Adaptive Hepatic and Intestinal Alterations in Mice after Deletion of NADPH-Cytochrome P450 Oxidoreductase (Cpr) in Hepatocytes

Xingguo Cheng, Jun Gu, and Curtis D. Klaassen

Department of Pharmaceutical Sciences, St. John's University, Queens, New York (X.C.); Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, Albany, New York (J.G.); and Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas (C.D.K.)

Received July 15, 2014; accepted August 21, 2014

ABSTRACT

Cytochrome P450 enzymes (P450) play an important role in first-pass metabolism in both the intestine and liver. NADPH-cytochrome P450 oxidoreductase (Cpr) is an essential electron transfer protein required for microsomal P450 activity. Mice with conditional knockout of Cpr in hepatocytes develop normally and survive even with complete loss of liver microsomal P450 activity. Our current studies were performed to determine whether alternative drug-metabolizing pathways increase in an attempt to maintain whole-body homeostasis. In addition to the liver, Cpr is mainly expressed in tissues such as lung, kidney, and gastrointestinal tract. In livers of H-Cpr-null mice, there is a marked increase in mRNA expression of phase I enzymes (Aldh1a1, 1a7, 3a2; Ces1b2, 2a6, and 2a12), antioxidant enzymes (Ho-1, Nqo1, and epoxide hydrolase), phase II enzymes (Ugt1a9; Gsta1/2, m3, m4, m6, t1, and t3; and Sult1a1 and

1d1), and drug transporters (Oatp1a4, Oct3, Mate1, Mdr1a, and Mrp3 and 4). In addition, glucuronide-conjugated bilirubin concentrations are doubled in serum of H-Cpr-null mice. Both constitutive androstane receptor (CAR) and nuclear factor erythroid 2-related factor 2 (Nrf2) protein in nuclei are higher in the livers of H-Cpr-null mice, indicating that CAR and Nrf2 are activated. In the small intestine of H-Cpr-null mice, mRNA expression of Cyp3a11 and Mdr1a, two genes critical for intestinal first-pass metabolism, are markedly up-regulated. In addition, nutrient (Pept1) and cholesterol (Npc1l1) transporters are induced in the small intestine of H-Cpr-null mice. In conclusion, in H-Cpr-null mice, adaptive regulation of alternative detoxification genes in liver and small intestine appear to partially compensate for the loss of microsomal P450 function in liver.

Introduction

Cytochrome P450 (P450) enzymes, especially Cyp3a/Cyp3A family members such as mouse Cyp3a11 and human Cyp3A4, plays a major role in the first-pass metabolism of drugs and other xenobiotics in both the liver and small intestine. All P450 enzymes in the endoplasmic reticulum (microsomes) of the liver receive electrons from a single donor, NADPH-cytochrome P450 oxidoreductase (Cpr, EC 1.6.2.4) (Black et al., 1979; Black and Coon, 1987; Shen et al., 2002). Loss of function of Cpr eliminates the activity of all P450 enzymes in the liver that metabolize drugs and thus is important for first-pass metabolism (Shen et al., 2002). In humans, dysfunctional CPR protein has been linked to disordered steroidogenesis, for example, in people with the Antley-Bixler syndrome (Adachi et al., 2006; Fukami et al., 2006). Whole body Cpr-null mice are embryonically lethal (Shen et al., 2002; Miller et al., 2005), but hepatocyte-specific Cpr knockout (H-Cpr-null) mice breed and develop normally, even though hepatic microsomal P450 activity is ablated in these mice (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003).

Gene microarrays of livers from H-Cpr-null mice have been reported (Gu et al., 2003; Wang et al., 2005; Weng et al., 2005). Even though the hepatic activity of microsomal P450 enzymes is ablated, H-Cpr-null mice show compensatory increased mRNA and protein expression of several hepatic P450s (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003), partially due to activation of the constitutive androstane receptor (CAR) (Weng et al., 2005) possibly by accumulated unsaturated fatty acids (Finn et al., 2009). In addition, although H-Cpr-null mice are fertile and grow normally, they have substantial changes in hepatic fatty acid profiles and enlarged fatty livers (Gu et al., 2003; Gonzalez et al., 2011). Moreover, in H-Cpr-null mice, serum levels of cholesterol, androgens, and triglycerides are markedly reduced (Gu et al., 2003; Henderson et al., 2003; Wang et al., 2005). However, the contributory mechanisms by which H-Cpr-null mice survive from loss of liver microsomal P450 activity are not fully understood. Therefore, we examined 1) the tissue distribution of Cpr in mice and 2) whether alteration of major drug-metabolizing enzymes and transporters in liver, kidney, and small intestine of H-Cpr-null mice occurs to compensate for the decrease in drug oxidation by the liver.

Materials and Methods

All chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies of anti-human nuclear factor erythroid

This work was supported by the National Institutes of Health National Institute of Environmental Health Sciences [Grant ES09649].
dx.doi.org/10.1124/dmd.114.060053.

ABBREVIATIONS: Aldh, aldehyde dehydrogenase; bDNA assay, branched DNA signal amplification assay; CAR, constitutive androstane receptor; Ces, carboxylesterase; Cpr, NADPH-cytochrome P450 oxidoreductase; Eh, epoxide hydrolase; Gst, glutathione S-transferase; Ho-1, heme oxygenase 1; Mate, multidrug and toxin extrusion protein; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Npc1l1, Niemann-pick C1 like 1 transporter; Nqo, NADPH:quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor 2; Oatp, organic anion transporting polypeptide; Oct, organic cation transporter; P450, cytochrome P450; Pept, peptide transporter; PXR, pregnane-X receptor; Sult, sulfotransferase; TBS-T, Tris-buffered saline containing 0.1% Tween-20; Ugt, UDP-glucuronosyltransferase; WT, wild type.

