Role of NO in endothelin-regulated drug transport in the renal proximal tubule

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1 Department of Pharmacology and Toxicology, University Medical Center Nijmegen, 6500 HB Nijmegen, The Netherlands; 2 Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Science, National Institutes of Health, Research Triangle Park, North Carolina 27709; and 3 Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

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Notenboom, Sylvia, David S. Miller, Paul Smits, Frans G. M. Russel, and Rosalinde Masereeuw. Role of NO in endothelin-regulated drug transport in the renal proximal tubule. Am J Physiol Renal Physiol 282: F458–F464, 2002; 10.1152/ajprenal.00173.2001.—We previously demonstrated in intact killifish renal proximal tubules that endothelin (ET), acting through an ETB receptor and protein kinase C (PKC), reduced transport mediated by multidrug resistance-associated protein 2 (Mrp2), i.e., luminal accumulation of fluorescein methotrexate (FL-MTX) (Masereeuw R, Terlouw SA, Van Aubel RAMH, Russel FGM, and Miller DS. Mol Pharmacol 57: 59–67, 2000). In the present study, we used confocal microscopy and quantitative image analysis to measure Mrp2-mediated transport of FL-MTX in killifish tubules as an indicator of the status of this ET-fired, intracellular signaling pathway. Exposing tubules to sodium nitroprusside (SNP), a nitric oxide (NO) donor, signaled a reduction in luminal accumulation of FL-MTX, which suggested pathway activation. Nω-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, blocked the action of ET-1 on transport. Because SNP effects on transport were blocked by bisindoylmaleide, a PKC-selective inhibitor, but not by RES-701–1, an ETB-receptor antagonist, generation of NO occurred after ETB receptor signaling but before PKC activation. NO generation was implicated in the actions of several nephrotoxicants, i.e., diatrizoate, gentamicin, amikacin, HgCl2, and CdCl2, each of which decreased Mrp2-mediated transport by activating ET signaling. For each nephrotoxicant, decreased FL-MTX transport was prevented when tubules were exposed to L-NMMA. ET-1 and each nephrotoxicant stimulated NO production by the tubules, as determined by a fluorescence-based assay. Together, the data show that NO generation follows ET binding to the basolateral ETB receptor and that, in activating the ET-signaling pathway, nephrotoxicants produce NO, a molecule that could contribute to subsequent toxic effects.

multidrug resistance-associated protein 2; calcium; endothelin signaling; protein kinase C; xenobiotic transport; nitric oxide

METABOLISM AND EXCRETION provide a first line of defense against the wide variety of potentially toxic chemicals to which we are continually exposed. In the kidney, the proximal tubule is responsible for the excretory transport from blood to urine of xenobiotics, xenobiotic metabolites, and waste products of metabolism. As a result of its rich transport function, the proximal tubule is also an important target for toxic effects (2, 27).

To accomplish this excretory function, epithelial cells in the proximal tubule possess multiple plasma membrane transporters that use ATP and transmembrane ion gradients to drive active drug secretion into urine. Among the proteins implicated in this process is a member of the ATP-binding cassette superfamily of membrane transporters: multidrug resistance protein 2 (Mrp2). This transport protein is present at high levels in the luminal membrane of proximal tubule cells and handles a wide range of chemicals, from large, lipophilic organic anions to polypeptides (12, 22).

Using intact killifish (Fundulus heteroclitus) renal proximal tubules, a fluorescein methotrexate (FL-MTX) derivative, and confocal microscopy, we recently demonstrated that Mrp2 was regulated by endothelin (ET), which acted through a basolateral ETB receptor and protein kinase C (PKC) (13). By activating this signaling pathway, ET rapidly decreased cell-to-lumen transport of the fluorescent Mrp2 substrate FL-MTX. Interestingly, a similar decrease in transport was seen when tubules were exposed to several known nephrotoxic drugs (aminoglycoside antibiotics and radiocontrast agents), and that decrease was found to be caused by activation of ET-ETB-PKC signaling (13, 25). The nephrotoxicants caused release of ET from the tubules, and the hormone acted by an autocrine mechanism to signal a decrease in Mrp2-mediated transport. ET release appeared to be Ca2+ dependent in that elevated medium Ca2+ triggered the pathway, and the effects of nephrotoxicants and elevated medium Ca2+ were abolished by nifedipine, a Ca2+ channel blocker (25).

