Caspase-dependent and -independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells

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Caspase-9 and -3 were activated only by 10 μM cadmium, as measured by immunofluorescence imaging and immunoblotting. Caspase-3 activity and apoptosis at 24 h, suggesting calpain-dependent death pathways, remained poorly defined. Using Hoechst 33342/ethidium bromide nuclear staining and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell death assays, 10–50 μM cadmium induced apoptosis of immortalized rat kidney cells derived from the S1-segment of PT at 6 and 24 h, but necrosis at 24 h only. Cadmium (10–50 μM) also caused mitochondrial cytochrome c (cyt. c-) and apoptosis-inducing factor release at 24 h, but not at 6 h, as measured by immunofluorescence imaging and immunoblotting. Caspases-9 and -3 were activated only by 10 μM cadmium for 24 h, and accordingly apoptosis was significantly reduced by the respective inhibitors (z-LEHD-fmk, z-DEVD-fmk; 10 μg/ml) at 24 h, but not at 6 h, without affecting necrosis. At 6 h, 10 μM cadmium increased the activity of the calcium-activated protease calpain, but not at 24 h, and calpain inhibitors (ALLN, PD 150606; 10–30 μM) blocked apoptosis by 10 μM cadmium at 3–6 h. However, PD-150606 also attenuated caspase-3 activity and apoptosis at 24 h, suggesting calpain-dependent caspase activation. Thus cadmium-induced apoptosis of PT cells involves a complex and sensitive interplay of signaling cascades involving mitochondrial proapoptotic factors, calpains and caspases, whose activation is also determined by cadmium concentration and the duration of cadmium exposure.

Any Cd^{2+} in the circulation that can be ultrafiltered in unbound or bound forms by the kidney glomeruli [e.g., as a high-affinity complex to metal-binding proteins, such as metallothioneins (18) or the tripeptide glutathione] passes the renal tubules. Filtered Cd^{2+} is mainly reabsorbed by the S1 segment of the proximal tubule (PT), the primary target of Cd^{2+}-induced nephrotoxicity (12, 35, 43), because it apparently possesses transport pathways, metabolizing enzymes, and receptors that are essential for Cd^{2+} uptake into the PT cells (35, 43). Accumulation of inorganic Cd^{2+}, the nephrotoxic moiety (35), in PT upregulates metallothioneins and glutathione once the balance of detoxifying peptides and Cd^{2+} is aberrant, damage to the kidneys ensues, resulting in a disease resembling Debré-Toni-Fanconi syndrome with proteinuria, aminoaciduria, polyuria, calciuria, glucosuria, and phosphaturia. However, the exact mechanisms by which Cd^{2+} mediates nephrotoxicity remain largely unclear.

At the cellular level, a wide variety of cytotoxic and metabolic effects can be mediated by Cd^{2+}, such as altering the activities of various enzymes, interfering with the normal protective actions of essential metals (Ca^{2+}, Zn^{2+}, Fe^{2+}, Se^{2-}), inducing oxidative stress, inhibiting mitochondrial ATP production, and altering gene expression that may all culminate in the triggering of cell death by either apoptosis or necrosis (2, 34, 35, 44). Apoptosis, or programmed cell death, is morphologically distinct from the degenerative process, necrosis. The end point of the cell death process in apoptotic cells is the condensation and fragmentation of chromatin within the nucleus, whereas the plasma membrane remains intact, preventing leakage of the cytosolic contents into the extracellular space. In contrast, necrotic cells swell and burst, spilling their contents into the extracellular space causing an inflammatory response.

In principle, apoptotic pathways can be divided into two main types: the death receptor, or extrinsic, pathway and the mitochondria-dependent, or intrinsic, pathway, which may cross talk under certain conditions (for review, see Refs. 15 and 45). The mitochondria-dependent pathway, which is thought to mediate Cd^{2+}-induced apoptosis (31, 35), entails the release of death-promoting factors, such as cytochrome c and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane space (IMS), which induce apoptosis in caspase-dependent and -independent manners, respectively (32). Caspases are cysteinyl proteases responsible for the cleavage of intracellular substrates, leading to apoptosis (10). Despite the differing upstream pathways, there is a convergence of extrinsic and...
intrinsinc pathways into two common downstream integrators, namely, mitochondrial dysfunction (9) and the activation of caspase-3. However, as aforementioned, apoptosis independent of the caspase cascade can also occur (3, 17, 42).

