Endoplasmic Reticulum Stress–Associated Caspase 12 Mediates Cisplatin-Induced LLC-PK1 Cell Apoptosis

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Reactive oxygen metabolites are important mediators in cisplatin-induced apoptosis in renal tubular epithelial cells (LLC-PK1). Mitochondria have been implicated to play a principal role in cisplatin-induced apoptosis. Caspase 12, an endoplasmic reticulum (ER)-specific caspase, participates in apoptosis under ER stress. Cytochrome P450 system is crucial to the generation of reactive oxygen metabolites and is present at high concentration in the ER. The direct role of caspase 12 in any model of renal injury has not previously been described. In this study, cleavage of procaspase 12 preceded that of caspases 3 and 9 after cisplatin treatment of LLC-PK1 cells. The active form of caspase 8 was not detected throughout the course of study. Preincubation of the LLC-PK1 cells with the caspase 9 inhibitor did not attenuate caspase 3 activation and provided no significant protection. Caspase 3 inhibitor provided only modest protection against cisplatin-induced apoptosis. LLC-PK1 cells that were transfected with anti–caspase 12 antibody significantly attenuated cisplatin-induced apoptosis. Taken together, these data indicate that caspase 12 plays a pivotal role in cisplatin-induced apoptosis. It is proposed that the oxidative stress that results from the interaction of cisplatin with the ER cytochrome P450 leads to activation of procaspase 12, resulting in apoptosis.

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Materials and Methods

Cell Culture and Treatment

LLC-PK1 cells, purchased from American Type Culture Collection (CRL 1392, Manassas, VA), were maintained in Medium 199 supplemented with 3% FBS in a humidified atmosphere of 95% air/5% CO2 at 37°C and fed at intervals of 3 d (27). The cells were maintained in 75-cm² tissue culture flasks, and the monolayer was subcultured using 0.05% trypsin, 0.53 mM EDTA, in HBSS. All experiments were carried out on confluent cell monolayer between passages 203 and 215. Apoptosis was induced in LLC-PK1 cells by incubation with 50 μM cisplatin at 37°C for 12 h (28). Caspase inhibitions were performed by preincubation of caspase 3 inhibitor DEVD-CHO (60 μM; Bachem, Torrance, CA) and caspase 9 inhibitor LEHD-CHO (60 μM; Peptide Inc., Osaka, Japan) for 1 h at 37°C.

Determination of Caspase Activity

Caspases 3, 8, and 9 activities were tested by use of the Caspase Colorimetric Activity Assay Kits (Chemicon International Inc., Temecula, CA) following the manufacturer’s protocols. Equal numbers of cells (0.5 to 2 x 10⁶) from each group were treated with cell lysis buffer on ice for 10 min and centrifuged at 10,000 x g for 5 min. The supernatants were incubated with respective caspase substrates (DEVD-pNA for caspase 3, IETD-pNA for caspase 8, and LEHD-pNA for caspase 9) at 37°C for 1 h. The activities were assayed by use of a spectrophotometer microtiter plate reader at 405 nm. The specificity of changes in the caspase activities was confirmed by addition of a specific inhibitor to the caspase in parallel experiments.

