Up-regulation of Multidrug Resistance P-glycoprotein via Nuclear Factor-κB Activation Protects Kidney Proximal Tubule Cells from Cadmium- and Reactive Oxygen Species-induced Apoptosis*

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Frank Thévenod‡‡, Jenny M. Friedmann‡, Alice D. Katsen†, and Ingeborg A. Hauser‡

From the Departments of ‡‡Physiology II and ‡\ Experimental Surgery, University of Saarland, 66421 Homburg and the †Department of Nephrology, J. W. Goethe University, Zentrum Innere Medizin, 60590 Frankfurt/Main, Germany

Cadmium-mediated toxicity of cultured proximal tubule (PT) cells is associated with increased production of reactive oxygen species (ROS) and apoptosis. We found that cadmium-dependent apoptosis (Hoechst 33342 and annexin V assays) decreased with prolonged CdCl₂ (10 μM) application (controls: 2.4 ± 1.6%; 5 h: +5.1 ± 2.3%; 20 h: +5.7 ± 2.5%; 48 h: +3.3 ± 1.0% and 72 h: +2.1 ± 0.4% above controls), while cell proliferation was not affected. Reduction of apoptosis correlated with a time-dependent up-regulation of the drug efflux pump multidrug resistance P-glycoprotein (mdr1) in cadmium-treated cells (∼4-fold after 72 h), as determined by immunoblotting with the monoclonal antibody C219 and measurement of intracellular accumulation of the fluorescent probe calcein ± the mdr1 inhibitor PSC833 (0.5 μM). When mdr1 inhibitors (PSC833, cyclosporine A, verapamil) were transiently added to cells with mdr1 up-regulation by pretreatment for 72 h with cadmium, cadmium-induced apoptosis increased significantly and to a percentage similar to that obtained in cells with no mdr1 up-regulation (72-h cadmium: 5.2 ± 0.9% versus 72-h cadmium + 1-h PSC833: 7.2 ± 1.4%; p < 0.001). Cadmium-induced apoptosis and mdr1 up-regulation depended on ROS, since co-incubation with the ROS scavengers N-acetylcysteine (15 mM) or pyrrolidine dithiocarbamate (0.1 mM) abolished both responses. Moreover, cadmium- and ROS-associated mdr1 up-regulation was linked to activation of the transcription factor NF-κB; N-acetylcysteine, pyrrolidine dithiocarbamate, and the IκB-α kinase inhibitor Bay 11-7082 (20 μM) prevented both, mdr1 overexpression and degradation of the inhibitory NF-κB subunit, IκB-α, induced by cadmium. The data show that 1) cadmium-mediated apoptosis in PT cells is associated with ROS production, 2) ROS increase mdr1 expression by a process involving NF-κB activation, and 3) mdr1 overexpression protects PT cells against cadmium-mediated apoptosis. These data suggest that mdr1 up-regulation, at least in part, provides anti-apoptotic protection for PT cells against cadmium-mediated stress.

The proximal tubule (PT)†† of the mammalian kidney is a major target of chronic cadmium-induced toxicity (1). The mechanisms of cadmium uptake by PT cells are not well understood. Cadmium may be partly taken up through nifedipine-sensitive Ca²⁺ channels (2) or via the proton-dependent divalent-cation transporter, DCT1, which is also expressed in rat PT (3). There is also some evidence that cadmium influx across the PT brush-border membrane occurs via a Cl⁻/HCO₃⁻ exchanger (4). Cadmium-induced nephrotoxicity may be mediated through the cadmium-metallothionein (Cd-MT) complex, which is synthesized in the liver, released into the circulation, and endocytosed by renal tubule cells (5). However, recent work with MT knock-out mice has provided evidence that inorganic cadmium is the nephrotoxic moiety (6). In the kidney, Cd-MT is degraded in endo/lysosomal compartments. The released cadmium as well as free cadmium transported by PT cells stimulate the intratubular synthesis of heavy heavy metal-detoxifying proteins, such as MT. Renal damage occurs once the cadmium concentration reaches or exceeds the binding capacity of these heavy metal-binding proteins (1, 5).

Clinically, cadmium nephropathy resembles acquired Fanco’s syndrome, i.e., it is associated with massive polyuria, glucosuria, amino aciduria, proteinuria, calcium, and phosphaturia (1), which are mainly caused by defects of PT Na⁺-dependent transporters (7). At the cellular level, evidence suggests an involvement of reactive oxygen species (ROS) in cadmium-mediated nephrotoxicity. Cadmium causes DNA strand breaks, lipid peroxidation, and generation of oxidatively modified proteins (8–10). Although cadmium, not being a Fenton metal, does not generate free radicals by itself (8), it has been shown to produce hydroxyl radicals in the presence of metallothioneins containing Fenton metals (11), probably as a consequence of displacement of Fenton metals and/or depletion of radical scavengers. Recently, we have reported ROS-mediated oxidative modification of Na⁺/K⁺-ATPase in a rat PT cell line with low micromolar cadmium concentrations, resulting in increased proteolysis of modified Na⁺/K⁺-ATPase molecules through the proteasome and lysosomal proteases (12). Furthermore, cadmium-induced degradation of Na⁺/K⁺-ATPase molecules was associated with an increase of cellular apoptosis, but not necrosis (12).