2-related factor 2 (Nrf2) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse CAR1/2 (Santa Cruz Biotechnology), β -actin antibody (Abcam, Cambridge, MA), and goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Sigma-Aldrich) are all commercially available.

Animals and Tissue Collection. Eight-week-old adult male and female C57BL/6 wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Breeding pairs of H-Cpr-null mice (Gu et al., 2003) in the C57BL/6 background were kindly provided by Dr. Xinxin Ding (Wadsworth Center, State University of New York, Albany, NY). All mice were maintained on an automatically timed 12-hour dark/light cycle in an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and were allowed water and rodent chow ad libitum (Teklad; Harlan, Indianapolis, IN).

To determine the tissue distribution of Cpr, 12 tissues—including liver, kidney, lung, stomach, duodenum, jejunum, ileum, colon, heart, brain, testis, and ovary—were collected from WT mice. The placenta was removed from pregnant mice on gestation day 17. The small intestine was longitudinally dissected, rinsed in saline, and divided into three equal-length sections (referred to as duodenum, jejunum, and ileum), before being snap-frozen in liquid nitrogen.

To determine the effect of deletion of Cpr from hepatocytes on the expression of drug processing genes, at approximately 8 weeks of age the liver, kidney, and small intestine from male H-Cpr-null mice and male C57BL/6 WT mice were collected, snap-frozen in liquid nitrogen, and stored in a -80°C freezer.

Total RNA Isolation. Total RNA was isolated with RNA-Bee reagents (Tel-Test, Friendswood, TX) per the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm.

Development of Specific Oligonucleotide Probe Sets for Branched DNA Analysis. For the branched DNA (bDNA) analysis, the gene sequences of interest were accessed from GenBank, and the target sequences were analyzed by ProbeDesigner Software Version 1.0 (Genospectra, Fremont, CA) as described previously elsewhere (Hartley and Klaassen, 2000; Cheng et al., 2005a). The probes were synthesized by Integrated DNA Technologies (Coralville, IA). The probe sets for mouse Cyp1a1, 2b10, 3a11, 4a14, and NADPH:quinone oxidoreductase (Nqo1) (Cheng et al., 2005b), organic anion transporting polypeptides (Oatp) (Cheng et al., 2005a), multidrug resistance-associated proteins (Mrp) (Maher et al., 2005b), organic anion transporters (Oat) (Buist and Klaassen, 2004), concentrative nucleoside transporters (Cnt) (Lu et al., 2004), equilibrative nucleoside transporters (Ent) (Lu et al., 2004), heme oxygenase 1 (Ho-1) (Aleksunes et al., 2005), sodium-phosphate cotransporter (Ntcp), bile salt export pump (Bsep) (Cheng et al., 2007), breast cancer resistance protein (Bcrp) (AbcG2) (Tanaka et al., 2005), Cyp7a1, ATP-binding cassette transporters (Abca1, Abcg5, Abcg8) (Dieter et al., 2004), organic cation transporters (Oct) (Alnouti et al., 2006), peptide transporters (Pept) (Lu and Klaassen, 2006), pregnane-X receptor (PXR), multidrug resistance proteins (Mdr), organic solute transporters α and β (Ost α , Ost β), Niemann-pick C1 like 1 transporter (Npc1l1), sodium-phosphate cotransporter 1 (Npt1), apical sodium dependent bile acid transporter (Asbt) (Cheng and Klaassen, 2006), sulfotransferases (Sult) (Alnouti and Klaassen, 2006), glutathione S-transferases (Gst) (Knight et al., 2007), and UDP-glucuronosyltransferases (Ugt) (Buckley and Klaassen, 2007) have been reported. Probe sequences of mouse Cpr are shown in Table 1.

bDNA Assay. Reagents required for RNA analysis (i.e., lysis buffer, amplifier/label probe buffer, and substrate solution) were supplied in the Quantigene bDNA signal amplification kit (Panomics, Fremont, CA). Pooled total RNA samples from five mice were used to determine the alteration of gene expression in wild-type and H-Cpr-null mice. Apparent altered levels of gene mRNA expression were confirmed by quantifying individual mouse total RNA samples. Data are expressed as relative mRNA expression of individual gene in the same amount of total RNA, specifically presented as relative light units (RLUs) per 10 μg total RNA.

Total and Direct Bilirubin Quantification in Mouse Serum. After collection, blood was allowed to coagulate and was centrifuged at 6000g for 15 minutes. The resulting supernatant (serum) was collected for analysis. Serum concentrations of direct (or conjugated) and total bilirubin were determined according to the manufacturer's protocol (Total or Direct Bilirubin Reagent Set Kit; Pointe Scientific, Canton, MI).

Nuclear Protein Extraction. Nuclear proteins from mouse livers were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions.