Presently, there is no strong evidence directly linking Cd2+- toxicity to cytochrome c liberation from mitochondria to induce apoptosis (1, 16, 19), and to our knowledge no data are available for PT cells. The source of proteolytic degradation in Cd2+-induced apoptosis is also one of the least resolved aspects: Caspase-dependent (16, 19, 39) and -independent (23, 33) proteolytic mechanisms have been reported in various nonrenal cells, but just one study in human lymphoma cells has investigated the role of calpains (24), another ubiquitously expressed family of cytosolic Ca2+-dependent cysteine proteases that are involved in the proteolytic degradation of cells undergoing apoptosis (5, 13). Furthermore, it is not known whether caspase activation leads to calpain activation or vice versa.

The aim of this study was to decipher the pathways activated by Cd2+ during apoptosis of kidney PT cells. The roles of cytochrome c and AIF release as well as activation of caspases and calpains were investigated.

MATERIALS AND METHODS

Materials

Etidium bromide (EtBr) and MTT were from Sigma (St. Louis, MO), N-acetyl-Leu-Leu-Nle-CHO (ALLN), Hoechst 33342 (H-33342), and 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD 150606) from Calbiochem (San Diego, CA), and digitonin (water soluble form) from Fluka (Dorset, UK). The mouse monoclonal antibody (mAb) against cytochrome c (clone 7H8.2C12) was from BD Pharmingen (Erembodegem, Belgium), donkey anti-mouse indocarbocyanin (Cy3)-coupled immunoglobulin G from Dianova (Hamburg, Germany), and sheep anti-mouse horseradish peroxidase- (HRP)-conjugated IgG from Amersham Life Sciences (Bucks, UK). The mAb against human AIF (no. sc-13116), benzoylxy carbonyl-Asp(Ome)-Glu(Ome)-Val-Asp(Ome)-fluoromethyl ketone (z-DEVD-fmk), and benzoylxy carbonyl-Leu-Glu(Ome)-His-Asp(Ome)-fluoromethyl ketone (z-LEHD-fmk) were from Santa Cruz Biotechnology (Santa Cruz, CA). Colorimetric assays of caspase-3 and -9 activities were purchased from Chemicon (Chemicon International) or BioCat (Heidelberg, Germany). The fluorometric assay of calpain activity was from Biovision Research Products (Mountain View, CA). Inhibitors and drugs were dissolved in either water, ethanol, or dimethyl sulfoxide, and solvents were used at ≤0.2% (vol/vol).

Methods

Cell culture. An immortalized cell line from the S1 segment of rat PT (WKPT-0293 Cl.2) (40) was cultured as described earlier (20, 37). Cells were passaged (passage number <70) twice a week on reaching confluence at a 1:20 dilution. Experiments with Cd2+ (CdCl2) were conducted essentially as described earlier (20, 37), but in serum-free medium.

Detection of apoptosis and necrosis with H-33342 and EtBr by fluorescence imaging. Staining was conducted as previously described (20, 37). Cells were visualized under λex/λem of 350/460 nm and 518/605 nm for H-33342 (apoptosis) and EtBr (necrosis), respectively, with a Visi-chrome High Speed Monochromator system (Visi-tron Systems, Puchheim, Germany) connected to a Zeiss Axiocam 100M microscope (Carl Zeiss, Jena, Germany). Images were captured using a digital Cool SONY ES CCD camera (Roper Scientific, Tucson, AZ) and acquired, processed, and analyzed with Metamorph software (Universal Imaging, Downingtown, PA). Cells from five random microscopic fields of view at ×200 magnification were counted per dish, and the average percentages of apoptotic and necrotic cells were calculated. Apoptotic cells (H-33342 positive), which were also necrotic (EtBr positive) were designated as “secondary necrotic.”

