Expression of multidrug resistance P-glycoprotein Mdr1 (Abcb1) in rat kidney proximal tubules is up-regulated by nephrotoxic metals

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ABSTRACT

Multidrug resistance P-glycoprotein Mdr1 (Abcb1), an ATP-driven efflux pump for potentially toxic metabolites and xenobiotics has been localized to the brush-border membrane of proximal tubule (PT) cells, where its expression may be affected by various stress-related factors, including heat-shock or cytotoxic metals. To test the effect of various metals upon renal Mdr1, we treated rats with 10 different metals for 2 weeks and compared the expression of Mdr1 protein in PT with the accumulation of these metals in the tissue. Whereas 6 of the tested metals (Cu, Mn, Zn, Ca, Mg, Al) showed a variable accumulation but no upregulation of Mdr1 expression, 4 well-known nephrotoxic metals (cisPt, Pb, Cd, and Hg) exhibited strong accumulation in the cortical tissue and strong upregulation of transporter expression (3-, 6-, 15-, and 18-fold, respectively). The immunostaining experiments showed that Cd affected all PT segments in both cortex (including medullary rays) and outer stripe, whereas Hg, Pb, and cisPt predominantly affected the PT S3 segments in the medullary rays and outer stripe. The observed metal-induced increase in renal Mdr1 expression may be part of a specific cytoprotective response of PT cells to oxidative stress in order to remove peroxidized lipids, other toxic substances or metals, and to escape cell death by apoptosis or necrosis.

Key Words: cadmium, cisplatin, heavy metals, lead, mammalian kidney, mercury, nephrotoxicity

INTRODUCTION

In mammals, the ~170 kDa-member of the ABC superfamily of transporters, Mdr1 (P-gp, Abcb1), functions as an ATP-driven efflux pump for various endogenous and exogenous organic cations (OC) and hydrophobic compounds (1-5). In mRNA and/or immunolocalization studies, the transporter was detected in epithelial cells of the small and large intestine, bile canaliculi and ducts of the liver, PT of the kidney, capillary endothelium of the blood-brain and blood-testis barriers, trophoblast of the placenta, as well as in leukocytes and various tumor cells (1, 6, 7). Mdr1 transports lipids, bile salts, drugs, and various toxic and environmental organic compounds out of cells, thus protecting the cell interior from potentially toxic effects of many endogenous and xenobiotic substances. On the other side, the protein may be responsible for multidrug resistance in cancer and other cells following chemotherapy, and for resistance of certain pest species to pesticides (2-4). In addition, up-regulation of Mdr1 expression by heat-shock, UV light, chemoterapeutics, steroid hormones, organ surgery, environmental toxicants, and various intracellular factors, indicates its behavior as a stress-inducible protein (2, 3, 8).

In the mammalian nephron, Mdr1 is localized to the brush-border membrane (BBM) of PT, where it mediates an ATP-driven efflux of various OC that enter the cell by other transporters at the apical and/or basolateral membrane (5). Studies in cultured cell lines of renal origin showed that Mdr1 expression and its function can be upregulated by heat-shock and some toxic metaloids and metals, such as arsenic (As), cisplatin (cisPt), and cadmium (Cd) (9-12). Only a few studies have investigated renal Mdr1 expression in experimental animals treated with toxic metals; in cisPt-treated rats, Mdr1 gene expression was upregulated in kidneys but not in the liver (13), whereas in our preliminary

immunochemical study, the expression of Mdr1 protein was strongly increased in the cortical PT of Cd-treated rats (10). As shown in details in a cell line derived from the immortalized rat PT S1 segment cells, Cd-induced upregulation of Mdr1 expression may be driven by reactive oxidative species (ROS)-mediated processes, and this upregulation may protect the cells from Cd-induced apoptosis (11). However, other nephrotoxic metals, including cisPt, lead (Pb), and mercury (Hg) may also exhibit a common, ROS-mediated mechanism of toxicity in PT cells, which *in ultima linea* can result in cell death by apoptosis or necrosis (14, 15), but their possible effect on the renal Mdr1 expression in experimental animals has not been reported. Accordingly, we have treated rats with 10 different metals, and compared the accumulation of metals in renal cortical tissue with the expression of Mdr1 protein in renal PT.