However, micromolar concentrations of cadmium have also been shown to enhance the expression of immediate early genes (c-fos, c-jun, c-myc) and of the tumor suppressor gene for p53 (Ref. 13; reviewed in Ref. 14) as well as to induce up-regulation of detoxifying proteins and cytoprotective stress proteins (14). Recently, we observed that cadmium-treatment of

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† To whom correspondence should be addressed. Tel.: 49-6841-166461; Fax: 49-6841-166655; E-mail: frank.thevenod@med-rz.uni- Fayette.de

‡ The abbreviations used are: PT, proximal tubule; H-33342, Hoechst 33342; mdr1, multidrug resistance P-glycoprotein; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; PSC833, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; PS, phosphatidylserine; MT, metallothionein; TNF, tumor necrosis factor.

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rats in vivo for up to 14 days increased the expression of the multidrug resistance P-glycoprotein (mdr1) in the brush-border of kidney PT (15). mdr1, a membrane transporter of 150–170 kDa, catalyzes the ATP-driven efflux of hydrophobic, potentially cytotoxic endogenous and xenobiotic compounds from PT cells into the primary filtrate (16). mdr1 expression has been shown to be up-regulated by many extracellular stimuli including UV irradiation, heat shock, and growth factors, and by a number of drugs (17). Interestingly, previous work had shown that cadmium increases the expression of MDR1 mRNA (18, 19).

The present study shows that micromolar cadmium concentrations increase apoptosis and mdr1 expression in a rat PT cell line by a process involving generation of ROS and activation of the transcription factor NF-κB. Cadmium-mediated apoptosis is time-dependent, with a maximum at 20 h and a reduction of apoptosis at later time points (48 and 72 h) that coincides with the kinetics of mdr1 up-regulation. Moreover, mdr1-selective transport blockers, such as PSC833, cyclosporine A, or verapamil, increased cadmium-induced apoptosis in PT cells over-expressing mdr1 (72-h cadmium), but not in control cells with low mdr1. The data suggest that NF-κB-mediated mdr1 up-regulation is part of the anti-apoptotic protection mechanism of PT cells against cadmium-induced oxidative stress and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following reagents were obtained from the listed sources and used at the concentrations indicated in the text. Stock solutions of calcine-AM ester (Molecular Probes, Eugene, OR), cyclosporine A (Sigma, Deisenhofen, Germany), and PSC833 (kind gift of Novartis, Basel, Switzerland) were made by solubilization in dimethyl sulfoxide (Me2SO). Verapamil (Alhrich Chemie, Steinheim, Germany) and cycloheximide (Sigma) were dissolved in ethanol. Rat tail collagen type I (Sigma) was dissolved in 100 mM acetic acid. Enhanced chemiluminescence reagents were purchased from Amersham-Buchler (Braunschweig, Germany). Nonfat dry milk and pre-stained protein standards were from Bio-Rad (Munich, Germany). Polyvinylidene difluoride membranes were from NEN Life Science Products (Bad Homburg, Germany). Hoechst 33342 (dissolved in H2O) was from Calbiochem (San Diego, CA). Ethidium bromide (dissolved in 150 mM HEPES, pH 7.4), N-acetyl-L-cysteine (dissolved in H2O), and pyrrolidine dithiocarbamate (PDTC) dissolved in Me2SO were from Sigma. Bay k706 (solubilized in Me2SO) was from Biomol (Hamburg, Germany). Annexin V-AlexaFluor680 was from Roche Molecular Biochemicals (Germany). Sytox® Green (solubilized in Me2SO) was from Molecular Probes. All other substances were from commercial sources and of analytical grade.

**Methods**

**Cell Culture**

Immortalized cells (WKPT-0293 Cl.2) of the S1-segment of the proximal tubule of normotensive Wistar-Kyoto rats were cultured as described previously (12, 17). Cells were plated on flasks coated with rat tail collagen type I (125 μg/ml 100 mM acetic acid), passaged at 80% confluence, and split 1:5 twice a week. For inhibitor studies, scavengers were added before starting experiments. Cells were stained intravitally with the substances were from commercial sources and of analytical grade. Ethidium bromide (dissolved in 150 mM HEPES, pH 7.4), N-acetyl-L-cysteine (dissolved in H2O), and pyrrolidine dithiocarbamate (PDTC) dissolved in Me2SO were from Sigma. Bay k706 (solubilized in Me2SO) was from Biomol (Hamburg, Germany). Annexin V-AlexaFluor680 was from Roche Molecular Biochemicals (Germany). Sytox® Green (solubilized in Me2SO) was from Molecular Probes. All other substances were from commercial sources and of analytical grade.

