulted in embryonic lethality through hepatocyte apoptosis, suggesting that GSK-3 $\beta$  is required for cell survival. As mentioned above, numerous previous reports have studied the effects of GSK-3 $\beta$  on type I cell death (apoptosis). However, the role of GSK-3 $\beta$  in type II cell death (autophagy) remains elusive.

In this study, we used mesangial cells as a cell model to determine the role of GSK-3 $\beta$  in Cd-induced autophagic cell death. We have shown that Cd induces an ROS burst, followed by GSK-3 $\beta$  activation, and then triggers autophagy. Combined with our previous report (Wang *et al.*, 2008), we demonstrated that Cd toxicity toward mesangial cells results in an autophagic cell death at least through calcium-ERK and ROS-GSK-3 $\beta$  signaling pathways.

## MATERIALS AND METHODS

**Cell culture, treatment, and chemicals.** SV40 transformed mouse kidney mesangial cells (MES-13) were obtained from the American Tissue Culture Collection (CRL-1927, Manassas, VA). Mesangial cells were grown at 37°C in medium containing Dulbecco's Modified Eagle's Medium (DMEM) and F-12 (3:1) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (pH 7.4) in a humidified atmosphere containing 5% CO<sub>2</sub>. In our previous publication, we have demonstrated that Cd induced a dose-dependent decline of cell viability, which reached to 48.5% after treatment with 6 $\mu$ M Cd (Wang *et al.*, 2008). Therefore, the dose for treating mesangial cells was determined to be 6 $\mu$ M for the following experiments. Mesangial cells were grown in complete medium for 24 h, followed by treatment with 6 $\mu$ M Cd for the indicated time periods. DMEM, FBS, penicillin, and streptomycin were purchased from HyClone (Logan, UT). Cadmium chloride, bovine serum albumin, acridine orange, 3-methyladenine (3-MA), vitamin E, SB 216763, and NAC were from Sigma

Chemical (St Louis, MO). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Rabbit monoclonal anti-p-Ser9-GSK-3 $\beta$  (clone E-8) and rabbit polyclonal anti-GSK-3 $\beta$  antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-LC3 (microtubule-associated protein 1 light chain 3) was from MBL International (Nagoya, Japan). Mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) was from Chemicon International (Temecula, CA). The secondary antibodies, including horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG, were from Pierce Biotechnology (Rockford, IL), and Jackson ImmunoResearch Laboratories (West Pine, PA), respectively. Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). Protein Assay Dye Reagent was from Bio-Rad Laboratories (Hercules, CA).

**Measurement of acidic vesicular organelles.** Cell staining was performed according to published procedures (Wang *et al.*, 2008). Briefly, acridine orange was added at a final concentration of 1  $\mu$ g/ml for a period of 20 min, and cells were removed from the plate by trypsinization and then collected in phenol red-free growth medium. 3-MA (2mM) or drugs indicated in the experiments were added 1 h before Cd treatment. Green (510–530 nm) and red (650 nm) fluorescence emissions from 1  $\times$  10<sup>4</sup> cells illuminated with blue (488 nm) excitation light were measured with a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

**Electron microscopic assay.** To morphologically demonstrate the induction of autophagy in Cd-treated mesangial cells, we performed an ultrastructural analysis according to published procedures (Wang *et al.*, 2008). Cells treated with or without 6 $\mu$ M Cd for 24 h were washed twice with phosphate-buffered saline and fixed with ice-cold glutaraldehyde (3% in 0.1M cacodylate buffer, pH 7.4) for 30 min. Next, cells were postfixed in OsO<sub>4</sub> and embedded in Epon; serial ultra thin sections (80nm) were cut and stained with uranyl acetate/lead citrate (Fluka, Chemie AG, Switzerland) and viewed in a Hitachi H600 electron microscope (Hitachi Instrument, Tokyo, Japan).