Key Words: cadmium, cisplatin, heavy metals, lead, mammalian kidney, mercury, nephrotoxicity

MATERIALS AND METHODS

Materials

The monoclonal anti-Mdr1 antibody (clone C219) was from Alexis Deutschland (Grünberg, FRG). The horseradish peroxidase-labeled sheep anti-mouse IgG (SAM-HRP) and the enhanced chemiluminescence (ECL) reagents were from Amersham-Buchler (Braunschweig, FRG). The CY3-labeled donkey anti-mouse IgG (DAM-CY3) was from Jackson ImmunoResearch Lab. (West Grove, PA, USA). Polyvinylidene difluoride (PVDF) membrane was from NEN-Dupont (Bad Homburg, FRG). The fluorescence fading retardant Vectashield was from Vector Laboratories Inc. (Burlingame, CA, USA). All other chemicals (analytical grade) were also purchased commercially.

Animals and Treatment

Male Wistar strain rats at an age of 3 months were used from the Institutional breeding colony in Zagreb. Before and during experiments, rats had free access to standard laboratory food and tap water *ad libitum*. The studies were approved by the Institutional Ethics Committee. Chloride salts of various metals were injected s.c. daily, excluding Sunday, at a dose of 5 mg/kg b.m. (Pb) or 2 mg/kg b.m. (other metals) for 14 days, whereas cisplatin (cisPt) was injected i.p. at a single dose of 5 mg/kg b.m. 5 days before sacrifice. Control animals were injected the same way with an equivalent volume of vehicle (physiological saline (0.9% NaCl), 3-5 mL/kg b.m.).

Determination of Metals in the Kidney Cortex Tissue

Rats were decapitated and opened by abdominal incision. The kidneys were removed, rinsed with an ice-cold saline, blotted on a filter paper, sagittally sliced, and the cortical tissue was dissected manually. The tissue from an animal was pooled, weighed, and dry-ashed at 450^{o}C for 24 hours. Ashed samples were dissolved in 2% nitric acid, the metal concentration was measured by atomic absorption spectrometry using commercial metal solutions as standards, and the data were recalculated per tissue wet mass.

Isolation of BBM, SDS-PAGE, Western Blotting

The renal cortical tissue was homogenized and the BBM were isolated by the Mg^{2+}/EGTA precipitation method (16). The final membrane preparation was dissolved in a buffer that comprised (in mM): 300 mannitol, 0.1 $MgSO_4$, 5 EDTA, 0.2 PMSF, 10 Hepes/Tris, pH 7.0. Protein was measured by the Bradford assay (17), using bovine serum albumin as a standard. Electrophoresis and blotting procedures were described in details previously (10, 11). Briefly, the BBM proteins were denatured in Laemmli buffer, separated by SDS-PAGE using 7.5% mini gels, and electrophoretically

transferred to PVDF-membranes. The transfer membrane was blocked with Tris-buffered saline, containing 3% nonfat dry milk and 0.05% Tween-20 for 6 h, followed by incubation in C219 (5 μg/mL) overnight at 4°C, washing, incubation in secondary antibody (SAM-HRP, 1:6,000) for 1 h at room temperature (RT), washing, and developing in ECL reagents. The chemiluminescence signals were visualized on X-ray films. The bands on the film were scanned, imported in and further enhanced with the Corel Photo-Paint 5.0 software, and quantified using a computer-assisted imaging system Bioprofil (Vilber-Lourmat, Marne La Vallee, France).

Tissue Fixation and Immunocytochemistry

Rats were anesthetized, and the organs were fixed by perfusing the circulatory system *in vivo* with PLP-fixative, as described in details previously (10, 18). Four μm-thick cryosections of the fixed tissue were cut and collected on Superfrost/Plus microscope slides. In order to expose cryptic antibody binding sites, the antigen retrieval steps, described in details previously for immunolocalization of organic anion transporter Oat3 in the rat kidney, were applied (19). Following retrieval, the sections were washed, incubated in C219 (15 μg/mL) for 3 h at RT, washed, incubated in DAM-CY3 (10 μg/mL) for 1 h at RT, washed, mounted in Vectashield, and the staining was examined by fluorescence microscopy. The images were captured using the Spot RT Slider camera and software (Diagnostic Instruments, Sterling Heights, MI, USA), and imported into Adobe Photoshop 6.0 for processing and labeling.

Presentation of the Data and Statistical Analysis

The figures represent results obtained in 3 or more independent experiments with different tissues (immunocytochemistry) and membrane preparations (Western blots). The numeric data, shown as mean ± SEM (n = 3-6), were statistically analyzed using unpaired Student's t-test at 5% level of significance.

RESULTS

As listed in Table 1, rats treated with Zn exhibited no metal accumulation in the cortex tissue, in rats treated with Mg, Al, and Ca the metal content was not determined, while rats treated with Cu, Mn, Cd, Hg, Pb, and cisPt exhibited strong accumulation of these metals in comparison with the respective concentrations in control animals, ranging from ~7-fold (Mn) up to ~3,000-fold (Cd).

