Effect of Experimental Kidney Disease on the Functional Expression of Hepatic Reductases

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ABSTRACT

Chronic kidney disease (CKD) affects the nonrenal clearance of drugs by modulating the functional expression of hepatic drug-metabolizing enzymes and transporters. The impact of CKD on oxidative and conjugative metabolism has been extensively studied. However, its effect on hepatic drug reduction, an important phase I drug-metabolism pathway, has not been investigated. We aimed to assess the effect of experimental CKD on hepatic reduction using warfarin as a pharmacological probe substrate. Cytosolic and microsomal cellular fractions were isolated from liver tissue harvested from five-sixths-nephrectomized and control rats (n = 10 per group). The enzyme kinetics for warfarin reduction were evaluated in both fractions, and formation of warfarin alcohols was used as an indicator of hepatic reductase activity. Selective inhibitors were employed to identify reductases involved in warfarin reduction. Gene and protein expression of reductases were determined using quantitative real-time polymerase chain reaction and Western blotting, respectively. Formation of RS/SS-warfarin alcohol was decreased by 39% (P < 0.001) and 43% (P < 0.01) in cytosol and microsomes, respectively, in CKD rats versus controls. However, RR/SS-warfarin alcohol formation was unchanged in the cytosol, and a trend toward its decreased production was observed in microsomes. Gene and protein expression of cytosolic carbonyl reductase 1 and aldo-keto reductase 1c3/18, and microsomal 11β-hydroxysteroid dehydrogenase type 1 were significantly reduced by >30% (P < 0.05) in CKD rats compared with controls. Collectively, these results suggest that the functional expression of hepatic reductases is selectively decreased in kidney disease. Our findings may explain one mechanism for altered nonrenal clearance, exposure, and response of drugs in CKD patients.

Introduction

Chronic kidney disease (CKD) is a major public health problem that affects approximately 26 million Americans (Weiner, 2009). CKD patients commonly exhibit unpredictable changes in drug disposition, largely resulting from decreased renal clearance of drugs excerted unchanged by the kidney, as well as altered nonrenal clearance (Nolin et al., 2008). Remarkable decreases in the functional expression of drug-metabolizing enzymes that mediate nonrenal drug clearance, particularly phase I oxidation by cytochrome P450 (P450) enzymes, have been well documented in the setting of kidney disease (Naud et al., 2012; Lalande et al., 2014; Yeung et al., 2014). Phase II conjugation via acetylation (Simard et al., 2008) is also decreased by kidney disease, but glucuronidation is not affected (Yu et al., 2006). However, the effect of CKD on other drug-metabolism pathways is less clear.

Carbonyl reductases are important phase I metabolizing enzymes. As depicted in Fig. 1, these enzymes can be classified into quinone reductases and carbonyl-reducing enzymes (Oppermann, 2007). Reduction by quinone reductases is mediated primarily by the NADPH quinone oxidoreductase type 1 (NQO1) (Oppermann, 2007; Siegel et al., 2012). The carbonyl-reducing enzymes are further separated into two major superfamilies: the short-chain dehydrogenase/reductases, which include primarily carbonyl reductase (CBR) and 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) (Skarydova and Wsol, 2012; Gathercole et al., 2013), and aldo-keto reductase (AKR) (Matsunaga et al., 2006; Malatkova et al., 2010). Collectively, reductase enzymes play key roles in the biotransformation of diverse endogenous compounds such as bile acids, glucocorticoids, and prostaglandins, and numerous drugs (Rosemond and Walsh, 2004; Matsunaga et al., 2006; Oppermann, 2007; Malatkova and Wsol, 2014). Although the pharmacokinetics of several drugs that are reductase substrates, including idarubicin (Camaggi et al., 1992) and bupropion (Turpeinen et al., 2007), are altered in patients with CKD, the effect of impaired kidney function on specific hepatic reductase enzymes has not been assessed to date.

