Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure

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1Institut National de la Santé et de la Recherche MédicaleU-426 and Institut Federatif de Recherche “Cellules Epithéliales,” Faculté Xavier Bichat, 75018 Paris; and 2Unité Propre de Recherche et Enseignement Supérieur 2706, Faculté de Pharmacie, 92290 Chatenay-Malabry, France

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Laouari, Denise, Ruchun Yang, Celine Veau, Isabelle Blanke, and Gérard Friedlander. Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure. Am J Physiol Renal Physiol 280: F636–F645, 2001.—Tubular function is altered in chronic renal failure (CRF). Whether drug secretion by renal tubules is modified in CRF is questioned because of frequent accumulation of various toxins in CRF. This function mainly involves ATP-dependent drug transporters, particularly P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) 2, both present in apical membrane of epithelial cells. The present study was aimed at determining the changes in P-gp and MRP2 expression induced by experimental CRF in kidney and liver. The relationship between MRP2 and glutathione metabolism changes was examined because MRP2 transports GSSG and glutathione conjugates. Rats underwent either 80% subtotal nephrectomy (Nx) or sham operation, and determinations were performed 3 and 6 wk later. CRF induced a 70–200% rise in protein and mRNA expression of MRP2 after 3 and 6 wk post-Nx in remnant kidney and after 6 wk in liver. However, P-gp expression was unchanged by CRF. Relative to whole kidney mass, total MRP2 levels decreased by only 27% in Nx rats whereas total P-gp levels were reduced by 60%. Renal GSSG and total glutathione levels were increased by 30% in Nx rats, but glutathione-S-transferase (GST) activity was normal; liver GSSG levels and GST activity were reduced in Nx rats. In conclusion, CRF resulted in specific overexpression of MRP2 in kidney and liver. This could be an adaptive response to some elevated circulating toxins. The later MRP2 induction and different glutathione changes in liver compared with kidney suggest different mechanisms for MRP2 induction and/or action in these two tissues.

THE PROGRESSION OF CHRONIC renal failure (CRF) is characterized by the development of glomerular and tubular lesions, which is influenced by multiple factors (14). Regarding renal tubules, numerous studies have been performed to understand the pathological process (39), but less is known about changes in tubular function. In the experimental model of subtotal nephrectomy, functional tubular changes are expected because of compensatory renal hypertrophy and elevated filtration rate per remaining nephron (17). This is demonstrated by the increase per nephron of several tubular activities (24, 38, 53). However, at the cellular level, evidence is provided that tubular dysfunction occurs in CRF. We previously showed that CRF induced a reduction in expression and/or activity of several enzymes located in brush-border membranes (BBM) and of type II Na-Pi cotransporter (33). These alterations were shown to be tissue specific and selective because other BBM enzymes such as 5′-nucleotidase were affected little by CRF (33). Other studies also reported reduced expression per cellular unit of other enzymes or transporters present in proximal and distal parts of the nephron (31, 32).

In CRF many waste products accumulate, in part due to reduced GFR, and these compounds are potentially toxic for cellular metabolism (43). Tubular function plays an important role in elimination of endogenous toxins and xenobiotics, but whether this process is altered in CRF is not known. Proximal tubular epithelial cells possess multiple membrane transporters that use ATP or transmembrane ion gradients to drive uphill solute transport from blood to urine (44). P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) 2, also called canalicular multispecific organic anion transporter, are two membrane ATP-dependent transporters that mediate the excretion of various toxins and xenobiotics (30, 48). In cancer cells, these proteins induce multidrug resistance (20, 29). In normal renal tissue, the two proteins are inserted in the BBM (46, 50), a location consistent with their secretory function. P-gp is also detected in BBM of other polarized cells and in plasma membrane of endothelial cells (5, 50). In rodents, P-gp exists as two functional isoforms, which are encoded by two different genes, namely, mdr1a and mdr1b, the latter being more abundant in kidney (6). MRP2 is

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exclusively found in apical membranes of polarized cells (30, 46), i.e., in kidney, intestine, and liver, the latter organ expressing MRP2 at high levels (30, 40). MRP2 has a homolog form called MRP1, which transports the same substrates as MRP2 (30) but is located in the basolateral side of epithelial cells (30, 42). This form, however, is weakly expressed in the proximal tubule (42, 46) and in the liver (30).

Up until now, the physiological role of P-gp and MRP2 remains unclear. Because of their role in drug excretion, we hypothesized that these two transporters could be affected in CRF while uremic toxins accumulate. The present study evaluates the changes in protein and mRNA expression of P-gp and MRP2 in experimental CRF by using the model of subtotal nephrectomy (Nx) in the rat. The determinations were done at two different times after Nx, namely, 3 and 6 wk. The tissue specificity of the response was examined by measuring P-gp and MRP2 expression in kidney by comparison to that in the liver. We found that CRF resulted in enhanced MRP2 but not P-gp expression in both tissues and that this effect occurred later in liver than in kidney. Because MRP2 transports glutathione conjugates and GSSG (30), we determined whether MRP2 changes were associated with changes in glutathione metabolism. We observed that CRF altered several parameters of glutathione metabolism differently in kidney and liver.

