Down-regulation of Hepatic CYP3A and CYP2C Mediated Metabolism in Rats with
Moderate Chronic Kidney Disease

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Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; ESRD, end-stage renal disease; CYP, cytochrome P450; KEEP, Kidney Early Evaluation Program; NHANES, National Health and Nutrition Examination Survey; Maximum reaction rate of enzyme, $V_{\text{max}}$; Michaelis-Menten constant, $K_m$
ABSTRACT

Expression and activity of drug metabolizing enzymes are decreased in severe kidney disease, however, only a small percentage of patients with chronic kidney disease (CKD) are at the final stage of the disease. This study aimed to determine the changes in drug metabolizing enzyme function and expression in rats with varying degrees of kidney disease. Sprague-Dawley rats were subjected to surgical procedures that allowed the generation of three distinct models of kidney function: normal kidney function, moderate and severe kidney disease. Forty two days following surgery, rats were sacrificed and hepatic CYP3A and CYP2C expression was determined. In addition, enzymatic activity was determined in liver microsomes by evaluating midazolam (CYP3A), testosterone (CYP3A and CYP2C) and tolbutamide (CYP2C) enzyme kinetics. Both moderate and severe kidney disease were associated with a reduction in CYP3A2 and CYP2C11 expression (p<0.05). Similarly, moderate kidney disease resulted in more than a 60% decrease in enzyme activity (V_{max}) for CYP2C11 and CYP3A, compared to controls (p<0.05). When the degree of kidney disease was correlated with metabolic activity, an exponential decline in CYP2C and CYP3A mediated metabolism was observed. Our results demonstrate that CYP3A and CYP2C expression and activity are decreased in both moderate and severe CKD. Our data suggests that drug metabolism is significantly decreased in earlier stages of CKD and imply that patients with moderate CKD may be subject to unpredictable pharmacokinetics.
INTRODUCTION

Chronic kidney disease (CKD) is a progressive condition characterized by the decrease in glomerular filtration rate (GFR) over time. The incidence of CKD is increasing at an alarming rate and these patients require more than seven medications to manage both their CKD and associated co-morbidities (Talbert, 1994). Drug therapy in patients with CKD is complicated as these patients frequently experience unpredictable pharmacokinetics resulting in an increased incidence of medication-related adverse events (Manley et al., 2005). Although it is appreciated that renal clearance of drugs is compromised in CKD, recent studies have demonstrated that kidney failure also affects non-renal drug clearance (Leblond et al., 2001; Naud et al., 2008; Nolin et al., 2009). The non-renal clearance of drugs is principally mediated by hepatic drug metabolism. CYP3A4, a member of the cytochrome P450 (CYP) family of drug metabolizing enzymes, is involved in the oxidative metabolism of up to 50% of all drugs on the market (Wrighton et al., 1996). Elegant studies in experimental animal models of severe CKD have demonstrated a reduction in the function and expression of CYP3A and other members of the CYP family (Leblond et al., 2000; Leblond et al., 2002).

The Kidney Early Evaluation Program (KEEP, 2000 to 2010) and the National Health and Nutritional Examination Survey (NHANES, 1996 to 2006) have estimated the prevalence of kidney disease to be greater than 14% in the United States (KEEP, 2011; McCullough et al., 2011; Stevens et al., 2011). The majority of animal and clinical studies investigating the effect of CKD on non-renal drug clearance have focused solely on severe CKD. Patients studied in the majority of these publications are on dialysis despite the fact that severe CKD patients comprise approximately 3% of the total
number of patients with CKD (KEEP 2000 to 2009, NHANES 1999 to 2006). According to KEEP, 11%, 19% and 67% of patients with CKD are in stages 1, 2 and 3, respectively (KEEP, 2011). Therefore, patients with mild and moderate kidney disease comprise the majority (97%) of the population of patients with CKD. Few studies have evaluated pharmacokinetics and drug metabolism in varying degrees of kidney disease despite the fact that CKD is known to be a progressive disorder whereby kidney function declines over time. Clinical studies in patients with varying degrees of CKD have demonstrated altered lidocaine and nicardipine pharmacokinetics; however, these drugs are metabolized by multiple CYP enzymes and are influenced by other pharmacokinetic factors in kidney disease, such as changes in plasma protein binding (Ahmed et al., 1991; De et al., 2006). Consequently, there is a lack of data investigating the effects of CKD on specific drug metabolizing enzymes in the majority of the patient population. This void in the literature has been highlighted in a recent review (Naud et al., 2012).

