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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 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.

Keywords

Cytochrome P450; P450 reductase; Gene knockout; Nephrotoxicity; Chloroform; Mice

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Conflict of interest statement
The authors declare that there are no conflicts of interest.

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. 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) (Wu et al., 2005; Wei et al., 2010), 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 (Wu et al., 2003; Gu et al., 2003; Wei et al., 2010). 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 (Wu et al., 2005; Wei et al., 2010; Wu et al., 2003). 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., 2008). 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., 2008). 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 visualization of the expression sites (Red) of CPR, Alexa Fluor 488 goat anti mouse IgG was used for 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 replace of the primary antibody.

For immunoblot analysis of CPR expression in the kidney and other tissues, microsomes were prepared as described previously (Fang et al., 2008). 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 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 18s and 28s 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 $\Delta(\Delta Ct) = \Delta Ct_{(PTCN,\ CL\ or\ XPT\text{-}CL)} - \Delta Ct_{(WT)}$.

Animal treatments with chloroform

Chloroform and other chemicals were purchased from Sigma-Aldrich unless stated otherwise. WT, PTCN, CL, and XPT-CL mice (male, 2-month-old) were treated with a single oral dose of chloroform at 200 mg/kg (20 mg/ml in olive oil). Control group mice were treated with vehicle (olive oil) only. The animals were sacrificed by CO_2 overdose at 24 h after treatment. The time point was selected based on our previous study (Fang et al., 2008a). Specimens of blood, liver and kidney were collected from individual mice for the analysis. The tissues were immediately frozen on dry ice and then stored at –80°C until analysis.

Biochemical analysis

The levels of blood urea nitrogen (BUN), creatinine (Scr) and alanine aminotransferase (ALT) were determined in serum samples collected at 24 h following chloroform treatment, using commercial kits (BUN from Diagnostic Chemicals, Oxford, CT; Scr from WAKO, Richmond, VA; ALT from Sigma-Aldrich, St. Louis, MO), according to the instructions provided by the manufacturers.

Determination of chloroform concentration in the tissues

Tissues and blood samples were processed as previously described (Fang et al., 2008), and chloroform levels in the blood, liver and kidney were determined using headspace solid-phase microextraction (HS-SPME) and gas chromatography with micro-electron capture detection on a model 6890 instrument (Agilent Technologies, Santa Clara, CA) equipped with a DB-5 capillary column (60 m × 0.25mm I.D. with 0.25 μm 5% polydiphenylsiloxane-95% polydimethylsiloxane phase; J&W Scientific, Folsom, CA). The injection port temperature was maintained at 225 °C, the initial oven temperature was 40 °C and was increased at a rate of 30 °C /min to a final temperature 270 °C. Calibration curves

for quantitative determination of chloroform were prepared by spiking the known amounts of chloroform standard into tissue homogenates tissue or serum samples.

Statistical analysis

All data are expressed as mean ± SD. A single-factor ANOVA was used for comparisons among multiple groups, and unpaired t-tests were used for comparisons between two groups. $P<0.05$ was considered statistically significant.

Results

General characterization of the proximal tubule-*Cpr*-null (PTCN) mice and extra proximal tubule-*Cpr*-low (XPT-CL) mice

The PTCN mice were viable, fertile, and normal in size and body weight. No obvious physical or behavioral abnormities were observed in PTCN mice in comparison with WT littermates. No embryonic lethality was apparent in the PTCN mice based on the genotype distribution analyses of pups derived from crossbreeding between KAP-$Cre^{+/-}$/$Cpr^{lox/lox}$ and $Cpr^{lox/lox}$ (data not shown). Histological examination of the kidneys from the PTCN mice did not reveal any structural abnormalities (data not shown).

The general characteristics of XPT-CL mice were similar to those of CL mice, as was observed in our previous study (Wei et al., 2010). Both CL and XPT-CL mice were normal in general appearance, and they showed significantly lower body weight (~ 10%) and lower organ weights (heart, lung, and kidney) (5–15%) compared with WT mice (data not shown).

Renal proximal tubular specific deletion of *Cpr* gene in PTCN mice or rescue of *Cpr* in XPT-CL mice

The renal proximal tubule specific *Cpr* gene deletion in male PTCN mice or *Cpr* rescue in male XPT-CL mice were examined by immunohistochemical analysis of the CPR protein. CPR protein was expressed predominantly in the proximal tubule and outer stripe of the outer medulla of male WT mice (Fig. 1A), as reported previously (Fang et al., 2008a) CPR staining in the cortex was co-localized with that of the proximal tubule marker aquaporin 1 (AQP1). In male PTCN mice, CPR expression was not detected in about 84% of the proximal tubular cells that were examined (Fig. 1B). In CL mice, CPR expression was markedly suppressed in whole kidney, while CPR expression was restored in about 76% of the proximal tubular cells visualized in XPT-CL mice (Fig. 1D). However, there was no overall decrease in CPR levels in microsomes prepared from whole kidney of PTCN mice determined by immunoblot analysis (Fig. 2), in comparison with the levels in WT mice. Both CL and XPT-CL mice showed much lower CPR expression than that observed in WT and PTCN mice. In comparison with CL mice, there was a significantly increased level of CPR protein expression in samples of whole kidney from XPT-CL mice. The levels of expression of CPR in the liver were not affected in PTCN mice in comparison with the levels in control mice (data not shown). Similar to CL mice, CPR expression in the liver of XPT-CL mice was significantly decreased in comparison with the level of WT mice (data not shown).

