Increased Apical Insertion of the Multidrug Resistance Protein 2 (MRP2/ABCC2) in Renal Proximal Tubules following Gentamicin Exposure

Sylvia Notenboom, Alfons C. Wouterse, Bram Peters, Leon H. Kuik, Suzanne Heemskerk, Frans G. M. Russel, and Rosalinde Masereeuw

Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Received March 14, 2006; accepted June 2, 2006

ABSTRACT
Multidrug resistance protein (MRP) 2 (MRP2; ABCC2), an organic anion transporter apically expressed in liver, kidney, and intestine, plays an important protective role through facilitating the efflux of potentially toxic compounds. We hypothesized that upon a toxic insult, MRP2 is up-regulated in mammalian kidney, thereby protecting the tissue from damage. We studied the effects of the nephrotoxicant gentamicin on the functional expression of MRP2 in transfected Madin-Darby canine kidney type II (MDCKII) cells and rat kidney. Transport of glutathione-methyl fluorescein by cells or calcein by isolated perfused rat kidney was measured to monitor MRP2 activity. MDCKII cells were exposed to gentamicin (0–1000 M) for either 1 h, 24 h, or for 1 h followed by 24-h recovery. No effect was observed on MRP2 after 1-h exposure. After 24-h gentamicin exposure or after a 24-h recovery period following 1-h exposure, an increase in MRP2-mediated transport was seen. This up-regulation was accompanied by a 2-fold increase in MRP2 protein expression in the apical membrane, whereas the expression in total cell lysates remained unchanged. In perfused kidneys of rats exposed to gentamicin (100 mg/kg) for seven consecutive days, an increase in Mrp2 function and expression was found, which was prevented by addition of a dual endothelin-receptor antagonist, bosentan. We conclude that an increased shuttling of the transporter to the apical membrane takes place in response to gentamicin exposure, which is triggered by endothelin. Up-regulation of MRP2 in the kidney may be interpreted as part of a protective mechanism.

The renal proximal tubule fulfills an important role in the elimination of anionic, cationic, and neutral waste products, varying from endogenous (metabolic) waste products to xenobiotics. One of the transporters involved in the active secretion of organic anions in the preurine is multidrug resistance protein (MRP) 2 (MRP2; ABCC2). MRP2 is located at the brush-border membrane of the proximal tubule (Schaub et al., 1997) and transports a variety of organic anionic conjugates, amphiphilic anions, and neutral substrates (for review, see Van de Water et al., 2005). Low expression or absence of MRP2 causes conjugated hyperbilirubinemia and pigment disposition in the liver, as observed in patients with the autosomal recessively inherited Dubin-Johnson syndrome, partly due to an impaired canalicular secretion of glutathione, glutathione conjugates, and bilirubin glucuronides (Paulusma et al., 1997; Smitherman et al., 2004). We found previously that the renal excretion capacity for a number of known Mrp2 substrates was decreased as well in an Mrp2-deficient rat (Masereeuw et al., 2003).

Functional expression of MRP2 may be influenced by exogenous factors, such as exposure to toxicants, cellular stress, and disease conditions. For example, cholestasis results in a decreased expression of Mrp2 in the liver, whereas the expression of the transporter protein in the kidney is up-regulated (Tanaka et al., 2002). Less dramatic changes in Mrp2 were observed after exposure to the nephrotoxic antibiotic agent gentamicin using a killfish renal model. Exposure to gentamicin results in a rapid reduction in Mrp2,

ABBREVIATIONS: MRP/Mrp, multidrug resistance protein; ABC, ATP-binding cassette; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ balanced salt solution; AM, acetoxymethylester; CMFDA, 5-chloromethylfluorescein diacetate; MK-571, (3-(3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl)-(3-dimethyl-amino-3-oxopropyl)-thio)-methyl(thio)propanoic acid); CDNB, 1-chloro-3,4-nitrobenzene; ET, endothelin; MDCKII, Madin-Darby canine kidney type II; wt, wild type/wild-type; OK, opossum kidney; GS-MF, glutathione-methylfluorescein; LDH, lactate dehydrogenase; WH, Wistar-Hannover; PBS, phosphate-buffered saline; NFDM, nonfat dried milk; E-64, N-(trans-epoxysucciny1)-L-leucine 4-guanidinobutylamide; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gent, gentamicin; WT, wild type.
triggered by an endothelin (ET) signaling pathway (Maser- eeuw et al., 2000; Terlouw et al., 2001; Notenboom et al., 2002, 2004). However, an increase in Mrp2-mediated transport and protein expression was observed in tubules after a 24-h recovery period following a short-term exposure (Noten- boom et al., 2005). Although the killifish model has been proven to be a reliable model (Miller and Pritchard, 1991), the regulatory mechanism of Mrp2 in mammalian kidneys is yet unknown. In addition, it is unclear whether the long-term effect changes during prolonged exposure, i.e., therapy.