The precise sequence of events by which ET regulates Mrp2 transport is unclear. However, there is reason to believe that nitric oxide (NO) could be part of
this ET-signaling cascade in the kidney (18). NO is produced by NO synthase (NOS). Both NOS and ET were found to be localized to the same nephron segments, including the proximal tubule (16, 18). In the vascular wall, it is well documented that ET can trigger the endothelial release of NO by means of stimulation of the ETB receptors (4, 5, 26). Recent data suggest comparable cross talk between ET and NO in renal tubular cells (19, 20). In addition, NO has been implicated in cyclosporin A- and FK506-induced nephrotoxicity in renal proximal tubules (6).

In the present study, we used killifish renal proximal tubules to demonstrate that release of NO is an intermediate step in ET signaling. The data also show that ET-activated NO production is also an early event in the action of nephrotoxicants, thus providing a possible mechanistic link between signaling and toxicity.

METHODS

Chemicals. The chemicals included FL-MTX, bisindolylmaleide (BIM), Nω-monomethyl-L-arginine (L-NMMA), 4-amino-5-methylamino-2,7’-difuoro fluorescein (DAF-FM) diacetate (Molecular Probes, Eugene, OR); RES-701–1, an ETB-receptor antagonist (Peninsula Laboratories, Belmont, CA); sodium nitroprusside (SNP; Calbiochem, San Diego, CA); and HgCl2, CdCl2, gentamicin, amikacin, and diatrizoic acid (Sigma, St. Louis, MO). All other chemicals were obtained at the highest purity available.

Animals and tissue preparation. Killifish were collected by local fishermen in the vicinity of Mount Desert Island, ME, and maintained at the Mount Desert Island Biological Laboratory in tanks with natural flowing seawater. Renal tubular masses were isolated in a marine teleost saline, based on that of Forster and Taggart (3), containing (in mM) 140 NaCl, 2.5 KCl, 1.5 CaCl2, 1.0 MgCl2, and 20 Tris at pH 8.0. All experiments were carried out at 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 and transferred to a foil-covered Teflon chamber (Bionique) 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 tissue 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 killifish proximal tubules were incubated for periods of at least 1 h (12, 23).

Confocal microscopy. The chamber containing renal tubules was mounted on the stage of an Olympus Fluoview inverted confocal laser scanning microscope and viewed through a ×40 water-immersion objective (numerical aperture 1.15). Excitation was provided by the 488-nm line of an argon ion laser. A 510-nm dichroic filter and 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 1,500–3,000 (on a scale of 0–4,096), 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 or Zip disk (Iomega). In previous studies, it has been shown that there is a linear relationship between fluorescence intensity and dye concentration (15). 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 Scion image version 1.8 for Windows (12, 14). Briefly, two or three adjacent cellular and luminal areas were selected from each tubule, and the average pixel intensity for each area was calculated. The values used for that tubule were the means of all selected areas.

NO production by tubules was measured using an indicator, DAF-FM, that increases fluorescence intensity on exposure to NO. For the experiments, tubules were loaded for 1 h in medium containing 10 μM DAF-FM diacetate. This non-fluorescent derivative is membrane permeant and, on entering cells, is hydrolyzed to DAF-FM. After loading, tubules were transferred to confocal chambers containing medium without (control) and with effectors. After 5 min, confocal images were collected (as for FL-MTX) and saved to a Zip disk. Average tubule fluorescence was measured from the stored images as described above.

Data analysis. Values are given as means ± SE. Mean values were considered to be significantly different when P < 0.05, by use of the unpaired t-test, or by a one-way ANOVA followed by Bonferroni’s multiple comparison test. Software used for statistical analysis was GraphPad Prism (version 3.00 for Windows; GraphPad Software, San Diego, CA).

RESULTS

The present experiments were conducted using isolated renal proximal tubules from a marine teleost fish, the killifish. This has proven a powerful model for the study of secretory transport in an intact proximal tubule (21). As in mammalian proximal 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, that can be visualized and measured using confocal microscopy (12–14). Figure 1A shows a typical confocal image of a control killifish tubule after 30-min (steady-state) incubation in medium with 1 μM FL-MTX. The fluorescence distribution pattern is the same as that shown previously, i.e., fluorescence intensity in lumen > cells > medium (12, 13). 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 Refs. 13 and 25).