Impact of *Cpr* gene deletion on the expression of renal P450s in PTCN, CL and XPT-CL mice

Given the previously reported compensatory changes in gene expression in the liver of the liver-*Cpr*-null mice (Wu et al., 2003), we used real-time PCR to analyze potential changes in mRNA levels of major P450 isoforms in the kidneys of these mice. As shown in Fig. 3, no changes in *Cyp1a1, Cyp1b1, Cyp2b9, Cyp2e1* or *Cyp3a11* mRNA levels were observed in kidneys of PTCN, CL or XPT-CL mice in comparison with those of WT mice.

Extent of chloroform-induced renal toxicity in WT, PTCN, CL and XPT-CL mice

We compared the extent of renal toxicity among WT, PTCN, CL and XPT-CL mice after treatment with chloroform. Diffuse cortical tubular dilation, tubular necrosis, tubular attenuation and hyaline eosinophilic protein in tubules were found in the proximal tubules of WT mice (Fig. 4; however, much milder lesions were found in kidney of the PTCN and CL mice. The extent of kidney injury in XPT-CL mice was similar to that in WT mice. The scores of tubular injury in various mice are presented in Table 1.

The blood parameters for assessment of renal function, blood urea nitrogen (BUN) and serum creatinine (Scr), were determined to further assess chloroform induced renal toxicity. As shown in Fig. 5, PTCN and CL mice showed lower BUN and Scr levels compared to WT mice following chloroform treatment, but XPT-CL mice showed the levels similar to that of WT mice.

Chloroform levels in blood, liver and kidney of WT, PTCN, CL and XPT-CL mice following chloroform treatment

The levels of chloroform in the blood, liver, and kidney in CL and XPT-CL mice were significantly higher than those in WT and PTCN mice following chloroform treatment (Fig. 6). There were no significant differences in chloroform levels in blood, liver and kidney between WT and PTCN mice or between CL and XPT-CL mice.

Discussion

Germline deletion of the *Cpr* gene resulted in embryonic lethality in mice, demonstrating the critical role of CPR in early development (Shen et al., 2003). To circumvent this problem, the Cre/Lox system provides a useful tool to study the in vivo functions of CPR in adult mice in a tissue specific fashion that is not embryonically lethal. In recent years, conditional deletion of *Cpr* gene was achieved in several major organs in mice, including the liver (Gu et al., 2003), lung (Weng et al., 2007), intestine (Zhang et al., 2009), heart (Fang et al., 2008b), brain (Conroy et al., 2010) and mammary gland (Lin et al., 2012). These tissue-specific *Cpr* null models offered opportunities to study the roles of total P450 activities in these tissues in chemical-induced toxicity and metabolism of endogenous substances. Additionally, we developed a complementary mouse model, *Cpr*-low (CL), in which CPR expression is decreased by 70-90% (Wu et al., 2005). Most recently, another similar mouse model, named reversible-*Cpr*-low (r-CL), was generated in our lab (Wei et al., 2010), in which the suppressed CPR expression could be recovered in an organ/tissue-specific fashion through crossbreeding r-CL mice with tissue-specific *Cre* transgenic mice. The restoration of CPR function was achieved in the hepatocytes through crossbreeding r-CL mouse with Alb-Cre transgenic mouse (Wei et al., 2010). The Cpr-low and r-CL mice could serve as disease models for patients harboring mutations that affect CPR functions. Therefore, tissue-specific rescue of the *Cpr* gene under a globally suppressed *Cpr* condition is a unique tool to study the functions of CPR/P450 system and its combined roles in chemical toxicity in the target tissues, including the kidney, along with tissue-specific *Cpr*-null mouse models.

Here we have successfully generated a proximal tubule-*Cpr*-null (PTCN) mouse by crossbreeding Cpr^{lox} with KAP-*Cre* mice, in which *Cpr* gene was deleted specifically in the proximal tubules. Additionally, we also generated an extra proximal tubule-*Cpr*-low (XPT-CL) mouse model by crossing r-CL with KAP-*Cre* mice, in which the function of *Cpr* gene was rescued specifically in the proximal tubules. Our conditional renal *Cpr* knockout models offer a powerful tool for studying the function of CPR-dependent enzymes in the kidney, and their roles in nephrotoxicity induced by chemical compounds. Several kidney-specific *Cre*-transgenic mouse models were reported previously using kidney-specific prompters,

including sodium glucose co-transproter-2 (Rubera et al., 2004), phosphoenolpyruvate carboxykinase (Rankin et al., 2006), γ-glutamyl transpeptidase (Dworniczak et al., 2007) or kidney androgen-regulated protein (KAP)(Li et al., 2008).

