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# 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*/*SR*-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 11$\beta$-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 excreted 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.

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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 11$\beta$-hydroxysteroid dehydrogenase type 1 (11$\beta$-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),

**ABBREVIATIONS:** 11$\beta$-HSD1, 11$\beta$-hydroxysteroid dehydrogenase type 1; AKR, aldo-keto reductase; CBR, carbonyl reductase; CKD, chronic kidney disease; P450, cytochrome P450; PBS, phosphate-buffered saline.

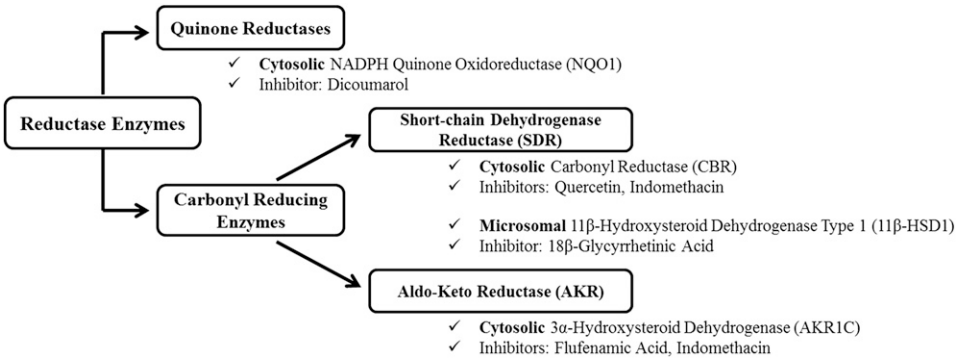


**Fig. 1.** Schematic representation of the hepatic reductase enzymes and inhibitors investigated in the current study. Cytosolic reductases can be classified into quinone reductases and carbonyl-reducing enzymes. Carbonyl-reducing enzymes comprise two major superfamilies: the short-chain dehydrogenase reductase (SDR) and aldo-keto reductase (AKR) enzymes. The main groups of these superfamilies implicated in ketone reduction of drugs are cytosolic carbonyl reductases (CBRs) and cytosolic 3$\alpha$-hydroxysteroid dehydrogenases (AKR1C), respectively. 11$\beta$-hydroxysteroid dehydrogenase type 1 (11$\beta$-HSD1) is the principal SDR enzyme that catalyzes drug reduction in the microsomes. The selective inhibitors used in this study are: dicoumarol for NQO1, and quercetin and flufenamic acid for CBR and AKR1C, respectively. Indomethacin is a non-specific inhibitor for both CBR and AKR1C. 18$\beta$-Glycyrrhetinic acid is selective for 11$\beta$-HSD1 in the microsomes.

and *RR*/*SS*-warfarin alcohol (alcohol 2) (Chan et al., 1972; Lewis et al., 1974; Alshogran et al., 2014). Moreover, recent evidence indicates that warfarin dosing requirements and response are altered in patients with impaired kidney function (Chan et al., 2009b; Limdi et al., 2009; Granger and Chertow, 2014; Shah et al., 2014), suggesting that warfarin disposition may also be altered in CKD. The mRNA and protein expression of selective hepatic reductases that mediate warfarin metabolism was assessed in CKD and control rats, and we performed enzyme kinetic studies to determine the metabolic activity of the reductases in rat liver cytosol and microsomes.

## Materials and Methods

**Chemical Reagents.** Warfarin, NADPH, magnesium chloride, tris(hydroxymethyl) aminomethane (Trizma base), quercetin, flufenamic acid, indomethacin, dicoumarol, and 18$\beta$-glycyrrhetinic acid were purchased from Sigma-Aldrich (St. Louis, MO). Warfarin alcohols were synthesized as previously described (Trager et al., 1970; Chan et al., 1972). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Primers used for PCR quantification were purchased from Eurofins MWG Operon (Huntsville, AL). Unless otherwise specified, all other chemicals and reagents were purchased from Sigma-Aldrich. All chemicals were liquid chromatography–mass spectrometry grade or of the highest purity available.

**Experimental Model.** Male Sprague-Dawley rats (Charles River, Saint-Charles, QC, Canada) that weighed 200–300 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 a standard two-stage five-sixths nephrectomy model as previously described (Leblond et al., 2000). Briefly, rats in the CKD group underwent a two-thirds nephrectomy of the left kidney that was followed by a complete right nephrectomy 7 days later. Rats in the control group were subjected to two sham laparotomies. Forty-two days after the initial surgery, rats were scarified by decapitation. Liver tissue was harvested and stored at –80°C. Blood was collected for the measurement of serum creatinine and urea concentrations.

