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The role of renal proximal tubule P450 enzymes in chloroform-induced nephrotoxicity: Utility of renal specific P450 reductase knockout mouse models

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ABSTRACT

The kidney is a primary target for numerous toxic compounds. Cytochrome P450 enzymes (P450) are responsible for the metabolic activation of various chemical compounds, and in the kidney are predominantly expressed in proximal tubules. The aim of this study was to test the hypothesis that renal proximal tubular P450s are critical for nephrotoxicity caused by chemicals such as chloroform. We developed two new mouse models, one having proximal tubule-specific deletion of the cytochrome P450 reductase (*Cpr*) gene (the enzyme required for all microsomal P450 activities), designated proximal tubule-*Cpr*-null (PTCN), and the other having proximal tubule-specific rescue of CPR activity with the global suppression of CPR activity in all extra-proximal tubular tissues, designated extra-proximal tubule-*Cpr*-low (XPT-CL). The PTCN, XPT-CL, *Cpr*-low (CL), and wild-type (WT) mice were treated with a single oral dose of chloroform at 200 mg/kg. Blood, liver and kidney samples were obtained at 24 h after the treatment. Renal toxicity was assessed by measuring BUN and creatinine levels, and by pathological examination. The blood and tissue levels of chloroform were determined. The severity of toxicity was less in PTCN and CL mice, compared with that of WT and XPT-CL mice. There were no significant differences in chloroform levels in the blood, liver, or kidney, between PTCN and WT mice, or between XPT-CL and CL mice. These findings indicate that local P450-dependent activities play an important role in the nephrotoxicity induced by chloroform. Our results also demonstrate the usefulness of these novel mouse models for studies of chemical-induced kidney toxicity.

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Introduction

The kidney is a primary target for numerous toxic xenobiotics including drugs, environmental chemicals and metals. The kidney concentrates solutes and xenobiotics during reabsorptive and secretive processes, and it has a variety of drug transporters and drug metabolizing enzymes that are involved in renal toxicity induced by xenobiotics. These physiological features make the kidney more susceptible to chemical insults than most other organs. It was reported that 19% of cases of critically ill patients with acute renal failure were drug-related (Uchino et al., 2005). Xenobiotics may cause kidney injuries through a variety of mechanisms. Studying the underlying mechanisms will provide molecular approaches for the prevention and intervention of chemical-induced renal toxicity.

Cytochrome P450 enzymes (P450 or CYP) play important roles in the biotransformation of both xenobiotics and endogenous compounds. Many xenobiotics require metabolic activation catalyzed by P450s to produce toxic metabolites. Hepatic P450s play the dominant role in catalyzing the biotransformation of a variety of xenobiotic compounds. The reactive intermediates resulting from P450-catalyzed metabolism are unstable, and therefore are less likely to be transported as activated forms from the liver to other tissues to exert toxicity. Thus, chemical-induced toxicity in extrahepatic tissues is believed to result from in situ metabolic activation. We have demonstrated that hepatic P450-dependent metabolism does not play a significant role in the renal injury induced by chloroform (Fang et al., 2008a). However, little is known about the specific contribution of renal P450s to the metabolic activation of renal toxicants, due to a lack of appropriate animal models.

The knockout approach of targeted gene disruption is a powerful tool to analyze the functions of genes and their products in vivo. However, P450s comprise a large gene family, and many P450s have overlapping substrate specificities and similar profiles in tissue distribution; thus it is not possible to knockout all the P450 isoforms in order to study the combined roles of P450s. However, NADPH-cytochrome P450 reductase (CPR) acts as the obligate redox partner for all P450 activities.

Abbreviations: P450 or CYP, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Scr, serum creatinine.

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Therefore, *Cpr* knockout mouse models allow us to study the combined roles of all microsomal P450s. The expression patterns of CPR and P450s in mouse kidney were investigated in our earlier study (Fang et al., 2008a). CPR and several major P450s were expressed primarily in the proximal tubules. The renal proximal tubules are also primary targets of most renal toxicants, including chloroform. Toxicity is attributed to, at least in part, the high expression levels of CPR and P450s in the proximal tubules, although direct evidence of this has yet to be obtained.

