Downregulation of liver drug metabolizing enzymes in a murine model of chronic renal failure

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Chronic renal failure (CRF)
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ABSTRACT

Drug metabolism could be altered in patients with chronic renal failure (CRF). In rat, this phenomenon is related to a decrease in liver cytochrome P450 (CYP450) and phase II enzymes, particularly N-acetyltransferase 2 (NAT2). This study attempted to determine the effects of CRF on liver CYP450 isoforms and NAT2 expressions using a CRF mouse model. Two groups of mice were studied: CRF induced by 3/4 nephrectomy and control. Liver protein expression and mRNA levels of the major CYP450 isoforms involved in drug metabolism (CYP1A2, 2C29, 2D, 2E1 and 3A11) and NAT2 were measured by Western blot and real-time PCR, respectively. CYP3A activity was also assessed by the N-demethylation of erythromycin. Results showed a significant reduction in the protein expression of CYP1A2 (56%), 2C29 (31%), and 3A11 (37%) in CRF mice compared with control animals. Real-time PCR revealed a similar reduction in mRNA levels of CYP1A2, 2C29, and 3A11 (59%, 56%, 37%, respectively), in CRF mice. There was no significant modification in protein expression and mRNA of CYP2D and 2E1. When compared with control animals, CRF mice displayed a 25% reduction in N-demethylation of erythromycin. As for NAT2, protein expression decreased by 33% and mRNA levels by 23%. In conclusion, this study demonstrates that protein expression of liver CYP1A2, CYP2C29 and CYP3A11 are downregulated in CRF mice, secondary to reduced gene expression. Phase II enzymes are similarly affected by CRF. Our results will allow the use of knockout mice to determine the mechanism underlying CRF-induced downregulation of liver drug metabolizing enzymes.
INTRODUCTION

In patients with chronic renal failure (CRF), several studies have shown a decrease in the metabolic clearance of drugs, particularly those metabolized by cytochrome P450 (CYP450) (Dreisbach and Lertora, 2003; Dreisbach and Lertora, 2008; Nolin et al., 2008), which could be explained by a decrease in liver CYP450 enzymes and/or by reduced liver uptake (Sun et al., 2006). Although the precise isoforms downregulated in humans are still unknown, animal studies have shown that CRF induced a downregulation of several CYP450 isoforms in the liver: CYP3A1, 3A2, and 2C11. The mechanism underlying this decrease in CYP450 protein expression appears to be related to reduced gene expression (Leblond et al., 2000; Leblond et al., 2001; Nolin et al., 2008). Phase II enzymes, namely N-acetyltransferases (NATs), are equally downregulated in CRF due to a decrease in gene expression (Simard et al., 2008).

Knowing which drug metabolizing enzymes are reduced by CRF is critical in order to predict which drugs are at risk of accumulation. Since all animal studies have been conducted in rats, the results may not necessarily be applicable to other species. Indeed, there may be species-specific differences in modulation of liver CYP450 and NATs by CRF. Moreover, there are no reports on the effects of CRF on drug metabolizing enzymes in the mouse, which is a good model to directly evaluate the role of different factors due to the availability of knockout mice.

In the present study, the effects of CRF on protein expression, gene expression, and activity of the major liver CYP450 isoforms involved in drug metabolism were investigated in a CRF mouse model. Similarly, the protein and gene expression of NAT2 were also investigated using this model. Renal failure was induced following a 3/4 nephrectomy, an adaptation of the 5/6 nephrectomy used on rats.
METHODS

Experimental Model

Male C57BL/6 mice (Charles River, Hollister, CA), weighing 20-25 g were housed in the Research Centre animal care facility and maintained on a Teklad diet (Harlan Laboratories Inc., Montreal, Québec, Canada) and water ad libitum. An acclimatization period of at least 7 days was provided to the animals before any experimental work was undertaken. All of the experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals. Our animal facilities are approved by the Canadian council for animal care. Studies were performed using two groups of mice: CRF (n=12) and control (n=12). Chronic renal failure was induced by two-stage three-quarters nephrectomy. To do so, we adapted the 5/6 nephrectomy model (Leblond et al., 2001; Leblond et al., 2002) commonly used for the rat with slight modifications. Briefly, a dorso-lumbar incision was made and apexes (two-quarters) of the left kidney were removed with a scalpel on day 1. A second dorso-lumbar incision was made on the opposite side on day 15 and a right total nephrectomy was performed. After surgery, CRF animals were fed mouse chow and water ad libitum. Mice from the control group underwent two sham laparotomies (day 1 and 15). Body weight was measured every other day for the duration of the study. Control mice were pair-fed the same amount of mouse chow that was ingested by the CRF mice on the previous day. On day 49, five weeks after the total nephrectomy, mice were sacrificed by decapitation. Sera were stored at -80°C for the measurement of serum creatinine and urea.

