NEPHROTOXICANTS ACTIVATE NITRIC OXIDE SYNTHASE AND INDUCE NITRIC OXIDE PRODUCTION IN KILLIFISH RENAL PROXIMAL TUBULES

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We previously found that activation of a receptor-mediated, autocrine signaling pathway was an early event in the action of several nephrotoxic chemicals in killifish (*Fundulus heteroclitus*) renal proximal tubules. Exposing tubules to radiocontrast agents, aminoglycoside antibiotics and certain heavy metals caused the following sequence of events: release of endothelin (ET) to the medium, activation of a basolateral ET B-type receptor, activation of protein kinase C (PKC) and decreases in transport mediated by two ATP-driven drug export pumps in the luminal membrane, p-glycoprotein and Mrp2 (Masereeuw et al, Mol. Pharm. 57:59-67, 2000; Terlouw et al, Mol. Pharm., in press). Signaling appeared to be Ca-dependent in that it was blocked by the Ca channel blocker, nifedipine, and it was activated (in the absence of nephrotoxins) by elevated medium Ca. Signaling was also specific in that active transport on the classical, Na-dependent, organic anion system was relatively insensitive to added ET-1 and was only reduced after exposure to much higher levels of nephrotoxins.

Nitric oxide (NO) has been implicated in ET signaling in renal vasculature and renal tubules (Plato and Garvin, Clin. Exp. Pharmacol. Physiol. 26:262-268, 1999). In addition, NO production has been implicated in toxic reactions to a number of chemicals that produce reactive oxygen species. Here we demonstrate that in renal proximal tubule NO production is an essential step in the ET signaling pathway activated by nephrotoxins.

Proximal tubules were isolated from killifish renal masses and the steady state accumulation of 1 μM fluorescein methotrexate (FL-MTX, a substrate for Mrp2, which is on the luminal plasma membrane of renal proximal tubule cells) in cells and tubular lumens was measured using confocal microscopy and quantitative image analysis as described previously (Masereeuw et al, Am. J. Physiol. 271:F1173-1182, 1996). Control tubules showed rapid accumulation of FL-MTX in cells and lumens with steady state lumen-to-cell ratios averaging 4-5. Incubation of tubules in medium containing 50-100 μM sodium nitroprusside (SNP, an NO donor) reduced luminal accumulation by about 50%. SNP acted rapidly, with significant reductions seen within 10 min. SNP did not affect cellular accumulation of FL-MTX, indicating action on the luminal transport step, i.e., on Mrp2. SNP effects on the luminal accumulation of FL-MTX were blocked by 1 μM bis-indolylmaleimide (BIM), a PKC-selective inhibitor. N-methyl-L-arginine (L-NMMA, a NO synthase inhibitor) at 50 μM blocked the reduction in luminal accumulation of FL-MTX caused by 10 nM ET-1 and by elevated medium Ca. Finally, RES 701-1, an ET B-type receptor antagonist that blocks ET-1 action in killifish tubules, did not affect the ability of SNP to reduce FL-MTX transport.

Taken together, these results indicate that activation of NOS and production of NO are intermediate steps in the ET signaling pathway in renal proximal tubule; NO release occurred after receptor activation, but before PKC activation. To determine whether nephrotoxins that

induce ET release from the tubules also cause NO production, we exposed tubules to a radiocontrast agent (diatrizoate), an aminoglycoside antibiotic (gentamicin) and a heavy metal salt (mercuric chloride) in the absence and presence L-NMMA and measured luminal accumulation of FL-MTX. Table 1 shows that each of the nephrotoxic chemicals significantly reduced luminal accumulation of the Mrp2 substrate, but that in every case L-NMMA protected against this effect. Thus, in intact renal proximal tubules, NO production is an early event in the action of several nephrotoxic chemicals. How NO release and the subsequent generation of reactive oxygen species contribute to nephrotoxicity in proximal tubule remains to be determined. Supported by the Dutch Kidney Foundation, the Dr. Saal van Zwanenberg Foundation and the MDIBL CMTS (ES 03828).

Table 1. Reversal of inhibition of Mrp2-mediated transport by 50 μM L-NMMA

Nephrotoxin	Control (No Additions)	+Nephrotoxin	+Nephrotoxin + L-NMMA
10 μM Gentamicin	2162 ±145	1553 ±126*	2253 ±231
10 μM Diatrizoate	1669 ±127	1021 ±162**	1635 ±165
0.1 μM $HgCl_2$	2143 ±162	1278 ±172**	2172 ±133

Tubules were incubated for 30 min in medium containing 1 μM FL-MTX and the indicated additions. Data given as mean luminal fluorescence intensity ± SE for 13-16 tubules. None of the treatments used altered cellular FL-MTX accumulation. L-NMMA by itself did not affect FL-MTX transport. *Significantly lower than control value, $P<0.05$; **significantly lower than control value, $P<0.01$.