METHODS

Experimental Protocol

Animal experimentation was performed in conformity with the National Research Council Guide for the Care and Use of Laboratory Animals (7th ed.; Washington, DC: Natl. Acad. Press, 1996). Adult male Sprague-Dawley rats (120–140 g, Iffa Credo, Saint Aubin Les Elboeuf, France) underwent either subtotal nephrectomy (n = 25) or sham operation (n = 18). Two studies were started at the same time and were stopped after 3 (3-wk study) or 6 wk (6-wk study) postsurgery. Subtotal nephrectomy consisted of 80% renal ablation performed in two steps: in the first step, animals were anesthetized under Nembutal (40 mg/kg); the two poles of the left kidney were excised and weighed to remove 60% of the left kidney mass, which was estimated from measurement of mean kidney weight/100 g body wt (BW) in five control rats. Bleeding was prevented by using collagen powder (Pangen Fournier). The second step was done under ether anesthesia and included removal of the right kidney 4 days later. Sham-operated rats underwent laparotomy and kidney decapsulation in parallel with nephrectomy. Experimental periods began on the day of the second nephrectomy. All Nx and control sham-operated (C) rats were fed ad libitum a standard diet containing 20% casein, 0.6% calcium, 0.45% phosphate, 0.5% potassium, and 0.25% sodium.

A metabolic study was performed at 3 wk after Nx. All rats were placed in metabolic cages to collect urine for 24 h and then were ether anesthetized for the collection of blood. Parameters of renal function were measured, and creatinine clearance (Cc1cr) was calculated. Subsequently, Nx rats were matched into two groups with similar mean Cc1cr; one group (n = 10) was killed 2 days later (3-wk study), and the second group (n = 12) was killed 3 wk later (6-wk study). C rats were also divided into two groups (n = 9 rats/group) having similar mean body weight and were studied in parallel with Nx rats. The rats that were kept for a 6-wk period underwent a second metabolic study at the end of the experimental period to measure parameters of renal function. Metabolic studies showed no significant difference in the amount of food ingested between Nx rats and C rats.

Euthanasia was performed by injection of Nembutal (50 mg/kg BW ip). The abdomen was opened, and the two kidneys for C rats or the remnant kidney for Nx rats was clamped and rapidly excised and weighed. The remnant kidney was cut on a sagittal plane, and half was used to prepare BBM to perform Western blot analyses for measuring P-gp and MRP2 expression. The other half was frozen in liquid N2 to be analyzed later. A similar protocol was used for each kidney of C rats. A lobe of liver was excised and frozen in liquid N2. The remaining liver tissue was weighed. All frozen tissues were pulverized in liquid N2 by using a Spex 6700 Freezer (Mill Industries, Edison, NJ). They were kept in liquid N2, and different aliquots were used for 1) RNA extraction for measuring mRNA expression of MRP2 and mdr1b, the main form of P-gp, along with measurement of mRNA expression of γ-glutamyl transpeptidase (γ-GT), which is involved in glutathione catabolism (8), and of the heavy subunit of γ-glutamyl-cysteine synthase (γ-GCS-HS), which is the limiting enzyme of glutathione synthesis (35); 2) crude membrane (CM) extraction from liver samples to perform Western blot analyses; 3) measurement of total glutathione and GSSG content and calculation of the oxidized-to-total glutathione ratio, which is an index of magnitude of oxidative stress (37); and 4) measurement of specific activity of γ-GT and of glutathione transferase, as the latter is involved in formation of glutathione conjugates (21). Parameters of glutathione metabolism were measured only in the 6-wk study because the amount of renal tissue was too small in the 3-wk study.

Techniques

Plasma and urine parameters were measured on a Hitachi automatic analyzer (Boehringer Mannheim, Meylan, France).

Western blot analyses. BBM from either whole kidney or half of the remnant kidney were prepared by Mg2+ precipitation (2). Renal tissue was homogenized in 300 mM mannitol buffer (pH 7.1) in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMsF). The homogenate was centrifuged at 2,500 g for 5 min, and supernatants were kept at −80°C until analyses. To assess that BBM recovery was not affected by CRF, BBM enrichment was compared in Nx and C rats at the two time periods after Nx. This was obtained by measuring γ-GT specific activity, as previously described (33), in homogenates and BBM and calculating the ratio of BBM to homogenate γ-GT specific activity. The ratios were comparable in C and Nx rats in the 3-wk study (10 ± 1 vs. 9 ± 1) and in the 6-wk study (10 ± 1 vs. 10 ± 1), thus confirming similar BBM recovery in Nx and C rats. CM from liver tissue were prepared by homogenizing 150 mg liver powder in 1.5 ml of isotonic medium containing 0.25 M sucrose, 10 mM Tris, 1 mM Na2EDTA, and 0.2 mM PMsF (pH 7.5). Homogenates were centrifuged at 1,000 g for 5 min, and supernatants were centrifuged again for 15 min at 2,500 g. The pellet was suspended in 0.5 ml buffer containing 10 mM Tris and 1 mM Na2EDTA (pH 7.5) and a cocktail of protease inhibitors.
Protein concentration in renal BBM and liver CM samples was determined by using Pierce Coomassie reagent.

