

Title page

**Short-term exposure of renal proximal tubules to
gentamicin increases long-term Mrp2 (Abcc2) transport function
and reduces nephrotoxicant sensitivity**

Sylvia Notenboom, David S. Miller, Leon H. Kuik, Paul Smits,
Frans G.M. Russel and Rosalinde Masereeuw

*Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences,
Radboud University Nijmegen Medical Centre, The Netherlands (SN, LHK, PS, FGMR, RM),
Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health
Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA (DSM),
and Mount Desert Island Biological Laboratory, Salisbury Cove, Maine, USA (SN, DSM,
LHK).*

Running Title Page

a) Running title: Gentamicin and long-term regulation of Mrp2

b) Corresponding author: Rosalinde Masereeuw, Ph. D.

Dept. Pharmacology and Toxicology 233

Nijmegen Centre for Molecular Life Sciences

Radboud University Nijmegen Medical Centre

P.O. Box 9101

6500 HB Nijmegen

The Netherlands

Phone: +31 24361 3730

FAX: +31 24361 4214

E-mail: R.Masereeuw@ncmls.ru.nl

c) Number of text pages: 15

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in: Abstract: 250

Introduction: 451

Discussion: 1492

d) **Abbreviations:** cGMP, guanosine 3',5'-cyclic monophosphate; Mrp2, multidrug resistance protein 2; ET, endothelin; ET_B receptor, endothelin receptor subtype B; NO, nitric oxide; NOS, nitric oxide synthase; FL-MTX, fluorescein methotrexate; L-NMMA, N^G-Methyl-L-arginine acetate salt (NOS inhibitor); ODQ, oxadiazole quinoxalin (guanylyl cyclase inhibitor)

e) Gastrointestinal, Hepatic, Pulmonary & Renal

Abstract

We previously showed that the function of renal Multidrug resistance protein 2 (Mrp2; Abcc2) is reduced by endothelin-1 (ET-1) signaling through an ET_B receptor, NOS, cGMP and protein kinase C and that this pathway was activated by several nephrotoxicants (Masereeuw et al., 2000; Notenboom et al., 2002; Notenboom et al., 2004; Terlouw et al., 2001). Here we determined the long-term effects on Mrp2-mediated transport (luminal fluorescein methotrexate accumulation) of short-term (30 min) exposure to ET-1 and the aminoglycoside antibiotic, gentamicin. Our data show that over the 3h following exposure proximal tubules recovered fully from the initial decrease in Mrp2-mediated transport and that transport activity was not changed 9h later. However, 24h after exposure, luminal accumulation of an Mrp2 substrate had increased by 50%. Increased transport at 24h was accompanied by an increased transporter protein content of the luminal plasma membrane as measured by immunostaining. Blocking ET-1 signaling at the ET_B receptor or downstream at NOS or guanylyl cyclase abolished both stimulation of transport and increased transporter expression. Thus, regardless of whether signaling was initiated by a short exposure to ET-1 or to a nephrotoxicant, the time course of Mrp2 response to ET_B signaling was the same and was multiphasic. Finally, when tubules were exposed to gentamicin for 30 min and removed to gentamicin-free medium for 24h they were less sensitive to acute gentamicin toxicity than paired controls not initially exposed to the drug. Thus, short-term exposure to ET-1 or gentamicin resulted in long-term protection against a second insult.

Introduction

ATP-driven drug efflux pumps, e.g., P-glycoprotein and Multidrug resistance protein 2 (Mrp2), play an important role in the absorption, distribution and excretion of endogenous compounds, drugs and their metabolites (Russel et al., 2002; Schinkel and Jonker, 2003). In vertebrate renal proximal tubule, Mrp2 has been localized to the luminal pole of the epithelial cells (Schaub et al., 1997), the correct location to provide the final step in transport of many anionic xenobiotics from blood to urine. The efflux pump handles a wide range of substrates and its activity and expression level in the kidney is influenced by hormones, local mediators, nuclear receptors, protein kinases, and disease conditions (Terlouw et al., 2003). As with drug metabolizing enzymes, e.g., cytochrome P450s and transferases (Korashy et al., 2004; Rushmore and Kong, 2002), pump activity is regulated in response to exposure to substrates and physiological state.

We previously showed in intact killifish renal proximal tubules that Mrp2 activity, as measured by luminal accumulation of a fluorescent MTX derivative (FL-MTX), is rapidly reduced by subnanomolar to nanomolar concentrations of ET-1 acting through an ET_B receptor, NOS, cGMP and PKC. Surprisingly, acute exposure of tubules to several nephrotoxicants, i.e. aminoglycoside antibiotics, radiocontrast agents and heavy metal salts, also reduces FL-MTX transport, and blocking ET signaling at any point in the chain abolishes the nephrotoxicant effects on transport (Notenboom et al., 2002; Notenboom et al., 2004; Terlouw et al., 2001). From these experiments, it was also clear that the nephrotoxicants caused Ca²⁺-dependent release of ET from the tubules and that released ET activated intracellular signaling by an autocrine mechanism. In contrast, after long-term, continuous exposure (6-24 hours) to the nephrotoxic heavy metal salt CdCl₂, transport activity and immunostaining of Mrp2 at the luminal membrane of the proximal tubules had increased (Terlouw et al., 2002). This long-term induction of Mrp2 may function as a compensatory mechanism for the initially reduced efflux of potentially toxic compounds, serving a protective route.

The present study addresses the issue of whether short-term signaling through the ET-activated pathway has longer-term consequences to tubular function. Our results show that after 30 min exposure to ET-1 or the aminoglycoside antibiotic, gentamicin, Mrp2-mediated transport initially declined; this is in agreement with previous studies (Notenboom et al., 2002; Terlouw et al., 2001). When tubules were removed to ET-1- and gentamicin-free medium, transport recovered over the next several hours. Twenty-four hours after exposure, Mrp2-mediated transport and Mrp2 protein expression were significantly higher than controls. These increases in transport and Mrp2 expression were abolished when ET signaling was disrupted. Finally, short-term gentamicin exposure and subsequent recovery for 24 h was protective against acute gentamicin tubular toxicity. Thus, short-term signaling has long-term consequences with regard to transport function and nephrotoxicant resistance.

Methods

Chemicals

Fluorescein methotrexate (FL-MTX), fluorescein, Mito Tracker® Red CM-H₂XRos and Alexa Fluor® 488 goat anti-rabbit IgG (rabbit polyclonal antibodies) were obtained from Molecular Probes (Eugene, OR). N^G-methyl-L-arginine (L-NMMA) was purchased from Alexis biochemicals (San Diego, Ca). The ET_B receptor antagonist, cyclo(-Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp)-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp-OH (RES-701-1), was purchased from Bachum Bioscience Inc. (King of Prussia, PA). Oxadiazole quinoxalin (ODQ; a guanylyl cyclase inhibitor) was obtained from Calbiochem (San Diego, Ca). Modified medium 199 with Earle's salts (M199) was purchased from Sigma Chemical (St. Louis, MO). Rabbit polyclonal antibodies against Mrp2 (k78 mrp2) and Mrp4 (anti-Mrp4) were obtained as described previously (Van Aubel et al., 1998; Van Aubel et al., 2002).

All other chemicals used, were obtained at the highest purity available commercially.