In the current study, we used a recently developed KAP-*Cre* transgenic mouse with enhanced expression of *Cre* under the control of the KAP promoter (Li et al., 2008). In the KAP-*Cre* mouse, Cre expression was localized in all three segments of the proximal tubules, but it was not expressed in extrarenal tissues. Since the expression of CPR was primarily localized in the proximal tubules, the cell-type-specific deletion or rescue of the *Cpr* gene can be confirmed by co-localization of CPR expression with staining for AQP1, a specific marker for the proximal tubular epithelial cells. In male PTCN and XPT-CL mice, the respective absence or recovery of CPR expression was observed in the AQP1-positive cells, and the deletion or rescue of the *Cpr* gene was achieved in about 80% proximal tubules viewed. The expression of the KAP transgene in male mice can be up-regulated by testosterone administration (Li et al., 2008), which could thus be used to further increase the extent of deletion or rescue of the *Cpr* gene in our two mouse models. There was no significant decrease in the levels of CPR expression in kidney microsomes of PTCN mice compared with WT mice determined by immunoblot analysis, while a significant recovery in the levels of CPR expression was observed in the kidney of XPT-CL mice compared with expression in CL mice. These observations can be explained by the fact that the proximal tubule is a minor cell population in the kidney, and thus loss of CPR expression in these cells would not cause a large decrease in overall CPR levels in whole kidney homogenate, a limitation that could be resolved by immunohistochemical analysis. In that connection, the data in Figure 1 show that the abundance of CPR-expressing proximal tubule cells, which are sensitive to chloroform toxicity, was clearly different among the four strains of mice. Additionally, it would be much more sensitive to detect an increased expression from a low expression background than to detect a decreased expression from the high basal level in tissue homogenates by immunoblot analysis. KAP-*Cre* mediated gene deletion in the proximal tubules could also be achieved in female mice via testosterone treatment (Ding et al., 2001; Li et al., 2008), which may expand its application in females although male mice are the representative for most renal toxicity studies.

The liver is the primary organ for P450-mediated metabolism of foreign compounds; however, many P450 isoforms are expressed in the kidney at high levels. CYP1A, 2B, 2C, 2E, 2J, 3A, 4A and 4F, were shown to be expressed predominantly in the proximal tubule in rodents (Cummings et al.,2000; Stern et al., 2005), and the expression of CYP3A, 4A11 and 4F2 was also detected in human kidney (Cummings et al., 1999). The exact role of renal P450s in chemical toxicity is largely unknown due to lack of appropriate animal models. It is possible that chemical metabolites formed in the liver may selectively target kidney through further metabolic activation in the kidney (Anders et al., 1993). Previously, we used the liver-*Cpr*-null mice to demonstrate that hepatic P450-dependent metabolism does not play a significant role in chloroform-induced renal toxicity (Fang et al., 2008), indirectly suggesting that renal P450 dependent metabolic activation may play an important role in chemical induced renal toxicity. Here, with the availability of PTCN and XPT-CL mice, we are able to provide the direct evidence to support our hypothesis.

Chloroform causes renal toxicity in a number of species through P450-dependent metabolic activation. Chloroform is an industry solvent and a by-product from water chlorination, making chloroform a potential risk for human exposure (Van Vleet et al., 2003). Chloroform is metabolically activated by P450s to form trichloromethanol and eventually phosgene, and the latter can react with GSH, cysteine, and cellular macromolecules to initiate toxicity (Schnellmann et al., 2001). *Cyp2e1*-null mice were resistant to chloroform toxicity, indicating that CYP2E1 plays an important role in the metabolic activation of chloroform

and the proximal tubule is the primary target for chloroform toxicity (Constan et al., 1999), while the glomerulus and the distal tubule are resistant (McMartin et al., 1981). It was proposed that the nephrotoxic metabolites of chloroform are produced within the kidney (Pohl et al., 1984). Our current results showed that deletion of the *Cpr* gene in the proximal tubules led to a significant decrease in chloroform renal toxicity in PTCN mice, while rescue of the *Cpr* gene in the proximal tubule resulted in the restoration of the renal toxicity in XPT-CL mice. These data from the two mouse models strongly support the notion that renal proximal tubule P450s play an important role in metabolic activation of nephrotoxicants, such as chloroform.

Hepatic P450s are believed to play a major role in the systemic clearance of foreign compounds. We previously showed that hepatic deletion of the *Cpr* gene caused the accumulation of chloroform in the blood and other tissues (Fang et al., 2008), but the contribution of renal P450s in local clearance of chemical compounds was unknown. In this study, the chloroform levels in the blood, liver, and kidney remained unchanged in PTCN mice compared to that of WT mice, and the restoration of CPR function in the proximal tubules did not alter the chloroform levels in XPT-CL mice compared to that of CL mice. Therefore, the two lines of evidence clearly demonstrate that renal P450s do not play a significant role in systemic or local clearance of chloroform. The high chloroform levels observed in CL and XPT-CL mice compared to WT mice were caused by the decreased P450 dependent clearance in extra-renal tissues, mostly in the liver.