MTT assay to detect apoptosis and necrosis. The MTT assay is a measurement of cell viability; hence it does not distinguish between apoptosis, necrosis, or inhibition of cell growth (26). MTT, a yellow tetrazolium salt, is metabolized by active succinate dehydrogenase in the mitochondria of living cells into a blue formazan product. Assays were conducted according to Denizot and Lang (8), as previously described (20). Reference wavelength values were subtracted from test wavelength values, and the differences obtained in controls were set to 100%, which was equivalent to %cell death.

Detection of cytochrome c or AIF release and apoptosis by immuno- and H-33342 staining using fluorescence imaging. Immunofluorescence staining was carried out as previously described (20). Briefly, 1 × 105 cells (or 4 × 104 cells for quantification experiments) were grown for 48 h on glass coverslips before Cd2+- treatment. All staining steps were performed at room temperature. Cells were fixed with 4% parafomaldehyde/PBS, permeabilized with 1% SDS/PBS, incubated with anti-cytochrome c (1:600) or anti-AIF antibodies (1:50) and with Cy3-conjugated secondary antibody (λex/λem = 550/630 nm) (1:600). Cells were counterstained with H-33342, and slides were viewed with Cy3 and H-33342 filters using fluorescence imaging and a >100 oil-immersion objective. Cy3 (red) and H-33342 (blue) planes of focus were selected, and images were merged using the Color Combine function in the Metamorph software. Cytochrome c staining was quantified by counting ≥200 cells in each of five microscopic fields of view at ×400 magnification, and the percentages of nonapoptotic or apoptotic cells with either punctate or diffuse cytosolic staining were calculated.

Measurement of caspase activity. Caspase-3 and -9 activities were measured through cleavage of a colorless substrate specific for caspase-3 (Ac-DEVD-pNA) or caspase-9 (Ac-LEHD-pNA) releasing the chromophore, p-nitroaniline (pNA). Assays were carried out according to manufacturer’s instructions with modifications. Cells (5 × 105) were lysed for 15 min on ice, with mixing every 5 min, and cytosolic extracts were mixed with either caspase-3 or caspase-9 substrate, incubated at 37°C for 2 h and measured at λ = 405 nm. The concentration of pNA released by caspases was calculated from a pNA standard curve, or caspase activity was expressed as the percentage of control condition when a caspase assay without pNA standards was used.

Measurement of calpain activity. Calpain activity was determined according to the manufacturer’s instructions by cleavage of a calpain-specific substrate conjugated to the fluorophore 7-amino-4-trifluoromethyl coumarin (AFC). Its cleavage shifts fluorescence emission. Cells (9 × 105) were grown for 48 h before treatment ± Cd2+ for 1–24 h. Cytosolic extracts (70 µg of protein) were mixed with Ac-LLY-AFC, incubated for 1 h at 37°C, and measured at λex/λem = 400/505 nm using an LS50B luminescence spectrophotometer (PerkinElmer, Wellesley, MA).

Digitonin permeabilization of confluent PT cell monolayers. Permeabilization of plasma membranes with digitonin was performed according to Leist et al. (22). Cells (6 × 105) were grown for 48 h before incubation ± CdCl2 for 6 or 24 h. Cold mannitol buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM sodium succinate, 0.2 mM EGTA, pH 7.2 with KOH) containing 80 µg/ml digitonin was added to selectively permeabilize apical cell membranes. The flask was vigorously agitated at 4°C for 15 min, supernatants containing cytosolic proteins were centrifuged at maximal speed in a microcentrifuhe, and the supernatant was lyophilized for immunoblotting experiments. Cell homogenate was used as a positive control for total cellular content of AIF.

SDS-PAGE and immunodetection. Lyophilized cytosolic proteins (140 µg) were separated by SDS-PAGE on 9% acrylamide Laemmli minigels and transferred onto polyvinylidene difluoride membranes
overnight at 4°C. Blots were blocked with 3% nonfat dry milk and incubated overnight at 4°C with primary anti-AIF (1:500–1:1,000) antibody. Following incubation with HRP-conjugated secondary antibody (1:10,000) for 1 h at 4°C, blots were developed using chemiluminescence reagents and signals were visualized on X-ray films. Blots were scanned digitally using a Bio-Rad GS 700 Densitometer apparatus and analyzed with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Statistical Analyses

Representative data are shown as means ± SE. Statistical analysis using unpaired Student’s t-test was carried out with Sigma Plot 8.0 (Chicago, IL). For more than two groups, one-way ANOVA was used assuming equality of variance with Levene’s test and Tukey’s post hoc test for pairwise comparison with SPSS 11.0. Results with P ≤ 0.05 were considered to be statistically significant.

