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Crosstalk Between Endoplasmic Reticulum Stress and Mitochondrial Pathway Mediates Cadmium-Induced Germ Cell Apoptosis in Testes

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Cadmium (Cd) is associated with male infertility and poor semen quality in humans. Increasing evidence demonstrates that Cd induces testicular germ cell apoptosis in rodent animals. However, the molecular mechanisms of Cd-induced testicular germ cell apoptosis remain poorly understood. In the present study, we investigated the role of endoplasmic reticulum (ER) stress on Cd-evoked germ cell apoptosis in testes. We show that spliced form of XBP-1, the target of the IRE1 pathway, was significantly increased in testes of mice injected with $CdCl_2$. GRP78, an ER chaperone, and CHOP, a downstream target of the PERK pathway, were upregulated in testes of Cd-treated mice. In addition, acute Cd exposure significantly caused eIF2α and JNK phosphorylation in testes, indicating that the unfolded protein response pathway in testes was activated by Cd. Interestingly, phenylbutyric acid (PBA), an ER chemical chaperone, attenuated Cd-induced ER stress and protected against germ cell apoptosis in testes. In addition, PBA significantly attenuated Cd-evoked release of cytochrome c from mitochondria to cytoplasm in testes. Taken together, these results suggest that crosstalk between ER stress signaling and mitochondrial pathway mediates Cd-induced testicular germ cell apoptosis.

Key Words: **cadmium; testis; apoptosis; germ cell; endoplasmic reticulum stress.**

Cadmium (Cd) is one of major occupational and environmental toxicants. The general population is exposed to Cd via drinking water, food, and cigarette smoking (Honda *et al.*, 2010). Cd is a reproductive toxicant in human. Increasing evidence demonstrated that Cd is associated with male infertility and the poor semen quality in humans (Pant *et al.*, 2003). According to several earlier studies, there was a significant inverse correlation between blood Cd level and semen quality (Telisman *et al.*, 2000; Xu *et al.*, 1993). Even a low level of Cd accumulation in semen might contribute to male infertility by reducing sperm quality (Wu *et al.*, 2008). Cd is a testicular toxicant in rodent animals (Siu *et al.*, 2009). Several studies have demonstrated that Cd induces testicular germ cell apoptosis (Kim and Soh, 2009; Ozawa *et al.*, 2002). However, the molecular mechanisms of Cd-induced testicular germ cell apoptosis remain poorly understood.

The signaling events leading to apoptosis can be divided into two major pathways, either Fas/FasL (extrinsic) or mitochondrial (intrinsic) pathway. The Fas system is an important regulator of germ cell apoptosis in testes. Several reports showed that the expression of Fas and FasL in testes was significantly increased following heat exposure (Miura *et al.*, 2002; Yin *et al.*, 2002). Other studies demonstrated that some reproductive toxicants, such as mono-(2-ethylhexyl) phthalate, diethylstilbestrol, bisphenol A, lindane, p,p'-DDE, and fenvalerate, evoked testicular germ cell apoptosis through the Fas/FasL pathway (Giammona *et al.*, 2002; Li *et al.*, 2009; Nair and Shaha, 2003; Saradha *et al.*, 2009; Shi *et al.*, 2010; Zhao *et al.*, 2011). Mitochondrial signaling is another important apoptotic pathway. Several earlier studies found that mitochondrial signaling pathway was involved in the process of heat-induced germ cell apoptosis in testes (Hikim *et al.*, 2003; Vera *et al.*, 2004). Recent reports showed that some reproductive toxicants induced testicular germ cell apoptosis through the mitochondrial pathway (Saradha *et al.*, 2009; Vaithinathan *et al.*, 2010; Yeh *et al.*, 2007).

Endoplasmic reticulum (ER) is an important organelle required for cell survival and normal cellular function (Ferri and Kroemer, 2001; Rao *et al.*, 2004). In the ER, nascent proteins are folded with the assistance of ER chaperones. If the client protein load is excessive compared with the reserve of ER chaperones, ER stress occurs. ER stress causes unfolded and misfolded proteins retained in the ER. Accumulation of unfolded and misfolded proteins that aggregate in the ER lumen causes activation of a signal response termed the unfolded protein response (UPR) (Wu and Kaufman, 2006). The UPR is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE)1, activating transcription factor (ATF)6, and double-stranded RNA-activated kinase (PKR)-like ER kinase

(PERK) (Kohno, 2007). Increasing evidence demonstrated that ER stress was involved in the process of apoptosis in somatic cells (Kim *et al*., 2008). However, whether ER stress mediates Cd-induced germ cell apoptosis in testes is unknown. In the present study, we found that acute Cd exposure caused ER stress in testes. We demonstrated for the first time that crosstalk between ER stress signaling and mitochondrial pathway might contributes to Cd-evoked germ cell apoptosis in testes.

MATERIALS AND METHODS

Chemicals and reagents. $CdCl_2$ and phenylbutyric acid (PBA) were from Sigma Chemical Co. (St Louis, MO). Caspase-3, Fas, FasL, Bcl-2, Bax, cytochrome c and caspase-9, XBP-1, and Lamin A/C antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). GRP78, caspase-3, caspase-9, caspase-12, CCAAT/enhancer binding protein (C/EBP), homologous protein (CHOP), phosphor-JNK, and phosphor-eIF2α antibodies were from Cell Signaling Technology (Beverley, MA). β-actin antibody was from Boster Bio-Technology Co. LTD (Wuhan, China). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, OH). RNase-free DNase was from Promega Corporation (Madison, WI). All other reagents were purchased from Sigma Chemical Co. if not otherwise stated.

Animals and treatments. Adult male CD-1 mice (8 week old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25°C) and humidity (50 ± 5%) environment. Mice were intraperitoneally (ip) injected with different doses of $CdCl_2$ (0.5, 1, or 2.0 mg/kg). Testes were collected at different time (0, 12, 24, and 48 h) after Cd. To investigate protective effects of PBA on Cd-induced testicular germ cell apoptosis, 48 mice were divided into four groups randomly. In Cd alone group, all mice were ip injected with $CdCl_2$ (2.0 mg/kg). In PBA+Cd group, mice were ip injected with PBA (100 mg/kg) every 8 h, beginning at 8 h before Cd (2.0 mg/kg, ip). In PBA alone group, mice were ip injected with PBA (100 mg/kg) every 8 h. Saline-treated mice served as controls. Testes were collected at 24 h after Cd. The testes were divided in two parts: left one was kept at –80°C for Western blotting and reverse transcription (RT)-PCR. The other part of the testes was immersed in modified Davidson's fluid (mDF) for 24 h for testicular histology and apoptosis analysis. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Terminal dUTP nick-end labeling (TUNEL) staining. For the detection of apoptosis, paraffin-embedded sections were stained with the TUNEL technique using an *in situ* apoptosis detection kit (Promega) according to the manufacturer's protocols. To assess apoptosis in testicular cells, 200 different seminiferous tubules were observed in predetermined different fields in each section at a magnification of ×400. A histogram of the number of TUNEL-positive germ cells per seminiferous tubule and the percentages of the number of seminiferous tubules containing TUNEL-positive germ cells were analyzed.