In order to study the in vivo role of proximal tubule P450s in chemical-induced nephrotoxicity, we have generated two novel mouse models with proximal tubule-specific alteration of the *Cpr* gene using the Cre/loxP approach. In the first model (designated proximal tubule-Cpr-null or PTCN), the *Cpr* gene was deleted specifically in the proximal tubules of the kidney. In the second model (designated extra-proximal tubule *Cpr*-low or XPT-CL), *Cpr* is expressed normally in the proximal tubules, but is at substantially lower levels elsewhere, throughout the body. We compared the PTCN and XPT-CL models to wild-type (WT) mice and a previously reported mouse model that has decreased levels of CPR in all tissues (designated *Cpr*-low or CL) (Wei et al., 2010; Wu et al., 2005), in order to examine the role of renal P450s in nephrotoxicity induced by chloroform, a renal toxicant.

Materials and methods

Generation of the proximal tubule-Cpr-null (PTCN) mice and extra proximal tubule-Cpr-low mice (XPT-CL) mice. Breeding pairs of hemizygous KAP-Cre transgenic mice (on a mixed B6/129 background), with *Cre* driven by the kidney androgen protein (KAP) promoter, were obtained from The Jackson Laboratory (Bar Harbor, ME) (Li et al., 2008). The *Cpr*-lox mice ($Cpr^{lox/lox}$; congenic on B6 background) having two "floxed" *Cpr* alleles and reversible *Cpr*-low mouse ($Cpr^{r\text{-}CL/r\text{-}CL}$) were created in our previous studies (Gu et al., 2003; Wei et al., 2010; Wu et al., 2003). KAP-*Cre* hemizygous transgenic mice were initially crossed with $Cpr^{lox/lox}$ mice or $Cpr^{r\text{-}CL/r\text{-}CL}$ mice to generate KAP-$Cre^{+/-}/Cpr^{lox/lox}$ mice and KAP-$Cre^{+/-}/Cpr^{r\text{-}CL/r\text{-}CL}$ mice, which were then crossed with $Cpr^{lox/lox}$ mice and $Cpr^{r\text{-}CL/r\text{-}CL}$ mice, producing KAP-$Cre^{+/-}/Cpr^{lox/lox}$ mice (proximal tubule-*Cpr*-null or PTCN), KAP-$Cre^{-/-}Cpr^{lox/lox}$ mice (WT), KAP-$Cre^{+/-}/Cpr^{r\text{-}CL/r\text{-}CL}$ (extra proximal tubule Cpr-low or XPT-CL) and KAP-$Cre^{-/-}/Cpr^{r\text{-}CL/r\text{-}CL}$ (Cpr-low or CL) mice. Genotype analyses for the Cre transgene and the *Cpr* allele were performed as described previously (Wei et al., 2010; Wu et al., 2003, 2005). Two- to three-month-old male mice from each of the four strains were used for the studies. All animal studies were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

Histopathologic examination. Kidneys were dissected from 2-month-old male WT, PTCN, CL and XPT-CL mice 24 h after treatment with vehicle (olive oil) or chloroform. The collected kidneys were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm thickness and the sections were stained with hematoxylin and eosin. The extent of tubular injury (dilatation, vacuolation and necrosis) was evaluated semi-quantitatively as previously described (Fang et al., 2008a). Briefly, the extent of tissue damage was graded from 0 to 4 according to the severity of tubular necrosis, tubular vacuolation and tubular dilatation. The scoring system was as follows: 0 = no change in the tubules; 1 = <25% of the tissue showing tubular injury (mild); 2 = 25% to 50% of tubular involvement (moderate); 3 = 50% to 75% of tubules showing characteristic change (severe) and 4 = >75% of tubular damage (very severe). Fifty fields were scored from each slide. All the assessments were done in a blinded fashion.

