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Nephrotoxicants Induce Endothelin Release and Signaling in Renal Proximal Tubules: Effect on Drug Efflux

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ABSTRACT

We previously used killifish proximal tubules, fluorescent substrates, and confocal microscopy to demonstrate that transport mediated by the multidrug resistance protein (Mrp2) and by P-glycoprotein was reduced by nanomolar concentrations of endothelin-1 (ET), acting through a basolateral B-type ET receptor and protein kinase C (PKC). Here we show that representatives of two classes of nephrotoxicants decrease transport by activating the endothelin-PKC signaling pathway. Exposing tubules to radiocontrast agents (iohexol, diatrizoate) or aminoglycoside antibiotics (gentamicin, amikacin) reduced Mrp2-mediated fluorescein methotrexate (FL-MTX) transport from cell to tubular lumen. Pretreating the tubules with an ET_B-receptor antagonist or with PKC-selective inhibitors abolished these effects. The nephrotoxicants activated signaling by inducing release of ET from the tubules, because adding of an antibody against ET to the medium abolished the effects. Elevating medium Ca^{2+} also reduced FL-MTX transport; this reduction was abolished when tubules were pretreated with an ET antibody, an ET_B-receptor antagonist, PKC-selective inhibitors, or the Ca^{2+} channel blocker, nifedipine. None of these drugs by themselves affected FL-MTX transport. Importantly, nifedipine also blocked the ET_B-receptor/PKC-dependent reduction in FL-MTX transport caused by gentamicin and diatrizoate. These results for two classes of structurally unrelated nephrotoxicants suggest that Ca^{2+}-dependent ET release and subsequent action through an autocrine mechanism may be an early response to tubular injury.

Endothelins (ETs) are polypeptide hormones that are potent vasoconstrictors. ET isoforms (ET-1, ET-2, and ET-3) are synthesized in many tissues and can affect the function of vascular and nonvascular tissues by interacting with two pharmacologically distinct, G protein-coupled receptors, ET_A and ET_B. ETs have been implicated in diseases involving the vasculature, such as acute myocardial infarction, atherosclerosis, congestive heart failure, and hypertension (Rubanyi and Polokoff, 1994; Hocher et al., 1997). In the kidney, ET regulates blood flow, glomerular hemodynamics, and sodium and water homeostasis (Rubanyi and Polokoff, 1994) but also plays a role in a number of renal diseases, including acute and chronic renal failure, renal glomerular and interstitial fibrosis, diabetic nephropathy, vascular rejection of the transplanted kidney, reperfusion injury, and nephrotoxicity induced by a variety of chemicals (e.g., cyclosporin A, cisplatin, and radiocontrast agents) (Clavell and Burnett, Jr. 1994; Rubanyi and Polokoff, 1994; Bruzzi et al., 1997; Hocher et al., 1997; Haug et al., 1998). Urinary ET-1 excretion increases in chronic renal failure from a variety of causes, including radiocontrast nephropathy and during cyclosporin and cisplatin administration (Bruzzi et al., 1997; Hocher et al., 1997). Under pathophysiological conditions, the ET receptor density in kidney changes dramatically, especially that of the ET_B receptor (Hocher et al., 1997). Finally, ET receptor antagonists have been used in animal models of acute renal failure to limit the effects of nephrotoxicants and of reperfusion injury (Bird et al., 1996; Krause et al., 1997).

Clearly, disruption of vascular function is an important element of the role of ET in renal disease. However, recent studies provide evidence for direct tubular effects of ET. For example, in renal proximal tubule, ET regulates Na^+/H^+ exchange, Na^+-HCO_3^- cotransport and fluid absorption (Garcia and Garvin, 1994; Guntupalli and DuBose, 1994). Moreover, an increase in ET production was observed in renal proximal tubules after exposure to cyclosporin A, mercury, high-molecular-weight proteins, and hypoxia, suggesting that these nephrotoxicants can act through ET to alter tubular function (Zoja et al., 1995; Bruzzi et al., 1997; Haug et al., 1998; Yanagisawa et al., 1998).

Recently, Masereeuw et al. (2000) demonstrated another

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ABBREVIATIONS: ET, endothelin; Mrp2, multidrug resistance protein 2; PKC, protein kinase C; FL, fluorescein; MTX, methotrexate; PMA, phorbol 12-myristate 13-acetate; BIM, bis-indolylmaleimide I.

tubular function of ET. Using isolated killifish proximal tubules and confocal microscopy, we found that ET acted through a basolateral ET_B receptor and protein kinase C (PKC) to regulate two luminal, ATP-driven xenobiotic transporters, P-glycoprotein, and multidrug resistance protein 2 (Mrp2). We also showed that when isolated tubules were exposed to the nephrotoxic radiocontrast agent iohexol, transport through P-glycoprotein and Mrp2 was reduced. Transport was restored, however, when tubules were pretreated with an ET_B-receptor antagonist, suggesting that iohexol had fired the ET signaling system and that such signaling could be an early event in tubular toxicity. In the present study, we use changes in Mrp2-mediated drug secretion to establish the generality of the finding and to begin to characterize the pathophysiological mechanisms involved. We demonstrate that representatives of two classes of nephrotoxicants caused ET release from the tubules followed by autocrine activation of ET signaling. Ca^{2+} influx seems to be one stimulus for ET release.