Figure 1 also shows confocal images of FL-MTX transport in tubules exposed to the NO donor SNP. In previous experiments using ET-1, a decrease in luminal accumulation of FL-MTX and no effect on cellular accumulation have been taken to indicate decreased transport on Mrp2 (13). Similar to the effects of ET-1,
100 μM SNP decreased luminal, but not cellular, accumulation of FL-MTX (Fig. 1B). Quantitation of images showed that 50–100 μM SNP reduced steady-state luminal accumulation of FL-MTX by about 50% (Fig. 2A). The action of SNP was rapid, causing a significant reduction of luminal fluorescence within 10 min of exposure and a sustained decrease over the 30-min experiment (Fig. 2B). SNP did not affect cellular fluorescence.

Figures 1 and 2 indicate that SNP and ET-1 have similar effects on Mrp2-mediated transport of FL-MTX. To determine whether NO release was a consequence of ET-1 exposure, tubules were exposed to 10 nM ET-1 in the absence or presence of L-NMMA, an NOS inhibitor. Figure 3 shows that L-NMMA by itself had no effect on FL-MTX transport but attenuated the effect of ET-1. Thus ET-1 activated NOS. In killifish tubules, ET-1 interacts with a basolateral ET<sub>B</sub> receptor and reduces Mrp2-mediated transport by acting through PKC (13). In this study the effects provided by ET-1 were prevented using the ET<sub>B</sub> receptor-antagonist RES-701–1 or PKC-selective inhibitors BIM, calphostin C, or staurosporine. To determine where NO generation was placed in the signaling pathway, we examined the abilities of RES-701–1 and the PKC-selective inhibitor BIM to attenuate the effects of SNP on FL-MTX transport. RES-701–1 did not alter the effects of SNP on transport (Fig. 4), but BIM protected completely (Fig. 5), indicating that NO generation came after ET<sub>B</sub> receptor binding but before PKC activation.
We recently found that ET release and subsequent signaling in killifish tubules could be activated by two types of external stimuli: elevated extracellular Ca\(^{2+}\) and several structurally unrelated nephrotoxicants (25). The present experiments indicate that each acts through NO. Figure 6 shows that L-NMMA prevented the decrease in luminal FL-MTX accumulation caused by increasing the medium Ca\(^{2+}\) concentration from 1.5 mM in controls to 3 mM in treated tubules. Table 1 shows the results of a series of experiments in which nephrotoxicants reduced FL-MTX transport mediated by Mrp2. The data for the aminoglycoside antibiotics gentamicin and amikacin and for the radiocontrast agent diatrizoate confirm previous findings (25). For these drugs, effects on Mrp2-mediated transport were shown to be caused by stimulation of ET release by the tubules and subsequent ET action through the ET\(_{B}\) receptor and PKC (25). Additional experiments with killifish tubules have disclosed a similar mechanism of action for low concentrations of heavy-metal salts HgCl\(_2\) and CdCl\(_2\) (Terlouw SA and Miller DS, unpublished observations). Table 1 shows that, for all these nephrotoxicants, including HgCl\(_2\) and CdCl\(_2\), inhibition of NOS by L-NMMA prevented the reduction in Mrp2-mediated transport. Thus, the effects of elevated medium Ca\(^{2+}\) and the nephrotoxicants appear to involve generation of NO.

To test this supposition directly, we used a fluorescence-based technique to measure NO generation in intact killifish tubules. The technique relies on the formation of a highly fluorescent product when DAF-FM reacts with NO. The indicator is introduced into the tubule as a nonfluorescent diacetate, which is membrane permeable and hydrolyzed intracellularly to DAF-FM. Figure 7 shows that the average fluorescence intensity was low in control tubules but increased by several times in tubules exposed to SNP (100 \(\mu\)M). Roughly the same magnitude of increase was also found for tubules exposed to diatrizoate, CdCl\(_2\), amikacin, and gentamicin at concentrations that reduce FL-MTX transport mediated by Mrp2. ET-1 also generated NO and the nephrotoxicants appear to involve generation of NO.