Immunohistochemical and immunoblot analysis of CPR expression. For immunohistochemical detection of CPR expression in the kidneys, paraffin sections (4 μm) of kidneys were processed according to a published protocol (Fang et al., 2008a). The sections were analyzed using the following polyclonal antibodies: rabbit anti-rat CPR antibody (Chemicon, 1:1000), mouse anti-human aquaporin antibody (Santa Cruz, 1:1000). Alexa Fluor 594 Tyramide Signal Amplification Kit (Molecular Probes, Eugene, OR) was used for the visualization of the expression sites (Red) of CPR, Alexa Fluor 488 goat anti-mouse IgG was used for the visualization of the expression sites (green) of aquaporin, and the nucleus was stained with DAPI (blue). The control sections were incubated with normal rabbit serum (Biogenex, San Ramon, CA) in replacement of the primary antibody.

For the immunoblot analysis of CPR expression in the kidney and other tissues, microsomes were prepared as described previously (Fang et al., 2008a,b). Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL), with bovine serum albumin as the standard. Microsomal samples were then subjected to electrophoresis on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). A polyclonal antibody to rat CPR (Chemicon, 1:2000), which recognized a single band (Lin et al., 2012), was used in the analyses. Peroxidase-labeled rabbit anti-goat IgG (Sigma-Aldrich, St. Louis, MO) was used as the second antibody, and immunoreactive proteins were detected using an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). The signal intensities of the detected bands were quantified by densitometry.

Real time PCR analysis of mRNA levels for P450 isoforms. Total RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Total RNA was quantified by the determination of optical density at 260 nm. All RNA samples used in these studies had a ratio of 260:280 between 1.9 and 2.2. The integrity of the RNA samples was confirmed by agarose gel electrophoresis, which showed the presence of intact 18 s and 28 s ribosomal RNA bands. Synthesis of cDNA was performed using the SuperScript III cDNA Synthesis Kit (Invitrogen, Grand Island, NY), and the cDNAs were stored at −20 °C until use. Quantitation of each target transcript was carried out using the SYBR® Green Master Mix kit (Invitrogen, Grand Island, NY). PCR was performed according to the kit instructions. Gene-specific primers for *Cyp1a1*, *Cyp1b1*, *Cyp2b9*, *Cyp2e1* and *Cyp3a11* are presented in Table 2. PCR reaction mixtures contained 10 μl of SYBR® Green Master Mix, 0.5 μM of each primer, and 4 μl of undiluted or diluted (10–1000 fold) RT product in a total volume of 20 μl. Reactions were initiated with a denaturation/Taq activation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 15 s, and 72 °C for 30 s. The specificity of the PCR reactions was confirmed by melting-curve analysis. The fold changes in the levels of target transcipts between PTCN, CL or XPT-CL and WT group, normalized to the levels of β-actin, were determined using the following equation: Fold change $= 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(PTCN,\ CL\ or\ XPT\text{-}CL)} - \Delta Ct_{(WT)}$.

Table 1
The extent of proximal tubular toxicity induced by chloroform in WT, PTCN, CL and XPT-CL mice.
Kidneys were dissected from 2-month-old male WT, PTCN, CL and XPT-CL mice at 24 h after vehicle or chloroform treatment. The severity of lesions in the kidney was graded: 0 = no change in the tubules; 1 = <25% of tubular injury; 2 = 25% to 50% of tubular injury; 3 = 50% to 75% of tubular injury, and 4 = > 75% of tubular injury.

Score	WT	PTCN	CL	XPT-CL
Vehicle treatment	0	0	0	0
Chloroform treatment	3.63 ± 0.52	1.50 ± 0.53*	1.75 ± 0.46*#	3.75 ± 0.46

Values are means ± S.D., n = 8.
* $P < 0.01$ versus WT + Chloroform group.
\# $P < 0.05$ versus XPT-CL + Chloroform group.