Protein MR2 and P-gp expression was determined by immunoblotting. The protein expression of 5'-nucleotidase was measured in parallel as it is a BBM marker, which has been shown to be unaffected by CRF (33). For each individual rat tissue, 30 μg of renal BBM or liver CM proteins were analyzed after incubation for 10 min in loading buffer containing 2% SDS, 10% glycerol, 0.5 mM EDTA, and 2% β-mercaptoethanol. Proteins were electrophoresed on 6.8% SDS-PAGE gel and were electrotransferred onto nitrocellulose membranes. The membranes were blocked in PBS buffer containing 0.05% Tween 20 (PBST) and 10% skim dried milk (SDM) and incubated overnight by using appropriate antibodies. For MR2, a rabbit polyclonal antibody (Ab) against the COOH-terminal part of the protein, kindly provided by Dr. D. Keppler (46), was used at a dilution of 1:4,000 in PBST buffer. This Ab recognizes the two renal P-gp isoforms (18).

For detection of P-gp protein, the monoclonal mouse C 219 Ab (Valbiotech) was used at 1:100 dilution in 7.5% SDM-PBST buffer. For 5'-nucleotidase detection, a rabbit polyclonal Ab against rat kidney 5'-nucleotidase, kindly provided by Dr. B. Kaiserling (11), was used at a 1:5,000 dilution. After incubation with primary Ab, the membranes were washed in PBST buffer and incubated for 90 min with secondary Ab diluted in PBST buffer containing 7.5% SDM. Anti-rabbit IgG from donkey or anti-mouse IgG from sheep linked to peroxidase was used as secondary Ab. Ab binding was revealed by chemiluminescence using enhanced chemiluminescence Western blot reagents (Amersham). The intensities of bands were quantified by densitometric scanning and integrated by a National Institutes of Health program.

mRNA quantification. RNA was extracted from kidney or liver powders by using a RNA Plus Kit (Quantum, Montreuil, France) on the basis of the phenol-guanidium thiocyanate method. mRNA expression was quantified by using RNase protection assay as previously described (33). Antisense riboprobes were labeled with 32PUTP and generated by in vitro transcription of cDNA fragments inserted in plasmids containing SP6 or T7 RNA polymerase promoters. For MR2, P-gp, and γ-GCS-HS, cDNA fragments were generated from rat kidney RNA by RT-PCR using specific primers. cDNA fragments (PCR products) coding for MR2 (251 bp) and for γ-GCS-HS (287 bp) were cloned into PGEM easy vector (Promega), which was linearized by ApcI. Plasmid linearization produced a riboprobe of 378 bp and a protected fragment of 251 bp for MR2 and a riboprobe of 414 bp and a protected fragment of 287 bp for γ-GCS-HS. A cDNA fragment (261 bp) of mdr1b coding for the main renal form of P-gp was cloned into PTag vector (RD Systems) and linearized by Bsu36I to produce a riboprobe of 158 bp and a protected fragment of 137 bp. PGEM-4 plasmid containing γ-GT was cut by Psp8I to obtain a riboprobe of 373 bp and a protected fragment of 353 bp. To perform the RNase protection assay, 30 or 20 μg of total RNA extracted from kidney or liver of each animal were hybridized overnight at 50°C with 3 × 106 cpm of each riboprobe (where cpm is counts/min), and cohybridization was done with GAPDH or β-actin riboprobes, which were used as internal standards. GAPDH mRNA was used as the internal standard in kidney because it was not affected by CRF (33) whereas β-actin was used as the internal standard in liver because GAPDH mRNA tended to increase in liver of Nx rats (D. Laouari, unpublished observations). The radioactive mRNA hybrids were separated by electrophoresis after RNAse digestion, and the radioactive bands corresponding to the protected fragments were quantified by electronic autodiarography (Instant Image, Packard).

Measurement of glutathione metabolism. Total glutathione and GSSG contents were determined enzymatically by the recycling method of Tietze (51). A 150-μg frozen sample of kidney or liver was homogenized by using a Ultra-Turrax homogenizer in 0.5 ml of M perchloric acid solution containing 2 mM EDTA. A 50-μl sample was kept for protein measurement, and the homogenate was kept protected from light at 5,000 g; from the resulting supernatant, 50 μl were kept and further diluted at 1:100 for measuring total glutathione content whereas the remainder was immediately mixed with 100 μM N-ethylmaleimide (NEM; 4 vol/1vol) and incubated for 60 min. NEM immediately forms a complex with GSH, thus avoiding spontaneous conversion into an oxidized form. The mixture was then neutralized with 3 M KOH/0.2 M MOPS buffer, and a clear extract was obtained after centrifugation. The supernatant was extracted 10 times with 2 vol of ether to eliminate NEM. The excess of ether was removed under vacuum. The resultant extract and the diluted non-treated supernatant were analyzed for GSSG and total glutathione content, respectively. Because NEM traces may inhibit enzymatic reaction, values for GSSG were corrected by the percentage of recovery of the known amount of GSSG added to duplicate samples. This percentage ranged between 70 and 90%. A sample (0.1 ml) was mixed with a reaction mixture (1 ml) containing 0.1 M KH2PO4/K2HPO4 (pH 7), 0.06 mM 5,5-dithio(2-nitrobenzoic acid), and 0.12 U/ml glutathione reductase. The reaction was initiated with NADPH (0.2 mM), and the changes in optical density at 412 nm over 5 min were followed. Standards were prepared from GSSG (0–10 μM). All data are expressed as nanomoles of equivalent GSH per milligram protein.