The objective of this study was to determine if similar decreases in hepatic drug metabolizing enzymes observed in severe CKD are also seen at earlier stages of disease progression. To address this, we employed a combination of surgical kidney resection and partial renal artery ligation in rats to establish a novel model of CKD with two different degrees of kidney disease. Upon establishing this model, we assessed hepatic expression and function of the predominant CYP isoforms, CYP3A and CYP2C. In order to fully elucidate the effect of CKD progression on enzyme function, full enzyme kinetics of selected probe drugs was used to characterize the metabolic activity of these enzymes across the continuum of disease progression.
MATERIALS AND METHODS

Chemical Reagents and Drugs

Midazolam, 1’OH-midazolam, 4’OH-midazolam, OH-tolbutamide and ketoconazole were purchased from Toronto Research Chemicals (Toronto, ON). Testosterone, 6βOH-testosterone, 16αOH-testosterone were purchased from Steraloids Inc. (Newport, RI). Flurazepam and carbamazepine were purchased from Cerilliant (Round Rock, TX) and tolbutamide was purchased from Sigma (St. Louis, MO).

Experimental Model

Seventeen male Sprague-Dawley rats, weighing 150 g, were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The animals were maintained on standard rat chow and water ad libitum on a 12 hour light cycle. Rats were allowed to acclimatize for at least 3 days before any experimental procedure was undertaken. The animal protocols were approved by the University of Western Ontario Animal Care Committee.

Surgical Induction of Kidney Disease

Studies were performed in three experimental groups, which simulated normal kidney function (n = 6) as well as moderate (n = 7) and severe (n = 4) kidney disease. Moderate and severe kidney disease were surgically induced by modification of a standard two-stage partial nephrectomy. Atropine (0.04 mg/kg SC) was given prior to anaesthesia in addition to intramuscular xylazine (5mg/kg) and ketamine (100 mg/kg). Anaesthesia was maintained with 2% isoflurane in oxygen via facemask throughout all surgical procedures. On day zero, rats in the moderate kidney disease group underwent a one-third partial nephrectomy of the left kidney. Rats in the severe kidney disease
group were subjected to the same procedure plus ligation of one branch of the left renal artery to mitigate the perfusion of blood to the remaining kidney. Seven days later, a complete right nephrectomy was performed on both moderate and severe kidney disease groups as previously reported (Leblond et al., 2000). Rats in the control group underwent sham laparotomies. All rats were given ketoprofen (5 mg/kg, IM) pre-operatively and every 24 hours post-operation for 3 days. All rats were weighed daily to monitor health. Control and moderate kidney disease rats were pair-fed the same amount of standard rat chow that was ingested by severe kidney disease rats on the previous day. Forty two days after the initial surgery rats were sacrificed and liver tissue was harvested, flash frozen in liquid nitrogen and stored at -80°C. Serum creatinine and urea concentrations were determined by the London Laboratory Services Group by standard methods (London, ON).

**Real-time PCR Analysis**

Total RNA was extracted using Trizol Reagent (Life Technologies Inc. Burlington, ON) according to the manufacturer's instructions. RNA concentration and integrity were measured by spectrophotometry. Total RNA was reverse transcribed using iScript RT-qPCR Supermix (Bio-rad Hercules, CA) in a 20 µL reaction volume. Primer pairs used were as follows: CYP3A2 (forward) 5'- GCTCTTGATGCGTGAAGGATTTG-3' and (reverse) 5'- ATCACAGACCTTGCCAACCTCCTT-3' (Baldwin et al., 2006), CYP2C11 (forward) 5'- CCCTGAGGACTTTTGGGATGGGC-3' and (reverse) 5'- AGGGGCACCTTTGCTCTCCT-3' and β-actin (forward) 5'- ACGAGGCCACAGAGCAAGA-3' and (reverse) 5'-TTGTTACAATGCCGTGTTCA-3'
(Sohi et al., 2011). Real-time PCR was performed using SsoFast Evagreen® (Bio-rad Hercules, CA) and expression was analyzed using the $\Delta \Delta C_T$ normalized to $\beta$-actin.