**Immunoblot analysis.** Cells were scraped and lysed with 50  $\mu$ l of lysis buffer (25mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 1.5% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.5M NaCl, 5mM ethylenediaminetetraacetic acid, and 0.1mM sodium deoxycholate) (Simizu *et al.*, 1998) containing a protease inhibitor cocktail (Roche, Boehringer Mannheim, Germany). After a 10-min incubation on ice, sampling buffer (60mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 140mM  $\beta$ -mercaptoethanol) was added to each lysate, which was subsequently boiled for 7 min and centrifuged at 15,000  $\times$  g for 5 min. The concentration of collected supernatant was determined using the Bio-Rad Protein Assay Dye Reagent and subjected to electrophoresis on an SDS-polyacrylamide gel (30  $\mu$ g protein per lane). Proteins were electrotransferred onto PVDF membranes and immunoblotted with anti-p-Ser9-GSK-3 $\beta$  (1:2000 dilution), anti-GSK-3 $\beta$  (1:5000 dilution), anti-LC3 (1:1000 dilution), or anti-GAPDH (1:10,000 dilution) antibodies. Detection was performed with appropriate HRP-conjugated secondary antibodies (1:10,000 dilution) and enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). The density of band was determined with Gel-Pro Analyzer densitometry software (Media Cybernetics, Bethesda, MD).

**Knockdown of GSK-3 $\beta$ .** Mesangial cells were transfected with 50nM small interfering RNA (siRNA) using lipofectamine RNAimax reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. In brief, 2  $\times$  10<sup>4</sup> cells were incubated with lipofectamine RNAimax reagent and 50nM GSK-3 $\beta$  siRNA for 6 h, and then the medium was refreshed, followed by incubation for a further 42 h. Transfected cells were treated with 6 $\mu$ M Cd for 24 h and analyzed by flow cytometry with acridine orange staining. Silencer-validated siRNA to GSK-3 $\beta$  (sense siRNA strand, 5'-GGACAAGCG-AUUUAAGAAGCTT-3'; antisense siRNA strand, 5'-GUUCUAAAUCG-CUUGUCCTG-3') was from Invitrogen.

**Overexpression of GSK-3 $\beta$ .** Plasmid pCMV6-XL4 is a mammalian cells expression vector driven by the cytomegalovirus (CMV) promoter. GSK-3 $\beta$  expression construct pCMV6-XL4-GSK-3 $\beta$  contains a full length of GSK-3 $\beta$

gene inserted into pCMV6-XL4 at *SalI* and *EcoRI* sites. Both plasmids were purchased from Origene Technology (Rockville, MD) and were transfected into mesangial cells ( $5 \times 10^4$  cells) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After transfection, cells were treated with  $6\mu\text{M}$  Cd for 24 h, and the percentage of autophagy was analyzed by flow cytometry with acridine orange staining.

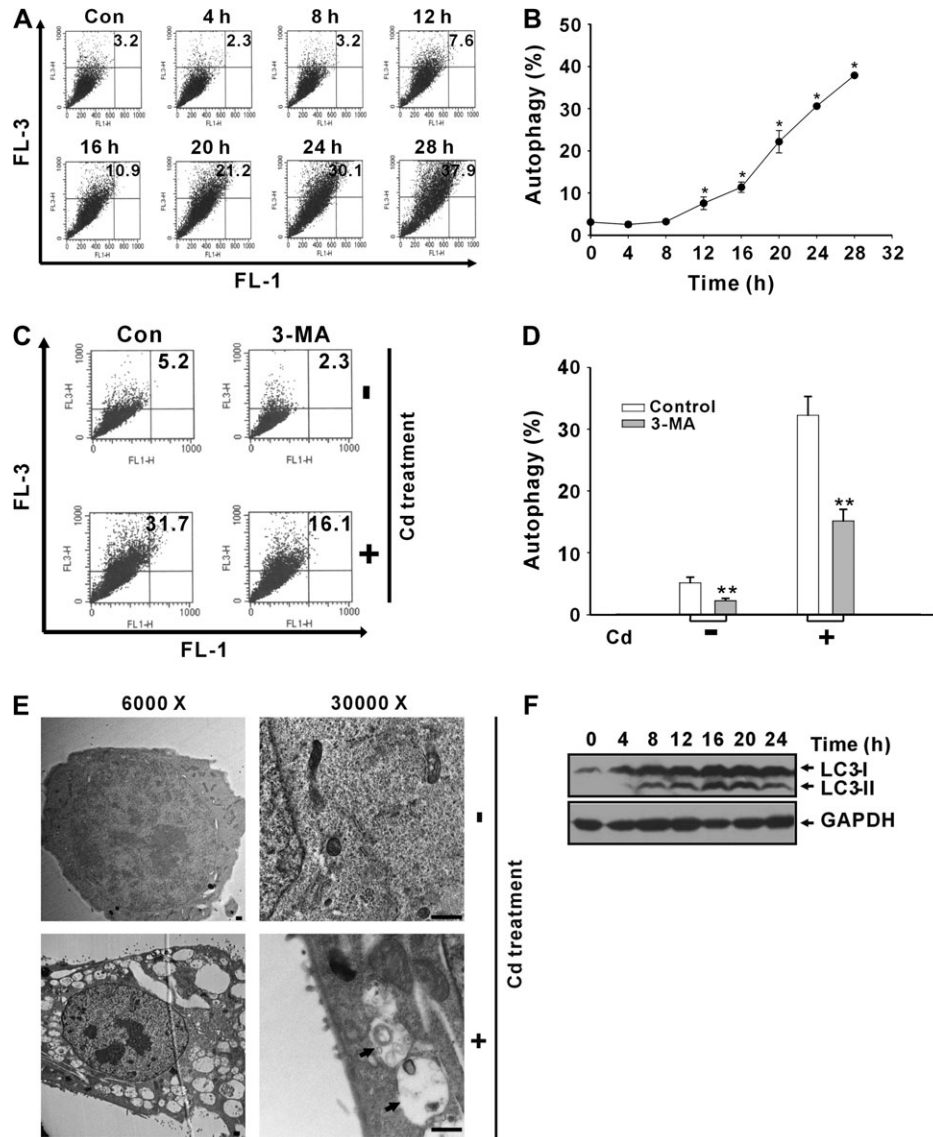
**Measurement of reactive oxygen species.** DCFH-DA was added to cells at a final concentration of  $20\mu\text{M}$ . After 30 min of incubation, cells were trypsinized and collected in phenol red-free RPMI medium. The fluorescence intensity of DCF, a compound formed in response to  $\text{H}_2\text{O}_2$ , was detected by flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA). The excitation and emission wavelengths were set at 488 and 530 nm, respectively.

