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Effects of chronic renal failure on kidney drug transporters and cytochrome P450 in rats

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Kidney drug transporters in renal failure

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List of non-standard abbreviations: CMPF: 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid, CRF: chronic renal failure, CTL: control, CYP: cytochrome P450, IS: indoxyl sulfate, Mrp: multidrug resistance related protein, Npt1: Sodium-

dependent phosphate transport protein 1, Oat: organic anion transporter, Oatp: organic anion transporting polypeptide, PAH: para-amino-hippuric acid, P_{app}: Apparent permeability, PBS: Phosphate Buffered Saline, PCR: polymerase chain reaction, P-gp: P-glycoprotein, PMSF: Phenylmethylsulfonylfluoride, URAT1: urate transporter.

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ABSTRACT

Chronic renal failure (CRF) leads to decreased drug renal clearance due to a reduction in glomerular filtration rate. However, little is known about how renal failure affects renal metabolism and elimination of drugs since both depend on the activity of uptake and efflux by renal transporters as well as enzymes in tubular cells. The purpose of this study was to investigate the effects of CRF on the expression and activity of select renal drug transporters and cytochrome P450. Two groups of rats were studied: control and CRF (induced by 5/6 nephrectomy). Compared to control rats, we observed reductions in the expression of both protein and mRNA of Cyp1a, Npt1, Oat1-2-3, OatK1/K2, Oatp1-4c1, P-gp and URAT1, while an induction in the protein and mRNA expression of Mrp2-3-4 and Oatp2-3 was observed. Cyp3a expression remained unchanged. Similar results were obtained by incubating a human proximal tubule cell-line (HK-2) with sera from CRF rats, suggesting the presence of uremic modulators. Finally, the renal elimination of ^{3}H-digoxin and ^{14}C-benzylpenicillin was decreased in CRF rats, compared to controls, as shown by a 4 and 9 fold accumulation respectively of these drugs in kidneys of rats in CRF. Our results demonstrate that CRF affects the expression and activity of several kidney drug transporters leading to the intrarenal accumulation of drugs and reduced renal clearance which could at least partially explain the tubular toxicity of many drugs.

INTRODUCTION

The kidneys play a major role in the elimination of endogenous and exogenous compounds from the bloodstream by the following three processes: glomerular filtration, tubular secretion and tubular reabsorption. It has been known for decades that chronic renal failure (CRF) reduces the renal clearance of filtrated drugs and endogenous substances through a decrease in the glomerular filtration rate. However, little is known about how renal failure affects renal secretion and reabsorption in the proximal tubule. Both these processes are dependent on the activity of uptake and efflux renal transporters expressed in tubular cells (Inui et al., 2000; Russel et al., 2002; Sekine et al., 2006; El-Sheikh et al., 2008; Feng et al., 2010; Giacomini et al., 2010; Masereeuw and Russel, 2010; Nagai and Takano, 2010).

Tubular transporters are members of two families: ATP-binding cassette (ABC) transporters and solute carriers (Slc). The former include multidrug resistance related proteins (Mrp) and P-glycoprotein (P-gp) which depend on ATP phosphorylation. They are efflux transporters expressed either on the apical brush-border membrane where they excrete drugs in the urine or on the basolateral membrane where they participate in the reabsorption of compounds from the urine. On the other hand, Slc transporters include the sodium-dependent phosphate transport protein 1 (Npt1), organic anion transporters (Oat), organic anion transporting polypeptides (Oatp) and urate transporters (URAT), and are mostly, but not exclusively, uptake transporters located on

either the apical or basolateral membrane where they participate in the uptake of compounds from the bloodstream or from the urine into the tubular cell. The most important drug transporters are illustrated in figure 1 (Hsiang et al., 1999; Russel et al., 2002; Masereeuw and Russel, 2010).

Recently, we have demonstrated that CRF can affect the expression of intestinal P-gp and Mrp2 and hepatic P-gp and Oatp2 in rats, thus increasing the bioavailability and decreasing the biliary elimination of various drugs (Naud et al., 2007; Naud et al., 2008). We have also demonstrated that CRF can decrease extrarenal elimination of drugs by decreasing intestinal and hepatic drug metabolism by cytochrome P450 (CYP) and phase II drug metabolism (Leblond et al., 2000; Leblond et al., 2001; Leblond et al., 2002; Simard et al., 2008). Finally, we have shown a nearly 100% decrease in the urinary clearance of rhodamine 123, a P-gp substrate, in nephrectomised rats (Naud et al., 2007; Naud et al., 2008). It is thus possible that CRF could affect drug transport and metabolism in the diseased kidney.

To this date, very few studies have been published concerning the effects of CRF on renal drug transport. Ji *et al*. have demonstrated a decrease in the protein expression of the organic cation transporter 2 (OCT2) two weeks after rats were submitted to 5/6 nephrectomy, while Oat1 and 3 expression remained unchanged (Ji et al., 2002). Also, Laouari *et al.* showed an increase in both the

protein and mRNA expression of Mrp2 six weeks post-5/6 nephrectomy, while P-gp expression remained stable (Laouari et al., 2001).

The objective of this study was to investigate the effects of CRF on the expression and activity of the major renal drug transporters and cytochrome P450. For this purpose, we have compared renal protein and mRNA expression in CRF and control rats of 1) Cyp1a, Cyp3a, Npt1, Oat1-2-3, Oatp2-3-4c1, Mrp2-3-4, P-gp and URAT1, and 2) the *in vivo* renal accumulation of ^{3}H-digoxin and ^{14}C-benzylpenicillin. Furthermore, we have studied the effects of uremic serum on the expression of drug transporters and cytochromes P450 in a proximal tubule cell-line as well as on the activity of P-gp in the same cell-line.

METHODS

Experimental model

Male Sprague-Dawley rats (Charles River, Portage, MI), weighing 176 to 225 g were housed in the Research Center animal care facility and maintained on Harlan Teklad rodent diet (Harlan Teklad Global Diets, Montreal, Canada) and water *ad libitum*. Animals were allowed an acclimatization period of at least 7 days before the first nephrectomy was performed. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals and under the supervision of our local animal care committee.

Experimental protocol

Studies were performed in two groups of animals: control pair-fed and CRF. The expression levels of protein and mRNA of kidney drug transporters were measured in 12 rats per group. Drug transporter activity was measured *in vivo* through the renal accumulation of ^{3}H-digoxin and ^{14}C-benzylpenicillin in at least 6 rats per group, per experiment.