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 seawater. Renal tubular masses were isolated in a marine teleost saline based on that of Forster and Taggart, containing (in mM) 140 NaCl, 2.5 KCl, 1.5 CaCl₂, 1.0 MgCl₂ and 20 tris(hydroxymethyl)-amino methane (TRIS) at pH 8.0 (Forster and Taggart, 1950). Isolation and short-term exposure 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 from several fish were dissected, pooled and transferred to a well plate containing 3 ml marine teleost saline in presence or absence of 10 μM gentamicin. After 30 min exposure tubules were washed in marine teleost saline and transferred to recovery medium, i.e. modified medium 199 with Earle's salts

(M199) supplemented with (in mM, unless stated otherwise) 30.0, NaCl, 4.2 NaHCO₃, 1.0 L-glutamine, 25.0 HEPES, 14.75, NaOH (pH 7.5, 347 mosmol/kg H₂O), and 20 mg/l tetracycline, 10 µg/ml insulin, 5 µg/ml hydrocortisone, and 10% flounder serum (Renfro et al., 1999) with added effectors. The viability of teleost tubules is not preserved at 18-20 °C past 6 h, but can be extended to 48 h by reducing the incubation temperature to 13.5°C (Miller, unpublished data). After 1.5, 3, 12, or 24 h of recovery at 13.5°C tubules were washed in marine teleost saline and transferred to a foil covered Teflon chamber containing 1 ml of marine teleost saline with 1 µM FL-MTX. The chamber floor was a 4 x 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 minutes until steady state was reached for FL-MTX. Analysis of tubule extracts by HPLC showed no metabolic degradation of FL-MTX when incubated with killifish proximal tubules for periods of at least 1 h (Masereeuw et al., 1996; Schramm et al., 1995).

In toxicity experiments, isolated killifish tubules were transferred to a well plate containing marine teleost saline in presence or absence of 10 µM gentamicin. After 30 min exposure, tubules were washed in marine teleost saline and transferred to the recovery medium. After 24 h of recovery at 13.5°C tubules were exposed to 100 µM gentamicin for 2 h and subsequently washed in marine teleost saline and transferred to a foil covered Teflon chamber containing 1 ml of marine teleost saline with an indicator of tubular function. We used transepithelial transport of the fluorescent agent fluorescein and the mitochondrial marker, Mito Tracker® Red CM-H₂Xros, as two functional indicators of tubule viability. Both were measured using confocal microscopy and quantitative image analysis.

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 40x water immersion objective (NA 1.15). Excitation was provided by the 488 nm line of an argon ion

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 auto-fluorescence 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 a percentage of average measured pixel intensity compared to control rather than estimated dye concentration. Fluorescence intensities were measured from stored images using Scion image version 1.8 for Windows as described previously (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 for measurement of fluorescein and FL-MTX transport. The values used for that tubule were the means of all selected areas.

Measurement of mitochondrial function

Mito Tracker® Red CM-H₂Xros was used for determination of mitochondrial functional integrity. We used the optical sectioning capabilities of the confocal microscope to measure the dye fluorescence intensity inside tubular cells. Essentially all of the cellular fluorescence was in discrete structures, suggesting mitochondrial accumulation (Pendergrass et al., 2004).

Immunohistochemistry

Isolated killifish proximal tubules were exposed to 10 μ M gentamicin or 10 nM ET-1 for 30 min and transferred to gentamicin and ET-1 free medium for a 24 h recovery period. Subsequently tubules were processed in PBS for immunostaining: fixation with 2% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde; permeabilization with 1% (v/v) Triton X-100; 90 min exposure to primary antibody (k78 1:50 for Mrp2, or anti-Mrp4, 1:10); 60 min exposure to secondary antibody (Alexa488-labeled goat anti-rabbit IgG, 1:20). Antibody binding was detected with the Zeiss confocal laser scanning microscope using an 20x objective and staining was quantified using ImageJ 1.30v (NIH, USA). A grid, consisting of 655.02 μ m² squares, was placed on top of the confocal images to semi-quantify the fluorescent staining. Only there where the grid crossed luminal staining fluorescence intensities were measured. The average of the fluorescence intensities measured for that tubule was used for quantitation.

Data analysis

Data are given as mean percentage of control fluorescence \pm SE, unless indicated otherwise. 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 Graph Pad Prism (version 3.00 for Windows; Graph Pad Software, San Diego CA, USA).

Results

Isolated renal tubules from killifish, have proven to be a powerful model for the study of excretory transport in an intact proximal tubule (Pritchard and Miller, 1991). Unlike mammalian proximal tubules, the broken ends of teleost proximal tubules reseal after isolation, thus, forming a closed fluid filled luminal compartment. This enables study of cellular uptake and luminal accumulation of fluorescent compounds using imaging techniques. As in mammalian proximal tubules, killifish express high levels of Mrp2 in the luminal membrane of renal proximal tubule cells. Moreover, killifish tubules exhibit Mrp2-mediated transport of a number of fluorescent substrates, e.g., FL-MTX, that can be visualized and measured in intact individual tubules using confocal microscopy (Masereeuw et al., 1996; Masereeuw et al., 2000; Miller et al., 1996). Figure 1A-B show a typical confocal image of a killifish tubule after 30 min (steady state) incubation in medium with 1 μ M FL-MTX following 24 h of recovery. The fluorescence distribution pattern is the same as shown previously after short-term exposure, i.e., fluorescence intensity in lumen>cells>medium (Masereeuw et al., 1996; Masereeuw et al., 2000). 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., 1996; Masereeuw et al., 2000; Terlouw et al., 2001). Using an Sf9 overexpression system, we previously showed that FL-MTX is a substrate for MRP2 (Terlouw et al., 2002). Although MRPs are known to share many substrates, interference of other members of the MRP family with FL-MTX transport in this model is unlikely. Mrp5 and Mrp6 are located in the basolateral membrane and not in the apical membrane of renal proximal tubules, whereas Mrp1 and Mrp3 are not expressed in renal proximal tubules (Russel et al., 2002). Furthermore, we can exclude the contribution of Mrp4 because results from our group show that FL-MTX is not a substrate for MRP4 (Smeets and Russel, unpublished data), and MRP4-mediated transport is insensitive to

leukotriene C₄ (Van Aubel et al., 2002), which is an excellent inhibitor of FL-MTX secretion in killifish proximal tubules (Masereeuw et al., 1996; Masereeuw et al., 2000).

To determine whether short-term ET-1 signaling has long-term effects, we exposed tubules to ET-1 for 30 min, removed them to ET-free medium and assayed Mrp2 transport function at several times after transfer. Figure 1C. shows that 1.5 h after transfer the tubules exhibit significantly reduced transport. This result is in agreement with previous experiments from this laboratory where tubules were exposed continuously to ET-1 in transport experiments lasting up to 90 min (Masereeuw et al., 2000). However, with time in the ET-free medium, transport increased, and 3 h and 12 h values were nearly identical to tubules not exposed to ET-1 (controls). Moreover, 24 h after ET-1 exposure, luminal accumulation of FL-MTX had increased by about 50% ($P < 0.001$; Fig. 1). Neither short-term exposure to ET-1 nor short-term exposure followed by recovery affected cellular accumulation of FL-MTX. Thus, both the short-term and long-term effects of 30 min ET-1 exposure on luminal accumulation were evident with FL-MTX as substrate.