TABLE 1

Oligonucleotide probes generated for analysis of mouse Cpr mRNA expression by Quantigene branched DNA signal amplification assay

Cpr (NM_008898) ^a	
CE ^b	ccctcttgagcaggtgctgaaTTTTTctcttgaaagaagt
CE	atctttggccatatttcgagcTTTTTctcttgaaagaagt
CE	ggacgtgattacaggagcgTTTTTctcttgaaagaagt
LE	catagaccttggtggcctgTTTTTaggcataggaccgtgtct
LE	ggcaccacctctggtgatcaTTTTTaggcataggaccgtgtct
LE	atccccgcagacatagatgtgTTTTTaggcataggaccgtgtct
LE	cgatgtcatagaatgtgtctgacTTTTTaggcataggaccgtgtct
LE	gtcatgagcttctaaccatagtcacTTTTTaggcataggaccgtgtct
LE	gcgagtagcggcccttgTTTTTaggcataggaccgtgtct
LE	cagctcctagctccatcacatccaTTTTTaggcataggaccgtgtct
LE	ggcaggaaaccacagaggtgTTTTTaggcataggaccgtgtct
BL	gtctccacaggtgctcttgt
BL	ggcccaactcggcca
BL	agcctgggtgtgtccatg
BL	agggtgtggggggcgg
BL	gaggtcgcgagaagaagttaa

CE, capture extender; LE, label extender; BL, blocker.

^aGenBank accession number for Cpr is given in parenthesis.

^bThe type of function of each bDNA oligonucleotide probe.

Western Blots. Protein samples mixed with sample loading buffer (50 μg cytoplasmic protein/lane or 75 μg nuclear protein/lane) were loaded after heating onto a 10% (for Nrf2 protein) or 4–20% (for CAR protein) sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins in the gel were electrotransferred to a nitrocellulose membrane for 4.5 hours at 34 V at room temperature. Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Blots were then incubated 4 hours with each antibody at 4°C . Polyclonal antibodies of anti-human Nrf2 and anti-mouse CAR1/2 were diluted (1:200) in 2.5% nonfat dry milk in TBS-T buffer. β -Actin protein was used as loading control using a β -actin antibody. After thorough washing (three 30-minute washes with excess TBS-T), blots were incubated with goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:5000 dilution with 2.5% nonfat milk in TBS-T) for 1 hour. Blots were washed again. Immunoreactive bands were detected with an enhanced chemical luminescence kit (Pierce Biotechnology). Protein bands of interest were visualized by exposure to Fuji Medical X-ray film (Fujifilm Medical Systems USA, Stamford, CT). Protein band intensities on the film were quantified with Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis. When individual RNA samples were used, data were expressed as mean $\pm$ S.E.M. Data from multiple groups were analyzed by one-way

Fig. 1. Messenger RNA expression of Cpr in mouse tissues. Total RNA from both adult male and female C57BL/6 mouse tissues ($n = 5/\text{gender}$) was analyzed by the bDNA assay for expression of Cpr mRNA. Data are presented as mean $\pm$ S.E.M. Asterisks indicate statistically significant differences between male and female mice ($P < 0.05$).

Fig. 2. Alteration of mRNA expression of phase I drug metabolizing enzymes in livers of H-Cpr-null mice. Pooled RNA samples from livers of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of hepatic mRNA levels of one gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was further determined by using individual RNA samples ($n = 5/\text{group}$). The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in WT mice per 10 μg total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5/\text{group}$). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

analysis of variance, followed by Duncan's post-hoc test. The differences between male and female mice as well as between WT and H-Cpr-null mice were analyzed by Student's t test. $P < 0.05$ was considered statistically significant.

Results

Tissue Distribution of Mouse Cpr. Messenger RNA expression of mouse Cpr was quantified in 13 major tissues (Fig. 1). Expression of Cpr mRNA was detectable in all tissues examined, with the highest levels in lung, followed by kidney, placenta, liver, ovary, and gastrointestinal tract, and low in heart, brain, and testes. A gender difference in Cpr mRNA expression was observed in mouse liver, lung, stomach, and duodenum, with higher levels in females.

Alteration of Major Phase I and II Drug-Metabolizing Enzymes in Livers of H-Cpr-Null Mice. The activity of a majority of microsomal P450 enzymes is completely abolished in the livers of H-Cpr-null mice (Wang et al., 2005; Weng et al., 2005; Wu et al., 2003). However, as shown in Fig. 2, the mRNA of some P450s, such as Cyp2b10, 3a11 and 4a14, which are hallmark target genes of CAR, PXR, and peroxisome proliferator-activated receptor α (PPAR α) signaling, respectively, are markedly increased in livers of H-Cpr-null mice. In contrast, the aryl hydrocarbon receptor (AhR) prototypical target gene Cyp1a1 is not altered.

The regulation of some other major phase I enzymes in the livers of H-Cpr-null mice is also shown in Fig. 2. Specifically, mRNA levels of three aldehyde dehydrogenases (Aldh1a1, 1a7, and 3a2), three carboxylesterases (Ces1b2, 2a6, and 2a12), and three Nrf2-target genes (heme oxygenase-1 [Ho-1], NADPH:quinone oxidoreductase [Nqo-1], and epoxide hydrolase [EH]) increased in livers of H-Cpr-null mice.

As shown in Fig. 3, mRNA expression of some phase II enzymes, such as UDP-glucuronosyltransferases (Ugt1a1, 1a7, 1a9, 2b34, and 2b35), glutathione S -transferases (Gsta1/2, m3, m4, m6, t1, and t3), and sulfotransferase (Sult1a1 and 1d1), were increased in the livers of H-Cpr-null mice. In contrast, mRNA expression of Gstp1/2 was down-regulated in the H-Cpr-null mice.