Chronic renal failure was induced by two-stage 5/6 nephrectomy as previously described (Leblond et al., 2001). Every animal had free access to water, but in order to limit the effects of CRF-induced malnutrition, control pair-fed rats were fed the same amount of chow that CRF rats ate on the previous day. We have previously demonstrated that the calorie reduction in control animals induced by pair-feeding did not influence the expression of drug metabolizing enzymes

(Leblond et al., 2001) and drug transporters (unpublished data). At day 41 post-nephrectomy, the rats were housed in metabolic cages and urine was collected for a 24 hour period to determine the clearance of creatinine. Rats were sacrificed by decapitation 42 days after nephrectomy for organ and blood collection, or underwent a radio-labeled drug accumulation study. Following sacrifice, the remnant kidney of CRF rats and the left kidney of CTL rats were immediately excised, rinsed in ice-cold saline then flash-frozen in liquid nitrogen. Samples were stored at -80°C until membrane preparation or mRNA extraction was performed. Blood was collected for the measurement of serum creatinine and urea and for bioassays with proximal tubule cells.

Preparation of kidney crude membranes

Rat kidney biopsies (200 mg) were homogenized in 1 mL of homogenizing buffer (250 mM sucrose,10 mM HEPES and 10 mM Tris-HCl, pH 7.4) containing 0.1 mM PMSF using a Potter-Elvehjem tissue grinder (Wheaton Science Products, Millville, NJ). Differential centrifugation was used to obtain a crude membrane fraction (Chow et al., 2010). Briefly, the kidney homogenates were centrifuged at 9 000 g for 10 min at 4 °C. The supernatant was then spun at 33 000 g for 60 min at 4°C and the resulting pellet was resuspended in phosphate buffered saline containing 0.1 mM PMSF. Samples were then sonicated on ice for 10 seconds to ensure homogeneity. Protein concentration was determined using the method of Lowry *et al*. (Lowry et al., 1951), using bovine serum albumin as a reference protein. Aliquots were stored at -80 °C until used in Western blot analysis.

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Western blot analysis

Major renal drug transporters were assessed by Western blot analysis following a previously described protocol (Leblond et al., 2002; Naud et al., 2007; Naud et al., 2008). Protein expression in control animals was arbitrarily defined as 100%.

P-gp and Mrp2 were detected using monoclonal antibodies from ID Labs (London, Ontario, Canada): C219 and M_2 III-6 respectively. Npt1, Oat2, Oat3, OatK1/K2, Oatp2 and Oatp3 were detected using antibodies from Alpha Diagnostic International (San Antonio, TX). Anti-Mrp3 was from Sigma (St-Louis, MO), anti-Mrp4 from Novus Biological (Littleton, CO), anti-Oat1 and anti-URAT1 from Abbiotec (San Diego, CA), anti-Oatp1 from Millipore (Billerica, MA) and anti-Oatp4c1 from Santa Cruz (Santa Cruz, CA). Cyp1a and Cyp3a were detected using primary antibodies from US Biological (Swampscott, MA) and Daiichi Pure Chemicals (Tokyo, Japan), respectively. Villin-1, a protein specific to tubular brush-border cells (Cartier et al., 1993), used as a loading control, was detected using rabbit anti-Villin-1 from Cell Signaling Technology (Beverly, MA). The antibodies used recognized rat and human proteins.

mRNA analysis

Total RNA was extracted from frozen tissue using Trizol reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's

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protocol. RNA concentration was determined by measuring absorbance at a wavelength of 260nm. One µg of total RNA was used to prepare cDNA by reverse transcription using SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen. The mRNA encoding for P-gp (mdr1a), Mrp2, Oatp2 and Villin-1 was measured by quantitative Real-Time PCR (qPCR) as previously described (Naud et al., 2007; Naud et al., 2008). Other genes were measured using appropriate TaqMan gene expression assays from Applied Biosystems (Carlsbad, CA). Table 1 shows the TaqMan gene expression assays used for the quantification of mRNA for each transporter. PCR products were analyzed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) using Villin-1 as a standard gene.

In vivo intra-renal accumulation of radio-labeled drugs

The intra-renal accumulation of radio-labeled drugs was used to determine the activity of the kidney drug transporters. Two different drugs were tested. ^{14}C-benzylpenicillin is an antibiotic and a known substrate for Oat3 and Mrps (Windass et al., 2007; Choi et al., 2009). ^{3}H-digoxin is a cardiac glycoside and a known substrate for Oatp4c1 and P-gp (Mikkaichi et al., 2004; Yamaguchi et al., 2010). Digoxin (0.5 mg/kg) was administered via intra-peritoneal injection and benzylpenicillin (5 mg/kg CTL, 1 mg/kg CRF) via intra-muscular injection. Both were injected with an approximate 1:1500 hot/cold ratio so the mg amount of radioactive compound wouldn't factor into the final dosage calculation. To evaluate renal accumulation, similar plasma concentrations of drugs need to be attained and therefore CRF rats received a lower dose of benzylpenicillin

because of its reduced clearance and acute accumulation in CRF. As for digoxin, there is no acute accumulation in CRF patients, thus, CRF patients receive the same loading dose as patients with normal renal function with an adjustment in their maintenance dose (Jelliffe, 1968; Jelliffe, 1969; Gault et al., 1980). Since this study with rats requires a single loading dose, no digoxin dose adjustment was necessary to obtain plasma concentrations similar to those observed in CTL rats. Rats were anesthetized using isoflurane 2 h post-injection and a cardiac puncture was performed to recover the maximum amount of blood in heparinised tubes to obtain plasma. Remnant left kidneys were harvested, rinsed in cold phosphate buffered saline, weighed, minced and placed in 2 mL of Solvable solution (PerkinElmer, Waltham, MA) at 56°C overnight. Dissolved kidney volumes were noted and radioactivity was counted on a Wallac 1409 beta-counter (Perkin Elmer) in 1 mL of dissolved kidney after addition of 4 mL ScintiSafe Plus Scintillant (Fisher, Ottawa, Ontario, Canada). Plasma radioactivity was counted in 2 mL of plasma with 4 mL Scintillant. The results were calculated as a ratio between the radioactivity count (CPM) for 1 g of kidney and the radioactivity count for 1mL of plasma. The mean for the CTL groups was arbitrarily defined as 100%.

Effects of uremic serum on kidney drug transporters

In order to determine whether uremic serum could affect the expression and function of kidney drug transporters, two sets of experiments were conducted using human kidney-2 (HK-2) cells, a proximal tubule cell line. The

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first studied the effects of uremic serum on drug transporters' protein expression, while the second studied the effects of uremic serum on P-gp activity.

Incubation of HK-2 cells with uremic serum

HK-2 cells were purchased from ATCC (Manassas, VA) and seeded into 25 mm² uncoated polystyrene flasks (Sarstedt, St-Leonard, Quebec, Canada) in DMEM (Invitrogen) containing 10% fetal bovine serum (PAA, Etobicoke, Ontario, Canada). The cells were subcultured four days later into two 75 mm² uncoated polystyrene flasks, then expended by 1:3 subcultures every three to four days. For an experiment, culture medium was replaced by DMEM containing 10% CTL or CRF rat serum (1 serum/flask). After 48 h of culture, the cells were trypsinized, pelleted and homogenized in phosphate buffered saline containing 0.1 mM PMSF. Protein concentrations were determined using the Micro BCA Protein Assay Kit from Thermo Fisher Scientific (Rockford, IL). One hundred micrograms were loaded for Western Blot analysis of each studied transporter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. It was detected using rabbit anti-GAPDH from Abcam (Cambridge, MA).

