Cadmium-Induced Autophagy in Rat Kidney: An Early Biomarker of Subtoxic Exposure

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Environmental exposures to Cadmium (Cd) are a major cause of human toxicity. The kidney is the most sensitive organ; however, the nature of injuries and of adaptive responses has not been adequately investigated, particularly in response to environmental relevant Cd concentrations. In this study, rats received a daily ip injection of low CdCl₂ dose (0.3 mg Cd/Kg body mass) and killed at 1, 3, and 5 days of intoxication. Functional, ultrastructural, and biochemical observations were used to evaluate Cd effects. We show that Cd at such subtoxic doses does not affect the tubular functions nor does it induce apoptosis. Meanwhile, Cd accumulates within lysosomes of proximal convoluted tubule (PCT) cells where it triggers cell proliferation and autophagy. By developing an immunohistochemical assay, a punctate staining of light chain 3-II is prominent in Cd-intoxicated kidneys, as compared with control. We provide the evidence of a direct upregulation of autophagy by Cd using a PCT cell line. Compared with the other heavy metals, Cd is the most powerful inducer of endoplasmic reticulum stress and autophagy in PCT cells, in relation to the hypersensitivity of PCT cells. Altogether, these findings suggest that kidney cortex adapts to subtoxic Cd dose by activating autophagy, a housekeeping process that ensures the degradation of damaged proteins. Given that Cd is persistent within cytosol, it might damage proteins continuously and impair at long-term autophagy efficiency. We therefore propose the autophagy pathway as a new sensitive biomarker for renal injury even after exposure to subtoxic Cd doses.

Key Words: cadmium; exposure to subtoxic doses; kidney; apoptosis; proliferation; autophagy.

Cadmium (Cd), a major industrial and environmental pollutant, raises serious public health concerns worldwide because of its toxic and carcinogenic effects on human (IARC 1993; Järup and Åkesson 2009; NTP 2000). It adversely affects several organs, including the kidneys, liver, and lungs. Given its persistence (10–30 years) and its low rate of excretion, this heavy metal is toxic even at low doses for kidneys where it accumulates. Chronic Cd exposure is responsible for a wide range of human diseases ranging from cancer, hepatotoxicity to severe kidney failure, and there is no effective therapy for Cd poisoning (Fowler 2009; Järup and Åkesson 2009; Satoh et al. 2002).

Human exposure occurs by inhalation (cigarette smoke) and by ingestion of Cd-contaminated food or water (Järup 2003). Following absorption, Cd accumulates primarily in the liver, where glutathione and metallothionein sequester the majority of intracellular Cd. Cd/metallothionein complex is slowly released from the liver in the blood circulation (Dudley et al. 1985). Because of its low molecular weight, Cd/metallothionein is freely filtered in the kidney and reabsorbed from the glomerular filtrate by megalin/cubilin receptors of PCT cells (Barbier et al. 2005; Christensen and Birn 2001). There, the Cd/metallothionein is rapidly degraded by lysosomal enzymes to release the Cd ions that cause then nephrotoxicity (Abouhamed et al. 2007).

The kidney is the most sensitive organ and responds to Cd intoxication by general transport defects of the proximal tubules characterized by proteinuria, glycosuria, acidoinimuria, phosphaturia, calciuria, and reduction in glomerulus filtration, mimicking the Fanconi syndrome (Brzoska et al. 2003; Buchet et al. 1990; Jacquillet et al. 2006; Järup and Åkesson 2009). However, the underlying cellular mechanisms of its nephrotoxic effects still remain elusive.

Cytotoxicity refers to the cell-killing potential of a compound and occurs mainly via induction of apoptosis (type I), autophagic cell death (type II), and necrosis (type III) (for a review see Galluzzi et al. 2007). Electron microscopy serves as a “gold standard” method for distinguishing apoptosis from autophagy.
and necrosis. Apoptosis is a noninflammatory cell death, characterized by cell shrinking, mitochondrial depolarization, chromatin condensation, nucleosomal DNA fragmentation, and finally the breakdown of the cell into smaller apoptotic bodies. At molecular level, it is mediated by the activation of caspases and other factors released from mitochondria.

Unlike apoptosis, autophagy is primarily an adaptive response upregulated to provide the supply of energy needed for cell survival and repair under environmental stress. This is a highly ordered pathway, which begins with the formation of a double-membrane vesicle termed “autophagosome” that engulfs a part of cytosol (for a review, see Xie and Klionsky 2007). Subsequently, an autophagosome fuses with a lysosome to become an “autolysosome” where the content is finally degraded for the synthesis of new molecules and organelles. Depending on the environmental stress and cell type, this pathway acts either as a survival or death safeguard mechanism. Indeed, autophagy first protects the cells by clearing the damaged organelles and toxic protein aggregates that would otherwise induce apoptosis. However, a massive and persistent autophagy, which depletes the cell of organelles and critical proteins, can kill the severely damaged cells by a caspase-independent form of cell death, termed type II cell death (for a review, see Scarlatti et al. 2009).