Materials and Methods

Chemicals. FL-MTX and phorbol 12-myristate 13-acetate (PMA) were purchased from Molecular Probes (Eugene, OR). The ET_A receptor antagonist (JKC-301) and ET_B receptor antagonist (RES-701-1) were obtained from Peninsula Laboratories (Belmont, CA). Bis-indolylmaleimide I (BIM), nifedipine, diatrizoate, amikacin, gentamicin, and monoclonal anti-ET-1 IgG (mouse-derived) were purchased from Sigma Chemical Co. (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 Lab, Inc. (Gaithersburg, MD). Iohexol was obtained from Nycomed (Oslo, Norway). All other chemicals were obtained from commercial sources at the highest purity available.

Animals and Tissue Preparation. Killifish (*Fundulus heteroclitus*) were collected by local fishermen 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 $CaCl_2$, 1.0 mM $MgCl_2$ and 20 mM Tris at pH 8.0. All experiments were carried out at 18 to 20°C. Under a dissecting microscope, each mass was teased with fine forceps to remove adherent hematopoietic tissue. Individual killifish proximal tubules were dissected 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- $\times$ 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 until steady state was reached for FL-MTX. Analysis of tubule extracts by high-performance liquid chromatography showed no metabolic degradation of FL-MTX when incubated with killifish proximal tubules for periods of at least 1 h (Schramm et al., 1995; Masereeuw et al., 1996).

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$\times$ water immersion objective (NA 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. In previous studies, it has been shown that there is a linear relationship between fluorescence intensity and dye concentration (Miller and Pritchard, 1991). However, because of the many uncertainties in relating cellular fluorescence to actual compound concentration in cells and tissues with complex geometry, data are reported here as average measured pixel intensity rather than estimated dye concentration. Fluorescence intensities were measured from stored images using National Institutes of Health Image 1.61 software as described previously in great detail (Masereeuw et al., 1996; Miller 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 the means of all the selected areas. For immunohistochemistry with anti-Mrp2 antibodies and a fluorescent secondary antibody, killifish renal tubules were treated as described previously (Masereeuw et al., 2000).

Data Analysis. Data are given as means $\pm$ S.E. Mean values were considered to be significantly different when $P < 0.05$ by use of the appropriate paired or unpaired t test, or by a one-way ANOVA followed by Bonferroni's multiple comparison test.

Results

Nephrotoxicants and Mrp2-Mediated Transport. The present experiments were conducted using isolated renal proximal tubules from a teleost fish, the killifish. This has proven a powerful model for the study of secretory transport in an intact proximal tubule. As discussed previously (Miller, 1987; Pritchard and Miller, 1991), renal tissue from certain marine teleosts offers several important advantages for the study of secretory transport in proximal tubule. Teleost kidneys contain a high proportion of proximal tubules that are easily isolated and that remain viable for long periods. When tubules are isolated, broken ends rapidly reseal to form a closed, fluid-filled, luminal compartment that communicates only with the medium through the tubular epithelium. Thus, this tissue has the appropriate geometry for the study of transepithelial secretion in intact tubules. Moreover, secretory transport mechanisms found in teleost tubules seem to be identical to those found in mammalian proximal tubules (Pritchard and Miller, 1991; Masereeuw et al., 1996; Miller et al., 1996). Finally, when teleost tubules are used along with fluorescent substrates and quantitative fluorescence microscopy, the mechanisms driving both uptake by the cells and secretion into the tubular lumen can be examined (Masereeuw et al., 1996; Miller et al., 1996).

Figure 1, A and B, show confocal images of control tubules after 30-min (steady-state) incubation in medium with 1 μM FL-MTX. The fluorescence distribution pattern is the same as shown previously (i.e., fluorescence intensity in lumen>cells>medium). 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 (for data on substrate and inhibitor specificities as well as immunostaining with Mrp2 antibodies, see Masereeuw et al., 2000). Figure 1, C–F, shows confocal images of tubules incubated in

media with FL-MTX and representatives of two classes of nephrotoxicants: the aminoglycosides, amikacin and gentamicin, and the radiocontrast agents, diatrizoate and iohexol. All nephrotoxic agents tested reduced the luminal accumulation of FL-MTX but had minimal effects on cellular accumulation. This inhibition pattern is the same as that seen with specific inhibitors of Mrp2, such as the competitor leukotriene C_4. As shown previously (Masereeuw et al., 1996, 2000), the absence of an increase in cellular fluorescence when luminal transport was blocked indicates that efflux into the lumen is not an important determinant of steady-state cellular FL-MTX accumulation. The steady-state cellular levels of FL-MTX seem to set independently of events at the luminal membrane.

To elucidate the mechanism by which these compounds reduced Mrp2-mediated transport, we measured steady-state cellular and luminal accumulation of FL-MTX (average cellular and luminal fluorescence intensities) in tubules exposed to the nephrotoxicants without and with pharmacological agents that act at specific cellular sites. Initially, we determined the concentration-dependence of inhibition of transport for each of the nephrotoxic compounds. Figure 2 shows data for the aminoglycoside antibiotic, gentamicin, which at 1 to 100 μM reduced luminal fluorescence in a concentration-dependent manner; cellular fluorescence was not affected by gentamicin. Similar experiments were conducted with the other nephrotoxicants (not shown); for each, we selected for further study a concentration that caused about 50% reduction in luminal fluorescence. Table 1 presents the inhibition data for each of the compounds. As with gentamicin (above), only luminal fluorescence was reduced, no significant reductions in cellular fluorescence were found. Thus, all of these chemicals reduced Mrp2-mediated transport of FL-MTX.

