Involvement of Selective Reactive Oxygen Species Upstream of Proapoptotic Branches of Unfolded Protein Response

Makiko Yokouchi1, Nobuhiko Hiramatsu2, Kunihiro Hayakawa, Maro Okamura, Shuqi Du, Ayumi Kasai, Yosuke Takeo, Akihiro Shitamura, Tsuyoshi Shimada, Jian Yao, and Masanori Kitamura

From the 1Department of Molecular Signaling, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Shimokato 1110, Chuo, Yamanashi 409-3898, Japan and the 2Department of Urology, First Affiliated Hospital of China Medical University, Shenyang 110001, China

Cadmium triggers apoptosis of LLC-PK1 cells through induction of endoplasmic reticulum (ER) stress. We found that cadmium caused generation of reactive oxygen species (ROS) and that cadmium-induced ER stress was inhibited by antioxidants. In contrast, suppression of ER stress did not attenuate cadmium-triggered oxidative stress, suggesting that ER stress occurs downstream of oxidative exposure. Exposure of the cells to either O2•−, H2O2, or ONOO− caused apoptosis, whereas ER stress was induced only by O2•− or ONOO−. Transfection with manganese superoxide dismutase significantly attenuated cadmium-induced ER stress and apoptosis, whereas pharmacological inhibition of ONOO− was ineffective. Interestingly, transfection with catalase attenuated cadmium-induced apoptosis without affecting the level of ER stress. O2•− caused activation of the activating transcription factor 6-CCAAT/enhancer-binding protein-homologous protein (CHOP) and the inositol-requiring ER-to-nuclear signal kinase 1-X-box-binding protein 1 (XBP1) proapoptotic cascades, and overexpression of manganese superoxide dismutase attenuated cadmium-triggered induction of both pathways. Furthermore, phosphorylation of proapoptotic c-Jun N-terminal kinase by O2•− or cadmium was suppressed by dominant-negative inhibition of XBP1. These data elucidated 1) cadmium caused ER stress via generation of ROS, 2) O2•− was selectively involved in cadmium-triggered ER stress-mediated apoptosis through activation of the activating transcription factor 6-CHOP and inositol-requiring ER-to-nuclear signal kinase 1-XBP1 pathways, and 3) phosphorylation of JNK was caused by O2•−-triggered activation of XBP1.

Endoplasmic reticulum (ER) stress plays a crucial role in cadmium-induced apoptosis of renal tubular cells. It is based on our following findings: 1) Cadmium chloride (CdCl2) induced expression of endogenous ER stress markers, 78-kDa glucose-regulated protein (GRP78), GRP94, and CCAAT/enhancer-binding protein-homologous protein (CHOP) in a dose-dependent manner. 2) Attenuation of ER stress by overexpression of GRP78 or 150-kDa oxygen-regulated protein (ORP150) significantly suppressed CdCl2-induced apoptosis. 3) Among three major branches of unfolded protein response (UPR), the activating transcription factor 6 (ATF6) pathway and the inositol-requiring ER-to-nuclear signal kinase 1 (IRE1)-X-box binding protein 1 (XBP1) pathway mediated apoptosis. 4) Induction of CHOP by ATF6, IRE1-initiated activation of XBP1, and phosphorylation of c-Jun N-terminal kinase (JNK) were responsible for the induction of apoptosis (1). However, several previous studies also indicated involvement of reactive oxygen species (ROS) in cadmium-induced renal tubular injury. For example, exposure of LLC-PK1 cells to cadmium caused generation of ROS (2), which was associated with a decrease in glutathione levels and consequent cellular death (3). Another report showed that cadmium-triggered apoptosis of tubular cells was inhibited by an antioxidant (4). However, currently, it is unknown whether and how oxidative stress is linked to ER stress and, if so, what kind of ROS are involved in the induction of apoptosis in cadmium-exposed cells.

Oxygen normally accepts four electrons and is converted to water. In biological systems, partial reduction of oxygen occurs, resulting in the generation of cytotoxic ROS. That is, sequential reduction of oxygen leads to generation of superoxide anion (O2•−) and hydrogen peroxide (H2O2) (5). Superoxide dismutase (SOD) scavenges O2•− by catalyzing conversion of O2•− to H2O2. O2•− also rapidly reacts with nitric oxide (NO), yielding another reactive species, peroxynitrite (ONOO−) (6). All of these ROS may be potential triggers of apoptosis (5–7).

