Reduced Hepatic Synthesis of Calcidiol in Uremia

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ABSTRACT
Calcidiol insufficiency is highly prevalent in chronic kidney disease (CKD), but the reasons for this are incompletely understood. CKD associates with a decrease in liver cytochrome P450 (CYP450) enzymes, and specific CYP450 isoforms mediate vitamin D3 C-25-hydroxylation, which forms calcidiol. Abnormal levels of parathyroid hormone (PTH), which also modulates liver CYP450, could also contribute to the decrease in liver CYP450 associated with CKD. Here, we evaluated the effects of PTH and uremia on liver CYP450 isoforms involved in calcidiol synthesis in rats. Uremic rats had 52% lower concentrations of serum calcidiol than control rats (P < 0.002). Compared with controls, uremic rats produced 71% less calcidiol and 48% less calcitriol after the administration of vitamin D3 or 1α-hydroxyvitamin D3, respectively, suggesting impaired C-25-hydroxylation of vitamin D3. Furthermore, uremia associated with a reduction of liver CYP2C11, 2J3, 3A2, and 27A1. Parathyroidectomy prevented the uremia-associated decreases in calcidiol and liver CYP450 isoforms. In conclusion, these data suggest that uremia decreases calcidiol synthesis secondary to a PTH-mediated reduction in liver CYP450 isoforms.


It has been known for decades that chronic renal failure (CRF) is associated with low serum 1,25-dihydroxyvitamin D3 [calcitriol, or 1,25(OH)2D3], the active metabolite of vitamin D3, because of a reduction in renal 1α-hydroxylase (CYP27B1). More recently, 25-hydroxyvitamin D3 [calcidiol, or 25(OH)D3] deficiency has also been demonstrated in patients with stages 3 and 4 chronic kidney disease (CKD) and in patients who are on dialysis.1–8 In fact, low serum 25(OH)D3 is so intimately associated with CRF that in one study, only 29 and 17% of patients with stages 3 and 4 CKD, respectively, had sufficient levels [defined as a serum 25(OH)D3 concentrations >75 nmol/L or 30 ng/ml].2 A more recent study showed a prevalence of calcidiol insufficiency and deficiency as high as 98% in predialysis patients with a mean GFR of 18.3 ml/min.4 Prevalence of low serum 25(OH)D3 was 78 and 89% in two large cohorts of hemodialysis patients,9,10 and 87% in a large cohort of peritoneal dialysis patients.11

The metabolic consequences of calcidiol deficiency are important, because low levels of 25(OH)D3 might contribute to low levels of 1,25(OH)2D3 and to secondary hyperparathyroidism.1–8 Moreover, in addition to its role in bone metabolism, there is increasing evidence that vitamin D3 is involved in the prevention of many chronic diseases, such as type 1 diabetes, hypertension, cardiovascular diseases, and cancer.5,9,12–14 As a consequence, according to the 2003 Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines, calcidiol levels should be measured in patients with CKD, and deficiency should be treated with ergocalciferol (vitamin
D₂) or cholecalciferol (vitamin D₃)³; however, a paucity of information exists concerning the effect of treatment of vitamin D₃ insufficiency in CRF on the frequency and severity of secondary hyperparathyroidism among patients with decreased 25(OH)D₃ concentrations. Furthermore, the efficacy of vitamin D₃ therapy on serum calcidiol levels of patients who experience kidney failure is variable and remains poor compared with patients without CKD.¹⁻⁷,¹⁵⁻¹⁸