ET Signaling. Previous experiments showed that the reduction of Mrp2-mediated transport caused by iohexol could be prevented by pretreating tubules with 100 nM concentrations of the ET_B receptor antagonist, RES-701-1. The ET_A receptor antagonist, JKC-301 was without effect and neither receptor antagonist by itself altered transport (Masereeuw et al., 2000). Figure 3A shows that 100 nM RES-701–1 was also protective when tubules were exposed to gentamicin. As with iohexol, JKC-301 had no such protective effect. In contrast, when Mrp2-mediated FL-MTX transport was reduced by leukotriene C_4, RES-701-1 was without effect, indicating that the receptor antagonist did not interact with the transporter (Fig. 3B). Results of experiments with all four nephrotoxic compounds are summarized in Table 2. In each case, pretreating the tubules with 100 nM RES-701-1 prevented the nephrotoxicant-induced decrease in luminal fluorescence. Although the reversal of RES-701-1 on the iohexol-treated tubules seems to be incomplete, the reversal was comparable with the control tubules and significantly higher than the iohexol treatment alone.

In killifish proximal tubules, ET acting through the ET_B receptor signals a decrease in Mrp2-mediated transport by activating PKC (Masereeuw et al., 2000). If the nephrotoxicants activated this signaling pathway, their effects should be abolished when PKC activation is prevented. To establish whether PKC activation is an intermediate step in the action of the nephrotoxic agents, we used a PKC-selective inhibitor, BIM. Although BIM by itself did not affect FL-MTX transport, for every nephrotoxicant, it did prevent the decrease in Mrp2-mediated FL-MTX transport (Fig. 3C and Table 2).

Together, these findings indicate that the four nephrotoxic compounds trigger a signaling pathway that involves the ET_B receptor and PKC activation. Because these compounds are so structurally diverse and so different from the natural ligands for the ET_B receptor, it is unlikely that the compounds themselves are receptor agonists. Rather, the data imply that nephrotoxicant exposure causes ET release from the tubules and that ET acts by an autocrine mechanism to fire the signaling pathway that ultimately reduces Mrp2-mediated transport. To test this directly, we pretreated tubules for 30 min with an excess of a monoclonal antibody against ET-1 (cross-reacts with ET-2 and ET-3) and then exposed tubules to gentamicin. Figure 4A shows that exposing tubules to the antibody alone did not affect FL-MTX transport, but that pretreating tubules with an ET antibody

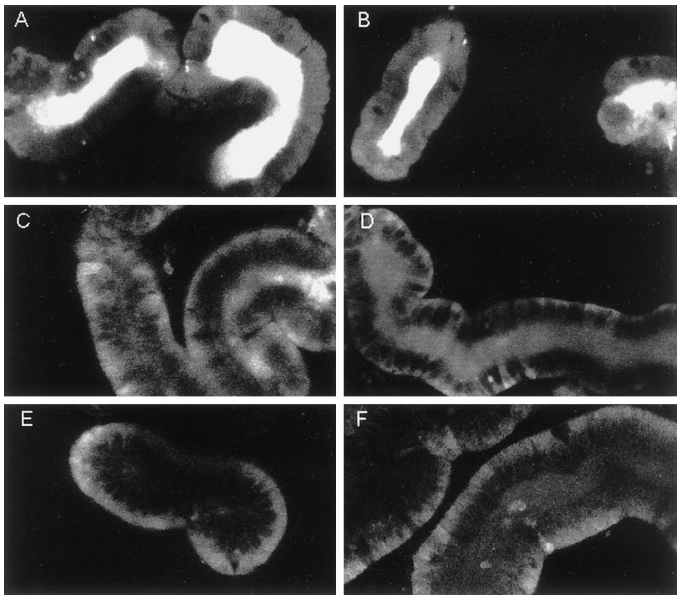

Fig. 1. Confocal images of killifish proximal tubules after incubation in medium with 1 μM FL-MTX for 30 min in absence or presence of nephrotoxicants. Controls (A and B), and in presence of 50 μM amikacin (C), 10 μM gentamicin (D), 100 μM diatrizoate (E), and 300 μM iohexol (F).