Cytosolic glutathione transferase activity was measured according to Habig’s method (21), and the samples were prepared as described by Benson et al. (1). Briefly, 100 mg of frozen powder were homogenized in 1 ml of sucrose buffer (0.25 M, pH 7.4) by using a Teflon Potter, and the homogenate was centrifuged for 5 min at 20,000 g. The supernatant was mixed with 0.2 vol of 0.1 M CaCl2 and incubated in ice for 30 min. The mixture was centrifuged for 30 min at 20,000 g, and the pellet containing the microsomal fraction was discarded. The supernatant was analyzed for protein content and for glutathione transferase activity; two different substrates were used, namely, dichlororibonobenzene (DCNB) and chlorodinitrobenzene (CDNB), which give measurements at initial rates and at maximal velocity (Vmax), respectively. A sample (0.1 ml) was mixed with either 1 mM DCNB plus 5 mM GSH in neutral 0.1 M KH2PO4 buffer or 1 mM CDNB plus 1 mM GSH in acidic KH2PO4 buffer (pH 6.5). The reaction was started by addition of the sample, and the changes in optical density at 340 nm were followed over 5 min. Linearity was observed by using 25 μg liver protein and 100 μg kidney protein for the DCNB assay and 100 μg liver protein for the CDNB assay. The activity was calculated from the molar extinction coeffi-
cient of the reaction product with DCNB (Δe = 8.5 mM) or CDNB (Δe = 9.6 mM) and expressed as milliunits or nanomoles per minute per milligram protein.

The activity of γ-GT, expressed in units or micromoles per minute per milligram protein, was measured in homogenates of C and Nx rats as previously described (33).

Expression of Results and Statistical Analyses

All data are expressed as means ± SE. Data of Western blot analyses are expressed in arbitrary units (au) of densitometry/30 μg protein. Data of mRNA abundance are expressed as the ratio of radioactive counts of each protected fragment to radioactive counts of protected fragment of either GAPDH (kidney) or β-actin (liver) used as internal standards. For statistical comparisons, the data of each study (3 or 6 wk) were considered separately because measurements were not done at the same time. Comparisons between Nx and C groups were done by using the unpaired t-test. P values <0.05 were considered significant.

RESULTS

Renal function, as expected, was impaired in Nx rats (Table 1) at 3 wk and further altered at 6 wk: plasma creatinine levels rose to 165% of control values at 3 wk and to 222% at 6 wk. Inversely, ClCr/100 g BW was reduced to 47% of C values at 3 wk and to 34% at 6 wk. Plasma urea levels were even more affected: they increased to 288 and to 405% of C values at 3 and 6 wk, respectively. The remnant kidney weight /100 g BW was reduced by 16% in Nx rats compared with C rats at 3 wk but it did not differ between the two groups at 6 wk (Table 1).

As illustrated in Fig. 1, renal P-gp expression was affected little by CRF. Protein P-gp expression in renal BBM (au of densitometry/30 μg BBM protein) was comparable in Nx and C rats at 3 and 6 wk; mRNA expression of P-gp did not differ between Nx and C rats at 3 wk but significantly rose above C values at 6 wk.

Table 1. Renal function and morphological data at 3 and 6 wk after Nx

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<th>3 Wk After Nx</th>
<th>6 Wk After Nx</th>
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<tr>
<td></td>
<td>C (n=9)</td>
<td>Nx (n=10)</td>
</tr>
<tr>
<td>Plasma creatinine, μM</td>
<td>40 ± 1</td>
<td>66 ± 2*</td>
</tr>
<tr>
<td>Plasma urea, mM</td>
<td>5.0 ± 0.4</td>
<td>14.4 ± 1.0*</td>
</tr>
<tr>
<td>ClCr, ml-min⁻¹ 100 g BW⁻¹</td>
<td>0.58 ± 0.03</td>
<td>0.27 ± 0.01*</td>
</tr>
<tr>
<td>BW, g</td>
<td>272 ± 9</td>
<td>244 ± 4*</td>
</tr>
<tr>
<td>Kidney weight, g/100 g BW</td>
<td>0.38 ± 0.02</td>
<td>0.49 ± 0.02*</td>
</tr>
<tr>
<td>Liver weight, g/100 g BW</td>
<td>4.43 ± 0.23</td>
<td>3.71 ± 0.19*</td>
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</table>

Values are means ± SE, n. No. of rats/group; Nx, nephrectomized; C, control; ClCr, creatinine clearance over 24 h; BW, body wt. Kidney weight is given for 1 kidney in C rats and for the remnant kidney in Nx rats. Significant differences between Nx and corresponding C rats, *P < 0.01.

(P < 0.05). Liver P-gp expression (Fig. 2) was unaffected by CRF at both periods in terms of protein (Fig. 2A) or mRNA levels (Fig. 2B). At variance with P-gp, MRP2 expression increased in kidney and liver of Nx rats (Figs. 3 and 4). However, the changes in MRP2 expression were not parallel in the two tissues: in kidney, protein MRP2 expression (au of densitometry/30 μg BBM protein) was increased in Nx rats to 224 and to 182% of C values at 3 and 6 wk, respectively (P < 0.001). Kidney mRNA expression also significantly increased in Nx rats to 165% of C values at 3 wk and to 300% of C values at 6 wk. In liver, protein MRP2 expression did not differ significantly between Nx and C rats at 3 wk whereas it increased in Nx rats at 6 wk to 170% of C values (P < 0.001). Liver mRNA expres-
sion was significantly higher in Nx than in C rats at 3 and 6 wk post-Nx ($P < 0.01$).