Subcellular fractionation and Western blotting. Cytosolic and mitochondrial fractions were prepared as described previously (Johnson *et al*., 2008; Vera *et al*., 2004). Briefly, saline-perfused testes were homogenized using a dounce homogenizer in 3 ml buffer A (0.25M sucrose, 50mM Hepes, 10mM NaCl, 10mM EDTA, and 2mM dithiothreitol) supplemented with protease inhibitors (Complete Protease Inhibitors; Roche, Indianapolis, IN). The crude homogenates were centrifuged at 1000 × g for 10 min at 4°C and the resultant supernatant centrifuged at 10,000 × g for 15 min at 4°C to sediment the low-speed fraction containing mainly mitochondria. The mitochondria were washed two times in buffer A and pelleted. The cytosolic and high-speed fractions were isolated following centrifugation of the 10,000 × g supernatant fraction at 100,000 × *g* for 60 min at 4°C. The resulting supernatant was the cytosolic fraction. Western blotting was performed using testicular lysates and subcellular fractions. In brief, protein extracts from each sample were added to a gel loading buffer (100mM Tris, pH 6.8, 20% glycerol, 200mM DTT, 4% SDS, and 0.03% bromophenol blue) and boiled for 10 min. Proteins (50 μg/sample) in loading buffer were subjected to electrophoresis in 10–15% SDS-polyacrylamide gel for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) and blocked in 5% nonfat powdered milk in Dulbecco's PBS (DPBS) overnight at 4°C. The membranes were incubated for 2 h with the following primary antibodies: Fas, FasL, Bcl-2, Bax, cytochrome c, caspase-3, caspase-9, caspase-12, CHOP, p-JNK, GRP78, and p-eIF2α. β-actin was used as a loading control for total protein. Prohibitin was used as a loading control for mitochondrial protein. Lamin A/C was used as a loading control for nuclear protein. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody (Santa Cruz Biotechnology) for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce (Pierce Biotechnology, Rockford, IL).

Isolation of total RNA and RT-PCR. Fifty milligrams testis was collected from each mouse. Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RNase-free DNase was used to remove genomic DNA. The integrity and concentration of RNA was determined by measuring absorbance at 260 nm followed by electrophoresis on agarose gels. For the synthesis of cDNA, 1.0 μg of total RNA from each sample was resuspended in a 20-μl final volume of reaction buffer, which contained 25mM Tris HCl, pH 8.3, 37.5mM KCl, 10mM dithiothreitol, 1.5mM $MgCl_2$, 10mM of each dNTP, and 0.5 mg oligo$(dT)_{15}$ primer. After the reaction mixture reached 42°C, 20 units of RT was added to each tube, and the sample was incubated for 60 min at 42°C. RT was stopped by denaturing the enzyme at 95°C. The final PCR mixture contained 2.5 μl of cDNA, 1 × PCR buffer, 1.5mM $MgCl_2$, 200μM dNTP mixture, 1U of Taq DNA polymerase, 1μM sense and antisense primers, and sterile water to 50 μl. The reaction mixture was covered with mineral oil. PCR for glyceraldehyde 3-phosphate dehydrogenase was performed on each individual sample as an internal positive-control standard. Specific primers were synthesized by Shanghai Sangon Biological Engineering Technology (Shanghai). Oligonucleotide sequence, annealing temperature, and the number of cycles were listed in Table 1. The amplified PCR products were subjected to electrophoresis at 75 V through 1.5% agarose gels (Sigma, St Louis, MO) for 45 min. The pBR322 DNA digested with Alu I was used for molecular markers (MBI Fermentas). Agarose gels were stained with 0.5 mg/ml ethidium bromide (Sigma) TBE buffer.

Testicular histology and immunohistochemistry. Two cross sections from each testis were embedded in parafilm using standard procedures performed by Pathological Lab at Anhui Medical University. Paraffin-embedded tissues were serially sectioned. At least two nonserial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses. For immunohistochemistry, 5 μm of sections were mounted onto coated slides, dewaxed, and rehydrated. Antigen retrieval was performed by pressure cooking slides for 5 min in 0.01M citrate buffer (pH 6.0). Slides were incubated for 30 min in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris-buffered saline (TBS; 0.05M Tris, 0.85% [wt/vol] NaCl [pH 7.4]). Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (wt/vol) bovine serum albumin before the addition of rabbit polyclonal antibody GRP78 (Cell Signaling Technology) and overnight incubation at 4°C. After washing in TBS, slides were incubated for 30 min with the appropriate secondary antibody conjugated to biotin goat anti-rabbit (Santa Cruz), diluted 1:500 in the blocking mixture. This was followed by 30 min incubation with horseradish peroxidase-labeled avidin-biotin complex (Dako). Immunostaining

TABLE 1
Primers, Annealing Temperature, and the Number of Cycles for PCR

Name	Sequence	Annealing (°C)	Number of cycles
GAPDH	F: 5′-GAGGGGCCATCCACAGTCTTC-3′ R: 5′-CATCACCATCTTCCAGGAGCG-3′	56	35
Bim	F: 5′-CATCGTCGCCGTCACCGGAT-3′ R:5′-GTCGTATGGAAGCTTGCGGTTCT-3′	62	32
Bax	F: 5′-ACAGATCATGAAGACAGGGG-3′ R: 5′-CAAAGTAGAAGAGGGCAACC-3′	54	32
Bad	F: 5′-TCCGGAGCCTGGGGAGCGACGCGGG3′ R: 5′-CTCATCGCTCATCCTTCGGAGCTC-3′	62	32
Bcl-2	F: 5′-TTCTCCTTCCAGCCTGAGAGCAA-3′ R: 5′-ATGACCCCACCGAACTCAAAG-3′	57	32
Bcl-xl	F: 5′-CATCAATGGCAACCCATCCTG-3′ R: 5′-TGTTCCCGTAGAGATCCACAAAAG-3′	57	32
Bcl-w	F: 5′-GATTCCGGATCCCGGCTGCG-3′ R: 5′-CATCGTCGCCGTCACCGGAT-3′	62	32

Note. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

was developed by application of diaminobenzidine (liquid DAB^+; Dako), and slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, U.K.).

Statistical analysis. All quantified data were expressed as means ± SEM. ANOVA and the Student-Newmann-Keuls *post hoc* test were used to determine differences among different groups.

RESULTS

A Single Dose of Cd Induces Germ Cell Apoptosis in Testes

To investigate the time-course for Cd-induced germ cell apoptosis, mice were injected with 2.0 mg/kg of $CdCl_2$, and germ cell apoptosis was detected at different times. As shown in Figure 1, a single dose of Cd induced germ cell apoptosis in seminiferous tubules in a time-dependent manner. Most of apoptotic cells were observed at 24 and 48 h after Cd (Figs. 1A and 1B). Next, the percentage of tubules with TUNEL+ germ cells and the number of apoptotic cells per tubules were analyzed. The percentage of tubules with TUNEL+ germ cells was highest at 24 h after Cd (Fig. 1C). In addition, the number of apoptotic cells per tubule was highest at 24 h after Cd (Fig. 1D). To investigate the dose-effect relationship for Cd-induced germ cell apoptosis, mice were injected with different doses of $CdCl_2$, and germ cell apoptosis was detected at 24 h after Cd. As shown in Figures 2A and 2B, most of apoptotic cells were observed in testes of mice treated with 2.0 mg/kg Cd. Further analyses showed that the percentage of tubules with TUNEL+ germ cells and the number of apoptotic cells per tubules were highest in mice treated with 2.0 mg/kg $CdCl_2$ (Figures 2C and 2D).