Alteration of Hepatic Uptake and Efflux Transporters in H-Cpr-Null Mice. Many compounds must be transported into hepatocytes by uptake transporters before being metabolized by phase I and II enzymes or becoming pharmacologically effective, such as the statins (Nishizato et al., 2003). Messenger RNA expression of the uptake transporters of nucleosides and nucleotides (equilibrative nucleoside transporter 1, Ent1), organic anions (Oatp1b2, 2a1, and 2b1), and organic cations (Oct1) were not altered in livers of the H-Cpr-null mice (Fig. 4). In contrast, Oatp1a4 and Oct3 mRNA expression increased but organic anion

Fig. 3. Alteration of mRNA expression of phase II drug metabolizing enzymes in livers of H-Cpr-null mice. Pooled RNA samples from livers of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of hepatic mRNA levels of one gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was evaluated by using individual RNA samples ($n = 5/\text{group}$). The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in WT mice per 10 μg total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5/\text{group}$). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

transporter 2 (Oat2) and Oatp1a1 decreased in the livers of the H-Cpr-null mice (Fig. 4).

In the liver, parent compounds and/or their metabolites are either excreted into bile by canalicular efflux transporters or transported back into blood by sinusoidal efflux transporters. As depicted in Fig. 5, the majority of efflux transporters are not altered in the livers of the H-Cpr-null mice. However, the canalicular transporters multidrug and toxin extrusion protein 1 (MATE1) and multidrug resistance protein 1a (MDR1a) as well as the sinusoidal efflux transporters Mrp3 and 4 were increased in the livers of H-Cpr-null mice.

Regulation of Total and Direct Bilirubin in Serum of WT and H-Cpr-Null Mice. After being taken into the liver, bilirubin is first converted to water-soluble glucuronide conjugates via UDP-glucuronyltransferases (UGT) in the presence of UDP-glucuronic acid. As shown in Fig. 3, UGT expression is higher in the livers of H-Cpr-null than WT mice. We further evaluated the physiologic consequences of alteration of these genes by determining the concentration of total bilirubin and bilirubin glucuronide in serum of H-Cpr-null and WT mice.

As shown in Fig. 6, the concentration of bilirubin glucuronide was doubled in the serum of the H-Cpr-null mice compared with the WT mice. In contrast, the serum concentrations of total bilirubin were similar between the H-Cpr-null and WT mice.

Regulation of Nuclear CAR and Nrf2 Protein in Livers of WT and H-Cpr-Null Mice. Activation of the CAR and Nrf2 signaling pathways is accompanied and achieved by enhanced nuclear translocation of CAR and Nrf2 protein. Therefore, we determined the nuclear levels of CAR and Nrf2 protein in the livers of WT and H-Cpr-null mice (Fig. 7). Nuclear levels of CAR protein were 8-fold higher in the livers of H-Cpr-null than WT mice. Nuclear levels of Nrf2 protein were 2.1-fold higher in the livers of H-Cpr-null than WT mice. Consequently, the signaling pathways of both CAR and Nrf2 are enhanced in the livers of H-Cpr-null mice.

Alteration of mRNA Expression of Drug-Metabolizing Enzymes and Transporters in Kidneys and Small Intestine of H-Cpr-Null Mice. In addition to the liver, the kidney and small intestine also play

Fig. 4. Alteration of mRNA expression of liver uptake transporters in livers of H-Cpr-null mice. Pooled RNA samples from livers of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of hepatic mRNA levels of one transporter gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was evaluated using individual RNA samples ($n = 5/\text{group}$). The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in WT mice per 10 μg total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5/\text{group}$). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

Fig. 5. Alteration of mRNA expression of liver efflux transporters in livers of H-Cpr-null mice. Pooled RNA samples from livers of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of hepatic mRNA levels of one transporter gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was evaluated in individual RNA samples ($n = 5/\text{group}$). The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in WT mice per 10 μg total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5/\text{group}$). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

important roles in drug disposition. Thus, we next determined whether the knockout of Cpr in hepatocytes of mice altered the expression of drug processing genes in other tissues such as the kidney and small intestine.

The mRNA expression of none of the drug-metabolizing enzymes or transporters in kidneys was increased in the H-Cpr-null mice. In contrast to the liver, the mRNA of Cyp3a11 and 4a14 was down-regulated in the kidneys of H-Cpr-null mice (Fig. 8). However, because both Cyp3a11 and 4a14 are lowly expressed in the kidneys, the down-regulation of these two Cyps is probably of little or no physiologic significance. The mRNA

Fig. 6. Quantification of total and direct bilirubin levels in sera of WT and H-Cpr-null mice. Total and direct (or conjugated) bilirubin levels in mouse serum were determined by a commercially available kit (Total or Direct Bilirubin Reagent Set Kit; Pointe Scientific). The data are reported as mean $\pm$ S.E.M. ($n = 5/\text{group}$). Asterisks indicate statistically significant differences between WT and H-Cpr-null mice ($P < 0.05$).