Necrotic cell death is characterized by organelle swelling, large-scale permeabilization, rupture of plasma membrane, and release of cellular contents that activate immune and inflammatory responses (for a review, see Golstein and Kroemer 2007). Thus, necrosis is viewed as inflammatory cell death, in sharp contrast to silent autophagic or apoptotic cell deaths.

The pathophysiological consequence of Cd intoxication in vivo is far from being elucidated, particularly at low-level environmental exposure. Indeed, most studies conducted in animal and cellular models have focussed on the induction of severe apoptosis and necrosis, however, mainly following long-term exposure (weeks) at high Cd concentrations (mg/kg) (Aoyagi et al. 2003; Brzoska et al. 2003; Lee et al. 2007; Mao et al. 2007; Tanimoto et al. 1993; Yokouchi et al. 2007). As result, the role of autophagy remains elusive, and an early biomarker for cytotoxicity that faithfully reproduces environmental exposures is still lacking.

Therefore, the present study was designed to explore in vivo the early effects of CdCl₂ intoxication on rat renal proximal tubule cells using subtoxic Cd doses (WHO 2004). The aim was to evaluate the degree of damages and determine the early defence mechanisms developed by renal PCT to maintain epithelium integrity, with a particular emphasis on the balance between apoptosis, mitosis, and autophagy.

MATERIALS AND METHODS

Animals. Fifteen female Wistar rats (2 months old, body weight 190–220 g) from the Tunisian pharmaceutical industries company (SIPHAT) were adapted during 6 days before experimentation. Rats were housed in controlled conditions of lighting (12 h light:12 h dark), temperature (22 ± 2°C), and humidity (55 ± 15%), were fed a standard laboratory diet, and had free access to tap water. Animals received an ip injection of 0.5 μg/ml anhydrous cadmium chloride (CdCl₂) dissolved in sterile 0.9% NaCl solution, corresponding to a dose of 0.3 mg/kg body mass. Control animals were injected with a physiological saline solution. Groups of five animals were killed after 1, 3, and 5 days of daily treatment. One group was daily treated during 5 days and killed 5 days after the end of treatment (“5 + 5” days group). All conducted experiments were approved by the animal care committee of Tunis medicine faculty.

Kidneys were removed; decapsulated and cortex fragments were weighed and quickly stored at –80°C until Cd measurement with atomic absorption spectrophotometry. Other cortex fragments were directly fixed for subsequent histology immunohistochemical staining and electron microscopy.

Cadmium determination. Cd levels in tissues were measured as previously described (Roberts and Clark 1986), with minor modifications. Briefly, kidney cortex fragments were digested with nitric acid (5N, 1 ml per 50 mg of wet tissue), overnight at 80°C, and centrifuged for 5 min at 12,000 × g. In parallel, reference materials from Cd-treated rat kidneys (Jacquillet et al. 2006) were similarly digested. The supernatant was then diluted 1:1000 with 5N nitric acid and Cd content was determined by atomic absorption using the Zeeman furnace system (thermo elemental, 969Z AA spectrometer) and Cd standard solutions (10 μg/l, serial dilution in nitric acid; Aldrich). Cd concentrations are expressed in microgram per gram of kidney cortex.

Kidney function. Clearance experiments were performed to analyze the effect of Cd intoxication on the whole kidney function on two groups of rats: control rats receiving 0.9% saline solution and Cd-treated group intoxicated by daily ip injection during 3 days. The animals had free access to water until the start of the experiment and were starved for 18 h prior to the surgical procedure. Anesthesia was induced by ip injection of sodium pentobarbital (Nembutal, 5 mg/100 g body weight). The animals were then placed on a heated table to maintain their body temperature between 37 and 39°C. One catheter (PE-20) was inserted into the right jugular vein for perfusion of solutions and another (PE-10) into the left ureter for urine collection. A third catheter (PE-50) was inserted into the right femoral artery for blood sampling and arterial blood pressure recording (research BP transducer, Harvard apparatus). Clearance experiments were carried out in rats perfused with 0.9% NaCl solution at a rate of 20 μl/min. [³H]-metoxy-inulin (TRA.324 specific radioactivity 120 μCi/mg inulin, 0.53 Ci/mmol; Amersham Pharmacia Biotech UK Limited) was used as glomerular indicator. Urine samples were collected serially over 20-min periods, and blood samples were taken halfway through each urine collection. In all experiments, a loading dose of [³H] inulin (4 μCi) was given, following by a continuous infusion of 0.4 μCi/min for the duration of the experiments. Urinary collection began 1 h after the administration of [³H] inulin priming dose. [³H] radioactivity was measured by liquid scintillation counting (Packard). In plasma and urinary samples, Na⁺ and P releases were determined by ion exchange chromatography (AS50/BioLC; Dionex).

The effects of Cd on PCT cell proliferation, apoptosis, and autophagy were studied both by transmission electron microscopy and immunohistochemical markers.