Effects of Acute Cd Exposure on Fas/FasL and Mitochondrial Pathway

The effects of acute Cd exposure on testicular Fas/FasL pathway were analyzed. As shown in Figure 3, acute Cd exposure did not affect the expression of Fas in testes (Figs. 3A and 3C). In addition, acute Cd exposure had little effect on the level of FasL in testes (Figs. 3B and 3D). The release of cytochrome c from mitochondria and subsequent activation of caspase-9 represent a key step in the mitochondrion-dependent apoptotic pathway (Jiang and Wang, 2004). The abundance of mitochondrial and cytosolic cytochrome c was analyzed. As shown in Figures 4A and 4C, acute Cd exposure significantly increased the level of cytosolic cytochrome c in a time- and dose-dependent manner. By contrast, the level of mitochondrial cytochrome c in testes was significantly decreased in Cd-treated mice (Figs. 4B and 4D). The effects of Cd on testicular caspase-9 and caspase-3 were then analyzed. As expected, the level of active caspase-9 in testes was significantly increased in Cd-treated mice (Figs. 4E and 4F). Correspondingly, the level of active caspase-3 in testes was significantly increased in Cd-treated mice (Figs. 4G and 4F). Bcl-2 family proteins regulate mitochondrial outer membrane permeabilization (Garrido *et al*., 2006). As shown in Figure 5, the expression of bim, bax and bad, several proapoptotic genes, was upregulated in testes of Cd-treated mice (Figs. 5A, 5B, and 5C). In addition, the level of testicular Bax protein was significantly increased in Cd-treated mice (Supplementary fig. 1A). Interestingly, testicular bcl-2, an antiapoptotic gene, was slightly upregulated in Cd-treated mice (Fig. 5D). Correspondingly, the level of testicular Bcl-2 protein was slightly increased in Cd-treated mice (Supplementary fig. 1B). Finally, the effects of Cd on the level of mitochondrial bax were analyzed. As shown in Figures 5G and 5H, acute Cd exposure significantly increased the level of mitochondrial bax in a dose- and time-dependent manner.

Acute Cd Exposure Induces ER Stress in Testes

To examine whether acute Cd exposure induces ER stress in testes, we analyzed the expression of testicular ER chaperones. The level of testicular GRP78, an ER chaperone and ATF6 target, was significantly increased in Cd-treated mice (Fig. 6A). Further analysis showed that GRP78 was expressed in germ cells

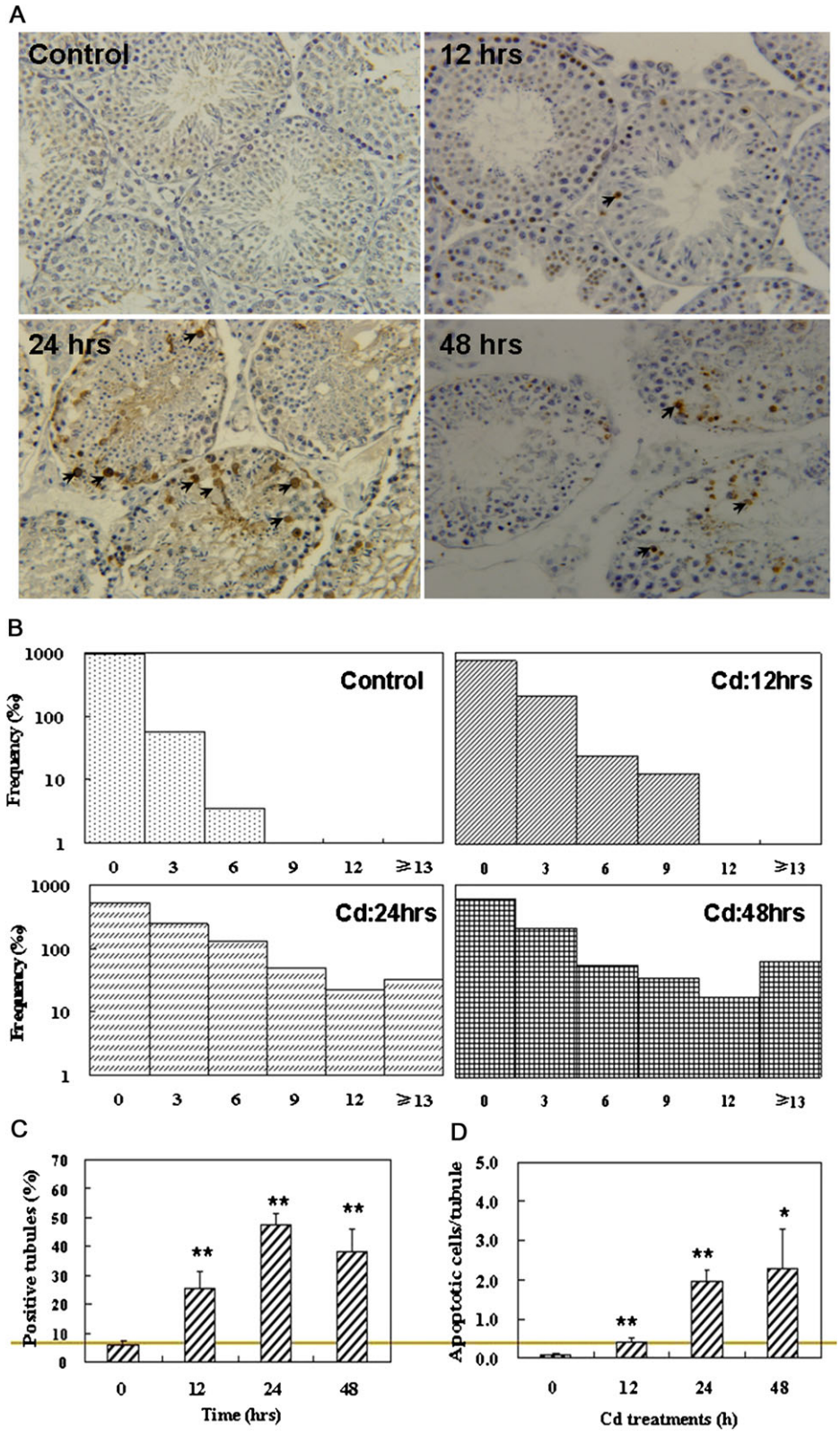

FIG. 1. Acute Cd exposure induces testicular germ cell apoptosis in a time-dependent manner. Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (12, 24, or 48h) after Cd. (A) Germ cell apoptosis was detected by TUNEL staining. Arrow showed apoptotic germ cells. (B) A histogram of the number of TUNEL-positive germ cells per seminiferous tubule. (C) The percentage of seminiferous tubules containing TUNEL-positive germ cells. (D) The number of apoptotic cells per tubule. All data were expressed as means ± SEM ($n = 12$). *$p < 0.05$, **$p < 0.01$ as compared with controls.