Preparation of Cellular Fractions

For the detection of caspases, cell fractions were prepared as described previously (25). In brief, LLC-PK1 cells or kidney cortex was lysed and homogenized in buffer A (50 mM Tris-HCl [pH 8.0], 1 mM mercaptoethanol, 1 mM EDTA, 0.32 μM sucrose, and 0.1 mM PMSF). Nuclear fraction was collected from the pellet after first centrifugation at 900 x g for 10 min. The supernatants were centrifuged again at 5000 x g for 10 min, and the pellet was collected as mitochondrial fraction. The resulting supernatants were centrifuged at 105,000 x g for 60 min, and the microsomal and soluble fractions were the pellet and the supernatant, respectively. The purity of these fractions was controlled by the presence of known compartment-specific proteins using Western blot analysis: Cytochrome c for mitochondria and grp78 (Bip) for ER (25). For measurement of cytochrome c release, cytosolic mitochondrial fractions were prepared as described elsewhere (29). In brief, cells were washed with PBS and collected, incubated in 200 μl of mitochondrial buffer (65 mM sucrose, 200 mM mannitol, 50 mM KCl, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail [P-8340; Sigma, St. Louis, MO]), homogenized, and centrifuged at 4°C at 800 x g. The supernatant was centrifuged at 14,000 x g for 10 min at 4°C, and the resulting pellet and supernatant were collected as mitochondrial and cytosolic fractions, respectively.

Western Blot

Western blot was performed by the chemiluminescence method as described elsewhere (30). The antibodies used were as follows: Rabbit anti–caspase 12 (AB3612, Chemicon) and mouse anti–actin (MAB1501; Chemicon); rabbit anti–caspase 3 (sc-7148), caspase 8 (sc-7890), caspase 9 (sc-8355), and rabbit anti–cytochrome C (sc-7159; Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit anti–grp78 (Stressgen, Victoria, BC, Canada).

Transferase-Mediated dUTP Nick-End Labeling Assay

Transferase-mediated dUTP nick-end labeling (TUNEL) was performed by using ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA), by which fluorescein-dUTP incorporation at the free ends of fragmented DNA is visualized by fluorescence microscopy. TUNEL stain was performed following the manufacturer’s protocol.

Detection of DNA Fragmentation

LLC-PK1 cells were homogenized and lysed with a buffer that contained 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM NaCl, and 0.5% SDS. The lysates were incubated with proteinase K (0.2 mg/ml) at 50°C overnight. DNA was isolated from the lysates following the procedures as described by Ramachandra and Studzinski (31). The DNA obtained was resuspended in a buffer (10 mM Tris-HCl and 1 mM EDTA) at 1 μg/ml and incubated with 0.1 U/10 μg DNA of DNase-free RNAse cocktail (cat. no. 2286; Ambion, Austin, TX). Electrophoresis was performed in 1.6% agarose gels, and DNA was visualized with ethidium bromide.

Immunocytochemistry

Cultured LLC-PK1 cells were fixed in B5 solution, and cell block was made by embedding in tissue medium. Sections were cut on a glass slide. After deparaffinization and antigen retrieval, the sections were immunolabeled and visualized according to an avidin-biotin complex (ABC) method (32).

Transfection of Anti–Caspase 12 Ab into LLC-PK1 Cells

Anti–caspase 12 Ab (AB3612, Lot 22101182; Chemicon) was delivered into LLC-PK1 cells by using transfection agent Chariot (3025; Active Motif, Carlsbad, CA) following the manufacturer’s protocol. Briefly, the initial transfection mixture was prepared by adding 100 μl of anti–caspase 12 Ab dilution (1.2 μl of stock Ab in 98.8 μl of PBS) to 100 μl of Charot dilution (6 μl of Chariot stock solution in 96 μl of distilled H2O). The mixture was incubated at room temperature for 30 min to allow the formation of Chariot-Ab complex. LLC-PK1 cells in six-well culture plate (40 to 50% confluence) were overlaid with the mixture (200 μl/well), added with 400 μl of serum-free culture medium (final Ab dilution 1:500), incubated at 37°C in a humidified atmosphere that contained 5% CO2 for 1 h, then added with 1 ml of completed growth medium and continued to

Figure 1. Cleavage of procaspases 12 and 3 in CYP2e1 knockout (KO) mice after cisplatin (Cis) treatment. Procaspase 12 cleavage was markedly attenuated (A) and there was no activation of caspase 3 (B) in the cisplatin-treated KO mice as compared with that of wild-type (WT) mice.
incubate at 37°C for 2 h. The cells were washed twice with PBS before the cisplatin treatment. The transfection efficacy of Chariot in LLC-PK1 cells was tested by using \( \beta \)-galactosidase in a pilot study, and the transfection rate for \( \beta \)-galactosidase was 90%.