Hepatic Microsome Isolation
Liver microsomes were isolated by differential centrifugation according to a slightly modified method by Kurosawa et al. (Kurosawa et al., 2009). Briefly, liver tissue was rinsed in 0.9% NaCl solution and homogenized in 1.15% KCl containing 1 mM ethylenediaminetetraacetic acid (EDTA) using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 9000 g for 20 min at 4°C and the subsequent supernatant was centrifuged at 105 000 g for 60 min at 4°C. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4 and protein concentration was determined by Pierce BCA protein assay (Fisher Scientific, Whitby, ON, Canada). Microsomal protein was stored at -80°C until analysis.

Western Blot Analysis
Protein expression of CYP3A2 and CYP2C11 was assessed in hepatic microsomal fractions using Western blot analysis. Twenty five micrograms of microsomal protein was electrophoresed on a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Proteins were transferred to nitrocellulose and immunoblots were performed according to antibody manufacturer’s recommendation. Immune complexes were revealed by horseradish peroxidase (Millipore, Billerica, MA) and band intensity was determined by densitometry (Quantity One 1-D Analysis Software on a VersaDoc Imaging System, Bio-Rad, Hercules, CA). Primary antibodies for CYP3A2 (monoclonal
rabbit anti-rat) were obtained from Millipore (Billerica, MA), CYP2C11 (monoclonal mouse anti-rat) from Detroit R&D Inc. (Detroit, MI) and actin (polyclonal rabbit anti-rat, chicken, mouse human) from Sigma (St. Louis, MO). HRP-linked secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Luminata™ Forte Western HRP substrate was obtained from Millipore (Billerica, MA). CYP450 antibody specificities were determined using Supersomes™ obtained from BD Biosciences (Mississauga, ON) (Supplemental Figure 1).

**Hepatic Metabolism of CYP3A and CYP2C Substrate Drugs**

Metabolic activity of CYP3A and CYP2C in hepatic microsomes was determined using specific probe substrates. Midazolam and testosterone were selected as probes for CYP3A, and testosterone and tolbutamide were selected as a probe for CYP2C enzyme activity. These probe substrates were selected based on their previously documented selective metabolism by specific rat P450 isozymes (Chovan et al., 2007). Reactions (final volume 250 µL) were conducted in 50 mM potassium phosphate buffer, 2 mM MgCl₂ (pH 7.4) with 1 mg/mL of hepatic microsomal protein. Prior to experimentation, linear rate of production of metabolites was determined by varying time, protein and relevant substrate concentrations. Metabolism was determined to be linear at 10 minutes for formation of testosterone metabolites (6β-OH testosterone and 16α-OH testosterone) and 30 minutes for formation of midazolam (1-OH midazolam, 4-OH midazolam) and tolbutamide (OH-tolbutamide) metabolites. All reactions were conducted at 37°C and were started by the addition of a relevant concentration of substrate drug (midazolam, testosterone or tolbutamide) followed by 1 mM NADPH. At the end of the incubation, reactions were terminated with 50 µL of ice-cold acetonitrile.
followed by a 15 minute incubation on ice and centrifugation to pellet precipitated protein (Chovan et al., 2007).