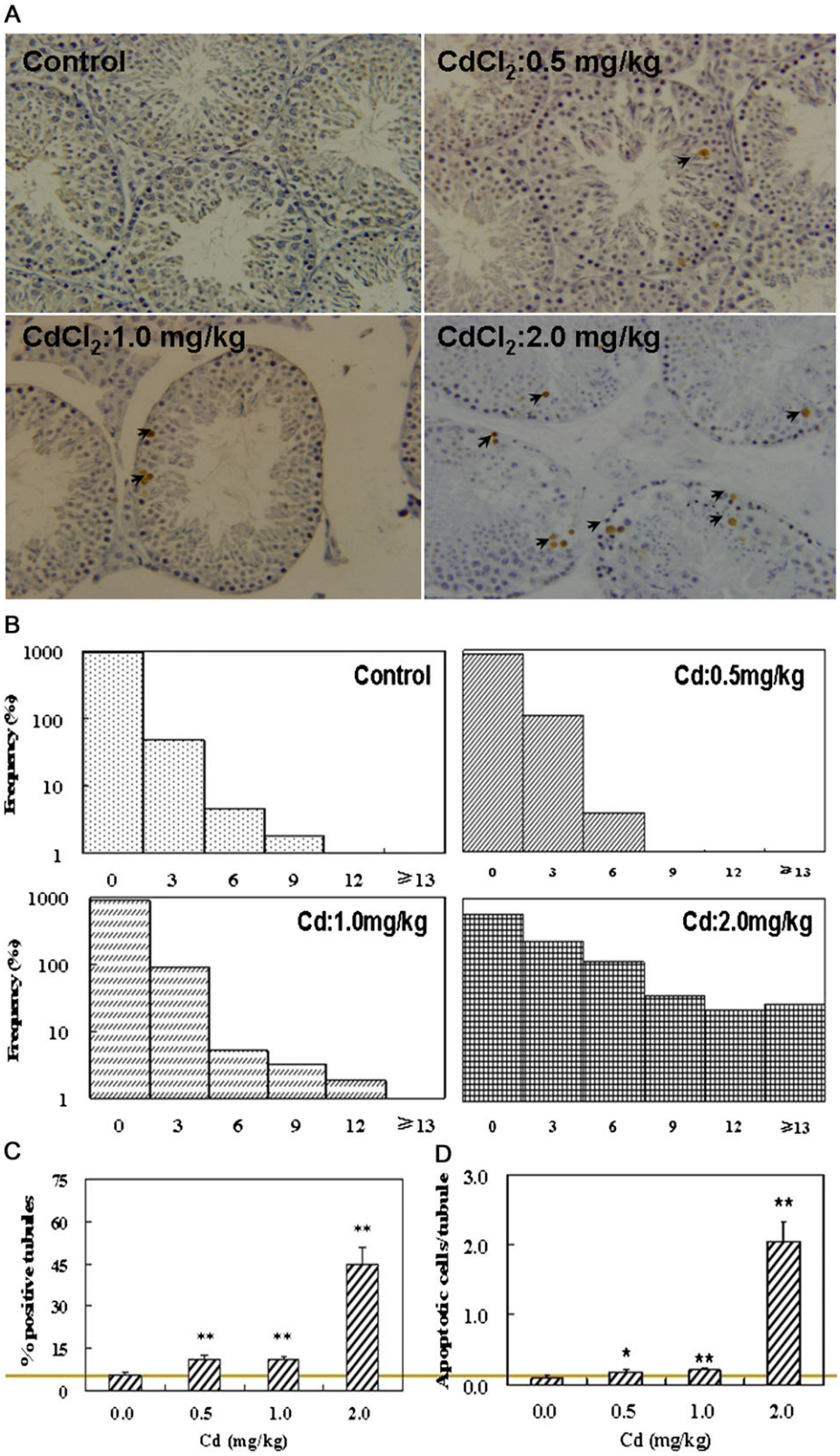

FIG. 2. Acute Cd exposure induces testicular germ cell apoptosis in a dose-dependent manner. Mice were injected with different doses of $CdCl_2$ (0.5, 1.0, or 2.0 mg/kg). Testes were collected at 24h after Cd treatment. (A) Germ cell apoptosis was detected by TUNEL staining. Arrow showed apoptotic germ cells. (B) A histogram of the number of TUNEL-positive germ cells per seminiferous tubule. (C) The percentage of seminiferous tubules containing TUNEL-positive germ cells. (D) The number of apoptotic cells per tubule. All data were expressed as means ± SEM ($n = 12$). *$p < 0.05$, **$p < 0.01$ as compared with controls.

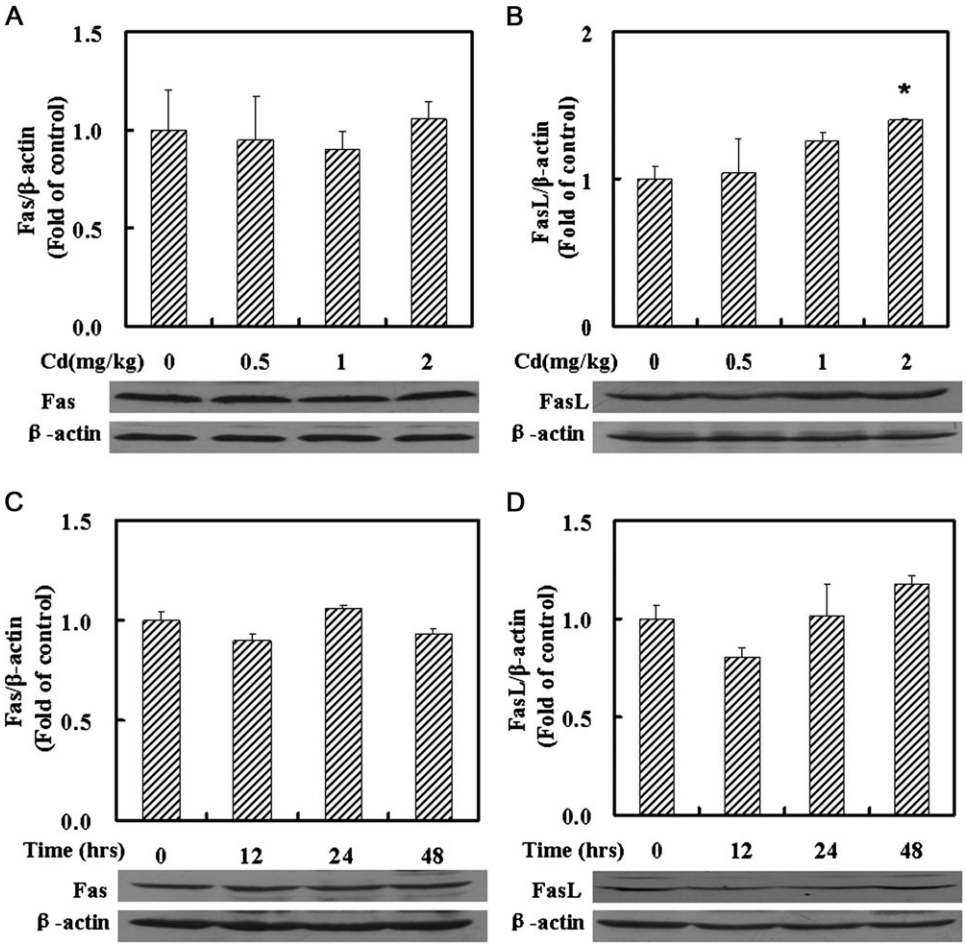

FIG. 3. Effects of acute Cd exposure on the expression of Fas and FasL in testes. (A and B) Mice were injected with different doses of $CdCl_2$ (0.5, 1.0, or 2.0 mg/kg). Testes were collected at 24h after Cd. (C and D) Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (12, 24, or 48h) after Cd. The expression of (A and C) Fas and (B and D) FasL in testes was detected using Western blotting. Fas and FasL were normalized to β-actin level in the same samples. The densitometry unite of the control was assigned as 1. $*p < 0.05$ as compared with controls.

(Fig. 6H). To investigate whether Cd can activate the PERK pathway in testes, phosphorylated eIF2α, a downstream target of the PERK pathway, was analyzed. As expected, the level of phosphorylated eIF2α was increased in testes of mice treated with Cd (Fig. 6B). In addition, testicular CHOP, another downstream target of the PERK pathway, was upregulated in Cd-treated mice (Fig. 6F). To investigate whether the IRE1 branch of the UPR pathway is activated by Cd, mRNA level of sliced X box-binding protein (sXBP)-1 in testes was measured. As shown in Figure 6C, mRNA level of sXBP-1 in testes was significantly increased in Cd-treated mice. In addition, a single dose of Cd significantly increased the level of nuclear XBP-1 (Fig. 6D). Finally, JNK, a downstream target of the IRE1 pathway, was analyzed. As shown in Figure 6E, the level of phosphorylated JNK in testes was significantly increased in Cd-treated mice.

Cd-Evoked Germ Cell Apoptosis Is Associated with ER Stress

To investigate whether Cd-induced germ cell apoptosis is associated with ER stress, PBA, an ER chemical chaperone, was administered to alleviate Cd-induced ER stress. As shown in Figures 7A and 7B, Cd-induced splicing of XBP-1 mRNA and eIF2α phosphorylation in testes were significantly alleviated by PBA. In addition, PBA almost completely inhibited Cd-evoked CHOP upregulation and JNK phosphorylation in testes (Figs. 7C and 7D), indicating that PBA is effective to reduce ER stress in testes. We next investigated the effects of PBA on Cd-induced germ cell apoptosis in testes. As shown in Figure 8A, PBA pretreatment almost completely inhibited Cd-induced testicular germ cell apoptosis. Further analyses showed that PBA pretreatment reduced the frequency of tubules with more than six TUNEL+ germ cells (Fig. 8B). In addition, PBA reduced the percentage of tubules with TUNEL+ germ cells and the number of apoptotic cells per tubules (Figs. 8C and 8D).