Rhodamine 123 transport on HK-2 cells

The effects of uremic serum on P-gp function was evaluated in HK-2 cells using the trans-epithelial transport of rhodamine 123 across HK-2 monolayers. Cells were subcultured into 12-wells, 0.4µm pore-size PET-transwells (Becton Dickinson, Mississauga, Ontario, Canada) and allowed 7 days to reach

confluence and form a polarized monolayer. Fourty-eight hours prior to the transport experiment, culture medium was replaced by culture medium containing 10% CTL or CRF rat serum. The transport experiment was conducted as previously reported with Caco-2 cells (Naud et al., 2007).

Other assays.

Blood and urine chemistries were determined with an Architect C1600 clinical analyzer (Abbott, Saint-Laurent, QC, Canada).

Statistical analysis.

Results are expressed as mean ± SD. Differences between groups were assessed using an unpaired Student's *t* test or an ANOVA test. Significant ANOVA was followed by a Scheffe *post hoc* comparison of groups. The threshold of significance was $p<0.05$.

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RESULTS

Biochemical parameters and body weight in control and CRF rats (Table 2)

Table 2 presents the biochemical parameters and body weights of both groups of rats. Compared to control animals, CRF rats had higher levels of serum creatinine and urea and lower creatinine clearance (reduced by 80%, $p<0.001$). There was no difference in body weight between control and CRF rats.

Protein expression of kidney drug transporters and cytochrome P450s in CRF rats (Figure 2).

Figure 2 presents the measured protein expression of select drug transporters and cytochrome P450s from a crude kidney membrane preparation from control and CRF rats. The level of expression of Mrp2, 3 and 4 and of Oatp2 and 3 were significantly increased by more than 2-fold in CRF rat kidneys compared to control rat kidneys as shown by Western blot. On the other hand, the expression of Cyp1a, Npt1, Oats, Oatp1, Oatp4c1, P-gp and URAT1 were significantly reduced from 50 to 85% in CRF rats compared to controls. The only measured protein that remained stable was Cyp3a. We found significant correlations ($p<0.05$) between protein expression and creatinine clearance for Cyp1a ($R = 0.787$), Mrp2 ($R = -0.662$), Mrp3 ($R = -0.719$), Mrp4 ($R = -0.626$), Npt1 ($R = 0.687$), Oat1 ($R = 0.716$), Oat2 ($R= 0.720$), Oat3 ($R = 0.723$), OatK1/K2 ($R = 0.861$), Oatp1 ($r = 0.717$), Oatp2 ($R = -0.586$), Oatp3 ($R = -0.684$), Oatp4c1 ($R = 0.757$), P-gp ($R = 0.811$) and URAT1 ($R = 0.603$).

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mRNA expression of kidney drug transporters and cytochrome P450 in CRF rats (Figure 3).

Figure 3 presents the results of the analysis of mRNA coding for kidney transporters and cytochrome P450. There was a significant increase in the mRNA expression of Mrp2, 3 and 4 (40%, 92% and 72% respectively) and of Oatp2 and 3 (125% and 180% respectively) in CRF rats compared to controls. On the other hand, there were significant decreases in the mRNA expression of Cyp1a (95%), Npt1 (45%), Oat1 (75%), Oat2 (95%), Oat3 (70%), Oatp1 (90%), Oatp4c1 (90%), Oatp5 (95%), P-gp (50%) and URAT1 (75%) in CRF rat kidneys compared to controls. Only Cyp3a mRNA remained stable between the groups.

***In vivo* accumulation of radio-labeled drugs in the kidney of rats with surgically-induced chronic renal failure (Figure 4).**

Two hours post-administration, plasma radioactivity of digoxin was similar in the control and CRF rats, while the plasma radioactivity of benzylpenicillin was slightly elevated by 17% in CRF rats compared to control animals. There was a 9-fold greater accumulation of ^{14}C-benzylpenicillin and a 4-fold accumulation of ^{3}H-digoxin in the kidney of CRF rats, compared to control rat kidneys. These data were obtained via a ratio of radioactivity for 1 g of kidney/1 mL of plasma.

Protein expression of renal drug transporters and cytochrome P450 in HK-2 cells incubated with the serum of CRF rats (Figure 5).

Protein expression of select drug transporters and cytochrome P450 were

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measured in HK-2 cells, a proximal tubule cell line, incubated for 48 h with serum from control or CRF animals. There was a significant decrease in the protein expression of Cyp1a (45%), Npt1 (30%), Oat3 (30%), Oatp1 (25%) and P-gp (35%) when incubated with CRF serum compared to control serum. On the other hand, the expression of Mrp2, Mrp4 and Oatp2 were significantly increased by 45%, 35% and 30% respectively. Only the expression of Cyp3a remained unaltered. The expression of the reporter protein GAPDH was unchanged between both groups. These results were similar in directional change to those obtained *in vivo* in CRF rats.

Apical to basolateral transport of rhodamine 123 in HK-2 cells incubated with the serum of CRF rats (Figure 6).

The effect of uremic serum on P-gp function was evaluated in HK-2 cells using the transepithelial transport of rhodamine 123 across HK-2 monolayers. We found a nearly 50% increase in the apical to basolateral apparent permeability (P_{app}) of rhodamine 123 across HK-2 cells pre-incubated with uremic sera compared to control. Moreover, the addition of P-gp inhibitor cyclosporine did not affect the P_{app} of rhodamine 123 in cells incubated with uremic sera but caused a great increase in permeability in HK-2 cells pre-incubated with control sera.

DISCUSSION

The purpose of this study was to evaluate the effect of chronic renal failure on the expression and activity of select drug transporters and cytochrome P450 in the rat kidney. We have demonstrated that CRF affects drug transporters in different manners. The protein and mRNA expression of many transporters were significantly reduced (Npt1, Oat1, Oat2, Oat3 OatK1/K2, Oatp1, Oatp4c1, P-gp and URAT1) while those of Mrp2, Mrp3, Mrp4, Oatp2 and Oatp3 were significantly upregulated. Also, the protein and mRNA expression of Cyp1a were significantly reduced while those of Cyp3a remained unchanged.

