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Atypical, bidirectional regulation of cadmium-induced apoptosis via distinct signaling of unfolded protein response

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Cadmium is a widely distributed nephrotoxic metal that causes renal tubular injury. In this report, we investigated involvement of endoplasmic reticulum (ER) stress and individual unfolded protein responses in cadmium-initiated apoptosis of tubular epithelial cells. Cadmium chloride ($CdCl_2$) induced expression of endogenous ER stress markers, *GRP78*, *GRP94* and *CHOP in vitro* and *in vivo*, and subsequently caused cytological changes typical of apoptosis. Attenuation of ER stress by transfection with ER chaperone *GRP78* or *ORP150* suppressed $CdCl_2$-triggered apoptosis. In response to $CdCl_2$, phosphorylation of RNA-dependent protein kinase-like ER kinase (PERK) and eukaryotic translation initiation factor 2α (eIF2α) was observed. Enhanced phosphorylation of eIF2α attenuated, whereas inhibition of eIF2α exacerbated $CdCl_2$-induced apoptosis. Activating transcription factor 6 (ATF6) was also activated by $CdCl_2$ and blockade of this process suppressed induction of *CHOP* and thereby improved cell survival. $CdCl_2$ also triggered activation of the inositol-requiring ER-to-nucleus signal kinase 1 (IRE1)–X-box-binding protein 1 (XBP1) pathway and inhibition of XBP1 attenuated apoptosis independent of *GRP78* and *CHOP*. c-Jun N-terminal kinase (JNK), another molecule downstream of IRE1, was also phosphorylated by $CdCl_2$ and its inhibition attenuated apoptosis. These results evidenced bidirectional regulation of apoptosis in cadmium-exposed cells. The ATF6 and IRE1 pathways cooperatively caused apoptosis via induction of CHOP, activation of XBP1 and phosphorylation of JNK, and the PERK–eIF2α pathway counteracted the proapoptotic processes.
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Exposure to cadmium via drinking water, foods and cigarette smoke causes accumulation of this metal in a variety of organs, especially in the liver and kidney.[1,2] In particular, the highest concentrations of cadmium are observed in the kidney, the main target of the cadmium-related toxicity.[3,4] In the kidney, accumulation of cadmium occurs mainly in the proximal tubules and other nephron segments are affected only at later stages of intoxication.[5]

Previous investigations suggested that toxic effects of cadmium on the proximal tubules are caused through several mechanisms. *In vitro*, short-term exposure of the cells to cadmium caused disruption of tight and gap junctions without cellular death.[6,7] Longer-term exposure resulted in a decrease in glutathione levels and consequent cellular death,[8] indicating involvement of oxidative stress. *In vivo* oral administration of cadmium in beagles caused apoptosis and regeneration in the proximal convoluted tubules where cadmium is localized predominantly.[9]

Although the accumulative evidence for tubulotoxic effects of cadmium, molecular mechanisms involved in its proapoptotic action are still unclear. Although roles of reactive oxygen species (ROS) have been postulated,[10] involvement of other mechanisms have not been elucidated. Alteration in the levels of c-Myc and Bcl-2 is not causative, as shown in previous reports.[11,12] Recently, Xie *et al.*[13] reported that cadmium suppressed activity of nuclear factor-κB, a well-known antiapoptotic transcription factor, and thereby induced apoptosis of tubular epithelial cells.

Endoplasmic reticulum (ER) stress is involved in the induction of apoptosis that occurs in a wide range of pathological situations including hypoxia and ischemia, viral infection, neurodegenerative disorders, drug-induced tissue injury and metabolic diseases such as diabetes mellitus.[14,15] In the central nervous system, some previous reports suggested a possibility that heavy metals may induce ER stress. For example, manganese, lead and mercury induced expression of 78 kDa glucose-regulated protein (*GRP78*), a marker of ER stress, in cultured dopaminergic neuronal cells, astrocytes and glioma cells, respectively.[16–18] Recently, it has been reported that ER stress was involved in

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Abbreviations: ROS, reactive oxygen species; ER, endoplasmic reticulum; GRP, glucose-regulated protein; UPR, unfolded protein response; PERK, RNA-dependent protein kinase-like ER kinase; ATF6, activating transcription factor 6; IRE1, inositol-requiring ER-to-nucleus signal kinase 1; eIF2α, eukaryotic translation initiation factor 2α; XBP1, X-box-binding protein 1; ERSE, ER stress response element; CHOP, CCAAT/enhancer-binding protein-homologous protein; GADD153, growth arrest and DNA damage-inducible protein 153; TRAF2, tumor necrosis factor receptor-associated factor 2; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; ES-TRAP, ER stress-responsive alkaline phosphatase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; ORP150, 150 kDa oxygen-regulated protein; EGFP, enhanced green fluorescence protein; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; XBP1(U), unspliced form of XBP1; DN, dominant-negative mutant
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paracetamol- and cisplatin-induced apoptosis of renal tubular cells.[19,20] However, roles of ER stress in cadmium-induced renal tubular injury have not been disclosed yet.

ER stress is defined as accumulation of unfolded or misfolded proteins in the ER, which induces a coordinated adaptive program called unfolded protein response (UPR). The UPR alleviates ER stress by suppression of protein synthesis, facilitation of protein folding via induction of ER chaperones (e.g., GRP78) and reinforced degradation of unfolded proteins. If the stress is beyond capacity of the adaptive machinery, cells undergo apoptosis.[21] Currently, three major transmembrane transducers for sensing ER stress are identified in the ER; RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring ER-to-nucleus signal kinase 1 (IRE1). Activation of PERK leads to phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), which causes general inhibition of protein synthesis. In response to ER stress, p90ATF6 transits to the Golgi where it is cleaved by the proteases S1P and S2P, yielding a free cytoplasmic domain that is an active transcription factor p50ATF6. Similarly, activated IRE1 catalyzes removal of a small intron from the mRNA of the gene encoding X-box-binding protein 1 (XBP1). This splicing event creates a translational frameshift in *XBP1* mRNA to produce an active transcription factor. Activated p50ATF6 and XBP1 subsequently bind to the ER stress response element (ERSE) and the UPR element, leading to expression of target genes including ER chaperones *GRP78* and *GRP94*.[21] Based on this current knowledge, the ATF6 and IRE1–XBP1 pathways are generally regarded as antiapoptotic UPR.

