

Short- and Long-Term Influences of Heavy Metals on Anionic Drug Efflux from Renal Proximal Tubule

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ABSTRACT

We recently demonstrated in isolated killifish renal proximal tubules that two classes of nephrotoxicants, aminoglycoside antibiotics and radiocontrast agents, rapidly decrease transport mediated by multidrug resistance protein 2 (Mrp2) by causing endothelin (ET) release and signaling through an ET_B receptor and protein kinase C (PKC) (Masereeuw et al., 2000; Terlouw et al., 2001). In the present study, we used killifish proximal tubules, fluorescein methotrexate, a fluorescent model substrate for Mrp2, and confocal microscopy to examine the effects of two heavy metal salts (CdCl₂ and HgCl₂) on Mrp2 function. Three patterns of effects were seen. First, exposing tubules to 10 μM CdCl₂ or 100 nM HgCl₂ for 30 min reduced Mrp2-mediated transport. This reduction was abolished by the ET_B receptor antagonist, RES-701-1, and by the PKC-selective in-

hibitor, bis-indolylmaleimide I; neither of these pharmacological tools by itself affected transport. As with aminoglycoside antibiotics and radiocontrast agents, the acute effects of 10 μM CdCl₂ or 100 nM HgCl₂ on transport were also blocked by nifedipine, suggesting that Ca²⁺ also initiated cadmium and mercury action. Second, exposure to higher concentrations of CdCl₂ and HgCl₂ appeared to be toxic. Third, exposing tubules for 6 to 24 h to lower levels of CdCl₂ increased Mrp2-mediated transport and Mrp2 immunostaining at the luminal membrane of the proximal tubule cells. Together, these findings indicate that exposure of renal proximal tubules to heavy metals initially leads to reduced Mrp2 function but is followed by an induction in Mrp2-mediated transport after long-term exposure.

Endothelins (ETs) are a family of potent vasoconstricting polypeptides (ET-1, -2, and -3) that can alter function of vascular and nonvascular tissues by interacting with two pharmacologically distinct, G protein-coupled receptors, ET_A and ET_B. In the kidney, ET regulates blood flow, glomerular hemodynamics, and sodium and water homeostasis (Rubanyi and Polokoff, 1994) but also has been implicated in a number of renal syndromes, including acute renal failure, reperfusion injury, and chemical nephrotoxicity (Rubanyi and Polokoff, 1994; Bruzzi et al., 1997; Haug et al., 1998). In some animal models of acute renal failure, ET receptor antagonists have been able to protect against the effects of nephrotoxicants and reperfusion injury (Bird et al., 1996; Krause et al., 1997).

Although altered vascular function is an important component of the role of ET in renal disease, recent studies provide evidence for a direct effect of ET on tubular function. For example, in proximal tubules, ET regulates Na⁺/H⁺ exchange, Na⁺/HCO₃⁻ cotransport, and fluid reabsorption (Garcia and Garvin, 1994; Guntupalli and DuBose, 1994), and ET production in proximal tubules increases after exposure to

cyclosporin A, mercury, high-molecular weight proteins, and hypoxia (Zoja et al., 1995; Bruzzi et al., 1997; Haug et al., 1998; Yanagisawa et al., 1998). In this regard, we recently demonstrated that in isolated killifish renal proximal tubules, ET, acting through an ET_B receptor and protein kinase C (PKC), rapidly down-regulates transport mediated by two luminal drug export pumps, P-glycoprotein and multidrug resistance protein 2 (Mrp2) (Masereeuw et al., 2000). Interestingly, representatives of two classes of nephrotoxicants, aminoglycoside antibiotics and radiocontrast agents, also decreased Mrp2-mediated transport by activating ET_B receptor-PKC signaling (Masereeuw et al., 2000; Terlouw et al., 2001). These nephrotoxicants caused Ca²⁺-dependent ET release from the tubules; ET acted by an autocrine mechanism then bound to its receptor and initiated signaling. These effects were specific in that transport of the small organic anion, fluorescein (FL), on the classical organic anion system was not affected by ET or the nephrotoxicants at the concentrations that reduced Mrp2-mediated transport (Terlouw et al., 2001).

ABBREVIATIONS: ET, endothelin; Mrp2, multidrug resistance-associated protein 2; PKC, protein kinase C; ET_A/ET_B receptor, endothelin receptor subtype A or B; NO, nitric oxide; NOS, NO synthase; FL, fluorescein; MTX, methotrexate; BIM, bis-indolylmaleimide I; Sf9, *Spodoptera frugiperda*.

Heavy metal salts are another class of nephrotoxicants that specifically target the proximal segment of the nephron (Conner and Fowler, 1993; Diamond and Zalups, 1998; Zalups, 2000). They are of particular interest in the context of ET signaling to drug export pumps because of 1) the links between ET production and mercury toxicity (Yanagisawa et al., 1998), 2) the ability of CdCl₂ and *cis*-platinum to up-regulate renal P-glycoprotein and Mrp2 (Demeule et al., 1999a; Thévenod et al., 2000; Liu and Brunner, 2001), and 3) the potential for interactions between classes of toxicants that share or alter function of xenobiotic transporters. In the present study, we investigated the effects of CdCl₂ and HgCl₂ on Mrp2-mediated transport in killifish proximal tubules. Our data show multiple effects, which include triggering of the ET-PKC signaling system after exposure to low metal salt concentrations for short times, toxicity with short-term exposure to higher concentrations, and induction of Mrp2 with long-term exposures to low concentrations of CdCl₂.