RESULTS

Cd²⁺ Induces Apoptosis and Necrosis of PT Cells

When cultured PT cells are incubated with Cd²⁺ at low micromolar concentrations, cell death occurs. The DNA dyes H-33342 and EtBr were used to distinguish between apoptosis and necrosis, respectively. Apoptosis was defined by condensation or fragmentation of the nucleus. Condensed nuclei have increased fluorescence intensity and are smaller in size compared with controls. Control cells at 6 and 24 h exhibited increased fluorescence intensity and are smaller in size compared with controls.

Next, we investigated whether apoptosis mediated by Cd²⁺ in PT cells is associated with mitochondrial cytochrome c release, as described for other cells (16, 19). Control cells display punctate cytosolic staining of cytochrome c and AIF, whereas punctate cytosolic staining is observed in AIF-exposed cells compared with controls using 1-way ANOVA.

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Fig. 1. Cd²⁺ induces apoptosis and necrosis in proximal tubule (PT) cells. Cells were treated with Cd²⁺ (10 or 50 μM) for 6 or 24 h in serum-free medium. Nuclei were stained with 2 μg/ml H-33342, and apoptotic nuclei were defined as condensed or fragmented. Necrotic cells were detected with 5 μg/ml ethidium bromide (EtBr). Values are means ± SE of 6 experiments. *P < 0.05, **P < 0.01, Cd²⁺-exposed cells compared with controls.

Apoptotic nuclei were identified as condensed or fragmented. Ne-
Fig. 3. Apoptosis-inducing factor (AIF) is released by Cd\(^{2+}\) from PT cells after 24 h only. 

A: immunostaining used an anti-AIF antibody (1:50). Micrographs are representative of 8 experiments. 

B: immunoblots of cytosolic supernatant with AIF antibody (for details, see MATERIALS AND METHODS). Homogenate represents total cellular content of AIF. Immunoblots are typical of 5 experiments.

Fig. 4. Caspase activity in PT cells after Cd\(^{2+}\) exposure for up to 24 h. 

A: caspase-9 and -3 enzyme activities per 10\(^6\) cells were plotted. Controls (17.6 ± 2.7 and 10.4 ± 1.6 \(\mu\)mol/10\(^6\) cells for caspases-3 and -9, respectively) were subtracted from Cd\(^{2+}\) data at 24 h incubation. Values are means ± SE of 4–7 experiments. **P < 0.01, Cd\(^{2+}\)-treated cells compared with controls using 1-way ANOVA. 

B: values are means ± SE of 4–6 experiments. **P < 0.01, Cd\(^{2+}\) (10 \(\mu\)M)-treated cells compared with respective controls at different time points up to 24 h using Student’s unpaired \(t\)-test. 

C: MTT assay of PT cells treated with 10 \(\mu\)M Cd\(^{2+}\) for 6 h ± caspase-9 inhibitor z-LEHD-fmk (10 \(\mu\)g/ml) or caspase-3 inhibitor z-DEVD-fmk (10 \(\mu\)g/ml). The MTT assay at 6 h reflects apoptosis. Inhibitors were preincubated for 1 h before coincubation with Cd\(^{2+}\) for 24 h. Values (means ± SE of 6–8 experiments) are normalized to controls without Cd\(^{2+}\). Cd\(^{2+}\) plus inhibitor compared with Cd\(^{2+}\) only and inhibitor with control condition were not significantly different from each other. *P < 0.01, Cd\(^{2+}\) and Cd\(^{2+}\) + inhibitor compared with control using 1-way ANOVA.
chromosome c localization. When the cells are treated with 10 μM Cd²⁺ for 6 h, apoptosis occurs, as exemplified by the fragmented nucleus, but cytochrome c staining remains largely particulate, as in the control; in contrast, after Cd²⁺ treatment for 24 h, cytochrome c distribution has changed from punctate to diffuse, indicating that cytochrome c has been released from mitochondria into the cytosol (data not shown). Quantification of cytochrome c release by 10 μM Cd²⁺ further confirmed that cytochrome c was mainly liberated after 24 h (Fig. 2). There were significantly more cells with punctate cytochrome c distribution (i.e., without cytochrome c release) and apoptotic nuclei at 6 h compared with 24 h. Accordingly, there was a significant increase in the percentage of cells with diffuse cytochrome c distribution (i.e., with cytochrome c release) and condensed or fragmented nuclei at 24 h compared with 6 h.