During the UPR, however, distinct death signals, as well as survival signals, may also be transduced. For example, expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) (also called growth arrest and DNA damage-inducible protein 153 (GADD153)) is caused by ATF4 that is induced by the PERK–eIF2α pathway. ER stress also activates ER stress-specific caspase-12 (or caspase-4) localized at the ER membrane through an interaction with IRE1 and tumor necrosis factor receptor-associated factor 2 (TRAF2), leading cells to undergo apoptosis. The IRE1–TRAF2 interaction also allows for recruitment and activation of apoptosis signal-regulating kinase 1 (ASK1) and downstream c-Jun N-terminal kinase (JNK), which is involved in a variety of apoptotic pathways.[21] Based on these findings, the PERK–eIF2α and IREI–JNK/caspase-12 pathways have been considered as proapoptotic UPR.

In the present investigation, we first demonstrate that ER stress plays a crucial role in cadmium-induced apoptosis of renal tubular cells. We further examine roles of individual UPR in the regulation of apoptosis and disclose atypical, bidirectional regulation of apoptosis in cadmium-exposed cells.

Results

Induction of ER stress by cadmium. To examine whether cadmium triggers ER stress in renal tubular cells, we investigated effects of cadmium chloride ($CdCl_2$) on the expression of endogenous biomarkers for ER stress. Exposure of LLC-PK1 cells to $CdCl_2$ resulted in the induction of *GRP78*, *GRP94* and *CHOP* in a dose-dependent manner (Figure 1a and b). Time-lapse experiments revealed that induction of these transcripts was observed within 1 h and sustained for at least 5–6 h (Figure 1c). The induction of *GRP78* mRNA was associated with accumulation of GRP78 protein (Figure 1d). Similarly, induction of *GRP78* (and *GRP94*) was also observed in response to maximum, non-toxic concentrations of $(CH_3COO)_2Pb_3$, $HgCl_2$, $CuSO_4$, $(CH_3COO)_2Ni_4$ and $CoCl_2$ (Supplementary Figure 1a and b).

We recently reported that ER stress-responsive alkaline phosphatase (ES-TRAP) is a sensitive, quantitative marker for ER stress.[22] Activity of ES-TRAP secreted by transfected cells is rapidly and sensitively downregulated in response to ER stress independent of transcriptional regulation. To confirm induction of ER stress by cadmium, ES-TRAP assay was performed. LLC-PK1 cells constitutively producing ES-TRAP were treated with $CdCl_2$ for 3 h and activity of ES-TRAP in culture medium was evaluated. By $CdCl_2$ treatment, activity of ES-TRAP was markedly reduced to 24.7% (20 μM $CdCl_2$) and 15.2% (50 μM $CdCl_2$) *versus* untreated control (100%) (Figure 1e, left). This reduction was not caused by cellular damage, because the number of viable cells estimated by formazan assay was not affected by $CdCl_2$ (Figure 1e, right).

We tested whether cadmium administered *in vivo* also induces ER stress in the kidney. Mice were injected with $CdCl_2$ intraperitoneally (12 mg/kg body weight), and after 8 and 16 h, the renal cortex was subjected to Northern blot analysis. In consistence with the result in LLC-PK1 cells, expression of *GRP78* and *CHOP* was markedly induced after 8 h. This induction was transient and the expression levels were returned to the basal level after 16 h (Figure 1f).

ER stress-mediated apoptosis after exposure to cadmium. To examine whether induction of ER stress by cadmium is associated with cellular damage, LLC-PK1 cells were exposed to $CdCl_2$ for 6 h and subjected to phase-contrast microscopy and Hoechst staining. As shown in Figure 2a, $CdCl_2$ induced detachment and rounding of the cells. Hoechst33258 staining exhibited nuclear condensation typical of apoptosis. Quantitative analysis revealed that this effect was dose-dependent (Figure 2b). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay also confirmed that even after short (3 h) exposure to $CdCl_2$ the number of TUNEL-positive apoptotic cells substantially increased (Figure 2c).

To examine whether apoptosis caused by cadmium is mediated by ER stress, LLC-PK1 cells were stably transfected with *GRP78* and ER stress-resistant LL/GRP78-3 and LL/GRP78-19 cells were established (Figure 3a). Overexpression of *GRP78* conferred resistance to tunicamycin-induced damage (Supplementary Figure 2a). Induction of apoptosis was abolished in LL/GRP78 cells when examined by Hoechst staining (Figure 3b). Using these clones, effects of $CdCl_2$ were evaluated. Similar to the response to tunicamycin, $CdCl_2$-induced injury was significantly attenuated in *GRP78*-transfected cells (Supplementary Figure 2b). In contrast to tunicamycin-induced apoptosis, however, the inhibition was partial, approximately 60% (Figure 3c). Similar results were