**Isolation of Hepatic Cytosolic and Microsomal Fractions.** Liver cytosol and microsomes were isolated by standard differential centrifugation as previously described with slight modification (Hermans and Thijssen, 1989). In brief, frozen tissue was homogenized in ice-cold buffer (50 mM Tris-HCl buffer, 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor and 0.113 mM butylated hydroxytoluene as an antioxidant. The homogenate was centrifuged at 20,000$g$ for 30 minutes at 4°C. The supernatant was then centrifuged at 140,000$g$ for 60 minutes at 4°C. The resulting 140,000$g$ soluble supernatant was collected as a cytosol, and the microsomal pellet was washed twice and resuspended in 0.02 M Tris-HCl (pH 7.4) containing 0.25 M sucrose. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Cytosolic and microsomal proteins were stored at –80°C until analysis.

**In Vitro Warfarin Reduction Using Cytosolic and Microsomal Fractions.** The metabolic activity of rat hepatic reductases that catalyze warfarin reduction was assessed using cytosolic and microsomal cellular fractions of control and CKD rat liver tissue. Incubations were conducted using 0.5 mg/ml of either cytosolic or microsomal protein in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and various concentrations of warfarin (20, 50, 100, 250, 500, 1000, 1500, and 2000 $\mu$M). Reactions were started by the addition of 1 mM NADPH and conducted for 30 minutes at 37°C. The final reaction volume was 500 $\mu$l. Substrates were added in acetone with a final concentration consistently maintained at 1%. Controls omitting substrate/NADPH were included in each incubation. Conditions were optimized during preliminary experiments to achieve linear formation of warfarin alcohol metabolites with respect to incubation time and protein concentration. Incubations were quenched by adding 500 $\mu$l ice-cold acetonitrile. Samples were briefly vortex-mixed, placed on ice for 15 minutes and centrifuged at 14,000 rpm for 10 minutes to pellet the protein. Metabolites were extracted using methyl *tert*-butyl ether, evaporated to dryness under nitrogen gas, and reconstituted with acetonitrile-water (25:75, v/v) prior to injection onto the liquid chromatography–mass spectrometry system. Warfarin alcohol metabolites were quantified by ultra-performance liquid chromatography–tandem mass spectrometry as we reported previously (Alshogran et al., 2014). The intraday accuracy and precision of analytes ranged from 90 to 108.8% and from 4 to 11%, respectively. The interday accuracy and precision ranged from 91 to 107.7% and from 5 to 8.2%, respectively.

**Identification of Enzymes Catalyzing Warfarin Reduction.** To identify rat hepatic reductases that are involved in the carbonyl 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 $\mu$M) 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 18$\beta$-glycyrrhetinic acid for microsomal 11$\beta$-HSD1 (Breyer-Pfaff 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

TABLE 1

Nucleotide sequence for the primers used in quantitative real-time polymerase chain reaction

| mRNA | Left Primer (5′–3′) | Right Primer (5′–3′) | Predicted Product Size (Base Pair) |
|---|---|---|---|
| CBR1 | TCCACATTCAAGCAGAGGTG | CACTCTGCCTTGGGGTTTTA | 98 |
| CBR3 | ACCACATGGTAGAGTGGTGA | AGTGTGTCACATCGGAACCT | 99 |
| AKR1C3 | CCTGTGTGCAACCAGGTAGA | CCATAGGCAACCAGAACGAT | 95 |
| AKR1C14 | TTCCTGTACTGGGGTTTGGA | CAGAGTCAAAATGGCGGAAT | 108 |
| NQO1 | GCTTGACACTACGATCCGC | CACAGCAGCCTCCTTCATG | 117 |
| 11$\beta$-HSD1 | AGCATTGCCATCATCTCCTC | GTGGAAAAGAACCCATCCAG | 98 |
| CYP1A2 | GGCAGGTCAACCATGATGAG | CTTGTCGATGGCCGTGTT | 92 |
| CYP2D1 | CTGCAGGTGGACCTCAGTAA | ATGGGCTTCCAACCCTTC | 95 |
| CYP2E1 | GGGACATTCCTGTGTTCCAG | GTCTCGGAGAATGCTTAGGG | 104 |
| GAPDH | TGCCACTCAGAAGACTGTGG | GGATGCAGGGATGATGTTCT | 85 |