The objectives of this study were to determine the effect of severe CKD on the expression and function of several hepatic reductases in rats. This was accomplished using warfarin as a pharmacological probe substrate on the premise that it undergoes reduction with formation of distinct alcohol metabolites, namely RS/SR-warfarin alcohol (alcohol 1), 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; AKR, aldo-keto reductase; CBR, carbonyl reductase; CKD, chronic kidney disease; P450, cytochrome P450; PBS, phosphate-buffered saline.
Studies to determine the metabolic activity of the reductases in rat liver was assessed in CKD and control rats, and we performed enzyme kinetic expression of selective hepatic reductases that mediate warfarin metabolism (Granger and Chertow, 2014; Shah et al., 2014), suggesting that warfarin impaired kidney function (Chan et al., 2009b; Limdi et al., 2009; Pittsburgh, PA). Primers used for PCR quantification were purchased from Chemical Reagents.

Experimental Model. Male Sprague-Dawley rats (Charles River, Saint-Charles, QC, Canada) that weighed 200–250 g were housed in the Research Center animal care facility and maintained on standard rat chow and water ad libitum on a 12-hour light/dark cycle. Rats were allowed to acclimatize for at least 3 days before any experimental procedure. 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 the Maisonneuve Rosemont Hospital Research Center Animal Care Committee.

Studies were performed in two experimental groups of rats: control (n = 10) and CKD (n = 10). Control rats were pair-fed the same amount of rat chow that was ingested by CKD rats on the previous day. Severe CKD was induced by nephrectomy of the left kidney that was followed by a complete right nephrectomy (Leblond et al., 2000). Briefly, rats in the CKD group underwent a two-thirds nephrectomy for the measurement of serum creatinine and urea concentrations.

Identification of Enzymes Catalyzing Warfarin Reduction. To identify rat hepatic reductases that are involved in the carboxyl reduction of warfarin, pooled liver cytosol or microsomes of control rats were incubated with warfarin (1 mM) and a chemical inhibitor under the optimized conditions described above. Figure 1 depicts the classes of reductase enzymes and the corresponding chemical inhibitors used in this study. The inhibitors and concentrations (10 and 100 μM) used and their selectivity for reductase enzymes were based on the previous reports as follows: quercetin for cytosolic CBRs (Hermans and Thijssen, 1992; Tong et al., 2010); flufenamic acid for cytosolic AKRs (Atalla et al., 2000; Rosemond et al., 2004; Molnari and Myers, 2012); indomethacin for both cytosolic CBRs and AKRs (Usami et al., 2001; Tong et al., 2010); dicoumarol for cytosolic NQO1 (Tong et al., 2010); and 18β-glycyrrhetinic acid for microsomal 11β-HSD1 (Breyer-Pafl et al., 2004; Molnari and Myers, 2012). All inhibitors were dissolved in a mixture of dimethylsulfoxide-methanol (50:50, v/v) except for dicoumarol, which was prepared in 1% sodium
hydroxide in water. Organic solvents were consistently maintained at a final concentration of 0.5% in all incubations. Control reactions were performed with the vehicles in the absence of the chemical inhibitors. All experiments were conducted in triplicate.

RNA Isolation and Quantitative Real-Time PCR Analysis. Total RNA was extracted from liver tissue using TRizol reagent (Invitrogen, San Diego, CA). RNA concentration and purity were measured by spectrophotometry at 260 nm. One microgram of total RNA was used to prepare the cDNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) using the SYBR Green master mix (Roche, Indianapolis, IN) and specific primers (Table 1). Hepatic reductase mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase genes was determined by quantitative real-time polymerase chain reaction performed with the ABI Prism 7300 system (Applied Biosystems, Foster City, CA) using the SYBR Green master mix (Roche, Indianapolis, IN) and specific primers (Table 1). Heparin reductase mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase and expressed relative to the controls using the 2-ΔΔCt method (Livak and Schmittgen, 2001).

Western Blot Analysis. The protein expression of cytosolic CBR1 and AKR1C18 [also known as 20 a-hydroxysteroid dehydrogenase (20α-HSD)] and microsomal 11β-HSD1 was determined using Western blotting. Samples (50 μg total protein) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Transferred membranes were blocked for 1 hour at room temperature with 5% nonfat powdered milk dissolved in phosphate-buffered saline (PBS). membranes were then incubated overnight at 4°C with specific primary antibodies diluted in PBS: 1:1000 rabbit polyclonal anti-CBR1 (Abcam, Cambridge, MA); 1:2000 rabbit polyclonal anti-CYP1A1 (Cayman Chemical Company, Ann Arbor, MI); or 1:1000 goat anti-mouse (Sigma-Aldrich). The membranes were then washed with PBS-Tween, and the immune complexes were revealed by chemiluminescence using Multi Gauge Software (Fujifilm) and normalized to β-actin.