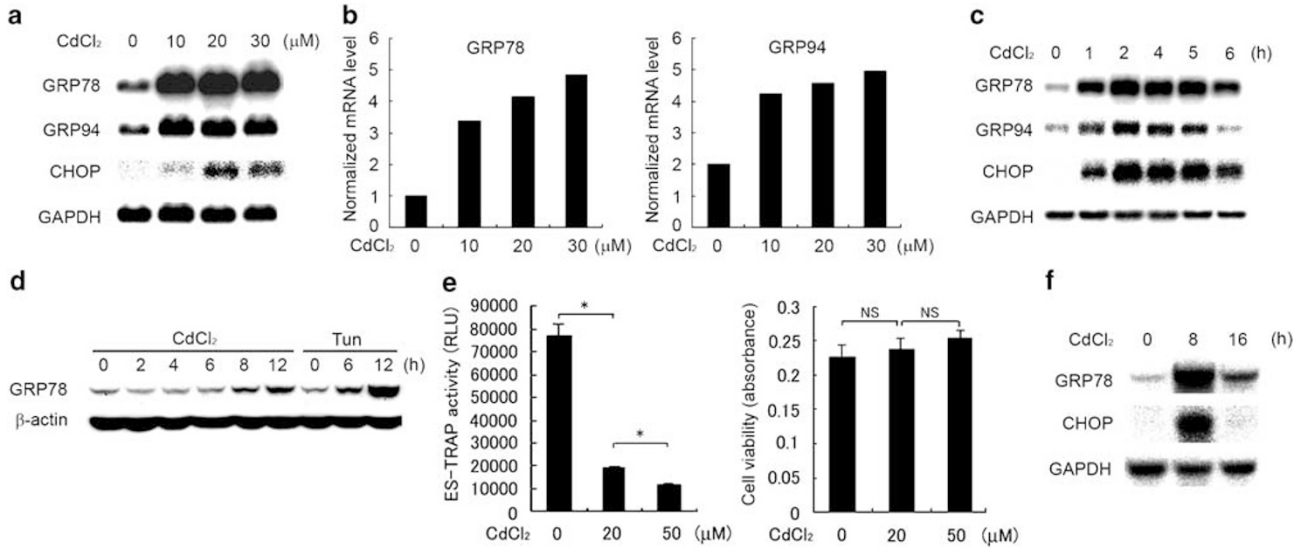

Figure 1 Induction of ER stress by cadmium. (**a** and **b**) LLC-PK1 cells were treated with 10–30 μM $CdCl_2$ for 4 h and expression of GRP78, GRP94 and CHOP was examined by Northern blot analysis. (**a**) Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The level of individual bands was normalized by the level of GAPDH and the results are shown in (**b**). (**c**) Cells were treated with 20 μM $CdCl_2$ for 1–6 h and subjected to Northern blot analysis. (**d**) LLC-PK1 cells were treated with 20 μM $CdCl_2$ or 5 μg/ml tunicamycin (Tun) for up to 12 h and subjected to Western blot analysis of GRP78. The level of β-actin is shown as a loading control. (**e**) LLC-PK1 cells constitutively producing ES-TRAP were treated with $CdCl_2$ (20–50 μM) for 3 h and activity of ES-TRAP in culture medium was evaluated by chemiluminescent assay (left). At the end of the study, viability of cells was evaluated by formazan assay (right). Data are presented as means $\pm$ S.E. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences ($P<0.05$). NS, not statistically significant. RLU, relative light unit. (**f**) Mice were administered with 12 mg/kg body weight of $CdCl_2$ intraperitoneally, and before and after 8 and 16 h, cortexes of the kidneys were subjected to Northern blot analysis of GRP78 and CHOP

obtained in both LL/GRP78-3 and LL/GRP78-19 cells. This result indicated a possibility that cadmium may induce apoptosis through ER stress-dependent and -independent mechanisms.

To confirm our conclusion, we attempted another set of transfection studies using 150 kDa oxygen-regulated protein (ORP150), an ER chaperone that protects cells from ER stress-induced injury.[23–25] Northern blot analysis confirmed abundant expression of *ORP150* in the established clones LL/ORP150-13 and LL/ORP150-20 (Figure 3d). Induction of ER stress by $CdCl_2$ was attenuated in these transfectants when the levels of *GRP78* was examined (Figure 3e). In these clones, the apoptogenic effect of $CdCl_2$ was attenuated (Supplementary Figure 2c). The percentages of apoptotic cells evaluated by Hoechst staining decreased from 78.2 ± 2.3 to $44.2\pm1.7\%$ ($P<0.05$) (Figure 3f). Consistent with this result, transient transfection with *ORP150* also reduced the percentages of round cells after the treatment with $CdCl_2$ (Supplementary Figure 2d).

Activation of the PERK–eIF2α pathway by cadmium. There are three proximal transmembrane proteins in the ER for sensing of ER stress. The first transducer is PERK, the activation of which leads to phosphorylation of eIF2α and blockade of protein synthesis.[21] We first examined involvement of the PERK–eIF2α pathway in the cadmium-induced apoptosis of tubular epithelial cells. Western blot analysis showed that treatment of LLC-PK1 cells with $CdCl_2$ rapidly induced phosphorylation of PERK and eIF2α within 1 h (Figures 4a, b). To examine whether this signaling pathway participates in the regulation of apoptosis, salubrinal, a selective inhibitor of eIF2α dephosphorylation, was used to activate the eIF2α pathway.[26] In LLC-PK1 cells, salubrinal effectively induced phosphorylation of eIF2α (Supplementary Figure 3a). LLC-PK1 cells were then pretreated with or without salubrinal for 1 h, stimulated by $CdCl_2$ for 4 h and subjected to microscopic analyses. As shown in Supplementary Figure 3b, salubrinal significantly inhibited $CdCl_2$-induced injury. Hoechst staining showed that the percentage of apoptotic cells was reduced from 90.9 ± 2.5 to $63.0\pm4.0\%$ (Figure 4c). Salubrinal alone did not affect the basal cell survival. In this experiment, cadmium-triggered phosphorylation of eIF2α was not obviously enhanced by salubrinal (data not shown). It may be owing to negative feedback via the ATF4–GADD34 pathway that dephosphorylates eIF2α.