We previously demonstrated that several tubular nephrotoxicants (aminoglycoside antibiotics, radiocontrast agents and heavy metal salts) were capable of mimicking the effects of ET-1 on FL-MTX transport. Each of these activated the ET signaling pathway by a Ca²⁺-dependent mechanism causing ET release from the tubules. None of them interacted with Mrp2 directly (Notenboom et al., 2002; Terlouw et al., 2001). When we incubated tubules in medium containing the aminoglycoside antibiotic, gentamicin (10 μ M), transferred them to gentamicin-free medium and monitored FL-MTX transport, we found the same pattern of effects as observed with ET-1 (Fig. 2). Thus, both ET-1 and gentamicin had a triphasic effect on FL-MTX: short-term reduction, followed by recovery, and finally significantly increased transport. Note that when fluorescein (FL) was used as a substrate, there was no initial decrease in transport with 10 μ M gentamicin (Fig 3A) and no increase 24 h after gentamicin exposure (Fig. 3B). However, with short-term exposure to higher concentrations of gentamicin (Fig 3A) and with 24 h continuous exposure to 10 μ M

gentamicin (preliminary data not shown), a decrease in FL transport was seen, indicating toxicity.

We used a pharmacological approach to further examine the intracellular events signaling the increase in FL-MTX transport 24 h after a short exposure to ET-1 or gentamicin. For this purpose, specific antagonists were used to block steps in the common signaling pathway. Figure 4 shows that blocking the ET_B receptor with RES701-1, blocking NOS with L-NMMA or blocking guanylyl cyclase with ODQ abolished the long term effects of short-term exposure to gentamicin. None of the inhibitors affected FL-MTX transport by itself (Figure 4). Apparently, basal efflux pump activity is not influenced by signaling components of the ET-triggered pathway, which has been a consistent finding in our studies over the past 5 years (Masereeuw et al., 2000; Notenboom et al., 2002; Notenboom et al., 2004; Terlouw et al., 2001; Terlouw et al., 2002). Thus, at a minimum, the long-term effects of short-term exposure to ET-1 or gentamicin appear to be a consequence of signaling through the ET_B receptor, NOS and cGMP.

The increase in luminal accumulation of FL-MTX 24 h after short-term exposures to ET-1 or gentamicin may have resulted from increased transporter expression or increased transport activity with no increase in expression. To determine whether the expression level was altered, we used immunofluorescence to examine the long-term effects on Mrp2. Figure 5 shows representative confocal images of killifish tubules immunostained for Mrp2. In agreement with previous studies in mammalian and killifish renal tissue, this transporter localized to the luminal (brush border) membrane of the tubule epithelial cells (Masereeuw et al., 2000; Schaub et al., 1997; Schaub et al., 1999; Terlouw et al., 2001; Terlouw et al., 2002). Figure 5 also shows representative confocal images of tubules exposed to gentamicin or ET-1 for 30 min and then assayed 24 h later. In these tubules, Mrp2 immunofluorescence appears to be more intense than in controls (no ET-1 or gentamicin exposure). Quantitation of Mrp2 immunofluorescence bears out this impression. Both ET-1 and gentamicin increased luminal membrane immunofluorescence by about 25% ($P < 0.01$). In contrast, no such increase was found when tubules were stained for Mrp4, which, as in mammalian renal

proximal tubules (Van Aubel et al., 2002), is also localized to the luminal membrane of killifish renal proximal tubule cells (Fig. 5E). Consistent with the transport data presented above, inhibiting NOS with L-NMMA blocked the increase in Mrp2 expression caused by 30 min exposure to ET-1 or gentamicin (Fig. 6).

Thus, one consequence of short-term exposure to ET-1 or gentamicin is an increase in Mrp2 expression and function 24 h later. To determine whether the response to nephrotoxicants was also affected, we used MitoTracker® Red CM-H₂XRos in tubules exposed to 100 μ M gentamicin to indirectly assess mitochondrial functional integrity. This concentration of nephrotoxicant is an order of magnitude higher than that which alters Mrp2-mediated transport through ET signaling, and was found to affect tubular viability (Figure 3). The advantage of using MitoTracker® Red CM-H₂XRos is that the probe is highly stable. It is oxidized within cells and then selectively sequestered in mitochondria of actively respiring cells. Probes often used for measuring mitochondrial membrane potential, e.g. JC-1 and Safranin O, cannot be used in our model because they are substrates for efflux carriers present in the apical membrane of the tubular cells. In this experiment, tubules were pre-exposed to 0 (controls) or 10 μ M gentamicin for 30 min and then incubated in gentamicin-free medium for 24 h. Subsequently, they were challenged with a 2 h exposure to 100 μ M gentamicin and changes in mitochondrial integrity were measured using 500 nM of Mito Tracker® Red CM-H₂XRos and confocal imaging after 30 min incubation with the probe. In tubules not pre-exposed to the low concentration of gentamicin (controls), 100 μ M gentamicin significantly decreased mitochondrial integrity ($P < 0.05$). This effect was roughly half of that seen when control tubules were exposed to 1 mM NaCN for 2 h (Fig. 7). In contrast, no such gentamicin-induced alteration in mitochondrial functional integrity was observed in tubules that had been pre-exposed to 10 μ M gentamicin. Thus, at least for one nephrotoxicant and one measure of toxicity, gentamicin pre-exposure and subsequent recovery was protective.

Discussion

In killifish renal proximal tubules, ET-1 signals a decrease in Mrp2 transport function by acting through an ET_B receptor, NOS, cGMP, and PKC. Moreover, exposure to any of several nephrotoxicants causes release of ET from the tubules with subsequent activation of signaling and reduced Mrp2 function (Masereeuw et al., 2000; Terlouw et al., 2001; Terlouw et al., 2002). These inhibitory effects on Mrp2-mediated transport were observed within 15 min of exposure and were maximal within 60 min. After 60 min exposure, the Mrp2 protein content of the luminal plasma membrane is not changed, suggesting that transporter activity in the luminal plasma membrane was modified or transporter was removed to a subapical compartment that was not resolved at the light microscope level (Terlouw et al., 2001). In the present study, we investigated the effects of exposing renal proximal tubules to ET-1 or to gentamicin for a short time (30 min) and then returning them to ET-1- and gentamicin-free medium. The data show that over the 3 h following exposure, tubules recovered fully from the initial decrease in Mrp2-mediated transport and that transport activity was not changed 9 h later. However, 24 h after exposure, luminal accumulation of FL-MTX had increased by 50%. Increased transport at 24 h was accompanied by an increased transporter protein content of the luminal plasma membrane measured by immunostaining. This finding could not be confirmed using immunoblots. Immunoblot analysis is not feasible in this situation, because of the small amount of membrane protein isolated from killifish tubules. A recent direct comparison of quantitative immunostaining with immunoblot analysis, however, indicates that the results obtained with the two methods are comparable (Bauer et al., 2004). Furthermore, preliminary data in rats treated with gentamicin *in vivo* showed a clear up-regulation of Mrp2 in the apical membrane of renal proximal tubule confirming the findings of our killifish experiment in a different species (Notenboom et al., unpublished findings).