More important, the mechanisms underlying calcidiol deficiency remain poorly understood. Lower diet intake and reduced sun exposure have been proposed but never demonstrated.¹²,⁴⁻⁶,⁷ Vitamin D₃ is normally synthesized in the skin under the influence of sunlight or taken orally as a vitamin supplement. It is hydroxylated in the liver to 25(OH)D₃, then hydroxylated in the kidney to form 1,25(OH)₂D₃, the most bioactive form of the vitamin (Figure 1). Both calcitriol and calcidiol are degraded in part by a C-24-hydroxylation specific, elevated PTH could downregulate liver CYP450 isozymes implicated in the C-25-hydroxylation of vitamin D₃ (Figure 1). Indirect evidence supporting such a hypothesis is that low serum levels of 25(OH)D₃ have also been reported in primary hyperparathyroidism and found to be corrected by parathyroidectomy (PTX).³⁷ The objectives of this study were to determine (1) the effect of CRF on calcidiol levels in rats, (2) the ability of CRF rats to C-25-hydroxylate vitamin D₃ after administration of vitamin D₃ or 1α-hydroxyvitamin D₃, (3) the role of liver CYP450 downregulation in calcidiol deficiency in CRF, and (4) the potential role of secondary hyperparathyroidism in calcidiol synthesis in rats with CRF.

RESULTS

Biochemical Parameters and Vitamin D₃ Concentrations in Control Rats and Rats with CRF

Table 1 presents the physiologic characteristics of studied animals. Body weights and calcium and phosphate concentrations were similar in control rats and rats with CRF. Compared with control rats, rats with CRF had higher concentrations of serum creatinine and a highly reduced creatinine clearance (P < 0.001). PTH serum concentrations were increased 10-fold in rats with CRF, indicating the presence of secondary hyperparathyroidism. Serum concentrations of 25(OH)D₃ were reduced by 52% (P < 0.01) in rats with CRF compared with control rats. 1,25(OH)₂D₃ serum concentrations were decreased by 73% (P < 0.01) in rats with CRF compared with controls. There was also a significant decrease in serum 24,25(OH)₂D₃ concentration (P < 0.01).

Correlation of 25(OH)D₃ Concentration with Renal and Parathyroid Functions

As shown on Figure 2A, we found a highly significant negative correlation between 25(OH)D₃ and creatinine concentration (P < 0.01).
in serum of rats with CRF ($r^2 = 0.78$, $P < 0.001$). We also found, as shown in Figure 2B, a significant negative correlation between PTH concentration and 25(OH)D$_3$ level in rats with CRF ($r^2 = 0.40$, $P < 0.05$).

**Impact of CRF on the Vitamin D$_3$ 25-Hydroxylase Activity in Rats**

The ability of rats with CRF and control rats to C-25-hydroxylate vitamin D$_3$ was assessed after the administration of vitamin D$_3$ or 1α-hydroxyvitamin D$_3$. As shown in Figure 3A, rats with CRF produced 71% ($P < 0.05$) less 25(OH)D$_3$ compared with controls, after the injection of vitamin D$_3$, suggesting a decrease in C-25-hydroxylation activity. Similarly, the formation of 1,25(OH)$_2$D$_3$ after the administration of 1α-hydroxyvitamin D$_3$ was reduced by 48% in rats with CRF compared with controls ($P < 0.05$), also suggesting a vitamin D$_3$-C-25-hydroxylation downregulation in rats with CRF (Figure 3B).

**Effect of Uremic Serum on Calcidiol Synthesis in Cultured Hepatocytes**

The objective of this experiment was to determine the effects of uremic serum on the synthesis of calcidiol by cultured hepatocytes incubated with its precursor, vitamin D$_3$, to confirm the role of uremic mediators in the downregulation of vitamin D$_3$-C-25-hydroxylation. Figure 4 demonstrates a reduction in the production of 25(OH)D$_3$ by isolated normal hepatocytes in the presence of serum obtained from animals with CRF. Production of 25(OH)D$_3$ was reduced by 54% ($P < 0.05$) in hepatocytes incubated with CRF serum compared with the production observed in controls.
Creatinine (\text{Total plasma calcium (mmol/L) 2.77})