Interestingly, a change in cytochrome c distribution also seems to occur in cells without condensed or fragmented nuclei (data not shown), confirming that release of proapoptotic factors from mitochondria takes place before DNA condensation/fragmentation (29, 32). Indeed, there was a significant increase in cells without nuclear signs of apoptosis but with subsequent cytochrome c localization. When the cells are treated with 10 μM Cd²⁺ for 6 h, apoptosis occurs, as exemplified by the fragmented nucleus, but cytochrome c staining remains largely particulate, as in the control; in contrast, after Cd²⁺ treatment for 24 h, cytochrome c distribution has changed from punctate to diffuse, indicating that cytochrome c has been released from mitochondria into the cytosol (data not shown). Quantification of cytochrome c release by 10 μM Cd²⁺ further confirmed that cytochrome c was mainly liberated after 24 h (Fig. 2). There were significantly more cells with punctate cytochrome c distribution (i.e., without cytochrome c release) and apoptotic nuclei at 6 h compared with 24 h. Accordingly, there was a significant increase in the percentage of cells with diffuse cytochrome c distribution (i.e., with cytochrome c release) and condensed or fragmented nuclei at 24 h compared with 6 h.

To complement the data with cytochrome c, the release of AIF was also investigated (Fig. 3A). In control cells, AIF staining also consisted of a punctate distribution (Fig. 3A, middle). When PT cells were treated with 10 μM Cd²⁺, apoptosis occurred after 6 h (Fig. 3A, top right), but there was no release of AIF as depicted by the lack of diffuse AIF distribution. However, after 24-h Cd²⁺ treatment AIF distribution was now diffuse (Fig. 3A, bottom right), indicating AIF release into the cytosol. Surprisingly, we could not detect any translocation of AIF from the cytosol to the nucleus, as described by others (7). The release of AIF at 24 h, but not at 6 h, was confirmed by immunodetection of cytosolic extracts of Cd²⁺-treated PT cells (Fig. 3B). After 24 h there was a Cd²⁺ concentration-dependent increase in cytosolic AIF with significant AIF release at 10 and 50 μM Cd²⁺ (10 μM Cd²⁺: 14.4 ± 3.0% of cellular AIF; n = 5, P < 0.05; 50 μM Cd²⁺: 54.4 ± 2.7% of cellular AIF, n = 5, P < 0.01).

Table 1. Effect of caspase inhibitors on Cd²⁺-induced apoptosis and necrosis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 μM Cd²⁺</th>
<th>z-DEVD-fmk, 10 μg/ml</th>
<th>z-DEVD-fmk, 10 μM Cd²⁺</th>
<th>z-LEHD-fmk, 10 μg/ml</th>
<th>z-LEHD-fmk, 10 μM Cd²⁺</th>
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<tr>
<td>Apoptosis</td>
<td>0.79±0.11</td>
<td>7.18±1.05†</td>
<td>0.84±0.12</td>
<td>2.93±0.60‡</td>
<td>0.87±0.20</td>
<td>2.98±0.51†</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0.25±0.08</td>
<td>6.20±1.57†</td>
<td>0.20±0.05</td>
<td>5.50±1.37</td>
<td>0.35±0.13</td>
<td>5.05±1.04</td>
</tr>
<tr>
<td>Secondary necrosis</td>
<td>0.00±0.00</td>
<td>0.82±0.28*</td>
<td>0.00±0.00</td>
<td>0.41±0.15</td>
<td>0.10±0.07</td>
<td>0.38±0.21</td>
</tr>
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Values are mean percentage ± SE of 3 experiments. Cell death was determined by H-33342 (apoptosis) and ethidium bromide (EtBr) staining (necrosis). The caspase-9 inhibitor z-LEHD-fmk or caspase-3 inhibitor z-DEVD-fmk was preincubated for 1 h before coincubation with Cd²⁺ for 24 h. Cd²⁺ treatment compared with controls (*P < 0.05, †P < 0.01) and Cd²⁺ + inhibitor compared with Cd²⁺ only (‡P < 0.01) by 1-way ANOVA.