Materials and Methods

Chemicals. FL methotrexate (MTX) and FL were obtained from Molecular Probes (Eugene, OR). The ET_A receptor antagonist, JKC-301, and the ET_B receptor antagonist, RES-701-1, were purchased from Peninsula Laboratories (Belmont, CA). Medium 199, CdCl₂, HgCl₂, bis-indolylmaleimide I (BIM), MgATP, and nifedipine were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies directed against Mrp2 (k78 mrp2) were obtained as described previously (van Aubel et al., 1998). Fluorescein-labeled anti-rabbit IgG was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Creatine kinase was purchased from Roche Diagnostics (Indianapolis, IN). Creatine phosphate was purchased from Roche Applied Science (Indianapolis, IN). All other chemicals were obtained from commercial sources at the highest purity available.

Animals and Tissue Preparation. Killifish, *Fundulus heteroclitus*, were collected by local fisherman in the vicinity of Mount Desert Island, Maine, and maintained at the Mount Desert Island Biological Laboratory in tanks with natural flowing sea water. Renal tubular masses were isolated in a marine teleost saline based on that of Forster and Taggart (1950), containing 140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, and 20 mM Tris at pH 8.0. For teleost fish, a blood pH of 7.8 at 18 to 20°C is physiological. The pH was adjusted with HCl. With Tris buffer, we have found that a pH of 8.0 gives optimal transport of a number of compounds (under which fluorescein, FL-MTX, *p*-aminohippurate, tetraethylammonium, and [*N*-ε-(4-nitrobenzofuranon-7-yl)-D-Lys⁸]cyclosporin A) in killifish and flounder renal proximal tubules (Schramm et al., 1995; Masereeuw et al., 1996; Miller et al., 1996; Pritchard and Miller, 1996). All tubule dissection and all short-term experiments were carried out at room temperature (18–20°C). Under a dissecting microscope, each mass was teased with fine forceps to remove adherent hematopoietic tissue. Individual killifish proximal tubules were dissected free of the masses and transferred to a foil-covered Teflon chamber (Bionique, Saranac Lake, NY) containing 1.5 ml of marine teleost saline with 1 μM FL-MTX and added effectors. The chamber floor was a 4 × 4-cm glass coverslip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted microscope. Tubules were incubated at room temperature for 30 min, which is the time for FL-MTX to reach a steady-state distribution within the tubules (Masereeuw et al., 1996).

For the long-term experiments, tubules were dissected as above and transferred to 12-well plates, of which each well contained 3 ml of medium 199 that was supplemented with 4.2 mM NaHCO₃, 25 mM HEPES, 17 mM NaCl, 1.0 mM Na₂SO₄, 1.2 mM CaCl₂, and 16 mM NaOH with or without added CdCl₂. Tubules were incubated for

6 or 24 h at 10°C and then transferred to confocal chambers containing marine teleost saline with 1 μM FL-MTX.

Confocal Microscopy. The chamber containing renal tubules was mounted onto the stage of an Olympus FluoView inverted confocal laser scanning microscope (Olympus, Tokyo, Japan) and viewed through a 40× water immersion objective (numerical aperture 1.15). Excitation was provided by the 488-nm line of an argon laser. A 510-nm dichroic filter and a 515-nm long-pass emission filter were used. Neutral density filters and low laser intensity were used to avoid photobleaching. With the photomultiplier gain set to give an average luminal fluorescence intensity of 1500 to 3000 (on a scale of 0–4096), tissue autofluorescence was undetectable. To obtain an image, dye-loaded tubules in the chamber were viewed under reduced, transmitted light illumination, and a single proximal tubule with well-defined lumen and undamaged epithelium was selected. The plane of focus was adjusted to cut through the center of the tubular lumen, and an image was acquired by averaging four scans. The confocal image was viewed on a high-resolution monitor and saved to an optical disk. Fluorescence intensities were measured from stored images using Scion Image software (Scion Corp., Frederick, MD) as described in detail previously (Masereeuw et al., 1996). Briefly, two or three adjacent cellular and luminal areas were selected from each tubule, and the average pixel intensity for each area was calculated after background subtraction. The values used for that tubule were means of all selected areas. Previous work (Miller and Pritchard, 1991) suggests that these fluorescence intensities provide a measure of the concentrations of FL-MTX in the cellular and luminal compartments of the tubules.

Uptake of FL-MTX in Membrane Fractions of *Spodoptera frugiperda* (Sf9) Cells Over-Expressing Mrp2. Cells from Sf9-expressing rabbit Mrp2 were generated by infection of cells using a recombinant baculovirus encoding Mrp2 as described previously (van Aubel et al., 1998). For controls, Sf9 cells were infected with a baculovirus encoding the β-subunit of rat H⁺/K⁺-ATPase. Crude membrane fractions from infected Sf9 cells were isolated. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Venendaal, The Netherlands). Uptake of FL-MTX was measured using a rapid filtration technique (van Aubel et al., 1998) followed by fluorescence spectrometry. Briefly, membrane vesicles (450 μg of protein equivalent) were rapidly thawed and incubated at 37°C in the presence of 4 mM MgATP, 10 mM MgCl₂, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 10 μM FL-MTX in a final volume of 400 μl of TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4). After 5 min, the reaction mixture was diluted with ice-cold TS buffer and filtered through GF/F filters (Whatman, Maidstone, England) using a filtration device (Millipore Corp., Bedford, MA). Filters were washed once with 5 ml of TS buffer and eluted in 1% SDS and 7.5 mM HEPES. Fluorescence was determined using a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). Samples were excited at 491 nm and emission was measured at 516 nm using a slit setting of 5 nm. In uptake experiments, net ATP-dependent transport was calculated by subtracting values in the absence of ATP from those in the presence of ATP.