**CYP2e1 KO Mice**

Mice (strain 129/SV) carrying a deletion of CYP2e1 gene were provided by Dr. Gonzalez (National Institutes of Health, Bethesda, MD). The CYP2e1 gene was isolated from a 129/SV mouse (CYP2e1 WT, CYP2e1+/+) genomic library that contained the complete coding region. The gene was disrupted by the replacement of exon 2 with the PGK-NEO cassette (33). Mice homozygous for the disrupted CYP2e1 allele were born and developed normally with no obvious phenotypic divergence from WT mice. The clone of KO mice was maintained by breeding CYP2e1−/− male mice with CYP2e1−/− female mice. Immunoblotting and Northern blot confirmed the complete absence of CYP2E1 protein and mRNA in the mice. The animal study was approved by the Institutional Review Board, and the experimental procedures were conducted in accordance with our institutional guidelines.

**Results**

**Cisplatin-Induced ER Stress Results in the Cleavage of Procaspase 12**

We recently demonstrated an important role of microsomal CYP2E1 as a source of ROM generation in cisplatin-induced renal tubular apoptosis (5). Treatment of CYP2e1 KO mice with cisplatin resulted in marked reduction of ROM generation and significant attenuation of apoptosis when compared with CYP2e1 WT mice. Hence, we looked at the role of caspase 12 in these animals that were treated with cisplatin. Indeed, procaspase 12 was markedly cleaved in the WT mice that were treated with cisplatin, and this cleavage was significantly diminished in the KO animals (Figure 1A). These results indicate that the ER-associated caspase 12 plays an important role in cisplatin-induced apoptosis. The activation of caspase 3 was also attenuated in these KO mice (Figure 1B).

**Detection and Localization of Procaspase 12**

Procaspase 12 was detected predominantly in the microsomal fraction of the normal kidney cortex but not in the nuclear, mitochondrial, and soluble fractions by Western blot analysis (Figure 2).

**Cisplatin Induced Apoptosis**

Cisplatin induced apoptosis in a dose-dependent manner as detected by TUNEL staining (17 ± 4.5% at 25 μM, 31 ± 6.7% at 50 μM, and 85 ± 10% at 100 μM). Significant apoptosis with intense TUNEL-positive nuclear staining was noted in the cells that were treated with 50 μM cisplatin for 12 h (Figure 3).

**Figure 2.** Localization of procaspase 12. Western blot analysis of cell fraction from normal rat kidney cortex using anti–caspase 12 antibodies (Ab) showed that procaspase 12 was present exclusively in the microsomal fraction but not in the nuclear, mitochondrial, and soluble fractions. The purity of these fractions was controlled by the presence of known compartment-specific proteins: Cytochrome c for mitochondria and grp78 (Bip) for endoplasmic reticulum (ER).

**Figure 3.** Representative sections of transferase-mediated dUTP nick-end labeling (TUNEL) staining for detection of apoptotic LLC-PK1 cells 12 h after cisplatin treatment. Apoptotic cells were identified by the nuclear fluorescence staining.

**Figure 4.** Time course of caspase activity after cisplatin treatment. Cells were treated with 50 μM cisplatin and assayed for caspase activity at different time points. Caspase 3 and caspase 9 were significantly activated at 9 h and continued to increase. There was only a modest increase in the caspase 8 activity at 24 h as compared with caspases 3 and 9. Values are means ± SEM from four separate measurements. *P < 0.05 versus control (0 h).
Time Course of Caspase Activation and Cytochrome C Release

We initially examined the activities of caspases 3, 9, and 8 in response to cisplatin treatment in the LLC-PK1 cells. Caspases 3 and 9 were significantly activated at 9 h and continued to increase up to 24 h (Figure 4). There was only a modest increase in the caspase 8 activity at 24 h (Figure 4).