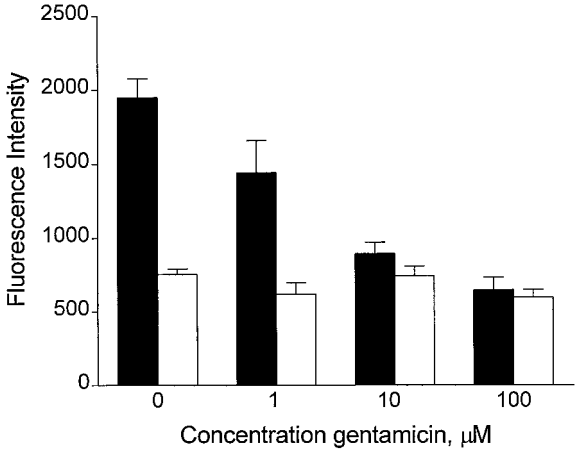

Fig. 2. Inhibition of FL-MTX transport by gentamicin. Tubules were incubated for 30 min in medium containing 1 μM FL-MTX and 0 to 100 μM gentamicin. Data are given as mean ± S.E. for 13 to 43 tubules from one to three fish. ■, lumen; □, cells.

prevented the decrease in transport induced by gentamicin. In contrast, pretreatment with a different, nonspecific, monoclonal antibody (a donkey-derived α-rabbit-HRP IgG), did not prevent the decrease in FL-MTX transport caused by gentamicin (Fig. 4B).

To study the mechanism by which the function of Mrp2 is regulated by ET, killifish tubules were preincubated for 30 min in the presence or absence of 10 nM ET-1 and were subsequently immunostained using antibodies to Mrp2. The control tubules showed a very bright luminal staining. However, the tubules treated with ET-1 showed the same pattern (Fig. 5). There was no difference in average fluorescence intensity or band thickness, suggesting that if ET signaled internalization of Mrp2, it was not detectable using confocal microscopy.

Involvement of Ca^{2+}. In the vascular endothelium, ET-1 release is signaled by an increase in intracellular Ca^{2+} (Tasaka and Kitazumi, 1994; Carlini et al., 1995; Marsen et al., 1996). Moreover, elevated intracellular Ca^{2+} is known to be one response of renal proximal tubule cells to acute injury (Humes, 1986; Smith et al., 1992; Rose et al., 1994). The Ca^{2+} concentration in the normal medium used for the present experiments was 1.5 mM. Increasing medium Ca^{2+} to 3 mM reduced secretion of FL-MTX by 60% (Fig. 6, A and B). Pretreating tubules with BIM or RES-701–1 abolished this reduction, indicating that the increase in medium Ca^{2+} affected a process that was upstream of both PKC and the ET_B receptor. To test whether increased medium Ca^{2+} induced ET release, tubules were pretreated with the monoclonal antibody against ET-1 (above) and then exposed to 3 mM Ca^{2+}. Figure 6C shows that exposure to the antibody prevented the Ca^{2+}-induced decrease in FL-MTX secretion.

The obvious experiment at this point was to determine whether nephrotoxin effects on FL-MTX transport could be seen when tubules were maintained in Ca^{2+}-free medium. Our attempts to do this were unsuccessful, because in the absence of nephrotoxicants, luminal accumulation of FL-MTX was nearly abolished when Ca^{2+} was removed from the medium. We suspect that, as suggested previously (Kottra and Fromter, 1983), Ca^{2+} depletion opened the tight junctions, resulting in increased leakiness of the tubule and run down of lumen-to-bath concentration gradients.

From these results, it was not clear how the increase in medium Ca^{2+} was causing ET release from the tubules. One possibility was that raising extracellular Ca^{2+} increased influx, which in turn increased intracellular free Ca^{2+}. We attempted to reduce Ca^{2+} influx using nifedipine, a drug that blocks Ca^{2+} channels (Matsunaga et al., 1994). By itself, 10 μM nifedipine did not significantly affect FL-MTX transport (Table 2). However, pretreating tubules with nifedipine did prevent the decrease in FL-MTX secretion caused by increased medium Ca^{2+} (Fig. 7A and Table 2). To determine whether the inhibition of FL-MTX secretion by the various nephrotoxic agents was caused by a Ca^{2+}-dependent mechanism, tubules were exposed to gentamicin or diatrizoate in presence or absence of nifedipine. Again, the nephrotoxicant-induced reduction in luminal FL-MTX accumulation was abolished by nifedipine (Fig. 7B; Table 2).

A recent report suggests that, in addition to blocking Ca^{2+} entry, nifedipine can also inhibit PKC (Hempel et al., 1999). If such inhibition occurred in our renal tubules, it would have acted downstream to block the effects of elevated medium Ca^{2+} and the nephrotoxicants. This is not the case. We previously demonstrated that activation of PKC with phorbol ester reduces FL-MTX secretion in killifish tubules (Masereeuw et al., 2000). Our additional experiments show that exposing tubules to 10 μM nifedipine did not alter the effects of 10 to 100 nM PMA on FL-MTX transport. For example, in one experiment with 14 tubules per treatment, fluorescence intensities in lumens and cells of control tubules averaged 2700 $\pm$ 190 and 740 $\pm$ 40 U, respectively; corresponding values for tubules exposed to 10 nM PMA were 1000 $\pm$ 130 and 600 $\pm$ 50 U; corresponding values for tubules exposed to 10 nM PMA plus 10 μM nifedipine were 870 $\pm$ 130 and 640 $\pm$ 30 U. Thus, in the experiments presented here, nifedipine did not inhibit PKC.