The PERK–eIF2α pathway has the ability to regulate apoptosis via induction of ATF4 that may affect expression of the proapoptotic gene *CHOP* or the antiapoptotic gene *GRP78*.[27,28]We examined expression of *GRP78* and *CHOP* in $CdCl_2$-stimulated LLC-PK1 cells in the presence or absence of salubrinal. Northern blot analysis revealed that $CdCl_2$ induced both *GRP78* and *CHOP*, whereas expression of these genes was unaffected by salubrinal (Figure 4d). Of note, treatment with salubrinal alone, which caused phosphorylation of eIF2α (Supplementary Figure 3a), also did not upregulate expression of *GRP78* and *CHOP*.

To confirm the antiapoptotic role of the PERK–eIF2α pathway, LLC-PK1 cells were transiently transfected with a gene encoding a dominant-negative mutant of eIF2α together with enhanced green fluorescence protein (*EGFP*) and exposed to $CdCl_2$ to induce apoptosis. Consistent with the result shown in Figure 4c, dominant-negative inhibition of

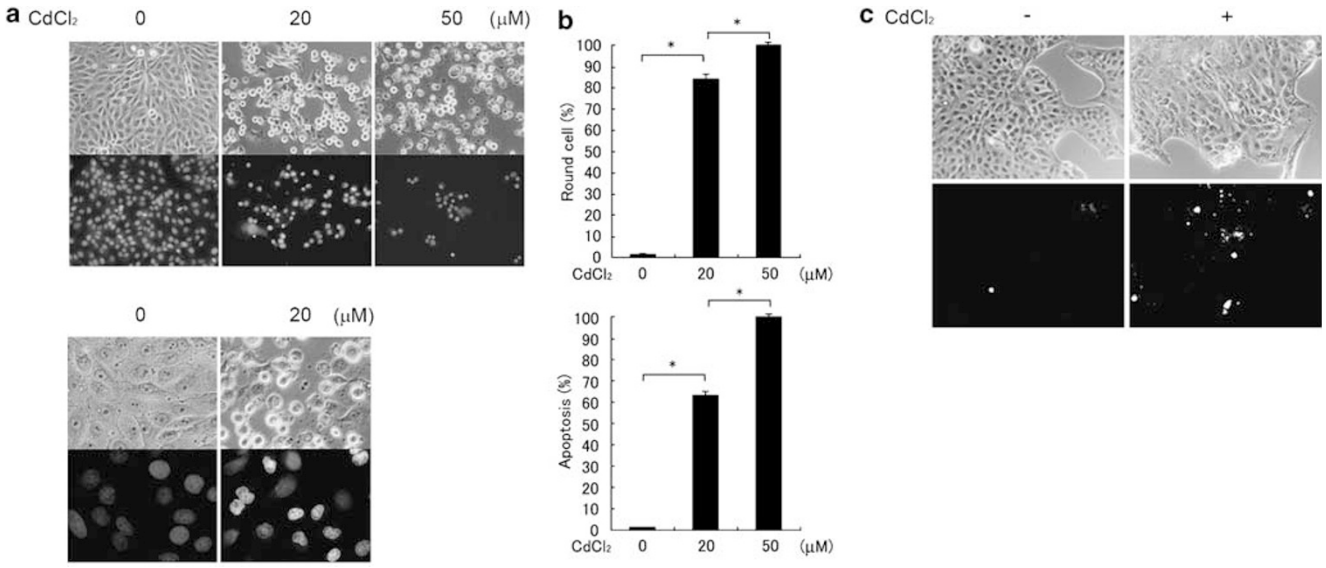

Figure 2 Induction of apoptosis after exposure to cadmium. (**a** and **b**) LLC-PK1 cells were exposed to 20 or 50 μM $CdCl_2$ for 6 h and subjected to phase-contrast microscopy (top) and Hoechst staining (bottom). High-power view of the cells is shown in the lower panel. (**b**) Percentages of round cells (top) and apoptotic cells (bottom). Data are presented as means $\pm$ S.E. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences ($P<0.05$). (**c**) Cells were treated with 20 μM $CdCl_2$ for 3 h and subjected to phase-contrast microscopy (top) and TUNEL assay (bottom)