Blocking ET-1 signaling at the ET_B receptor, or downstream at NOS, abolished both stimulation of transport and increased transporter expression. Thus, regardless of whether signaling was initiated by a short exposure to ET-1 or by a nephrotoxicant, the time course of the Mrp2 response to ET_B signaling was the same. It was multiphasic, involving reduced

Mrp2-mediated transport, recovery to control levels, and a delayed increase over control levels 24 h after exposure. Non-specific leakage of the fluorescent dye can be excluded because luminal FL-MTX accumulation is concentrative with respect to medium, and leakage of the dye at the tight junctions would decrease the luminal concentrating ability of the tubules. Note that FL transport in killifish renal tubules was constant over the entire exposure/recovery time course (Figure 3) as observed previously (Terlouw et al., 2002). This fluorescent organic anion is avidly transported from bath to tubular lumen by an organic anion transport system that does not include Mrp2 (Masereeuw et al., 1996). Fluorescein may be transported by members of the SLC superfamily of transporters, organic anion transporters (Oat's) and organic anion transporting polypeptides (Oatp's). However, the finding that fluorescein transport was unchanged argues for the lack of an effect on other carriers. In addition, in agreement with the zebrafish genome unpublished findings demonstrate that killifish tubules express only one Oat which is localized to the basolateral membrane (Pritchard and Miller, personal communication). Concerning the Oatp carriers, a large species difference exists in this subfamily of transporters, and it is unknown whether an isoform is present in killifish renal proximal tubules. Furthermore, 24 h after exposure we did not detect any qualitative change in luminal plasma membrane content of Mrp4. This suggests no increase in expression of luminal membrane transporters in general or in the expression of MRP subfamily members in particular.

The increase in Mrp2 transport function (~50%) 24 h after exposure to ET-1 or gentamicin was accompanied by a less than proportional increase in transporter content in the plasma membrane (~25%). Assuming a one-to-one correspondence between transport activity and transporter content, this difference suggests that multiple mechanisms contribute to the increase in transporter function. These might include: 1) de novo synthesis of Mrp2, 2) increased insertion of Mrp2 into the apical membrane, 3) reduced Mrp2 retrieval from the apical membrane and 4) functional activation of membrane-bound transporter. Mrp2 activity is known to be modulated by transcriptional and post-transcriptional mechanisms. For example, several ligand activated nuclear receptors have been shown to transcriptionally

regulate the activity of xenobiotic metabolizing enzymes and xenobiotic transporters, including Mrp2, in liver, intestine and blood-brain barrier (Bauer et al., 2004; Kast et al., 2002; Kullak-Ublick and Becker, 2003). These include the pregnane X nuclear receptor (PXR) and the constitutive androstane receptor (CAR), both of which are activated by a wide range of xenobiotics. Teleost fish do express a PXR homolog (Maglich et al., 2003; Moore et al., 2002), but it is not known to what extent (if any) gentamicin or ET-1 affects PXR activity in any species. In addition, in mammalian hepatocytes, both PKC and PKA have been implicated in bidirectional, regulated trafficking of Mrp2 between intracellular stores and the canalicular membrane (Beuers et al., 2001; Kubitz et al., 2001; Roelofsen et al., 1998). Furthermore, (Hegedus et al., 2003) suggested that PKC is involved in the Mrp2 targeting and recycling through phosphorylation of the PDZ domain, which influences the interaction between Mrp2 and its anchoring PDZ proteins and thereby its transport function. PKA activation does not appear to be involved in short-term exposure, since we previously found no effect of a PKA-selective inhibitor on ET-1 signaling (Masereeuw et al., 2000). Preliminary data using a canine kidney cell line over-expressing MRP2 pointed to an increased insertion of the transport protein in the luminal membrane after 1 h gentamicin exposure and 24 h recovery or 24 h gentamicin exposure, whereas overall MRP2 expression remained unchanged (Notenboom et al, unpublished). Additional experiments are needed to further characterize in the killifish renal proximal tubule model the mechanisms that link ET-NOS-cGMP-PKC signaling and Mrp2 up- and down-regulation.

Up-regulation of Mrp2 may be interpreted as part of a mechanism called preconditioning. Thus an up-regulation of Mrp2 and other adaptive mechanisms after recovery protects the tissue against a second exposure to gentamicin. This phenomenon has been observed for ischemia in heart, liver and kidney (Andreucci et al., 2003; Jaeschke, 2003; Juhaszova et al., 2004; Park et al., 2003). Mrp2 serves a protective function through the elimination of potentially harmful chemicals. Indeed, Mrp2 up-regulation was also observed after long-term exposure to cadmium (Terlouw et al., 2002) and after ischemia (Laouari et al., 2001). Since Mrp2 handles many potentially toxic compounds, like

xenobiotics and their metabolites (Russel et al., 2002), up-regulation of the transport protein may be part of a protective pathway of proximal tubules following harmful events.

Finally, use of gentamicin and other aminoglycoside antibiotics has been associated with severe proximal tubular nephrotoxicity, which limits their clinical use. Although such irreversible toxicity has been historically associated with multiple administration of high doses (Bennett, 1989), gentamicin can alter renal function even at low dose levels (Foster et al., 1992). The present data show for the first time that low-level, short-term, gentamicin exposure could have beneficial side effects, possibly by triggering a survival/protective pathway. Certainly, ET signaling and gentamicin preconditioning affect more than Mrp2 expression and function. It will be important to determine which of these changes in gene expression or enzyme/transporter function confers increased resistance to aminoglycoside antibiotic toxicity. Note that the present experiments, in which Mrp2 transport function was the end-point, provide an additional example of context-dependent signaling, as demonstrated recently for protein kinases (Bhalla et al., 2002; Ingolia and Murray, 2002). In this regard, we have been able to document three patterns of effects following tubule exposure to gentamicin (and other nephrotoxicants): 1) inhibition of transport with short-term exposure (Notenboom et al., 2002; Notenboom et al., 2004; Terlouw et al., 2001; Terlouw et al., 2002), 2) toxicity with long-term, continuous exposure (unpublished results), and 3) increased transport with long-term exposure to low concentrations of nephrotoxicants (Terlouw et al., 2002) or with short-term exposure followed by a period of recovery (present study).

In conclusion, renal proximal tubules exposed to gentamicin or ET-1 and allowed to recover for 24h, display an increase in Mrp2-mediated transport and Mrp2 expression in the luminal membrane. These increments are a result of intracellular signaling consequences caused by short-term nephrotoxicant exposure in the renal proximal tubule. Several signaling molecules were identified as participants in this pathway leading to the following sequence of events: Short-term exposure to gentamicin triggers ET-1 release, ET-1 binds to the ET_B receptor, subsequently NOS is stimulated resulting in the activation of soluble guanylyl

cyclase. The produced cGMP might cause de novo synthesis of Mrp2, stimulation of Mrp2 insertion in the luminal membrane and/or inhibition of Mrp2 retrieval from the luminal membrane, eventually leading to increased Mrp2-mediated transport through increased Mrp2 insertion in the luminal membrane in the renal proximal tubule. Thus, short-term signaling (nephrotoxicant exposure) has long-term consequences for renal proximal tubule functioning.

References

- Andreucci M, Michael A, Kramers C, Park KM, Chen A, Matthaeus T, Alessandrini A, Haq S, Force T, and Bonventre JV (2003) Renal ischemia/reperfusion and ATP depletion/repletion in LLC-PK(1) cells result in phosphorylation of FKHR and FKHL1. *Kidney Int* **64**:1189-1198.
- Bauer B, Hartz AM, Fricker G, and Miller DS (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood-brain barrier. *Mol Pharmacol* **66**:413-419.
- Bennett WM (1989) Mechanisms of aminoglycoside nephrotoxicity. *Clin Exp Pharmacol Physiol* **16**:1-6.
- Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, and Dombrowski F (2001) Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. *Hepatology* **33**:1206-1216.
- Bhalla US, Ram PT, and Iyengar R (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science* **297**:1018-1023.
- Forster R.P and Taggart JV (1950) Use of isolated renal tubules in the estimation of metabolic processes associated with active cellular transport. *J Cell Comp Physiol* **36**:251-270.
- Foster JE, Harpur ES, and Garland HO (1992) An investigation of the acute effect of gentamicin on the renal handling of electrolytes in the rat. *J Pharmacol Exp Ther* **261**:38-43.
- Hegedus T, Sessler T, Scott R, Thelin W, Bakos E, Varadi A, Szabo K, Homolya L, Milgram SL, and Sarkadi B (2003) C-terminal phosphorylation of MRP2 modulates its interaction with PDZ proteins. *Biochem Biophys Res Commun* **302**:454-461.