**Detection of Apoptosis and Necrosis by Fluorescence Microscopy**

Hoechst 33342 and Ethidium Bromide Staining Assays—Cells (4 x 10^4) were seeded into 35-mm tissue culture dishes and grown for 3 days before starting experiments. Cells were stained intravitally with the DNA dyes Hoechst 33342 (H33342; 2 μg/ml) for 20 min and ethidium bromide (EtBr; 5 μg/ml) for 10 min, as described previously (20). Under ultraviolet (UV) epifluorescence (λex, 350–380 nm; λem, 490 nm), necrotic cells fluoresce pink due to EtBr, whereas normal and apoptotic cells emit blue fluorescence due to H-33342. EtBr stains nuclei from cells that lost their plasma membrane integrity, i.e., that underwent necrosis. In contrast, the lipophilic DNA dye H-33342 freely enters as well as apoptotic cells. Apoptotic cells can be distinguished from viable cells by their nuclear morphology with nuclear condensation and fragmentation, as well as by the higher intensity of blue fluorescence of the nuclei. Following washing out of the dyes, cells were examined under a UV-visible fluorescence microscope (IMT-2 Olympus). Cells from five microscopic fields (magnification, ×200) were counted per dish. Cell proliferation was estimated semiquantitatively by counting cell numbers and mitotic figures in five microscopic fields.

**Annexin V and Sytox Green Assays—For early detection of apoptosis, we made use of alterations of the plasma membrane structure associated with exposition of phosphatidylserine (PS) to the external cellular environment (21). We determined annexin V binding, a Ca<sup>2+</sup>-dependent protein with high affinity for PS, using annexin conjugated to the fluorescent label Alexa<sup>488</sup>568, a stable fluorochrome, which emits red fluorescence (λex > 500 nm). Cells were seeded into 35 mm tissue culture dishes and grown onto 12-mm glass slides for 3 days. To distinguish vital and apoptotic cells on the basis of nuclear staining, cells were intravitally stained with the blue fluorescent DNA dye H-33342, and to separate necrotic from viable and apoptotic cells, the cultures were additionally stained with the green fluorescent DNA dye Sytox Green (λex, 504 nm; λem, 523 nm). Vial cells had a blue fluorescence due to H-33342, and necrotic cells green fluorescence (Sytox Green), and apoptotic cells both blue (H-33342) and red fluorescence (annexin V-Alexa<sup>488</sup>568 staining). For staining, the growth medium was replaced by fresh medium containing Ca<sup>2+</sup> (in our case Dulbecco’s modified Eagle’s medium), annexin V-Alexa<sup>488</sup>568 (dilution 1:50) and 2 μg/ml H-33342. After 20 min of incubation, the cells were centrifuged at 12,000×g for 5 min, resuspended in 50 μg/ml calcein AM, and the suspension was examined under a UV-visible fluorescence microscope (IMT-2 Olympus), and five microscopic fields (magnification, ×200) were counted per dish.

**Antibodies**

The C219 monoclonal antibody (Alexis Deutschland, Grünberg, Germany) was used for the detection of mdr1 P-glycoprotein. The rabbit polyclonal antibody to IκBα (C21) and NF-κB p65/RelA (C20) were from Novocastra Biotechnologies (Novocastra, CA). Horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit IgG were purchased from Amersham-Buchler (Braunschweig, Germany).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**

WKPT-0293 Cl.2 cells were solubilized by addition of 3-fold concentrated SDS sample buffer and sonicated for 30 s on ice. Following centrifugation at 12,000×g for 5 min, proteins (50 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide laemmlini minigels, transferred onto polyvinylidene difluoride membranes, and the membranes blocked essentially as described earlier (12, 17, 22). Blots were incubated at 4 °C with primary antibodies against mdr1 (0.5 μg/ml), IκBα (0.05 μl/ml), or NF-κB p65/RelA (0.2 μg/ml) overnight, incubated with horseradish peroxidase-conjugated second-ary antibodies, and visualized on x-ray films, and processed for documentation and quantitative analysis as described elsewhere (12, 17, 22, 23).

**Fluorescence Measurements**

Chambers containing cells grown on glass coverslips were placed on an inverted microscope (IMT2-F, Olympus, Hamburg, Germany) and imaged with a 20×, 0.85 or 40×, 0.85 objective (Fluar 20 or 40; Nikon, Düsseldorf, Germany). A variable monochromatic light source (Till Photonics, Munich, Germany), was coupled to the microscope with a light guide. Images were recorded with an integrating slow scan camera (Theta System, Munich, Germany) and digitized to 8 bits/pixel (23). Camera and scanner were controlled by TILLvision version 3.02 software, which was also used to analyze the stored images. For evaluation of individual cells, areas of interest were defined on an image, and the fluorescence intensities were expressed as the sum of pixel values per 100 ms of exposure time per area. The excitation wavelength used for measurements of calcine-AM fluorescence was 495 nm. Emitted light was collected with the appropriate dichroic mirrors.