Preparation of Liver Microsomes and Cytosols

Mouse livers were immediately excised after death, and microsomes isolated by differential centrifugation (Tindberg et al., 1996) as previously described (Leblond et al., 2001).
The pellets containing the microsomes were stored at -80°C in 0.1 M Tris (pH 7.4), 20% glycerol, 10 mM ethylenediamine tetraacetate (EDTA) until analysis. Microsomal protein content was measured using micro BCA Protein Assay, using bovine serum albumin as standard protein (Pierce, Rockford, IL). Cytosolic proteins were dosed using the Bradford method (Coomassie Plus Protein Assay Reagent), with bovine serum albumin as standard (Pierce).

**Western Blot Analysis**

Protein expression of the major drug metabolizing enzymes was analyzed by Western Blot. Mouse CYP1A2, CYP2C29 and CYP2E1 were detected using polyclonal goat anti-rat 1A1, 2C11 and 2E1 (Daiichi Pharmaceutical Co. Ltd., Japan), respectively. CYP2D was detected using a rabbit anti-human 2D (Oxford Biochemical Research Inc., Oxford, MI), CYP3A11 was detected using a polyclonal goat anti-mouse 3A (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and NAT2 was detected using specific polyclonal rabbit anti-rat NAT2 as described previously (Stanley et al., 1996; Simard et al., 2008). β-Actin was detected using a mouse anti-chicken β-actin (Neo-Markers, Fremont, CA). Immune complexes were revealed by corresponding secondary antibodies: swine anti-goat IgG (Biosource International, Cammarillo, CA), donkey anti-goat IgG (US Biological, Swampscott, MA), goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and goat anti-mouse IgG (Sigma-Aldrich Corp., St. Louis, MO) coupled to peroxidase and the Luminol derivative of Lumi-Light Western blotting substrate (Roche Diagnostics, Laval, Que, Canada).

**mRNA Isolation and Real-Time PCR Analysis**

Liver RNA extractions were done using the RNeasy Mini Kit (Qiagen, Mississauga, Ont, Canada). One µg of total RNA was used to synthesize cDNA by reverse transcription using Omniscript RT kit (Qiagen) and random primers (Invitrogen, Burlington, Ont, Canada).
Multiplex quantitative PCR analysis was performed using a iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, Ont, Canada) with Bio-Rad’s IQ Multiplex Powermix. Specific primers for Cyp3a11 and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were previously published (Mikula et al., 2005; Richardson and Morgan, 2005). Probe for Cyp3a11 was dye-labelled with FAM™ while probe for Gapdh was dye-labelled Yakima Yellow®. For Cyp1a2, Cyp2c29, Cyp2d22, Cyp2e1 and Nat2, the primer-probe sets used were pre-designed TaqMan® Gene Expression Assays obtained from Applied Biosystems (Foster City, CA). PCR conditions were optimized to 95°C for 15 sec and 60°C for 60 sec. Resulting data were processed by the delta Ct method (Livak and Schmittgen, 2001).

Ex Vivo Metabolism of Erythromycin

In order to evaluate the metabolic activity of CYP3A in liver microsomes, erythromycin N-demethylation was determined as described elsewhere (Wang et al., 1997; Guevin et al., 2002; Michaud et al., 2005). Briefly, 1mM erythromycin (Sigma-Aldrich Corp.) was incubated with 1.5 mg of mouse liver microsomal proteins (either from control or CRF) at 37°C for 30 min in the presence of an NADPH-generating system consisting of the following: 20 mM glucose 6-phosphate, 2 mM NADP, and 0.14 units/mL glucose-6-phosphate dehydrogenase (Sigma-Aldrich Corp.) in a total volume of 0.5 mL. Resulting production of formaldehyde was measured by the NASH reaction and read on spectrophotometer at 405 nm.