Data Analysis. Data are expressed as mean ± S.E. Statistical differences between means were first assessed by one-way analysis of variance, followed by Student's *t* test with Bonferroni correction. Differences were considered significant when *P* < 0.05.

Results

Short-Term Heavy Metal Exposure and Mrp2-Mediated Transport. The present experiments were conducted using isolated renal proximal tubules from a marine teleost fish, the killifish. This has proven to be a powerful model for the study of secretory transport in an intact proximal tubule (Miller and Pritchard, 1991). As in mammalian proximal

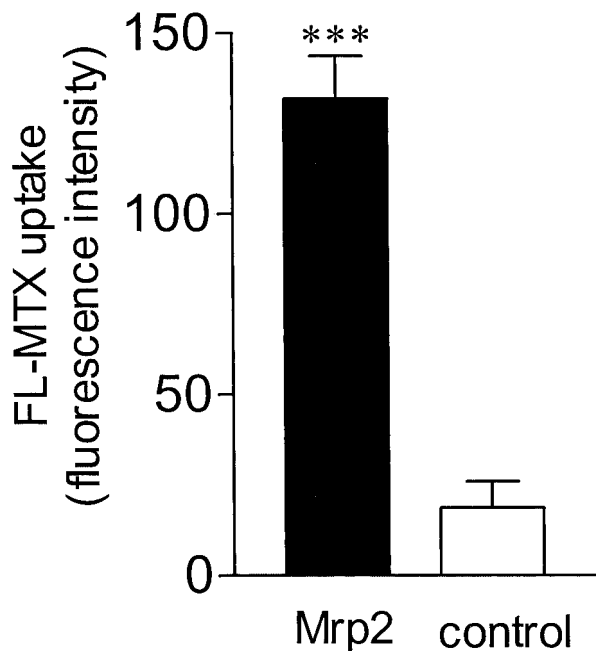


Fig. 1. Net ATP-dependent uptake of FL-MTX in Sf9-Mrp2 and Sf9-control vesicles. Membrane vesicles were incubated at 37°C for 5 min with 10 μ M FL-MTX and an ATP regenerating system (as described under *Materials and Methods*). Data are given as mean \pm S.D., $n = 3$. ***, significantly higher than control, $P < 0.0001$.

tubules, killifish express high levels of Mrp2 in the luminal membrane of renal proximal tubule cells. Moreover, intact killifish tubules exhibit Mrp2-mediated transport of a number of fluorescent substrates, e.g., FL-MTX and sulforhodamine 101, which can be visualized and measured using confocal microscopy (Masereeuw et al., 1996, 2000). Although we cannot completely rule out the involvement of other transporters present in the apical membrane of renal proximal tubules, the high inhibitory potencies of excellent Mrp2 substrates and inhibitors (viz. leucotriene C_4 , estradiol-17- β -D-glucuronide, and bromosulphophthalein) suggest that FL-MTX is transported by Mrp2 in killifish renal proximal tubules (Masereeuw et al., 1996, 2000). Figure 1 provides the first direct demonstration that FL-MTX is indeed an Mrp2 substrate. Membrane vesicles from Sf9 cells over-expressing recombinant rabbit Mrp2 have provided a useful tool to investigate Mrp2-mediated transport (van Aubel et al., 1998, 1999, 2000). Figure 1 shows the net ATP-dependent uptake (5 min) of 10 μ M FL-MTX in vesicles isolated from Sf9 cells over-

expressing Mrp2 and Sf9 control vesicles. ATP-driven FL-MTX uptake was 7-fold higher in Sf9-Mrp2 vesicles compared with controls.

Figure 2A shows a typical confocal image of a control killifish tubule after 30 min of incubation (steady state) in medium with 1 μ M FL-MTX. The fluorescence distribution pattern is the same as shown previously, i.e., fluorescence intensity in the lumen is higher than in the cells, which in turn is higher than the intensity in the medium (Masereeuw et al., 1996). We have demonstrated that this pattern is indicative of a two-step process, involving uptake at the basolateral membrane mediated by an as yet uncharacterized transporter for large organic anions and secretion into the lumen mediated by a teleost form of Mrp2 (Masereeuw et al., 1996, 2000).

Tubules exposed to 10 μ M CdCl₂ or 100 nM HgCl₂ for 30 min exhibited a clear reduction in luminal fluorescence intensity, but no apparent change in cellular fluorescence was observed (Fig. 2, B and C). As shown previously, the steady-state cellular levels of FL-MTX seem to set independently of events at the luminal membrane (Masereeuw et al., 2000). Quantitation of luminal and cellular fluorescence showed significantly reduced luminal fluorescence with 10 to 50 μ M CdCl₂ but no change in cellular fluorescence (Fig. 3A). Tubules exposed to HgCl₂ for 30 min exhibited a concentration-dependent decrease in FL-MTX accumulation, but effects were found at much lower heavy metal concentrations than for cadmium. With 100 nM HgCl₂, luminal fluorescence fell by about 60%, but cellular fluorescence was unaffected (Fig. 3B). With 250 to 500 nM HgCl₂, luminal fluorescence fell further and cellular fluorescence decreased significantly.