**Calcine Accumulation Assay**

Cells grown on collagen-coated coverslips and incubated for 48 h with or without 5 μM CdCl<sub>2</sub> were superfused for 15–20 min with 1 μM calcine-AM (Molecular Probes). Calcine-AM is a nonfluorescent, highly lipophilic dye that rapidly penetrates the plasma membrane of cells. Once inside the cell, ester bonds are cleaved by endogenous esterases, transforming calcine-AM into hydrophilic and intensely fluorescent calcine. Cells expressing high levels of mdr1 rapidly extrude non-fluorescent calcine-AM from the plasma membrane, thereby reducing accumulation of fluorescent calcine in the cytosol (24). The amount of
mdr1 activity is therefore inversely proportional to the accumulation of intracellular calcein fluorescence (17, 23, 24). The gradual increase in fluorescence intensity in the cells was recorded by taking an image every minute. After 15–20 min, the section was superfused by replacing the chamber content with a solution of the previous concentration of calcein-AM containing, in addition, 0.5 μM non-fluorescent mdr1 inhibitor, PSC 833, a non-immunosuppressive cyclosporine A analog (25), or the solvent Me2SO used as a control. All experiments were carried out at room temperature and repeated at least three times with cells grown on different glass coverslips. Images were stored on disk for further analysis.

Statistics

All experiments were repeated at least three times with different batches of cell preparations, and representative data or means ± S.D. are shown. Statistical analysis was carried out with the Statgraphics program using unpaired or paired Student’s t test. Results with levels of p ≤ 0.05 were considered significant.

RESULTS

Biphasic Time Dependence of Cadmium-induced Apoptosis in Rat PT Cells—In the rat kidney, cadmium has been shown to induce apoptosis (6, 26). In cultured immortalized cells (WKPT-0293 Cl.2) from the S1 segment of the rat PT, we have shown that application of 10 μM CdCl2 for 20 h induces apoptosis, but not necrosis, by a mechanism involving the production of ROS (12). In the present study, we first wanted to characterize the concentration dependence and kinetics of cadmium-mediated apoptosis in the rat kidney PT cell line in more detail. By subsequent incubation with the vital bisbenzimidazole dye H-33342 and the DNA intercalating agent EtBr, apoptotic cells, whose plasma membrane integrity is still intact, can easily be distinguished from cells that underwent necrosis and therefore can be labeled by the plasma membrane-impermeable charged dye EtBr (20). Incubation of PT cells with 2.5–10 μM CdCl2 for 5 h induced typical features of apoptosis, such as chromatin condensation (arrows) or fragmentation (asterisk) (see Fig. 1A). The basal rate of apoptosis under control conditions of 2.2 ± 1.4% (n = 8) was significantly increased by cadmium concentrations as low as 2.5 μM (6.8 ± 1.6%; n = 6; p ≤ 0.001). The percentage of apoptotic cells did not further increase after 5 h of incubation with 5 or 10 μM CdCl2 (6.8 ± 1.0% and 6.9 ± 2.1%; n = 7; p ≤ 0.001) (Fig. 1B). In contrast, the percentage of necrotic cells was almost negligible and not significantly differ-
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Fig. 3. Time-dependent changes of cadmium-induced apoptosis in rat proximal tubule cells. Cells were incubated with 10 μM CdCl₂ for various time periods, stained with ethidium bromide and H-33342, and analyzed as described under “Methods.” Cadmium-induced apoptosis was calculated by subtracting the percentage of apoptosis in time-matched controls from the percentage of apoptosis in cadmium-treated cells. Means ± S.D. of 3–12 different experiments are shown. Asterisks identify the values that are significantly different from those obtained in cells incubated for 20 h with 10 μM CdCl₂ (*, p ≤ 0.05; **, p ≤ 0.001 using unpaired Student’s t test. n.s., not significant.

Table I

| Time (hours) | 0 h | 3 h | 5 h | 20 h | 48 h | 72 h |
|-------------|-----|-----|-----|------|------|------|
| Cell number (n) | 150.6 ± 51.4 | 123.2 ± 4.40 | 109.0 ± 14.4 | 116.8 ± 69.5 | 146.3 ± 27.3 | 122.6 ± 28.0 |
| Mitotic index (%) | 1.27 ± 0.55 | ND | ND | 1.33 ± 0.91 | 1.10 ± 0.36 | ND |

The values were obtained by counting five microscopic fields. Means ± S.D. from 3–12 different experiments are shown. ND, not determined.

ent from control values (0.4 ± 0.4%; n = 8) (data not shown).