Data and Statistical Analysis. The formation rate of alcohol metabolites was calculated from their measured concentrations. A nonlinear regression was normalized to intensity was quantified by densitometry using Multi Gauge Software (Fujifilm) and Lumi-Light (Roche, Indianapolis, IN) detection system on the Fujifilm intelligent with PBS-Tween, and the immune complexes were revealed by chemiluminescence using Multi Gauge Software (Fujifilm) and normalized to β-actin.

Results

Characteristics of Control and CKD Rats. Biochemical parameters and weights of the two groups of rats are presented in Table 2. Successful induction of severe kidney disease was confirmed by measuring the serum concentrations of creatinine and urea. Serum creatinine concentrations were significantly elevated in CKD group compared with controls. Likewise, the serum concentrations of urea were significantly higher in CKD rats compared with controls. Body weight was not different between the two groups.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Characteristics of control pair-fed and CKD rats</th>
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<tr>
<td></td>
<td>Control Paired-Fed (n = 10)</td>
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<tr>
<td>Body weight (g)</td>
<td>386.0 ± 38.4</td>
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<tr>
<td>Serum creatinine (μM)</td>
<td>40.4 ± 8.7</td>
</tr>
<tr>
<td>Creatinine clearance (μl/100 g of body weight per minute)</td>
<td>359.7 ± 79.3</td>
</tr>
<tr>
<td>Serum urea (mM)</td>
<td>3.6 ± 1.1</td>
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NS, non-significant.
alcohol 1 and alcohol 2 in the presence of inhibitors as normalized to control incubations is depicted in Fig. 3, A and B, respectively. Cytosolic reduction of warfarin to its alcohols was inhibited by quercetin, flufenamic acid, indomethacin, and dicoumarol in a concentration-dependent manner. The magnitude of inhibition at chemical inhibitor concentrations of 10 mM ranged from 7 to 26% for formation of alcohol 1 and 9 to 28% for alcohol 2. The extent of inhibition at inhibitor concentrations of 100 mM was markedly increased, ranging from 33 to 81% and 32 to 70% for formation of alcohol 1 and alcohol 2, respectively. All inhibitions in the cytosol were statistically significant except for the formation of alcohol 1 at 10 mM indomethacin, and alcohol 2 at 10 mM of both flufenamic acid and dicoumarol. Quercetin, an inhibitor of CBR, exhibited the most potent inhibition. Microsomal reduction of warfarin to alcohol 1 and alcohol 2 was significantly inhibited by 15 and 44%, respectively at 100 mM 18β-glycyrrhetinic acid.

mRNA and Protein Expression of Hepatic Reductase Isoforms. To determine whether the decrease in the metabolic activity of hepatic reductases in CKD rats was secondary to reduced gene expression, mRNA levels encoding cytosolic and microsomal reductase isoforms were measured by quantitative real-time polymerase chain reaction (Fig. 4). Although the expression of hepatic reductases was differentially affected by CKD, many of the investigated reductases were downregulated. The mRNA expression of cytosolic CBR1 was significantly decreased by 34% (P < 0.001) in CKD rats compared with controls, but CBR3 expression was not changed. While the mRNA expression of cytosolic AKR1C3 significantly decreased, by 93% (P < 0.001), AKR1C14 expression increased by 53% in CKD compared with control rats (P < 0.05). Cytosolic NQO1 expression remained unchanged. Lastly, mRNA expression of the microsomal 11β-HSD1 isoform was significantly reduced, by 35% (P < 0.05) in CKD rats, compared with controls. To

<table>
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<th>Alcohol 1 (RS/RR)</th>
<th>Alcohol 2 (RR/SS)</th>
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<tr>
<td><strong>K_m</strong> (μM)</td>
<td><strong>V_max</strong> (pmol/mg protein per minute)</td>
</tr>
<tr>
<td>Cytosol Control</td>
<td>352.0 ± 59.65</td>
</tr>
<tr>
<td>CKD</td>
<td>359.3 ± 64.06</td>
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<tr>
<td>Micromesomes</td>
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<tr>
<td>Control</td>
<td>446.9 ± 174.7</td>
</tr>
<tr>
<td>CKD</td>
<td>365.6 ± 95.82</td>
</tr>
</tbody>
</table>