Crosstalk Between ER Stress Signaling and Mitochondrial Pathway

Increasing evidence demonstrated that caspase-12 plays a key role in ER stress-mediated apoptosis (Morishima *et al.*,

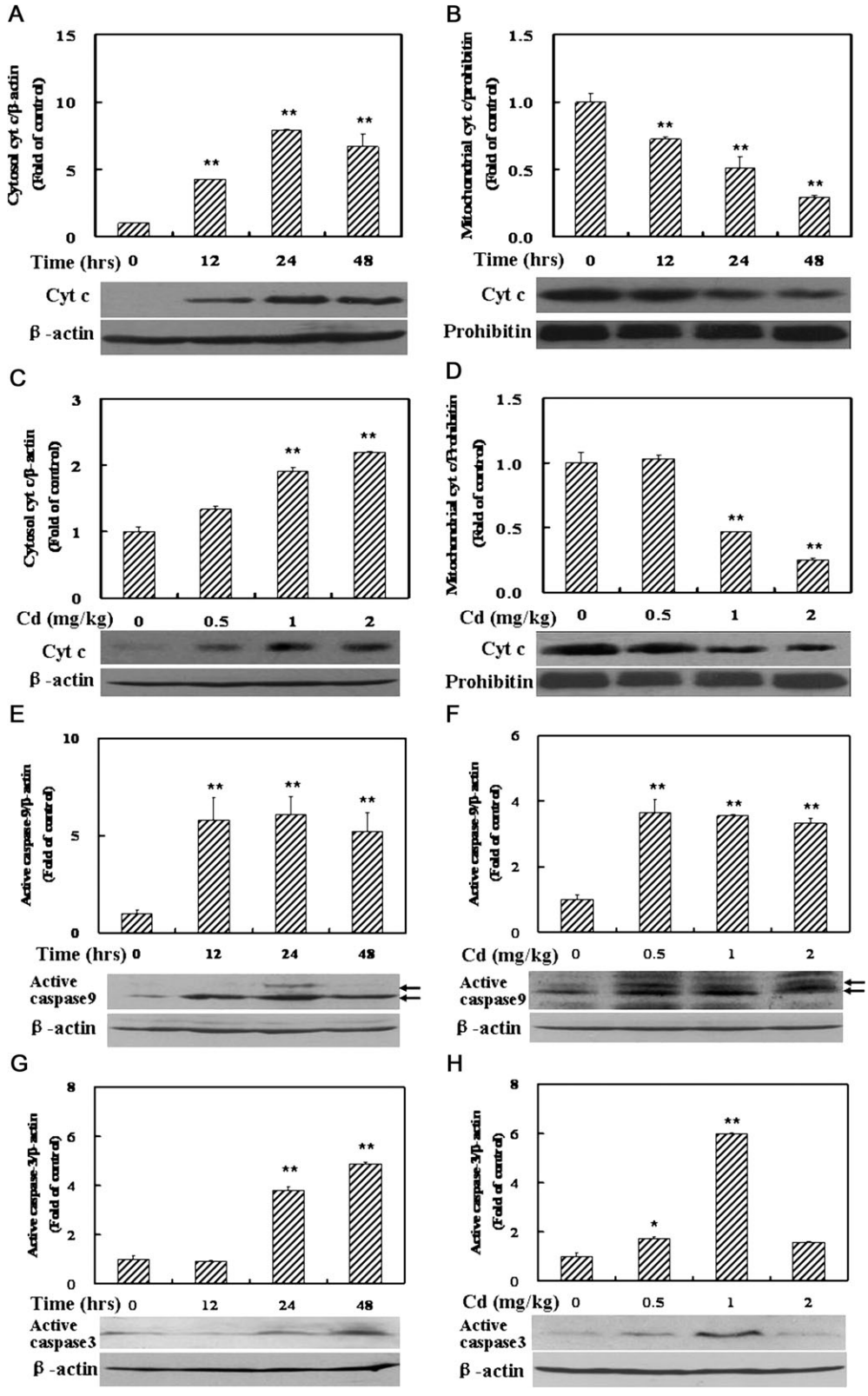

FIG. 4. Effects of acute Cd exposure on mitochondrial signaling pathway. (A, B, E, and G) Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (12, 24, or 48h) after Cd. (C, D, F, and H) Mice were injected with different doses of $CdCl_2$ (0.5, 1.0, or 2.0 mg/kg). Testes were collected at 24h after Cd. (A and C) Cytosol and (B and D) mitochondrial cytochrome c were measured using Western blotting. (E and F) Active caspase-9 and (G and H) active caspase-3 were measured using Western blotting. All data were expressed as means ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$ as compared with controls.