CBR1, carbonyl reductase type 1; CBR3, carbonyl reductase type 3; AKR1C3, aldo-keto reductase 1C3; AKR1C18, aldo-keto reductase 1C18; NQO1, NADPH quinone oxidoreductase type 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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) in a 20-$\mu$l reaction volume. Expression of rat CBR1, CBR3, AKR1C3, AKR1C14, NQO1, 11$\beta$-HSD1, CYP1A2, CYP2D1, CYP2E1, and 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). Hepatic reductase mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase and expressed relative to the controls using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

**Western Blot Analysis.** The protein expression of cytosolic CBR1 and AKR1C18 [also known as 20 $\alpha$-hydroxysteroid dehydrogenase (20$\alpha$-HSD)] and microsomal 11$\beta$-HSD1 was determined using Western blotting. Samples (50 $\mu$g total protein) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Transferred membranes were blocked for 1 hour at room temperature using 5% (w/v) nonfat powdered milk dissolved in phosphate-buffered saline (PBS). Membranes were then incubated overnight at 4°C with specific primary antibodies diluted in 0.5% nonfat powdered milk dissolved in PBS: 1:1000 rabbit polyclonal anti-CBR1 (Abcam, Cambridge, MA); 1:2000 rabbit polyclonal anti–20$\alpha$-HSD (Kera Fast, Boston, MA); 1:200 rabbit anti–11$\beta$-HSD (Cayman Chemical Company, Ann Arbor, MI); or 1:1000 mouse monoclonal anti–$\beta$-actin (GeneTex, Irvine, CA). After washing four times with PBS-Tween (0.1% Tween-20 in PBS), membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibodies diluted in 0.5% milk: 1:4000 goat anti-rabbit (Sigma-Aldrich), or 1:1000 goat anti-mouse (Sigma-Aldrich). The membranes were then washed with PBS-Tween, and the immune complexes were revealed by chemiluminescence Lumi-Light (Roche, Indianapolis, IN) detection system on the Fujifilm intelligent dark box equipped with the LAS-4000 camera (Fujifilm, Piscataway, NJ). Band intensity was quantified by densitometry using Multi Gauge Software (Fujifilm) and normalized to $\beta$-actin.

**Data and Statistical Analysis.** The formation rate of alcohol metabolites was calculated from their measured concentrations. A nonlinear regression Michaelis-Menten kinetic model was used to fit the formation of alcohol metabolites, and the maximum velocity of enzymes ($V_{max}$) and the affinity constant ($K_m$) were estimated and compared between groups using GraphPad Prism (Version 5; GraphPad Software Inc., San Diego, CA). Formation rate of alcohol metabolites in the inhibition studies was expressed as percent of control incubations, and statistical differences in formation of alcohols in the presence or absence of inhibitors were determined using Student's $t$ test. Reductase gene and protein expression in CKD rats were expressed relative to that of controls, and statistical differences between the two groups were assessed using Student's $t$ test. $P < 0.05$ was considered significant for all comparisons. The results are expressed as mean ± S.E.M.

## 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.

**In Vitro Warfarin Reduction in Cytosolic and Microsomal Fractions.** To determine the impact of experimental kidney disease on the activity of hepatic reductases, we assessed warfarin reduction in vitro using rat liver cytosol and microsomes. Both cellular fractions generated warfarin alcohol metabolites, and the enzyme kinetics for alcohols were determined.

In the cytosolic fraction, $V_{max}$, for formation of warfarin alcohol 1 was significantly decreased, by 39% in CKD compared with control rats (23.3 ± 1.29 versus 38.0 ± 1.97 pmol/mg protein per minute, $P < 0.001$; Fig. 2A). $V_{max}$ for formation of warfarin alcohol 2 was not different between the two groups (8.74 ± 0.47 versus 8.43 ± 0.61 pmol/mg protein per minute, $P$ = NS), and virtually superimposable Michaelis-Menten curves were observed (Fig. 2B).