In the present report, we first describe that cadmium induces generation of ROS and consequent ER stress and apoptosis in ER-to-nuclear signal kinase 1; XBP1, X-box binding protein 1; JNK, c-Jun N-terminal kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; MnTM-2-PyP, manganese(III)-5,10,15,20-tetrakis(N-methylpyridinium-2)-pyrophosphophenyl chlorohydroxide; DN, dominant-negative mutant; NAC, N-acetylcycteine; SIN-1, 3-morpholinosydnonimine; L-NAME, N-nitro-L-arginine methyl ester; ASK1, apoptosis signal-regulating kinase 1; TRAF2, tumor necrosis factor receptor-associated factor 2; MnSOD, manganese SOD.
renal tubular cells. We show that, among ROS generated, O$_2^-$, but not downstream substances H$_2$O$_2$ and ONOO$^-$, plays a crucial role in cadmium-triggered, ER stress-mediated apoptosis. We further demonstrate that cadmium induces both ER stress-dependent and -independent proapoptotic pathways, the latter of which involves H$_2$O$_2$, but not NO or ONOO$^-$. We also provide evidence that O$_2^-$ has the potential to induce a novel, atypical proapoptotic pathway, i.e. the XBP1-JNK pathway, as well as the ATF6-CHOP pathway, and thereby critically contributes to cadmium-triggered apoptotic cell death.

**EXPERIMENTAL PROCEDURES**

**Reagents**—CdCl$_2$ was purchased from Wako Pure Chemical Industries (Osaka, Japan), manganese(III)-5,10,15,20-tetrakis(N-methylpyridinium-2yl)porphyrin pentachloride (MnTM-2-PyP) was from Calbiochem, and other reagents were from Sigma-Aldrich.

**Cells and Stable Transfectants**—The porcine renal proximal tubular cell line LLC-PK1 was obtained from American Type Culture Collection (Manassas, VA). Experiments were performed in the presence of 1% fetal bovine serum. LLC-PK1 cells constitutively expressing GRP78, ORP150, manganese SOD (MnSOD), catalase or a dominant-negative mutant of XBP1 (XBP1-DN) were established by stable transfection (electroporation) with pcDNA3.1-GRP78 (8), pCIneo-ORP150 (9), pcDNA3-MnSOD (10), pCIneo-catalase (11), or pdn-XBP1 (12) and designated as LL/GRP78, LL/ORP150, LL/MnSOD, LL/Catalase, and LL/XBP1-DN, respectively. LL/Mock cells were established by stable transfection with neo alone (pcDNA3.1, Invitrogen).

**Northern Blot Analysis**—Total RNA was extracted by the single-step method, and Northern blot analysis was performed as described before (13). cDNAs for GRP78 (14), ORP94 (15), CHOP (16), c-Fos (17), catalase (11), and MnSOD (10) were used for preparation of radiolabeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Densitometric analysis was performed using Scion Image (Scion Corp., Frederick, MO).

**Detection of ROS**—Cells were washed twice with phosphate-buffered saline and loaded with ROS-responsive fluorescent probe 2′,7′-dichlorofluorescein (100 μM) for 3 h in the absence of fetal bovine serum. After washing with phosphate-buffered saline, cells were stimulated with CdCl$_2$ or H$_2$O$_2$ for 2 h and subjected to fluorescent microscopy.

**Assessment of Apoptosis**—After induction of apoptosis, morphologic examination was performed by phase-contrast microscopy and fluorescent microscopy for Hoechst 33258 staining, as described before (1). Because, in some situations, round cells were easily detached from the bottoms of the plates, percentages of round cells were first evaluated by phase-contrast microscopy and then subjected to Hoechst staining. Apoptosis was identified using morphological criteria, including shrinkage of the cytoplasm (round shape), nuclear condensation, and membrane blebbing. Assays were performed in quadruplicate.

**Transient Transfection**—Using GeneJuice Transfection Reagent (Novagen, Madison, WI), cells were transiently transfected with pcDNA3-FLAG-XBP1(S) (18), pCMV-3xFLAG-ATF6 (19), or pCAX-F-XBP1ADBD-Luc (20), treated with test reagents and subjected to Western blot analysis or chemiluminescent assay.

**Western Blot Analysis**—Western blot analysis of ATF6 and GRP78 was performed using anti-FLAG antibody (1:1000 dilution, Sigma-Aldrich Japan) and anti-KDEL antibody (1:1000 dilution; Stressgen, Victoria, Canada). As a loading control, the level of β-actin was evaluated using anti-β-actin antibody (1:3000 dilution, Sigma-Aldrich Japan). Phosphorylation of JNK was assessed using PhosphoPlus stress-activated protein kinase/JNK (Thr-183/Tyr-185) Antibody Kit (Cell Signaling, Beverly, MA), as described before (21). Blots were visualized using Western blotting Detection Reagents (Amersham Biosciences).