The question remained as to how Cd²⁺ induces apoptosis at early time points. Thus the time-dependent activity of the protease calpain in response to 10 μM Cd²⁺ was studied. Figure 5 shows that calpain activity started to increase after 3 h, reached a maximum at 6 h, and was back to basal levels at 24 h. Measurements of calpain activity were complemented by 10.22.33.4 on October 20, 2017 http://ajprenal.physiology.org/ Downloaded from
by MTT assays with calpain inhibitors. Cell death (reflecting apoptosis) induced by 10 μM Cd2+ for 3 or 6 h was prevented when PT cells were preincubated with the general calpain and cathepsin inhibitor ALLN (10–20 μM; Fig. 6A) or the specific calpain inhibitor PD 150606 (10–30 μM; Fig. 6, B and C). The inhibitory effect of PD 150606 on apoptosis was further confirmed using H-33342/EtBr staining. Preincubation of PT cells with 30 μM PD 150606 significantly inhibited apoptosis induced by 10 μM Cd2+ after 3 and 6 h (Table 2).

To find out whether calpains and caspases are intertwined in a feedback mechanism, the effect of PD-150606 was studied on Cd2+-induced apoptosis after 24 h using H-33342/EtBr staining. Indeed, there was significant attenuation of apoptosis, but not of necrosis, induced by 10 μM Cd2+ after 24 h with 30 μM PD-150606 (Fig. 7A). Thus the partial inhibition of Cd2+-induced apoptosis by PD-150606 at 24 h suggests that calpain activity at 3–6 h plays a role in caspase-mediated apoptosis at 24 h. Indeed, when the activity of the effector caspase, caspase-3, was determined after 24-h incubation with 10 μM Cd2+, the calpain inhibitor PD-150606 (30 μM), was found to significantly inhibit Cd2+-induced caspase-3 activity by ~60% (Fig. 7B), which is the same order of magnitude as the inhibition of Cd2+-induced apoptosis by PD-150606 (Fig. 7A).

**DISCUSSION**

The objective of the present study was to determine the underlying mechanisms by which Cd2+ induces apoptosis in cultured kidney PT cells. We show that apoptotic pathways activated by Cd2+ and involving calpains and caspases are dependent on exposure time and Cd2+ concentration. Activation of the caspase-dependent intrinsic pathway is also enhanced by calpains, demonstrating cross talk between both pathways, which culminate in apoptosis.

**An Early Event in Cd2+ Apoptosis: Calpain Activation**

Caspase-independent mechanisms of Cd2+-induced apoptosis have been primarily attributed to the release of AIF and endonuclease G from mitochondria (23). Although apoptosis occurs at 6 and 24 h, AIF was released at 24 h only (Fig. 3). Hence, we hypothesized that additional proteases could be responsible for caspase-independent apoptosis at early time points (Fig. 4). Calpains are activated by Ca2+ and also thought to mediate apoptosis (13). Cd2+ has been previously reported to release Ca2+ from intracellular pools (23, 24), and because Cd2+ and Ca2+ have similar ionic radii, Cd2+ may also directly activate calpains (21). Indeed, calpain activity was significantly enhanced at 6 h of Cd2+ treatment (Fig. 5) and 10 μM Cd2+-induced apoptosis at 3–6 h was attenuated by calpain inhibitors (Fig. 6, Table 2). The specific calpain inhibitor PD-150606 acts by preventing Ca2+ binding to calpains (38); thus inhibition of Cd2+-mediated apoptosis by PD-150606 could result from the block of Ca2+ and/or Cd2+ with binding sites on calpains. So far, only one other study has described calpain activation as a mechanism for Cd2+-induced apoptosis. However, the study was performed using human lymphoma U397 cells and very high Cd2+ concentrations (100 μM for 12 h) were used to achieve apoptosis (24). Further studies will be necessary to determine the mechanism by which calpains are activated by Cd2+.