Western blot analysis was performed to examine the cleavage of procaspase 12 and its relationship to caspases 3, 9, and 8 during cisplatin-induced injury to the LLC-PK1 cells. As shown in Figure 5A, the cleaved form of procaspase 12 was noted at 9 h after cisplatin treatment and increased progressively thereafter up to 24 h. The densitometric analysis of the blot demonstrated a gradual decrease in procaspase 12 density and an increase in the cleaved form of caspase 12 starting at 9 h (Figure 5B). The active form of caspase 3 was observed at 12 h after the cisplatin treatment, with progressive increase up to 24 h (Figure 5A). The proform of caspase 9 showed a decrease in density of the band at 12 h with absence at 24 h. This is because the Ab to caspase 9 was probably specific to the proform only and did not recognize the active form of caspase 9. The anti–caspase 9 Ab specificity was confirmed by a study using a known caspase 9 activator, staurosporine. As shown in Figure 5C, the procaspase 9 was significantly reduced in staurosporine-treated (5 μM) cells as compared with the control, whereas no cleaved caspase 9 was observed. There was no activation of caspase 8 as indicated by neither presence of active form nor decrease in the band of its proform (Figure 5A). The weak activity of caspase 8 coincided with the lack of its activation by way of Western blot, indicating that the extrinsic pathway may not play a significant role in cisplatin-mediated apoptosis. Western blot analysis indicated that cytochrome c was released from the mitochondria into the cytosol in a time-dependent manner (Figure 6), thus implicating the caspase 9–dependent mitochondrial pathway. Our data thus suggest that the cleavage of the ER-associated procaspase 12 precedes that of caspases 3 and 9.

Cisplatin-Induced Apoptosis Is Caspase 12–Dependent

Effect of Caspase 9 Inhibitor. Pretreatment of the cells with caspase 9 inhibitor prevented cisplatin-induced caspase 9 activation as measured by its activity and Western blot analysis (Figure 7, A and C). Caspase 9 inhibitor, however, did not completely attenuate the caspase 3 activity (Figure 7B) and the formation of the active form of caspase 3 as determined by Western blot analysis. The decrease in the density of cytochrome c band from mitochondrial fraction corresponded with the increase in the density of the band in cytosolic fraction, indicating cytochrome c release beginning at 12 h after cisplatin treatment.
Western blot (Figure 7D), thus indicating that the activation of caspase 3 may also be from an unknown pathway or caspase 12. The cleavage of procaspase 12 after pretreatment with caspase 9 inhibitor was no different from that induced by cisplatin alone (Figure 7E). Caspase 9 inhibitor had no protective effect on cisplatin-induced apoptosis as indicated by DNA laddering (Figure 8A) and TUNEL staining (Figure 8D).

**Effect of Caspase 3 Inhibitor.** Cisplatin-induced caspase 3 activation as measured by Western blot analysis and its activity was markedly attenuated by pretreatment of the cells with caspase 3 inhibitor (Figure 9, A and B). Pretreatment of the cells with caspase 3 inhibitor did not alter the cleaved form of procaspase 12 when compared with cisplatin alone (Figure 9C). Caspase 3 inhibitor provided modest protection against cisplatin-induced apoptosis as demonstrated by TUNEL staining (Figure 8E). However, there was no effect on cisplatin-induced DNA damage as measured by DNA laddering (Figure 8A). This suggests that there exists another pathway or caspase 12 that may act directly on the cells and result in apoptosis.