Cellular Energy Metabolism. The reduction in Mrp2-mediated transport of FL-MTX seen in response to short-term exposure of killifish proximal tubules to 10 μ M CdCl₂ and 100 nM HgCl₂ could be the result of specific effects on the luminal transporter or of cellular toxicity, e.g., reduced ATP levels. To determine whether cellular function was compromised by short-term exposure of tubules to heavy metal salts, we monitored transport of the organic anion, FL. Basolateral uptake of FL is mediated by the classical organic anion transport system via indirect coupling to Na⁺ and, subsequently, to cellular metabolism through Na⁺, K⁺-ATPase, and ATP (Pritchard and Miller, 1993). Transepithelial transport of FL is very sensitive to changes in cellular metabolism (Miller and Pritchard, 1991; Miller et al., 1993) but is not regulated by ET in concentrations up to 1 nM (Terlouw et al.,

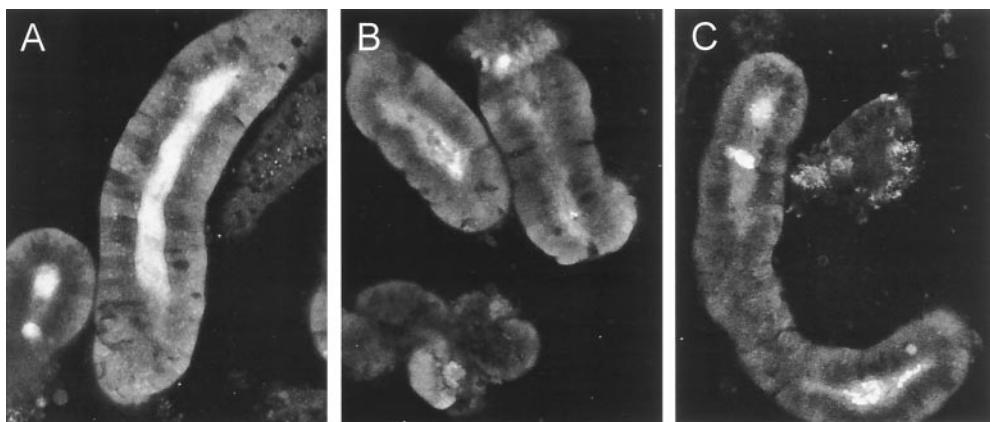


Fig. 2. Representative confocal images of killifish proximal tubules after incubation in medium with 1 μ M FL-MTX for 30 min in the absence or presence of heavy metal salts. A, control; B, in the presence of 10 μ M CdCl₂; and C, in the presence of 100 nM HgCl₂.

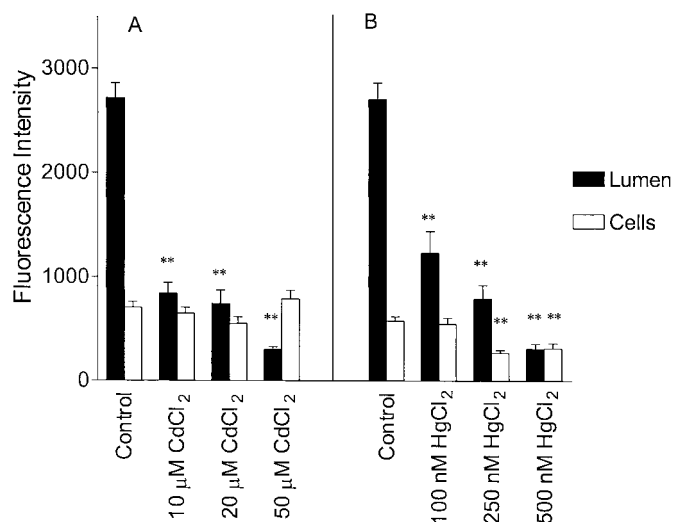


Fig. 3. Short-term inhibition of FL-MTX transport by CdCl₂ (A) and HgCl₂ (B). Tubules were incubated for 30 min in medium containing 1 μM FL-MTX and 0 to 50 μM CdCl₂ (A) or 0 to 500 nM HgCl₂ (B). Data are given as mean ± S.E. for 14 to 30 tubules from one to two fish. **, significantly lower than controls, $P < 0.01$.

2001), which makes FL a useful tool for detecting cytotoxic effects. Previous studies showed that concentrations of 0.5 μM HgCl₂ or below did not alter cellular energy metabolism as measured by tissue sodium and potassium or oxygen consumption (Miller, 1981; D. S. Miller, unpublished observations). Figure 4 shows that neither 10 μM CdCl₂ nor 100 nM HgCl₂ affected FL transport, although higher concentrations clearly reduced both cellular and luminal accumulation.

Signaling of Reduction in Mrp2-Mediated Efflux.

Taken together, the data for CdCl₂ and HgCl₂ effects on FL-MTX and FL transport indicate that at low concentrations, these compounds rapidly reduced Mrp2-mediated transport without disrupting overall cellular function. At higher concentrations, function was clearly altered. This is the same pattern of effects seen previously in killifish renal proximal tubules exposed to two other classes of nephrotoxics, radiocontrast agents and aminoglycoside antibiotics (Terlouw et al., 2001). With those chemicals, reduced Mrp2-mediated transport was caused by firing of a Ca²⁺-dependent signaling system that rapidly modulates Mrp2 function. Signaling in this system is initiated by Ca²⁺-dependent release of ET from the tubules; ET activates a basolateral ET_B receptor, which in turn activates PKC; PKC activation reduces transport mediated by Mrp2. To determine whether low concentrations of CdCl₂ and HgCl₂ reduced FL-MTX transport by triggering this signaling system, we measured the ability of pharmacological agents that block specific links in the signaling chain to attenuate the effects of 10 μM CdCl₂ and 100 nM HgCl₂ on FL-MTX transport. We previously demonstrated that each of the pharmacological agents by itself had no effects on FL-MTX transport (Masereeuw et al., 2000; Terlouw et al., 2001).