**Fig. 6.** Calpain inhibitors abolish Cd2+-induced cell death at 3- and 6-h exposure. MTT assay of PT cells treated with 10 μM Cd2+ for 3 (A and B) or 6 h (C) ± calpain inhibitors ALLN (A) or PD-150606 (B and C). The MTT assay at 3–6 h reflects apoptosis. Values (means ± SE of 3–12 experiments) were normalized to controls without Cd2+. *P < 0.05, **P < 0.01, Cd2+ and/or inhibitor compared with control and Cd2+ plus inhibitor compared with Cd2+ condition using 1-way ANOVA.
A Late Event in Cd²⁺ Apoptosis: Caspase Activation

Caspase-3 and -9 activities were measured at various time points but were only significantly increased after 24 h (Fig. 4, A and B). In agreement with these data, the inhibitors of caspases-9 and -3, z-LEHD-fmk and z-DEVD-fmk, did not inhibit apoptosis induced by 10 μM Cd²⁺ at 6 h (Fig. 4C) but significantly inhibited apoptosis at 24 h (Table 1). Significant elevation of caspase-3 and -9 activities was observed with 10 μM Cd²⁺, but not with 50 μM Cd²⁺. This was unexpected and may either be due to inhibition of caspase activation by high Cd²⁺ or be related to the increase in necrosis (Fig. 1). The activation of caspases after 24-h exposure to 10 μM Cd²⁺ occurs concomitantly with the observation of cytochrome c release (Fig. 2) and correlates with the kinetics of accumulation of 10⁷Cd²⁺ by PT cells (11). We have recently shown that direct application of 2–10 μM Cd²⁺ to isolated kidney cortex mitochondria triggers mitochondrial swelling and the release of cytochrome c (20). This suggests that cytosolic Cd²⁺ exceeding a certain threshold contributes to apoptosis by direct damage of mitochondria and release of proapoptotic factors (20). The release of cytochrome c into the cytosol will typically result in apoptosome complex formation and in caspase-9 and -3 activation (32). Interestingly, cytochrome c was also released in PT cells not displaying morphological signs of apoptosis when treated with Cd²⁺ for 24 h (data not shown). This confirms data by Nur et al. (29), who showed that the peak number of cells, which had released cytochrome c from mitochondria, occurred 8 h before the maximum number of apoptotic nuclei was observed.

A Late Event in Cd²⁺ Apoptosis: Release of AIF

The simultaneous release of cytochrome c and AIF from mitochondria at 24 h shows that caspase-dependent and caspase-independent pathways, respectively, may be activated in parallel. Caspase inhibitors only partially inhibited Cd²⁺-induced apoptosis after 24 h (Table 1), which provides further evidence that caspase-independent mechanisms are also operative at this time point. The release of AIF, a mitochondrial flavoprotein with oxidoreductase activity (25), bypasses downstream caspase activation to cause DNA fragmentation and
apoptosis (Fig. 3A). It has been shown through structure-based mutagenesis studies that AIF has DNA binding capacity, which is essential to its cell death-inducing properties (41). It has also been suggested that AIF may activate a DNA-cleaving enzyme (4). However, at this stage the mechanisms by which AIF could contribute to Cd\(^{2+}\)-induced apoptosis remain speculative.