Metabolite Analysis by UPLC-PDA

Metabolite analysis was conducted by solid phase extraction followed by ultra-performance liquid chromatography with photodiode array detection (UPLC-PDA). Solid phase extraction cartridges (C18, Strata-X Polymeric Reverse Phase 33 µm, Phenomenex, Torrance, CA) were conditioned according to manufacturer’s specifications. Flurazepam was used as an internal standard for midazolam and tolbutamide quantification and carbamazepine was used as an internal standard for testosterone quantification. Analytes and internal standard were passed across the packing by gravity after which the cartridges were washed with 1 mL of nano-pure water followed by 1 mL of 50/50 methanol/water (for midazolam and testosterone extraction) or 1 mL of 5% methanol/water (for tolbutamide extraction). Analytes were eluted into clean glass test tubes with 1 mL of methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid. The eluent was dried, reconstituted in mobile phase and injected on either a Waters Acquity UPLC™ BEH C18 column (1.7 µm particle size, 50 X 2.1 mm) for testosterone analyte separation or a Waters Acquity UPLC™ BEH Shield RP C18 column (1.7 µm particle size, 50 X 2.1 mm) for midazolam and tolbutamide analyte separation. The columns were maintained at 40°C in a Waters Acquity UPLC™ H-Class System. The mobile phase flow was maintained at 0.8 mL/min and consisted of 10 mM KH2PO4 (pH = 3.0), acetonitrile and methanol. Mobile phase ratios and gradients for each assay are summarized in Supplemental Table 1. An ACQUITY UPLC® PDA detector was used to detect midazolam (254nm), tolbutamide (230nm) and testosterone
(245 nm for testosterone and 290 nm for carbamazepine) for quantification. The inter-assay coefficients of variation for 1’OH-midazolam, 4’OH-midazolam, 6β-OH testosterone, 16α-OH testosterone, and OH-tolbutamide were 10.7%, 23.1%, 3.1%, 3.2%, and 5.6%, respectively. The intra-assay coefficients of variation for 1’OH-midazolam, 4’OH-midazolam, 6β-OH testosterone, 16α-OH testosterone and OH-tolbutamide were 2.7%, 5.0%, 3.2%, 3.2% and 8.6%, respectively.

**Data Analysis and Statistical Procedures**

A Michaelis-Menten model was used to fit the formation of midazolam, testosterone and tolbutamide metabolites. Maximum reaction rate of enzyme (V<sub>max</sub>) and Michaelis-Menten constant (K<sub>m</sub>) were calculated using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA). Correlations were performed using Spearman’s rank test. Statistical analysis between control, moderate and severe kidney disease groups was performed using one-way ANOVA followed by Tukey’s multiple comparison tests. Data are presented as mean ± SEM and a p value less than 0.05 was considered significant.

**RESULTS**

**Serum Biochemistry and Body Weight**

A rat 5/6 nephrectomy model has been used extensively as an animal model for severe kidney disease (Leblond et al., 2000; Michaud et al., 2006; Michaud et al., 2010). We employed variations of this surgical procedure to generate rats with moderate or severe CKD. Rats that underwent sham laparotomy (control), 2/3 nephrectomy and 2/3 nephrectomy plus vessel ligation surgeries resulted in three distinct degrees of kidney
function (control, moderate CKD and severe CKD; respectively). This is represented by 1.65-fold and 4.78-fold higher serum creatinine levels in moderate and severe CKD, respectively, compared to control ($p < 0.05$, Table 1). Rats in the severe kidney disease group also had significantly higher serum creatinine levels than those with moderate kidney disease ($p < 0.05$). Serum urea concentrations were higher in severe kidney disease compared to control and moderate CKD rats ($p < 0.05$). There was no difference in body weight between the groups (Table 1).

**CYP3A2 and CYP2C11 mRNA and Protein Expression**

An 88% and 99.6% decrease in hepatic CYP3A2 mRNA expression was demonstrated in moderate and severe kidney disease rats, respectively, compared to controls (Figure 1A, $p < 0.05$). Similarly, CYP2C11 mRNA expression in moderate and severe CKD was decreased by 77% and 95%, respectively, compared to controls (Figure 1B). When mRNA expression was correlated with serum creatinine, there was a significant exponential decrease in both CYP3A and CYP2C mRNA expression as serum creatinine levels increased (Figure 1C $r_s = -0.785$ and Figure 1D $r_s = -0.809$, respectively; $p < 0.05$).