Electron microscopy. The ultrastructural changes caused by Cd in kidney were analyzed by transmission electron microscopy. Briefly, kidney cortex fragments from control and treated rats were immediately fixed with ice-cold 3% glutaraldehyde in 0.1M Na cacodylate, pH 7.4, for 2–3 h. Tissues were then rinsed in cacodylate buffer, postfixed in a 1% OsO₄ for 1 h at 4°C, dehydrated through graded ethanol washes, and embedded in epon 812 resin. Oriented 1-mm semi-thin sections were obtained with diamond knives, and representative areas were chosen for ultrathin sectioning. Thin 1-μm sections were mounted on copper mesh grids, stained with uranyl acetate and lead citrate, and examined on a Jeol 1010 electron microscope. Some ultrathin sections were not contrasted in order to visualize Cd deposits in tissues; 20–25 micrographs, primary magnification ×10,000, were taken randomly from each sample.
Immunofluorescence and immunohistochemistry. Kidney cortex fragments were fixed in 4% paraformaldehyde in PBS overnight at 4°C, washed in PBS, and half were embedded in paraffin. The other half fragments were dehydrated by overnight incubation in 30% sucrose, embedded in Optimal Cutting Temperature medium, snap-frozen, and stored at −20°C until cryosectioning.

Sucrose infiltrated tissues were freeze sectioned and processed for megalin detection using indirect immunofluorescence. Briefly, nonspecific binding was blocked with 1% bovine serum albumin-PBS for 15 min. Then, goat anti-rat megalin antibody (1/100; Santa Cruz Biotechnology) was applied overnight at 4°C. After two washes (PBS—2.7% NaCl, PBS), sections were incubated with FITC-conjugated anti-goat secondary antibody (1/150; Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactivity was analyzed with a fluorescence microscope (Nikon Eclipse E600).

Immunohistochemistry was performed on serial paraffin sections using the envision+ kit (Dako). Sections (5 μm) from paraformaldehyde-fixed tissues were dewaxed with xylene (5 min) and ethanol (100, 95, and 70%, 2 min each) and then washed in distilled water. Antigens were retrieved by microwave heat treatment (5 min, 370 W) in citrate buffer (0.01M, pH 6). After a wash with PBS, endogenous peroxidase was blocked by incubating the sections with the 3% hydrogen peroxide (5 min). Sections were blocked in 4% goat serum for 20 min, then incubated overnight at 4°C with the specific primary antibodies against rat Ki67 (Dako, clone MB5, 1:50, mouse) and human and rat active caspase-3 (Promega, 1:50, rabbit), for detection of proliferative and apoptotic cells, respectively. After washing with PBS containing 0.1% Tween 20, sections were incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (envision+ kit, Dako) for 20 min. Sections were then washed with distilled water, incubated with developing solution (diaminobenzidine-hydrogen peroxide; Dako), and counterstained with hematoxylin. Immunoreactivity was identified as brown nuclear or cytoplasmic labelling in kidney sections counterstained with hematoxylin. Negative controls were run in parallel by replacing the primary antibody with nonimmune immunoglobulins. Sections were observed using a light microscope (Olympus) with a digital camera (Nikon). For each section, at least 200 PCT were randomly examined and positive cells counted. Results are expressed as an apoptotic index (AI) and proliferative index (PI) and represent the median number of colored cells per 1000 PCT.

In situ detection of autophagy by immunohistochemistry. LC3 immunostaining was performed on a Benchmark XT automated immunostainer (Ventana) using a specific antibody against light chain 3-II (LC3-II; clone 5F10, Nanotools, mouse) and Ventana immunohistochemistry kits (Ventana medical systems, Roche). Briefly, deparaffinized tissue sections were pretreated with cell conditioner #1 (EDTA buffer, pH 8.4, Ventana) for 60 min to unmask antigen targets, followed by incubation with anti-LC3-II at a 1/100 dilution for 32 min at 37°C. Incubation with nonimmune immunoglobulins under the same condition served as negative control. Wash steps with reaction buffer (Tris pH 7.6, Ventana) were performed at 37°C. Sections were then incubated with biotinylated secondary antibody for 8 min at 37°C, rinsed, and incubated with streptavidin biotinylated peroxidase complex (XT iView DAB V.1 detection kit, Ventana) for 8 min at 37°C. Diaminobenzidine was the chromogen and tissue sections were counterstained with hematoxylin II (Ventana) for 8 min. LC3-II immunoreactivity was identified as brown dots in kidney sections counterstained with hematoxylin.