Blood Chemistries and Statistical Analysis

Blood chemistries (urea, creatinine) were determined with an Architect C1600 clinical analyser (Abbott, Saint-Laurent, Que, Canada). The results are expressed as mean ± SEM. Differences between groups were assessed using an unpaired t test. The threshold of significance was p<0.05.
RESULTS

Biochemical Parameters and Body Weight in Control and CRF Mice

When compared with control animals, CRF mice had higher levels of plasma creatinine (93.1 ± 7.6 compared to 56.7 ± 1.5 µmol/L, p<0.01) and blood urea nitrogen (BUN) (97.6 ± 6.3 compared to 28.3 ± 1.2 mg/dL, p<0.01). There was no significant difference in body weight between control and CRF mice (27.1 ± 1.4 vs 23.6 ± 0.8 g).

Protein Expression of Liver Cytochrome P450 Isoforms in Control and CRF Mice

The level of protein expression of CYP1A2, 2C29 and 3A11 in CRF mice (n=8) were reduced by 56% (p<0.05), 31% (p<0.05), and 37% (p<0.01), respectively, in comparison with control animals (Figure 1). On the other hand, the levels of CYP2D and 2E1 were not modified in CRF mice in comparison with control animals.

mRNA Encoding Liver Cytochrome P450 Isoforms in Control and CRF Mice

In order to evaluate whether cytochrome P450 isoforms in CRF were downregulated due to a decrease in their synthesis or an increase in their degradation, mRNA encoding the different isoforms were evaluated by real-time PCR. A significant decrease in mRNA levels of CYP1A2 (59%, p<0.01), 2C29 (56%, p<0.01) and 3A11 (37%, p<0.01) isoforms was observed in CRF mice (n=12) when compared to control mice (Figure 2). Thus, this decrease in mRNA encoding for CYP450 isoforms could explain the decrease in their protein expression. On the other hand, no significant change was observed in mRNA levels of CYP2D22 and 2E1 between CRF and control mice.

Ex vivo Metabolism of Erythromycin

The ex vivo N-demethylation of erythromycin was assessed in liver microsomes to determine the repercussion of cytochrome CYP450 reduction in CRF on the metabolism of drugs.
The CYP3A family primarily mediates this enzymatic reaction. The N-demethylation of erythromycin was reduced by 25% (p<0.05) in mice with CRF (n=8) when compared to control animals (1.3 ± 0.1 vs 1.7 ± 0.1 nmol/mg of microsomal protein/min, p<0.01).

**Protein and mRNA expression of NAT2 in Control and CRF mice**

NAT2 protein expression was decreased by 33% in CRF mice (n=8) compared with control animals (p<0.05; **Figure 3**). To evaluate whether the downregulation of NAT2 in the liver of CRF mice was related to a decrease in protein synthesis, we evaluated mRNA levels by multiplex quantitative PCR. A 23% decrease in mRNA encoding NAT2 was revealed in CRF animals (n=12) compared with controls (p<0.05; **Figure 3**). Thus, the decrease in NAT2 protein levels observed in CRF is caused by reduced gene expression.
DISCUSSION

This study demonstrates that, in the mouse, CRF is associated with an important reduction of selected liver CYP450 isoforms (CYP1A2, 2C29 and 3A11) secondary to reduced gene expression. The repercussions on drug metabolism are significant since we observed a 25% reduction of erythromycin N-demethylation by the CYP3A family. Moreover, diminished protein and mRNA levels of NAT2 suggest that, in addition to CYP450, phase II enzymes are also altered in CRF.

Although several reports have demonstrated a decrease of liver CYP450 in CRF, very few have focussed on which specific isoforms were affected by the disease. Past studies have shown that uremic rats have reduced CYP2C11, 3A1 and 3A2 (Leblond et al., 2000; Leblond et al., 2001; Nolin et al., 2008). Similarly, the present study has illustrated decreased CYP2C29 and 3A11 in CRF mice, confirming the results obtained in rats. It is interesting to note that these two aforementioned isoforms are responsible for the metabolism of many drugs and could thus explain the reduction in the metabolic clearance described in CRF patients (Sun et al., 2006; Dreisbach and Lertora, 2008; Nolin, 2008; Nolin et al., 2008). Indeed, some of these drugs, such as warfarin and erythromycin, are less metabolized in renal failure patients in comparison with controls (Dowling et al., 2003; Dreisbach et al., 2003; Nolin et al., 2006). This probably reflects a decrease in CYP2C and 3A in patients, as shown in CRF animals. However, we also observed a reduction of CYP1A2 in CRF mice, something that was not found in rats. This may be attributed to species-specific differences in regulation of CYP450 isoforms (Martignoni et al., 2006).