Urea (mmol/L) 5.9

Urine 25(OH)D3 (nmol/L) 81.8

24,25(OH)2D3 (nmol/L) 26.8

1,25(OH)2D3 (pmol/L) 91.6

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dc

b

a

25(OH)D3 (nmol/L) 88.2

Weight (g) 372.5

Creatinine (\mu mol/L) 53.4

Creatine clearance (\mu l/100 g per min) 370.5

Urea (mmol/L) 5.9

25(OH)D3 (nmol/L) 88.2

1,25(OH)2D3 (pmol/L) 91.6

24,25(OH)2D3 (nmol/L) 26.8

Urine 25(OH)D3 (nmol/L) 81.8

PTH (1-84) (pg/ml) 2.49

Total plasma calcium (mmol/L) 2.77

>10-fold, and 25(OH)D3 and 1,25(OH)2D3 were decreased in rats with CRF. Most parathyroidectomized animals have undetectable levels of PTH; therefore, the PTH values in Table 2 are the mean for those with a detectable (>1.6 pg/ml) level of PTH (n = 4 in control parathyroidectomized rats and n = 3 in parathyroidectomized rats with CRF). In parathyroidectomized rats with CRF, the 25(OH)D3 concentrations were found to be similar to those of control rats. Thus, PTX prevents the downregulation of 25(OH)D3 production.

Effects of CRF on Liver CYP450 Isoforms Involved in the C-25-Hydroxylation of Vitamin D3 and the Role of PTH in Their Downregulation

This experiment was designed to evaluate whether CRF induced a downregulation of enzymes implicated in the synthesis of calcidiol and also to confirm the implication of PTH in the decrease of liver CYP450. As shown in Figure 5A, the protein levels of CYP450 2C11, 2J3, 3A2, and 27A1, which are known to play a role in the C-25-hydroxylation of vitamin D3, were reduced by 66, 32, 72, and 36%, respectively (\(P < 0.05\)), in rats with CRF compared with control rats. Results also show that preventing secondary hyperparathyroidism by PTX partially prevents the negative effect of CRF on protein expression levels of these isoforms. No significant differences were observed in CYP2R1 expression. Similar results have been obtained for mRNA levels (Figure 5B), except for CYP27A1, for which gene expression was unaffected in rats with CRF.

Effects of CRF on Renal CYP450 Isoform Involved in the C24–25-Hydroxylation of Vitamin D3

Although we found a decrease in 24,25(OH)2D3 concentration in rats with CRF (Tables 1 and 2), suggesting that increased catabolism of calcidiol was not involved in the observed low levels of 25(OH)D3, we measured protein expression and mRNA levels of CYP24A1 in the kidney of...
Figure 5. CRF downregulates the protein and mRNA expression of various CYP450s implicated in 25(OH)D₃ hydroxylation and this downregulation can be prevented by PTX. (A and B) Impact of CRF on CYP450 protein expression (A) and mRNA expression (B) with or without previous PTX. Protein and mRNA encoding for various cytochrome isoforms are compared in control rats (), rats with CRF (), control rats with PTX (), and rats with CRF and PTX () after quantification by Western blot or quantitative PCR in the liver. (C) Protein and mRNA encoding for CYP24A1 are compared in control rats () and rats with CRF () after quantification by Western blot or quantitative PCR in the kidney. *P < 0.05 versus control rats; †P < 0.05 versus rats with CRF; ‡P < 0.05 versus control rats with PTX.

Figure 6. Serum PTH concentration regulates 25-hydroxylation of vitamin D₃. (A) Effect of PTH infusion on the in vivo production of 25(OH)D₃ in rats. Serum 25(OH)D₃ concentrations are measured at T = 0 () and T = 48 hours () on either control rats (CTL) or rats with PTX. Infusion pumps deliver either rat PTH (1-34) in 2% cysteine-HCl at 0.06 nmol/kg per h or 2% cysteine-HCl (vehicle) intravenously in rats with PTX. *P < 0.05 versus T = 0. (B and C) Effect of PTH infusion on CYP450 protein (B) and mRNA expression (C) in CTL rats (), rats with PTX and vehicle (), rats with PTX and PTH (), or rats with PTX and PTH pumps () after quantification by Western blot or quantitative PCR in the liver. *P < 0.05 versus CTL rats.
control rats and rats with CRF. We found that CYP24A1 protein expression and mRNA were not significantly different between groups (Figure 5C).