Fig. 7. Nuclear levels of CAR and Nrf2 protein in livers of WT and H-Cpr-null mice. (A) Protein levels of nuclear CAR and β -actin in mouse liver were analyzed by Western blotting (top panel); the protein level of CAR is expressed as ratio of CAR to β -actin protein levels per 75 μ g nuclear protein (bottom panel). (B) Protein levels of nuclear Nrf2 and β -actin in mouse liver were analyzed by Western blotting (top panel); the protein level of Nrf2 is expressed as ratio of Nrf2 to β -actin protein levels per 75 μ g nuclear protein (bottom panel). Data are presented as mean $\pm$ S.E.M. Asterisks indicate statistically significant differences between WT and H-Cpr-null mice ($P < 0.05$).

of Oatp1a1, which is responsible for reabsorption of substrates such as perfluorooctanoate from the filtrate (Yang et al., 2009), was also decreased in the kidneys of H-Cpr-null mice.

Alterations of mRNA expression of drug-metabolizing enzymes and transporters in the small intestine of H-Cpr-null mice are depicted in Fig. 9. The PXR-target gene Cyp3a11 was increased 440% in the small intestine of H-Cpr-null mice. In addition, the mRNAs of peptide transporter (Pept) 1 (300%), cholesterol uptake transporter Npc1l1 (280%), and efflux transporter Mdr1a (430%) were all increased in the small intestine of H-Cpr-null mice.

Discussion

Our studies have shown that in response to ablation of P450 enzyme activity in the livers of H-Cpr-null mice there is compensatory increased expression of some major phase I and II drug-metabolizing enzymes and transporters in the liver and small intestine, as summarized in Fig. 10. In addition, H-Cpr-null mice probably have increased absorption of cholesterol (Npc1l1) and nutrients (Pept1) from the intestine, as the mRNA of these two transporters is also increased in the small intestine.

Similar to previous reports (Wu et al., 2003; Zhang et al., 2009), Cpr mRNA is detected in multiple investigated mouse tissues, including the liver, lung, kidney, small intestine, and placenta. Interestingly, there is

a marked gender difference in the expression of Cpr in mouse livers, with females having more than twice the expression of males. In contrast, in the rat liver, Cpr expression and activity is apparently higher in males than females (Ram and Waxman, 1992). However, the underlying mechanism responsible for female-dominant Cpr expression in mouse liver is not known, which merits further investigation.

In the liver of H-Cpr-null mice, certain drug-metabolizing enzymes, such as Cyp1a1/2, Cyp2b10, Aldh1a7, Ces2a6, Gsta2, and Ho-1, as well as uptake transporters such as Oatp1a4, are up-regulated, similar to what has been previously described elsewhere (Wang et al., 2005; Weng et al., 2005). In addition, we also showed that several efflux transporters, including Mrp3, Mrp4, Mdr1, and Mdr1a, are up-regulated in the liver of H-Cpr-null mice. The induction of certain specific drug-processing genes indicates characteristic activation of specific signaling pathways. Cyp2b10 (about 17-fold) and Cyp3a11 (about 3-fold), which are characteristic target genes of CAR and PXR, are increased in the livers of H-Cpr-null mice (Fig. 2) (Gu et al., 2003; Weng et al., 2005), suggesting activation of CAR and PXR in the livers of H-Cpr-null mice. Nqo1, Ho-1, and epoxide hydrolase (Eh) are well-characterized target genes of the transcription factor Nrf2 (Kwak et al., 2001; Owuor and Kong, 2002; Chen et al., 2004; Cheng et al., 2005b; Slitt et al., 2006), and are increased in livers of H-Cpr-null mice (Fig. 2), indicating activation of the Nrf2 signaling

Fig. 8. Regulation of mRNA expression of drug-processing genes in the kidneys of H-Cpr-null mice. Pooled RNA samples from kidneys of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of kidney mRNA levels of one gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was evaluated using individual RNA samples ($n = 5$ /group). The data are represented as the ratio of kidney mRNA expression in H-Cpr-null mice to that in WT mice per 10 μ g total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5$ /group). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

Fig. 9. Regulation of mRNA expression of drug-processing genes in small intestine of H-Cpr-null mice. Pooled RNA samples from small intestines of five WT and five H-Cpr-null mice were initially analyzed for mRNA expression of each gene by bDNA assay. When the ratio of intestinal mRNA levels of one gene in H-Cpr-null mice to that in WT mice was greater than 1.5 or less than 0.75, the mRNA expression of that gene was evaluated in individual RNA samples ($n = 5/\text{group}$). The data are represented as the ratio of intestinal mRNA expression in H-Cpr-null mice to that in WT mice per 10 μg total RNA. Gray bars indicate the data from pooled RNA samples. Striated bars indicate the data from individual RNA samples ($n = 5/\text{group}$). The asterisk indicates statistically significant differences of mRNA expression between H-Cpr-null mice and WT mice ($P < 0.05$).

pathway. Activation of CAR, PXR, and Nrf2 alters the expression of a large battery of genes. For instance, activation of CAR leads to up-regulation of Mrp3, Mrp4 (Slitt et al., 2006), Gstm3, Gstt1 (Knight et al., 2008), Ces2a6, and Ces2a12, but down-regulation of Oatp1a1 (Cheng et al., 2005b). PXR activation leads to increased mRNA expression of Oatp1a4 and 1a6 (Cheng et al., 2005b), Mdr1a (Cheng and Klaassen, 2006), Mrp3 (Maher et al., 2005a), Gstm3 (Knight et al., 2008), Ugt1a1 (Chen et al., 2003), and Ces2a12. Nrf2 activation might explain the up-regulation of Mrp3 and 4 (Maher et al., 2007) as well as Gsta1/2, m3, and m4 (Hayes et al., 2000) in the livers of H-Cpr-null mice (Fig. 10).