These results differ from those previously published by Ji *et al.* (Ji et al., 2002) who had shown no changes in the expression of Oat1 and Oat3 two weeks post-nephrectomy. We hypothesize that two-weeks was too short to fully develop the long term effects of renal failure. Our results also confirm and differ from those obtained by Laouari *et al.* (Laouari et al., 2001) who had demonstrated an increase in the protein and mRNA expression of Mrp2 three and six weeks post-nephrectomy, but no change in the expressions of P-gp at 3 weeks and an increase in P-gp mRNA after six weeks. We confirmed the results obtained for Mrp2 expression, but we have demonstrated a 50% decrease in P-gp protein and mRNA expressions six weeks post-nephrectomy. The reasons for this discrepancy remain unclear.

We found significant correlations between the clearance of creatinine and

the protein expression of transporters and Cyp1a (data not shown) indicating that changes in transporter expression are directly related to the severity of renal failure. We also found significant correlations between the protein and mRNA expression of transporters and Cyp1a (data not shown), suggesting that changes in protein expression are the result of a decrease in mRNA levels. Also, we have demonstrated that the modifications in drug transporter expression and activity occurred in response to one or many factors present in the sera from uremic rats, as demonstrated by the changes in protein expression of transporters and CYPs in HK-2 cells, which are similar to those observed *in vivo,* although they are of smaller magnitude, probably due to the shorter length of exposure to uremic toxins.

Several uremic toxins accumulate in renal failure. They are breakdown products of metabolic pathways which are no longer being eliminated by the kidneys. Those that were most studied in relations to drug transport are indoxyl sulfate (IS), para-amino-hippuric acid (PAH) and 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid (CMPF). They are known substrates and inhibitors of organic anion transporters including Oat1, Oat3 and Mrps (Leier et al., 2000; Deguchi et al., 2002; Tsutsumi et al., 2002; Deguchi et al., 2004; Deguchi et al., 2005; Sun et al., 2006; Enomoto and Niwa, 2007) and even play a role in the progression of renal failure as demonstrated by the induction of renal damage by indoxyl sulfate (Niwa and Ise, 1994; Niwa et al., 1994; Niwa et al., 1997; Sun et al., 2006). The downregulation of uptake transporters Oat1, Oat2 and Oat3, and

of bidirectional transporters OatK1/K2, Oatp1, Oatp4c1 and URAT1, combined with the upregulation of efflux transporters Mrp2, Mrp3 and Mrp4, and of bidirectional transporters Oatp2 and Oatp3, all of which are anion transporters, could thus be an adaptative and protective response against uremic toxin accumulation and renal toxicity. Still, these changes do not seem to be sufficient to prevent the accumulation of organic anions as demonstrated by the 9-fold accumulation of ^{14}C-benzylpenicillin, a substrate of OATs and Mrps, in the kidneys of CRF-suffering animals compared to controls and, thus, could probably be insufficient to prevent the progression of renal failure by uremic toxins toxicity. Competitive inhibition of the transporters by uremic toxins could explain this phenomenon.

While most efflux transporter expression was increased in the current study, P-gp expression and its *in vitro* activity were significantly reduced by chronic renal failure and uremic sera. P-gp differs from most other studied transporters as it transports mostly cationic or neutral substrates (Masereeuw et al., 2000). We have thus evaluated the *in vivo* accumulation of the P-gp substrate ^{3}H-digoxin. Digoxin is also a substrate of the uptake transporter Oatp4c1(Mikkaichi et al., 2004). Although plasma concentrations of digoxin were similar between the control and CRF animals, there was a significant 4-fold increase in the intra renal accumulation of digoxin in CRF kidneys. This indicates a reduced secretion of digoxin most likely due to the reduced expression of its efflux transporter P-gp. This result could be reproduced *in vitro* by the incubation

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of a HK-2 monolayer with sera from uremic rats where we demonstrated a significant increase in the P_{app} of rhodamine 123 from the apical to the basolateral side of the monolayer. This argues in favor of a decrease in the transport activity of P-gp, facilitating the migration of rhodamine-123 toward the basolateral side. The addition of the P-gp inhibitor cyclosporine to both sides of the monolayer caused an increase in the P_{app} of rhodamine in the CTL group, demonstrating that rhodamine 123 transport is P-gp-dependent, but induces no significant change in the CRF group, indicating a very weak P-gp activity even before the addition of cyclosporine. This could explain the nearly 100% reduction in rhodamine 123 urinary clearance that we previously reported (Naud et al., 2007; Naud et al., 2008) and strongly suggests that a factor present in uremic serum has a modulatory effect on P-gp protein expression and activity, similar to what we previously showed in the intestine and liver (Naud et al., 2007; Naud et al., 2008).

We also measured the protein and mRNA expression of Cyp1a and Cyp3a in the kidneys of control and CRF rats. They are drug metabolizing enzymes with wide substrate variability. CYP3A is implicated in the biotransformation of nearly 60% of drugs metabolized by CYPs in humans, like acetaminophen, cyclosporine, erythromycin, etc.(Guengerich, 2003), while Cyp1a is mostly implicated in the biotransformation of environmental pollutants like polycyclic aromatic hydrocarbons and tobacco smoke or substances like caffeine and tamoxifen(Guengerich, 2003). In the kidney, Cyp1a and Cyp3a are expressed in

the tubular cells, where they can activate or deactivate their substrates prior to urinary secretion. We have demonstrated a significant decrease in the expression of Cyp1a in CRF which could cause intra-renal toxicity of Cyp1a active substrates, if they where to accumulate in the kidney because of reduced drug transporters. On the other hand, Cyp3a expression seems unaffected by CRF which would allow normal activation or deactivation of substrates within the renal tubular cells. Toxicity could occur if activated metabolites where to remain trapped inside the tubular cells because of reduced drug transport.

In conclusion, this study demonstrates that CRF significantly alters the expression and activity of select renal drug transporters which could contribute to reduced renal clearance as well as renal drug accumulation and nephrotoxicity. The modifications in drug transporters induced by uremic serum were similar to that produced by CRF in our animal model, suggesting that uremic toxins such as indoxyl sulphate, para-amino-hippuric acid and 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid, which are known to modulate drug transporters(Leier et al., 2000; Deguchi et al., 2002; Tsutsumi et al., 2002; Deguchi et al., 2004; Deguchi et al., 2005; Sun et al., 2006; Enomoto and Niwa, 2007), could be responsible for the alterations in renal transporters *in vivo*.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Naud, Hébert, Roger, Lefrancois, Pichette

Conducted experiments: Naud, Michaud, Beauchemin, Leblond

Performed data analysis: Naud, Leblond, Pichette

Wrote or contributed to the writing of the manuscript: Naud, Hébert, Roger, Lefrancois, Leblond, Pichette

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Renal drug transporters. Most of these transporters are located in the proximal tubular cells, except Mrp3 which is found in distal tubular cells and OatK2 which can also be found in cortical collecting duct cells. Mrp: multidrug resistance related protein, Npt1: Sodium-dependent phosphate transport protein 1, OA: organic anion, Oat: organic anion transporter, Oatp: organic anion transporting polypeptide, P-gp: p-glycoprotein, URAT1: urate transporter. Oatps and OatK1/K2 are widely known to be bidirectional transporters and it is not yet determined whether they favor organic anion reabsorption or secretion, hence the arrows indicating both directions(Masuda et al., 1997; Inui et al., 2000; Deguchi et al., 2002; Masereeuw and Russel, 2010). It is also believed that URAT1 can participate in organic anion secretion and reabsorption via exchange mechanisms(Masereeuw and Russel, 2010). Npt1 acts as an organic anion uptake transporter as well as a urate exporter(Iharada et al., 2010).