**Effect of In Vivo PTH Infusion on 25(OH)D₃ Production and on Liver CYP450 Isoforms**

To determine further the involvement of PTH in the downregulation of the C-25-hydroxylation of vitamin D₃, we used an implantable osmotic minipump to infuse PTH (1-34) in control rats after PTX. Figure 6A shows a reduction in the production of 25(OH)D₃ in rats after a 48-hour PTH infusion (P < 0.05). No significant changes in 25(OH)D₃ concentrations were observed in rats infused with vehicle alone or in control rats. Significant (P < 0.05) decreases in CYP2C11, 2J3, and 3A2 protein expression (Figure 6B) as well as mRNA (Figure 6C) expression were also observed in rats infused with PTH (1-34) for 48 hours.

**DISCUSSION**

This study demonstrates that calcidiol deficiency in rats with CRF is secondary to a decrease in its synthesis by the liver. Our results show that calcidiol formation, after vitamin D₃ injection or calcitriol production after 1α-hydroxyvitamin D₃ administration, is significantly reduced in rats with CRF, demonstrating that CRF impedes the C-25-hydroxylation of vitamin D₃. The decrease in biosynthesis of calcidiol in CRF is explained by a downregulation in key liver CYP450 isoforms (namely, CYP2C11, 2J3, 3A2, and 27A1) responsible for the C-25-hydroxylation of vitamin D₃. This study also suggests that PTH is a major regulator of calcidiol biosynthesis. Indeed, PTX in rats with CRF not only prevented calcidiol deficiency but also prevented the downregulation of several liver CYP450 isoforms involved in the C-25-hydroxylation of vitamin D₃. Furthermore, injection of PTH in rats mimics the effect of CRF by inducing a decrease in calcidiol levels and also a reduction in CYP450 isozymes.

Although controversial, dietary restriction or reduced sun exposure have been proposed to explain the very high prevalence of inadequate calcidiol concentrations in the CKD population. In this study, to avoid the effect of CRF-induced malnutrition, we used control animals that were pair-fed, and we still can demonstrate a significant reduction in calcidiol synthesis in rats with CRF, ruling out that lower dietary intake is involved. Furthermore, all of our study animals were submitted to strictly controlled light exposure. This also rules out a potential difference in biosynthesis of 25(OH)D₃ precursor by skin cells.

Because CRF, especially that induced by 5/6 nephrectomy, is frequently associated with proteinuria, another hypothesis to explain the observed calcidiol deficiency is an increase in urinary losses of calcidiol. We thus measured the levels of 25(OH)D₃ in urine of control rats and rats with CRF. As shown in Table 1, there was only a slight difference in urinary calcidiol concentrations between the two groups, ruling out the role of urinary loss in the observed calcidiol deficiency in CRF. Furthermore, calcidiol deficiency could be secondary to an increased degradation of 25(OH)D₃ into 24,25(OH)₂D₃, which is not the case, as illustrated in Table 1.

Our results clearly demonstrate for the first time that CRF impedes the vitamin D₃ 25-hydroxylase activity. Indeed, we found that rats with CRF have a reduced capacity to C-25-hydroxylate not only vitamin D₃ but also 1α-vitamin D₃, leading to a decrease in calcidiol and calcitriol formation, respectively. This decrease in 25-hydroxylase activity explains the calcidiol deficiency observed in CRF, but it could also explain why the efficacy of vitamin D₃ therapy on serum calcidiol levels is variable and remains poor compared with patients without CKD.

In this study, we tested the hypothesis that uremic mediators were involved in the decrease in calcidiol concentrations. The results clearly demonstrate that CRF serum contains factors that are able to downregulate hepatic vitamin D₃ 25-hydroxylase activity. The results obtained in hepatocytes incubated with uremic serum closely mimic the data obtained in vivo when we administered vitamin D₃ to rats with CRF.