**Reverse Transcription-PCR**—Splicing of XBP1 mRNA was examined by reverse transcription-PCR using following primers; 5′-AACAGATGACAGATAGCTAGTCG-3′ and 5′-CTCTTTCTGGGTAGACCTCTCTGGGA-3′. As an internal control, expression of glyceraldehyde-3-phosphate dehydrogenase mRNA was examined using the primers as follows: 5′-ACCACTGTCATGCACTAC-3′ and 5′-TCCACACCCTGTTGCTGTA-3′.

**Luciferase Assay**—Activity of luciferase was evaluated by Luciferase Assay System (Promega) following the protocol provided by the manufacturer. Assays were performed in quadruplicate.

**Statistical Analysis**—Data were expressed as means ± S.E. Statistical analysis was performed using the non-parametric Mann-Whitney U test to compare data in different groups. p value <0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**ER Stress as an Event Downstream of Oxidative Stress in Cadmium-exposed Cells**—Previous reports indicated involvement of ROS in cadmium-induced renal tubular injury (2–4). We first confirmed generation of ROS in LLC-PK1 cells exposed to cadmium. Cells were loaded with an ROS-responsive fluorescence probe 2′,7′-dichlorofluorescein and stimulated with CdCl$_2$ or H$_2$O$_2$ (positive control). Fluorescence microscopy showed that, under a basal culture condition, LLC-PK1 cells exhibited little fluorescence. Exposure of the cells to cadmium caused substantial and dose-dependent increases in the number of fluorescence-positive cells (Fig. 1A, bottom row). To examine whether the generation of ROS is causative of cadmium-induced apoptosis, cells were pretreated with antioxidant N-acetylcysteine (NAC) or SOD-like antioxidant MnTM-2-PyP, stimulated with CdCl$_2$, and subjected to phase-contrast microscopy and Hoechst staining. As shown in Fig. 1B, CdCl$_2$ induced rounding and detachment of the cells with nuclear condensation typical of apoptosis. Treatment with NAC or MnTM-2-PyP attenuated these morphological changes. Quantitative analysis revealed that percentages of round cells and apoptotic cells with condensed nuclei were significantly reduced by NAC from 43.5 ± 0.5% to 39.2 ± 0.5% (Fig. 1C, left) and from 39.2 ± 3.9% to 4.5 ± 1.0% (Fig. 1C, right), respectively (means ± S.E., p < 0.05). Similarly, percentages of round cells and apoptotic cells were reduced by MnTM-2-PyP from 43.5 ± 1.9% to 13.9 ± 0.9% and from 39.2 ± 3.9% to 12.1 ± 2.5%.
Cadmium-induced apoptosis of LLC-PK1 cells is mediated, at least in part, by ER stress (1). To investigate relationship between oxidative stress and ER stress, LLC-PK1 cells were treated with CdCl₂ in the absence or presence of NAC, and expression of endogenous ER stress markers GRP78 and GRP94 was examined. Northern blot analysis revealed that induction of GRPs by CdCl₂ was partially attenuated by NAC (Fig. 2A, top), similarly, induction of CHOP by CdCl₂ was also inhibited by NAC (Fig. 2A, bottom), suggesting that ER stress is an event downstream of oxidative stress. Of note, induction of c-fos, a marker of oxidative stress (22, 23), by CdCl₂ was completely abolished by NAC (Fig. 2B), indicating involvement of ROS-dependent and -independent induction of ER stress by CdCl₂.

To exclude a possibility that ER stress is located upstream of oxidative stress, LLC-PK1 cells were stably transfected with the ER chaperone GRP78, which attenuates ER stress. The established ER stress-resistant LL/GRP78 cells exhibited high levels of GRP78 mRNAs (supplemental Fig. S1) and resistance to tunicamycin-induced apoptosis (supplemental Fig. S2). Using LL/GRP78 cells, induction of c-fos by CdCl₂ was compared with that in mock transfected cells. As shown in Fig. 2C, expression of c-fos in LL/GRP78 cells was not attenuated when compared with that in LL/Mock cells. Similarly, attenuation of ER stress by overexpression of another ER chaperone ORP150 (1, 9) did not affect induction of c-fos by CdCl₂ (Fig. 2D). Taken together, these results evidenced

**FIGURE 1. Involvement of oxidative stress in cadmium-induced apoptosis of renal tubular epithelial cells.** A, LLC-PK1 cells were loaded with reactive oxygen species-responsive fluorescent probe 2',7'-dichlorofluorescein (100 μM) for 3 h, stimulated with 20–50 μM CdCl₂ or 100 μM hydrogen peroxide (H₂O₂) for 2 h, and subjected to phase-contrast microscopy (top row) and fluorescent microscopy (bottom row). B, cells were pretreated with NAC (1 mM) or manganese(III)-5,10,15,20-tetakis(N-methylpyridinium-2-yl)porphyrin pentachloride (MnTM, 50 μM) for 1 h, stimulated by 20 μM CdCl₂ for 5 h, and subjected to phase-contrast microscopy (top row) and Hoechst 33258 staining (bottom row). C, percentages of round cells evaluated by phase-contrast microscopy (left) and apoptotic cells assessed by Hoechst staining (right) are shown. Data are presented as means ± S.E. Assays were performed in quadruplicate. Asterisks indicate statistically significant differences versus CdCl₂-exposed, antioxidant-untreated control (p < 0.05).