Similar to mRNA expression, CYP3A2 and CYP2C11 protein expression were significantly decreased in both moderate and severe CKD. CYP3A2 protein expression was decreased by 75% and 91% in moderate and severe kidney disease rats, respectively, compared to controls (Figure 2A, $p < 0.05$). Likewise, a reduction in CYP2C11 expression was also demonstrated in moderate (41%) and severe (68%) kidney disease compared to controls (Figure 2B, $p < 0.05$). Both CYP3A2 and CYP2C11 protein expression showed a significant inverse exponential relationship with
serum creatinine levels. (Figure 2C \( r_s = -0.820 \) and Figure 2D \( r_s = -0.665 \), respectively; \( p < 0.05 \)).

**Hepatic CYP3A and CYP2C Mediated Drug Metabolism**

To determine the effect of varying degrees of kidney disease on hepatic CYP3A function, we evaluated the metabolism of CYP3A probe drugs, midazolam and testosterone, using rat liver microsomes. Both 1`-OH midazolam and 4`-OH midazolam are produced by CYP3A (Chovan et al., 2007) and full enzyme kinetics of these metabolites were determined in this study. \( V_{max} \) values for 1`-OH midazolam production was significantly reduced by 62% and 72%, respectively, in moderate and severe kidney disease groups compared to control (Figure 3A; \( p < 0.05 \)). 4`OH-midazolam production was reduced by 63% and 75%, respectively, in moderate and severe CKD compared to controls (Figure 3B; \( p < 0.05 \)). The formation of 6\( \beta \)OH-testosterone is also specifically catalyzed by CYP3A dependant mechanisms (Chovan et al., 2007). A 66% and 68% decrease in 6\( \beta \)OH-testosterone \( V_{max} \) was demonstrated in moderate and severe kidney disease groups, respectively, compared to controls (Figure 3C). Michaelis-Menten kinetic parameters for 1`-OH midazolam, 4`-OH midazolam and 6\( \beta \)OH-testosterone are summarized in Table 2. A significant inverse correlation was found between \( V_{max} \) values for 1`-OH midazolam, 4`-OH midazolam and 6\( \beta \)OH-testosterone formation and serum creatinine levels (Figure 3D \( r_s = -0.818 \), Figure 3E \( r_s = -0.659 \) and Figure 3F \( r_s = -0.750 \), respectively; \( p < 0.05 \)).

Hepatic CYP2C enzyme activity was evaluated using the probe drugs testosterone and tolbutamide. Testosterone is specifically metabolized to 16\( \alpha \)OH-testosterone by CYP2C11. CYP2C11 \( V_{max} \) was significantly decreased by 67% and 82% in moderate
and severe kidney disease rats, respectively (Figure 4A). An inverse correlation was also demonstrated between $V_{\text{max}}$ values for $16\alpha$OH-testosterone production and serum creatinine (Figure 4C $r_s = -0.742$, $p < 0.05$). Hepatic CYP2C function was also characterized by evaluating tolbutamide metabolism. Formation of OH-tolbutamide was unchanged between control, moderate and severe kidney disease groups (Figure 4B) and there was no correlation between OH-tolbutamide $V_{\text{max}}$ and serum creatinine (Figure 4D). Michaelis-Menten kinetic parameters for $16\alpha$OH-testosterone and OH-tolbutamide are presented in Table 2.