As indicated in Table 2, 5'-nucleotidase protein expression (au of densitometry/30 $\mu$g protein) was unaffected by CRF in renal BBM as in liver CM for each period considered. This confirms the specificity of Nx-induced changes in renal and liver MRP2 expression.

The consequences of reduced renal mass on renal expression of the two transporters were evaluated by calculating protein expression of each transporter for the whole kidney mass, also named total transporter levels. As shown in Table 3, the amount of BBM protein for the whole kidney was reduced by 50% or more after Nx. By contrast, total renal MRP2 levels (Table 3) were not significantly reduced (~27%) at 6 wk post-Nx. On the other hand, total renal P-gp levels were reduced by ~65% and by 55% at 3 and 6 wk, respectively. By comparison, total liver MRP2 levels were comparable in Nx and C rats at 3 wk whereas they increased by 60% in Nx compared with C rats at 6 wk. Total liver P-gp levels were unaltered in Nx compared with C rats.

Tables 4 and 5 show several parameters of glutathione metabolism determined in the 6-wk study. Renal total glutathione content per milligram protein was increased by 28% in Nx compared with C rats ($P < 0.01$). Renal GSSG content per milligram protein was increased in the same proportion so that the ratio of GSSG to total glutathione was unchanged by CRF.
Renal glutathione transferase activity was similar in Nx and C rats (Table 4). At variance with kidney, liver total glutathione content was unchanged by Nx and liver GSSG content was significantly reduced in Nx compared with C rats ($P < 0.05$, Table 4). Consequently, the ratio of GSSG to total glutathione in liver was also significantly lower in Nx rats ($P < 0.05$). The activity of glutathione transferase in liver was significantly reduced in Nx compared with C rats ($P < 0.05$) regardless of the substrate used (Table 4). Table 5 indicates the renal changes in the two main enzymes involved in catabolism and synthesis of glutathione. Renal γ-GT activity and mRNA expression (Table 5) were reduced by half in Nx compared with C rats. Also, renal γ-GCS-HS mRNA expression was reduced by 48% in Nx rats.

### DISCUSSION

The present study shows that MRP2, a multidrug transporter present in BBM of secretory epithelial cells, was upregulated in CRF, at the two time points in the study in the remnant kidney and after a longer duration of CRF in liver of Nx rats. The specificity of these changes was demonstrated by the lack of changes in protein expression of 5′-nucleotidase, an enzyme that is considered as a BBM marker, and the unchanged protein expression of P-gp, another drug transporter that is colocalized and shares common substrates with MRP2 (7, 16). There was a rough parallelism between protein and mRNA changes, suggesting that changes in mRNA stability and/or transcriptional process were in part involved in those of MRP2 expression.

The finding that MRP2 expression increased not only in remnant kidney but also in liver suggests that the response may be mediated, at least partly, by circulating factors. In the liver, increased MRP2 protein expression was seen only after 6 wk post-Nx when renal function was aggravated, as indicated by the decrease in creatinine clearance (Table 1). Renal deterioration is closely associated with increased uremic toxicity, which is characterized by accumulation of end products of protein metabolism and various hormonal peptides (43). In our study, plasma urea levels, usually considered as an index of uremic toxicity, were increased by twofold between 3 and 6 wk after Nx, suggesting worsening of uremic toxicity. Because MRP2, like P-gp, is involved in the defense of tissues against the stress caused by drugs and toxins, it is conceivable that some endogenous uremic toxins may induce MRP2 expression. This is suggested by in vitro and in vivo studies that showed MRP2 is inducible in terms of mRNA and/or protein levels by various drugs, such as chemical carcinogens, protein synthesis inhibitors, chemotherapeutic agents, barbiturates, and even corticosteroids (13, 26). Studies on MRP2 promoter have identified several sequences that mediated MRP2 gene induction and contained putative binding sites for transcriptional factors (27).

In our study, the lack of parallelism in MRP2 changes between kidney and liver suggests that the factors involved in MRP2 induction may differ between

### Table 2. Protein expression of 5′-nucleotidase in renal brush-border and liver crude membranes at 3 and 6 wk after Nx

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<th>3 Wk After Nx</th>
<th>6 Wk After Nx</th>
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<tr>
<td></td>
<td>C ($n = 9$)</td>
<td>Nx ($n = 10$)</td>
</tr>
<tr>
<td>Renal BBM 5′Nu,</td>
<td>1.017 ± 0.49</td>
<td>1.097 ± 0.54</td>
</tr>
<tr>
<td>C ($n = 9$)</td>
<td>1.044 ± 0.56</td>
<td>1.045 ± 0.48</td>
</tr>
<tr>
<td>Liver CM 5′Nu,</td>
<td>612 ± 32</td>
<td>649 ± 31</td>
</tr>
<tr>
<td>C ($n = 9$)</td>
<td>726 ± 62</td>
<td>785 ± 60</td>
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Values are mean ± SE expressed as in arbitrary units (au) of densitometry/30 μg protein from renal brush-border membranes (BBM) or liver crude membranes (CM). $n$, No. of rats/group; 5′Nu, 5′-nucleotidase. There was no difference between C and Nx rats at each period.
Differential modulation of drug transporters in uremia