**Statistics.** Three independent experiments were performed and statistics were evaluated by Student's *t*-test (for two groups) or one-way ANOVA (for three or more groups). A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Cadmium Induces an Autophagy

Cadmium has been shown to cause cell damage through apoptotic cell death. To investigate whether autophagy is involved in the cytotoxicity of Cd, we first examined Cd-treated



**FIG. 1.** Autophagy induced by Cd in mesangial cells. (A) Cells were treated with  $6\mu\text{M}$  Cd for the indicated periods of time and then analyzed for autophagy by acridine orange staining using flow cytometry. Three independent experiments were performed, and the statistical results are presented in (B). Significantly different compared with the control was evaluated using one-way ANOVA test ( $*p < 0.05$  vs. the control). (C) Cells were pretreated with 2mM 3-MA and incubated with  $6\mu\text{M}$  Cd for another 24 h. Treated cells were analyzed by acridine orange staining to determine the ratio of autophagy. Three independent experiments were performed, and the statistical results are presented in (D). ( $**p < 0.01$  vs. the respective control, Student's *t*-test). (E) Transmission electron microscopic analysis showing mesangial cells with or without Cd for 24 h. Autophagosomes were indicated by arrows. (F) Cell lysates ( $30\mu\text{g}$  per lane) were analyzed using immunoblotting with anti-LC3 or anti-GAPDH antibodies. The GAPDH was used as an internal control to normalize the amount of proteins applied in each lane. Con, control.

mesangial cells for autophagy using flow cytometry with acridine orange staining. The increasing percentage of autophagy was detectable as early as 12 h after treatment with Cd, and it rose as high as  $37.9 \pm 0.4\%$  at 28 h (Figs. 1A and 1B). Besides, Cd-induced autophagy was inhibited effectively in the presence of 3-MA, an autophagy inhibitor, as demonstrated in Figs. 1C and 1D). In addition, transmission electron microscopy examination of Cd-treated mesangial cells revealed the cytoplasm to be full of double-membraned vacuolar structures containing visible cytoplasmic contents (Fig. 1E, as indicated by arrows). Furthermore, to confirm the occurrence of autophagy, we examined the processing of full-length LC3-I to LC3-II, a hallmark of autophagy, using immunoblot to detect cell-extracted lysates from mesangial cells treated with or without cadmium for every 4 h. As shown in Figure 1F, we observed that the amounts of LC3-II proteins increased after treatment with Cd for 8 h. Thus, these data suggested that Cd induced an autophagy in mesangial cells.