Since cadmium induced apoptosis as early as after 5 h using the DNA dye H-33342, we decided to measure apoptosis at an earlier time point. Changes in the plasma membrane occur very early during apoptosis; the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a Ca²⁺-dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS (21). Annexin V may be conjugated to fluorochromes, such as the fluorescent label Alexa™568, and thus serve as a sensitive probe for analysis of cells that are undergoing apoptosis (Fig. 2A). When cells were incubated for 3 h with 10 μM CdCl₂, the percentage of annexin V-positive apoptotic cells increased from 1.2 ± 0.2% in controls to 5.9 ± 0.4% in cadmium-treated cells (n = 3; p ≤ 0.01) (Fig. 2B). The percentage of necrotic cells was determined using the green fluorescent nucleic acid stain Sytox®, and was not different in controls (1.7 ± 0.2%) and cadmium-treated cells (1.3 ± 0.4%) (Fig. 2B).

We then investigated apoptosis and necrosis in PT cells that had been incubated with cadmium for longer time periods using the DNA staining dyes H-33342 and EtBr, respectively. After a 20-h cadmium incubation, the percentage of apoptotic cells was significantly increased (2.5 μM cadmium: 9.1 ± 3.80%; 5 μM cadmium: 8.6 ± 1.8%; 10 μM cadmium: 8.1 ± 1.90%; n = 7) (Fig. 1B), compared with the respective controls (2.4 ± 1.6%; n = 12; p ≤ 0.001). Notice that there was no concentration dependence of apoptosis in the tested concentration range of 2.5–10 μM cadmium. Moreover, although the percentage of apoptotic cells was higher at 20 h compared to 5 h, the differences did not reach statistical significance. Following incubation of PT cells with 10 μM CdCl₂ for 48 or 72 h the apoptosis was also significantly increased. Interestingly, the percentage of apoptotic cells was smaller at 48 and 72 h, compared with cells that had been exposed to cadmium for 20 h. Fig. 3 summarizes the results of cadmium-dependent apoptosis, i.e. the percentage of apoptosis above control levels, obtained by incubating PT cells with 10 μM CdCl₂ for 5, 20, 48, and 72 h. These data clearly show that cadmium-dependent apoptosis was not different at 5 and 20 h (5.1 ± 2.3% and 5.7 ± 2.5% above controls, respectively) (see also Fig. 1). However, cadmium-dependent apoptosis was significantly decreased at 48 h (3.3 ± 1.0% above controls, p ≤ 0.05) and even more at 72 h (2.1 ± 0.4% above controls, p ≤ 0.001) compared with 20 h (Fig. 3), although apoptosis after 72 h of cadmium exposure was still significantly higher than in the respective controls (cadmium: 3.9 ± 0.4% versus controls: 1.9 ± 0.6%; n = 7; p ≤ 0.001). In contrast, the percentage of necrotic cells was not increased by cadmium exposure, either at 48 h (controls: 0.8 ± 0.6%; cadmium: 0.5 ± 0.3%) or at 72 h (controls: 0.2 ± 0.1%; cadmium: 0.3 ± 0.3%). Importantly, Table I shows that at all incubation periods tested, CdCl₂ (10 μM) did not affect cell proliferation, as estimated by comparison of cell numbers and percentage of mitotic figures.

Reduced Apoptosis of Rat PT Cells with Prolonged Cadmium Exposure Is Associated with Up-regulation of mdr1—The above results were intriguing; however, the essential cellular processes involved in this decrease of cadmium-dependent apoptosis with prolonged cadmium exposure were not defined. Recently, we have described that cadmium increases the expression of the multidrug resistance P-glycoprotein (mdr1) in the brush-border of the S1 and S2 segments from kidney PT of cadmium-intoxicated rats (15). As such, we hypothesized that overexpression of mdr1 could represent a protective mechanism for cells against cadmium-mediated nephrotoxicity. We therefore determined the expression of mdr1 in cultured PT cells as a function of the duration of cadmium-exposure. mdr1 expression was determined in whole cell lysates by immuno blotting with an mdr1-specific monoclonal antibody (C219; 0.5 μg/ml). In control cells, mdr1 expression was low. As shown in the representative immunoblot of Fig. 4A, incubation of PT
cells with 5 \( \mu \text{M} \) CdCl\(_2\) for 24 h (or 10 \( \mu \text{M} \) CdCl\(_2\), not shown) increased mdr1 expression, and it increased further to statistical significance at 48 and 72 h of CdCl\(_2\) incubation (see Fig. 4B).

The presence of the multidrug resistance P-glycoprotein (mdr1) transporter in the plasma membrane of PT cells was further assessed by measurement of the time course of intracellular accumulation of the dye calcein in the absence or presence of the potent inhibitor of mdr1, PSC833 by means of fluorescence cell imaging (25). The dye calcein has the advantage that its acetoxymethyl (AM) ester form is non-fluorescent, while the intracellular hydrolysis product calcein is fluorescent and practically insensitive to changes in pH, Ca\(^{2+}\), and Mg\(^{2+}\). Calcein-AM, but not calcein, is a known substrate of mdr1 (24) (see inset of Fig. 5).