In vitro PCT model. To confirm the direct upregulation of autophagy by Cd, renal epithelial cells derived from S1 and S2 segments of PCT were isolated from mice and immortalized with the pSV3 neo vector, as described (L’hoste et al. 2009). After G418 selection, PCT cell clones were picked and expanded. The PCT cell line was maintained in a 5% CO2 atmosphere at 37°C in DMEM/Ham’s F12 (1:1, Invitrogen) supplemented with 15mM NaHCO3, 20mM HEPES (pH 7.4), 1% fetal calf serum, 2mM glutamine, 5 mg/l insulin, 50mM dexamethasone, 10 μg/ml epidermal growth factor, 5 mg/l transferrin, 30mM sodium selenite, and 10mM triiodothyronine (Sigma). Exponentially growing cells were incubated for the indicated times in fresh DMEM/Ham’s F12 supplemented with Insulin Transferin Selenium-A (Gibco) with Cd (5μM) or other heavy metals (i.e., CoCl2 [100μM], FeCl2 [100μM], HgCl2 [5μM], Pb(NO3)2 [5μM], or Zn(NO3)2 [100μM]). Where required, cells were treated with Cd (5μM) in the absence or the presence of autophagy inhibitors: bafilomycin A1 (Baf; 100nM; Sigma) or 3-methyladenine (3-MA, 10mM; Sigma). Controls were incubated with vehicle. Cell toxicity was examined under light microscopy. Representative pictures were taken with a ×40 magnification lens.

Enhanced Green Fluorescent Protein-LC3 transfection. Cells seeded on glass coverslips were transiently transfected with enhanced green fluorescent protein (EGFP)-LC3 expression plasmids (Kabeya et al. 2000) using Fugene HD reagent (Boehringer Mannheim). After 3 h transfection, cells were washed and allowed to recover for 24 h before Cd treatment. Pictures were taken with a ×63 magnification lens using a confocal laser-scanning microscope (Zeiss LSM510 Meta) fitted with a 405 and 488 krypton/argon laser allowing simultaneous analyses of 4',6-diamidino-2-phenylindole and EGFP fluorescence’s.

Western blotting. Tissues and cells were solubilized by sonication in Laemmli buffer and analyzed by Western blotting as previously described (Corello et al. 2006) with antibodies that specifically recognize LC3-II (1:100, clone 5F10; Nanotools), ubiquitinated proteins (1/6000, rabbit; Dako) and endoplasmic reticulum (ER) stress markers (RNA-dependent protein kinase–like ER kinase [phospho-PERK]; glucose-regulated protein [Grp78]; and protein disulfide isomerase [PDI], 1:1000, rabbit; Cell Signaling Technology). After washing, the presence of primary antibodies were revealed with HRP-conjugated anti-mouse (1:3000; Santa Cruz Biotechnology) or anti-rabbit (1:10000; Santa Cruz Biotechnology), respectively, and visualized by enhanced chemiluminescence detection system (Perkin Elmer). After stripping, equal loadings of proteins were verified by reprobing the same blots with anti-actin (Santa Cruz Biotechnology).

Statistics. Results of Cd accumulation are given in standard error. Apoptotic and proliferate indexes are given in medians, and U Mann-Whitney nonparametric test was used in order to compare different groups. Non-parametric Spearman correlation was applied to study correlations between different results; p < 0.05 was considered statistically significant.

RESULTS

Cadmium Accumulation in Kidney Cortex

Renal cortex concentrations of Cd in control and treated rats are shown in Figure 1A. Little if any Cd content was detected in control rats (4 ± 0.8 ng Cd/g of kidney cortex, n = 3). Daily administration of CdCl2 increased Cd level with time from 0.38 ± 0.03 μg/g after 1 day of treatment to 1.16 ± 0.005 μg/g after 5 days. No significant decrease in Cd content was observed 5 days after the end of the intoxication (group 5 + 5 d: 1.27 ± 0.04 μg/g). Concomitant electron microscopy observations of non-contrasted sections evidenced Cd deposits in treated rats (Fig. 1C). They were located in lysosomes and appeared as dark agglomerated fine and short needles. Moreover, at 5 days and at 5 + 5 days, Cd-containing lysosomes were packed together and fusing (Fig. 1C).

Nephrotoxic Effects of Cadmium

Tubular functions, megalin labeling, and structural and ultrastructural observations were carried out in order to highlight the consequences of Cd at tubular, cellular, and molecular levels.

Subtoxic Cd dose fails to affect glomerular and tubular functions. Using inulin clearance as a marker of glomerular...

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filtration rate (GFR), we observed that GFR was not significantly modified in Cd-treated animals after 3 days of intoxication compared with control animals (Fig. 1B, left panel). Furthermore, to evaluate the effects of Cd intoxication on tubular dysfunction, fractional excretions of Na^+^ and PO_4^{3−} were estimated for each experimental group: no differences between control and intoxicated group could be shown (right panel). Na^+^ and PO_4^{3−} plasma concentrations remained unchanged (data not shown). Under this low dose of Cd, this therefore indicated that the glomerular and tubular functions were not or barely affected.

Cd does not affect megalin distribution. Metal protein complexes, such as Cd/metallothionein, are apically endocytosed partly via megalin by kidney PCT cells (Barbier et al. 2005; Christensen and Birn 2001). As shown in Figure 1D, megalin immunoreactivity was mainly located at the apical side of PCT cells and formed a thick continuous line following the brush border irregularities. Distal convoluted tubules were not labeled. The same labeling was seen on control, and treated rat kidney sections, indicating that Cd treatment did not affect megalin distribution.