In microsomes, $V_{max}$ of microsomal reductases producing warfarin alcohol 1 was significantly decreased, by 43% in CKD versus control rats (5.49 ± 0.45 versus 9.57 ± 1.25 pmol/mg protein per minute, $P < 0.01$; Fig. 2C). The results for the metabolic production of warfarin alcohol 2 in the CKD group were unable to be fit to the Michaelis-Menten model. However, the formation of alcohol 2 at the maximum substrate concentration (2000 $\mu$M warfarin) was markedly reduced, by 87% in CKD rats compared with controls (Fig. 2D). Estimated $V_{max}$ and $K_m$ of cytosolic and microsomal studies are presented in Table 3.

**Identification of Enzymes Catalyzing Warfarin Reduction.** Selective inhibitors were employed to identify reductase enzymes involved in warfarin reduction. The relative formation of warfarin

TABLE 2

Characteristics of control pair-fed and CKD rats

| | Control Paired-Fed ($n$ = 10) | CKD ($n$ = 10) | $P$ |
|---|---|---|---|
| Body weight (g) | 386.0 ± 38.4 | 333.9 ± 75.0 | NS |
| Serum creatinine ($\mu$M) | 40.4 ± 8.7 | 203.4 ± 83.9 | <0.001 |
| Creatinine clearance ($\mu$l/100 g of body weight per minute) | 359.7 ± 79.3 | 68.8 ± 41.3 | <0.001 |
| Serum urea (mM) | 3.6 ± 1.1 | 38.3 ± 26.1 | <0.001 |

NS, non-significant.