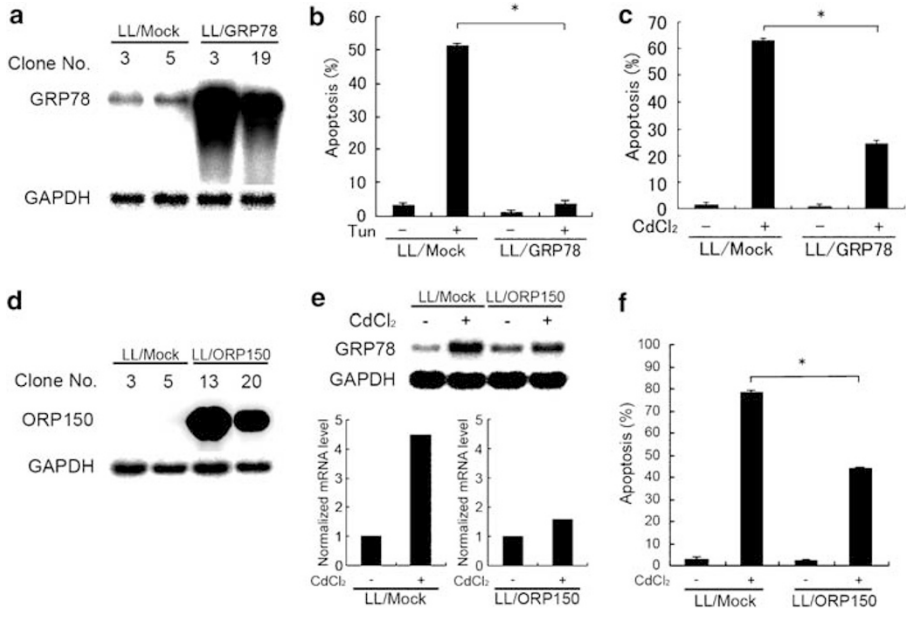

Figure 3 Involvement of ER stress in cadmium-induced apoptosis. LLC-PK1 cells were stably transfected with a gene coding for (**a**–**c**) ER chaperone GRP78 or (**d**–**f**) ORP150 and LL/GRP78 cells and LL/ORP150 cells were established. (**a** and **d**) Northern blot analysis of *GRP78* and *ORP150* expression in mock-transfected cells (LL/Mock) and established transfectants. (**b** and **c**) Cells were treated with (**b**) 10 μg/ml tunicamycin (Tun) for 6 h or (**c**) 20 μM $CdCl_2$ for 4 h and subjected to fluorescence microscopy. Percentages of apoptotic cells evaluated by Hoechst staining are shown. Asterisks indicate statistically significant differences ($P<0.05$). (**e**) Cells were treated with $CdCl_2$ for 4 h and induction of *GRP78* was examined by Northern blot analysis (top) and densitometric analysis (bottom). (**f**) Cells were treated with $CdCl_2$ for 4 h and subjected to Hoechst staining to evaluate percentages of apoptotic cells

eIF2α significantly enhanced cadmium-induced apoptosis (Figure 4e).

Activation of the ATF6 pathway by cadmium. We next examined involvement of the ATF6 pathway in the cadmium-induced apoptosis of tubular cells. LLC-PK1 cells were transiently transfected with FLAG-tagged p90ATF6, exposed to $CdCl_2$ and subjected to Western blot analysis. As shown in Figure 5a, treatment with $CdCl_2$ rapidly caused generation of p50ATF6 within 2 h. To examine whether this signaling pathway is involved in the regulation of apoptosis, a selective inhibitor of ATF6, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), was used. AEBSF is a serine protease inhibitor and prevents ER stress-induced cleavage of p90ATF6.[29]

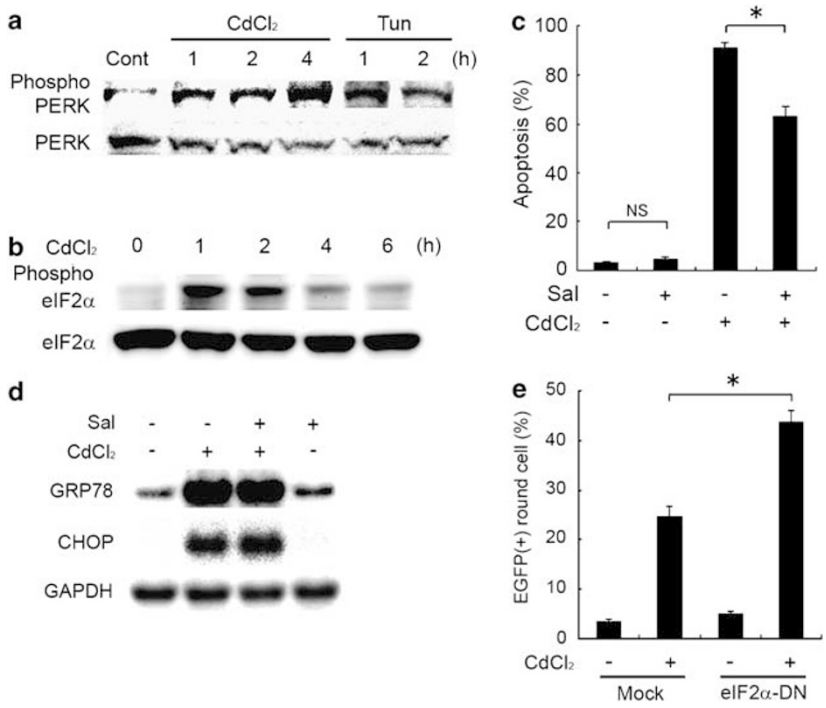

Figure 4 Activation of the PERK–eIF2α pathway by cadmium. (**a**) LLC-PK1 cells were treated with 20 μM $CdCl_2$ or 10 μg/ml tunicamycin (Tun) for up to 4 h and phosphorylation of PERK was evaluated by Western blot analysis. Cont, untreated control. (**b**) Cells were treated with $CdCl_2$ for up to 6 h and phosphorylation of eIF2α was evaluated by Western blot analysis. (**c**) LLC-PK1 cells were exposed to $CdCl_2$ in the presence (+) or absence (–) of 50 μM salubrinal (Sal) for 4 h and subjected to Hoechst staining. An asterisk indicates a statistically significant difference ($P<0.05$). NS, not statistically significant. (**d**) Cells were exposed to $CdCl_2$ in the presence (+) or absence (−) of salubrinal for 4 h and subjected to Northern blot analysis. (**e**) LLC-PK1 cells were transiently transfected with *EGFP* together with an empty plasmid (Mock) or an expression plasmid for a dominant-negative mutant of eIF2α (eIF2α-DN) and exposed to $CdCl_2$ for 4 h. Percentages of EGFP-positive round cells against total EGFP-positive cells were evaluated by fluorescence microscopy. An asterisk indicates a statistically significant difference ($P<0.05$)

Treatment of LLC-PK1 cells with AEBSF abrogated increase of p50ATF6 in response to $CdCl_2$ (Figure 5b). Under this experimental condition, AEBSF inhibited $CdCl_2$-induced injury (Supplementary Figure 3c). Quantitative analysis by Hoechst staining showed that AEBSF significantly inhibited cadmium-induced apoptosis in a dose-dependent manner (Figure 5c). To confirm that the ATF6 pathway is proapoptotic, cells were transiently transfected with a gene encoding a dominant-negative mutant of ATF6 together with *EGFP* and the effect of $CdCl_2$ on apoptosis was retested. As shown in Figure 5d, dominant-negative inhibition of ATF6 significantly suppressed cadmium-induced apoptosis.