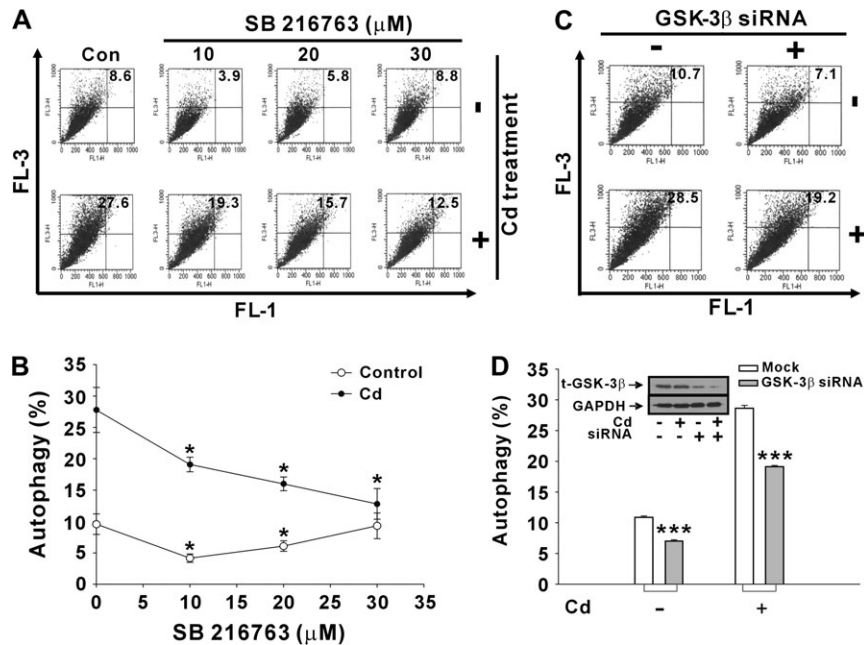
#### Cadmium-Induced Autophagy is Mediated by GSK-3 $\beta$

It has been reported that activation of GSK-3 $\beta$  is involved in various types of stimuli-triggered apoptosis. However, its role in Cd-induced autophagic cell death is unclear. Therefore, we tried to examine whether GSK-3 $\beta$  plays a major role in the regulation of Cd-induced autophagy. To evaluate this, we compared the ratio of autophagy after treatment with Cd for 24 h in cells pretreated

