JPET Fast Forward. Published on July 17, 2014 as DOI: 10.1124/jpet.114.216796
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Title: Decreased Bile-Acid Synthesis in Livers of Hepatocyte-Conditional NADPH-Cytochrome-p450 Reductase-Null Mice Results in Increased Bile Acids in Serum

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JPET Fast Forward. Published on July 17, 2014 as DOI: 10.1124/jpet.114.216796
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Running title: Alteration of bile-acid profile in H-Cpr-null mice

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Number of text pages: 26

Number of Tables: 1

Number of Figures: 9

Number of References: 38

The number of words in the Abstract: 245

The number of words in the Introduction: 1008

The number of words in the Discussion: 1444

List of nonstandard abbreviations: Abc, ATP-binding cassette; Asbt/Ibat, apical sodium-dependent bile acid transporter/ ileal bile acid transporter; BA, bile acid; BAL, bile acid CoA ligase; BAT, bile acid CoA: amino acid *N*-acyltransferase; bDNA assay, branched DNA signal amplification assay; Bsep, bile salt export pump; CA, cholic acid; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; Cpr, NAD(P)H-cytochrome p450 reductase; Cyp, cytochrome p450 enzyme; Cyp7a1, cholesterol 7α-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; Cyp27a1, sterol 27-hydroxylase; DCA, deoxycholic acid; Fgf15, fibroblast growth factor 15; FXR, farnesoid X receptor; Ibabp, intestinal bile-acid binding protein; LCA, lithocholic acid; Lrh, liver receptor homolog; MCA, muricholic acid; Mrp, multidrug resistance-associated protein; Ntcp, Na^+/taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Ost, organic solute transporter; PXR, pregnane-X receptor; Shp, small heterodimer partner; StAR, steroidogenic acute regulatory protein; TGR5, G protein-coupled bile acid receptor; UDCA, ursodeoxycholic acid; WT, wild-type.

Abstract:

NADPH-cytochrome p450 reductase (Cpr) is essential for the function of microsomal cytochrome p450 mono-oxygenases (Cyp), including those Cyps involved in bile acid (BA) synthesis. Mice with hepatocyte-specific deletion of NADPH-cytochrome p450 reductase (H-Cpr-null) have been established to understand the *in vivo* function of hepatic Cyps on the metabolism of xenobiotics and endogenous compounds. However, the impact of hepatic Cpr on BA homeostasis is not clear. The present study revealed that H-Cpr-null mice had a 60% decrease of total BA concentration in liver, whereas the total BA concentration in serum was almost doubled. The decreased level of cholic acid (CA) in both serum and livers of H-Cpr-null mice is likely due to diminished enzyme activity of Cyp8b1 that is essential for CA biosynthesis. Feedback mechanisms responsible for the reduced liver BA concentrations and/or increased serum BA concentrations in H-Cpr-null mice included: 1) enhanced alternative BA synthesis pathway, as evidenced by the facts that classical BA synthesis is diminished but chenodeoxycholic acid (CDCA) still increases in both serum and livers of H-Cpr-null mice; 2) inhibition of FXR activation, which increased the mRNA of Cyp7a1 and 8b1; 3) induction of intestinal BA transporters to facilitate BA absorption from intestine to the circulation; 4) induction of hepatic Mrp transporters to increase BA efflux from liver to blood; and 5) increased generation of secondary BAs. In summary, the present study reveals an important contribution of the alternative BA synthesis pathway and BA transporters in regulating BA concentrations in H-Cpr-null mice.

Introduction

Cytochrome p450 enzymes (Cyps) not only have a major impact on the metabolism of drugs and nutrients. But they also regulate the homeostasis of many endogenous compounds, such as cholesterol and bile acids (BAs). All microsomal Cyps receive electrons from a single donor, namely NADPH-cytochrome p450 reductase (Cpr, EC 1.6.2.4), a house-keeping gene widely expressed in various tissues with the highest level in liver (Black and Coon, 1987; Black et al., 1979; Shen et al., 2002). Whole-body deletion of the Cpr gene causes embryonic lethality in mice, whereas mice with hepatocyte-specific deletion of Cpr (H-Cpr-null) can breed and develop normally, even though their hepatic microsomal Cyp activity is ablated (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003). Therefore, H-Cpr-null mice have been characterized extensively and used for studying the *in vivo* function of Cyps in drug metabolism. It was reported that H-Cpr-null mice have a 90% reduction in bile volume in the gallbladder, accompanied with a 65% reduction of serum cholesterol (Henderson et al., 2003). However, the impact of hepatocyte-specific Cpr-depletion on individual BA homeostasis in serum and livers of mice is still not clear.