Figure 2: Protein expression of select kidney drug transporters and cytochrome P450s in CRF rats. A. Protein bands are expressed in densitometry units. The densitometry units measured for transporters and CYPs were standardized by dividing them by the value obtained for villin-1. The standardized densitometry units of control rats were arbitrarily defined as 100% (dotted line). Graph shows the mean expression in chronic renal failure (CRF) rats expressed as percent of controls ± SD of 12 rats in each group. **p<0.01, ***p<0.001 compared to control (CTL) rats. **B.** Representative western blots for

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each transporter or CYP. Each blot presents bands for two CRF (left) and two control (right) rats.

Figure 3: mRNA expression of select kidney drug transporters and cytochrome P450s in CRF rats. mRNA encoding renal drug transporters and CYPs in control and chronic renal failure (CRF) rats were measured by quantitative real time PCR. mRNA levels are expressed in relative quantities using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) with villin-1 as a reference gene. The mean relative quantity of control rats was arbitrarily defined as 100% (dotted line). Graphic shows the mean expression in CRF rats expressed as percent of controls ± SD of 12 rats in each group. *p<0.05, **p<0.01, ***p<0.001 compared to control rats.

Figure 4: *In vivo* accumulation of radio-labeled drugs in the kidney of rats with surgically-induced chronic renal failure. Kidney/plasma radioactivity (counts per minute) ratio for 1g kidney/1mL plasma in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats, after a 2h latency period post drug injection. CRF rats received a fifth of the CTL rats' dose of benzylpenicillin, but had a 17% (p<0.05) higher plasma radioactivity of benzylpenicillin while, with equal dosing between the groups, digoxin plasma radioactivity was similar in the CTL and CRF rats though it had a tendency to be about 15% increased in CRF, but this result did not reach statistical significance (data not shown). The mean ratio for CTL animals was arbitrarily defined as 100%. Data are expressed as

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mean ± SD of at least 6 rats in each group. **p<0.01, ***p<0.001 compared to CTL rats.

Figure 5: Protein expression of select renal drug transporters and cytochrome P450s in HK-2 cells incubated with the serum of CRF rats. Protein bands are expressed in densitometry units. The densitometry units measured for transporters and CYPs were standardized by dividing them by the value obtained for GAPDH. The standardized densitometry units of control rats were arbitrarily defined as 100% (dotted line). Graphic shows the mean expression in chronic renal failure (CRF) rats expressed as percent of controls ± SD of 10 different sera in each group. *p<0.05, **p<0.01, ***p<0.001 compared to control rats.

Figure 6: Apical to basolateral transport of rhodamine in HK-2 cells incubated with the serum of CRF or CTL rats. Apparent permeability (P_{app}) of rhodamine across a HK-2 cells monolayer pre-incubated with sera from control (white bars) and chronic renal failure (CRF) (black bars) rats, in the absence or presence of the P-gp inhibitor cyclosporine. P_{app} was determined using the equation $P_{app} = (dC_r/dt)/(AC_0)$ were dC_r/dt represents the rate at which the substrate appears in the receiving (basolateral) compartment, A is the monolayer surface and C_0 is the initial substrate concentration in the donor (apical) compartment. The mean P_{app} of controls without cyclosporine was arbitrarily defined as 100%. Data are presented as the mean compared to

controls ± SD of 8 different sera in each group. NS: no significant difference.

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Table 1: TaqMan gene expression assays used for Real-time PCR

	Gene	TaqMan Gene Expression Assay
Cyp1a1	*cyp1a1*	Rn00487218_m1
Cyp3a1	*cyp3a23*	Rn01412959_g1
Mrp3	*abcc3*	Rn00589786_m1
Mrp4	*abcc4*	Rn01465702_m1
Npt1	*slc17a1*	Rn00591970_m1
Oat1	*slc22a6*	Rn00568143_m1
Oat2	*slc22a7*	Rn00585513_m1
Oat3	*slc22a8*	Rn00580082_m1
OatK1/K2	*slc21a4*	Rn00755673_m1
Oatp1	*slco1a1*	Rn00755148_m1
Oatp3	*slco1a5*	Rn00578150_m1
Oatp4c1	*slco4c1*	Rn01427754_m1
Oatp5	*slco1a6*	Rn00590562_m1
URAT1	*slc22a12*	Rn01479631_m1

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Table 2. Characteristics of control and CRF rats

	Control (n=20)	CRF (n=20)	p-value
Body Weight (grams)	342,7 ± 30.9	327.4 ± 55.1	0.29
Serum creatinine (μmol/L)	62.5 ± 4.0	229.5 ± 86.2	p<0.001
Creatinine clearance (μL/100 g of body weight/min)	313.1 ± 49.9	63.7 ± 35.4	p<0.001
Serum urea (mmol/L)	4.5 ± 1.0	38.2 ± 19.0	p<0.001

Data are the mean ± SD. Measurements were made at the time of sacrifice. Urinary collection was begun the day before. Blood urea and creatinine, and urine creatinine were determined with an Architect C1600 clinical analyzer (Abbott, Saint-Laurent, QC, Canada).