It is well known that ATF6 induces expression of ER chaperones via binding to ERSE.[30] However, the regulatory region of *CHOP* also contains ERSE and ATF6 may induce expression of *CHOP* in some cell types.[30] We examined a role of the ATF6 pathway in the induction of *CHOP* by $CdCl_2$. Northern blot analysis revealed that inhibition of ATF6 by AEBSF blunted induction of *CHOP* in response to $CdCl_2$ (Figure 5e). Furthermore, transient transfection with a gene coding for a dominant-negative mutant of CHOP significantly attenuated cadmium-induced apoptosis (Figure 5f). These results suggested that the ATF6 pathway activated by $CdCl_2$ participated in the induction of apoptosis, at least in part, via induction of CHOP.

Activation of the IRE1–XBP1 pathway and JNK by cadmium. The third proximal transducer for ER stress is IRE1. Activated IRE1 catalyzes removal of a small intron from *XBP1* mRNA, leading to production of the active transcription factor. We first evaluated activation of the IRE1–XBP1 pathway by cadmium using the frameshift of XBP1 as an indicator. LLC-PK1 cells were stably transfected with a gene encoding FLAG-tagged, unspliced form of XBP1 (XBP1(U)) and treated with $CdCl_2$. Abundant expression of XBP1(U) was confirmed by Northern blot analysis (Supplementary Figure 4a). Western blot analysis revealed that $CdCl_2$ rapidly reduced the level of XBP1(U) within 30 min, reached to the bottom at 1 h and recovered to the basal level after 3 h (Figure 6a).

To examine whether the IRE1–XBP1 pathway is involved in the regulation of apoptosis by $CdCl_2$, LLC-PK1 cells were stably transfected with a gene coding for a dominant-negative mutant of XBP1 (XBP1-DN) and LL/XBP1-DN cells were established. Northern blot analysis confirmed abundant expression of *XBP1-DN* in the established LL/XBP1-DN-7 and LL/XBP1-DN-20 cells (Figure 6b). Phase-contrast microscopy showed that cadmium-induced injury was significantly attenuated in these LL/XBP1-DN cells (Supplementary Figure 4b). The percentage of apoptotic cells was markedly reduced from 65.0 ± 2.5 to 8.6 ± 1.5% ($P<0.05$) by the dominant-negative suppression of XBP1 (Figure 6c). The proapoptotic effect of XBP1 was independent of GRP78 and CHOP because induction of *GRP78* and *CHOP* by $CdCl_2$ was observed to the same extent in LL/Mock cells and LL/XBP1-DN cells (Figure 6d).

We next examined whether the IRE1–JNK proapoptotic pathway[31] is involved in the $CdCl_2$-triggered apoptosis of

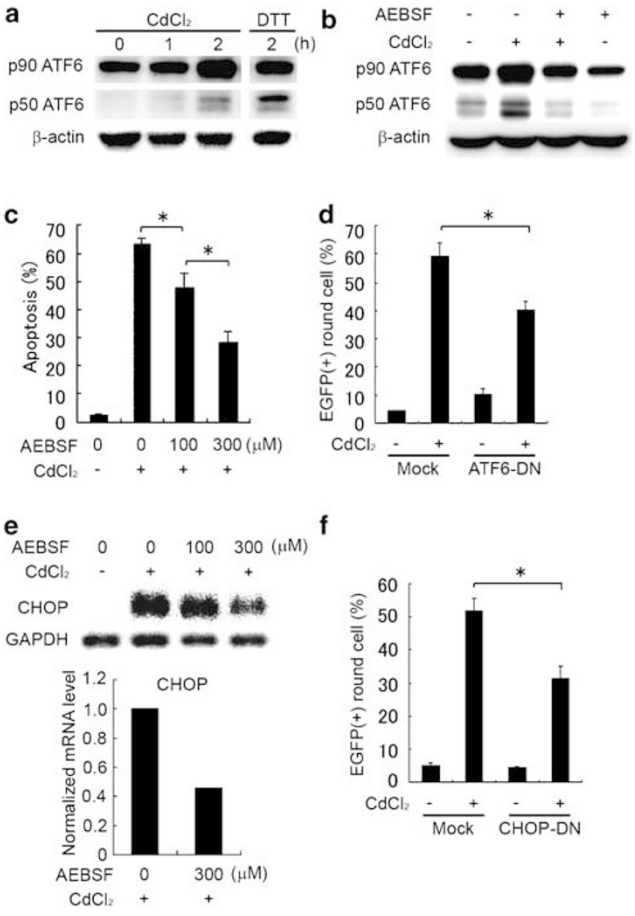

Figure 5 Activation of the ATF6 pathway and consequent induction of *CHOP* by cadmium. (**a**) LLC-PK1 cells transiently transfected with a gene encoding FLAG-tagged p90ATF6 were treated with 20 μM $CdCl_2$ or 5 mM dithiothreitol (DTT) for up to 2 h and levels of p90ATF6 and p50ATF6 were evaluated by Western blot analysis. Protein level of β-actin is shown at the bottom as a loading control. (**b**) Cells were exposed to $CdCl_2$ in the absence ($-$) or presence ($+$) of 300 μM AEBSF for 3 h and subjected to Western blot analysis of ATF6. (**c**) Cells were exposed to $CdCl_2$ in the presence of 0–300 μM AEBSF for 4 h and subjected to Hoechst staining. Asterisks indicate statistically significant differences ($P<0.05$). (**d**) LLC-PK1 cells were transiently transfected with *EGFP* together with an empty plasmid (Mock) or an expression plasmid for a dominant-negative mutant of ATF6 (ATF6-DN) and exposed to $CdCl_2$ for 6 h. Percentages of EGFP-positive round cells against total EGFP-positive cells were evaluated by fluorescence microscopy. An asterisk indicates a statistically significant difference ($P<0.05$). (**e**) Cells were exposed to $CdCl_2$ in the presence of 0–300 μM AEBSF for 4 h and subjected to Northern blot analysis of *CHOP*. Densitometric analysis of individual mRNAs is shown at the bottom. (**f**) LLC-PK1 cells were transiently transfected with *EGFP* together with a gene coding for a dominant-negative mutant of CHOP (CHOP-DN) and exposed to $CdCl_2$ for 6 h. Percentages of apoptotic cells were evaluated by fluorescence microscopy