Table 1. Inhibition of FL-MTX transport by nephrotoxins and protection by 50 \(\mu\)M L-NMMA

<table>
<thead>
<tr>
<th>Nephrotoxicant</th>
<th>Control</th>
<th>Nephrotoxicant + L-NMMA</th>
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<tbody>
<tr>
<td>Gentamicin (10 (\mu)M)</td>
<td>2,160 ± 140</td>
<td>1,550 ± 130(^{a})</td>
</tr>
<tr>
<td>Amikacin (10 (\mu)M)</td>
<td>2,650 ± 120</td>
<td>1,460 ± 120(^{\dagger})</td>
</tr>
<tr>
<td>Diatrizoate (10 (\mu)M)</td>
<td>1,670 ± 130</td>
<td>1,020 ± 160(^{\dagger})</td>
</tr>
<tr>
<td>HgCl(_2) (0.1 (\mu)M)</td>
<td>2,140 ± 160</td>
<td>1,280 ± 170(^{\dagger})</td>
</tr>
<tr>
<td>CdCl(_2) (10 (\mu)M)</td>
<td>2,300 ± 180</td>
<td>1,130 ± 150(^{\dagger})</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE luminal fluorescence intensity of 11–21 tubules. L-NMMA, \(\text{N}^{\text{G}}\)-monomethyl-\(\text{L}\)-arginine. None of the nephrotoxicants altered cellular fluorescence methoxetate (FL-MTX) accumulation. \(^{a}\)Significantly lower than the control value (\(P<0.05\)). \(\dagger\)Significantly lower than the control value (\(P<0.001\)).

**Fig. 4.** The ET\(_{B}\) receptor antagonist RES-701–1 does not protect against inhibition of Mrp2-mediated FL-MTX transport by NO. Tubules were incubated for 30 min in medium containing 1 \(\mu\)M FL-MTX alone (control) or with 100 \(\mu\)M SNP, or SNP plus 100 \(\mu\)M RES-701–1. Confocal images were collected and analyzed as described in METHODS. Values are means ± SE for 10–11 tubules from a single fish. **Significantly lower than the control value, \(P<0.001\).

**Fig. 5.** A protein kinase C (PKC)-selective inhibitor bisindolmaleide (BIM) protects against inhibition of Mrp2-mediated FL-MTX transport by NO. Tubules were incubated for 30 min in medium containing 1 \(\mu\)M FL-MTX alone (control) or with 100 \(\mu\)M SNP, or SNP plus 100 \(\mu\)M BIM, or SNP plus BIM. Confocal images were collected and analyzed as described in METHODS. Values are given as means ± SE for 9–15 tubules from a single fish. **Significantly lower than the control value, \(P<0.01\).

**Fig. 6.** L-NMMA protects against inhibition of Mrp2-mediated FL-MTX transport by elevated medium Ca. Tubules were incubated for 30 min in medium containing 1.5 mM Ca and 1 \(\mu\)M FL-MTX (control); 5 mM Ca and 1 \(\mu\)M FL-MTX; and 5 mM Ca, 1 \(\mu\)M FL-MTX, and 50 \(\mu\)M L-NMMA. Confocal images were collected and analyzed as described in METHODS. Values are given as means ± SE for 18 tubules from a single fish. **Significantly lower than the control value, \(P<0.001\).
gether these data demonstrate that ET-1 and the nephrotoxicants stimulated NO production in the tubules.

DISCUSSION

Using killifish renal proximal tubules, we previously demonstrated that ET, acting through an ET<sub>B</sub> receptor and PKC, reduced transport mediated by Mrp2, i.e., luminal accumulation of FL-MTX (13). In the present study, we used Mrp2-mediated transport of FL-MTX to monitor activity of this ET-fired, intracellular signaling pathway; the purpose was to examine further the chain of events that connect ET<sub>B</sub> receptor binding at the basolateral membrane to reduction of transport at the luminal membrane. We show here that exposing tubules to SNP, an NO donor, also signaled a reduction in transport. Importantly, exposure to l-NMMA, an NOS inhibitor, blocked the action of ET-1 on transport. Because the effects of SNP on transport were blocked by BIM, a PKC selective inhibitor, but not by RES-701-1, an ET<sub>B</sub> receptor antagonist, generation of NO occurred after receptor signaling but before PKC activation. Several additional observations are consistent with this proposed sequence of events. Experiments using a fluorescence-based NO assay showed that ET-1 and the nephrotoxicants stimulated NO production by intact tubules. For ET-1, NO production was blocked by an inhibitor of NOS; for the nephrotoxicant, gentamicin, NO production was blocked by an ET<sub>B</sub> receptor antagonist. A scheme of the proposed signaling pathway is shown in Fig. 9.