Bile formation is a major function of liver. More than 90% of total cholesterol in the body is excreted after conversion into BAs. Detergent properties of BAs not only make them critical for absorption of lipids and fat-soluble vitamins from the intestine, but also contribute to their cytotoxicity during various liver diseases. Recently, BAs also have emerged as signaling molecules by serving as ligands of the farnesoid X receptor (FXR) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999), pregnane X receptor (PXR) (Staudinger et al., 2001), and a G-protein-coupled BA receptor (TGR5), activation of which in turn regulates the homeostasis of xenobiotics and endogenous compounds, such as glucose and lipids (Kawamata et al., 2003). Therefore, the synthesis, transport and metabolism of BAs have to be tightly regulated to ensure that a sufficient amount of cholesterol is metabolized to provide adequate emulsification of lipids in the intestine, and meanwhile prevent cytotoxic accumulation of BAs in the liver.

BA biosynthesis is initiated by oxidation of cholesterol through two different pathways: the classical (or neutral) pathway and the alternative (or acidic) pathway (Fig. 1). The classical pathway is initiated by

microsomal cholesterol 7α-hydroxylase (Cyp7a1), which catalyzes the rate-limiting step in the classical pathway (Ishibashi et al., 1996; Schwarz et al., 1996). The alternative pathway of BA biosynthesis is initiated by sterol 27-hydroxylase (Cyp27a1), which is located in the inner mitochondrial membrane. The rate-limiting step in the alternative pathway is cholesterol delivery to the inner mitochondrial membrane, carried out by the steroidogenic acute regulatory protein (StAR, also named mitochondria cholesterol transporter) (Pandak et al., 2002). The classical pathway of BA synthesis is thought to occur exclusively in the liver, whereas the alternative pathway functions in numerous tissues. Two important microsomal enzymes for bile acid biosynthesis are Cyp7a1 and sterol 12α-hydroxylase (Cyp8b1), the later is required for the biosynthesis of cholic acid (CA).

In addition to BA biosynthetic enzymes, BA transporters are also critical to maintain BA homeostasis by regulating BA enterohepatic circulation (Fig. 1). Approximately 95% of total BAs in humans and 87% in mice (Dawson et al., 2003) undergo enterohepatic circulation. After BA biosynthesis in liver, the bile salt export pump (Bsep) transports BAs into bile, whereas multidrug resistance-associated protein (Mrp) 3, 4, and organic solute transporters Ostα/β export BAs out of hepatocytes into the blood. Once excreted into bile, BAs either flow directly into the duodenum (rats) or are stored in the gall bladder (humans and mice). In the intestinal lumen, primary BAs (CA, muricholic acid [MCA], and chenodeoxycholic acid [CDCA]) are converted by bacterial enzymes into secondary BAs (deoxycholic acid [DCA], lithocholic acid [LCA], and ursodeoxycholic acid [UDCA]). The majority of BAs are then taken up into enterocytes by the apical sodium bile-acid transporter (Asbt) in the ileum. Once in the enterocytes, BAs bind to the intestinal bile-acid binding protein (Ibabp) and translocate from the apical to the basolateral membrane of enterocytes. Organic solute heterodimer transporter alpha/beta (Ostα/β), located in the basolateral membrane of enterocytes, exports BAs from enterocytes into the circulation, where they return to the liver. Sodium-taurocholate cotransporting polypeptide (Ntcp) transports conjugated BAs, and organic anion transporting polypeptide (Oatp) 1b2 transports unconjugated BAs into hepatocytes from the sinusoidal blood (Csanaky et al., 2011). BAs then return to the liver, and another cycle of enterohepatic circulation of BAs begins.

The classical pathway of BA biosynthesis is considered more important for maintaining normal BA concentrations; the relative importance of the alternative pathway of BA biosynthesis during altered circumstances is not known. A Cyp7a1-null mouse model was engineered (Ishibashi et al., 1996; Schwarz et al., 1996), which eliminated the classical bile-acid biosynthesis pathway. The Cyp7a1-null mice died within 18 days of age, unless supplemented with lipid-soluble vitamins and BAs (Ishibashi et al., 1996). Intriguingly, the Cyp7a1-null mice improved after three-weeks of age, co-incident with expression of the alternative BA biosynthesis pathway involving Cyp7b1, the expression of which is not initiated until 3 weeks of age (Ishibashi et al., 1996). As mentioned above, in the H-Cpr-null mouse, the classical pathway of BA biosynthesis is abolished, because of the loss of function of Cpr-dependent microsomal Cyp7a1 and 8b1. In contrast to Cyp7a1-null mice, H-Cpr-null mice breed and develop normally. This suggests H-Cpr-null mouse model might be a useful model to understand the contribution of the alternative pathway of BA biosynthesis, because mitochondrial Cyp27a1 does not require Cpr to function, and extrahepatic BA biosynthesis should be intact in H-Cpr-null mice.