Figure 1

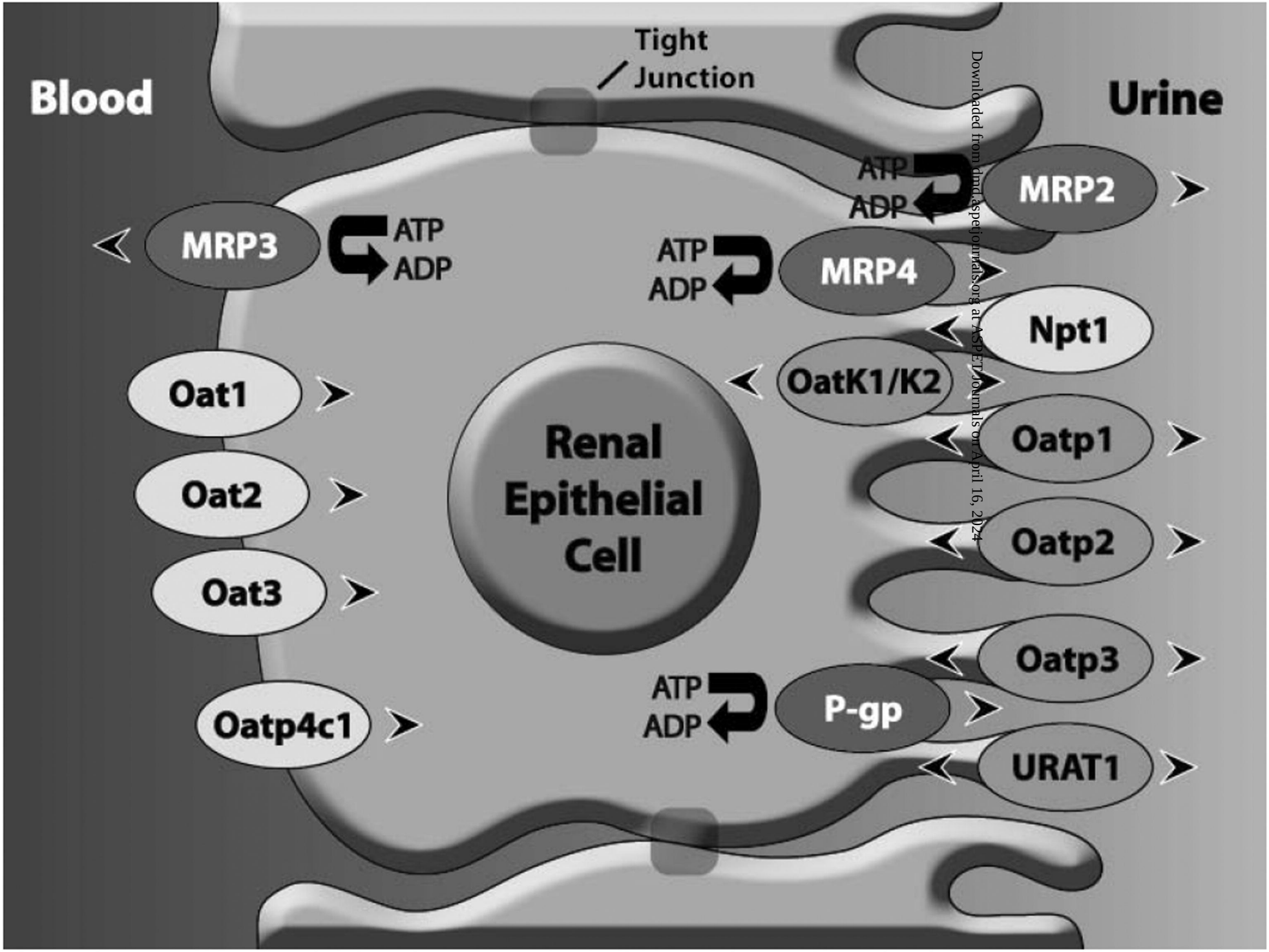


Figure 2A

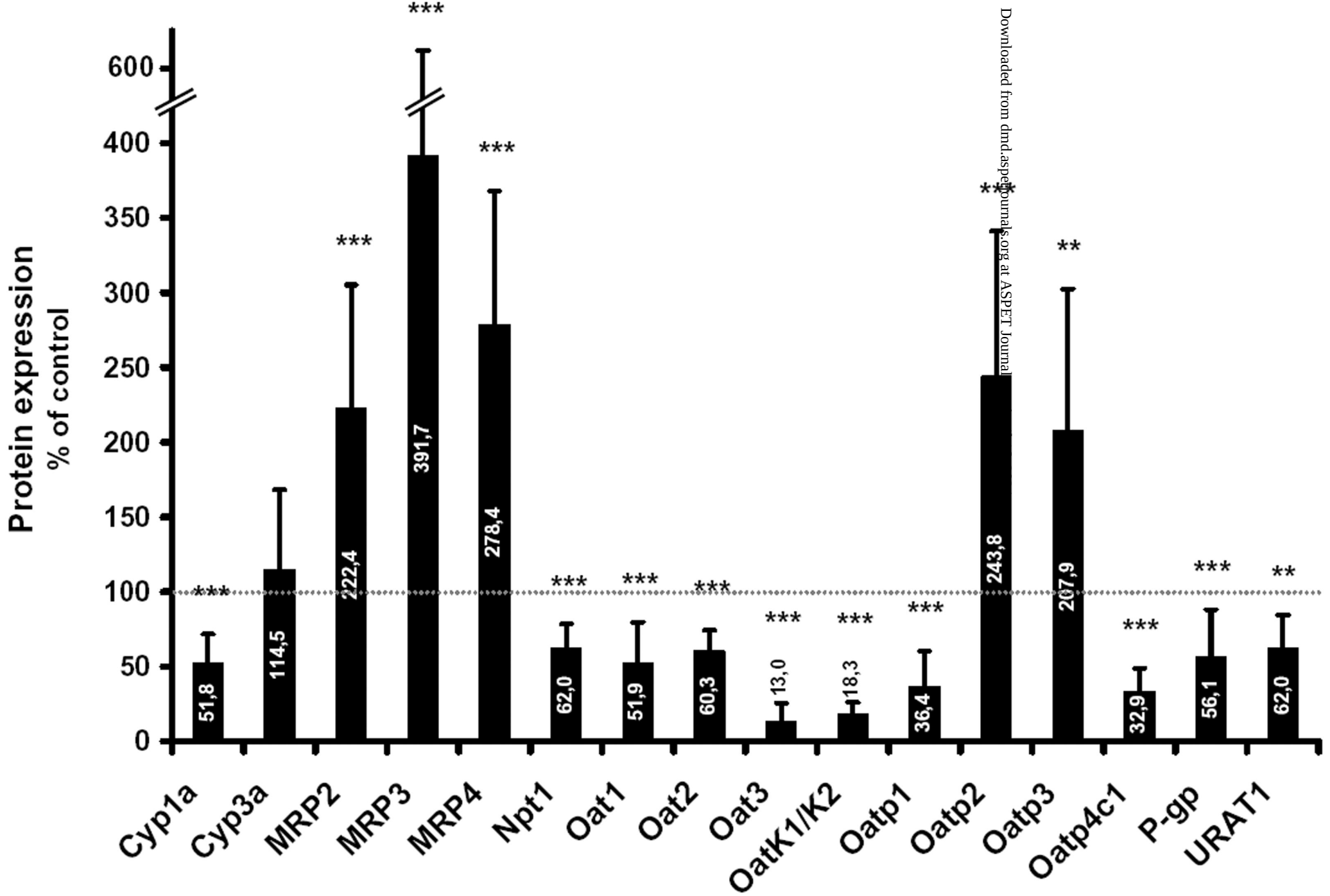


Figure 2B

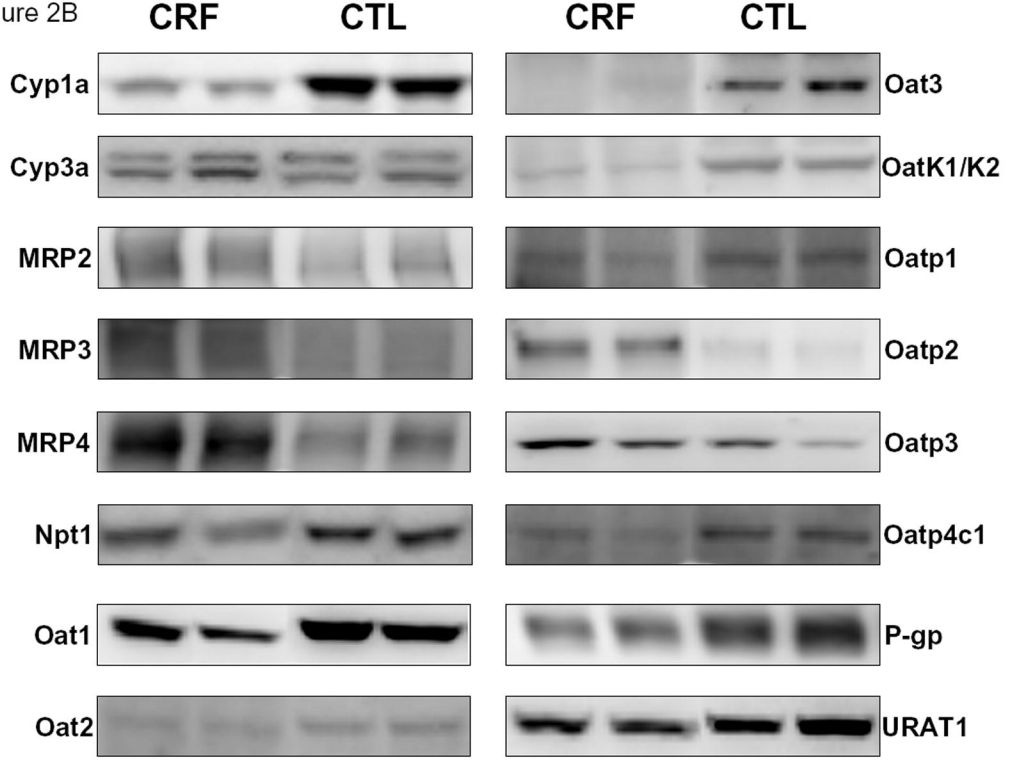

CRF
CTL
CRF
CTL
Cyp1a
Cyp3a
MRP2
MRP3
MRP4
Npt1
Oat1
Oat2
Oat3
OatK1/K2
Oatp1
Oatp2
Oatp3
Oatp4c1
P-gp
URAT1

Figure 3