**Calpain-Caspase Interactions**

There is increasing evidence that caspase and calpains can cross talk during apoptosis (27, 28). Calpains can activate (27) or inhibit (6) procaspases by proteolysis, but calpains are also able to cleave the physiological calpain inhibitor, calpastatin, thus decreasing its binding ability to calpains and thereby increasing calpain activity (30). Thus we hypothesized that Cd\(^{2+}\) first activates calpains that subsequently cleave procaspases to activate or inhibit their proteolytic abilities. As a matter of fact, after 24 h of Cd\(^{2+}\) exposure, apoptosis (Fig. 7A) and caspase-3 activity (Fig. 7B) could be partially inhibited by the calpain inhibitor PD-150606, indicating that calpains are partly responsible for the caspase activation. The fact that basal levels of calpain activity were measured after 24-h Cd\(^{2+}\) incubation (Fig. 5) suggests that calpain-caspase cross talk is not bidirectional and that an additional calpain-independent apoptotic pathway mediates caspase activation at 24 h. Cytochrome c release, which occurs mainly at 24 h (Fig. 2), is likely to account for calpain-independent caspase activation.

**Possible Mechanisms Underlying Cd\(^{2+}\)-Induced Necrosis**

Although apoptosis and necrosis are mediated through distinct pathways, the same insult can lead to either apoptosis or necrosis depending on its intensity and duration of exposure. Necrotic cell death is normally associated with increased intracellular Ca\(^{2+}\), decreased ATP levels, cellular swelling, and disruption of the plasma membrane. There are a number of observations in the present study, which may play a role in Cd\(^{2+}\)-induced necrosis. The basal levels of caspase-9 and -3 activities observed with 50 \(\mu\)M Cd\(^{2+}\) at 24 h (Fig. 4A) could be related to the increase in necrosis (Fig. 1). Decreased ATP levels may cause the failure of caspase activation because ATP is required for the formation of the apoptosome complex, which normally leads to the cleavage of the dormant proteases (9, 10). Interestingly, AIF release was also increased when PT cells were incubated with 50 \(\mu\)M Cd\(^{2+}\) for 24 h (Fig. 3B). We may speculate that with higher concentrations of Cd\(^{2+}\) the caspase-dependent apoptotic pathway may redirect to either a calpain-independent apoptotic pathway or to a necrotic pathway involving AIF. AIF has oxidoreductase activity and may play a role in scavenging ROS generated in mitochondria (for review, see Ref. 25). Therefore, the increased release of AIF from mitochondria may consequently lead to a further increase in ROS levels, depletion of cellular antioxidants, culminating in necrosis. In future work, we intend to measure ATP levels at different Cd\(^{2+}\) concentrations and time points used in the present study and to investigate the role of AIF in necrosis.

**A Proposed Sequence of Apoptotic Events Induced by Cd\(^{2+}\) in PT Cells**

It is well established that Cd\(^{2+}\) has apoptosis-inducing capacity in vitro (37) as well as in vivo (14) in kidney PT cells. How Cd\(^{2+}\) causes apoptosis is only partially understood. The present study has shown the differential pathways involved in the execution of apoptosis by Cd\(^{2+}\) in cultured rat kidney PT cells (Fig. 8). Apoptotic cell death by 10 \(\mu\)M Cd\(^{2+}\) increases calpain proteolytic activity after 3–6 h, whereas the caspases remain dormant. As the cytosolic Cd\(^{2+}\) concentration rises over time, other apoptotic pathways may come into play. Of particular importance is the role of mitochondria. The liberation of cytochrome c leads to caspase-dependent apoptosis, whereas AIF release may underlie caspase-independent apoptosis at 24 h. The activation of the caspase pathway is additionally enhanced by calpains. At higher Cd\(^{2+}\) concentrations, toxicity also becomes associated with increased rates of necrotic cell death. The involvement of caspase-dependent (16, 19, 39) as well as caspase-independent apoptosis induced by Cd\(^{2+}\) (23, 24, 33) has been previously reported in various nonrenal cellular models. From the data obtained in the present study, it is apparent that the role of caspases in Cd\(^{2+}\) apoptosis of PT cells is also determined by Cd\(^{2+}\) concentration and duration of Cd\(^{2+}\) exposure and that caspase-dependent and -independent pathways can be concurrently activated. Hence, delineating these alternative pathways for apoptosis induced by Cd\(^{2+}\) will help us to better understand Cd\(^{2+}\) nephrotoxicity.

**GRANTS**

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