For the first time, the renal specific *Cpr* knockout or rescue mouse models were applied to study the mechanisms of kidney toxicity induced by chemical compounds. P450s are the most important drug metabolizing enzymes, responsible for disposition and metabolic activation of nearly 70% of drugs and numerous other foreign compounds including nephrotoxicants. The role of renal P450s in the local metabolism of therapeutic agents and toxicants is unclear. Our two models provided a unique approach for studying the mechanisms of kidney toxicity induced by drugs and other chemical compounds that are metabolized by P450s. Moreover, P450s are also responsible for the synthesis/degradation of many important endogenous compounds. Renal P450s are supposedly to be involved in the metabolism of arachidonic acid, whose metabolites contribute to the regulation of blood pressure and the pathogenesis of ischemic kidney injury. The two renal conditional *Cpr* knockout models would be useful for studying the functions of proximal tubule P450s and their roles in renal toxicity and injury.

In conclusion, we investigated the role of renal P450s in renal toxicity induced by chloroform using the two novel mouse models having the proximal tubule-specific knockout or rescue of the *Cpr* gene. Our data strongly support the hypothesis that renal P450-mediated metabolic activation plays an important role in renal toxicity induced by chloroform. This is the first direct in vivo evidence to demonstrate the essential role of renal P450s in nephrotoxicity induced by chemical compounds, which could promote future studies on novel approaches for the prevention and intervention of chemical-induced renal injury. Our results also demonstrate the usefulness of the two novel mouse models for studying kidney toxicity induced by chemical compounds.

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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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Highlights

- New mouse models were developed with varying P450 activities in the proximal tubule
- These mouse models were treated with chloroform, a nephrotoxicant
- Studies showed the importance of local P450s in chloroform-induced nephrotoxicity

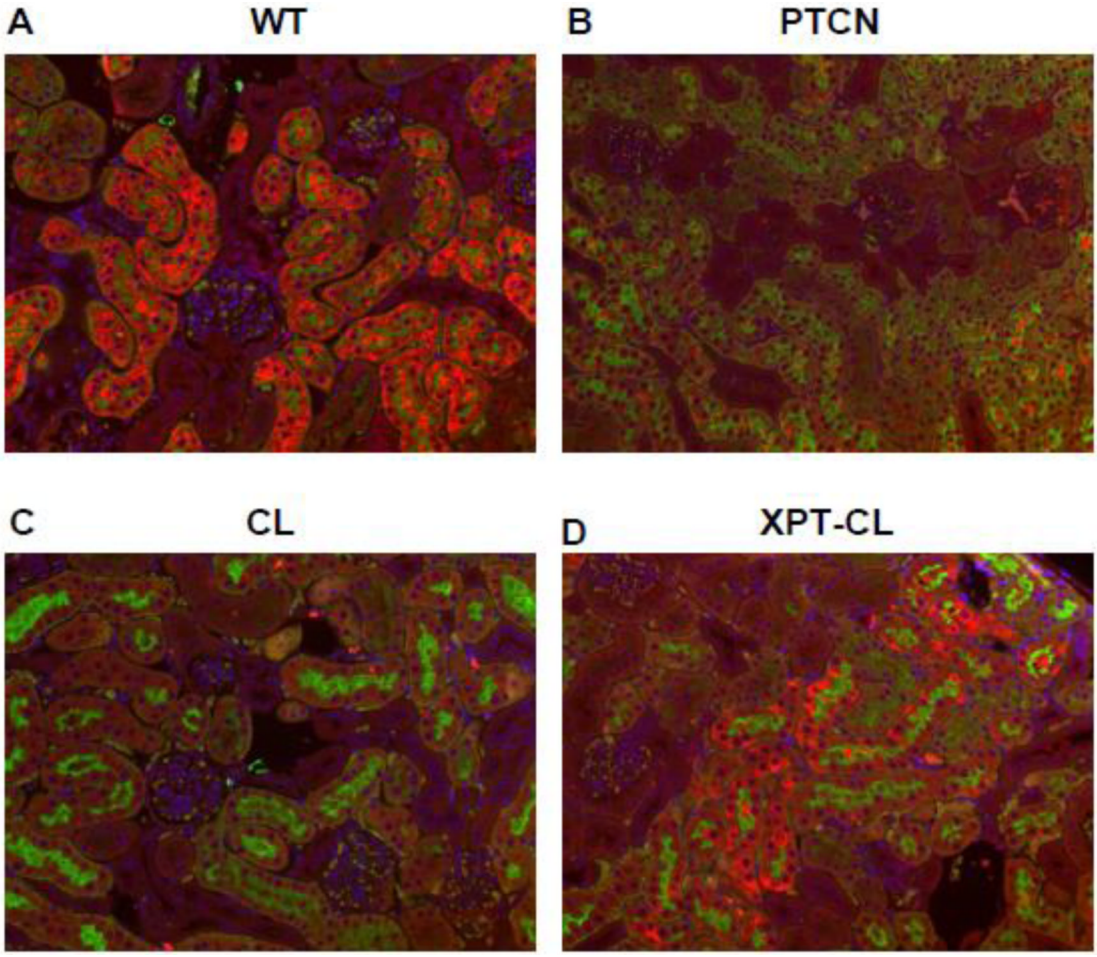

Fig. 1. Immunohistochemical analysis of CPR protein expression in the kidneys of WT, PTCN, CL and XPT-CL mice