To study uptake of calcein as a function of time in individual cells, areas of interest were defined on an image, and the fluorescence intensities were expressed as the sum of pixel...
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The mdr1 Inhibitor PSC833 Increases Cadmium-induced Apoptosis in Rat PT Cells Overexpressing mdr1, but Not in Control Cells—The decrement of cadmium-induced apoptosis (Fig. 3) observed in cells that showed overexpression of mdr1 after prolonged exposure to cadmium (Fig. 4) is merely correlative. A stronger footing to the hypothesis that overexpression of mdr1 might protect PT cells against cadmium-mediated apoptosis, was obtained with the inhibitor of mdr1, PSC833. If this hypothesis is valid, inhibition of mdr1 by PSC833 should result in increased apoptosis in cells overexpressing mdr1, but not in cells with low mdr1 expression. To that end, we compared the effect of PSC833 on cells that had been pre-exposed to 10 μM CdCl2 for 72 h and therefore showed increased mdr1 expression (see Fig. 4), with its effect on time-matched control cells. PSC833 by itself caused apoptosis, when it was incubated for 6–24 h at concentrations of 1–10 μM (data not shown).

Therefore, the preincubation medium (72 h without or with 10 μM PSC833: 7.2 ± 0.32%, 2.65 ± 0.43% for MeSO and PSC833, respectively. Data show six to eight different experiments. Statistical analysis compares conditions without and with inhibitor using unpaired Student’s t test; *, p ≤ 0.05; **, p ≤ 0.01.

Table II

| Acceptor cells after CdCl2 exposure (10 μM; 4 h) | CdCl2 | +200 μM verapamil | CdCl2 | +10 μM cyclosporine A |
|-----------------------------------------------|-------|-------------------|-------|----------------------|
| %                                             | %     | %                 | %     | %                    |
| 5.96 ± 0.66                                   | 6.96 ± 0.79* | 5.99 ± 0.81       | 7.86 ± 0.92**          |
| (5)                                           | (5)   | (8)               | (8)   |                      |

Fig. 6. Effect of PSC833 on apoptosis of PT cells induced by CdCl2. Cells were left untreated (A) or exposed to 10 μM CdCl2 for 72 h (B). The cadmium-containing medium was replaced by a medium containing either 0.1% v/v MeSO (controls) or the mdr1 inhibitor PSC833 (10 μM). After 1 h, the medium was removed and replaced by a solution containing 10 μM CdCl2, and cells were incubated for additional 4 h in the cadmium-containing medium. The respective controls without CdCl2 were 2.64 ± 0.32%, 2.65 ± 0.92%, and 2.65 ± 0.43% for MeSO/ethanol, cyclosporine A, and verapamil, respectively. Data show means ± S.D.

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of fluorescent calcein in the cytosol (17, 23, 24). Addition of 0.5 μM PSC833 accelerated the net accumulation of calcein, resulting in a slope of calcein accumulation that resembled that of control cells (133.4 ± 7.5 counts/area × min−1) (Fig. 5). This indicates that the inhibitor of mdr1 PSC833, prevented dye extrusion by inhibiting the transport function of mdr1 and thereby accelerated the net uptake of the fluorescent dye calcein (see inset of Fig. 5).

The mdr1 Inhibitor PSC833 Increases Cadmium-induced Apoptosis in Rat PT Cells Overexpressing mdr1, but Not in Control Cells—The decrement of cadmium-induced apoptosis (Fig. 3) observed in cells that showed overexpression of mdr1 after prolonged exposure to cadmium (Fig. 4) is merely correlative. A stronger footing to the hypothesis that overexpression of mdr1 might protect PT cells against cadmium-mediated apoptosis, was obtained with the inhibitor of mdr1, PSC833. If this hypothesis is valid, inhibition of mdr1 by PSC833 should result in increased apoptosis in cells overexpressing mdr1, but not in cells with low mdr1 expression. To that end, we compared the effect of PSC833 on cells that had been pre-exposed to 10 μM CdCl2 for 72 h and therefore showed increased mdr1 expression (see Fig. 4), with its effect on time-matched control cells. PSC833 by itself caused apoptosis, when it was incubated for 6–24 h at concentrations of 1–10 μM (data not shown).

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| (5)                                           | (5)   | (8)               | (8)   |                      |

values per 100 ms of exposure time per area. Means ± S.D. of four different experiments are shown in Fig. 5. In the presence of calcein-AM (1 μM), the increase in fluorescence was linear or only slightly curved over 15 min in control cells. A rate (slope) of net calcein accumulation of 112.6 ± 7.4 counts/area × min−1 was calculated from linear regression. A solution change with readaptation of 1 μM calcein-AM and PSC833 (0.5 μM, arrow in Fig. 5) did not increase further the slope of the fluorescence curve in control cells (103.6 ± 8.2 counts/area × min−1) (Fig. 5, control curve). Solution change with readaptation of 1 μM calcein-AM and MeSO had a similar effect on the slope of the fluorescence time course (data not shown). This suggests that no or very low levels of functional mdr1 are expressed in the plasma membrane of these cells. In PT cells that had been incubated for 48 h with 5 μM CdCl2, the accumulation rate of calcein was diminished (48.8 ± 3.5 counts/area × min−1) (Fig. 5, CdCl2 curve). This is expected for cells that overexpress mdr1 in their plasma membranes, because mdr1 extrudes non-fluorescent calcein-AM from the cell, thereby reducing accumu-

2 F. Thévenod, unpublished results.
itors of mdr1-mediated transport, verapamil (200 μM) or cyclosporine A (10 μM) (23–25) (Table II).