**DISCUSSION**

A number of studies have shown altered hepatic CYP expression in severe CKD (Leblond *et al.*, 2001; Leblond *et al.*, 2000; Uchida *et al.*, 1995). Major rat drug metabolizing isoforms investigated have included CYP1A2, CYP2C6, CYP2C11, CYP2D, CYP2E1 and CYP3A1/2. Of these isoforms, CYP2C11 and CYP3A1/2 continually appear down-regulated in severe CKD (Guevin *et al.*, 2002; Leblond *et al.*, 2001; Rege *et al.*, 2003; Uchida *et al.*, 1995). Our results indicate that CYP3A2 and CYP2C11 protein expression are decreased by 75% and 40% in moderate kidney disease, respectively. This profound decrease in protein expression is similar to the decrease seen in severe kidney disease. Clinically, this suggests that patients with earlier stages of CKD are likely to experience variable and unpredictable pharmacokinetics and drug response when taking standard doses of drugs that are substrates for CYP3A and CYP2C. These results may help explain the increased incidence of medication related adverse events in patients with CKD (Manley *et al.*, 2005).
Previous studies have suggested that protein down-regulation is the result of a reduction in mRNA for hepatic CYP3A2 and CYP2C11 (Leblond et al., 2001). Our study provides further evidence of this notion as both moderate and severe kidney disease resulted in reduced CYP3A2 and CYP2C11 protein expression that is secondary to decreased mRNA. A possible cause for this decrease may be due to uremic toxin accumulation in moderate and severe CKD. Recent studies have shown that uremic toxins can cause direct inhibition or down-regulation of drug metabolizing enzymes and drug transporters (Reyes and Benet, 2011; Tsujimoto et al., 2010). In this study, it appears that uremic toxins are interfering with the regulation of drug metabolizing enzyme transcription. Indeed, rat hepatocytes treated with pre-dialysis serum from human ESRD patients exhibit down-regulation of CYP3A and CYP2C. This affect was alleviated by the addition of an NF-κB inhibitor suggesting that uremic toxins may be activating the NF-κB pathway (Michaud et al., 2005). The uremic toxin, indoxyl sulfate, has been shown to activate NF-κB in proximal tubule cells and enhance the infiltration of monocytes to uremic kidneys (Miyazaki et al., 1997). Furthermore, down-regulation of hepatic CYP expression has been noted in inflammation-associated pathological states occurring in other organs (Renton and Nicholson, 2000).

The majority of studies demonstrating decreased hepatic CYP3A function in severe CKD have used erythromycin as a CYP3A probe (Leblond et al., 2001; Leblond et al., 2000; Sun et al., 2004). Although erythromycin is metabolized by CYP3A, its disposition is determined by the interplay between transport and metabolism (Sun et al., 2010). This confounds its use as a specific CYP3A probe for whole cell and in vivo studies (Sun et al., 2004). Pharmacokinetic studies in patients with varying degrees of kidney
function have also used a variety of non-selective probe substrates for CYP enzymes (Ahmed et al., 1991; De et al., 2006). Although these studies clearly highlight that drug metabolism is differentially affected as kidney function declines, it is impossible to draw mechanistic conclusions about which specific CYP isozymes are affected. An alternate approach is to use midazolam, a selective phenotypic probe for CYP3A, which is not a substrate for uptake or efflux drug transporters (Nolin et al., 2009). In the present study, we evaluated the function of CYP3A using the well-established probe substrates, midazolam and testosterone (Dostalek et al., 2011; Guengerich, 1999). Our results demonstrate that as kidney function declines, there is a rapid decrease in CYP3A enzymatic activity.

CYP2C function has been assessed in severe CKD using the aminopyrine breath test (Leblond et al., 2000). Although aminopyrine is metabolized by CYP2C enzymes, it is also metabolized by many other CYP isoforms, such as CYP1A2 and CYP3A (Tanaka and Breimer, 1997). In our study, CYP2C mediated metabolism of tolbutamide to OH-tolbutamide was unchanged in CKD. Tolbutamide is a CYP2C family drug probe but is not specific for CYP2C11 (Brown et al., 2007; Cribb et al., 1995; Dostalek et al., 2007). Therefore, other CYP2C isoforms that are unaffected by CKD may have accounted for the lack of effect observed with regard to tolbutamide metabolism. This theory is supported by studies which show no effect of severe CKD on CYP2C6 protein expression (Leblond et al., 2001). To characterize the effect of progressive decline in kidney function on CYP2C11 activity, we analyzed the formation of 16αOH-testosterone, a specific metabolite of CYP2C11. The formation of 16αOH-testosterone was decreased in hepatic microsomes of both moderate and severe CKD. This data
supports other studies, which demonstrate that CYP2C11 function is diminished in severe kidney disease (Leblond et al., 2000; Uchida et al., 1995). This also suggests that earlier stages of kidney disease result in a significant decrease in CYP2C11 function, similar to the outcome seen in severe kidney disease.