Paraffin sections (4 μm) of kidneys from WT, PTCN, CL and XPT-CL mice (male, 2-month-old) were processed for immunohistochemistry analyses. The tissue sections were incubated with a polyclonal rabbit anti-rat CPR antibody. Alexa Fluor 594 Tyramide Signal Amplification Kit was used for visualization of CPR expression sites (Red). Then the tissue sections were incubated with a monoclonal mouse anti-AQP1 antibody, Alexa Fluor 488 Goat anti-mouse IgG was used for visualization of AQP1 protein (green). The nucleus was stained with DAPI (Blue). No signal was detected when the primary antibody was replaced by a normal rabbit serum (data not shown). Results shown are typical of eight mice per strain analyzed. A: WT; B: PTCN; C: CL; D: XPT-CL. × 200 in magnification.

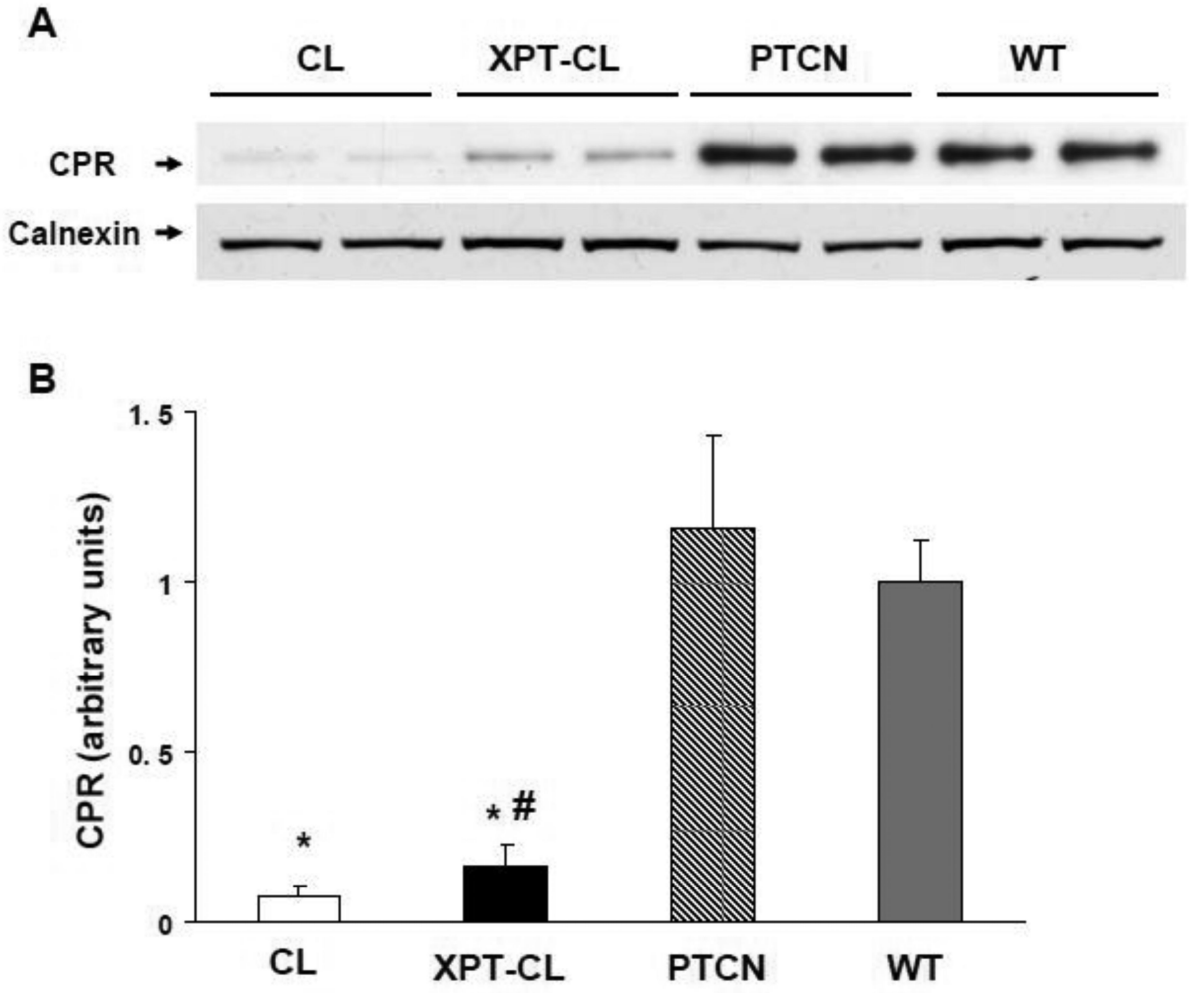

Fig. 2. Immunoblot analysis of CPR protein expression in the kidney of WT, PTCN, CL, and XPT-CL mice