Cd-induced PCT cell damages. Histology of the kidney cortex was compared among the groups. Analysis of toluidine blue colored sections showed a normal aspect for control kidney cortex (Fig. 2A). In this group, PCT cells presented a round mediobasal nucleus with a prominent nucleolus and discrete heterochromatin. A brush border was visible in a round mediobasal nucleus with a prominent nucleolus (Fig 2A, 1d). After 3 days of Cd treatment (Fig. 2A, 3d), degenerated vacuolated cells were present in the lumen after Cd treatment. In 1 day–treated rats, first injuries kidney cortex (Fig. 2A). In this group, PCT cells presented cortex was compared among the groups. Analysis of toluidine labeled. The same labeling was seen on control, and treated rat kidney sections, indicating that Cd treatment did not affect megalin distribution.

By contrast, dramatic renal cellular damages were observed after Cd treatment. In 1 day–treated rats, first injuries concerned the PCT cell nuclei that were irregular in shape. Degenerated vacuolated cells were present in the lumen (Fig 2A, 1d). After 3 days of Cd treatment (Fig. 2A, 3d), identical adverse effects were seen in the PCT lumina and discrete dilatations were present at the basal cellular sides. Furthermore, capillary lumina were barely detectable in control rats, whereas enlarged and oedemas were present between tubules of treated kidney (Fig. 2A, 3d). After 5 days of Cd treatment, PCT morphology returned to normal, except for nuclei still protruding to the lumen (Fig. 2A, 5d). “Five + five” day’s rat sections showed dramatic modifications: lumina blood capillaries were enlarged and numerous large intercellular spaces were seen, sometimes extending from PCT cell basis to the lumen. Numerous large vacuoles were also present toward the PCT lumina (Fig. 2A, 5 + 5 d).

These Cd-induced injuries were confirmed using transmission electronic microscopy. Basolateral sides as well as intercellular spaces were enlarged after 3 days of treatment (Fig. 2B, 3d) and extensive at 5 + 5 days (Fig. 2B, 5 + 5 d). Interestingly, the PCT cells with dilated intercellular spaces remained adherent to the basal lamina by hemi-desmosomes (Fig. 2B) and joined together by apical junction complexes (Fig. 2B, 5 + 5 d). By contrast, Cd did not induce visible alteration of the apical PCT brush border. Similarly, mitochondria and endocytosis apparatus were not altered by Cd treatment and numerous clathrin-coated pits and vesicles were present between microvilli (Fig. 2B).

Subtoxic Cd Dose Triggers In Vivo PCT Cell Proliferation but Not Apoptosis

Apoptotic response. To elucidate whether such Cd-induced toxicity was accompanied by an increased apoptosis, immunohistochemical hallmarks of apoptosis were studied in kidney cortices of Cd-treated rats. Caspase-3, the last executive apoptotic enzyme, was examined in cytosol of Cd-exposed PCT cells (Fig. 3A). As shown in Figure 3B, active caspase-3–positive cells were rare in control and Cd-treated rats as the AI (number of active caspase-3–positive cells per thousand PCT) barely increased two times (20 positive cells/thousand PCT) the first day, three times the third day (31 positive cells/thousand PCT), and then it gradually decreased the fifth day of intoxication to almost returned to control level at day 5 + 5. However, the differences between these values were not statistically significant (U Mann-Whitney test).

Proliferation process. Cd-induced PCT cell proliferation was revealed using Ki67 immunohistochemistry (Fig. 3C). Ki67-labeled nuclei were rare (Fig. 3C) with only one positive cell per 1000 PCT in control rats (Fig. 3D). In treated rats, renal cortex presented numerous Ki67-positive nuclei in PCT, distal convoluted tubules, loop of Henle, glomeruli, stromal, and endothelial cells (Fig. 3C, 5 + 5 d). The positive cells were frequently found inside or protruding into the PCT lumen. A time-related variation was observed for cell mitosis rate. After Cd treatment, the PI (number of positive nuclei per thousand PCT) significantly increased until the third day and then it decreased significantly at fifth day of Cd treatment (Fig. 3D). The differences between the day 1 and day 5 and between day 3 and day 5 were statistically significant (p = 0.03). Five days after the end of intoxication (5 + 5), the proliferation index was higher than during intoxication. The difference of PI between the fifth day and 5 + 5 days of intoxication was highly significant (p < 0.001).