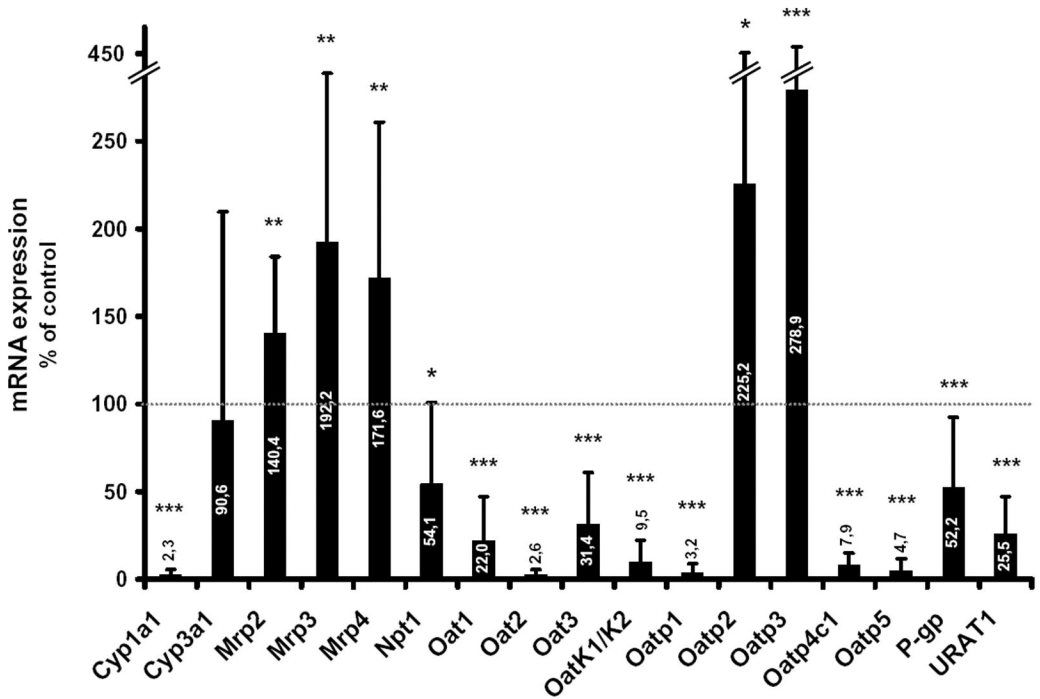

mRNA expression
% of control
450
250
200
150
100
50
0
2,3
90,6
140,4
192,2
171,6
54,1
22,0
2,6
31,4
9,5
3,2
225,2
278,9
7,9
4,7
52,2
25,5
Cyp1a1
Cyp3a1
Mrp2
Mrp3
Mrp4
Npt1
Oat1
Oat2
Oat3
OatK1/K2
Oatp1
Oatp2
Oatp3
Oatp4c1
Oatp5
P-gp
URAT1