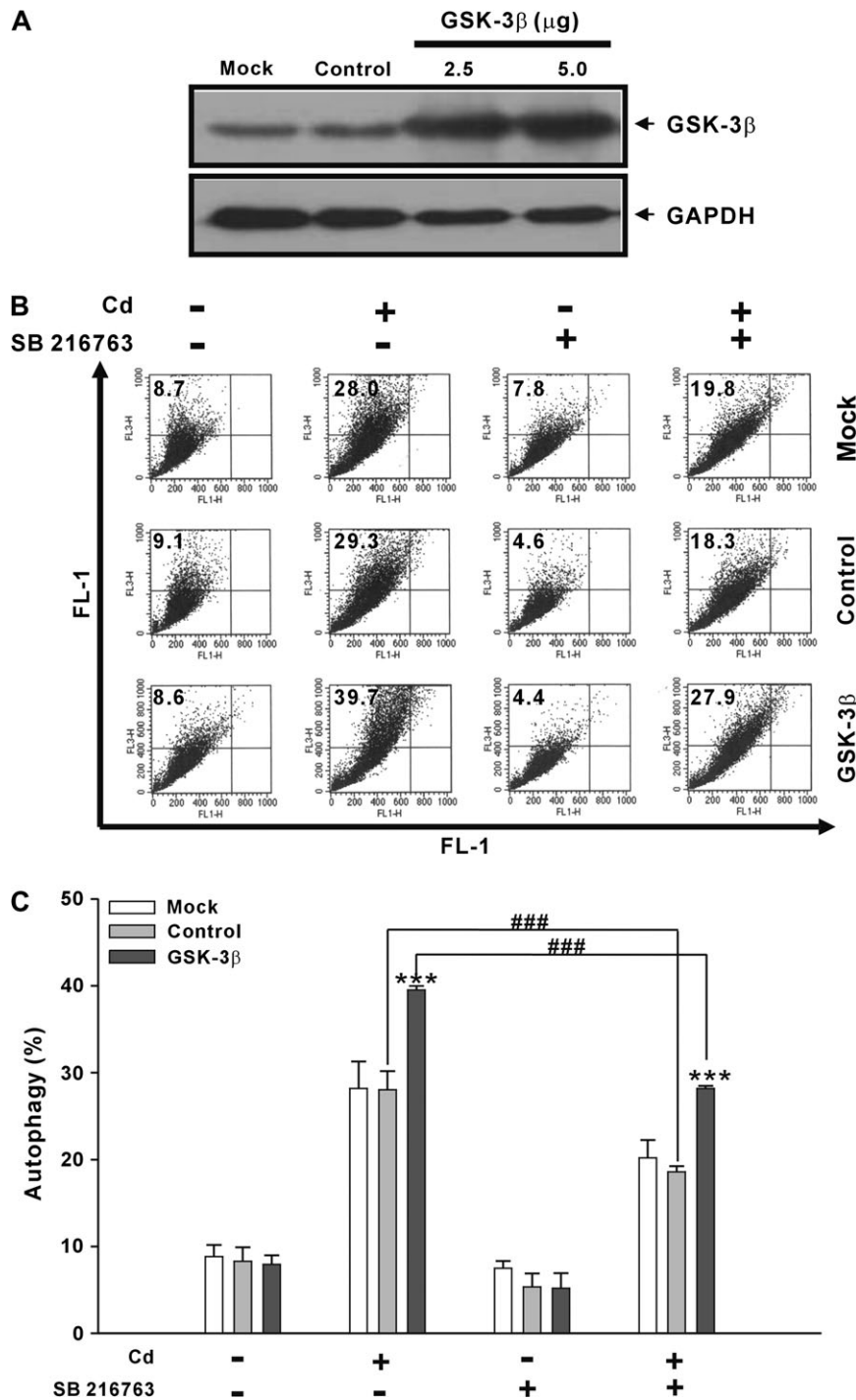
with or without GSK-3 $\beta$  inhibitor, SB 216763. As revealed in Figures 2A and 2B, SB 216763 significantly decreased the percentage of autophagy induced by Cd in a dose-dependent manner. To further confirm the role of GSK-3 $\beta$  in Cd-induced autophagy, cells were treated with siRNA against the GSK-3 $\beta$  coding sequence. The knockdown of GSK-3 $\beta$  resulted in a reduction in the ratio of Cd-induced autophagy (Figs. 2C and 2D). Furthermore, cells harboring the plasmid for the overexpression of GSK-3 $\beta$  significantly augmented the ratio of Cd-induced autophagy, and SB 216763 abrogated the effects (Fig. 3). Collectively, these findings suggest that Cd-induced autophagy in mesangial cells is mediated by the GSK-3 $\beta$  signaling pathway.

#### Cadmium-Induced Autophagy Occurs through the ROS-GSK-3 $\beta$ Signaling Pathway

The activity of GSK-3 $\beta$  is regulated by site-specific phosphorylation, which is increased by the phosphorylation at Tyr216 or dephosphorylation at Ser9, and emerging evidence has shown that dephosphorylation at Ser9 is much more dominant for activating GSK-3 $\beta$  than the phosphorylation level of Tyr216 (Bhat *et al.*, 2000). Therefore, to investigate the relationship between ROS and GSK-3 $\beta$ , we detected the level of Ser9 phosphorylation of GSK-3 $\beta$  using an immunoblot assay. As shown in Figure 4, we observed that this level had decreased to 52% after treatment with Cd for 12 h. ROS-mediated GSK-3 $\beta$