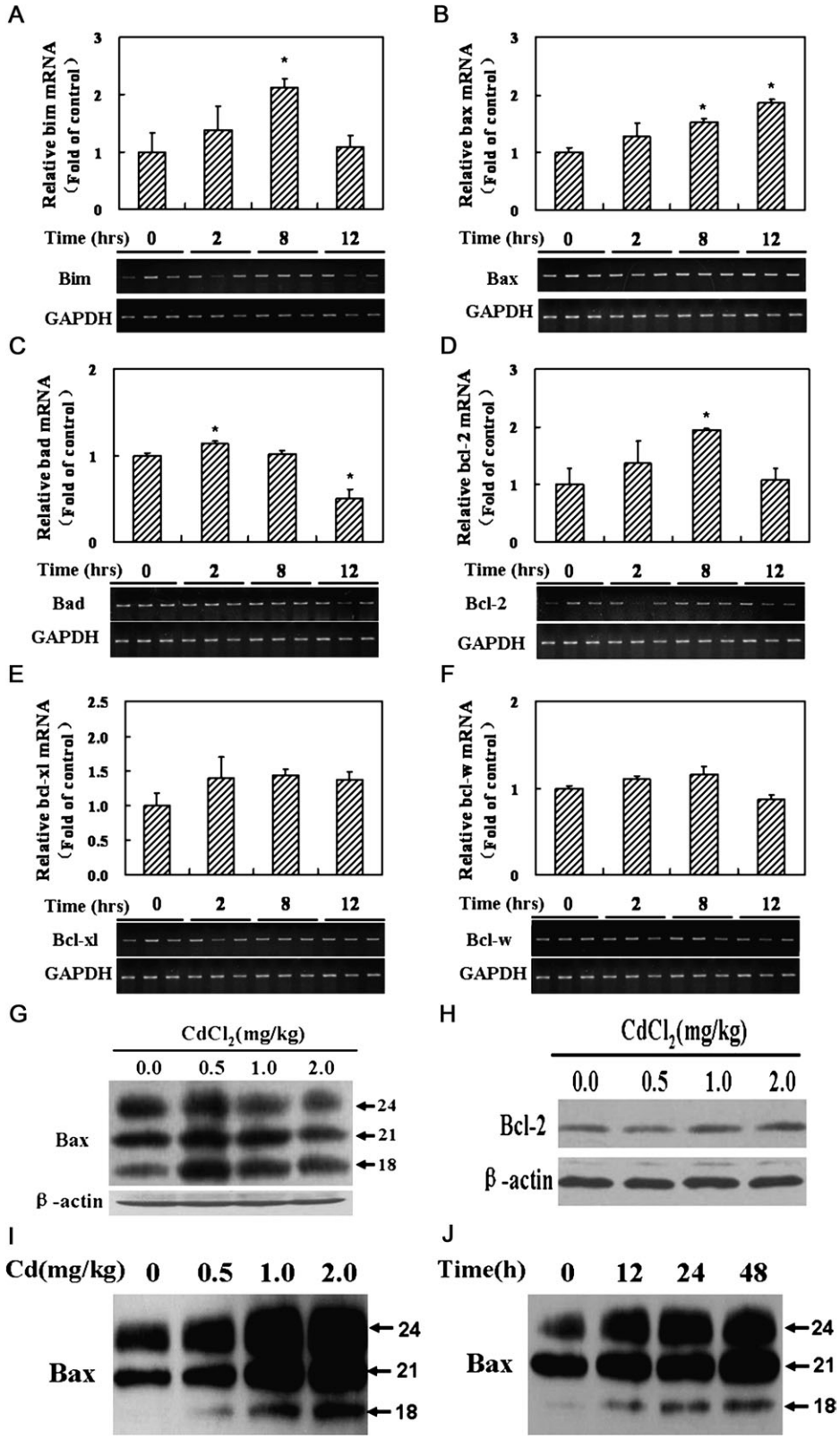

FIG. 5. Effects of acute Cd exposure on the expression of Bcl family in testes. (A–F) Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (2, 8, or 12h) after Cd. The expression of testicular bim, bax, bad, bcl-2, bcl-xl, and bcl-w was measured using RT-PCR. All data were expressed as means ± SEM ($n = 6$). (G-I) Male mice were injected with different doses of $CdCl_2$ (0.5, 1.0, or 2.0 mg/kg). Testes were collected at 24 h after Cd treatment. (G and H) The expression of testicular Bax and Bcl-2 protein was measured using Western blotting. (I) Mitochondrial bax was measured using Western blotting. Data were expressed as means ± SEM ($n = 4$). (J) Male mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (12, 24, or 48h) after Cd. Mitochondrial bax was measured using Western blotting. All data were expressed as means ± SEM ($n = 4$). *$p < 0.05$ as compared with controls.

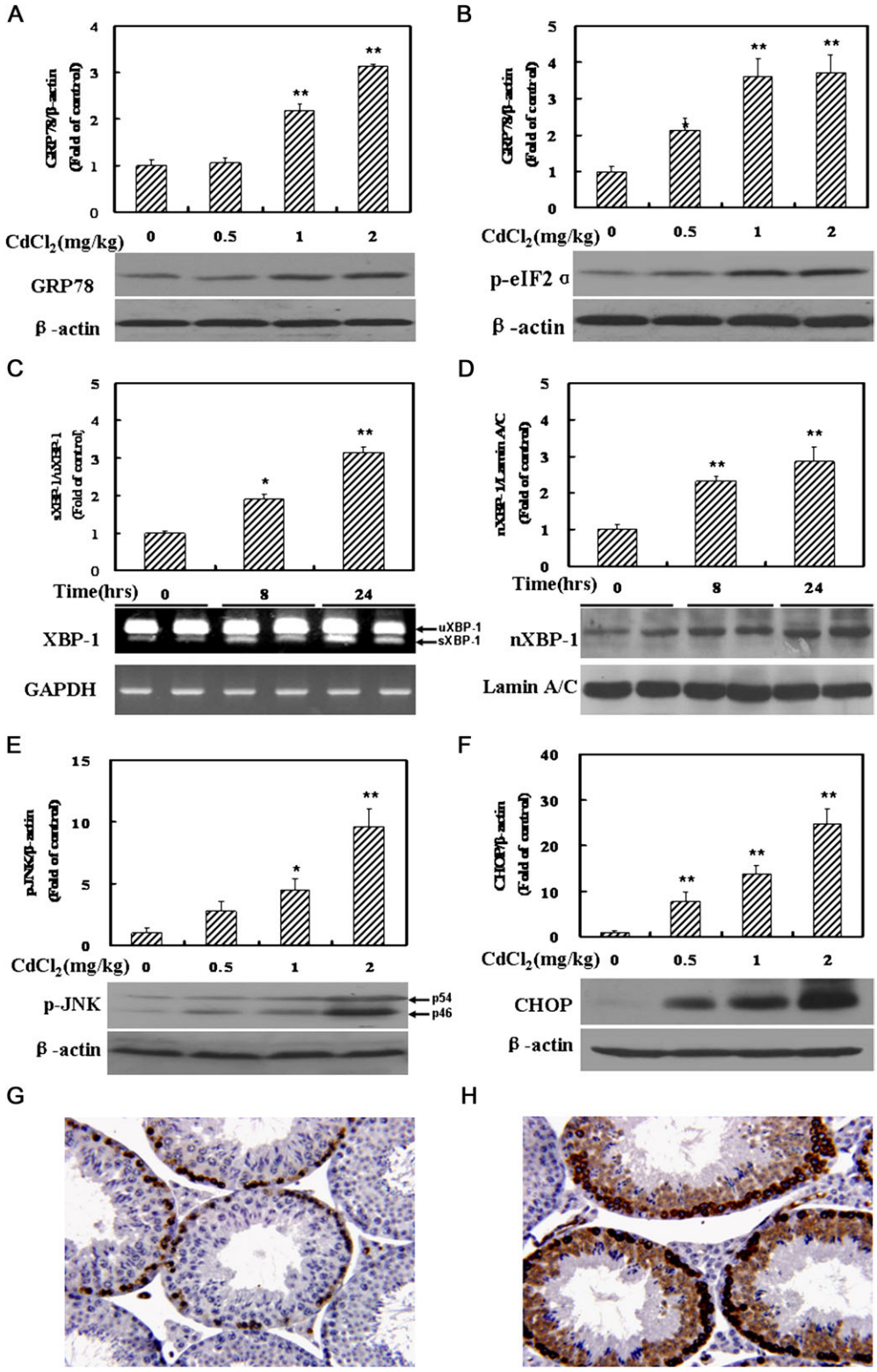

FIG. 6. Cd-induced ER stress in testes. (A, B, E, and F) Mice were injected with different doses of $CdCl_2$ (0.5, 1.0, or 2.0 mg/kg). Testes were collected at 24h after Cd. (A) GRP78, (B) p-eIF2α, (E) p-JNK, and (F) CHOP in testes were measured using Western blotting. Data were expressed as means ± SEM ($n = 4$). (C and D) Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at different times (8 or 24h) after Cd. (C) uXBP-1 and sXBP-1 were measured using RT-PCR. (D) Nuclear XBP-1 was measured using Western blotting. Data were expressed as means ± SEM ($n = 6$). (G and H) Mice were injected with $CdCl_2$ (2.0 mg/kg). Testes were collected at 24h after Cd. Testicular GRP78 protein was measured using immunohistochemistry. (G) control; (H) Cd. $^*p < 0.05$, $^{**}p < 0.01$ as compared with controls.