Table 3. MRP2 and P-gp protein expressions in whole kidney and liver mass at 3 and 6 wk after Nx

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<th>3 Wk After Nx</th>
<th>6 Wk After Nx</th>
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<tr>
<td></td>
<td>C (n = 9)</td>
<td>Nx (n = 11)</td>
</tr>
<tr>
<td>Renal BBM protein, mg/100 g BW</td>
<td>1.42 ± 0.08</td>
<td>0.47 ± 0.04†</td>
</tr>
<tr>
<td>Liver CM protein, mg/100 g BW</td>
<td>138 ± 7</td>
<td>104 ± 5†</td>
</tr>
<tr>
<td>Total renal MRP2, au/100 g BW × 10⁻²</td>
<td>183 ± 19</td>
<td>132 ± 11†</td>
</tr>
<tr>
<td>Total renal P-gp, au/100 g BW × 10⁻²</td>
<td>397 ± 37</td>
<td>145 ± 16†</td>
</tr>
<tr>
<td>Total liver MRP2, au/100 g BW × 10⁻²</td>
<td>1,761 ± 420</td>
<td>1,791 ± 328</td>
</tr>
<tr>
<td>Total liver P-gp, au/100 g BW × 10⁻²</td>
<td>1,444 ± 213</td>
<td>1,346 ± 130</td>
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</table>

Values are means ± SE, n. No. of rats/group; MRP2, multidrug resistance protein 2; P-gp, P-glycoprotein. Renal BBM protein mass is for 2 kidneys in C rats and for the remnant kidney in Nx rats; this is the product of kidney weight/100 g BW (Table 1) by renal BBM protein amount/g kidney sample used for BBM preparation. Liver CM protein mass is the product of liver weight/100 g BW (Table 1) by liver CM protein amount/g liver sample used for CM preparation. Total MRP2 and P-gp levels are expressed in au of densitometry/100 g BW; they were calculated from the values given in Figs. 1–4 (au/30 μg protein) and values for renal BBM or liver CM protein mass given above. Significant differences between Nx and corresponding C rats: *P < 0.05, †P < 0.01.

Table 4. Total and oxidized glutathione levels and glutathione transferase activity in kidney and liver in the 6-wk study

<table>
<thead>
<tr>
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<th>Kidney</th>
<th>Liver</th>
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<tr>
<td></td>
<td>C (n = 9)</td>
<td>Nx (n = 11)</td>
</tr>
<tr>
<td>GSH + GSSG, nmol/mg protein</td>
<td>19.4 ± 0.4</td>
<td>26.2 ± 1.0†</td>
</tr>
<tr>
<td>GSSG, nmol/mg protein</td>
<td>0.28 ± 0.02</td>
<td>0.36 ± 0.03†</td>
</tr>
<tr>
<td>GSSG/GSH, × 100</td>
<td>1.43 ± 0.09</td>
<td>1.41 ± 0.15</td>
</tr>
<tr>
<td>GST activity, mU/mg protein</td>
<td>3.10 ± 0.30</td>
<td>2.30 ± 0.20†</td>
</tr>
<tr>
<td>+CDNB</td>
<td>129 ± 5</td>
<td>131 ± 7</td>
</tr>
<tr>
<td>+DCNB</td>
<td>NA</td>
<td>NA</td>
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Table 5. Activity and mRNA abundance of γ-glutamyl transferase and mRNA expression of γ-glutamyl-cysteine synthase heavy subunit in kidney of C and Nx rats in the 6-wk study

<table>
<thead>
<tr>
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<th>C (n = 9)</th>
<th>Nx (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-GT activity, U/mg protein</td>
<td>1.70 ± 0.10</td>
<td>0.80 ± 0.09†</td>
</tr>
<tr>
<td>γ-GT mRNA, relative radioactivity</td>
<td>3.85 ± 0.16</td>
<td>1.83 ± 0.16†</td>
</tr>
<tr>
<td>γ-GCS-HS mRNA, relative radioactivity</td>
<td>0.92 ± 0.08</td>
<td>0.48 ± 0.05†</td>
</tr>
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</table>

Values are means ± SE, where γ-glutamyl (GT) activity is expressed in U/ per mg protein, where 1 U is 1 mol/min of product. mRNA abundance of each enzyme is expressed as the ratio of radioactivity corresponding to mRNA hybridized to the enzyme riboprobe to radioactivity corresponding to mRNA hybridized to the glyceraldehyde-3-phosphate dehydrogenase riboprobe. n. No. of rats/group; γ-GCS-HS, γ-glutamyl-cysteine synthase heavy subunit. Significant differences between Nx and C rats: *P < 0.05, †P < 0.01.

the two tissues. Two explanations may be proposed. One possibility is that MRP2 expression in remnant kidney is stimulated synergistically by circulating toxins and local factors, thus resulting in greater and earlier MRP2 overexpression than in liver. Renal hypertrophy after Nx is associated with production of various cytokines and growth factors that may affect gene transcription (28, 49). In particular, activator protein-1 expression was shown to be stimulated in CRF (49), and a regulatory element, PEA3, located in MRP2 promoter is known to be activated by this factor (27). Another possibility is that the nature of toxins considered as potential inducers of MRP2 expression differs between kidney and liver. Some specific toxins associated with worsening of renal deterioration could be preferentially metabolized and transported by the liver and thus enhance liver MRP2 expression. As an example, cisplatin, which is transported by MRP2 (7) and is eliminated mainly by the kidney (10), was shown to induce MRP2 expression in the kidney but not in the liver of cisplatin-treated rats (12).