As illustrated in Figure 1, liver CYP450 is the major enzymatic family responsible for the synthesis of calcidiol. Several human and animal studies have revealed that CRF downregulates many hepatic CYP450 isoforms involved in the metabolism of drugs, and, interestingly, some of them are also involved in the C-25-hydroxylation of vitamin D₃. Our study demonstrates that CRF decreases several CYP450 isoforms (CYP2C11, 2J3, 3A2, and 27A1), whereas CYP2R1 was found to remain unchanged. The relative importance of these various CYP450 isoforms in the synthesis of calcidiol remains controversial; however, many studies suggested that 2C11, 2J3, and 3A2 are the most important isoforms implicated in the synthesis of calcidiol in the rodent liver. This could explain why our rats with CRF had reduced calcidiol synthesis.

We attempted to determine the mechanism leading to the downregulation of these enzymes. Because PTH has been shown to impair protein synthesis not only by reducing gene expression, in several organs including the liver, but also by reducing hepatic CYP450s, we tested the hypothesis that CRF-induced secondary hyperparathyroidism could be linked to the decrease in vitamin D₃ 25-hydroxylase activity in renal failure. First, we evaluated whether PTX could prevent the decrease in calcidiol levels in rats with CRF. As shown in Table 2, rats with CRF and PTX have essentially the same calcidiol concentrations as control rats. Furthermore, CYP450s in rats with CRF were significantly improved by PTX. Moreover, our data demonstrate that PTH infusion in control rats with PTX also led to decreased calcidiol concentrations to levels similar to those observed in rats with CRF.

Fibroblast growth factor 23 (FGF23) is a novel circulating factor implicated in phosphate and vitamin D regulation. In the kidney, FGF23 increases phosphate excretion and inhibi-
its 1α-hydroxylase, thereby decreasing 1,25(OH)2D3 synthesis. In patients with CRF, FGF23 rises progressively, and it has been linked not only to reduced calcitriol synthesis but also to an increase in morbidity and mortality.50,51 Whether FGF23 could also be implicated in calcidiol deficiency remains to be tested.

Although this study has been conducted in rats, our data could be applied to humans. First, in humans, hyperparathyroidism seems to decrease 25(OH)D3, a finding that has been reported in primary hyperparathyroidism and shown to be corrected by PTX.37 Second, we found a significant correlation between the decrease in renal function and the reduction in calcidiol concentrations (Figure 2A), suggesting that as CRF worsens, calcidiol synthesis is less efficient. This observation could explain why some human studies have reported a lower efficacy of vitamin D3 supplementation in stages 4 and 5 compared with stage 3 CKD.16,17 Conversely, major differences in the CYP450 isoforms are implicated in the synthesis of calcidiol between rats and humans. Indeed, in humans, CYP2R1 is a key isoform responsible for the 25-hydroxylation of vitamin D, whereas in the rats, its role is secondary.24 It remains to be demonstrated in patients with CRF whether CYP2R1 is also decreased to explain calcidiol deficiency.

In conclusion, CRF per se in an animal model of CKD causes a significant decrease in serum calcidiol concentration. CRF is associated with a decreased calcidiol synthesis from natural precursor and is associated with a downregulation of liver CYP450 isoforms involved in vitamin-D3-C-25-hydroxylation. An uremic humoral mediator seems to be involved, and as PTX abolishes CRF-associated calcidiol deficiency and PTH infusion decreases calcidiol synthesis, parathyroid hormone could be a potential culprit.

CONCISE METHODS

Experimental Model

All 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. Male Sprague-Dawley rats (Charles River, Saint-Constant, Québec, Canada) that weighed 176 to 225 g were housed in the Research Centre animal care facility and maintained on Harlan Teklad rodent diet (Harlan Teklad Global Diet, Montréal, Québec, Canada) and water. An acclimatization period of 7 days was instituted before any experimental work was undertaken.

CRF was induced by a two-stage 5/6 nephrectomy protocol as described previously.12 Control rats underwent two sham laparotomies (days 1 and 8) and were pair-fed with a quantity of rat food similar to that ingested by rats with CRF on the previous day. At day 41 after surgery, rats were housed in metabolic cages and urine was collected for 24 hours for determination of the clearance of creatinine. When rats were killed, tissue and blood samples were collected. Biopsy samples were stored at −80°C up to analysis.