Figure 5 shows that (as with radiocontrast agents and aminoglycoside antibiotics) CdCl₂ effects on transport were prevented when tubules were pretreated with a Ca²⁺ channel blocker, nifedipine, a specific ET_B receptor antagonist, RES-701-1, or a PKC-selective inhibitor, BIM. Similarly, Fig. 6 shows that each of these agents also prevented the reduc-

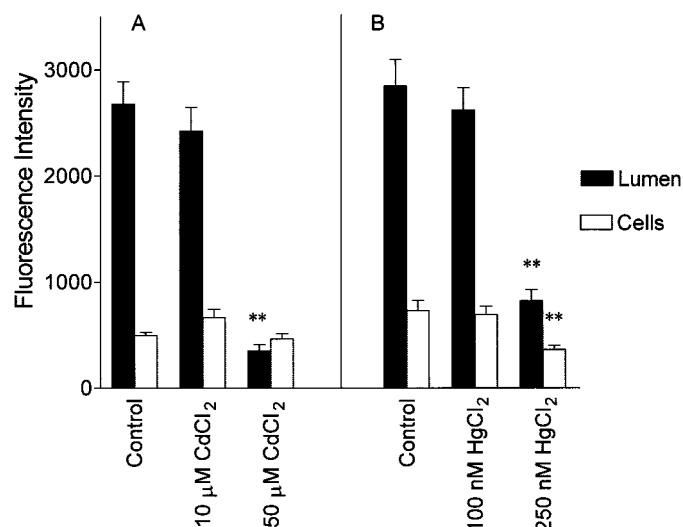


Fig. 4. Short-term effect of CdCl₂ (A) and HgCl₂ (B) on FL transport. Proximal tubules were incubated for 30 min in medium containing 1 μM FL without (control) or with the indicated additions. Data are given as mean ± S.E. for 10 to 14 tubules from a single fish. **, significantly lower than controls; $P < 0.01$.

tion in FL-MTX transport caused by 100 nM HgCl₂. For both 10 μM CdCl₂ and 100 nM HgCl₂, pretreatment of tubules with an ET_A receptor antagonist was without effect (data not shown). Also, the decrease in luminal fluorescence intensity seen after treating tubules with higher concentrations of HgCl₂ could not be prevented by the ET_B receptor antagonist or by the PKC-selective inhibitor (data not shown), indicating action through a signaling-independent mechanism, most likely disruption of cellular function.

Long-Term Exposure to CdCl₂. The present results indicate that acute exposure of killifish proximal tubules to heavy metals leads to a reduction in luminal accumulation of FL-MTX. However, recent reports have shown that longer term exposure to low levels of cytotoxic agents can cause an increase in expression of Mrp2 (Kauffmann and Schrenk, 1998; Demeule et al., 1999a,b). One of the advantages of using killifish tubules as a test system is that both morphology and function can be preserved in intact tubules for extended periods of time at reduced temperature (Maack and Kinter, 1969). Indeed, initial experiments demonstrated that transport of FL and FL-MTX transport (30-min uptake) in control tubules was not reduced when tubules were maintained for up to 2 days in a physiological saline (D. S. Miller, unpublished observations). Because tissue damage may occur during rewarming of the tissue, all treatments were accompanied by matched controls of the same batch of tubules. To determine whether low concentrations of CdCl₂ increased expression of Mrp2 in killifish tubules, we incubated tubules at 10°C for 6 h in medium with 0 (control), 0.5, or 1 μM CdCl₂ and assayed FL-MTX transport in 30-min experiments carried out at room temperature. Figure 7 shows that both concentrations of CdCl₂ significantly increased luminal fluorescence intensity, whereas cellular fluorescence remained the same; similar results were obtained with 24-h exposures (data not shown).

In agreement with previous reports (Masereeuw et al., 2000), control tubules immunostained for Mrp2 showed clear localization of the transporter to the luminal plasma membrane (Fig. 8, A and B). Exposure of tubules to 0.5 μM CdCl₂

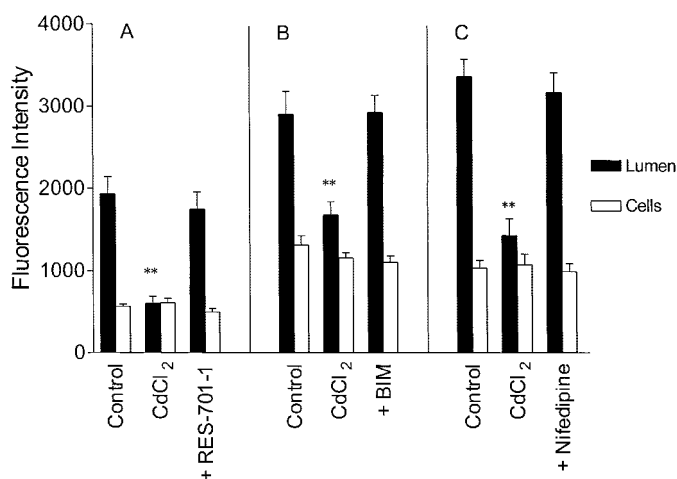


Fig. 5. Protection against short-term CdCl₂-induced reduction in FL-MTX transport by 100 nM concentrations of the ET_B-receptor antagonist, RES-701-1 (A), 1 μM concentrations of the PKC inhibitor, BIM (B), and 10 μM concentrations of the Ca²⁺ channel blocker, nifedipine (C). Tubules were incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with 10 μM CdCl₂ and the indicated additions. Data are given as mean ± S.E. for 11 to 14 tubules from a single fish. **, significantly lower than controls; *P* < 0.01.