Figure 4

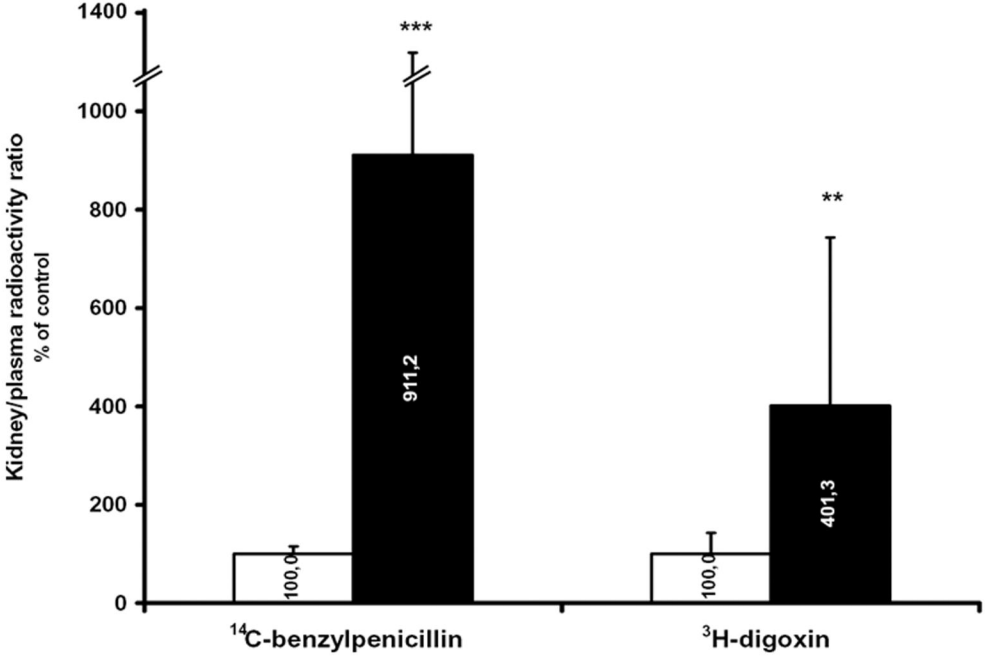

1400
1000
800
600
400
200
0
Kidney/plasma radioactivity ratio
% of control
***
**
911,2
100,0
401,3
100,0
14C-benzylpenicillin
3H-digoxin

Figure 5

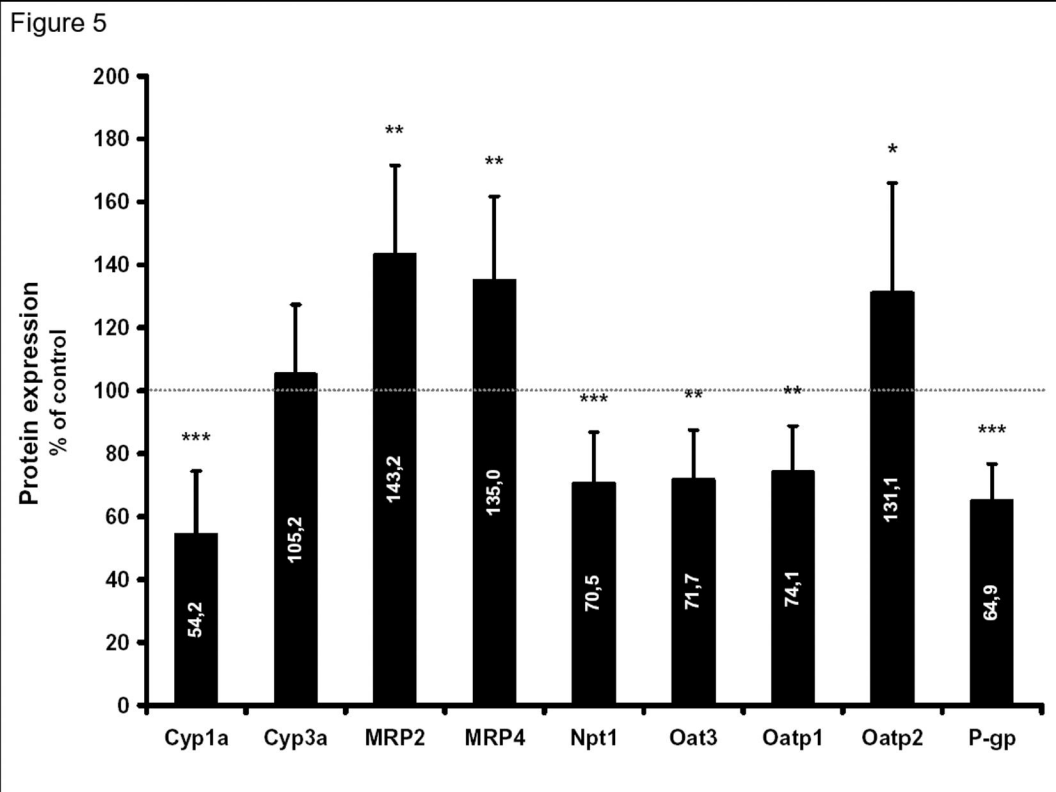
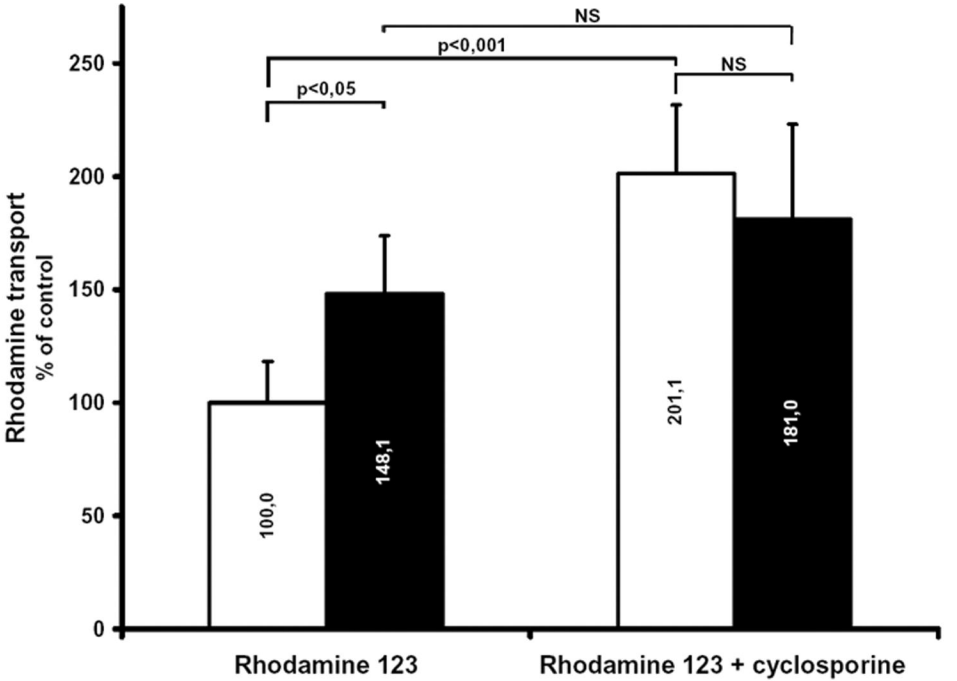

Protein expression
% of control
200
180
160
140
120
100
80
60
40
20
0
***
**
**
***
**
**
*
***
54,2
105,2
143,2
135,0
70,5
71,7
74,1
131,1
64,9
Cyp1a
Cyp3a
MRP2
MRP4
Npt1
Oat3
Oatp1
Oatp2
P-gp

Figure 6


NS
p<0,001
NS
p<0,05
250
200
150
100
50
0
Rhodamine transport
% of control
100,0
148,1
201,1
181,0
Rhodamine 123
Rhodamine 123 + cyclosporine