Gentamicin is widely used because of its broad-spectrum, low levels of resistance and low cost. Its clinical use, however, is hampered by its nephrotoxic and ototoxic potential (Ben- nett, 1989; Edson and Terrell, 1999). Renal damage is pre- dominantly a result of specific accumulation of gentamicin within the cells lining proximal tubules. The mechanism of nephrotoxicity that is supported by most data starts with binding of gentamicin to acidic phospholipids or megalin at predominantly the brush-border membrane and subsequent endocytosis (Nagai and Takano, 2004). Once inside the cell, gentamicin may be routed to the GoGi apparatus (Sandoval et al., 2000), where it is known to cause disruption of ion gradients across the plasmalemma, including excessive calcium influx (Foster et al., 1992; Ward et al., 2002), reduction of the activity of lysosomal enzymes, and inhibition of membrane-bound transporters (Domínguez et al., 1996; Skopicki et al., 1996; Terlouw et al., 2001). However, in experimental models in general, relatively high doses of gentamicin and/or multiple-day dosing were used to cause toxicity.

In the present study, we used cultured renal cell lines (Madin-Darby canine kidney type II (MDCKII) and opossum kidney (OK) cells) and a rat renal perfusion set-up as model systems to investigate the effect of gentamicin on MRP2/ Mrp2 regulation in more detail. The data show that long- term treatment of renal tubule cells with gentamicin and/or short-term treatment followed by 24 h recovery resulted in significantly higher MRP2/Mrp2-mediated transport and protein expression compared with controls. This functional up-regulation results from an increased insertion of the transport protein in the apical membrane.

Materials and Methods

Chemicals. Gentamicin, fetal bovine serum, and nonessential amino acids were obtained from ICN Biomedicals (Zoetermeer, The Netherlands). Dulbecco’s modified Eagle’s medium (DMEM), with Glutamax-I, 25 mM HEPES, and pyridoxine, and Hanks’ Balanced Salt Solution (HBSS) were purchased from Invitrogen (Breda, The Netherlands). Calcein-acetoxymethylster (AM) and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Invitrogen. N-acetylcysteine (NAC), 3-methyladenine, and gentamicin were procured from Sigma-Aldrich (StLouis, MO). Glucose-6-phosphate dehydrogenase (G6PDH) was purchased from Roche Diagnostics (Mannheim, Germany). Calcein and CMFDA were obtained from Invitrogen. HRP-conjugated secondary antibodies and HRP substrate (TMB) were purchased from Roche Diagnostics (Mannheim, Germany). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma-Aldrich. Gentamicin, fetal bovine serum, and nonessential amino acids were obtained from ICN Biomedicals (Zoetermeer, The Netherlands). Dulbecco’s modified Eagle’s medium (DMEM), with Glutamax-I, 25 mM HEPES, and pyridoxine, and Hanks’ Balanced Salt Solution (HBSS) were purchased from Invitrogen (Breda, The Netherlands). Calcein-acetoxymethylster (AM) and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Invitrogen. N-acetylcysteine (NAC), 3-methyladenine, and gentamicin were procured from Sigma-Aldrich (StLouis, MO). Glucose-6-phosphate dehydrogenase (G6PDH) was purchased from Roche Diagnostics (Mannheim, Germany). Calcein and CMFDA were obtained from Invitrogen. HRP-conjugated secondary antibodies and HRP substrate (TMB) were purchased from Roche Diagnostics (Mannheim, Germany). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma-Aldrich.
rescence intensity (in photomultiplier units) with a calibration curve of spiked samples of blank perfusion fluid with different concentrations of calcein.