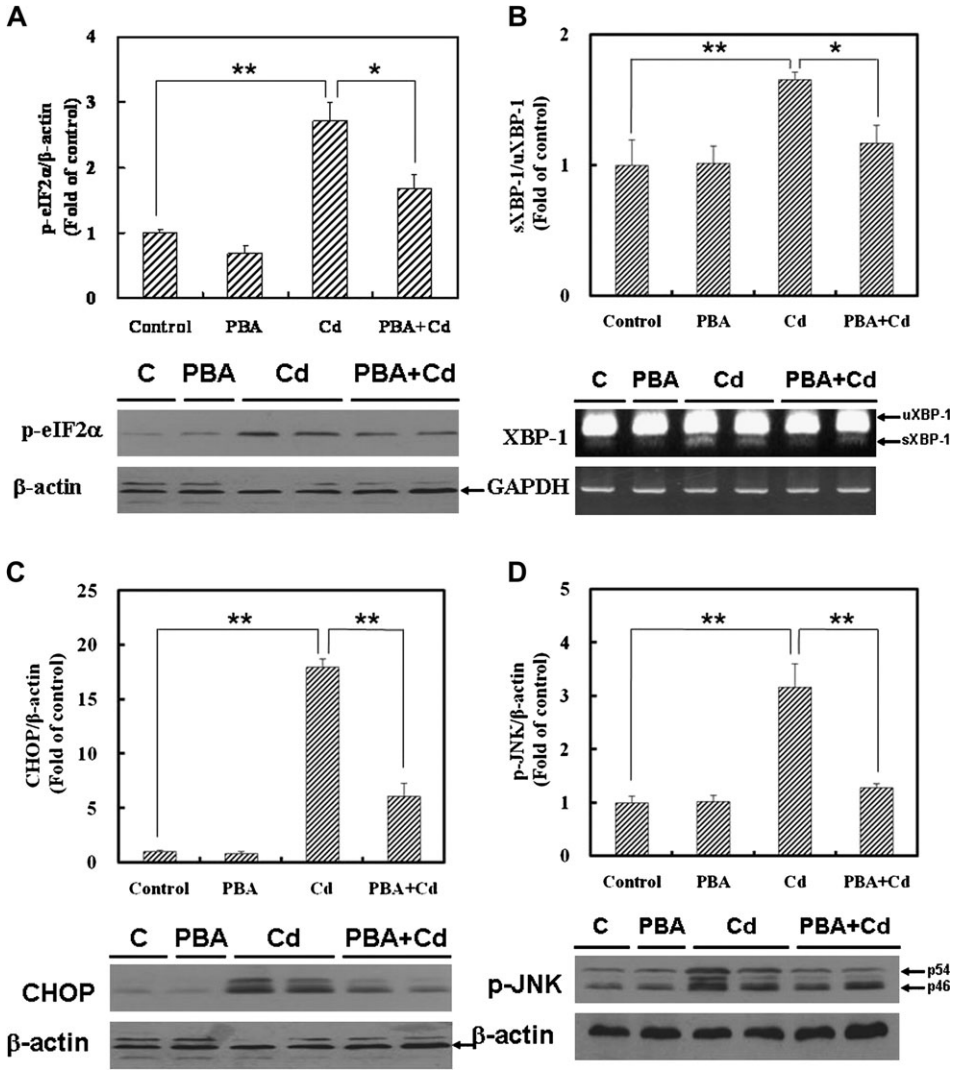

FIG. 7. Effects of PBA on Cd-induced UPR in testes. Mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with PBA as in the ''Materials and Methods.'' Testes were collected at 24h after Cd. Testicular (A) p-eIF-2α, (C) CHOP, and (D) p-JNK were measured using Western blotting. (B) uXBP-1 and sXBP-1 were measured using RT-PCR. All data were expressed as means ± SEM ($n = 3$–6). *$p < 0.05$, **$p < 0.01$ as compared with controls or Cd group.

2002; Nakagawa *et al*., 2000). In the present study, no cleaved caspase-12 was detected in testes of Cd-treated mice (data not shown). In addition, no significant difference on the level of procaspase-12 in testes was observed among different groups (data not shown). An earlier study showed that Leydig cell–derived heme oxygenase-1 regulates testicular germ cell apoptosis. The present study showed that testicular HO-1 expression was upregulated in Cd-treated mice (Supplementary fig.1). To investigate whether there is an association between ER stress and HO-1 upregulation, the effects of PBA on Cd-induced HO-1 upregulation were analyzed. As shown in Supplementary fig. 1, PBA had no effect on Cd-induced HO-1 upregulation. To investigate whether there is an association between ER stress and Fas/FasL pathway, the effects of PBA on the expression of Fas and FasL in testes were analyzed. As shown in Supplementary fig. 2, PBA had no effect on the expression of Fas and FasL in testes. To investigate whether there is an association between ER stress and mitochondrial pathway, we analyzed the effects of PBA on Cd-induced release of cytochrome c from mitochondria in testes. As shown in Figure 9A, Cd-induced elevation of cytosolic cytochrome c was almost completely inhibited by PBA. In addition, Cd-induced reduction of mitochondrial cytochrome c was significantly alleviated when mice were pretreated with PBA (Fig. 9B).

DISCUSSION

Increasing evidence demonstrated that ER stress was involved in the process of β-cell apoptosis in the pancreas

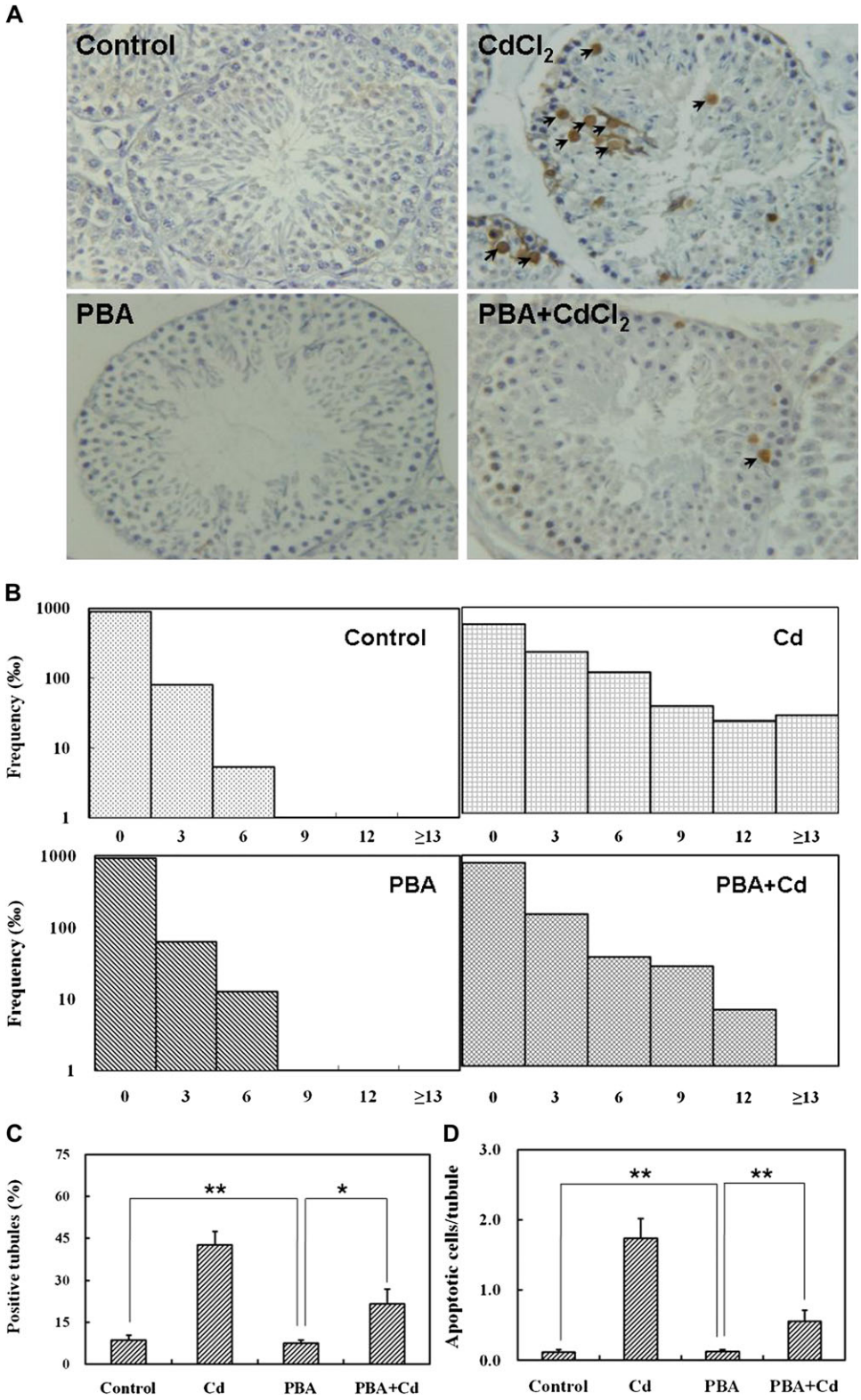

FIG. 8. Effects of PBA on Cd-induced testicular germ cell apoptosis. (A) Mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with PBA as in the ''Materials and Methods.'' Testes were collected at 24h after Cd. Germ cell apoptosis was detected by TUNEL staining. Arrow showed apoptotic germ cells. (B) A histogram of the number of TUNEL-positive germ cells per seminiferous tubule. (C) Percentages of the number of seminiferous tubules containing TUNEL-positive germ cells. (D) The number of apoptotic cells per tubule. All data were expressed as means ± SEM ($n = 12$). *$p < 0.05$, **$p < 0.01$ as compared with controls or Cd group.