- Ingolia NT and Murray AW (2002) Signal transduction. History matters. *Science* **297**:948-949.
- Jaeschke H (2003) Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* **284**:G15-G26.
- Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, and Sollott SJ (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* **113**:1535-1549.
- Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, and Edwards PA (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* **277**:2908-2915.
- Korashy HM, Elbekai RH, and El Kadi AO (2004) Effects of renal diseases on the regulation and expression of renal and hepatic drug-metabolizing enzymes: a review. *Xenobiotica* **34**:1-29.
- Kubitz R, Huth C, Schmitt M, Horbach A, Kullak-Ublick G, and Haussinger D (2001) Protein kinase C-dependent distribution of the multidrug resistance protein 2 from the canalicular to the basolateral membrane in human HepG2 cells. *Hepatology* **34**:340-350.
- Kullak-Ublick GA and Becker MB (2003) Regulation of drug and bile salt transporters in liver and intestine. *Drug Metab Rev* **35**:305-317.
- Laouari D, Yang R, Veau C, Blanke I, and Friedlander G (2001) Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure. *Am J Physiol Renal Physiol* **280**:F636-F645.
- Maglich JM, Caravella JA, Lambert MH, Willson TM, Moore JT, and Ramamurthy L (2003) The first completed genome sequence from a teleost fish (*Fugu rubripes*) adds significant diversity to the nuclear receptor superfamily. *Nucleic Acids Res* **31**:4051-4058.

- Masereeuw R, Russel FGM, and Miller DS (1996) Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. *Am J Physiol Renal Physiol* **271**:F1173-F1182.
- Masereeuw R, Terlouw SA, Van Aubel RA, Russel FG, and Miller DS (2000) Endothelin B receptor-mediated regulation of ATP-driven drug secretion in renal proximal tubule. *Mol Pharmacol* **57**:59-67.
- Miller DS, Letcher S, and Barnes DM (1996) Fluorescence imaging study of organic anion transport from renal proximal tubule cell to lumen. *Am J Physiol Renal Physiol* **271**:F508-F520.
- Miller DS and Pritchard JB (1991) Indirect coupling of organic anion secretion to sodium in teleost (*Paralichthys lethostigma*) renal tubules. *Am J Physiol Regul Integr Comp Physiol* **261**:R1470-R1477.
- Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH, and Moore JT (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* **16**:977-986.
- Notenboom S, Miller DS, Smits P, Russel FGM, and Masereeuw R (2002) Role of NO in endothelin-regulated drug transport in the renal proximal tubule. *Am J Physiol Renal Physiol* **282**:F458-F464.
- Notenboom S, Miller DS, Smits P, Russel FGM, and Masereeuw R (2004) Involvement of guanylyl cyclase and cGMP in the regulation of Mrp2-mediated transport in the proximal tubule. *Am J Physiol Renal Physiol* **287**:F33-F38.
- Park KM, Byun JY, Kramers C, Kim JI, Huang PL, and Bonventre JV (2003) Inducible nitric-oxide synthase is an important contributor to prolonged protective effects of ischemic preconditioning in the mouse kidney. *J Biol Chem* **278**:27256-27266.
- Pendergrass W, Wolf N, and Poot M (2004) Efficacy of Mito Tracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry* **61**:162-169.

- Pritchard JB and Miller DS (1991) Comparative insights into the mechanisms of renal organic anion and cation secretion. *Am J Physiol Regul Integr Comp Physiol* **261**:R1329-R1340.
- Renfro JL, Maren TH, Zeien C, and Swenson ER (1999) Renal sulfate secretion is carbonic anhydrase dependent in a marine teleost, *Pleuronectes americanus*. *Am J Physiol Renal Physiol* **276**:F288-F294.
- Roelofsen H, Soroka CJ, Keppler D, and Boyer JL (1998) Cyclic AMP stimulates sorting of the canalicular organic anion transporter (Mrp2/cMoat) to the apical domain in hepatocyte couplets. *J Cell Sci* **111 (Pt 8)**:1137-1145.
- Rushmore TH and Kong AN (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3**:481-490.
- Russel FGM, Masereeuw R, and Van Aubel RAMH (2002) Molecular aspects of renal anionic drug transport. *Annu Rev Physiol* **64**:563-594.
- Schaub TP, Kartenbeck J, Konig J, Spring H, Dorsam J, Staehler G, Storkel S, Thon WF, and Keppler D (1999) Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. *J Am Soc Nephrol* **10**:1159-1169.
- Schaub TP, Kartenbeck J, Konig J, Vogel O, Witzgall R, Kriz W, and Keppler D (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. *J Am Soc Nephrol* **8**:1213-1221.
- Schinkel AH and Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55**:3-29.
- Schramm U, Fricker G, Wenger R, and Miller DS (1995) P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. *Am J Physiol Renal Physiol* **268**:F46-F52.
- Terlouw SA, Graeff C, Smeets PHE, Fricker G, Russel FGM, Masereeuw R, and Miller DS (2002) Short- and long-term influences of heavy metals on anionic drug efflux from renal proximal tubule. *J Pharmacol Exp Ther* **301**:578-585.

Terlouw SA, Masereeuw R, and Russel FGM (2003) Modulatory effects of hormones, drugs, and toxic events on renal organic anion transport. *Biochem Pharmacol* **65**:1393-1405.

Terlouw SA, Masereeuw R, Russel FGM, and Miller DS (2001) Nephrotoxicants induce endothelin release and signaling in renal proximal tubules: effect on drug efflux. *Mol Pharmacol* **59**:1433-1440.

Van Aubel RAMH, Smeets PHE, Peters JGP, Bindels RJM, and Russel FGM (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol* **13**:595-603.

Van Aubel RAMH, van Kuijk MA, Koenderink JB, Deen PMT, van Os CH, and Russel FGM (1998) Adenosine triphosphate-dependent transport of anionic conjugates by the rabbit multidrug resistance-associated protein Mrp2 expressed in insect cells. *Mol Pharmacol* **53**:1062-1067.

Footnotes

a) This study was supported by the Dutch Kidney Foundation and the MDIBL Center for Membrane Toxicity Studies

Data presented on the ASN Renal Week 2003 SU-PO137 and ASN Renal Week 2004 F-PO130

b) Rosalinde Masereeuw, Ph. D.

Dept. Pharmacology and Toxicology 233

Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical

Centre

P.O. Box 9101

6500 HB Nijmegen

The Netherlands

Legends for figures

Figure 1: Changes in Mrp2-mediated transport in response to a short exposure to ET-1. Tubules were incubated for 30 min in the absence (control) or presence of 10 nM ET-1 and then transferred to ET-free medium for the indicated time. Representative confocal images of control (A) and ET exposed (B) killifish proximal tubules after 24h in ET-free medium after incubation in marine teleost saline with 1 μ M FL-MTX for 30 min. FL-MTX accumulation in cells and tubular lumens was then determined (C). At every time point measured, the luminal values of control tubules were set at 100%. Data are given as mean \pm SE for 24-128 tubules from 9-10 fish (***: significantly different from the control value; $P < 0.001$).