Cellular Energy Metabolism. It is clear from the above that short exposures to nephrotoxic compounds as well as increased medium Ca^{2+} results in a reduction in transport mediated by Mrp2. It is not clear from these experiments whether those reductions result from specific regulation of transporter function or from toxicity (e.g., reduced ATP levels). Previous experiments with killifish proximal tubules showed that Mrp2 was not the only transporter under PKC control. Activation of PKC also reduced transport by P-glycoprotein at the luminal membrane (Miller et al., 1998) and by an organic anion transporter at the basolateral membrane (Miller, 1998). The latter transporter is part of the classic Na^+-dependent and ouabain-sensitive organic anion system that handles small organic anions [e.g., *p*-aminohippurate and fluorescein (Pritchard and Miller, 1993)]. FL secretion mediated by this transporter is particularly sensitive to changes in cellular metabolism and ion gradients (Miller and Pritchard, 1991; Miller et al., 1993). However, unlike FL-MTX transport (Masereeuw et al., 2000), FL transport was not affected by 0.5 to 1 nM ET-1 (Fig. 8A). Our unpublished data indicate that this transporter is regulated by parathyroid hormone rather than ET. Thus, FL transport provides a

TABLE 1

Effect of various nephrotoxic agents on the transport of 1 μM FL-MTX from cell to tubular lumen in killifish proximal tubules

Luminal and cellular fluorescence intensities of control tubules and tubules exposed to various nephrotoxicants. All four nephrotoxic chemicals significantly reduced luminal FL-MTX accumulation ($P < 0.01$), whereas cellular accumulation was not affected. Values are expressed as mean $\pm$ S.E., $N = 24$ to 45 from two to three fish.

	Nephrotoxicant			
	–		+	
	Lumen	Cells	Lumen	Cells
Amikacin (50 μM)	2200 $\pm$ 200	500 $\pm$ 70	1100 $\pm$ 190	640 $\pm$ 80
Gentamicin (10 μM)	2600 $\pm$ 130	790 $\pm$ 60	1500 $\pm$ 110	780 $\pm$ 50
Diatrizoate (100 μM)	2000 $\pm$ 130	410 $\pm$ 50	740 $\pm$ 90	440 $\pm$ 60
Iohexol (300 μM)	3000 $\pm$ 240	820 $\pm$ 70	2100 $\pm$ 270	880 $\pm$ 100

sensitive but ET-independent tool to monitor toxic effects. Figure 8B shows that at concentrations that substantially reduced FL-MTX secretion, neither gentamicin nor diatrizoate reduced transport of 1 μM FL. In contrast, when nephrotoxicant concentrations were raised 5 to 10 fold, all of the treatments did reduce FL secretion (data not shown), suggesting toxicity with acute exposure to higher levels. These data indicate that the lower concentrations of nephrotoxicants used in FL-MTX experiments did not disrupt cellular energy metabolism. Thus, the observed nephrotoxicant-induced reductions in FL-MTX transport resulted from altered transporter function.

Discussion

The renal proximal tubule is both the segment of the nephron responsible for the active excretion of toxic chemicals, and an important target tissue for many of those same chemicals. Thus, it should not be surprising to find a convergence of these aspects of proximal tubule function and dysfunction. Using a comparative model and confocal microscopy, we recently demonstrated that two ATP-driven xenobiotic efflux pumps on the luminal membrane of proximal tubule epithelial cells, P-glycoprotein and Mrp2, were under the short-term control of ET acting through a basolateral ET_B receptor and PKC (Masereeuw et al., 2000). Moreover, we also found that the nephrotoxic radiocontrast agent, iohexol, decreased transport mediated by Mrp2 and P-glycoprotein by firing this signaling system. This observation provided a pathophysiological context in which to view regulation of drug export ATPases by ET, a hormone that has been implicated in a number of nephropathies.

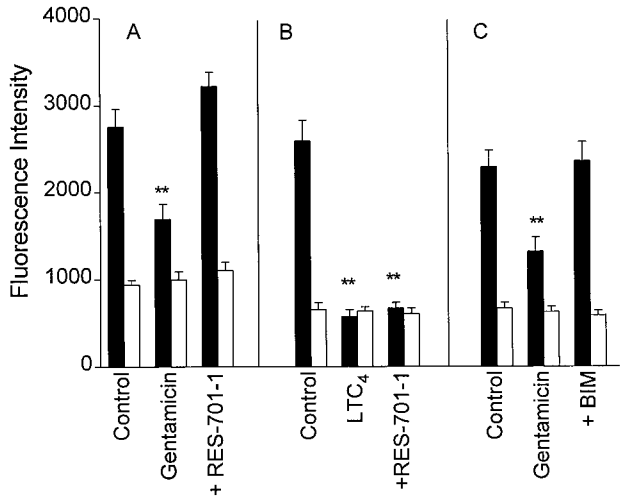

Fig. 3. Effect of the ET_B receptor antagonist, RES-701-1 (100 nM) on the gentamicin-induced (A) and leukotriene C_4-induced (B) reduction in FL-MTX transport. C, effect of 1 μM BIM (PKC inhibitor) on FL-MTX transport in gentamicin-treated tubules. Tubules were incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with the indicated additions. Data are given as mean ± S.E. for 10 to 18 tubules from a single fish. **, Significantly lower than controls, $P < 0.01$. ■, lumen; □, cells.