**FIGURE 2.** Endoplasmic reticulum (ER) stress as an event downstream of oxidative stress in cadmium-exposed cells. A and B, LLC-PK1 cells were pretreated with (+) or without (−) 1 mM NAC and exposed to 20 μM CdCl₂ for 4 h. Expression of endogenous ER stress markers 78-kDa glucose-regulated protein (GRP78), GRP94, and CCAAT/enhancer-binding protein-homologous protein (CHOP) (A) and an oxidative stress marker c-fos (B) was examined by Northern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. C, LL/Mock cells and LL/GRP78 cells overexpressing GRP78 were treated with CdCl₂, and expression of c-fos was evaluated. D, LL/Mock cells and LL/ORP150 cells expressing 150-kDa oxygen-regulated protein were treated with CdCl₂ and subjected to Northern blot analysis of c-fos.
that ER stress is an event downstream of oxidative stress in cadmium-exposed cells.

**Involvement of O$_2^.$ in Cadmium-triggered, ER Stress-dependent Apoptosis**—The sequential reduction of oxygen leads to generation of O$_2^.$ and H$_2$O$_2$ (5). O$_2^.$ also rapidly reacts with NO, yielding another reactive species ONOO$^-$ (6). All of these ROS have the potential for triggering apoptosis (5–7). A previous report indicated that, in HepG2 cells, cadmium triggered production of ROS including O$_2^.$ and H$_2$O$_2$ (24). However, the major ROS that contribute to CdCl$_2$-induced apoptosis have not been identified. It is also unknown which ROS contribute to the induction of ER stress. To address these issues, we first examined involvement of O$_2^.$ in the induction of ER stress and apoptosis. LLC-PK1 cells were treated with serial concentrations of menadione, a generator of O$_2^.$, and expression of GRPs was examined by Northern blot analysis. Northern blot analysis (E and G) and Western blot analysis (F). In E, the level of GRP78 was normalized by the level of glyceraldehyde-3-phosphate dehydrogenase, and its relative induction (versus untreated control) is shown on the right ($n = 4$). An asterisk indicates a statistically significant difference ($p < 0.05$).
Selective ROS Upstream of Proapoptotic UPR

Involvement of H2O2 in cadmium-triggered, ER stress-independent apoptosis. A, LLC-PK1 cells were exposed to H2O2 (50–200 μM) for 4 h, and expression of c-fos and GRP78 was examined by Northern blot analysis. B, cells were stably transfected with a cDNA coding for catalase, and transgene expression in the established transfectants (LL/Catalase clone 9 and clone 11) was examined by Northern blot analysis. C, LL/Mock cells and LL/Catalase cells were exposed to 200 μM H2O2 for 4 h and subjected to phase-contrast microscopy (left). Percentages of apoptotic cells evaluated by Hoechst staining are shown on the right. An asterisk indicates a statistically significant difference (p < 0.05). D, LL/Mock cells and LL/Catalase cells were treated with 20 μM CdCl2, and induction of GRP78 was examined. E and F, LL/Mock cells and LL/Catalase cells were exposed to CdCl2 for 4 h and subjected to phase-contrast microscopic analysis (E and F, left) and Hoechst staining to evaluate percentages of apoptotic cells (F, right).