Figure 2: Changes in Mrp2-mediated transport in response to a short exposure to gentamicin (Gent). Tubules were incubated for 30 min in the absence (control) or presence of 10 μ M gentamicin and then transferred to gentamicin-free medium for the indicated time. FL-MTX accumulation in cells and tubular lumens was then determined. At every time point measured, the luminal values of control tubules were set at 100%. Data are given as mean \pm SE for 14-138 tubules from 4-14 fish (***: significantly different from the control value; $P < 0.001$).

Figure 3: Effect of gentamicin exposure on fluorescein (FL) transport. A. Tubules were incubated for 30 min in the absence (control) or presence of different concentrations of gentamicin, after which FL accumulation in cells and tubular lumens was determined. B. Tubules were incubated for 30 min in the absence (control) or presence of 10 μ M gentamicin and then transferred to gentamicin-free medium for the indicated time. FL accumulation in cells and tubular lumens was then determined. Data are given as mean \pm SE for 11-39 tubules from 3 fish (significantly different from the control value: * $P < 0.05$; ** $P < 0.01$).

Figure 4: ET_B receptor, NO, and cGMP are involved in the upregulation of Mrp2 mediated transport of FL-MTX after short-term exposure to gentamicin and recovery. Tubules were incubated for 30 min in the absence (control) or presence of 10 μM gentamicin and then transferred to gentamicin-free medium (recovery) for 24h. As indicated, the recovery medium contained 100 nM of an ET_BR antagonist, RES701-1 (RES), 50 μM of an NOS inhibitor, L-NMMA, or 10 μM ODQ, a soluble guanylyl cyclase inhibitor. FI-MTX accumulation in cells and tubular lumens was then determined. The up-regulation of FL-MTX transport after 24 h recovery caused by 30 min exposure to 10 μM gentamicin was prevented by RES701-1 and L-NMMA to the recovery medium (A). Also ODQ was able to prevent this up-regulation (B). The inhibitors alone had no effect (A,B). The luminal values of the control tubules were set to 100%. Data are given as mean ± SE for 12-48 tubules from 3-6 fish (***: significantly higher than the control value; P<0.001).

Figure 5: Increased luminal plasma membrane Mrp2 protein 24 h after 30 min exposure to 10 μM gentamicin or 10 nM ET-1. (A) Quantitation of luminal plasma membrane immunostaining of Mrp2; (B) Representative image of control tubule; (C) tubule exposed to gentamicin; (D) tubule exposed to ET-1; (E) quantitation of Mrp4 immunostaining. Functional up-regulation of Mrp2-mediated transport was confirmed by an increased expression of Mrp2 in the luminal membrane of the proximal tubule after 24 h recovery of short-term exposure to 10 μM gentamicin or 10 nM ET-1 (C and D, resp.) compared to control tubules (B). In (A) this is semi-quantified. Mrp4, however, was not affected by recovery to short-term exposure to 10 μM gentamicin or 10 nM ET-1 (E). Data are given as mean ± SE for 31-108 tubules from 10-31 fish (**: significantly different from the control value; P<0.01).

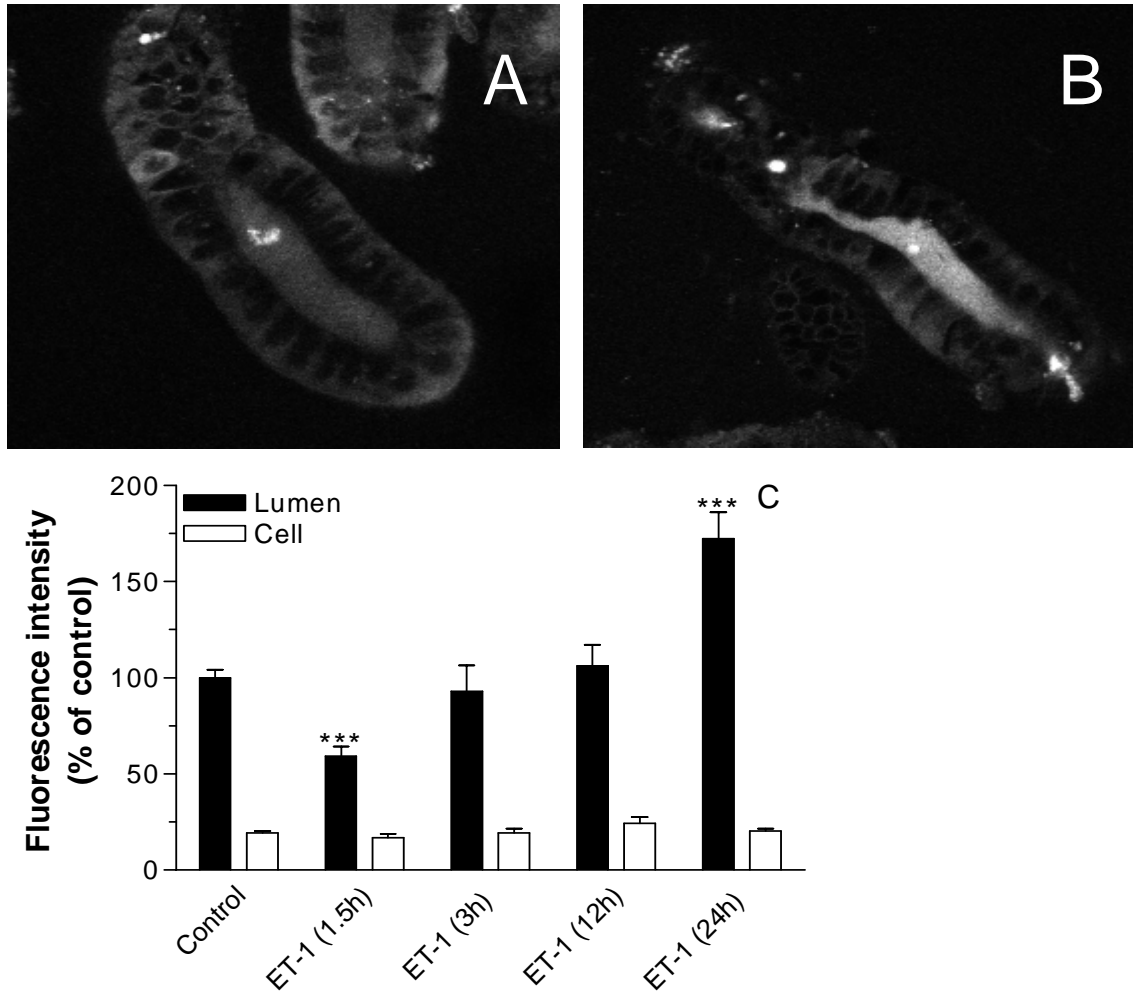
Figure 6: Blocking NOS prevents the increase in Mrp2 immunostaining 24 h after 30 min exposure to 10 μ M gentamicin (A) or 10 nM ET-1 (B). Data are given as mean \pm SE for 12-48 tubules from 12-31 fish (***: significantly higher than the control value; $P < 0.001$).

Figure 7: A short exposure to gentamicin protects against acute gentamicin toxicity 24 h later. Tubules were incubated for 30 min in the absence (control) or presence of 10 μ M gentamicin and transferred to gentamicin-free medium for 24 h. Tubules were then exposed to 100 μ M gentamicin or 1 mM NaCN (positive control) for 2 h. Mitochondrial respiration was measured using Mito Tracker[®] Red CM-H₂XRos. Data are given as mean \pm SE for 10-15 tubules from 3 fish (*: significantly lower than the control value ($P < 0.05$); ***: significantly lower than the control value ($P < 0.001$)).