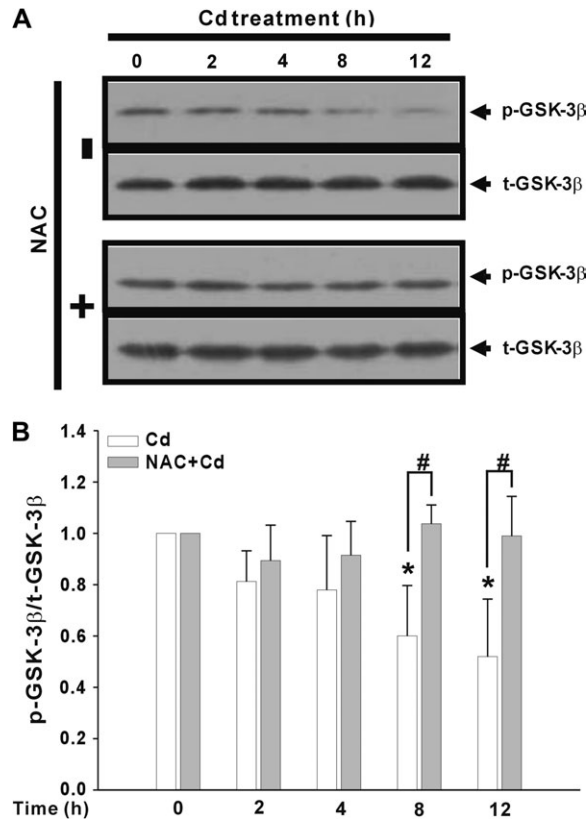
**FIG. 2.** GSK-3 $\beta$  involved in the induction of autophagy induced by Cd. (A) Cells pretreated with SB 216763, an inhibitor of GSK-3 $\beta$ , were incubated with Cd for another 24 h. After incubation, treated cells were trypsinized and collected to determine the ratio of autophagy using flow cytometry staining with acridine orange dye. Three independent experiments were performed, and the statistical results are presented in (B). Significantly different compared with the respective control was evaluated using one-way ANOVA test (\* $p < 0.05$  vs. the respective control). (C) Cells were transfected with siRNA against GSK-3 $\beta$  using the lipofectamine RNAiMax reagent. After transfection, cells were treated with Cd for another 24 h, trypsinized, and collected to determine the percentage of autophagy using acridine orange staining. Three independent experiments were performed, and the statistical results are presented in (D). (\*\* $p < 0.001$  vs. the mock, Student's *t*-test). The inset of (D) indicated a parallel immunoblot assay using anti-GSK-3 $\beta$  and -GAPDH antibodies to monitor the efficiency of siRNA. GAPDH was used as an internal control. Con, control.



**FIG. 3.** Cells overexpressing GSK-3 $\beta$  sensitive to Cd. (A) Cells were transfected with either the plasmid vehicle pCMV6-XL4 (Control) or pCMV6-XL4-GSK-3 $\beta$  (GSK-3 $\beta$ ) using the Lipofectamine 2000 reagent for 24 h. Transfected cells were treated with Cd for another 24 h. Cell lysates (20  $\mu$ g per lane) were analyzed using immunoblot with anti-GSK-3 $\beta$  or -GAPDH antibodies. The GAPDH was used as an internal control to normalize the amount of proteins applied to each lane. (B) Mesangial cells transfected with either the GSK-3 $\beta$  plasmid or control plasmid were treated with or without 30 $\mu$ M SB 216763 for 1 h, followed by treatment with Cd for 24 h. Autophagy was evaluated by flow cytometry with acridine orange dye staining. Three independent experiments were performed, and the statistical results are presented in (C) (\*\* $p$  < 0.001 vs. the mock; ### $p$  < 0.001 vs. the indicated group).

activation has been observed in neuroblastoma cells (Pizarro *et al.*, 2008). Our previous results also showed that Cd-induced cell death occurs through an ROS-dependent signaling pathway

(Shih *et al.*, 2004). Therefore, we proposed that Cd-induced ROS bursts may be involved in regulating GSK-3 $\beta$  activity. To confirm this possibility, we sought to determine the effect of ROS on



**FIG. 4.** Cd-induced activation of GSK-3 $\beta$  is mediated by generation of ROS. (A) Mesangial cells were pretreated with 4mM NAC or not, further incubated with Cd for indicated periods of time, and analyzed by immunoblot with anti-p-Ser9-GSK-3 $\beta$ (p-GSK-3 $\beta$ ) or anti-GSK-3 $\beta$ (t-GSK-3 $\beta$ ). Three independent experiments were performed, and the statistical results are presented in (B) (\* $p < 0.05$ , vs. the respective control, one-way ANOVA test; # $p < 0.05$  vs. the indicated group, Student's  $t$ -test).

GSK-3 $\beta$  activity. Pretreatment with the general ROS scavenger NAC significantly reversed downregulation of the Ser9 phosphorylation induced by Cd, suggesting ROS may play a major role in Cd-induced GSK-3 $\beta$  activation (Fig. 4).