for 6 h produced a small increase in staining intensity (Fig. 8, C and D). However, exposure to 1 μM CdCl₂ increased staining substantially (Fig. 8, E and F). Quantitative analysis of the images indicated a 46% increase in staining intensity with 0.5 μM CdCl₂ (*P* < 0.05) and a 132% increase in staining intensity with 1 μM CdCl₂ (*P* < 0.01). Luminal membrane fluorescence averaged 897 ± 109 units for control tubules, 1307 ± 138 units for tubules exposed to 0.5 μM CdCl₂, and 2087 ± 225 units for tubules exposed to 1 μM CdCl₂ (data from 10 tubules in each group). With these tubules, light microscopy showed no evidence of increased tubule damage or death after short- or long-term exposure to low concentrations of heavy metal salts. Thus, it is possible, but not likely,

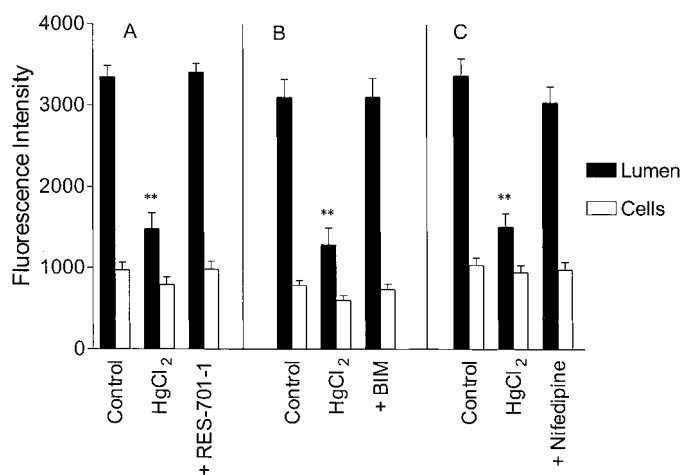


Fig. 6. Protection against short-term HgCl₂-induced reduction in FL-MTX transport by 100 nM concentrations of the ET_B-receptor antagonist, RES-701-1 (A), 1 μM concentrations of the PKC inhibitor, BIM (B), and 10 μM concentrations of the Ca²⁺ channel blocker, nifedipine (C). Tubules were incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with 100 nM HgCl₂ and the indicated additions. Data are given as mean ± S.E. for 10 to 13 tubules from a single fish. **, significantly lower than controls; *P* < 0.01.

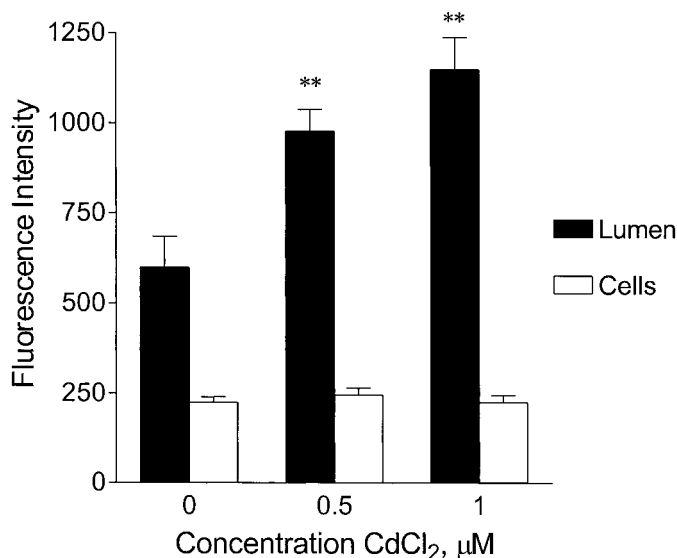


Fig. 7. Long-term stimulation of FL-MTX transport by CdCl₂. Tubules were pretreated for 6 h with normal medium (control) or medium with 0.5 or 1 μM CdCl₂ at 10°C. Proximal tubules were subsequently incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with the indicated additions. Data are given as mean ± S.E. for 10 to 12 tubules from a single fish. **, significantly higher than controls, *P* < 0.01.

that CdCl₂ exposure selected tubules with the greatest capacity to increase Mrp2 expression.