Cadmium Is a Powerful Inducer of Autophagy In Vivo

Prompted by the accumulation of Cd within lysosomes and the absence of Cd-induced apoptosis, we next investigated whether upregulation of autophagy was correlated with Cd-induced kidney injuries. The ultrastructural analysis showed little, if any, autophagic vesicles in control kidney. Of particular interest, Cd treatment resulted first in the formation of dense protein aggregates and inclusion bodies containing Cd deposits (Fig. 4A, 1d). These structures appeared in the perinuclear region of 1 day–treated cells. In rats treated for 3–5 days with Cd, PCT cells were found to contain many vesicles with typical morphological features of autophagosomes. These...
FIG. 1. Cd accumulation, tubular function, and megalin distribution in renal cortex. Rats received either physiological saline solution (controls, 0) or a daily ip injection of 0.3 mg Cd per kg body mass (1d, 3d, 5d, and 5 + 5 d). Groups 1d, 3d, and 5d treated, respectively, during 1, 3, and 5 days and killed directly after treatment. Group 5 + 5 d, treated during 5 days and killed 5 days later. (A) Cd concentrations in kidney cortex were determined by atomic absorption spectrophotometry. The differences between control and Cd-treated groups were significant (*p = 0.05). (B) Effects of Cd intoxication on GFR, fractional excretions of PO4$^{3-}$ and Na$^+$, and sodium excretion. Experiments were performed with ip injection of 0.9% NaCl (0, white bars) or Cd (gray bars) for 3 days (n = 3 for each condition). Values are ±SE for each clearance period. Not significant by Student’s t-test. (C) Cd deposits into lysosomes were visualized by transmission electron microscopy in uncontrasted sections. Arrowheads show Cd deposits into clustered lysosomes (L) that contain endosomes (e). (D) Megalin distribution in control and Cd-treated renal PCT. Arrows show megalin-positive staining on brush borders. Note that the aspects were similar in all cases. The star points to nonlabeled convoluted distal tubules (×400).
vesicles engulfed content identical to cytosol to form double-membrane or multilamellar autophagosomes. Meanwhile, the hallmarks of apoptosis, such as degeneration of mitochondria and chromatin condensation, were not observed in these cells. Importantly, even though the Cd treatment was stopped, such formation of autophagic vesicles remained elevated for an additional 5-day period (5 + 5 days).

We therefore developed a rapid immunohistochemical method for in situ detection of autophagic vesicles (Fig. 4B). We used in this purpose an antibody that recognizes the

**FIG. 2.** Cd-induced PCT damages. (A) PCT histology was observed after coloration with toluidine blue of semi-thin sections under optical microscope. Large arrows show degenerated cells with damaged nuclei and vesicles in tubule lumen; stars indicate dilatations on tubule basal side (×1000). (B) Cd effects on brush border and basolateral sides of PCT cells visualized by transmission electron microscopy. Note that brush borders were not modified in all cases. In contrast, basal invaginations were extensive and enlarged at days 3 and 5 + 5. Hemi-desmosomes (→) on basal lamina were present in all cases. “■” indicates coated vesicle; ‘‘□’’ indicates coated pit; and ‘‘→’’ indicates tight junction.
microtubule-associated protein LC3-II, a specific and sensitive marker that specifically associates with autophagosome membranes (Klionsky et al. 2008). In agreement with the above detection of autophagosomes by electron microscopy, we observed the characteristic punctuate vesicular staining of LC3-II following Cd treatment. Strikingly, accumulation of autophagic vesicles was prominent in over 80% of renal cells of Cd-treated rat (Fig. 4B).

Detection of LC3-positive dots is the most commonly used method to detect autophagic vesicles by microscopy. However, we observed in Cd-treated kidneys the formation of ubiquitinated proteins (Fig. 4C) and LC3 can also be incorporated into ubiquitinated inclusion bodies, independently of autophagy (Kuma et al. 2007). We therefore analyzed biochemically the induction of autophagy by western blotting of LC3-II (Kabeya et al. 2000). In agreement with the above detections of autophagosomes, LC3 was detected as an 18-kDa protein (LC3-I) that was converted to the 16-kDa LC3-II in Cd-treated kidney (Fig. 4D).

**PCT Cells Are Direct and Specific Targets of Cd Toxicity**

Using an *in vitro* model of PCT cells, we provide further proof of a dramatic upregulation of autophagy in Cd-treated PCT cells. Indeed, Cd treatment (5μM, 5 h) directly induced in PCT cells the dramatic accumulation of LC3-positive vesicles (Fig. 5A) and the conversion of LC3-I into LC3-II, even after 1 h of Cd treatment (Fig. 5B). At the same time, Cd induced an important accumulation of ubiquitin-conjugated proteins (Fig. 5C), consistently with the *in vivo* detection of ubiquitinated inclusion bodies.

Accumulation of ubiquitinated proteins engages an adaptive response called ER stress that, in many cases, is coupled with autophagy induction (Kim et al. 2010). As shown in Figure 6A, Cd-treated PCT cells elicited a dramatic ER stress response as evidenced by the rapid phosphorylation of PERK and the accumulation of the glucose-regulated protein (Grp78), in agreement with Yokouchi et al. (2008). The levels of the PDI were not affected by Cd treatment. Remarkably, Cd-induced ER stress started at 1 h and lasted up to 5 h, reminiscent of the time course observed for Cd-induced ubiquitylation and autophagy. This highly suggests that ER stress and autophagy may be interlinked for disposal of aggregated ubiquitinated proteins in Cd-exposed PCT cells.