**Immunohistochemistry.** MDCKII-MRP2 cells were cultured on glass slides, and confluent cell monolayers were fixed with 1.0% formaldehyde in PBS at room temperature for 20 min. Subsequently, cells were washed in PBS/0.1% Triton X-100 on ice for 5 min twice and in PBS supplemented with 1.5 mg/ml glycine and 5 mg/ml bovine serum albumin for 15 min. Thereafter, cells were incubated with a monoclonal antibody against MRP2 (k22-MRP2, 1:100 diluted; Smeets et al., 2004). After incubation, the washing steps were repeated, and cells were incubated with an Alexa Fluor 488-labeled goat-α-rabbit IgG (1:100 diluted; Invitrogen) for another 90 min at room temperature. For counterstaining, an Alexa Fluor 594-labeled lectin GS-II marker (Invitrogen) was used. After staining, cells were washed again with PBS/0.1% Triton X-100 and PBS and mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Slides were analyzed using a confocal laser scanning microscope (MRC1024; Bio-Rad, Hercules, CA).

**Membrane Isolation and Immunoblotting.** A specific biotinylation and immunoblotting assay was used to detect MRP2 in the apical membrane of MDCKII cells, as described by van Balkom et al. (2002). For this purpose, gentamicin-treated transwell monolayers were used and apical membrane samples were denatured following isolation by incubation in 1× Laemmli buffer for 10 min at 65°C. Samples were subjected to 6% SDS-polyacrylamide gel electrophoresis and transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Hoevelaken, The Netherlands). Reversible staining of the membrane with Ponceau Red was used to confirm transfer of the proteins. Next, the nitrocellulose membrane was blocked in TBS-T (20 mM Tris-HCl, 73 mM NaCl, and 0.15% Tween 20, pH 7.6) containing 5% nonfat dried milk (NFDM), for 1 h at room temperature, after which the membranes were incubated with a primary antibody directed against hMRP2 (k22-MRP2) (Smeets et al., 2004), 1:1000 in TBS-T overnight at 4°C. Subsequently, the membranes were washed twice in TBS-T, blocked in TBS-T containing 5% NFDM, washed twice in TBS-T again, and incubated with affinity-purified horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) diluted 1:5000 in TBS-T for 1 h at room temperature. After washing the membranes twice with TBS-T, Mrp2 proteins were visualized with enhanced chemiluminescence (Pierce Chemical, Rockford, IL). For semiquantification, we measured the pixel intensity of the bands using Scion Image version beta 4.02 for Windows (Scion Corporation, Frederick, MD).

For determination of Mrp2 expression in rats, frozen kidneys were pulverized using a Mikro-dismembrator U (B. Braun Biotech Int., Allentown, PA) set at 2000 rpm for 30 s and quickly dissolved in TS buffer containing protease inhibitors (250 mM sucrose and 10 mM Tris-HCl at pH 7.4, supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μM E-64 (cysteine proteinase inhibitor). After slow defrosting on ice, kidney samples were vortexed and centrifuged for 30 min (12,000 g; 4°C). The supernatant was centrifuged again at 105,000 g for 75 min at 4°C using a Beckman XL-80 ultracentrifuge after which...
Fig. 2. Effect of different gentamicin exposures in MDCKII wild type (wt) and MRP2-transfected MDCKII (MDCKII + MRP2) for either 1 h (A and B), 1 h followed by 24-h recovery (C and D), or 24 h (E and F) on Mrp2-mediated transport. Efflux is increased after 24-h recovery following 1-h exposure to high concentrations of gentamicin in both cell lines. In addition, a similar increase in efflux was observed after 24-h exposure to gentamicin in MDCKII-MRP2 (F) and opossum kidney (OK) cells (G). Values are means ± S.E., n = 8 to 23, where GS-MF efflux was set to 100% in cells exposed to 0 μM gentamicin. Significantly different from 0 μM gentamicin (*, P < 0.05, **, P < 0.01; and ***, P < 0.001).
the pellet containing membrane fraction was dissolved in TS buffer with protease inhibitors. These membrane samples were analyzed using immunoblotting with a monoclonal antibody detecting rat Mrp2 (M III-6, 1:1000; Alexis Biochemicals, San Diego, CA) as described above.