In the present study, we found that representatives of two classes of nephrotoxic compounds decreased Mrp2-mediated transport by activating ET_B receptor/PKC signaling. That is, each of the four nephrotoxicants tested reduced Mrp2-mediated FL-MTX secretion into the tubular lumen and this reduction in transport was abolished when an ET_B receptor-specific antagonist or a PKC-selective inhibitor blocked signaling. Figure 9 gives an overview of this sequence of events. Note that nephrotoxicant-induced signaling altered transporter function rather than energy supply to the transporter. Neither gentamicin nor diatrizoate affected secretion of FL at concentrations that clearly reduced FL-MTX transport. FL secretion is mediated by the classic, Na^+-dependent organic anion system, which is particularly sensitive to changes in cellular energy metabolism (Miller and Pritchard, 1991; Miller et al., 1993).

Given the wide range of chemical structures involved, it is unlikely that the four nephrotoxic compounds interacted directly with the ET_B receptor. Rather, they seemed to induce ET release from the tubules, with the hormone subsequently acting by an autocrine mechanism to trigger the pathway. In support of this supposition, the ability of the aminoglycoside antibiotic, gentamicin, to reduce Mrp2-mediated transport was abolished when tubules were pretreated with an antibody against ET. Another monoclonal antibody, which does not bind ET, had no such effect. Thus, the tubules released ET in response to gentamicin. Because an activation of the ET_B receptor was shown for the other three nephrotoxic agents as well, we speculate that this activation is a result of a release of ET by the tubules. Experiments in the presence of an antibody against ET should resolve this issue.

Released ET seems to have acted locally. In the experiments, each chamber contained micrograms of tubules in 1.5 ml of medium. The threshold for ET-1 action on FL-MTX transport in these tubules is about 500 pM (Masereeuw et al., 2000). Because the amount of hormone released from the tubules would be small and the overall dilution factor great, the average concentration of hormone in the bulk solution must have been well below this threshold. However, after ET release, concentrations in the vicinity of the tubules could

TABLE 2

Reversal of the effect of various nephrotoxic agents on the transport of 1 μM FL-MTX from cell to tubular lumen in killifish proximal tubules

Data are expressed as percentage of FL-MTX transported compared with control tubules. The PKC-selective inhibitor BIM (1 μM), the ET_B receptor antagonist RES-701-1 (100 nM), and the Ca^{2+} channel blocker nifedipine (10 μM) significantly reduced the inhibition mediated by the various nephrotoxicants ($P < .01$). Data are given as mean ± S.E., N = 10 to 45 from one to three fish.

	Nephrotoxicant	Nephrotoxicant		
		+ BIM	+ RES-701-1	+ Nifedipine
		%		
Control		110 ± 7	90 ± 8	100 ± 6
Amikacin (50 μM)	50 ± 8	110 ± 6	80 ± 13	N.D.
Gentamicin (10 μM)	60 ± 4	110 ± 7	120 ± 6	100 ± 5
Diatrizoate (100 μM)	40 ± 4	90 ± 8	90 ± 15	100 ± 8
Iohexol (300 μM)	70 ± 9	100 ± 7	80 ± 11	N.D.

N.D., not determined.

have easily exceeded the threshold and provided sufficient hormone at the basolateral membrane to activate signaling through the receptor.

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. A decrease in the luminal amount of Mrp2 could not be detected using confocal microscopy. However, the resolution of the confocal microscope (about 0.25 μm) could be insufficient to measure redistribution of the transporter, leaving the question of the regulatory mechanism unanswered. Evidently, more research on this subject is needed.

As in endothelial cells (Tasaka and Kitazumi, 1994; Carlini et al., 1995; Marsen et al., 1996), ET release from proximal tubules seemed to be Ca^{2+}-dependent. Increasing medium Ca^{2+} decreased FL-MTX secretion and this decrease was abolished when tubules were pretreated with a PKC-selective inhibitor, an ET_B receptor antagonist, or the anti-ET IgG (present study). Thus, ET release and activation through its receptor and PKC was triggered by elevated extracellular Ca^{2+}. Consistent with increased Ca^{2+} influx being one stimulus for ET release, the L-type Ca^{2+} channel blocker, nifedipine, abolished the reduction in FL-MTX transport seen in response to elevated Ca^{2+} and to gentamicin and diatrizoate. Influx of Ca^{2+} by other channels than the L-type cannot be excluded, because relatively high concentrations of nifedipine were used. Moreover, the exact localization of the Ca^{2+} channel is still controversial (Zhang and O'Neil, 1996); however, O'Neil et al. (1997) showed the expression of high-affinity dihydropyridine receptors at the apical as well as the basolateral membrane. These binding sites seem to be part of