At that stage, it was therefore of interest to address whether the induction of ER stress-induced autophagy is a general response of PCT cell intoxication to heavy metals. Although ER stress has been well documented in response to heavy metals (Hiramatsu et al. 2007), there is no information on the...
ability of nonessential (Cd, Pb, and Hg) and essential trace elements (Co, Zn, and Fe) to stimulate simultaneously autophagy. As shown in Figure 6B, Cd and Zn differed qualitatively from the other heavy metals examined as they were powerful inducers of both ER stress (P-PERK and Grp78) and autophagy (LC3-II). By contrast, Pb at concentration that induced LC3-II conversion was not sufficient to upregulate ER stress. Likewise, the ER stress induced by cobalt was not accompanied by autophagy. Such difference might be related to the hypersensitivity of PCT cells to Cd. Altogether, these data indicate that the activation of ER stress and autophagy was a specific response of PCT cells to Cd.

Sustained Induction of Autophagy Participates in Cd Toxicity

In response to stress, autophagy ensures cell survival through removal of damaged proteins and organelles but a massive and sustained autophagy can commit the severely damaged cells to cell death (Galluzzi et al. 2007; Scarlatti et al. 2009). We found that incubation with low Cd concentration (5µM) for more than 5 h triggered sustained activation of autophagy (Fig. 5B) and eventually led to PCT cell detachment, likely cell death (Fig. 7A). To clarify whether autophagy is required in Cd toxicity, we tested the effects of various autophagy inhibitors. 3-MA is a class III phosphatidylinositol-3-kinase inhibitor that inhibits autophagy at the earliest step of autophagosome formation. Strikingly, cotreatment of PCT cells with 3-MA (10mM) and low Cd concentration (5µM) completely suppressed both Cd-induced LC3 conversion and cell detachment (Figs. 7A and 7B). By contrast, coincubation of Cd-treated cells with Baf (100nM), a late-stage autophagy inhibitor that prevents the degradation of autolysosome did not protect against Cd-induced effects. These results suggested that the sustained induction of autophagy did participate in Cd toxicity in PCT cells, in agreement with previous reports in mesangial cells (Wang et al. 2008; Wang et al. 2009).

DISCUSSION

In this study a 5-day subchronic Cd intoxication was performed using low Cd concentrations (0.3 mg/kg body mass). Cd accumulated in kidney cortex with time, however, less than 1 µg, that is, 1% of total administered dose in total agreement with Zalups (2000). At such doses, Cd did not adversely affect glomerular and tubular functions. These results are in line with previous observations where a critical threshold concentration of 100–200 µg Cd/g kidney was required for tubular dysfunction (WHO 2004). Our data reveal novel and key consequences of Cd intoxication to such low doses: PCT cells presented dramatic morphological changes that were statistically associated with proliferation and autophagy but not apoptosis.
Indeed, we show here that low doses of Cd did not induce apoptosis in vivo in rat PCT cells, using both electron microscopy and active caspase-3 immunodetection. Several hypotheses could be proposed for the low apoptosis we found. One possibility is linked to the low Cd dose and the short intoxication duration studied in our experiments. Consistently, higher Cd concentrations were indeed reported to induce apoptosis both in vivo (2–5 mg/kg) and in vitro (50–160 μM) (Aoyagi et al. 2003; Brzoska et al. 2003; Chuang et al. 2000; Lee et al. 2007; Mao et al. 2007; Tanimoto et al. 1993; Yokouchi et al. 2007). Alternatively, it is well known that Cd induces the expression of repair and detoxifying enzymes that render cells more resistant to apoptosis (Koizumi and Yamada 2003).

A deeper understanding of Cd nephrotoxicity requires the identification of its intracellular localization and targets, which are very sensitive to Cd exposures. Although there is extensive literature on Cd toxicity, very little information is currently available about its uptake pathways. To be toxic, Cd must enter cells and be present as a free ion in the cytosol. Current models assume that Cd is taken up either by apical transport proteins/channels or by receptor-mediated endocytosis. Consistently, there is evidence of the presence of Cd in lysosomes, based on subcellular partitioning (Abouhamed et al. 2007; Kamunde 2009). However, although informative, these fractionation studies are insufficient to formally establish the distribution of Cd before cell lysis. We confirm here by electron microscopy the accumulation of Cd within the lysosomes of PCT cells. Lysosomal Cd uptake and increases in lysosomal numbers may reflect sequestration and detoxification of this metal.

Consistently, we provide here the first evidence that the main intracellular target for Cd in vivo was autophagy, an adaptive and catabolic mechanism that involves the lysosomal compartment. The autophagic vesicles were barely detectable in untreated rats and rapidly increased upon Cd exposure. Moreover, the conversion of the autophagy marker LC3 to LC3-II, together with the appearance of punctuate LC3 staining provided further evidence for upregulation of autophagy in response to Cd. Autophagy is a housekeeping process that ensures the turnover of damaged protein aggregates and organelles to maintain cellular homeostasis (Xie and Klionsky 2007). Interestingly, electron dense cytosolic inclusions that contained aggregated proteins and Cd deposits were close or surrounded by autophagic vesicles. Binding of Cd to the sulphydryl groups of proteins as well as the oxidative damage generated by this metal are two explanations for the
upregulation of autophagy (L’hoste et al. 2009; Thévenod and Friedmann 1999; Wang et al. 2009). Other mechanisms by which Cd exposure upregulates autophagy may include ceramides, ERK, and as we evidenced herein ER stress (for a review see Thévenod 2009), which are well-known inducers of autophagy. Along this line, resistance to Cd exposure has been associated in vitro with improved recycling of damaged cell components by autophagy in fibroblasts (Ord et al. 1990) and endothelial cells (Dong et al. 2009).