tubular cells. Western blot analysis revealed that phosphorylation of JNK was substantially induced within 1 h, peaked at 1.5–2 h and declined after 4 h (Figure 6e). To exclude a possibility that activation of JNK could be caused through other proapoptotic UPR, especially the ATF6 pathway, LLC-PK1 cells were treated with $CdCl_2$ in the absence or presence of AEBSF and activation of JNK was retested. As shown in Supplementary Figure 4c, phosphorylation of JNK by $CdCl_2$ was not attenuated by blockade of the ATF6 pathway.

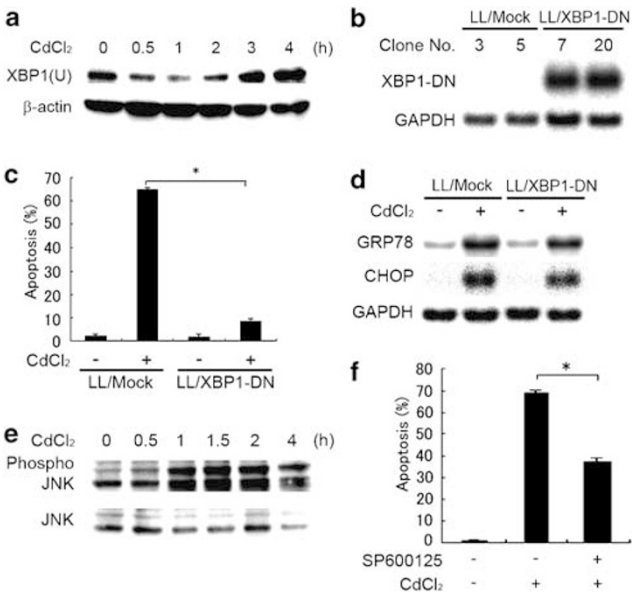

Figure 6 Activation of the IRE1–XBP1 pathway and JNK by cadmium. (**a**) LLC-PK1 cells were stably transfected with a gene encoding FLAG-tagged XBP1(U) and established transfectants were treated with 20 μM $CdCl_2$ for up to 4 h. Western blot analysis of XBP1(U) was performed using an anti-FLAG antibody. (**b–d**) LLC-PK1 cells were stably transfected with a gene coding for XBP1-DN and LL/XBP1-DN cells were established. (**b**) Northern blot analysis of *XBP1-DN* expression. (**c**) Cells were treated with $CdCl_2$ for 4 h and subjected to Hoechst staining. An asterisk indicates a statistically significant difference ($P<0.05$). (**d**) Transfectants were treated with $CdCl_2$ for 6 h and subjected to Northern blot analysis of *GRP78* and *CHOP*. (**e**) LLC-PK1 cells were treated with $CdCl_2$ for up to 4 h and subjected to Western blot analysis of phosphorylated JNK and total JNK. (**f**) LLC-PK1 cells were treated with $CdCl_2$ for 4 h in the absence ($-$) or presence ($+$) of 10 μM SP600125 and subjected to Hoechst staining. An asterisk indicates a statistically significant difference ($P<0.05$)

To examine a role of JNK in cadmium-induced apoptosis, LLC-PK1 cells were pretreated with an inhibitor of JNK, SP600125, and exposed to $CdCl_2$. Microscopic analysis revealed that SP600125 substantially attenuated $CdCl_2$-induced cellular damage (Supplementary Figure 4d). Hoechst staining showed that blockade of JNK activation significantly inhibited induction of apoptosis approximately by 46% (from 69.0 ± 1.5 to 37.0 ± 2.0%; $P<0.05$) (Figure 6f). These results suggested crucial, major roles of the IRE1–XBP1 pathway and JNK in $CdCl_2$-induced apoptosis of renal tubular cells.

Discussion

In the present study, we demonstrated involvement of ER stress in cadmium-induced apoptosis. In LLC-PK1 cells, cadmium induced ER stress and attenuation of ER stress by ER chaperones inhibited apoptosis. After exposure to cadmium, PERK, ATF6 and IRE1 were activated and the ATF6 and IRE1 pathways contributed to apoptosis. Induction of CHOP, activation of XBP1 and phosphorylation of JNK were identified as possible downstream events responsible for apoptosis. In contrast, the PERK–eIF2α pathway was found to be antiapoptotic and counteracted the proapoptotic signals. These results evidenced for the first time that cadmium-induced apoptosis of renal tubular cells is mediated,

at least in part, by ER stress and that atypical, bidirectional regulation of apoptosis via distinct UPR is implicated in the apoptotic process. The outline of our current hypothesis is illustrated in Figure 7.