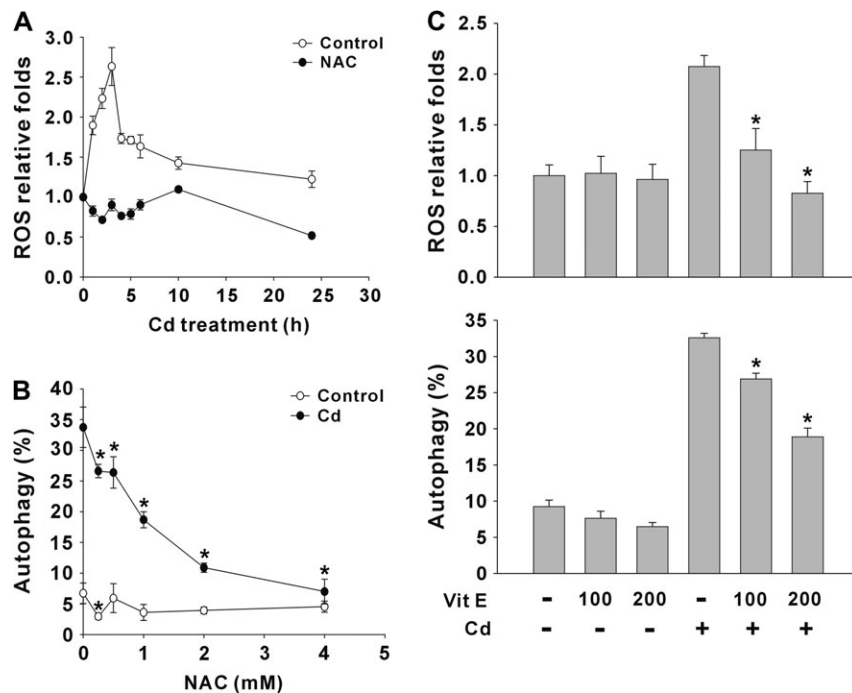
To further address whether autophagy is a consequence of the increase in ROS, we studied the effects of Cd on ROS production at different times using a flow cytometer and cells stained with DCFH-DA dye. As shown in Figure 5A, ROS production increased by 2.6-fold at 3 h, but fell to 1.2-fold after treatment with Cd for 24 h. In addition, the ROS scavenger NAC effectively decreased the elevation in ROS. Cd-induced autophagy was also reduced by NAC in a dose-dependent manner (Fig. 5B). The reduction of Cd-induced autophagy and ROS bursts was also observed in vitamin E-pretreated cells (Fig. 5C), suggesting that ROS production may play a pivotal role in Cd-induced autophagy.

## DISCUSSION

Autophagy has been intensively studied as a response to various stresses, such as limited nutrients and trophic factor

withdrawal, and recently, a growing body of evidence has implicated the process in some neurological diseases. A more recent report indicated that hematopoietic stem/progenitor cells underwent autophagy after treatment with Cd (Di-Gioacchino *et al.*, 2008). However, the key mediator of autophagy in the toxicity of cadmium in mesangial cells is not completely understood. In this study, we examined the underlying mechanisms of Cd-induced autophagy. We showed that it occurs through an ROS-GSK-3 $\beta$  mediated signaling pathway. First, we observed increases in the percentage of acidic vesicular organelle, in the processing of LC3, and in autophagosome formation that followed treatment with Cd. Next, SB 216763, an inhibitor of GSK-3 $\beta$ , and siRNA of GSK-3 $\beta$  effectively reduced the percentage of autophagy induced by Cd. Moreover, cells harboring the plasmid overexpressing GSK-3 $\beta$  were more sensitive to treatment with Cd, suggesting Cd-induced autophagy is mediated by a GSK-3 $\beta$  signaling pathway. The production of ROS was implicated in the regulation of Cd-induced autophagy as NAC and vitamin E, scavengers of ROS, were able to decrease the proportion of autophagy induced by Cd. Additionally, we observed that NAC was able to inhibit the Cd-induced dephosphorylation of GSK-3 $\beta$  on ser9, suggesting that the autophagy induced by Cd is mediated by an ROS-GSK-3 $\beta$  signaling pathway. As a matter of fact, our previous study has shown that Cd induced a calcium-mediated autophagy and apoptosis through ERK and mitochondria pathway, respectively (Wang *et al.*, 2008). However, the percentage of autophagy was not fully inhibited by treatment with 2-aminoethoxydiphenyl borate to suppress the ER-released calcium or by treatment with a specific ERK kinase 1/2 inhibitor to attenuate the ERK activity, suggesting that Cd induces multiple signalings to conduct mesangial cells autophagy including ROS-GSK-3 $\beta$  and calcium-ERK pathway.