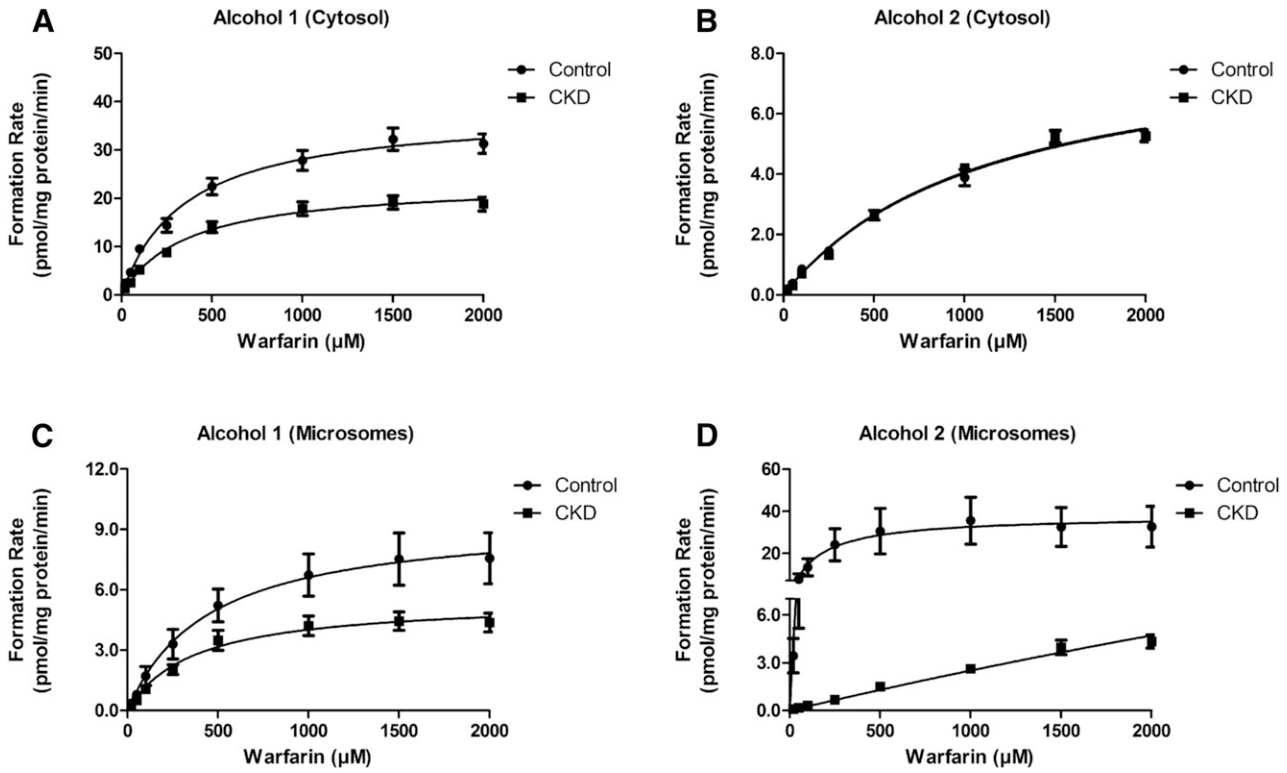


**Fig. 2.** Michaelis-Menten plots for the formation of warfarin alcohol 1 (A) and alcohol 2 (B) in rat liver cytosol, and alcohol 1 (C) and alcohol 2 (D) in rat liver microsomes of both control and CKD rats. Liver cytosolic or microsomal protein (0.5 mg/ml) was incubated with various concentrations of warfarin (20–2000 $\mu$M) for 30 minutes at 37°C in the presence of 1 mM NADPH and 5 mM $MgCl_2$. Each point presents the mean ± S.E.M. of 10 rats in each group.

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 $\mu$M 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 $\mu$M 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 $\mu$M indomethacin, and alcohol 2 at 10 $\mu$M 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 $\mu$M 18$\beta$-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 11$\beta$-HSD1 isoform was significantly reduced, by 35% ($P < 0.05$) in CKD rats, compared with controls. To

TABLE 3

Michaelis-Menten kinetic parameters for warfarin reduction in control and CKD rat liver cytosol and microsomes

Data are presented as mean ± S.E.M. of 10 rats in each group.

| | Alcohol 1 (*RS/SR*) | | Alcohol 2 (*RR/SS*) | |
|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ |
| | *μM* | *pmol/mg protein per minute* | *μM* | *pmol/mg protein per minute* |
| Cytosol | | | | |
| Control | 352.0 ± 59.65 | 38.0 ± 1.97 | 1091 ± 167.4 | 8.43 ± 0.609 |
| CKD | 359.3 ± 64.06 | 23.3 ± 1.29*** | 1149 ± 127.1 | 8.74 ± 0.466 |
| Microsomes | | | | |
| Control | 446.9 ± 174.7 | 9.57 ± 1.25 | 158.0 ± 105.6 | 37.7 ± 6.17 |
| CKD | 365.6 ± 95.82 | 5.49 ± 0.45** | Not fit to model | Not fit to model |

**$P < 0.01$ compared with control, ***$P < 0.001$ compared with control.

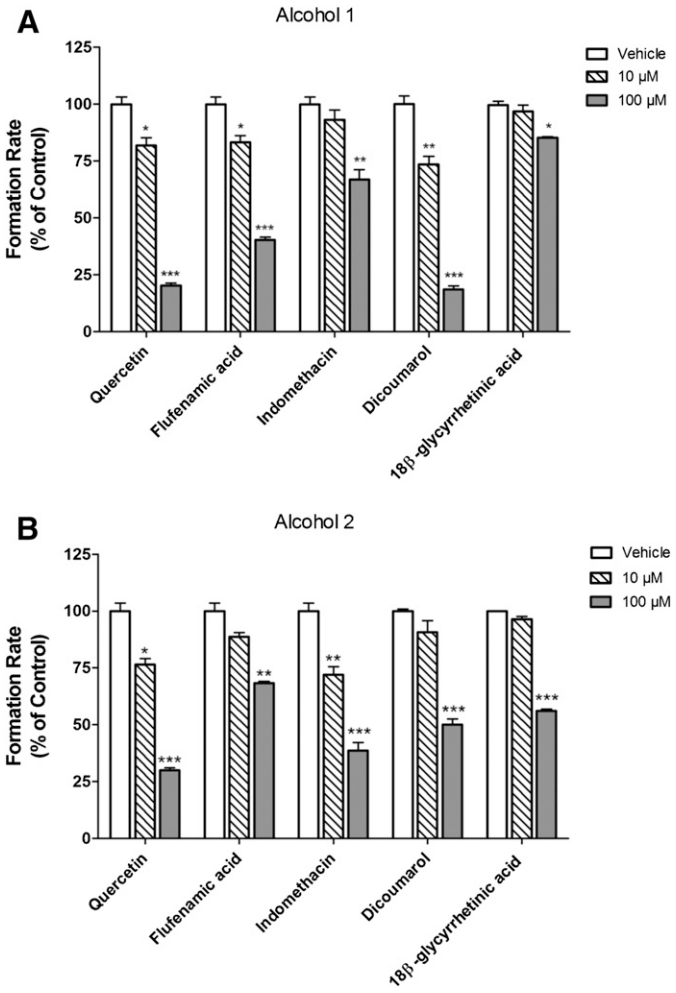


**Fig. 3.** The effect of carbonyl-reducing enzyme inhibitors on the formation of warfarin alcohol 1 (A) and alcohol 2 (B) in pooled rat liver cytosol (quercetin, flufenamic acid, dicoumarol, and indomethacin) and microsomes (18$\beta$-glycyrrhetinic acid). Incubations were conducted at two different inhibitor concentrations (10 and 100 $\mu$M). Results are presented as mean ± S.E.M. of triplicate incubations. Statistical differences are as follows: *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$, compared with control incubations which were arbitrarily defined as 100%.