Activation of CAR and Nrf2 is featured by enhanced nuclear translocation or increased nuclear levels of CAR and Nrf2 protein. In our present study, CAR protein was increased in nuclei of livers of H-Cpr-null mice (Fig. 7), suggesting that CAR is apparently activated in the livers of H-Cpr-null mice. Consistent with up-regulation of Nqo1 and Ho1, Nrf2 protein is also increased in the nuclei of livers of H-Cpr-null mice. These protein data further demonstrate that CAR and Nrf2 are activated in livers of H-Cpr-null mice. In addition, we have demonstrated that depletion of Cpr in mouse hepatocytes leads to increased level of lithocholic acid (LCA) in mouse serum and liver (Cheng et al., 2014). Lithocholic acid is an activator of PXR nuclear receptor (Staudinger et al., 2001).

In accordance with the higher expression of Ugt and Mrp3 in the livers of H-Cpr-null mice, bilirubin glucuronide was increased in the serum of H-Cpr-null mice (Fig. 6). In the liver, unconjugated bilirubin is conjugated with UDP-glucuronic acid by Ugt enzymes, especially Ugt1A1, making it water-soluble and ready for excretion. Much of conjugated bilirubin is excreted into bile through Mrp2. However, a small portion of conjugated bilirubin is transported back into blood through Mrp3 (Belinsky et al., 2005). In our present study, Ugt1A1 and Mrp3 were much higher in the livers of H-Cpr-null than WT mice, indicating that bilirubin glucuronidation is enhanced and more conjugated bilirubin is excreted into blood.

Cholesterol is an essential precursor of bile-acid biosynthesis and steroidogenesis. Whole-body cholesterol homeostasis is maintained through de novo hepatic and extrahepatic biosynthesis, dietary cholesterol absorption, and biliary excretion. In H-Cpr-null mice, de novo biosynthesis of cholesterol in the liver is impaired (Gu et al., 2003; Henderson et al., 2003; Wang et al., 2005) because of loss of function of lanosterol demethylase (CYP51a1) (Li and Porter, 2007). Therefore, absorption of

dietary cholesterol from the intestine and extrahepatic biosynthesis of cholesterol are important to maintain whole-body cholesterol homeostasis in H-Cpr-null mice. In the intestine, Npc1l1 is critical for the apical absorption of exogenous cholesterol from the intestinal lumen into enterocytes (Altmann et al., 2004). As shown in Fig. 9, intestinal Npc1l1 mRNA is 2.5-fold higher in H-Cpr-null mice than in WT mice. Therefore, a compensatory increase of cholesterol absorption from the small intestine probably helps to maintain cholesterol homeostasis in the H-Cpr-null mice. In addition, to meet the demands of increased expression of drug-processing genes, the peptide transporter (Pept1), a di- and tri-peptide uptake transporter in the intestine, is also markedly increased to enhance the absorption of amino acids and peptides in H-Cpr-null mice (Fig. 9). However, the mechanism responsible for up-regulation of Npc1l1 and Pept1 is not known, which merits further investigation.

Fig. 10. Proposed regulatory mechanisms associated with alterations of gene expression observed in liver and small intestine of H-Cpr-null mice. ↑ means up-regulation of target gene expression. ↓ means down-regulation of target gene expression.

In addition to absorption of compounds into the body, many transporters are involved in the elimination of xenobiotics from the body or to reduce the drug burden of a tissue. In H-Cpr-null mice, up-regulation of Mdr1a may reduce the bioavailability of some xenobiotics from the small intestine. Similarly, in livers of H-Cpr-null mice, up-regulation of efflux transporters Mrp3, 4, and Mdr1 probably reduce the liver burden of bile acids and xenobiotics by enhancing the excretion of compounds from the liver (Fig. 5).

In H-Cpr-null mice, Cyp3a11 and Mdr1a are markedly up-regulated in the small intestine (Fig. 9), indicating intestinal first-pass metabolism is enhanced. First-pass metabolism occurs in both liver and small intestine in an attempt to reduce the systemic bioavailability of a drug. Both P450 enzymes and efflux transporters in the liver and intestine play important roles in first-pass metabolism. A majority of P450 enzymatic activity is ablated in the livers of H-Cpr-null mice (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003), leading to decreased oxidation of drugs in these mice. Up-regulation of Cyp3a11 and Mdr1a in the small intestine of H-Cpr-null mice indicates that in response to compromised P450 enzyme activity in livers of H-Cpr-null mice, first-pass metabolism in the small intestine is enhanced. As discussed earlier, the nuclear receptor PXR is activated in H-Cpr-null mice, which can lead to increased expression of Cyp3a11 and Mdr1a in the small intestine (Cheng and Klaassen, 2006). In the small intestine of H-Cpr-null mice, even though Cyp3A11 is increased, Cpr expression is not altered (Fig. 9). This indicates that mRNA does not necessarily indicate the amount of protein or activity.

Taken together, our present studies offer insight into the adaptive regulatory mechanisms in response to Cpr deficiency in the livers of mice. In the livers of H-Cpr-null mice, the transcription factors CAR, PXR, and Nrf2 are apparently activated to up-regulate alternative drug-processing genes. In addition, increased mRNA levels of Cyp3a11 and Mdr1a indicate that first-pass metabolism is likely enhanced in the small intestine of H-Cpr-null mice. The present study also supports the hypothesis that compensatory mechanisms aim to restore and maintain homeostasis in the H-Cpr-null mice.