Few studies have evaluated the correlation between the progression of kidney disease and extent of drug metabolism. After demonstrating that CYP3A and CYP2C11 enzyme function and expression were decreased in moderate CKD, we examined the correlation of this affect with the degree of kidney function. Our study shows a significant inverse exponential correlation between kidney function and CYP3A2 and CYP2C11 protein and mRNA expression. A similar inverse correlation was also demonstrated between kidney function and CYP3A and CYP2C11 enzyme function. Surprisingly, these correlations are not linear as our data clearly demonstrates that a small decrease in kidney function during earlier stages of CKD produces a pronounced decrease in enzyme function and expression. The mechanism by which this occurs is unknown but may be due to the exponential increase in serum uremic mediators as CKD progresses.

For example, the uremic toxin indoxyl sulfate increases exponentially as kidney disease progresses (Barreto et al., 2009; Niwa and Ise, 1994). A similar trend has also been demonstrated for the uremic toxin p-cresylsulfate (Liabeuf et al., 2010). Our results suggest that the rapid decrease in kidney function may coincide with the exponential increase in uremic toxins that occurs in earlier stages of CKD.

Altered non-renal drug clearance has been studied in patients with varying levels of kidney disease including ESRD (Nolin et al., 2006; Nolin et al., 2009; Sun et al., 2010). A recent study by Nolin et al. (2009) administered oral midazolam to ESRD patients on
hemodialysis. Surprisingly, the metabolism of midazolam was unchanged in ESRD patients compared to healthy controls. The authors suggest that as ESRD patients undergo routine hemodialysis, it is possible that uremic toxins modulating the expression and/or direct inhibition of CYP3A4 are removed and restore drug metabolism to levels observed in healthy controls (Nolin et al., 2009). This is supported by other clinical studies that show no change in nicardipine metabolism in ESRD patients on dialysis, yet significant reductions in patients with CKD not requiring dialysis (Ahmed et al., 1991).

In conclusion, this study demonstrates that changes in drug metabolism are not restricted to severe CKD and that, in fact, milder forms of CKD significantly decrease the expression and activity of CYP3A and CYP2C enzymes. To our knowledge this is the first study to systematically evaluate the effect of varying degrees of CKD on hepatic drug metabolism using variations of the commonly used remnant kidney model. In addition, this is the first study to evaluate CYP3A and CYP2C activity in CKD by performing full enzyme kinetics of substrate drugs. The results of our study suggest that metabolism and disposition of drugs that are substrates of CYP3A or CYP2C may be altered in earlier stages of kidney disease. This may have profound implications in the variable pharmacokinetics of medications in kidney disease. A recent assessment by the US Food and Drug Administration highlights the importance of evaluating the impact of kidney impairment on the pharmacokinetics of drugs (Zhang et al., 2009). Our data are in agreement with this assessment and suggest that the pharmacokinetics of drugs in all stages of kidney impairment should be investigated, not just ESRD.
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AUTHOR CONTRIBUTIONS

Participation in research design: Velenosi, Fu, Luo, Wang, Urquhart

Conducted experiments: Velenosi, Fu, Luo

Contributed new reagents or analytic tools: Urquhart

Performed data analysis: Velenosi, Fu, Luo, Wang, Urquhart

Wrote or contributed to the writing of the manuscript: Velenosi, Urquhart
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Footnotes

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FIGURE LEGENDS

Figure 1 mRNA expression of hepatic CYP3A2 (A) and CYP2C11 (B) in control, moderate and severe kidney disease rats. Correlation between hepatic CYP3A2 ($p < 0.001$) (C) and CYP2C11 ($p < 0.001$) (D) mRNA expression with serum creatinine levels of control (○), moderate (□) and severe (Δ) kidney disease rats. Expression of CYP3A2 and CYP2C11 was normalized to expression of the housekeeping gene β-actin. Analysis was performed in triplicate and results are presented as mean ± SEM, $n \geq 4$ (A, B) or means of triplicates (C, D), **$p < 0.01$ compared to control.