A. Microsomal proteins from the kidneys (10 μg) of WT, PTCN, CL, and XPT-CL mice (male, 2-month-old) were analyzed for CPR expression with a rabbit anti-rat CPR antibody. Each lane is the pool of three mice per strain. Calnexin was used as internal standard. B. Densitometry analysis of the immunoblot results. CPR expression of WT group was set at 1. Values present means ± S.D. n=3. $^{*}P<0.05$, compared with WT group; $^{\#}P<0.05$, compared with CL group.

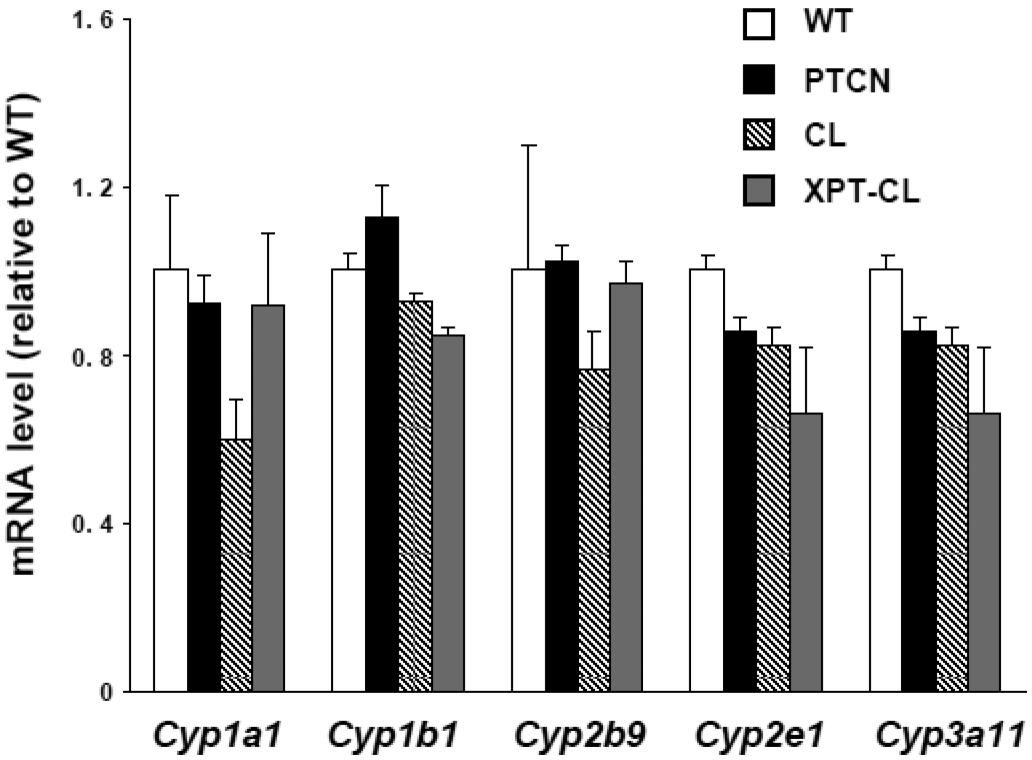

Fig. 3. Real-time quantitative RNA-PCR analysis of P450 mRNA levels for *Cyp1a1, Cyp1b1, Cyp2b9, Cyp2e1* and *Cyp3a11* in the kidney of WT, PTCN, CL and XPT-CL adult male mice

Total RNA was extracted from the kidney of 2-month-old WT, PTCN, CL and XPT-CL male mice, and then reversely synthesized. Duplicate reactions were performed for each experiment, and the values presented are the means ± S.D. of three independent experiments. The results were normalized by the levels of β-actin. $P>0.05$, one-way ANOVA