The present data are the first to describe an ET-NO-PKC signaling axis in renal proximal tubules. In agreement with our findings, activation of NOS was found after ET<sub>B</sub> receptor stimulation in the thick ascending limb, and this was associated with inhibited chloride transport (19, 20). In OK cells, an opossum kidney proximal tubule cell line, Liang and Knox (9) described an NO-PKC-Na<sup>+</sup>-K<sup>+</sup>-ATPase signaling pathway. Such a pathway was not present in LLC-PK<sub>1</sub> cells, a proximal tubule cell line from pig (9). Obviously, species differences or differences in culture conditions could underlie the differences in signaling found in the two cell lines.

Liang and Knox (9) have suggested that NOS might activate PKC through guanylate cyclase. Results indicating cGMP generation after NO synthesis confirm this suggestion (24, 30). Interestingly, it was recently shown that one member of the MRP family, viz. MRP5, transports cGMP (7). It is possible that Mrp2 transports the cyclic nucleotide as well, and cGMP might compete with FL-MTX for efflux, resulting in a decreased FL-MTX transport. In this regard, our preliminary experiments show that, in killifish tubules, 8-bromoguanosine 3',5'-cyclic monophosphate is a potent inhibitor of FL-MTX transport from cell to lumen, and at least a portion of that inhibition is removed when tubules are pretreated with an
inhibitor of protein kinase G (Notenboom S and Miller DS, unpublished observations). The latter suggests activation of guanylate cyclase by PKC rather than the opposite, because the effect of NO generation by the NO donor SNP was completely reversed by inhibiting PKC. Whether guanylate cyclase is directly or indirectly responsible for a diminished transport of organic anions by Mrp2 will be investigated in further research.

Although it is not clear whether proximal tubules are capable of generating NO under basal conditions, they certainly can produce large amounts of the substance when stimulated (10). In this regard, proximal tubules express several NOS isoforms, including inducible and endothelial NOS, and NO production increases on exposure to a variety of stimuli, including lipopolysaccharide, cytokines, hypoxia, and several nephrotoxic chemicals (10). The present results indicate that, when killiﬁsh proximal tubules were exposed to radiocontrast agents, aminoglycoside antibiotics, or heavy-metal salts, NO production increased. In addition, Mrp2-mediated transport decreased for each compound and these decreases were prevented when the tubules were also exposed to an NOS inhibitor, L-NMMA. We have recently found that each tested nephrotoxicant reduced Mrp2-mediated transport in killiﬁsh tubules by inducing ET release and activating ET_b receptor-PKC signaling (13, 25). Nephrotoxicant action appeared to be Ca^{2+} dependent, because nifedipine, an L-type Ca^{2+} channel blocker, protected its action and because elevated medium Ca^{2+} also stimulated ET release and signaling. The present results indicate that exposure of tubules to nephrotoxicants or elevated medium Ca^{2+} also produces NO as an intermediate step in signaling (Fig. 9).

Production of NO as a consequence of nephrotoxicant-triggered signaling provides a toxicological context in which to consider the present and previous results. NO production has been implicated in renal diseases and the action of several nephrotoxic compounds (8). For example, hypoxic/ischemic injury is prevented by NG-nitro-L-arginine methyl ester and enhanced by SNP (29), and inducible NOS appears to be the critical isoform involved (11, 17). In renal proximal tubule cells, NO induces apoptosis and potentiates immunosuppressant-induced apoptosis (1, 6). ET signaling and NO production are implicated in HgCl_2-induced acute renal failure (28). In this regard, although short-term exposure of killiﬁsh tubules to the relatively low concentrations of nephrotoxic compounds used in the present study produced no evidence of cellular toxicity [as measured by the ability to actively transport fluorescein on the classical organic anion system (Ref. 25 and Notenboom S. and Miller DS, unpublished observations)], our initial experiments show that longer exposures are clearly toxic and that L-NMMA can protect (Notenboom S and Miller DS, unpublished observations). Experiments are under way to determine whether toxicity is a result of signaling through the ET-ET_b receptor-NOS-PKC pathway Fig. 9. Scheme illustrating the proposed sequence of events by which nephrotoxicants reduce Mrp2-mediated transport in isolated renal proximal tubules. Nephrotoxicants cause a transient opening of calcium channels, which increases intracellular Ca concentration and stimulates ET release. The hormone binds to a basolateral ET_b receptor, which activates NOS, increases NO production, and activates PKC. PKC activation rapidly reduces transport on Mrp2.
or whether a separate sequence of events leads to NO production and subsequent toxicity.

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REFERENCES