Compared with the other heavy metals, Cd was the most powerful inducer of ER stress and autophagy in PCT cells. Only one essential element, Zn, was similarly able to induce ubiquitination (data not shown), ER stress, and autophagy. Both Cd and Zn are group IIb metals that share common uptake mechanisms, affinity to sulfur, and likely the same molecular targets. By displacing Zn, one might therefore assume that Cd would interfere with Zn-regulated ubiquitination, leading to ER stress and autophagy as a defence mechanism for the disposal of ubiquitinated proteins. By contrast, LC3 conversion was not accompanied by ER stress in responses to lead, iron, cobalt, and mercury. Such difference might be relevant to the hypersensitivity of PCT cells to Cd.

A massive autophagy would commit the severely damaged cells to demise. Indeed, the presence of autophagic vesicles has long been blamed in dying cells as a second form of programmed cell death, in addition to apoptosis. Whereas considerable progress has defined the importance of apoptosis, the relationships between autophagy and cell death remain unclear (Galluzzi et al. 2007; Scarlatti et al. 2009). This is in part because autophagy coexists with apoptosis in many pathologic tissues and in response to various death signals. Along this line, Cd-induced autophagy has been associated in vitro primarily with cell death of human hematopoietic stem cells (Di Gioacchino et al. 2008) and renal cell lines (Buffalo Green Monkey cells and mesangial cells; Romero et al. 2003; Wang et al. 2008). However, little, if any, dead cells in vivo showed that Cd did not induce autophagy cell death in kidney cortex. This therefore supports the notion that autophagy is an adaptive response that protects rather than commits cell to death under such low doses of Cd.

One of the major features of autophagy is its reversibility. Indeed, this pathway is dramatically and transiently upregulated in response to an environmental stress and upon stress removal, the autophagic vesicles rapidly disappear and the pathway returns to its basal low rate (Xie and Klionsky 2007). But if the stress is sustained, autophagy ultimately fails in preserving cell viability and turns to induce cell death. Importantly, 5 days after Cd removal, we still observed a massive accumulation of autophagic vesicles. This is in line with the persistent accumulation of Cd within lysosomes. The lysosomes are central in autophagy as they fuse with autophagosomes and degrade via their acid hydrolases the autophagic content. Interestingly, Cd has been reported to inhibit the v-ATPase, an essential proton pump that maintains acidic lysosomal pH (Herak-Kramberger et al. 1998). Altogether, this raises the possibility that Cd could inhibit the last
step of autophagy due to lysosomal injury, resulting in the accumulation of autophagic vesicles.

Cd has been classified as a human carcinogen by the International Agency for Research on Cancer and by the US National Toxicology Program (IARC 1993; NTP 2000). Several human cancers including renal cancers are linked with occupational Cd exposure (Hu et al. 2002). Interestingly, we show here that exposure to low Cd doses significantly increased in vivo the growth of PCT cells. Recent in vitro analyses have evidenced that Cd stimulates cell growth at low concentration (10 μM) in neuronal cells (Gulisano et al. 2009), fibroblasts (Jiang et al. 2009), and Leydig cells (Singh et al. 2009). At molecular level, we and others have documented that in vitro Cd shares with phorbol ester the remarkable ability to upregulate the MAPK ERK and the expression of proto-oncogenes in hepatoma cells (Kawata et al. 2009), breast cancer cells, mesangial cells, and PCT cells (for a review see Thévenod 2009).

Although additional studies are needed to explore mechanistic aspects, our study unravels unexpected early upregulation of autophagy and proliferation in response to low doses of Cd. Even at such subtoxic Cd doses, that is, that approach environmental levels and did not elicit apoptosis or tubular dysfunction, we provide the first evidence that activation of autophagy was prominent in more than 80% of Cd-treated PCT in vitro. In this respect, autophagy, by enhancing the cell’s tolerance, could provide an early adaptive mechanism avoiding apoptosis. Given that Cd is persistent within cytosol, it might damage proteins continuously and impair the efficiency of the autophagy process. A defective autophagy has been involved in growing list of diseases ranging from cancer, neurodegeneration, and aging (Mizushima et al. 2008), that are interestingly associated with Cd occupational exposure. We therefore propose that the upregulation of autophagy we evidenced herein in vivo in PCT cells could contribute to Cd-induced nephrotoxicity and serve as a reliable and early biomarker of Cd exposure to low subtoxic doses.

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