Figure 2 Protein expression of hepatic CYP3A2 (A) and CYP2C11 (B) in control, moderate and severe kidney disease rats. Correlation between hepatic CYP3A2 ($p < 0.001$) (C) and CYP2C11 ($p < 0.01$) (D) protein expression with serum creatinine of control (○), moderate (□) and severe (Δ) kidney disease rats. Protein bands are expressed in densitometry units (%) and are standardized to actin. Control bands were arbitrarily defined as 100%. Experiments were performed in duplicate and results are presented as mean ± SEM, $n \geq 4$ (A, B) or means of duplicates (C, D), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control. Representative blots are shown.

Figure 3 Michaelis-Menten plots of 1’OH-midazolam (A), 4’OH-midazolam (B) and 6β-OH testosterone (C) after incubation of rat liver microsomes with 1mM NADPH and various concentrations of midazolam (A and B) or testosterone (C). Correlation between 1’OH-midazolam $V_{\text{max}}$ (D) ($p < 0.001$), 4’OH-midazolam $V_{\text{max}}$ (E) ($p < 0.01$) and 6β-OH testosterone $V_{\text{max}}$ (F) ($p < 0.001$) with serum creatinine of control (○), moderate (□) and
severe (Δ) kidney disease rats. Experiments were performed in duplicate and results are presented as mean ± SEM, n ≥ 4 (A, B, C) or means of duplicates (D, E, F).

**Figure 4** Michaelis-Menten plots of 16αOH-testosterone (A) and OH-tolbutamide (B) after incubation of rat liver microsomes with 1mM NADPH and various concentrations of testosterone and tolbutamide, respectively. Relationship between 16αOH-testosterone $V_{\text{max}}$ (C) ($p < 0.001$) and OH-tolbutamide $V_{\text{max}}$ (D) with serum creatinine of control (○), moderate (□) and severe (Δ) kidney disease rats. Experiments were performed in duplicate and results are presented as mean ± SEM, n ≥ 4 (A, B) or means of duplicates (C, D).
Table 1: Physical and biochemical characteristics of control, moderate and severe kidney disease rats.

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<th>Moderate CRF</th>
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<td>448 ± 17</td>
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</table>

Data are presented as means ± SEM.

* p < 0.05 compared to control.

*** p < 0.001 compared to control.

††† p < 0.001 compared to moderate CRF.
Table 2. Michaelis-Menten kinetic values for P450 probe substrates in control, moderate and severe kidney disease rat liver microsomes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>Moderate CRF</th>
<th>Severe CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol min$^{-1}$ mg protein$^{-1}$)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>1’OH-midazolam</td>
<td>39.98 ± 7.33</td>
<td>19.64 ± 2.38</td>
<td>16.63 ± 7.41***</td>
</tr>
<tr>
<td>4’OH-midazolam</td>
<td>29.11 ± 2.78</td>
<td>20.80 ± 5.07</td>
<td>178.7 ± 26.9</td>
</tr>
<tr>
<td>6β-OH Testosterone</td>
<td>215.1 ± 178.7</td>
<td>186.10 ± 17.76</td>
<td>64.6</td>
</tr>
<tr>
<td>16α-OH Testosterone</td>
<td>674.4 ± 178.7</td>
<td>432.6 ± 75.7</td>
<td>1408 ± 72.1</td>
</tr>
<tr>
<td>OH-Tolbutamide</td>
<td>1408 ± 674.4</td>
<td>1408 ± 115</td>
<td>1239 ± 68.3</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

* $p < 0.05$ compared to control.

** $p < 0.01$ compared to control.

*** $p < 0.001$ compared to control.
FIGURE 1
FIGURE 3
FIGURE 4