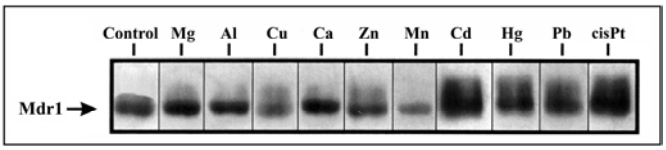

Figure 1: Representative immunoblots of the Mdr1-related ~170 kDa protein band in renal cortical BBM isolated from rats treated with the indicated metals.

The Mdr1-related ~170 kDa protein band in isolated cortical BBM from animals treated with different metals is shown in a representative immunoblot in Fig. 1, whereas the data of Mdr1 abundance in the experimental groups relative to their respective controls, are summarized in Table 1. In BBM from the rats treated with Mg, Al, Ca, and Zn, the abundance of Mdr1 protein remained similar to that in controls. The same is valid for Cu and Mn, which exhibited a marked accumulation in the tissue but no change in Mdr1 abundance. In contrast, the treatment with Cd, Hg, Pb, and cisPt caused strong up-regulation of Mdr1 protein abundance, that ranged between 3- (cisPt) and 18-fold (Hg).

Table. 1. Metal concentrations in kidney cortex tissue and relative abundance of Mdr1 protein in cortical BBM isolated from vehicle- (control) and metal-treated rats

Metal	Metal concentration (μg/g w.m.)			Mdr1 abundance (Relative units; Control = 1)
	Control	Treated	Fold increase	
Mg	ND	ND	-	1.7 ± 0.8
Al	ND	ND	-	1.5 ± 0.6
Cu	12.7 ± 1.4	156 ± 2.9**	12.3 ± 0.2	1.3 ± 0.5
Ca	ND	ND	-	1.7 ± 0.7
Zn	45.5 ± 5.5	21.8 ± 2.9	-	0.8 ± 0.3
Mn	0.89 ± 0.06	6.0 ± 0.7**	6.7 ± 0.8	1.4 ± 1.0
Cd	0.084 ± 0.01	250 ± 4.9**	2,976 ± 58	15.1 ± 6.3*
Hg	0.28 ± 0.09	121 ± 7.0**	432 ± 25	18.3 ± 5.3*
Pb	0.096 ± 0.02	17.3 ± 0.3**	180 ± 3	6.3 ± 2.1*
cisPt	0.43 ± 0.04	4.76 ± 0.44**	11.1 + 1	3.0 ± 0.3*

Metal concentration: Shown are means ± SEM of metal concentrations in the kidney cortex tissue from 3-6 animals in each experimental group. **Mdr1 abundance:** Shown are means ± SEM of relative densitometric units of Mdr1-related ~170 kDa-protein band in Western blots of isolated BBM from 3 (Mg, Al, Ca, Mn), 6 (Cu, Zn, Hg, Pb, cisPt), and 12 (Cd) animals after comparison with the average density of the bands obtained in membranes from 3-4 vehicle-treated rats in each experimental group (control density = 1). w.m., tissue wet mass. ND, not determined. Vs. Control, *P<0.025 or **P<0.01.

The immunolocalization of renal Mdr1 protein was tested in tissue cryosections from vehicle-treated rats (controls) and from rats treated with 4 nephrotoxic metals that exhibited the highest density of Mdr1-related ~170 kDa protein band in immunoblotting experiments (Fig. 2).