analysis confirmed abundant expression of MnSOD mRNA in LL/MnSOD-6 cells and LL/MnSOD-7 cells (Fig. 3B). Microscopic analysis revealed that these transfectants were resistant to menadione-induced apoptosis (Fig. 3C). Quantitative analysis showed that percentages of round cells and apoptotic cells with condensed nuclei (evaluated by Hoechst staining) were reduced by MnSOD from 52.6 ± 2.3% to 15.9 ± 0.3% (Fig. 3D, left), and from 44.4 ± 2.7 to 25.3 ± 2.0% (Fig. 3D, right), respectively. Using these transfectants, induction of ER stress by cadmium was evaluated. As shown in Fig. 3E, expression of GRP78 mRNA in response to CdCl2 was significantly reduced in LL/MnSOD cells (p < 0.05). The induction was approximately one-third of that in LL/Mock cells. Similar results were obtained in both LL/MnSOD-6 cells and LL/MnSOD-7 cells. The attenuated induction of GRP78 was associated with blunted accumulation of GRP78 protein (Fig. 3F). Furthermore, attenuation of cadmium-induced ER stress in LL/MnSOD cells was also evidenced by blunted induction of CHOP (Fig. 3G). Subsequent experiments revealed that apoptosis induced by CdCl2 was attenuated in LL/MnSOD cells, when compared with that in LL/Mock cells (Fig. 3H). Quantitative analysis showed that percentages of round cells and apoptotic cells were significantly reduced by MnSOD from 55.0 ± 2.9% to 16.4 ± 0.7% (Fig. 3I, left), and from 57.6 ± 1.9% to 19.8 ± 1.3% (Fig. 3I, right), respectively. These results suggested involvement of O2− in cadmium-triggered ER stress and consequent apoptotic cell death.

Involvement of H2O2 in Cadmium-triggered, ER Stress-independent Apoptosis—We next examined involvement of H2O2 in the induction of ER stress and apoptosis by cadmium. LLC-PK1 cells were treated with serial concentrations of H2O2, and expression of GRP78 as well as c-fos was examined by Northern blot analysis. As shown in Fig. 4A, H2O2 induced expression of c-fos in a dose-dependent manner. In contrast, expression of GRP78 was not increased by H2O2 at any concentration tested. This result indicated lack of involvement of H2O2 in the induction of ER stress and ER stress-dependent apoptosis by cadmium. To further examine this possibility, we created LLC-PK1 cells stably overexpressing catalase. Northern blot analysis confirmed abundant expression of catalase mRNA in LL/Catalase-9 cells and LL/Catalase-11 cells (Fig. 4B). Microscopic analysis revealed that these transfectants were resistant to H2O2-induced apoptosis (Fig. 4C, left). Quantitative analysis by Hoechst staining showed that the percentage of apoptotic cells with condensed nuclei (supplemental Fig. S3) was markedly reduced by catalase from 50.4 ± 4.2% to 7.3 ± 0.5% (Fig. 4C, right). Using these transfectants, induction of ER stress by cadmium was re-tested. As shown in Fig. 4D, induction of GRP78 by CdCl2 was not different between LL/Mock cells and LL/Catalase cells. The similar results were obtained in both LL/Catalase-9 cells and LL/Catalase-11 cells. This result, together with the finding shown in Fig. 4A, indicated that H2O2 did not contribute to the induction of ER stress and ER stress-dependent apoptosis in response to cadmium. Interestingly, however, apoptosis induced by CdCl2 was significantly less in LL/Catalase cells, when compared with that in LL/Mock cells (Fig. 4E). Percentages of round cells and apoptotic cells were reduced by catalase from 55.0 ± 2.6% to 21.5 ± 2.9% (Fig. 4F, left), and from 57.6 ± 1.9% to 37.6 ± 5.1% (Fig. 4F, right), respectively. These results indicated that H2O2 was generated in response to CdCl2.
and contributed to cadmium-induced apoptosis independent of ER stress.

Lack of Involvement of ONOO\(^{-}\) in Cadmium-triggered ER Stress and Apoptosis—We further examined involvement of ONOO\(^{-}\) in the induction of ER stress and apoptosis by cadmium. LLC-PK1 cells were treated with serial concentrations of 3-morpholinosydnonimine (SIN-1), a generator of ONOO\(^{-}\), and expression of GRP78 and CHOP, as well as c-fos, was evaluated by Northern blot analysis. Treatment with SIN-1 induced expression of c-fos, indicating induction of oxidative stress (supplemental Fig. S4). As shown in Fig. 5A, SIN-1 also induced expression of GRP78 and CHOP in a dose-dependent manner. Microscopic analyses revealed that treatment with SIN-1 induced membrane blebbing and nuclear condensation typical of apoptosis (Fig. 5B), suggesting the potential of ONOO\(^{-}\) to induce ER stress and apoptosis in LLC-PK1 cells. To examine involvement of ONOO\(^{-}\) in the effects of cadmium, we used uric acid, an inhibitor of ONOO\(^{-}\), as well as N\(^{\omega}\)-nitro-\(\lambda\)-arginine methyl ester (\(\lambda\)-NAME), an inhibitor of NO that is required for the generation of ONOO\(^{-}\). As shown in Fig. 5C, both inhibitors did not affect induction of the ER stress markers by CdCl\(_2\). Furthermore, apoptosis induced by CdCl\(_2\) was not attenuated by uric acid or \(\lambda\)-NAME (Fig. 5D). Quantitative analysis showed that, even at the high concentration (5 mM), uric acid and \(\lambda\)-NAME did not reduce percentages of round cells (Fig. 5E, left) and apoptotic cells (Fig. 5E, right). These results indicated that ONOO\(^{-}\) was not involved in the induction of ER stress and apoptosis by cadmium in LLC-PK1 cells.