**P < 0.01 compared with control, ***P < 0.001 compared with control.
further confirm the validity of our reductase gene–expression findings in CKD rats, we measured the expression of selective P450 isoforms that are not affected by kidney disease as additional controls. Consistent with previous findings (Leblond et al., 2001; Rege et al., 2003; Michaud et al., 2005), mRNA levels of CYP1A2, CYP2D1, and CYP2E1 were not statistically different between CKD and control rats. Consistent with our mRNA expression findings, protein expression of CBR1, AKR1C18 (encoded by AKR1C3), and 11β-HSD1 was decreased by 43, 76, and 70%, respectively, in CKD rats versus controls (*P < 0.05, **P < 0.01, and ***P < 0.001, compared with control incubations which were arbitrarily defined as 100%).

Discussion

The present study investigated the impact of experimental kidney disease on hepatic reduction using warfarin as a probe substrate. The enzyme kinetics of warfarin in both cytosolic and microsomal fractions of rat liver tissue were assessed. In addition, the effect of kidney disease on gene and protein expression of hepatic reductases was evaluated. This study demonstrates that the metabolic activities of selective hepatic reductases catalyzing warfarin reduction are decreased in CKD secondary to differential downregulation of gene and protein expression of the enzymes. These findings suggest that CKD alters the nonrenal clearance of drugs mediated by hepatic reduction.

Carbonyl-reducing enzymes catalyze the reduction of a diverse range of endogenous and exogenous xenobiotics. This includes numerous drugs representing several classes, including anticoagulants, antidiabetics, antihypertensives, anticancer agents, opiate antagonists, nonsteroidal anti-inflammatory drugs, antihistamines, diuretics, and many others (Rosemond and Walsh, 2004; Malatkova and Wsol, 2014). Several pharmacokinetic studies showed that the disposition of drugs that are primarily metabolized by reduction is altered in patients with kidney disease. For instance, bupropion exposure is dramatically increased by 126% in patients with impaired kidney function, suggesting reduced metabolic clearance (Turpeinen et al., 2007). Moreover, a 30% decrease in the metabolic clearance of idarubicin was reported in patients with creatinine clearance of <60 ml/min (Camaggi et al., 1992). This suggests that phase I reduction of drugs may be affected by kidney disease.

In the present study we evaluated the impact of experimental kidney disease on the metabolic activity of cytosolic and microsomal enzymes.
reductases. Our results indicate that warfarin reduction is significantly decreased in both cytosolic and microsomal liver fractions of CKD rats. The metabolic activity (represented by $V_{\text{max}}$) of cytosolic reductases generating warfarin alcohol 1 was decreased by 39% ($P < 0.001$; Fig. 2), and formation of alcohol 2 was not affected. This suggests that there are multiple reductase enzymes implicated in warfarin reduction that are differentially affected by CKD, and demonstrates that warfarin reduction is stereoselectively altered in kidney disease. Dramatic decreases in the metabolic activity of microsomal reductases generating alcohol 1 and alcohol 2 was also observed (Fig. 2). However, a definitive conclusion regarding the total activity of microsomal reductases generating alcohol 2 cannot be drawn owing to an unsuccessful fit of the model to the CKD data. This can be explained, in part, by the linear formation of alcohol 2 observed in the microsomes over the range of the substrate concentrations used. Our results are in agreement with previous findings that alcohol 1 is the major metabolite of warfarin metabolism in the cytosol (Moreland and Hewick, 1975; Hermans and Thijssen, 1989). Overall, our results suggest that kidney disease may impact hepatic reductases as it does other phase I and II metabolic pathways.