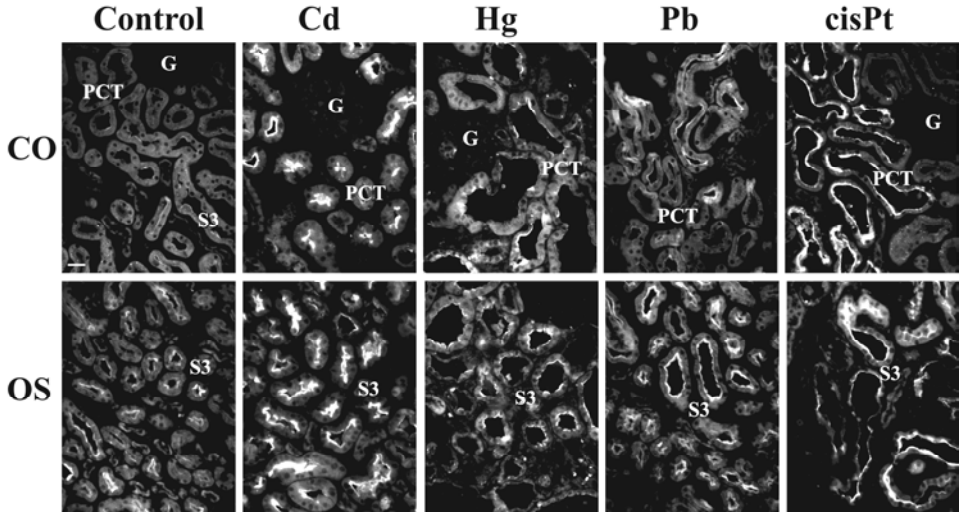

Figure 2. Immunostaining of Mdr1 protein in tissue cryosections of the rat kidney cortex (CO) and outer stripe (OS) from the vehicle- (control) and metal-treated rats. G, glomerulus; PCT, proximal convoluted tubules (= S1/S2 segments); S3, proximal straight segment. Bar = 20 μm.

In control rats, a weak staining of Mdr1 was observed exclusively in the BBM of PT in the cortex and outer stripe. In the cortex, stained were the proximal convoluted tubules (PCT) around the glomeruli (the initial PT S1 segment was negative (not shown)) and S3 segments in the medullary

rays, whereas in the outer stripe, stained were the PT S3 segments. The pattern of staining intensity was S3>PCT. In comparison with the staining in controls, in Cd-treated rats many PT exhibited structural damage and most of them had an enhanced BBM staining intensity in both cortex (PCT) and outer stripe (S3). In Hg-treated rats, in both tissue zones most PT profiles were heavily damaged and distended, and the intensity of Mdr1 staining in residual BBM was largely increased. In Pb-treated rats, the structure of PT in both zones was retained, and Mdr1 staining was visibly stronger in many PT segments. In cisPt-treated rats, most S3 segments in the outer stripe were largely destroyed and exhibited low or no Mdr1 staining (not shown), whereas the staining in the remaining, less damaged S3 segments in the outer stripe and medullary rays, as well as in some PCT in the cortex, was heterogeneously enhanced.

DISCUSSION

Previously we demonstrated in detail the up-regulation of Mdr1 expression in PT after treating rats or cell lines with Cd (10, 11). The present data show that, in addition to Cd, other nephrotoxic metals (Hg, Pb, cisPt) are also strong enhancers of Mdr1 protein expression in the BBM of rat renal PT. However, we observed no correlation between the tissue level of accumulated metals and the abundance of Mdr1 protein in membranes from the same tissue; Mn and Cu accumulated ~7- and ~12-fold above control values, respectively, but showed no increase of Mdr1 protein abundance, whereas similar accumulation of cisPt (11-fold) was associated with a strong enhancement of Mdr1 abundance. Furthermore, Cd and Hg were accumulated 3000- and 432-fold, respectively, yet with similar abundance of this protein. This means that accumulation of the metal itself was not a direct stimulus for the induction of Mdr1 protein. Rather, the stimulus may be ROS generated by metal ion-induced oxidative stress. Cd, Hg, Pb, and cisPt are well known nephrotoxic metals, which in target cells exhibit common mechanisms of action that include depletion of antioxidants and free radical scavengers and inhibition of free radicals-detoxifying enzymes. The resulting enhanced levels of ROS then cause a variety of deleterious intracellular actions, which in the PT cells accelerate lipid peroxidation, perturb Ca^{2+} homeostasis and mitochondrial energy production, and damage cell structure and function. These effects initially result in secretory and reabsorptive defects in PT, and can finally cause cell death by apoptosis or necrosis (14, 15, 20). Our previous studies of Mdr1 expression in a Cd-treated PT cell line showed that: a) upregulated Mdr1 expression was associated with decreased intracellular accumulation of its substrate calcein, b) treating the cells with ROS scavengers prevented up-regulation of the protein, and c) Mdr1 overexpression protected the cells from ROS-mediated apoptosis (10, 11). Since Hg, Pb, and cisPt also induce oxidative stress and production of ROS in the kidney (reviewed in (15)), and up-regulate the expression of Mdr1 (this report), the pattern of their nephrotoxic actions may be similar to that of Cd. Having a broad substrate specificity, the up-regulated Mdr1 may function as: a) „lipid translocase“, by mediating efflux of peroxidized lipids and their toxic metabolites in the urine; the secretion of such compounds was shown to be strongly elevated in the urine of Cd-treated rats (21), and b) „extruder of heavy metals“, by actively transporting toxic metals complexed with intracellular organic substances; such an efflux mechanism was shown for the complex cisPt-glutathione in human leukemic cells that was mediated by the ABC transporter MRP1 (22). Therefore, the up-regulation of Mdr1 in PT cells of toxic metal-treated rats may have an important cytoprotective function in removing metals and various toxic substances from the cells, thus preventing from structural and functional damages and cell death by apoptosis or necrosis.

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