At day 42, rats were either killed by decapitation or injected with vitamin D3 (250 μg/kg) or 1α-hydroxyvitamin D3 (1.2 nmol/kg). Vitamin D3 (Sigma, St. Louis, MO) was prepared in 95% ethanol and dissolved in saline:propylene glycol:ethanol (20:65:15). A maximum of 200 μl was injected intravenously via tail veins. Blood samples were collected before the injection and 48 hours later, when rats were killed. 1α-Hydroxyvitamin D3 (Leo Pharma, Thornhill, Ontario, Canada) was diluted in sterile saline and injected intravenously via tail veins. Blood samples were collected before injection and 3 hours later (when killed).

PTX was performed as previously reported,35,52 7 days before the first step of the 5/6 nephrectomy protocol. To avoid hypocalcemia, rats were supplemented in calcium by addition of calcium gluconate in drinking water (2.5% for rats with CRF and 5% for control rats). Control rats were subjected to a neck sham surgery.

Hepatocyte Isolation and Culture

Normal rat hepatocytes were isolated according to the two-step liver perfusion method as previously reported,23 with slight modifications: Cells were incubated in HAM/F12, 10% FBS, and 10,000 U/ml Pen-Strep. After preincubation (2 hours), the medium was changed for a 24-hour conditioning period. Cells were then conditioned by serum deprivation for 24 hours by incubation in HAM/F12 and Pen-Strep, containing growth factors (EGF [20 μg/ml], insulin/transferrin/selenium [25 mg:25 mg:25 μg/ml], prostaglandin E2 [100 μg/ml], hydrocortisone [5 mg/ml], thyroxine [4 mg/ml], IFN-γ [10,000 U/ml], and nonessential amino acids). Preconditioned cells were then incubated for 24 h with 10% serum from CRF or control rats with or without 20 nmol/ml vitamin D3. Supernatants were recovered and were stored at −80°C until 25(OH)D3 assay. All reagents were provided by Sigma Chemical Co.

PTH Infusion

Mini osmotic pumps (1003D; Alzet, Cupertino, CA) were implanted in the jugular vein for a period of 48 hours. Pumps were filled with 0.06 nmol/kg per h rat PTH (1-34) (Bachem, Torrance, CA) in 2% Cysteine-HCl (ICN Biomedical, Costa Mesa, CA). Pumps were implanted 14 days after PTX surgery, and rats received 1% calcium gluconate in drinking water. Control rats received only 2% Cysteine-HCl in their pumps. Rats were killed, and blood was stored at −80°C up to analysis.

Biochemical and Hormonal Analyses

Serum urea, creatinine, calcium, phosphate, and albumin and urine creatinine concentrations were measured with an Architect Ci6000 clinical analyzer (Abbott, Saint-Laurent, Québec, Canada). Rat intact serum PTH (1-84) was determined with an ELISA kit (Alpco Diagnostics, Windham, NH). Serum and supernatant 25(OH)D3 and serum 1,25(OH)2D3 were evaluated using RIA kit (IDS Inc., Medicoorp, Montréal, Québec, Canada). Supernatants (1 ml) were lyophilized overnight and were resuspended in 50 μl of solution 1 of RIA kit. The assay sensitivity for PTH is 1.6 pg/ml.

Measurement of 24,25(OH)2D3 in Sera and 25(OH)D3 in Urine

Quantification of vitamin D3 metabolites 24,25(OH)2D3 and 25(OH)D3 was performed according to a previously published method53 with some
modifications. In the first step, samples were extracted. Sera (500 μl) were spiked with 10,000 DPM of tritiated 24,25(OH)D₃ and 10,000 DPM of tritiated 25(OH)D₃ (Amersham Biosciences, Baie d’Urfé, Québec, Canada) then extracted with 500 μl of acetonitrile. The aqueous phase was separated by centrifugation then rinsed with 500 μl of 0.4 M K₂HPO₄ (pH 10.5). The recovered aqueous phase was passed through a preconditioned C18 column (Bond Elut C18-OH; Varian, Montréal, Québec, Canada) according to the manufacturer’s instructions and eluted with 2 ml of water and 5 ml of methanol:H₂O (70:30). Samples were recovered in 5 ml of hexane:methylene chloride (90:10) and dried under nitrogen. Samples were reconstituted in 100 μl of hexane:isopropanol (92:8), then injected on an Agilent 1100 HPLC mounted with a Zorbax silica column 5 μm 4.6 × 250 mm and eluted with hexane:isopropanol (92:8) at a flow rate of 2 ml/min. Fractions were collected between 4 and 5 minutes after injection for the quantification of 25(OH)D₃ and between 7.5 and 9.0 minutes for the quantification of 24,25(OH)₂D₃. These fractions were pooled and dried under nitrogen. Quantification of both metabolites was performed with an RIA assay (IDS Inc.). Dried samples were reconstituted in 275 μl of buffer from the kit, and the assay was performed on a 50-μl aliquot.