Previous reports indicated that cadmium may induce apoptosis through altered activity of protein kinases, phosphatases and transcription factors, generation of ROS, induction of mitochondrial pathways or activation of caspases.[32] Mechanisms involved may be different from cell type to cell type, but among those, mitochondria have been regarded as the crucial target of cadmium. For example, although cadmium has the potential for activating various protein kinases, it induced apoptosis through disruption of the mitochondrial membrane, cytochrome *c* release and activation of caspase-9 independent of protein kinase A, protein kinase C and mitogen-activated protein kinases in glioma cells.[33] Similarly, in proximal renal tubular cells, the mitochondrial pathway is also considered as a pathway responsible for cadmium-triggered apoptosis.[10] However, no information was available regarding whether and how the ER, another crucial organelle that transduces proapoptotic signals, is involved in cadmium-induced apoptosis of tubular cells. In the present study, we provided evidence that ER is also a critical player. Currently, the relationship between the mitochondrial pathway and the ER pathway is unclear. Both proapoptotic pathways may be independently induced, as suggested in glioma cells treated by N-(4-hydroxyphenyl)retinamide.[34] Alternatively, ER stress might be an upstream or a downstream event involved in the mitochondria–ROS pathway. Recently, we found that antioxidant N-acetylcysteine significantly attenuated ER stress and apoptosis caused by cadmium, supporting the latter possibility (our unpublished observation).

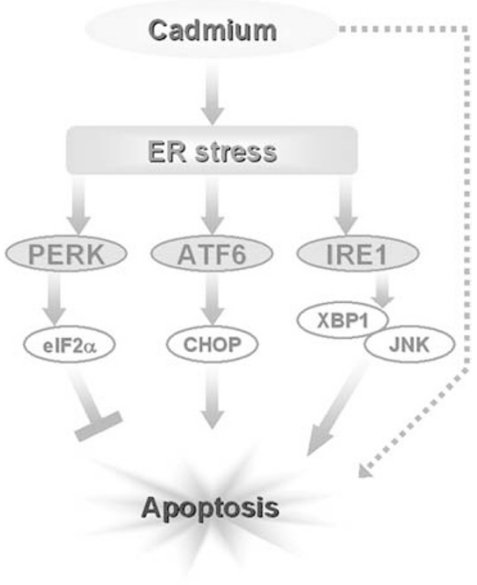

Figure 7 Current hypothesis on the induction of ER stress and consequent apoptosis by cadmium in renal tubular epithelial cells. Cadmium induces ER stress and causes activation of PERK, ATF6 and IRE1. The ATF6 pathway contributes to apoptosis via induction of CHOP and the IRE1 pathway induces apoptosis via activation of XBP1 and phosphorylation of JNK. In contrast, the PERK–eIF2α pathway is antiapoptotic and counteracted the proapoptotic signals

Induction of *GRP78* by cadmium may be observed in some tumor cells.[35,36] However, to date, little has been disclosed about which UPR is transduced by cadmium and whether and how individual signals contribute to apoptosis. In the present study, we first showed that, in response to $CdCl_2$, phosphorylation of PERK was induced in tubular epithelial cells. This is consistent with a previous report showing that eIF2α was phosphorylated by cadmium *in vitro* using reticulocyte lysate.[37] In general, the PERK–eIF2α pathway transduces proapoptotic signals, for example, induction of *CHOP* via translation of ATF4.[27] However, unexpectedly, we found that the PERK–eIF2α pathway was antiapoptotic in cadmium-exposed tubular cells. In some particular situations, GRP78 may be upregulated by ATF4. However, the cytoprotective effect of the PERK–eIF2α pathway was independent of upregulation of *GRP78*, as shown in this report. It is in contrast to a recent report implying that phosphorylation of eIF2α and resultant translation of ATF4 could be responsible for the induction of *GRP78* by cadmium in renal tubular cells.[38] It is known that the PERK–eIF2α pathway can relieve ER stress via translational suppression.[15] The antiapoptotic effect of the PERK–eIF2α pathway may be ascribed to the attenuation of ER stress simply by general inhibition of protein production.

We showed that the ATF6 pathway was induced by cadmium and that, in contrast to several other previous reports, this pathway was found to be proapoptotic in cadmium-exposed tubular cells. In general, the ATF6 pathway is considered as an important antiapoptotic machinery against ER stress because it induces several ER resident chaperones, including GRP78, via activation of ERSE.[21] Indeed, suppression of ATF6 by AEBSF reduced induction of *GRP78* by cadmium in a dose-dependent manner (Supplementary Figure 5). Currently, molecular mechanisms involved in the proapoptotic action of ATF6 has not been elucidated completely, but we found that induction of *CHOP* was caused not by the PERK–eIF2α pathway but by the ATF6 pathway and thereby contributed to cadmium-triggered apoptosis.

In the present report, we also showed the IRE1 pathway contributed to cadmium-induced apoptosis. In general, the IRE1 pathway is considered as a proapoptotic pathway via activation of ASK1 and JNK. Indeed, we observed that JNK was rapidly activated following exposure to cadmium and its suppression attenuated apoptosis. However, we also found that cadmium induced activation of the IRE1–XBP1 pathway and that dominant-negative inhibition of XBP1 markedly attenuated apoptosis. The proapoptotic effect of XBP1 was dependent neither on suppression of *GRP78* nor on induction of *CHOP*. This was unexpected because, generally, XBP1 is considered as an antiapoptotic transcription factor that triggers induction of ER chaperones and other genes involved in ER-associated protein degradation.[39,40] To our knowledge, this is the first to demonstrate the proapoptotic property of XBP1 in mammalian cells. Currently, it is unclear how XBP1 contributes to cadmium-triggered apoptosis. Our preliminary data indicated a possibility that activation of JNK may be an event downstream of XBP1 (unpublished observation).

In this report, we elucidated contribution of ER stress to cadmium-induced apoptosis of tubular cells. Further investigation will be required to elucidate relationship among

oxidative, mitochondrial and ER stress during the process of cadmium-induced apoptosis. In addition, the fact that ER stress was triggered by several other nephrotoxic metals (Supplementary Figures 1a and b) indicates a possibility that the similar mechanism identified here may be applied generally for metal-induced renal injury.

Materials and Methods

Data are provided as Supplementary materials.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)