To identify the carbonyl-reductase enzymes that play a role in warfarin reduction, we used selective chemical inhibitors for cytosolic and microsomal reductases. Alcohol formation in the cytosol was inhibited up to 81% by quercetin, flufenamic acid, and dicoumarol, suggesting the contribution of CBRs, AKRs, and NQO1, respectively, to warfarin reduction. In fact, the observed potent inhibition by quercetin demonstrates the significant contribution of CBRs to warfarin reduction. We also showed that indomethacin, a nonselective inhibitor for both cytosolic CBRs and AKRs (Atalla et al., 2000; Rosemond and Walsh, 2004), significantly inhibited the formation of alcohols by up to 60%. This provides further evidence of the involvement of the two families in warfarin reduction. Our results are in agreement with previous reports that showed that 4-nitrowarfarin reduction in liver cytosol of human and rabbit is strongly inhibited by quercetin and indomethacin (Hermans and Thijssen, 1992, 1993). Our results also demonstrate that 11β-HSD1 is involved in the microsomal reduction of warfarin. Together, our findings suggest that the microsomal 11β-HSD1 and cytosolic isoforms of CBR, AKR, and NQO are responsible for warfarin reduction in rats, with major contributions possibly by CBR isoforms. However, further investigations using recombinant enzyme preparations are required to confirm the specific reductase isoforms involved in warfarin reduction.

Previous studies have demonstrated that the decrease in enzyme activity in CKD is partially attributable to the downregulation of protein and gene expression of drug-metabolizing enzymes (Leblond et al., 2001, 2002; Simard et al., 2008; Velenosi et al., 2012). On the basis of inhibition data, we measured the gene expression of cytosolic CBR1, CBR3, AKR1C3, AKR1C14, and NQO1, and microsomal 11β-HSD1 in CKD and control rats. Our study showed, for the first time, that the mRNA expression of hepatic reductases catalyzing warfarin reduction is decreased in CKD rats. In fact, we were able to detect decreased expression of various carbonyl reductases implicated in drug metabolism, in both cytosolic (CBR1 and AKR1C3) and microsomal cellular fractions (11β-HSD1). The observed negligible effect on the expression of CBR3 and NQO1, and the upregulation observed for AKR1C14 indicates an isoform-selective effect of kidney disease on reductase expression. Our results also showed that protein levels of CBR1, AKR1C18, and 11β-HSD1 are significantly reduced in CKD rats. These findings are similar to those observed for the corresponding genes, suggesting that reduced protein levels could be explained by the decrease in gene expression. Collectively, our results suggest that reduced metabolic activity of hepatic reductases in CKD could be, in part, secondary to decreased gene and protein expression.

Our findings may have important clinical implications, as they may explain why the disposition of drugs that are metabolized through phase I reduction pathways (e.g., bupropion, idarubicin) is altered in patients with impaired kidney function. Moreover, our results may provide insight into warfarin treatment, which is challenging because of the high interindividual variability observed in the warfarin dose-response relationship. Besides differences in the genetic and clinical background of patients, recent studies have shown that patients with severe CKD require lower warfarin maintenance dosages compared with those with mild or no CKD (Limdi et al., 2009). Moreover, multiple studies suggest that warfarin use in CKD patients is associated with increased risk of poor outcomes such as hemorrhage, stroke, and mortality (Chan et al., 2009a, b; Shah et al., 2014). Although unproven, our findings of decreased warfarin reduction owing to decreased metabolic activity of hepatic reductases may contribute to, and could provide a novel mechanistic explanation for, altered warfarin dose requirements and response in CKD patients.

In conclusion, CKD selectively decreases the metabolic activity of cytosolic and microsomal hepatic reductases, secondary to downregulation in gene and protein expression. Collectively, our data suggest that kidney disease changes the nonrenal clearance of drugs mediated by hepatic reduction and may provide a novel mechanistic explanation for reduced warfarin dose requirements and altered responses observed in CKD patients.

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Authorship Contributions

Participated in research design: Alshogran, Leblond, Pichette, Nolín.
Conducted experiments: Alshogran, Naud, Leblond.
Contributed new reagents or analytic tools: Ocque, Leblond, Nolín.
Performed data analysis: Alshogran, Naud, Leblond.
Wrote or contributed to the writing of the manuscript: Alshogran, Naud, Ocque, Leblond, Pichette, Nolín.

References

Chan KE, Lazarus JM, Thadhani R, and Hakim RM (2009a) Anticoagulant and antiplatelet usage with major contributions possibly by CBR isoforms. However, further investigations using recombinant enzyme preparations are required to confirm the specific reductase isoforms involved in warfarin reduction.


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