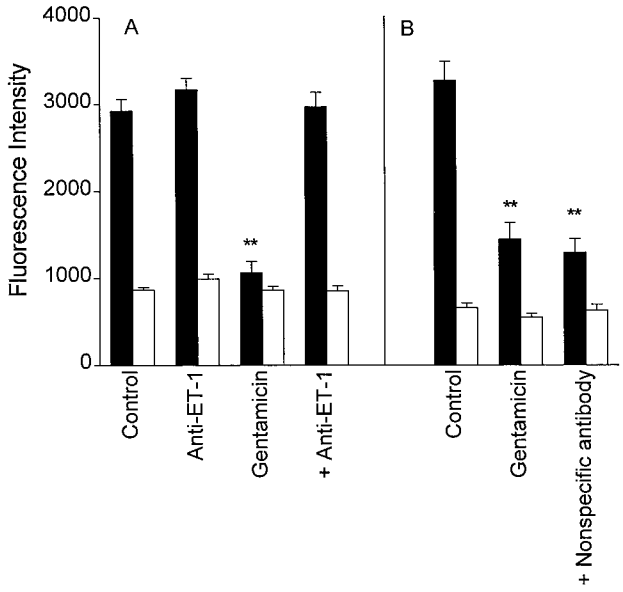

Fig. 4. Effect of 10 μM gentamicin (B) on FL-MTX transport and reversal by 1% (v/v) antibody against ET-1 (A), but not by a monoclonal antibody not specific for ET (B). Tubules were pretreated without (control) or with the antibody for 30 min and were subsequently incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with 10 μM gentamicin or gentamicin plus the antibody. Data are given as mean ± S.E. for 14 to 15 tubules from a single fish. **Significantly lower than controls, $P < 0.01$. ■, lumen; □, cells.

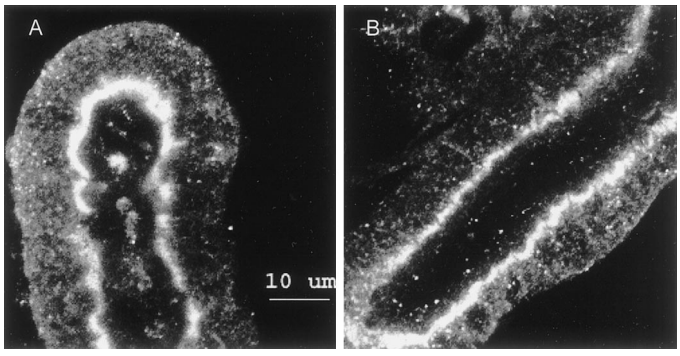

Fig. 5. Confocal micrographs of killifish tubules immunostained with anti-Mrp2 antibodies and a fluorescent secondary antibody in the absence (A) and presence of 10 nM ET-1 for 30 min (B).

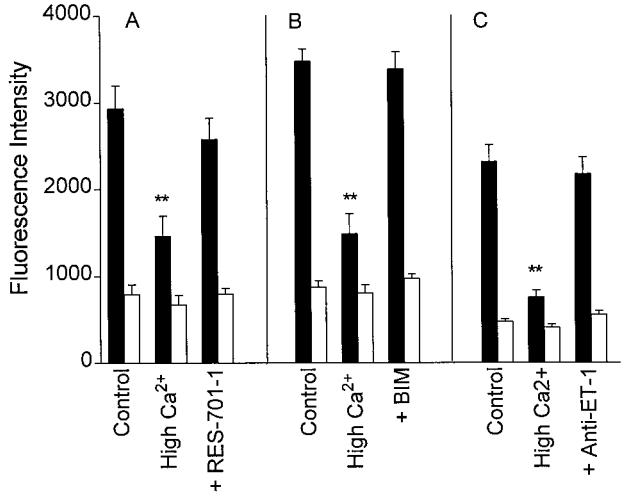

Fig. 6. Protection against Ca^{2+}-induced reduction in FL-MTX transport by the ET_B receptor antagonist RES-701-1 (A), by 1 μM BIM (B), and by 1% (v/v) antibody against ET-1 (C). Tubules were pretreated for 30 min with normal medium (control, 1.5 mM Ca^{2+}) or medium with 3 mM Ca^{2+} (in the presence or absence of the antibody, 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 11 to 15 tubules from a single fish. **, Significantly lower than controls, $P < 0.01$. ■, lumen; □, cells.

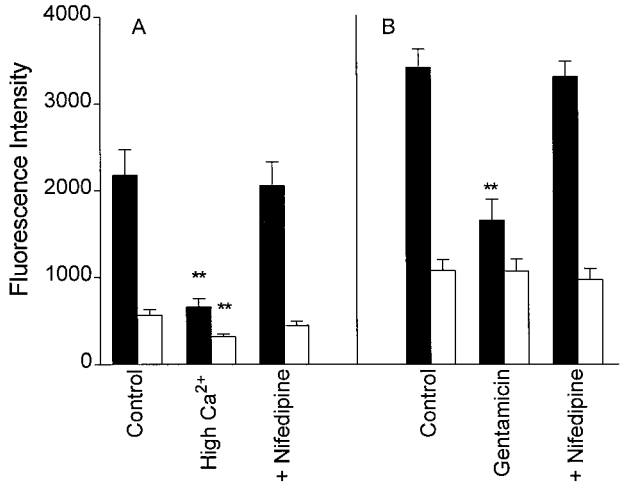

Fig. 7. Protection against Ca^{2+}-induced (A) and gentamicin-induced (B) reduction in FL-MTX transport by 10 μM nifedipine. A, tubules were pretreated for 30 min with normal medium (control with 1.5 mM Ca^{2+}) or medium with 3 mM Ca^{2+}. Tubules were subsequently incubated for 30 min in medium containing 1 μM FL-MTX without (control) or with the indicated additions. B, tubules were incubated for 30 min without (control) or with 10 μM gentamicin or gentamicin plus 10 μM nifedipine. Data are given as mean ± S.E. for 14 to 17 tubules from a single fish. **, Significantly lower than controls, $P < 0.01$. ■, lumen; □, cells.

distinct Ca^{2+} channels, indicating that it is reasonable to assume that a nifedipine-sensitive Ca^{2+} channel is present at the basolateral membrane of proximal tubule cells. Experiments are currently in progress to determine the extent to which elevated extracellular Ca^{2+} and nephrotoxicants alter intracellular Ca^{2+} within the tubular epithelium.