**RNA Isolation and mRNA Expression.** For mRNA isolation, pulverized kidneys were resuspended in TRIzol reagent (Invitrogen). Reverse transcription was performed on 1 μg of total RNA using random primers in a final volume of 100 μl (Reverse Transcription System; Promega, Madison, WI). Synthesized cDNA was used for quantitative real-time polymerase chain reaction (real-time quantitative-PCR) according to the TaqMan protocol in optical tubes (Applied Biosystems, Foster City, CA). Rat Mrp2 and GAPDH were amplified with a Gene Expression Assay (Applied Biosystems; Mrp2: Rn00563231_m1; GAPDH: Rn99999916_s1), in which 12.5 μl of PCR Master Mix, 5 μl of cDNA, 18 μl of each primer, and 5 μM TaqMan with a FAM reporter dye at the 5′ end and a nonfluorescent quencher at the 3′ end were used. The amplification was performed after an initial warm-up phase of 2 min at 50°C for optimal PCR Master Mix activity and 10 min at 95°C, which served as denaturing step. Forty amplification cycles were completed at 95°C for 15 s and 60°C for 1 min. Finally, the cDNA was subjected to reverse transcription-PCR quantification using the ABI Prism 7700 single reporter sequence detection system (Applied Biosystems). All experiments were performed in duplicate.

**Data Analysis.** Data are given as means ± S.E. Mean values are considered to be significantly different when P < 0.05 by use of a two-way analysis of variance corrected for repeated measurements or by a one-way analysis of variance followed by Bonferroni’s multiple comparison test. Software used for statistical analysis was GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego CA) and SPSS version 10 for Windows (SPSS Inc., Chicago, IL).

**Results**

**MRP2 Function and Expression in Cells.** Transport studies with WT MDCKII and MRP2-transfected MDCKII cells were performed to confirm that GS-MF is a good substrate for MRP2. To be confident that MRP2 was routed to the apical membrane, all cell layers used for transport were cultured to confluence (Evers et al., 1998). The transport capacity is expressed as the relative fluorescence intensity in the supernatant compared with the total amount of fluorescence present, measured after 30 min (Fig. 1A). Transport of GS-MF is roughly fourfold increased in MRP2-transfected MDCKII cells compared with WT cells at early time points, and it is still 1.1-fold higher after 30 min. At 10°C, transport of GS-MF is clearly diminished (Fig. 1A). This indicates that a large proportion of the transport measured at 37°C in both cell lines is actively driven. A known inhibitor of MRP-mediated transport, MK-571 (Van de Water et al., 2005), inhibited initial GS-MF efflux in WT and MRP2-transfected cells (Fig. 1B), although not synergistically. Furthermore, another MRP2 inhibitor, the glutathione conjugate of CDNB, decreased GS-MF efflux from WT cells (Fig. 1B).

To determine the effects of different gentamicin treatments on MRP2-mediated transport, we measured initial GS-MF efflux in wells. No significant increases in efflux were observed after 1-h exposure to gentamicin (Fig. 2, A and B). Note that initial transport rates in wild-type cells were less than 10% of the rates of GS-MF efflux observed in transfected cells. When cells were allowed to recover for 24 h, an up-regulation of GS-MF efflux was seen after exposures to 500 and 1000 μM gentamicin (Fig. 2, C and D). After 24-h exposure to several concentrations of gentamicin, a dose-dependent increase in GS-MF transport was observed for both cell lines, which was significant for MDCKII-MRP2 cells (Fig. 2, E and F). Furthermore, another kidney cell line often used for renal drug transport studies, OK cells, showed a similar dose-dependent increase in GS-MF transport upon exposure to gentamicin for 24 h (Fig. 2G). An LDH assay was carried out after the 24-h exposure to gentamicin to ensure that leakage of GS-MF due to toxicity did not occur. LDH levels in the supernatant ranged between 1 and 3.6% for both MDCKII cell types and between 6.8 and 8.0% for OK cells, which suggest that gentamicin does not affect cell viability.