Discussion

The results of the present study show that, depending on exposure conditions, CdCl₂ and HgCl₂ can induce very different effects on killifish renal proximal tubules. First, exposure to low concentrations for short periods decreased Mrp2-mediated FL-MTX secretion. Importantly, these low concentrations of heavy metal salts did not appear to disrupt cellular function, as demonstrated by their lack of effect on FL secretion. Rather, they activated a specific receptor-mediated signaling pathway, since heavy metal action was abolished when signaling was blocked. This latter observation also rules out competitive inhibition of Mrp2 by metal-glutathione conjugates, which was suggested for Mrp1 (Ishikawa et al., 1996; Zalups, 2000) as the mechanism of cadmium and mercury action, since direct effects of conjugates on the transporter should be insensitive to altered cell signaling. Second, acute exposure to higher concentrations did appear to be toxic in that other energy-dependent transporters mediating FL secretion were affected and blocking signaling no longer protected FL-MTX transport. Finally, longer term exposure of tubules to even lower CdCl₂ concentrations increased both Mrp2-mediated FL-MTX transport and Mrp2 expression as measured by immunostaining. Since the first and last effects are of particular interest, they will be the focus of the present discussion.

We recently used isolated killifish renal proximal tubules to demonstrate that transport on luminal Mrp2 and P-glycoprotein rapidly decreases when ET-1, acting through a basolateral ET_B receptor, activates PKC (Masereeuw et al., 2000). At present, it is not known how PKC activation reduces transporter function. It is known, however, that several nephrotoxicants (aminoglycoside antibiotics and radiocontrast agents) trigger this signaling pathway by inducing ET

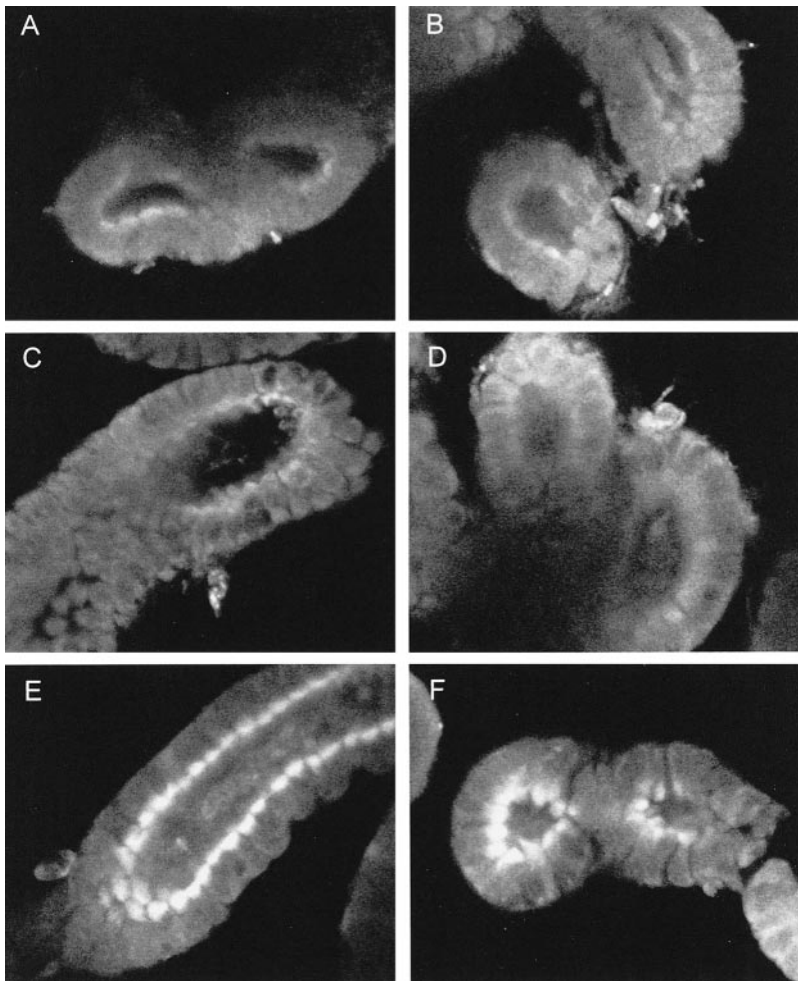


Fig. 8. Representative confocal micrographs of killifish tubules immunostained with anti-Mrp2 antibodies and a fluorescent secondary antibody after pretreatment with normal medium (A and B) or medium with 0.5 (C and D) or 1 μM (E and F) CdCl_2 for 6 h at 10°C.

release from the tubules. The hormone then acts by an auto-crine mechanism to signal decreased transport. Nephrotoxicant-induced ET release is Ca^{2+} -dependent in that increasing extracellular Ca^{2+} initiates signaling and nifedipine, a Ca^{2+} -channel blocker, abolishes nephrotoxicant-induced signaling (Terlouw et al., 2001).

In the present study, we have extended these findings to include two nephrotoxic heavy metals, cadmium and mercury. We show that exposing killifish tubules to 10 μM CdCl_2 or 100 nM HgCl_2 for 30 min activated the basolateral ET_B receptor and PKC. That is, cadmium and mercury reduced FL-MTX efflux, and this reduction was abolished by the ET_B receptor antagonist, RES-701-1, and by the PKC-selective inhibitor, BIM; neither of these pharmacological tools by itself affected transport. As with aminoglycoside antibiotics and radiocontrast agents, the acute effects of 10 μM CdCl_2 or 100 nM HgCl_2 on transport were also blocked by nifedipine, suggesting that Ca^{2+} also initiated cadmium and mercury action. Ca^{2+} has been previously implicated in the action of inorganic mercury and cadmium on renal cells. For example, Smith et al. (1987) showed that treatment of primary cultures of renal tubular cells with inorganic mercury increased the intracellular Ca^{2+} concentration. However, cellular uptake of cadmium appears to be in part through dihydropyridine-sensitive Ca^{2+} channels (Flanagan and Friedman, 1991; Friedman and Gesek, 1994; Souza et al., 1997), so it is

not clear to what extent nifedipine blocked cadmium entry in our tubules.