Activation of the ATF6-CHOP Pathway and the XBP1-JNK Pathway via Cadmium-triggered Generation of O\(_2\)\(^{-}\)—Cadmium activates the ATF6-CHOP pathway and the IRE1-XBP1 pathway, both of which contribute to the induction of apoptosis (1). We therefore examined roles of O\(_2\)\(^{-}\) in the activation of these proapoptotic cascades. First, LLC-PK1 cells were transiently transfected with FLAG-tagged p90 ATF6, exposed to menadione for up to 6 h, and subjected to Western blot analysis. As shown in Fig. 6A, treatment of the cells with menadione generated active p50 ATF6, suggesting the potential of O\(_2\)\(^{-}\) to activate the ATF6 pathway. To examine whether O\(_2\)\(^{-}\) produced by cadmium is causative of ATF6 activation, LL/Mock cells and LL/MnSOD cells were transiently transfected with FLAG-tagged p90 ATF6, exposed to CdCl\(_2\), and subjected to Western blot analysis. Compared with LL/Mock cells, the increase in the level of p50 ATF6 was attenuated in LL/MnSOD cells (Fig. 6B). Furthermore, induction of CHOP in LL/MnSOD cells, but not in LL/Catalase cells, was lower than that in LL/Mock cells (Fig. 6C). These results suggested that the ATF6-CHOP proapoptotic pathway was activated, at least in part, by O\(_2\)\(^{-}\) generated in cadmium-exposed cells.

We next examined a role of O\(_2\)\(^{-}\) in the activation of the IRE1-XBP1 pathway. LLC-PK1 cells were treated with menadione for up to 4 h, and splicing of XBP1 mRNA was examined by reverse transcription-PCR. As shown in Fig. 6D, following the exposure to O\(_2\)\(^{-}\), the spliced form of XBP1 mRNA (\(\beta\text{XBP1}\)) appeared within 1 h, which was sustained for at least 4 h, confirming the potential of O\(_2\)\(^{-}\) to activate the IRE1-XBP1 pathway. To examine whether cadmium-triggered O\(_2\)\(^{-}\) is causative of activation of this proapoptotic pathway, LL/Mock cells and LL/MnSOD cells were transiently transfected with a reporter plasmid pCAX-F-\(\beta\)XBP1\(\Delta\)DBD-Luc. As described previously, the transfected cells produce luciferase exclusively under the conditions that trigger splicing of XBP1 mRNA (20). In CdCl\(_2\)-exposed LL/Mock cells,
Selective ROS Upstream of Proapoptotic UPR

**Figure 6. Activation of the ATF6-CHOP and XBP1-JNK proapoptotic pathways via cadmium-triggered generation of O$_2^\cdot$.** A, LLC-PK1 cells were transiently transfected with FLAG-tagged p90ATF6, exposed to menadione (10 $\mu$m) for up to 6 h, and subjected to Western blot analysis of p90 ATF6 and p50 ATF6. Dithiothreitol (DTT, 5 mM) was used as a positive control. The level of $\beta$-actin is shown at the bottom as a loading control. B, LL/Mock cells and LL/MnSOD cells were transfected with FLAG-tagged p90ATF6, exposed to CdCl$_2$, and subjected to Western blot analysis. C, LL/Mock cells, LL/Catalase cells and LL/MnSOD cells were treated with CdCl$_2$, for 6 h, and induction of CHOP was examined by Northern blot analysis. D, LLC-PK1 cells were treated with menadione for up to 4 h and subjected to reverse transcription-PCR to evaluate splicing of XBP1 mRNA. XBP1(U) and XBP1(S) indicate unspliced and spliced forms of XBP1 mRNA, respectively. RT(-), reaction without reverse transcriptase. E, LL/Mock cells and LL/MnSOD cells were transiently transfected with a luciferase-based reporter plasmid for quantification of XBP1 mRNA splicing, treated with 20 $\mu$m CdCl$_2$, for 4 h, and subjected to chemiluminescent assay. The values are shown as relative percentages of luciferase activity versus cadmium-untreated, individual control cells. Assays were performed in quadruplicate, and data are presented as means $\pm$ S.E. An asterisk indicates a statistically significant difference (p < 0.05). F, G, and I, LL/Mock cells and LL/XBP1-DN cells expressing a dominant-negative mutant of XBP1 were treated with 10 $\mu$m menadione (F), 20 $\mu$m CdCl$_2$ (G), or 200 $\mu$m H$_2$O$_2$ (I) for 1–2 h and subjected to Western blot analysis of phosphorylated JNK (P-JNK). Total protein of JNK is shown at the bottom as a loading control. H, LLC-PK1 cells were transiently transfected with XBP1(S), treated with (+) or without (-) CdCl$_2$, for 1 h, and subjected to Western blot analysis. J, LL/Mock cells and LL/Catalase cells were treated with CdCl$_2$, for 1–2 h, and phosphorylation of JNK was evaluated.