The increase in GS-MF efflux was accompanied by a clear up-regulation of MRP2 in the plasma membrane of the transfected cells after 1-h exposure to 1000 μM followed by 24-h recovery (Fig. 3). After Western blotting, approximately a 2-fold increase (P < 0.05) in Mrp2 protein in the apical membrane was observed (Fig. 4, B and D). The amount of MRP2 in total cell lysates remained unchanged (Fig. 3, A and C), suggesting a functional up-regulation of MRP2 through increased shuttling toward the apical membrane after exposure to gentamicin for 24 or 1 h followed by a recovery period of 24 h. Although the Western blot in Fig. 4D shows an increased trend of MRP2 insertion into the membrane after 1-h exposure to 1000 μM gentamicin, this difference is not statistically proven and is in accordance with the functional data where a similar, although smaller, increased trend in MRP2-mediated transport is observed.

**Mrp2 Function and Expression in Rat Kidney.** Previous studies with isolated perfused kidneys have shown that the urinary excretion of calcine is significantly reduced in mutants transport-deficient rats lacking Mrp2 (TR−) compared with normal rats (Masereeuw et al., 2003). To elucidate the effects of gentamicin, isolated kidneys were exposed to gentamicin for 45 min after which urinary calcine excretion rates were assessed. Figure 5A shows that this short-term exposure did not affect Mrp2 function. In contrast, an
increase in calcein transport was observed after exposing rats to 100 mg/kg gentamicin for seven consecutive days (Fig. 5B). This effect is in agreement with the present findings in cells and our previous studies in killifish renal tubules on the longer term. In killifish renal tubules, intracellular events signaling the increase in Mrp2-mediated transport involved ET release, binding to the ETB receptor, and activation of subsequently nitric-oxide synthase and cGMP (Notenboom et al., 2005). Here, the addition of bosentan, a dual ET receptor antagonist, prevented the up-regulation of Mrp2-mediated transport, whereas bosentan itself did not affect calcein excretion rates (Fig. 5C). Long-term treatment of rats with gentamicin resulted in diminished glomerular filtration rates and fractional water reabsorption (Table 1). These effects were not prevented by bosentan. No changes in glucose transport and alkaline phosphatase excretion were found, suggesting intact proximal tubular functioning, although the decrease in alkaline phosphatase excretion by bosentan alone is unclear.

The increase in calcein transport in perfused rat kidneys was accompanied by an increase in Mrp2 protein expression (Fig. 6, A and B). The addition of bosentan decreased the gentamicin induced up-regulation, which is in agreement with its effect on calcein excretion. Gentamicin exposure did not affect expression of Mrp2 on the mRNA level, excluding de novo synthesis of the transporter protein (Fig. 6C).

Discussion

MRP transporters play an important role in the effective elimination of toxic compounds and metabolites and in the conservation of essential substances. The expression and transport activity of MRPs are highly regulated by signals such as circulating hormones, activation of protein kinases and nuclear receptors, and disease conditions, enabling the kidney to adapt to altered physiological conditions. The adaptive capacity of the kidney contributes to an effective elimination of endogenous compounds, xenobiotics, and their metabolites, thereby reducing their potential toxicity (for review, see Van de Water et al., 2005). In contrast to Prime-Chapman et al. (2005) using a human intestinal cell line (Caco-2), we showed in the present study that the nephrotoxicant gentamicin is able to influence Mrp2-mediated transport and expression in two mammalian models. Exposure to gentamicin for 24 h or 1 h followed by 24 h of recovery in MDCKII cells resulted in an increased transport by both endogenous canine Mrp2 and human MRP2. This induction is due to increased expression of the transporter protein in the apical membrane. These results were confirmed in a whole organ perfusion model, where long-term in vivo exposure to gentamicin resulted in enhanced Mrp2-mediated transport and increased expression of the transport protein as well. In good agreement with previous studies, using killifish renal proximal tubule (Notenboom et al., 2005), these effects were triggered by ET; since receptor blocking prevented this functional up-regulation. The discrepancy with our study and the study by Prime-Chapman et al. (2005) may be caused by organ differences (intestine versus kidney) or the sensitivities of the cell lines used (Caco-2 versus MDCKII).