Together, the present and previous results (Masereeuw et al., 2000; Terlouw et al., 2001) have disclosed what appears to be a general mechanism by which low levels of nephrotoxics rapidly alter transport function of proximal tubule cells. An ET-signaled reduction in Mrp2-mediated transport could be the result of internalization of the transporter or a reduced intrinsic activity of Mrp2 caused by phosphorylation. Earlier attempts to unravel the mechanism of regulation failed, leaving the question still unanswered (Terlouw et al., 2001). Consistent with an early common step in nephrotoxicant action, recent experiments now implicate activation of nitric oxide synthase (NOS) and generation of NO in the action of aminoglycoside antibiotics, radiocontrast agents, and heavy metal salts (Notenboom et al., 2002). In these experiments, Mrp2-mediated transport was reduced when killifish tubules were exposed to sodium nitroprusside, a reagent that generates NO. ET-1 was shown to stimulate NO production by the tubules, and ET-1 action was blocked by a NOS inhibitor. Sodium nitroprusside action was blocked by PKC-selective inhibitors but not by an ET_B receptor antagonist. Thus, ET signaling to Mrp2 involved the following sequence: activation of the ET_B receptor, NOS, and then PKC. Significantly, aminoglycoside antibiotics, radiocontrast agents, and heavy metal salts also stimulated NO production by the

tubules, and nephrotoxicant-induced reductions in Mrp2-mediated transport were blocked when NOS was inhibited (Notenboom et al., 2002).

Activation of NOS and generation of NO by the tubules provides a pathophysiological context within which to view the present results. Proximal tubules express several NOS isoforms, and NO production is known to increase upon exposure to LPS, cytokines, hypoxia, and several nephrotoxic chemicals (Liang and Knox, 1999). Moreover, both ET signaling and NO production have been implicated in HgCl₂-induced acute renal failure (Yanagisawa et al., 1998). Thus, nephrotoxicants, acting through rapid, ET-based signaling, have the potential to impair tubular function in two ways: through the generation of NO and subsequent formation of reactive oxygen species and through decreased function of xenobiotic export pumps. Additional research is needed to determine to what extent NO generation and decreased transporter function lead to further tubular dysfunction.

In contrast to the reduction in Mrp2-mediated transport seen after short-term exposure to CdCl₂, exposing proximal tubules to lower levels of cadmium for longer periods increased transport of FL-MTX across the luminal membrane. This increase was accompanied by a significant increase in Mrp2 immunostaining at the luminal membrane, suggesting increased Mrp2 expression. At present, it is not clear at what level Mrp2 expression is regulated in these proximal tubules. Regulation could be either at the level of gene expression or the result of an induced exocytic insertion of Mrp2 into the luminal membrane. In support of transcriptional regulation, increased levels of Mrp2 mRNA in kidney were found after exposure to several different toxic compounds and in chronic renal failure (Kauffmann et al., 1997; Kauffmann and Schrenk, 1998; Laouari et al., 2001). Different regions of the 5'-flanking region of the Mrp2 gene responsible for induction of rat gene expression were identified, containing several putative binding sites for transcription factors (Kauffmann and Schrenk, 1998). One of the transcription factors thought to be involved in the regulation of the multidrug resistance 1 (*mdr1*) gene is nuclear factor- κ B (Zhou and Kuo, 1997; Thévenod et al., 2000). Nuclear factor- κ B is a transcription factor composed of members of the Rel family and is implicated in the expression of several inducible genes. This pathway may also be involved in the regulation of the gene encoding Mrp2. Another family of oxidative stress-responsive transcription factors possibly involved in the up-regulation of Mrp2 is activator protein-1. Laouari et al. (2001) showed that the expression of activator protein-1 was stimulated in rats with chronic renal failure in parallel with an induction of Mrp2. More research on the mechanism of Mrp2 regulation after long-term exposure to toxic compounds is evidently needed.

Mrp2 and P-glycoprotein perform important excretory and barrier functions, protecting cells, tissues, and organisms from potentially toxic chemicals. However, additional functions have been proposed for P-glycoprotein, including the protection of cells against a range of different stimuli inducing caspase-dependent cell death (Johnstone et al., 2000). In this regard, a recent study demonstrated that the up-regulation of P-glycoprotein in response to cadmium exposure decreased apoptosis in cultured proximal tubule cells (Thévenod et al., 2000). A similar protective role has not yet been demonstrated for Mrp2. However, the present study and others have shown that Mrp2 is inducible by toxic stimuli

(Kauffmann et al., 1997; Kauffmann and Schrenk, 1998; Laouari et al., 2001), suggesting a protective role for Mrp2. If this were the case, the reduction in Mrp2-mediated transport seen after short-term exposure to nephrotoxicants could make proximal tubules more susceptible to damage by harmful compounds and the use of ET_B-receptor antagonists immediately after exposure to nephrotoxicants could prevent some of their early detrimental effects.

In conclusion, the present study shows that short-term exposure to low salt concentrations of the heavy metals, cadmium and mercury, triggers the autocrine/paracrine ET-PCSK signaling cascade. Exposure to higher concentrations of the metal salts for short times results in toxicity. However, long-term exposures to low concentrations of CdCl₂ resulted in a concentration-dependent increase in Mrp2 function. Immunostaining with antibodies against Mrp2 demonstrated that the elevation in function was at least partly due to an increased amount of transporters in the luminal membrane.

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