Luciferase activity was increased by 73.2% compared with untreated cells. However, this induction was significantly less in LL/MnSOD cells (39.3%) (Fig. 6E). These results suggested that O$_2^\cdot$ was also involved in the activation of the IRE1-XBP1 pathway in cadmium-exposed cells.

In general, the IRE1 pathway is proapoptotic via activation of apoptosis signal-regulating kinase 1 (ASK1) and JNK. Indeed, in our previous report, we demonstrated that JNK was rapidly activated following exposure to cadmium, and pharmacological suppression of JNK substantially attenuated apoptosis (1). However, we also found that dominant-negative inhibition of XBP1, which is generally not involved in the IRE1-JNK pathway, markedly attenuated cadmium-induced apoptosis independently of GRP78 or CHOP (1). We speculated that activation of JNK could be an event downstream of XBP1 in cadmium-exposed cells. To examine this possibility, LLC-PK1 cells were stably transfected with a gene coding for XBP1-DN, and LL/XBP1-DN cells were established. Northern blot analysis confirmed substantial suppression of the transgene in the established transfectants (supplemental Fig. S5). LL/Mock cells and LL/XBP1-DN cells were then treated with menadione or CdCl$_2$ and subjected to Western blot analysis of phosphorylated JNK. As shown in Fig. 6, F and G (left), both menadione and CdCl$_2$ induced rapid phosphorylation of JNK in LL/Mock cells. In contrast, the activation of JNK was markedly suppressed in LL/XBP1-DN cells (Fig. 6, F and G, right). Furthermore, transfection of LLC-PK1 cells with XBP1(S) enhanced cadmium-triggered phosphorylation of JNK (Fig. 6H), confirming involvement of XBP1 in the activation of JNK. Interestingly, the role of XBP1 was specific to the O$_2^\cdot$-triggered JNK activation, because 1) phosphorylation of JNK by H$_2$O$_2$ was observed to the same extent in LL/Mock cells and LL/XBP1-DN cells (Fig. 6I and 2) CdCl$_2$-induced phosphorylation of JNK was similarly observed in LL/Catalase cells (Fig. 6J). Taken together, these results evidenced that cadmium caused activation of the ATF6-CHOP and IRE1-XBP1 proapoptotic pathways via generation of O$_2^\cdot$ and that activation of JNK was located downstream of XBP1.

**DISCUSSION**

ROS is implicated in heavy metal-induced renal injury. However, cadmium-induced apoptosis of renal tubular cells is also dependent on ER stress (1). The present study was performed to elucidate roles of individual ROS and downstream events in cadmium-triggered, ER stress-mediated apoptosis. Our results elucidated that 1) cadmium caused ER stress via generation of ROS, 2) O$_2^\cdot$, but not H$_2$O$_2$ and ONOO$^-$, mediated cadmium-triggered, ER stress-induced apoptosis via activation of the
Selective ROS Upstream of Proapoptotic UPR

ATF6-CHOP and IRE1-XBP1 pathways, 3) phosphorylation of JNK, a mediator of cadmium-induced apoptosis, was caused via O$_2^-$-triggered activation of XBP1, and 4) H$_2$O$_2$ was involved in cadmium-induced apoptosis independently of ER stress. The outline of our current hypothesis is summarized and illustrated in Fig. 7.

Consistent with our current results, some reports indicated that O$_2^-$ may be selectively involved in ER stress-associated pathologies. Ischemic injury is a well known pathological situation in which both oxidative stress and ER stress are involved (25, 26). Using copper/zinc SOD transgenic rats, Hayashi et al. examined induction of ATF4 and CHOP, markers of ER stress, in the brain after cerebral ischemia (27). In wild-type rats, ischemia induced expression of ATF4 and CHOP in the hippocampus, leading to neuronal apoptosis. In contrast, transgenic rats overexpressing SOD showed only a modest increase of these molecules and exhibited minimal neuronal degeneration, indicating that O$_2^-$ is involved in both induction of ER stress and consequent neuronal death.