RNA Isolation
Total RNAs were extracted from liver and kidney with guanidine isothiocyanate and lithium chloride following Chirgwin’s method. All samples were digested by proteinase K to remove protein contaminants, then extracted with phenol-chloroform-isooamyl alcohol and precipitated with sodium acetate and ethanol. Isolated RNAs were further purified by digestion with DNase and purification with RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada). cDNA was synthesized with 1 μg of total RNA by reverse transcription (First-Strand cDNA Synthesis Kit, Amersham Biosciences) following the manufacturer’s instructions using random primers Pd(N)₆ (Invitrogen, Mississauga, Ontario, Canada). cDNAs were purified with ChargeSwitch PCR Clean-Up kit (Invitrogen) and resuspended in 30 μl of Tris buffer (10 mM, pH 8.5).

Quantitative PCR Analysis
Specific primers for the studied cytochrome CYP450 isoforms (CYP2C11, 2J3, 2R1, 3A2, and 27A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 3) were obtained from Invitrogen. PCR mix (25 μl) was made of cDNA (2.5 μl for GAPDH or 6 μl for CYP isoforms), 20 μM of specific primers, and 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Samples were amplified in Rotor-Gene 3000 Real-Time DNA Amplification system (Corbett Research, Sydney, Australia) using the following PCR conditions: 95°C for 3 minutes, followed by 45 amplification cycles (95°C for 10 seconds, 59°C for 30 seconds, and 72°C for 30 seconds). Standard curves were realized with an arbitrary chosen sample of liver RNA obtained from a normal animal. Results were expressed as a ratio of CYP to GAPDH. The mean ratio value for the control group was arbitrarily set to 1. TaqMan gene expression assay for CYP24 was obtained from Applied Biosystems (Foster City, CA). PCR was realized following the manufacturer’s protocol. Results were expressed as a ratio of CYP24 to GAPDH.

Western Blot Analysis
Protein expression of CYP2C11, 2J3, 2R1, 3A2, and 27A1 was assayed in the liver of different groups of rats using Western blot analysis as described previously. Protein expression of CYP24A1 has been measured in the kidney of control rats and rats with CRF by Western blot as previously reported. Antibodies for CYP2J3, 2R1, and 27A1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CYP24A1 was detected with an antibody from Abnova (Jhongli City, Taiwan), CYP2C11 and 3A2 antibodies came from Gentest (Woburn, MA), β-actin came from Neo-Markers (Fremont, CA), and GAPDH came from Abcam (Cambridge, MA). All secondary antibodies were coupled to peroxidase and were from Sigma.

Statistical Analysis
All results are expressed as means ± SD. Differences between group means were assessed using an unpaired t test or an ANOVA. All significant ANOVA was followed by a post hoc Scheffe analysis. All statistically significant values were set at P < 0.05.

ACKNOWLEDGMENTS
This work was presented in part at the annual meeting of the American Society of Nephrology; October 31 through November 5, 2007, San Francisco, CA; and October 27 through November 1, 2009, San Diego, CA.

This work was supported by the Canadian Institute of Health Research and Fonds de la recherche en Santé du Québec (FRSQ) and la Fondation de l’Hôpital Maisonneuve-Rosemont. V.P. is a scholar of the FRSQ, and J.M. as well as J.N. are fellows of the FRSQ.

DISCLOSURES
None.
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