JPET #89094

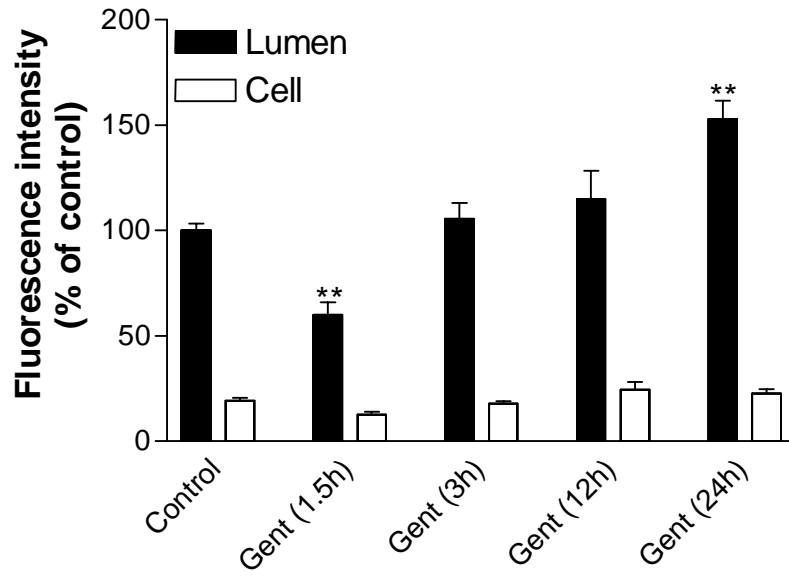
Figures

Figure 1



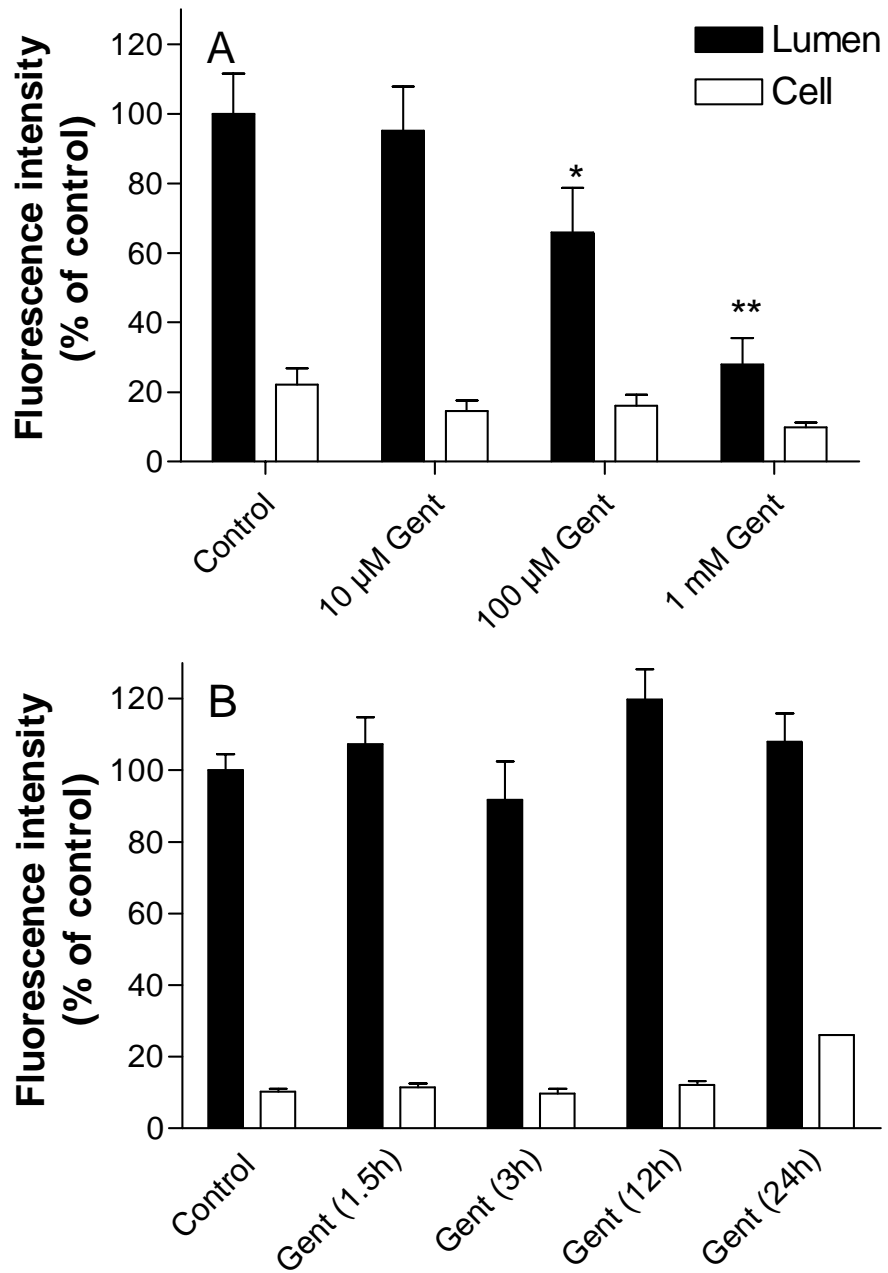
JPET #89094

Figure 2



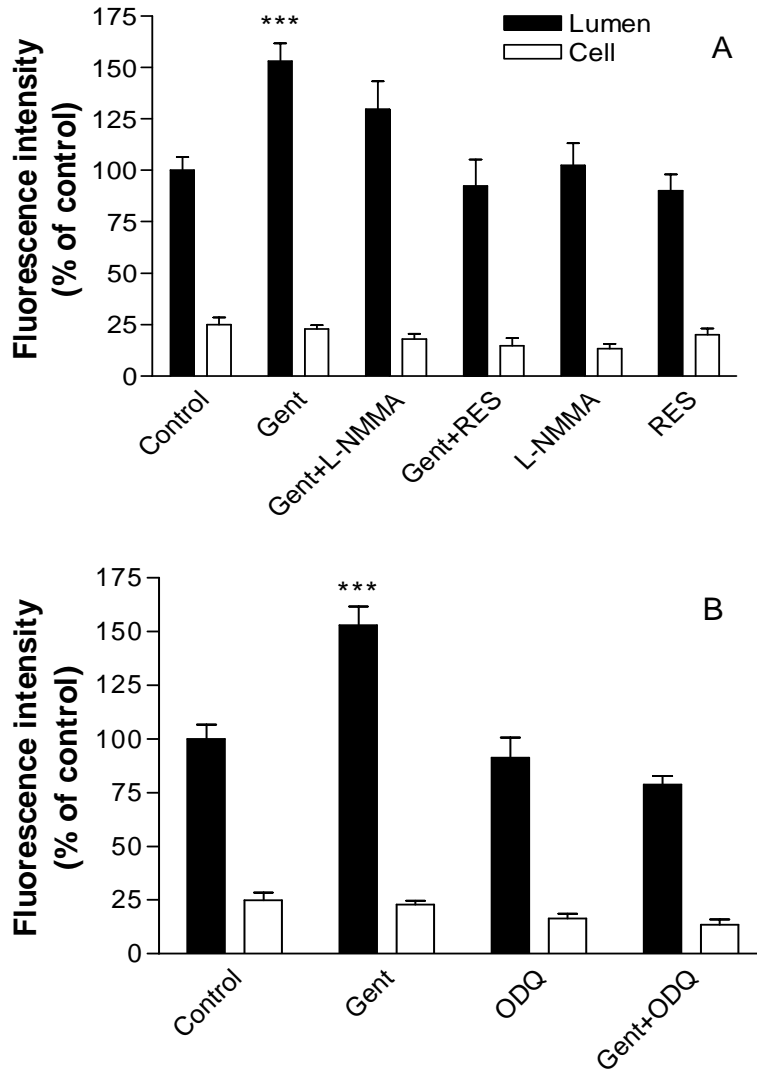
JPET #89094