In biological systems, O$_2^-$ is rapidly reduced by SOD to H$_2$O$_2$. O$_2^-$ also rapidly reacts with NO, yielding ONOO$^-$ (27). Once O$_2^-$ is generated, therefore, H$_2$O$_2$ and ONOO$^-$ may be subsequently produced and could contribute to the apoptotic process. In the present study, we found that all these ROS have the potential to induce apoptosis in LLC-PK1 cells. However, H$_2$O$_2$ did not cause ER stress in this cell type. Although ONOO$^-$ triggered ER stress, scavenging of ONOO$^-$ did not attenuate cadmium-induced ER stress and apoptosis. These results evidenced selective and differential roles of ROS in the cadmium-triggered, ER stress-mediated apoptotic pathway. Interestingly, overexpression of catalase significantly attenuated cadmium-induced apoptosis without affecting the level of ER stress. This result indicated that, although H$_2$O$_2$ is not involved in ER stress-dependent apoptosis, it does contribute to cadmium-induced apoptosis independently of ER stress, possibly via the mitochondrial pathway (28).

Our current data indicated that cadmium induces apoptosis of tubular cells through ER stress-dependent and -independent mechanisms. The fact that cadmium-induced apoptosis was markedly attenuated by antioxidants (Fig. 1, B and C) suggested that oxidative stress is the major mechanism for cadmium-induced apoptosis, as was reported previously (4, 24). However, the mechanisms involved were found to be not so simple, i.e. oxidative stress-induced apoptosis is mediated by, at least in part, ER stress, and only O$_2^-$ participates in the ER stress-dependent apoptotic process. H$_2$O$_2$ mediates apoptosis independently of ER stress. Furthermore, ONOO$^-$ is not involved in both ER stress-dependent and -independent apoptotic processes. NAC completely inhibited oxidative stress but only partially attenuated the level of ER stress (Fig. 2, A and B), indicating a possibility that cadmium could also induce ER stress independently of oxidative stress.

The relationship between oxidative stress and ER stress is not well understood. Haynes et al. recently reported that prolonged activation of UPR resulted in oxidative stress and consequent cellular death in Saccharomyces cerevisiae. Accumulation of ROS by UPR may be caused through two mechanisms; the oxidative folding machinery in the ER and the mitochondria-dependent ROS generation (29). However, our current results evidenced that oxidative stress was upstream, but not downstream of ER stress in cadmium-exposed cells. Consistent with our results, some recent reports also indicated that ER stress may be involved downstream of ROS. For example, in rat brain tumor cells, the anticancer agent geldanamycin caused expression of GRP78 via a ROS-dependent mechanism (30). In human vascular endothelial cells, ONOO$^-$ caused modest increases in GRP78 and GRP94 proteins (31). The source of ROS in cadmium-exposed cells was not investigated in the present study, but the mitochondrial pathway is possibly responsible for the generation of ROS in cadmium-exposed tubular cells (28).

Currently, it is still unclear how ROS induces ER stress. Previous reports showed that oxidative stress caused inhibition of Ca$^{2+}$-ATPase (32, 33), a known trigger of ER stress. One possibility is, therefore, that ROS may cause depletion of the calcium store in the ER via inhibition of Ca$^{2+}$-ATPase (34). Another possibility is that ROS might cause ER stress through generation and accumulation of oxidatively modified, abnormal proteins. Unfolded proteins may also be accumulated in the ER through ROS-induced functional perturbation of ER foldases and/or chaperones (35).

Among three major UPR branches, the ATF6 pathway and the IRE1 pathway mediate cadmium-triggered apoptosis, and induction of CHOP by ATF6 and activation of both XBP1 and JNK by IRE1 are involved in the apoptotic process (1). In the present investigation, we disclosed that O$_2^-$ produced in...
cadmium-exposed cells can trigger the ATF6-CHOP and the IRE1-XBP1 proapoptotic pathways. An interesting finding is that activation of JNK, an important mediator of cadmium-induced apoptosis (1), was located downstream of XBP1. In general, it is believed that, in response to ER stress, activation of JNK occurs following recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) by IRE1. ASK1 is also required for the TRAF2-dependent JNK activation, and the IRE1-TRAF2-ASK1-mediated phosphorylation of JNK plays a crucial role in ER stress-induced apoptosis independently of the IRE1-XBP1 pathway (36). To our knowledge, this is the first report demonstrating the existence of the XBP1-JNK proapoptotic pathway involved in ER stress-induced apoptosis.

ROS are involved in a wide range of cellular events under various pathophysiological circumstances. Our current finding that ER stress can locate downstream of oxidative stress raises a possibility that ER stress and UPR may also be involved not only in cadmium-induced apoptosis but also in a variety of other biological responses. Further investigation will be required to elucidate the spectrum of pathophysiological significance of the oxidative stress-ER stress axis in living cells and animals.

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