Two fluorescent substrates were used to monitor Mrp2/ Mrp2 function. Although these substrates are not selective for Mrp2, involvement of other efflux transporters seems highly unlikely. Because the renal cell cultures were grown in wells, basolateral transporters, such as Mrp1, Mrp3, and Mrp6, do not contribute to GS-MF efflux. This also holds true for urinary calcein efflux in perfused rat kidneys. Mrp4, in contrast, is apically expressed and colocalizes with Mrp2 in renal proximal tubule (Van Aubel et al., 2002); however, we showed previously that calcein is not a substrate for this transporter (Masereeuw et al., 2003). Concerning GS-MF efflux, involvement of canine Mrp4 cannot be excluded, but
its contribution is likely to be of minor importance, because increased GS-MF transport is accompanied by an increase in Mrp2 expression upon gentamicin treatment. Moreover, our findings in killifish renal tubules indicated that gentamicin did not affect Mrp4 expression (Notenboom et al., 2005).

In contrast with our previous findings (Terlouw et al., 2001; Notenboom et al., 2002, 2004), we did not observe an initial decrease in MRP2-mediated transport after a short-term exposure to gentamicin. After short-term exposure, concentrations of up to 1000 \( \mu \)M gentamicin in cells or 100 \( \mu \)M in perfused rat kidney did not affect Mrp2-mediated transport. Remarkably, these concentrations were found to be nontoxic for these mammalian models even after long-term exposure, whereas killifish renal proximal tubules already showed

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 11)</th>
<th>100 mg/kg Gentamicin (n = 6)</th>
<th>10 mg/kg Bosentan (n = 4)</th>
<th>Gentamicin + Bosentan (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFR (l/min)</td>
<td>308 ± 16</td>
<td>186 ± 23**</td>
<td>288 ± 22</td>
<td>164 ± 26**</td>
</tr>
<tr>
<td>Diuresis (l/min)</td>
<td>19.0 ± 0.9</td>
<td>23.1 ± 2.3</td>
<td>17.7 ± 1.1</td>
<td>22.5 ± 5.3</td>
</tr>
<tr>
<td>CE (_{\text{Alp}}) (µM PNP)</td>
<td>1.3 ± 0.3°</td>
<td>0.9 ± 0.3°</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>FE(_{\text{glucose}}) (%)</td>
<td>4.0 ± 0.5°</td>
<td>6.9 ± 0.8°</td>
<td>4.3 ± 0.5</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>FR(_{\text{glomer}}) (%)</td>
<td>93.7 ± 0.2</td>
<td>87.0 ± 1.3°</td>
<td>93.8 ± 2.7</td>
<td>85.8 ± 2.6°</td>
</tr>
<tr>
<td>RPP (mm Hg)</td>
<td>81.6 ± 1.6</td>
<td>78.0 ± 1.4</td>
<td>81.0 ± 2.0</td>
<td>81.5 ± 3.8</td>
</tr>
</tbody>
</table>

CE\(_{\text{Alp}}\), cumulative excretion of alkaline phosphatase; FE, fractional excretion; FR, fractional reabsorption; GFR, glomerular filtration rate; PNP, para-nitrophenyl; RPP, renal perfusion pressure.

\( * P < 0.001 \), significantly different from perfused control WH rat kidney.

\( * n = 3 \).
toxic signs after 24-h exposure to 10 μM gentamicin (S. Notenboom, D. S. Miller, F. G. M. Russel, and R. Maseereeuw, unpublished observations). Glomerular filtration rate and fractional water reabsorption were decreased in rat kidney after long-term gentamicin treatment, apparently by an ET receptor-independent mechanism, because bosentan was unable to prevent these effects. However, proximal tubular function remained unaltered, indicating minor toxicity. This large difference in sensitivity between killifish and mammalian models can be explained by species differences and exposure routes. Gentamicin is well known for its nephrotoxicity in various in vitro and in vivo models, and in patients. But it is known that the doses of aminoglycoside antibiotics needed in animals, such as mouse and rat, are higher than the doses described for patients to experience nephrotoxicity (Suzuki et al., 1995). Nevertheless, the mechanisms of toxicity are thought to be similar in all species studied, including humans (Kaloyanides, 1992). In patients, effective serum levels usually range between 10 and 50 μM, which are much lower than the concentrations used in our study. Yet, it should be taken into account that the kidney is an organ that concentrates compounds; therefore, local concentrations of gentamicin in the kidney proximal tubule are likely to be much higher than the serum concentrations.