Phase II enzymes, especially NATs, metabolize several commonly used drugs in CRF patients. Previous studies have shown that CRF was associated with a decrease in acetylation of drugs, implying that phase II enzymes could also be modified in CRF (du Souich and Erill, 1978;
Kim et al., 1993). The results of the present study clearly support this hypothesis since we found a decrease in NAT2 expression in CRF animals. Yet, the mechanism that initiates CYP450 and NAT2 downregulation in CRF remains unclear. This study confirms nonetheless the correlation between reduced protein expression and mRNA, suggesting that CRF inhibits CYP450 and NAT2 gene expression in the liver.

Very few CRF studies using a murine model have been published. Therefore, no specific procedure to induce CRF has been generally adopted. The possible techniques are diverse: bilateral electrocauterization of the renal cortex (Gagnon and Duguid, 1983), unilateral cauterization of the right renal cortex followed by a contralateral nephrectomy (Gagnon and Ansari, 1990), or unilateral nephrectomy and partial infarction of the remnant kidney (Kren and Hostetter, 1999). When choosing a CRF model, one must consider its simplicity, reproducibility, and similarity to the human pathology. Since the previous models do not reflect the most observed cases of CRF in humans, we induced CRF by 3/4 nephrectomy. This was an adaptation of the 5/6 nephrectomy model, most commonly used in the rat. We clearly demonstrated the efficacy and reproducibility of this model to induce CRF.

In conclusion, this study demonstrates that protein expressions of liver CYP1A2, 2C29 and 3A11 are downregulated in mice with CRF, along with NAT2. These effects are all secondary to reduced gene expression. This will allow the use of knockout mice to clarify the mechanism underlying CRF-induced inhibition of hepatic drug metabolizing enzymes. For instance, knockout animals for factors suspected to downregulate CYP450s in CRF (e.g. parathyroid hormone, cytokines) (Dreisbach, 2009) or factors involved in CYP450s regulation (e.g. nuclear receptors like PXR) could be studied (Zimmermann et al., 2009).
REFERENCES


FOOTNOTES

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Vincent Pichette is scholar of the FRSQ and Mélina Dani, Josée Michaud and Judith Naud are fellows of the FRSQ.

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LEGEND FOR FIGURES

**Figure 1**: Protein expression of hepatic cytochrome P450 isoforms in microsomes from control (white bar) and CRF mice (black bar). Protein bands are expressed in densitometry units (%). The densitometry units of control mice were arbitrarily defined as 100%. Data are the mean ± SEM of eight experiments in each group. *p<0.05 when compared with control. **p<0.01 when compared with control. Representative blots are also shown.

**Figure 2**: mRNA encoding hepatic cytochrome P450 isoforms in control (white bar) and CRF mice (black bar). Control results were defined as 100%. Data are the mean ± SEM of twelve experiments in each group. **p<0.01 versus CTL mice.

**Figure 3**: Expression of liver NAT2 protein (white bar) and mRNA (black bar) in control and CRF mice. Protein expression was measured by western blot in cytosols and specific mRNA was quantified by real-time PCR. Control results were defined as 100%. Data are the mean ± SEM of eight experiments in each group for protein expression, and twelve experiments in each group for mRNA levels. *p<0.05 when compared with control. Representative blots are also shown.
Figure 1
Figure 3

The diagram shows the expression levels of NAT2 and β-actin in CTL and CRF conditions. The bars represent the percentage of control values, with error bars indicating the standard deviation.

- In CTL, NAT2 expression is 100 ± 7, while β-actin expression is 100 ± 10.
- In CRF, NAT2 expression is 77 ± 5, and β-actin expression is 67 ± 11.

Significant differences are indicated by asterisks (*) for NAT2 and β-actin in CRF compared to CTL.