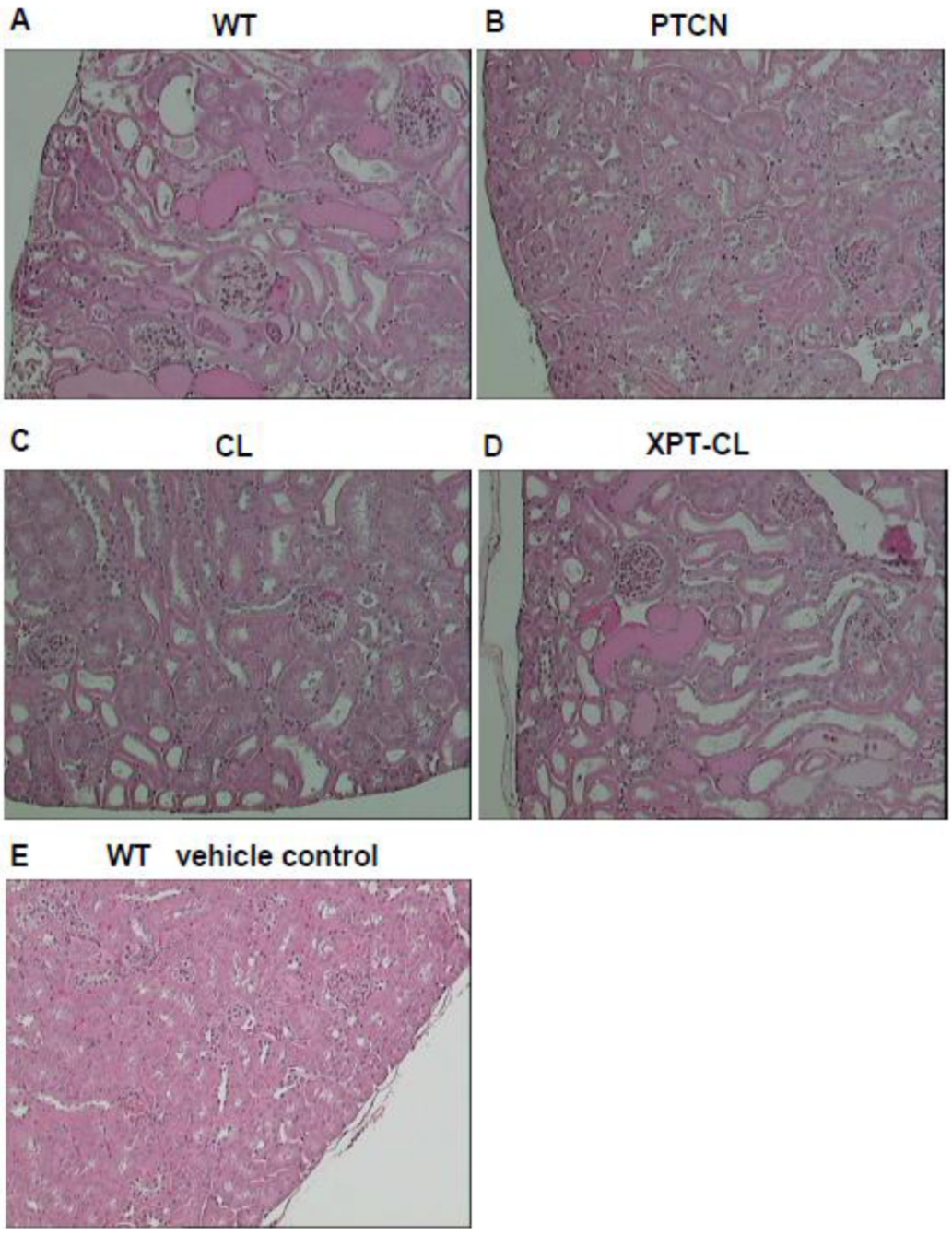

Fig. 4. Chloroform induced renal injury in WT, PTCN, CL and XPT-CL mice

The kidneys were collected from 2-month-old WT, PTCN, CL and XPT-CL male mice for pathological examination at 24 h following a single oral dose of chloroform at 200 mg/kg in olive oil. Results shown are typical of 8 mice in each group. The vehicle control mice were treated with olive oil alone (data not shown). A: WT + chloroform; B: PTCN + chloroform; C: CL + chloroform; D: XPT-CL + chloroform; E: WT + vehicle.

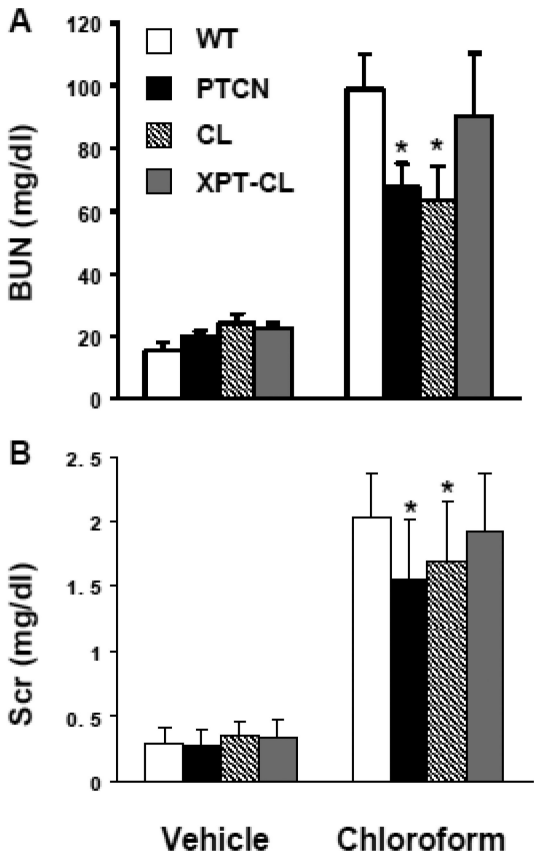

Fig. 5. Increases in the levels of serum BUN and creatinine in WT, PTCN, CL and XPT-CL mice following chloroform treatment