**Effect of Caspase 12 Ab.** Transfection of the anti–caspase 12 Ab using a Chariot kit significantly attenuated the cisplatin-induced apoptosis as demonstrated by TUNEL staining (Figure 8F) and DNA laddering (Figure 8B). Chariot itself had no effect on cisplatin-induced apoptosis as confirmed by both DNA fragmentation and TUNEL staining (Figure 8, C and F). The procaspase 12 was significantly decreased in the cisplatin-treated LLC-PK1 cells associated with the presence of cleaved caspase 12, and this cleavage was prevented in the cells that were transfected by caspase 12 Ab (Figure 10A). The induction of the active form of caspase 3 was also markedly reduced (Figure 10B) in anti–caspase 12 Ab-transfected cells that were treated with cisplatin. There were no significant differences in the cytochrome c release in the cytosol and the activation of caspase 9 in the caspase 12 Ab–transfected cells and the control cells that were treated with cisplatin (Figure 10, C and D).

**Discussion**

ER is one of the largest cell organelles that is responsible for the production of cellular components, proteins, lipids, and sterols (34). Its proper function is essential for the cell. Various agents, including oxidants, can interfere with the ER function, leading to ER stress and cell death (34). Caspase 12 is an ER-specific caspase that is localized to the cytosolic face of the ER, making it vulnerable to the ER stress and the activation of the caspase cascade (25). In our study, procaspase 12 was localized predominantly to the microsomal fraction of the kidney cortex but not in the nuclear, mitochondrial, and soluble fractions by Western blot analysis.

**Figure 7.** Effect of caspase 9 inhibitor LEHD-CHO on caspase activation. Pretreatment of the cells with LEHD-CHO completely prevented cisplatin-induced caspase 9 activation as measured by its activity (A) and Western blot (C). Administration of cisplatin to the cells that were pretreated with LEHD-CHO did not completely abolish the activation of caspase 3 (B and D). The cleavage of procaspase 12 was no different in the cisplatin-treated cells with or without pretreatment by the caspase 9 inhibitor (E). *P < 0.05 versus control; **P < 0.05 versus cisplatin alone.
cisplatin had significant reduction of ROM generation and provided marked protection against apoptosis (5). Procaspase 12 was markedly cleaved in the WT mice, with significant reduction of the cleavage in the KO animals that were treated with cisplatin, indicating that the ER stress activates caspase 12 that plays a prominent role in cisplatin-induced apoptosis.

Having demonstrated the link between ER stress and procaspase 12 cleavage resulting in apoptosis, we next examined the downstream targets that were yet to be identified in cisplatin-induced apoptosis after procaspase 12 cleavage. The cleavage of procaspase 12 preceded that of caspases 3 and 9, whereas the activation of caspase 8 was not detected, indicating the lack of association with the death receptor pathway. Cytochrome c was released into the cytosol in a time-dependent manner, thus implicating the involvement of the mitochondrial apoptotic pathway. Caspase 9 inhibitor did not completely attenuate caspase 3 activity, and the formation of the active form of the enzyme thus suggests that the activation of caspase 3 may result from an unknown pathway, possibly through caspase 12. This result is supported by the observation of Kaushal et al. (28), in which caspase 3 activation preceded that of caspase 9. Caspase 9 inhibitor had no protective effect as indicated by DNA laddering and TUNEL staining. However, caspase 3 inhibitor provided only modest protection as demonstrated by TUNEL staining with no effect on cisplatin-induced DNA fragmentation. However, caspase 3 inhibitor provided only modest protection as demonstrated by TUNEL staining with no effect on cisplatin-induced DNA fragmentation as tested by DNA laddering, thus suggesting that there exists another pathway or caspase 12 that may act directly on these cells. Our results are similar to those of Schnellman et al. (35), who demonstrated that cisplatin-induced apoptosis is partly caspase 3 dependent and that the activation of caspase 3 could be independent of the mitochondrial dysfunction as well as caspases 8 and 9 activation.