Acknowledgments

The authors thank Dr. Xinxin Ding (Wadsworth Center, State University of New York at Albany) for providing the H-Cpr-null mouse model.

Authorship Contributions

Participated in research design: Cheng, Klaassen.

Conducted experiments: Cheng.

Performed data analysis: Cheng.

Wrote or contributed to the writing of the manuscript: Cheng, Gu, Klaassen.

References

- Adachi M, Asakura Y, Matsuo M, Yamamoto T, Hanaki K, and Arlt W (2006) POR R457H is a global founder mutation causing Antley-Bixler syndrome with autosomal recessive trait. *Am J Med Genet A* **140**:633–635.
- Aleksunes LM, Slitt AM, Cherrington NJ, Thibodeau MS, Klaassen CD, and Manautou JE (2005) Differential expression of mouse hepatic transporter genes in response to acetaminophen and carbon tetrachloride. *Toxicol Sci* **83**:44–52.
- Alnouti Y and Klaassen CD (2006) Tissue distribution and ontogeny of sulfotransferase enzymes in mice. *Toxicol Sci* **93**:242–255.
- Alnouti Y, Petrick JS, and Klaassen CD (2006) Tissue distribution and ontogeny of organic cation transporters in mice. *Drug Metab Dispos* **34**:477–482.
- Altmann SW, Davis HR, Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, and Zeng M, et al. (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**:1201–1204.
- Belinsky MG, Dawson PA, Shchavaleva I, Bain LJ, Wang R, Ling V, Chen ZS, Grinberg A, Westphal H, and Klein-Szanto A, et al. (2005) Analysis of the in vivo functions of Mrp3. *Mol Pharmacol* **68**:160–168.
- Black SD and Coon MJ (1987) P-450 cytochromes: structure and function. *Adv Enzymol Relat Areas Mol Biol* **60**:35–87.
- Black SD, French JS, Williams CH, Jr, and Coon MJ (1979) Role of a hydrophobic polypeptide in the N-terminal region of NADPH-cytochrome P-450 reductase in complex formation with P-450LM. *Biochem Biophys Res Commun* **91**:1528–1535.
- Buckley DB and Klaassen CD (2007) Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. *Drug Metab Dispos* **35**:121–127.
- Buist SC and Klaassen CD (2004) Rat and mouse differences in gender-predominant expression of organic anion transporter (Oat1–3; Slc22a6–8) mRNA levels. *Drug Metab Dispos* **32**:620–625.
- Chen C, Pung D, Leong V, Hebbur V, Shen G, Nair S, Li W, and Kong AN (2004) Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Radic Biol Med* **37**:1578–1590.
- Chen C, Staudinger JL, and Klaassen CD (2003) Nuclear receptor, pregnane X receptor, is required for induction of UDP-glucuronosyltransferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug Metab Dispos* **31**:908–915.
- Cheng X, Buckley D, and Klaassen CD (2007) Regulation of hepatic bile acid transporters Ntcp and Bsep expression. *Biochem Pharmacol* **74**:1665–1676.
- Cheng X and Klaassen CD (2006) Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. *Drug Metab Dispos* **34**:1863–1867.
- Cheng X, Maher J, Chen C, and Klaassen CD (2005a) Tissue distribution and ontogeny of mouse organic anion transporting polypeptides (Oatps). *Drug Metab Dispos* **33**:1062–1073.
- Cheng X, Maher J, Dieter MZ, and Klaassen CD (2005b) Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug Metab Dispos* **33**:1276–1282.
- Cheng X, Zhang Y, and Klaassen CD (2014) Decreased bile-acid synthesis in livers of hepatocyte-conditional NADPH-cytochrome-p450 reductase-null mice results in increased bile acids in serum. *J Pharmacol Exp Ther* **351**:1–9.
- Dieter MZ, Maher JM, Cheng X, and Klaassen CD (2004) Expression and regulation of the sterol half-transporter genes ABCG5 and ABCG8 in rats. *Comp Biochem Physiol C Toxicol Pharmacol* **139**:209–218.
- Finn RD, Henderson CJ, Scott CL, and Wolf CR (2009) Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway. *Biochem J* **417**:43–54.
- Fukami M, Hasegawa T, Horikawa R, Ohashi T, Nishimura G, Homma K, and Ogata T (2006) Cytochrome P450 oxidoreductase deficiency in three patients initially regarded as having 21-hydroxylase deficiency and/or aromatase deficiency: diagnostic value of urine steroid hormone analysis. *Pediatr Res* **59**:276–280.
- Gonzalez M, Sealls W, Jesch ED, Brosnan MJ, Ladunga I, Ding X, Black PN, and DiRusso CC (2011) Defining a relationship between dietary fatty acids and the cytochrome P450 system in a mouse model of fatty liver disease. *Physiol Genomics* **43**:121–135.
- Gu J, Weng Y, Zhang QY, Cui H, Behr M, Wu L, Yang W, Zhang L, and Ding X (2003) Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem* **278**:25895–25901.
- Hartley DP and Klaassen CD (2000) Detection of chemical-induced differential expression of rat hepatic cytochrome P450 mRNA transcripts using branched DNA signal amplification technology. *Drug Metab Dispos* **28**:608–616.
- Hayes JD, Chanas SA, Henderson CJ, McMahon M, Sun C, Moffat GJ, Wolf CR, and Yamamoto M (2000) The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem Soc Trans* **28**:33–41.
- Henderson CJ, Otto DM, Carrie D, Magnuson MA, McLaren AW, Rosenwell I, and Wolf CR (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *J Biol Chem* **278**:13480–13486.
- Knight TR, Choudhuri S, and Klaassen CD (2007) Constitutive mRNA expression of various glutathione S-transferase isoforms in different tissues of mice. *Toxicol Sci* **100**:513–524.
- Knight TR, Choudhuri S, and Klaassen CD (2008) Induction of hepatic glutathione S-transferases in male mice by prototypes of various classes of microsomal enzyme inducers. *Toxicol Sci* **106**:329–338.
- Kwak MK, Itoh K, Yamamoto M, Sutter TR, and Kensler TW (2001) Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1,2-dimethiole-3-thione. *Mol Med* **7**:135–145.
- Li L and Porter TD (2007) Hepatic cytochrome P450 reductase-null mice reveal a second microsomal reductase for squalene monooxygenase. *Arch Biochem Biophys* **461**:76–84.
- Lu H, Chen C, and Klaassen C (2004) Tissue distribution of concentrative and equilibrative nucleoside transporters in male and female rats and mice. *Drug Metab Dispos* **32**:1455–1461.
- Lu H and Klaassen C (2006) Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. *Peptides* **27**:850–857.
- Maher JM, Cheng X, Slitt AL, Dieter MZ, and Klaassen CD (2005a) Induction of the multidrug resistance-associated protein family of transporters by chemical activators of receptor-mediated pathways in mouse liver. *Drug Metab Dispos* **33**:956–962.
- Maher JM, Dieter MZ, Aleksunes LM, Slitt AL, Guo G, Tanaka Y, Scheffer GL, Chan JY, Manautou JE, and Chen Y, et al. (2007) Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. *Hepatology* **46**:1597–1610.
- Maher JM, Slitt AL, Cherrington NJ, Cheng X, and Klaassen CD (2005b) Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. *Drug Metab Dispos* **33**:947–955.
- Miller WL, Huang N, Pandey AV, Flück CE, and Agrawal V (2005) P450 oxidoreductase deficiency: a new disorder of steroidogenesis. *Ann N Y Acad Sci* **1061**:100–108.
- Nishizato Y, Ieiri I, Suzuki H, Kimura M, Kawabata K, Hirota T, Takane H, Irie S, Kusuhara H, and Urasaki Y, et al. (2003) Polymorphisms of OATP-C (SLC21A6) and OAT3 (SLC22A8) genes: consequences for pravastatin pharmacokinetics. *Clin Pharmacol Ther* **73**:554–565.
- Owior ED and Kong AN (2002) Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol* **64**:765–770.
- Ram PA and Waxman DJ (1992) Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues. Regulation by multiple mechanisms. *J Biol Chem* **267**:3294–3301.
- Shen AL, O'Leary KA, and Kasper CB (2002) Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem* **277**:6536–6541.
- Slitt AL, Cherrington NJ, Dieter MZ, Aleksunes LM, Scheffer GL, Huang W, Moore DD, and Klaassen CD (2006) trans-Stilbene oxide induces expression of genes involved in metabolism and transport in mouse liver via CAR and Nrf2 transcription factors. *Mol Pharmacol* **69**:1554–1563.
- Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, and Reinhard J, et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* **98**:3369–3374.