WT, PTCN, CL and XPT-CL mice (male, 2-month-old) were treated with a single dose of 200 mg/kg chloroform (in olive oil) by oral gavage. Blood samples were collected at 24 h after dosing. Control mice were given olive oil alone. The serum levels of BUN and creatinine were determined. A: BUN levels of various mice; B: Serum creatinine (Scr) levels of various mice. **P*<0.05, compared with WT + chloroform group. Values present means± S.D., n=8.

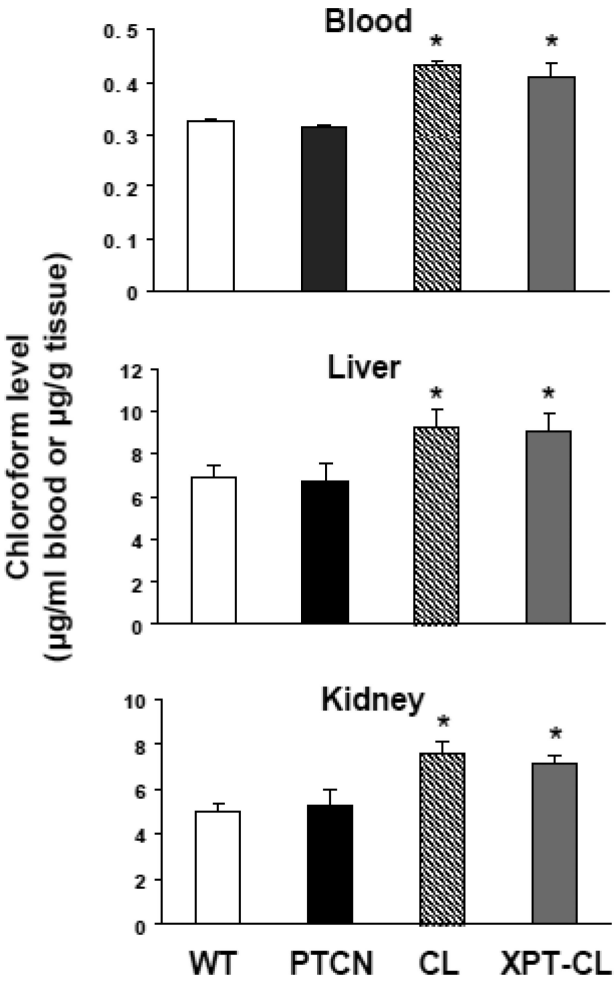

Fig. 6. Chloroform levels in blood, liver and kidney of WT, PTCN, CL and XPT-CL mice following chloroform treatment

WT, PTCN, CL and XPT-CL mice (male, 2 to 3-month-old) were treated with a single dose of 200 mg/kg chloroform (in olive oil) by oral gavage. Blood, liver and kidney samples were collected at 24 h after dosing. Control mice were given olive oil alone. The levels of chloroform were determined. A: Blood chloroform levels of various mice; B: Liver chloroform levels of various mice; C: Kidney chloroform levels of various mice. **P*<0.05, compared with WT + chloroform group. Values present means± S.D., n=8.

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 24h after vehicle or chloroform treatment. The severity of lesions in the kidney was graded: 0 = no changein the tubules; 1 = < 25% of tubular injury; 2 = 25% to 50% of tubular injury; 3 = 50% to 75% oftubular 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.

Table 2

Sequences of primers for real time PCR analysis of P450 gene expression in the kidney of WT, PTCN, CL and XPT-CL mice

Gene	Sequence	Reference
Cyp1a1	F 5′- CACCATCCCCCACAGCAC -3′ R 5′- ACAAAGACACAGCACCCCTT -3′	Xu et al., 2004
Cyp1b1	F 5′- TTGACCCCATAGGAAACTGC -3′ R 5′- GCTGTCTCTTGGTAGGAGGA -3″	Xu et al., 2004
Cyp2b9	F 5′-TGAAGCTTTTCTGCCCTTCT-3′ R 5′-GTGTGAGCAGCTACCAATGG-3′	Damon et al., 1996
Cyp2e1	F 5′- AAGCGCTTCGGGCCAG -3′ R 5′- TAGCCATGCAGGACCACGA -3′	Richardson et al., 2005
Cyp3a11	F 5′-GGATGAGATCGATGAGGCTCTG -3′ F 5′-CAGGTATTCCATCTCCATCACAGT-3′	Richardson et al., 2005
β-actin	F 5′-ATTGCTGACAGGATGCAGAA-3′ R 5′-CAGGAGGAGCAATGATCTGA-3′	Xu et al., 2004