Longer term exposure to gentamicin resulted in an increase in MRP2-mediated transport, which is, at least in part, due to an increased amount of protein in the apical membrane. The expression and transport activity of MRPs can be modulated by transcriptional and post-transcriptional mechanisms, of which the latter is in favor of MRP2 (Jones et al., 2005). Mechanisms that may lead to an increased apical expression include de novo synthesis of Mrp2, increased insertion of Mrp2 into the apical membrane or a reduced Mrp2 retrieval from the apical membrane. Important signals in these events are provoked by hormones, protein kinases, and nuclear receptors. Gentamicin is known to trigger several signaling molecules involved in the short-term and long-term regulation of Mrp2, including endothelin, nitric oxide, cGMP, and protein kinase C (Masereeuw et al., 2000; Terlouw et al., 2001; Notenboom et al., 2002, 2004). In mammalian hepatocytes, both protein kinase C and protein kinase A have been implicated in bidirectional, regulated trafficking of MRP2 between intracellular stores and the canalicular membrane (Roelofsen et al., 1998; Beuers et al., 2001; Kubitz et al., 2001). This is in accordance with our data, since MRP2 expression was increased in our cell system in the apical membrane, but not in total cell lysates. The absence of changes in the mRNA levels of Mrp2 in rat kidneys supports a similar conclusion. Future studies will be directed to investigate the mechanism of translational regulation of Mrp2 in the kidney upon gentamicin exposure, leading to an increased expression of the protein in the apical membrane.

Up-regulation of Mrp2 in the kidney may be interpreted as part of a protective mechanism, because the efflux pump serves a defensive function through the elimination of potentially harmful compounds. An up-regulation as observed after recovery following short-term gentamicin exposure (Notenboom et al., 2005; this study), after long-term exposure to cadmium (Terlouw et al., 2002), or after ischemia (Laouari et al., 2001) strongly supports the hypothesis that induction of Mrp2 is triggered to prevent (further) tubular injury. The prevention of toxicity due to a second toxic event following a recovery period of a short-term exposure is in support of a protective function as well (Notenboom et al., 2005). Furthermore, Mrp2 expression in the liver of rats was found to be down-regulated during cholestasis, but the expression of the basolateral proteins Mrp1, Mrp3, and Mrp4 was induced as was Mrp2 in the kidney (Pei et al., 2002; Tanaka et al., 2002; Denk et al., 2004). These up-regulated proteins may offer an alternative elimination route for accumulating compounds. In addition, one might argue that subtle changes in Mrp2-mediated transport promote cell survival through Mrp2-me-
diated transport of oxidized glutathione. This shifts the reduced glutathione/oxidized glutathione ratio in favor of protection against oxidative stress (Ballatori et al., 2005). However, it should be noted that induced Mrp2-mediated transport after gentamicin exposure seems to be associated with protection of the proximal tubule, but not of all renal functional parameters, as judged by the impaired glomerular filtration rate and fractional water reabsorption in the perfused kidney.

In conclusion, cultured mammalian renal proximal tubule cell lines exposed to gentamicin for 24 h or for 1 h followed by 24-h recovery showed an increase in Mrp2-mediated transport and protein expression in the brush-border membrane. This is caused by an increased expression of the transport protein in the apical membrane. In perfused rat kidney, a similar functional and expression effect was observed for Mrp2 after prolonged exposure to gentamicin in vivo, in which ET signaling seems to be implicated. Blocking the ET receptor prevented this functional up-regulation. An up-regulation of Mrp2 in the kidney may be interpreted as part of a protective mechanism through enhanced elimination of toxic compounds and metabolites during oxidative stress.

References


Beuers U, Bilzer M, Chittatou A, Kullak-Ublick GA, Keppler D, Paulartner G, and Dombrowski F (2001) Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion se-
cretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology 33:1206–1216.

Denk GU, Soroka CJ, Takeyama Y, Chen WS, Schuetz JD, and Boyer JL (2004) Multidrug resistance-associated protein 4 is up-regulated in liver but down-


ular multispecific organic anion transporter gene causes the Dubin-Johnson syn-

Foster JE, Harpur ES, and Garland HO (1992) An investigation of the acute effect of


ular multispecific organic anion transporter gene causes the Dubin-Johnson syn-

ular multispecific organic anion transporter gene causes the Dubin-Johnson syn-