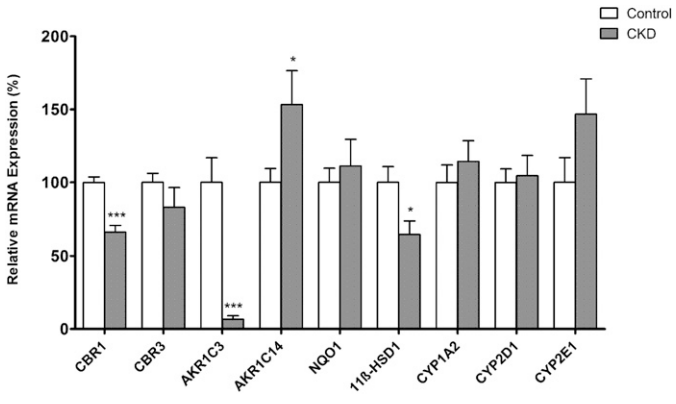


**Fig. 4.** The mRNA expression of cytosolic (CBR1, CBR3, AKR1C3, AKR1C14, and NQO1) and microsomal (11$\beta$-HSD1) rat hepatic reductases, as well as CYP1A2, CYP2D1, and CYP2E1 in control and CKD rats. Gene expression of reductase isoforms was normalized to that of glyceraldehyde 3-phosphate dehydrogenase gene. The results of CKD group were normalized to the control, which was arbitrarily defined as 100%. Experiments were conducted in duplicates, and results are presented as mean ± S.E.M. of 10 rats in each group. *$P < 0.05$ and ***$P < 0.001$ 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 11$\beta$-HSD1 was decreased by 43, 76, and 70%, respectively, in CKD rats versus controls ($P < 0.05$, Fig. 5).

## 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

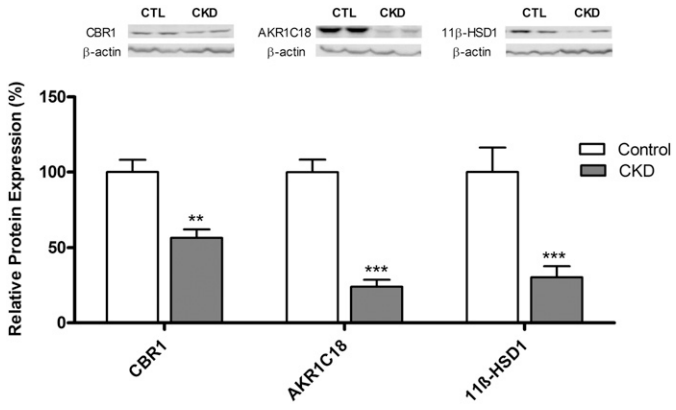


**Fig. 5.** Protein expression of cytosolic (CBR1 and AKR1C18) and microsomal (11$\beta$-HSD1) reductases in control (CTL) and CKD rat livers. The densitometry units of reductase protein expression were normalized to that of $\beta$-actin. The standardized densitometry units of control rats were arbitrarily defined as 100%. Experiments were conducted in duplicates and results are presented as mean ± S.E.M. of 10 rats in each group. The upper panels represent blots of two control and two CKD rats. **$P < 0.01$ and ***$P < 0.001$ compared with control.

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 $V_{max}$) 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 11$\beta$-HSD1 is involved in the microsomal reduction of warfarin. Together, our findings suggest that the microsomal 11$\beta$-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 11$\beta$-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 (11$\beta$-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 11$\beta$-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 suggests 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, Nolin.
*Conducted experiments:* Alshogran, Naud, Leblond.
*Contributed new reagents or analytic tools:* Ocque, Leblond, Nolin.
*Performed data analysis:* Alshogran, Naud, Nolin.
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