Figure 3



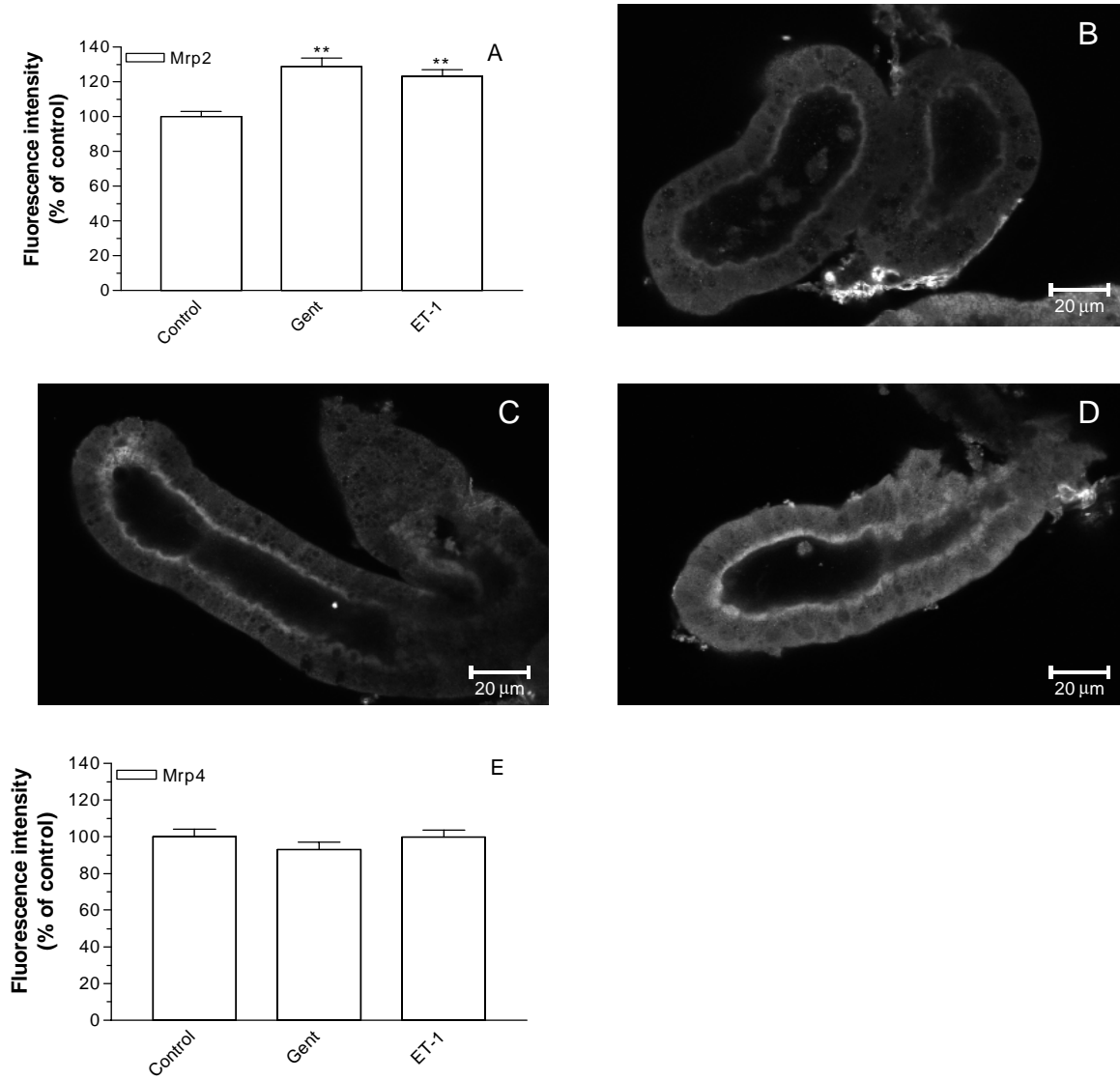
JPET #89094

Figure 4



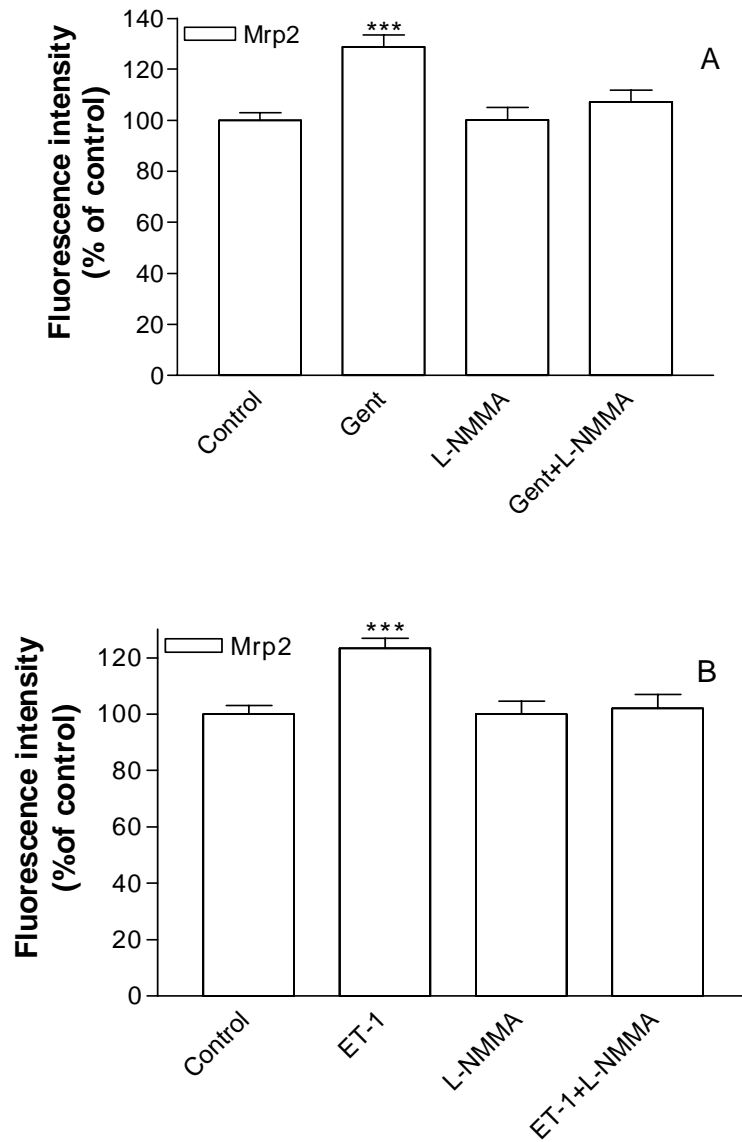
JPET #89094

Figure 5



JPET #89094

Figure 6



JPET #89094

Figure 7