ROS are oxygen compounds containing unpaired electrons, including singlet oxygen, hydroxyl radicals, superoxide, and hydrogen peroxides, which are signaling molecules under various conditions. Emerging evidence has shown that disturbance of the homeostasis of the oxidative condition of cells by the inhibition of caspase or starvation leads to the induction of autophagy (Scherz-Shouval *et al.*, 2007; Yu *et al.*, 2006), suggesting that ROS may be one of the major mediators in the regulation of autophagy. In this study, we observed that the levels of intracellular ROS increased by 2.6-folds compared with the control after treatment with Cd for 3 h, and that both NAC and vitamin E could effectively inhibit the increasing percentage of autophagy, suggesting that ROS play a pivotal role in the induction of Cd-induced autophagy. Consistent with our results, Scherz-Shouval *et al.* (2007) reported that the induction of autophagy by starvation occurs through inactivation of HsAtg4A, an oxidant-sensitive cysteine protease, by ROS, resulting in accumulation of Atg8-PE. In addition, NAC and catalase can decrease the ratio of autophagy, suggesting that ROS are necessary for the induction of



**FIG. 5.** ROS serve as a major mediator to regulate Cd-induced autophagy. (A) Cells were pretreated or not with 4mM NAC, a scavenger of ROS, incubated with Cd for the indicated periods of time, and then analyzed by DCFH-DA staining using flow cytometry. Three independent experiments were performed in (A). (B) Cells were pretreated with NAC or not, followed by incubation with Cd for 24 h. They were then analyzed by flow cytometry and acridine orange staining. Three independent experiments were performed, and the statistical results are presented. Significantly different compared with the respective control was evaluated using one-way ANOVA test ( $*p < 0.05$  vs. the respective control). (C) Two sets of MES-13 cells were pretreated with vitamin E (0, 100, or 200 $\mu$ M) for 1 h. One of which was further incubated with Cd for 3 h and then subjected for determination the extent of ROS generation using flow cytometry with DCFH-DA staining. Another set of cells was further incubated with Cd for 24 h and then analyzed the percentage of autophagy on a flow cytometer as described in Materials and Methods. Three independent experiments were performed, and the statistical results are presented. Vit E, vitamin E ( $*p < 0.05$  vs. the Cd-treated only, one-way ANOVA test).

autophagy (Scherz-Shouval *et al.*, 2007). As mentioned above, ROS have been confirmed to play a major role in regulating autophagy; however, knowledge of how the production of ROS affects its induction is scarce. In this study, we found that Cd-mediated GSK-3 $\beta$  activation was reversed by pretreatment with NAC, implying that GSK-3 $\beta$  activation may be a downstream event of Cd-induced ROS burst and eventually autophagy is conducted.

GSK-3 $\beta$ , an enzyme catalyzing the synthesis of glycogen, has been found to be capable of regulating many cellular functions and signaling pathways, the deregulation of which can lead to the development of cancer, diabetes, neurodegenerative disease, and bipolar disorder (Dugo *et al.*, 2007). GSK-3 $\beta$  has a dual function in the regulation of cell death in various cells. Activation of GSK-3 $\beta$  may lead to cell death by the mitochondrion-dependent apoptotic pathway, but it may reverse the death induced by the death receptor-mediated signaling pathway through impeding the activation of caspase-8 (Beurel and Jope, 2006). Though the role of GSK-3 $\beta$  in apoptosis has been extensively studied, knowledge of its role in regulating autophagy is scant. We showed that GSK-3 $\beta$  was activated in Cd-treated mesangial cells as revealed by images of immunoblotting showing the dephosphorylation of GSK-3 $\beta$  at Ser9 site during treatment with Cd. Consistent with our results,

cathepsin D-deficient brains exhibited a significant accumulation of autophagosomes and accompanied by a dephosphorylated level at Ser9 of GSK-3 $\beta$  (Walls *et al.*, 2007), suggesting that GSK-3 $\beta$  may play an important role in regulating autophagy. However, there is still no direct evidence to demonstrate the importance of GSK-3 $\beta$  in the induction of autophagy. In our results, a GSK-3 $\beta$  inhibitor and siRNA of GSK-3 $\beta$  effectively reduced Cd-induced autophagy. Moreover, cells harboring plasmid overexpressing GSK-3 $\beta$  augmented Cd-induced autophagy, indicating that autophagy induced by Cd is mediated directly by activation of GSK-3 $\beta$ . However, the downstream factors of GSK-3 $\beta$  involved in regulating autophagy need to be further elucidated.

To the best of our knowledge, this is the first report to demonstrate the role of GSK-3 $\beta$  in Cd-induced autophagy. Furthermore, we determined that ROS play the major role in the activation of GSK-3 $\beta$  that induces autophagy.

## FUNDING

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