When we first reported that the radiocontrast agent, iohexol, reduced transport mediated by Mrp2 and P-glycoprotein by activating the ET_B receptor/PKC signaling pathway, we questioned whether this phenomenon represented a protective mechanism (an attempt to conserve ATP, perhaps to maintain low intracellular Ca^{2+}) or an early event in nephrotoxicity (Masereeuw et al., 2000). With regard to the latter possibility, recent evidence suggests a role for P-glycoprotein in modulating cell death (reviewed in Johnstone et al., 2000). Multidrug resistant tumor cells overexpressing P-glycoprotein were protected from multiple forms of caspase-dependent apoptosis. However, protection was not extended to caspase-independent forms of cell lysis. Inhibition of P-glycoprotein, whether by an antibody or the drug verapamil, reduced resistance to apoptosis, indicating that overexpression of the transporter was not sufficient to protect; rather, the transporter had to be functional. Further evidence for a protective role of P-glycoprotein in renal cells comes from a recent study using a renal cell line where Thévenod et al. (1999) found that up-regulation of P-glycoprotein protected cells from cadmium- and reactive oxygen species-induced apoptosis. Given these recent findings for P-glycoprotein, our previous results for P-glycoprotein and Mrp2 (Masereeuw et al., 2000), and our present results for Mrp2, it is tempting to speculate that 1) both multidrug resistance transporters serve protective functions, and 2) a reduction in rate of Mrp2 transport could make proximal tubule cells more susceptible to damage by nephrotoxic compounds through an early loss of this protective mechanism.

Finally, taken together, the present data provide evidence for a new common mechanism by which nephrotoxic chemicals can disrupt proximal tubule function. Given the high incidence of nephrotoxicity associated with use of radiocontrast agents and aminoglycoside antibiotics, our results for a comparative model suggest that blockade of tubular ET_B receptors could be of value in the clinic. More research is evidently needed.

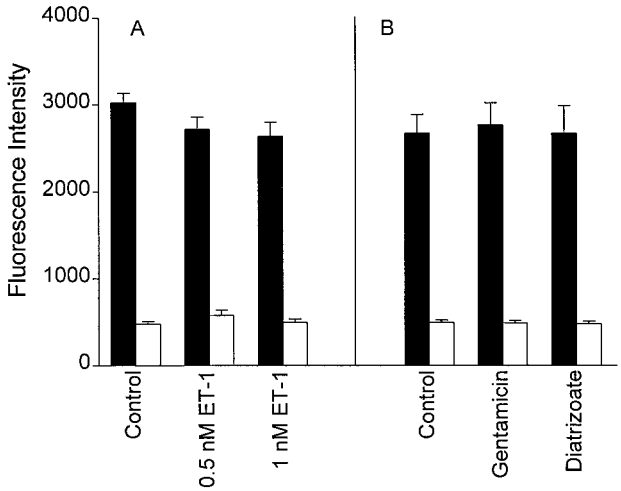

Fig. 8. Lack of effect of ET-1 (A) or the nephrotoxic agents gentamicin (10 μM) and diatrizoate (100 μM) on FL transport. Proximal tubules were incubated for 30 min in medium containing 1 μM FL without (control) or with the indicated additions. Data are given as mean ± S.E. for 11 to 33 tubules from one to three fish. ■, lumen; □, cells.

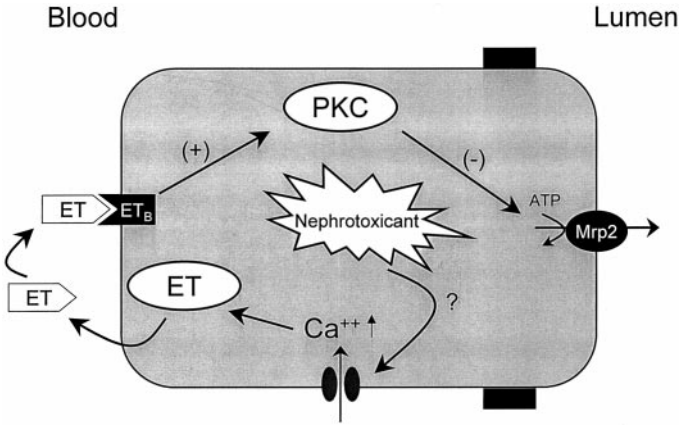

Fig. 9. Scheme illustrating the mechanism of nephrotoxicant-induced inhibition of transport by Mrp2. Various nephrotoxic agents cause an opening of Ca^{2+} channels. The resulting increase in intracellular Ca^{2+} concentration stimulates the release of ET, which subsequently activates the basolateral ET_B receptor. This leads to a reduction in Mrp2 transport activity through stimulation of PKC.

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