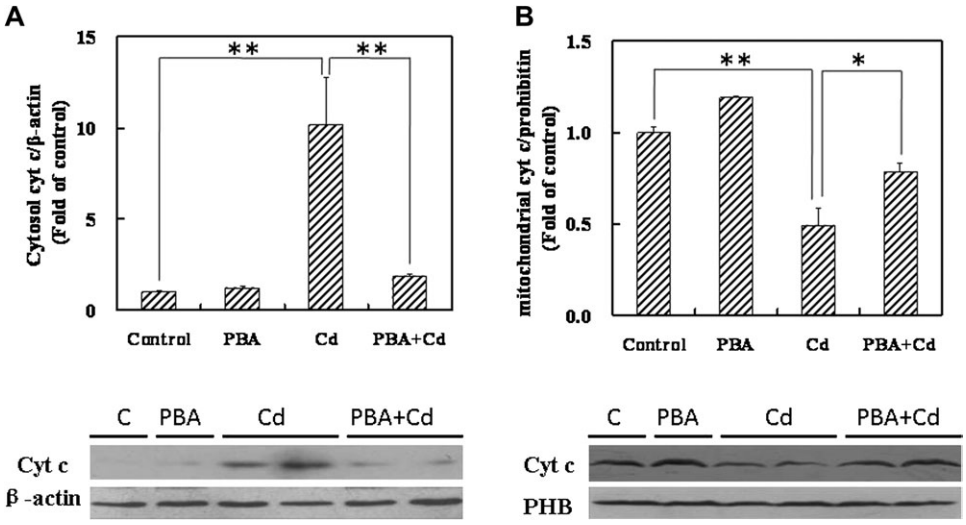

FIG. 9. Effects of PBA on Cd-induced release of mitochondrial cytochrome c in testes. Mice were injected with $CdCl_2$ (2.0 mg/kg). Some mice were administered with PBA as in the ''Materials and Methods.'' Testes were collected at 24h after Cd. (A) Cytosol and (B) mitochondrial cytochrome c were measured using Western blotting. All data were expressed as means ± SEM ($n = 3–6$). *$p < 0.05$, **$p < 0.01$ as compared with controls or Cd group.

(Oyadomari *et al*., 2001; Song *et al*., 2008), neuronal cell apoptosis in the brain (Galehdar *et al*., 2010; Higo *et al*., 2010), preadipocyte apoptosis in adipose tissue (Guo *et al*., 2007), renal epithelial cell apoptosis in the kidney (Timmins *et al*., 2009; Yokouchi *et al*., 2007), and myocardial cell apoptosis in the heart (Avery *et al*., 2010). The present study found for the first time that a single dose of $CdCl_2$ could activate the UPR signaling in testes in a dose- and time-dependent manner. First, GRP78, an important ER molecular chaperone, was significantly upregulated in testes of Cd-treated mice. Second, the level of phosphorylated eIF2α, a downstream target of the PERK pathway, was significantly increased, indicating that PERK pathway was activated by Cd. Third, the level of the sliced XBP-1 mRNA in testes was significantly increased. In addition, nuclear XBP-1 level was significantly increased in nuclear extracts of testes, suggesting that IRE-1α signaling pathway was activated by Cd. PBA is a chemical chaperone that stabilizes protein conformation and improves ER protein folding capacity. Several studies showed that PBA inhibited ER stress-mediated apoptosis (Qi *et al*., 2004; Wiley *et al*., 2010). Indeed, the present study found that PBA obviously attenuated Cd-induced ER stress in testes. Importantly, PBA significantly alleviated Cd-evoked germ cell apoptosis in testes. These results suggest that ER stress is mediated, at least in part, in Cd-induced germ cell apoptosis in testes.

Caspase-12 is known to be essential for ER stress-induced apoptosis in rodent animals. An earlier study showed that caspase-12 was activated in ER stress-mediated apoptosis (Nakagawa *et al*., 2000). Additional study demonstrated that ER stress triggered a specific cascade involving caspase-12, -9, and -3 in a cytochrome c-independent manner (Morishima *et al*., 2002). By contrast, recent studies found that ER stress-mediated apoptosis is a caspase-dependent process that does not require the expression of caspase-12 (Di Sano *et al*., 2006; Obeng and Boise, 2005). To investigate whether caspase-12 is involved in Cd-evoked and ER stress-mediated testicular germ cell apoptosis, procaspase-12 and cleaved caspase-12 were measured in testes of Cd-treated mice. Surprisingly, testicular caspase-12 in testes was not activated by Cd. These results suggest that Cd-evoked and ER stress-mediated testicular germ cell apoptosis is independent of caspase-12 activation.

CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is a downstream target of the PERK pathway (Oyadomari and Mori, 2004). Numerous reports have demonstrated that CHOP is one of the components in the ER stress-mediated apoptotic pathway (Lin *et al*., 2007; Oyadomari *et al*., 2002; Tajiri *et al*., 2004; Tsutsumi *et al*., 2004; Zinszner *et al*., 1998). The present study found that acute Cd exposure obviously upregulated testicular CHOP expression in a dose-dependent manner. To investigate whether CHOP is involved in Cd-evoked and ER stress-mediated testicular germ cell apoptosis, mice were pretreated with PBA. Interestingly, PBA, a well-known chemical chaperone that could protect against Cd-evoked germ cell apoptosis, significantly attenuated Cd-induced upregulation of CHOP in testes. These results suggest that CHOP might play an important role in Cd-evoked and ER stress-mediated germ cell apoptosis in testes.

How CHOP contributes Cd-evoked and ER stress-mediated testicular germ cell apoptosis remains obscure. Several earlier studies showed that CHOP could promote translocation of Bax from the cytosol to the mitochondria (Gotoh *et al*., 2004). A recent study found that CHOP is involved in ER stress-mediated apoptosis through upregulating the expression of Bim (Puthalakath *et al*., 2007). Indeed, Bim and Bax, two well-known proapoptotic

proteins, play an important role in the release of cytochrome c from the mitochondria to the cytoplasm (Precht *et al*., 2005; Yamaguchi and Wang, 2002). The present study found that acute Cd exposure significantly upregulated the expression of Bim and Bax mRNAs in testes. Importantly, acute Cd exposure exacerbated translocation of Bax from the cytosol to the mitochondria in testes. Moreover, acute Cd exposure evoked the release of cytochrome c from the mitochondria to the cytoplasm. Importantly, PBA, an ER chemical chaperone, not only alleviated Cd-induced ER stress but also attenuated the release of cytochrome c from the mitochondria to the cytoplasm in testes. Taken together, these results suggest that crosstalk between ER stress signaling and mitochondrial pathway mediates Cd-induced testicular germ cell apoptosis. Bax and Bim might function as an executioner in Cd-induced and ER stress-mediated testicular germ cell apoptosis.

In summary, the present study demonstrated for the first time that crosstalk between ER stress signaling and mitochondrial pathway mediates Cd-induced testicular germ cell apoptosis. CHOP-mediated Bim induction and translocation of Bax from the cytosol to the mitochondria might play an important role in the release of cytochrome c from the mitochondria to the cytoplasm.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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