We hypothesized that the changes in MRP2 expression in CRF could be associated with alterations in glutathione metabolism. First, MRP2 induction could directly affect GSSG levels because this metabolite is transported by MRP2 (30). Second, GSH, which represents >95% of total glutathione (51, Table 4), could be facilitated in remnant kidney. This effect might be associated with worsening of renal deterioration could be preferentially metabolized and transported by the liver and thus enhance liver MRP2 expression. As an example, cisplatin, which is transported by MRP2 (7) and is eliminated mainly by the kidney (10), was shown to induce MRP2 expression in the kidney but not in the liver of cisplatin-treated rats (12).

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Differential Modulation of Drug Transporters in Uremia

In conclusion, the specific increase in MRP2 expression in remnant kidneys and liver of CRF rats suggests a role for this multidrug transporter in enhanced excretion of xenobiotics and toxins transported by MRP2 was nearly preserved in our Nx model and that liver unlikely compensated for kidney in MRP2 effects. By contrast, total renal protein Pgp levels (au/100 g BW) were reduced by ~25% in Nx compared with control rats at 3 wk post-Nx. A nonsignificant difference in total renal MRP2 was seen at 6 wk post-Nx (Table 3) whereas total liver MRP2 levels significantly increased at this point. This suggests that the capacity of remaining tubular mass for excreting xenobiotics and toxins transported by MRP2 was nearly preserved in our Nx model and that liver unlikely compensated for kidney in MRP2 effects. Changes in P-gp and MRP2 levels could also affect the pharmacokinetics of administered drugs that are transported by either P-gp or MRP2. This is demonstrated experimentally for the cardiac glycoside digoxin, which is a substrate of P-gp (47), and the anticancer drug methotrexate, which is a substrate of MRP2 (36). The elimination of digoxin is reduced in mice invalidated for P-gp (mdr1a−/− mice) (47), and the excretion of methotrexate is decreased in mutant rats deficient for MRP2 (EHBR rats) (36). In patients with CRF, the pharmacokinetics of digoxin and of methotrexate are altered, as indicated by a reduction in the total clearance of these drugs (3, 4, 15, 45). These effects are attributable to impaired renal excretion, which is the major route of elimination of these drugs and involves both glomerular filtration and tubular secretion (15, 45). A significant correlation was found between GFR and digoxin or methotrexate clearance, but, interestingly, the contribution of GFR to the total clearance of these drugs decreased with the severity of CRF (3, 4). This suggests that extrarenal clearance of digoxin and methotrexate may increase in CRF. In addition, the proportion of tubular secretion to renal excretion could be augmented. Therefore, it may be speculated that a shift in digoxin and methotrexate excretion from kidney to other tissues may occur in CRF. This could involve an increased secretion of these drugs in nonrenal tissues, mediated by higher P-gp and MRP2 levels. Further studies are needed to support this hypothesis.

In our study, mRNA expression of γ-GCS-HS was reduced in remnant kidneys. As this parameter was shown to vary in proportion to γ-GCS activity (35), increased GSH synthesis cannot account for the elevation of renal glutathione levels observed in our study. However, there was a severe reduction in renal γ-GT activity and expression in CRF. This effect may contribute to increased GSH levels in CRF because recent studies showed a prooxidant effect of γ-GT whereas its inhibition induced a rise in intracellular GSH levels (9). Also, other pathways not investigated here could play a role in renal GSH elevation. As for GSH, GSSG levels increased in remnant kidneys, but the glutathione redox ratio (GSSG/GSH + GSSG) remained constant. The maintenance of this ratio is critical for normal cellular function (19, 25), and its increase under oxidative stress (37) may lead to cell injury (19, 22, 37). The glutathione redox ratio mainly depends on the reversible conversion of GSH to GSSG, but enhanced GSSG efflux may also contribute to maintain the steady-state level of GSSG (25). Therefore, the unchanged glutathione redox ratio despite higher GSSG levels in remnant kidneys suggests that GSSG produced from GSH by antioxidant reactions was efficiently recycled to GSH, and/or excess of GSSG may be prevented by MRP2-mediated efflux of GSSG. Our data agree with those of Nath et al. (38), who showed the maintenance of the glutathione ratio in CRF, except when rats were fed high-protein diets.

MRP2 expression increased in both kidney and liver at 6 wk post-Nx, but different changes in glutathione metabolism were seen in the two tissues. At variance with kidney, liver glutathione levels were unchanged by CRF, but liver GSSG levels, glutathione ratio, and glutathione transferase activity were reduced by CRF. This suggests that the formation of glutathione conjugates may be reduced in liver of CRF rats and could be a limiting factor for MRP2-mediated efflux of these compounds. The lowering of liver GSSG levels is in agreement with MRP2 induction, but the lack of functional studies preclude an assessment that MRP2 was directly responsible for this effect.