LLC-PK1 cells that were stably transfected with caspase 12 Ab prevented the cleavage of procaspase 12 and significantly attenuated the cisplatin-induced apoptosis as demonstrated by both TUNEL staining and DNA laddering, thus indicating the key role of caspase 12 in cisplatin-induced apoptosis. Several mechanisms have been considered to be responsible for the cytotoxic effects of cisplatin, including DNA damage by intra- and interstrand cross-link (36). However, Shoshan et al. (37) demonstrated the ability of cisplatin to induce ER stress–mediated apoptosis in enucleated tumor cells independent of the direct DNA damaging activity. This report thus further strengthens our observation on the role of ER-specific caspase 12 in this model of injury. As mentioned earlier, inhibition of caspase 3 provided only modest protection; in contrast, caspase 12 Ab–transfected cells significantly attenuated cisplatin-induced apoptosis. This suggests that caspase 12 could execute apoptosis directly or through unknown intermediates.

Apoptotic agents that perturb ER functions can also induce release of cytochrome c from the mitochondria (22,38). It is possible that the modulation of calcium homeostasis and ROM generation can mount an oxidative stress response that damages membranes, including that of the mitochondria (39). This is substantiated by the fact that caspase 3 activation was attenuated in the CYP2e1 KO mice, in which ROM generation was markedly reduced. In our study, no significant differences were observed in the cyto-

Figure 8. Effect of caspase inhibitors on DNA fragmentation. Cisplatin (50 μM, 12 h) induced apoptosis as indicated by DNA fragmentation (A and B). Both caspases 3 and 9 inhibitors did not significantly reduce the DNA damage from cisplatin treatment (A). However, transfection of anti–caspase 12 Ab to LLC-PK1 cells prevented cisplatin-induced DNA fragmentation (B). Chariot by itself did not have any effect on cisplatin-induced DNA fragmentation (C). Effect of caspase inhibitors on cisplatin (Cis)-induced apoptosis by TUNEL staining. Pretreatment of caspase 9 inhibitor did not significantly decrease the cisplatin-induced apoptosis as indicated by the percentage of TUNEL positive staining (D). There was only modest protection by caspase 3 inhibitor (E). LLC-PK1 cells that were transfected with anti–caspase 12 Ab demonstrated marked resistance to cisplatin-induced DNA damage (F). Chariot by itself did not have any effect on cisplatin-induced apoptosis as measured by TUNEL staining (F). The percentage of apoptotic cells was calculated by the cells with positive nuclear staining in five to six randomly chosen microscopy fields. Values are means ± SEM. *P < 0.05 versus controls; †P < 0.05 versus cisplatin alone.

Previous studies using renal tubular epithelial cells have shown that cisplatin-induced apoptosis is caspase dependent and that mitochondria play a prominent role (28,30). We recently demonstrated an important role of the microsomal CYP 2E1 as the source of ROM in cisplatin-induced nephrotoxicity and apoptosis (5). CYP 2e1 KO mice that were treated with...
chrome c release and the activation of caspase 9 are not the down-
stream effects of procaspase 12 cleavage.

Our data thus indicate the existence of a novel apoptotic
pathway in which caspase 12 functions as the initiator
caspase in response to a toxic insult to the LLC-PK1 cells. The
ER-specific caspase 12 not only plays a critical role but also
helps to define the precise mechanism of apoptosis in this
model of renal injury. We propose that the oxidative stress
that results from the interaction of cisplatin with CYP leads
to the activation of caspase 12 that acts directly on the cell
and also via the effector caspase 3 resulting in apoptosis. The
generation of ROM also leads to the mitochondrial mem-
brane damage with cytochrome c release that activates
caspase 9. These two arms of the ER stress response operate
independent of each other, leading to apoptosis. Recent ob-
servation indicates that caspase 4 in humans is homologous
to murine caspase 12, and it is activated in an ER stress–
specific manner, suggesting that it might be the human form
of caspase 12 (40). This is certainly encouraging as it will
provide new strategies or insights to prevent or ameliorate
cisplatin-induced injury to the renal tubular epithelial cells.

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