Cadmium-induced Apoptosis of PT Cells and mdr1 Up-regulation Are Mediated by ROS—To counteract the damaging effects of increased ROS production and to restore cellular homeostasis, cells often activate genes encoding regulatory transcription factors, antioxidant defense enzymes and/or structural proteins (28). We therefore wondered whether during cadmium-induced apoptosis, cells increase mdr1 expression to compensate for the deleterious effects of ROS on cell function. To that end, we used two different thiol antioxidants, N-acetylcysteine (NAC) and PDTC; NAC can raise intracellular

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**Fig. 7.** Effect of NAC on apoptosis of PT cells exposed to CdCl2. Cells were left untreated or exposed to 5 μM CdCl2 for 20 h in the absence or presence of 15 mM NAC added 2 h before CdCl2. Cells were stained with ethidium bromide and Hoechst 33342 and examined as described under “Methods.” Data are means ± S.D. of five different experiments. The statistical analysis compares the conditions without and with NAC using unpaired Student’s t test.

**Fig. 8.** Immunoblotting of mdr1, IκB-α, and p65/RelA in cadmium-treated PT cells incubated without or with antioxidants. Cells were incubated for 2 IκB-α, RelA) and 48 h (mdr1) without or with 5 μM CdCl2. The antioxidants NAC (15 mM) or PDTC (0.1 mM) were added 2 h before starting CdCl2 incubation, where indicated. B, statistical analysis of four experiments. Expression (optical density) of mdr1 in control cells was set to 100%. Quantification of the immunoblot was by image analysis as described under “Methods.” Data are means ± S.D. Asterisks identify the values that are significantly different from control cells; *, p ≤ 0.01 using unpaired Student’s t test.
glutathione levels and thereby protect cells from the effects of ROS (29). In addition, the SH group of the agent can directly react with radicals. PDTC, another thiol antioxidant (30), is also a potent chelator of various metals, including cadmium (31). Both NAC (15 mM) and PDTC (0.1 mM) have previously been shown to prevent cadmium-induced production of ROS in PT cells (12). NAC incubation for 20 h alone had no significant effect on apoptosis (controls: 1.7 ± 0.9% versus NAC: 2.5 ± 1.0%). In contrast, the increase of apoptosis caused by exposure of PT cells to 5 μM CdCl2 for 20 h (5.2 ± 1.3%) was nearly suppressed to control levels by co-incubation with 15 mM NAC (2.7 ± 0.6%; n = 5; p = 0.01) (Fig. 7). Similar results were obtained with 0.1 mM PDTC (data not shown). As shown in Fig. 8A, mdr1 expression was increased in PT cells that had been exposed to 5 μM CdCl2 for 48 h, compared with controls. However, when PT cells were co-incubated with 15 mM NAC or 0.1 mM PDTC, cadmium-induced mdr1 overexpression was abolished, suggesting that cadmium-induced production of ROS provides the signal linking both, apoptosis (Fig. 7) and mdr1 overexpression (Fig. 8).

FIG. 9. Immunoblotting of mdr1 and IκB-α in cadmium-treated PT cells incubated without or with Bay 11-7082. Cells were incubated for 2 (IκB-α) or 72 h (mdr1) in the absence or presence of 5 μM CdCl2. The IκB-α kinase inhibitor Bay 11-7082 (20 μM) or 0.1% Me2SO (v/v) were added 2 h before starting CdCl2 incubation, where indicated (A). B, statistical analysis of three experiments similar to that shown in panel A. Image analysis was carried out as described in Fig. 8. Data are means ± S.D. Asterisks identify the values that are significantly different from control cells; *, p ≤ 0.01 using unpaired Student’s t test.

**Cadmium-induced ROS Increase mdr1 Expression in PT Cells via Activation of NF-κB**—A NF-κB-binding site has been recently found in the promoter region of the mdr1 gene that is probably involved in basal as well as in inducible transcriptional regulation of human and rat mdr1 genes (32, 33). In the present study, we found that incubation of PT cells with 5 μM CdCl2 resulted in a rapid degradation of IκB-α, with a maximum 1–2 h after addition of cadmium (Fig. 8 and data not shown), but had no major effect on the respective p65/RelA transcription factor (Fig. 8). This effect could be prevented by both NAC (15 mM) or PDTC (0.1 mM) (Fig. 8), suggesting that cadmium-induced NF-κB activation is mediated by ROS. Further evidence for a link between cadmium-mediated production of ROS, NF-κB activation and mdr1 overexpression was provided using the inhibitor of IκB-α phosphorylation, Bay 11-7082 (20 μM) (34). Bay 11-7082 prevented both, cadmium-induced IκB-α degradation and the increase of mdr1 expression in PT cells (Fig. 9).