Contrasting with MRP2 induction, P-gp expression was affected little or was unaffected by CRF. P-gp is colocalized to, and shares common substrates with, MRP2 (16). These two proteins have ~20% homology, which is mainly confined to their ATP domains and characterizes the ABC family, i.e., ATP-dependent function (52). In addition, the expression of the two transporters can be induced in vivo or in vitro by the same drugs (12, 26), which could act on the same regulatory sequences of gene promoter (27). However, there are two major differences between the two transporters: 1) P-gp transports neutral or cationic substrates (48) whereas MRP2 mainly transports anionic substrates particularly as a conjugated form (30); and 2) there is a widespread distribution of P-gp, which is found in various cells and in all parts of the nephron (5, 50) whereas MRP2 is found only in polarized cells and, inside the kidney, it is confined to proximal tubules (46). To what extent these particularities could account for the selective changes in MRP2 expression during CRF cannot be stated. Different responses in P-gp and MRP2 were also reported in rat kidneys after dexamethasone treatment, but the mechanisms involved are unknown (13). In our study, the lack of effects of CRF and associated uremic toxicity on protein P-gp expression suggests that endogeneous toxins assumed to be inducers of MRP2 are ineffective on P-gp. However, a small stimulatory effect by these factors on renal P-gp cannot be excluded because its mRNA expression was increased after 6 wk post-Nx. For unknown reasons, this was unassociated with an increase in protein P-gp levels.

We found that total protein MRP2 levels relative to whole renal mass (au/100 g BW) were reduced by ~25% in Nx compared with control rats at 3 wk post-Nx. A nonsignificant difference in total renal MRP2 was seen at 6 wk post-Nx (Table 3) whereas total liver MRP2 levels significantly increased at this point. This suggests that the capacity of remaining tubular mass for excreting xenobiotics and toxins transported by MRP2 was nearly preserved in our Nx model and that liver unlikely compensated for kidney in MRP2 effects. By contrast, total renal protein Pgp levels (au/100 g BW) were reduced by >50% in Nx rats. This could substantially alter the renal capacity for eliminating substrates of P-gp and account for some accumulated uremic toxins found in CRF.

Changes in P-gp and MRP2 levels could also affect the pharmacokinetics of administered drugs that are transported by either P-gp or MRP2. This is demonstrated experimentally for the cardiac glycoside digoxin, which is a substrate of P-gp (47), and the anticancer drug methotrexate, which is a substrate of MRP2 (36). The elimination of digoxin is reduced in mice invalidated for P-gp (mdr1a−/− mice) (47), and the excretion of methotrexate is decreased in mutant rats deficient for MRP2 (EHBR rats) (36). In patients with CRF, the pharmacokinetics of digoxin and of methotrexate are altered, as indicated by a reduction in the total clearance of these drugs (3, 4, 15, 45). These effects are attributable to impaired renal excretion, which is the major route of elimination of these drugs and involves both glomerular filtration and tubular secretion (15, 45). A significant correlation was found between GFR and digoxin or methotrexate clearance, but, interestingly, the contribution of GFR to the total clearance of these drugs decreased with the severity of CRF (3, 4). This suggests that extrarenal clearance of digoxin and methotrexate may increase in CRF. In addition, the proportion of tubular secretion to renal excretion could be augmented. Therefore, it may be speculated that a shift in digoxin and methotrexate excretion from kidney to other tissues may occur in CRF. This could involve an increased secretion of these drugs in nonrenal tissues, mediated by higher P-gp and MRP2 levels. Further studies are needed to support this hypothesis.

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Differential modulation of drug transporters in uremia

CREATION OF SOME ENDODGENOUS SUBSTANCES, WHICH MAY BE OVERPRODUCED OR ACCUMULATED IN CRF. HOWEVER, DIFFERENT MECHANISMS IN MRPs2 INDUCTION AND MRPs2-MEDIATED TRANSPORT COULD OCCUR IN KIDNEY AND LIVER BECAUSE OF THE LACK OF PARALLEL CHANGES IN MRPs2 EXPRESSION AND GLUTATHIONE METABOLISM IN THESE TWO TISSUES. IN PARTICULAR, THE INCREASED GLUTATHIONE LEVELS IN REMNANT KIDNEY COULD FACILITATE TOXIN EXPORT THROUGH GSH CONJUGATES. THE FACTORS INVOLVED IN MRPs2 UPREGULATION HAVE TO BE IDENTIFIED. THE ABSENCE OF TISSUE SPECIFICITY IN THIS RESPONSE SUGGESTS THE INVOLVEMENT OF SOME CIRCULATING ENDODGENOUS TOXINS. FURTHER STUDIES ARE REQUIRED TO DEMONSTRATE THAT SOME UREMIC TOXINS PLAY A ROLE IN MRPs2 INDUCTION AND CAN BE TRANSPORTED BY MRPs2. IN CLINICAL TERMS, CHANGES IN TOTAL RENAL P-GP LEVELS MAY HAVE IMPLICATIONS IN THERAPEUTIC TREATMENT OF UREMIC PATIENTS.


REFERENCES