- Tanaka Y, Slitt AL, Leazer TM, Maher JM, and Klaassen CD (2005) Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice. *Biochem Biophys Res Commun* **326**:181–187.
- Wang XJ, Chamberlain M, Vassieva O, Henderson CJ, and Wolf CR (2005) Relationship between hepatic phenotype and changes in gene expression in cytochrome P450 reductase (POR) null mice. *Biochem J* **388**:857–867.
- Weng Y, DiRusso CC, Reilly AA, Black PN, and Ding X (2005) Hepatic gene expression changes in mouse models with liver-specific deletion or global suppression of the NADPH-cytochrome P450 reductase gene. Mechanistic implications for the regulation of microsomal cytochrome P450 and the fatty liver phenotype. *J Biol Chem* **280**:31686–31698.
- Wu L, Gu J, Weng Y, Kluetzman K, Swiatek P, Behr M, Zhang QY, Zhuo X, Xie Q, and Ding X (2003) Conditional knockout of the mouse NADPH-cytochrome p450 reductase gene. *Genesis* **36**:177–181.
- Yang CH, Glover KP, and Han X (2009) Organic anion transporting polypeptide (Oatp) 1a1-mediated perfluorooctanoate transport and evidence for a renal reabsorption mechanism of Oatp1a1 in renal elimination of perfluorocarboxylates in rats. *Toxicol Lett* **190**:163–171.
- Zhang QY, Fang C, Zhang J, Dunbar D, Kaminsky L, and Ding X (2009) An intestinal epithelium-specific cytochrome P450 (P450) reductase-knockout mouse model: direct evidence for a role of intestinal p450s in first-pass clearance of oral nifedipine. *Drug Metab Dispos* **37**:651–657.

Address correspondence to: Dr. Curtis D. Klaassen, 2617 West 112th Street, Leawood, KS 66211. E-mail: curtisklaassenphd@gmail.com