**DISCUSSION**

**Putative Mechanisms of Cadmium-induced Apoptosis in PT Cells**—The proximal tubule cell line used in this study (WKPT-0293 Cl.2) is derived from the S1 segment of rat proximal tubule cells and has been immortalized through transfection and permanent transfer of the SV40 large T antigen gene (35). The SV40 large T antigen binds to p53 that becomes permanently inactivated (35). For that reason, it has to be assumed that the apoptotic pathway responsible for cadmium-induced apoptosis is p53-independent. Previously, we have shown that toxicity of the WKPT-0293 Cl.2 cell line induced by micromolar cadmium concentrations is associated with increased production of ROS and apoptosis (12) suggesting that ROS initiate p53-independent apoptosis (36). The observation that cadmium-mediated ROS formation causes apoptosis has been confirmed and extended with the antioxidants NAC and PDTC, which prevented cadmium-induced apoptosis (Fig. 7). In our previous study (12), we showed that cadmium-mediated damage of PT cells is associated with an increased degradation of the α1 subunit of Na+/K+-ATPase. Movements of Na+ and K+ accompanying apoptotic loss of cell volume play a pivotal role in the activation of the cell death program (37). It is therefore possible that cadmium-induced changes of Na+/K+-ATPase ac-
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Mechanisms of Cadmium-induced mdr1 Overexpression in PT Cells—One of the key transcription factors activated by ROS is NF-κB (30). NF-κB is a dimeric transcription factor composed of members of the Rel family that is kept in the cytoplasm of non-stimulated cells through interaction with the inhibitory protein IκB-α (39). Phosphorylation of IκB-α by various stimuli (possibly also ROS) may result in polyubiquitination of IκB-α and its degradation by the 26 S proteasome (40). This results in liberation of NF-κB, its nuclear translocation, and activation of target genes (40). NF-κB is known to turn on protective genes that can help resist p53-dependent and -independent cell death induced by various stressors, such as TNF-α, radiation, chemotherapeutic agents or oncogenic ras (41–44). Since recent studies have provided evidence that the cellular stress induced by TNF-α, ceramides are generated by stimulation of sphingomyelinase following apoptosis by triggering mitochondria to release caspase-9. They have proposed that mdr1 might function as a lipid-transferring protein (Mdr1). MDR1 and mouse mdr1a can translocate a wide variety of drugs supporting a cytoprotective role of mdr1 against apoptosis has been demonstrated. Verapamil reversed resistance to apoptosis. Further evidence that mdr1 is involved in the regulation of mdr1 genes (32, 33), we suspected that cadmium-induced increase of mdr1 expression could be mediated by ROS and NF-κB activation. Indeed, inhibitors of NF-κB activation, such as anti-oxidants (30) or the inhibitor of IκB-α phosphorylation, Bay 11-7082 (20 μM) (34), prevented overexpression of mdr1 induced by cadmium (Figs. 8 and 9). Moreover, addition of the non-immunosuppressive cyclosporine A derivative PSC833, which inhibits mdr1 (24), enhanced the apoptotic response of PT cells to cadmium (Fig. 6). Since mdr1 overexpression induced by NF-κB activation could be a part of a “specific” cytoprotective response of PT cells to cadmium-mediated oxidative stress and apoptosis through a physiologic transport function of mdr1, however, at this stage, it cannot be ruled out that other ABC transporters, such as the multispecific organic anion transporter MR2 (cMrp/cMOAT), which is also expressed in kidney PT (45), could also contribute to protection of PT cells against cadmium-mediated toxicity.

How Could mdr1 Protect PT Cells against Cadmium-induced Apoptosis?—The most obvious explanation for a cytoprotective role of mdr1 is that mdr1 may transport cadmium out of PT cells. In a preliminary report, Canillo and co-workers (46) have proposed that in shock rectal gland cells cadmium is released with ATP through an ATP-permeable channel associated with mdr1. Increased mdr1 expression could contribute to cadmium detoxification through release of Cd-ATP associated with the bile salt sodium-dependent transporter MRP2, but not MDR1, likely represents the role of mdr1 is that mdr1 may transport cadmium out of PT Cells. Through a physiologic transport function of mdr1. However, at this stage, it cannot be ruled out that other ABC transporters, such as the multispecific organic anion transporter MR2 (cMrp/cMOAT), which is also expressed in kidney PT (45), could also contribute to the protection of PT cells against cadmium-mediated toxicity.

References—The WKPT-0293 CL2 rat renal proximal tubule cell line was generously provided by Dr. U. Hopfer (Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH).
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