H-Cpr-null mice have been useful in the pharmaceutical industry for determining the *in vivo* importance of hepatic Cyps in the safety and efficacy of drugs. While much effort has been made to understand the phenotype of the H-Cpr-null mice, BA homeostasis in these mice is still not well characterized. In the present study, individual BA concentrations, BA biosynthetic enzymes, and BA transporters were determined in H-Cpr-null mice in order to further understand the impact of hepatic Cpr on BA homeostasis.

Materials and Methods

Animals and Tissue Collection: Breeding pairs of H-Cpr-null mice (Gu et al., 2003), which were backcrossed over 10 generations with C57BL/6 mice, were kindly provided by Dr. Xinxin Ding (Wadsworth Center, State University of New York at Albany, NY). All mice were bred and maintained on an automatically timed 12-hr dark/light cycle in an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and allowed water and rodent chow ad libitum (Teklad; Harlan, Indianapolis, IN). At approximately two months of age, blood, liver, and small intestine (duodenum, jejunum, and ileum) from male H-Cpr-null mice and their corresponding male wild-type mice (n=5/genotype) were collected.

Quantification of Individual Bile Acids in Mouse Serum and Liver. Blood was allowed to coagulate, and centrifuged at 10,000 x g for 15 min. The resulting supernatant (serum) was collected for analysis. Total BAs were extracted from mouse serum and liver by a previously described method (Alnouti et al., 2008). Individual BAs (CA, α-MCA, β-MCA, CDCA, UDCA, DCA, and LCA) and their glycine- and taurine-conjugates were quantified by a UPLC-MS/MS method (Alnouti et al., 2008).

Total RNA Isolation. Total RNA was isolated using RNA Bee reagent (Fisher Scientific Inc., Waltham, MA) 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 (bDNA) Analysis. Gene sequences of interest were accessed from GenBank, and the target sequences were analyzed by ProbeDesigner Software Version 1.0 as described previously (Cheng et al., 2005; Hartley and Klaassen, 2000). Probes were synthesized by Integrated DNA Technologies (Coralville, IA). Probe sets for mouse Oatps (Cheng et al., 2005), Mrps (Maher et al., 2005), Ntcp, Bsep (Cheng et al., 2007), Cyp7a1 (Dieter et al., 2004), Ostα, Ostβ, and Asbt (Cheng and Klaassen, 2006), Shp, Cyp27a1, Cyp39a1, BAL, and BAT (Csanaky et al., 2009) have been reported previously. Probe sequences of mouse Cyp7b1 and StAR are shown in Table 1.

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Branched DNA Signal Amplification (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 Inc., Fremont, CA). Individual total RNA samples from 5 mice were used to determine the alteration of gene expression in wild-type and H-Cpr-null mice. Data are presented as relative light units (RLUs) per 10 µg total RNA.

Statistical Analysis. Data were expressed as mean ± S.E.M. The differences between wild-type and H-Cpr-null mice were analyzed by Student's T-test. Statistical significance was set at $p < 0.05$.

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Results

BA concentrations in serum of adult male H-Cpr-null and WT mice. As shown in Fig. 2a, total serum BA concentrations in H-Cpr-null mice were twice those in wild-type mice. In regard to the individual BAs in the serum, the concentration of unconjugated BAs was approximately 50% lower, glycine-conjugated BAs were not altered, but taurine-conjugated BAs were doubled in serum of H-Cpr-null mice compared to WT mice (Fig. 2a). The majority of unconjugated BAs, such as α-MCA, β-MCA, CA, and DCA, were decreased in serum of H-Cpr-null mice (Fig. 2b). The concentrations of glycine-conjugated BAs are very low in serum of mice, and were not different between H-Cpr-null and WT mice (Fig.2c). The serum concentration of taurine-conjugated MCA was decreased, but taurine-conjugated CDCA, UDCA, DCA, and LCA were increased 18.9-, 11.3-, 10.6-, and 1.6-fold in H-Cpr-null mice, respectively (Fig. 2d).

BA concentrations in liver of adult male H-Cpr-null and WT mice. As shown in Fig. 3a, total BA concentrations in livers of H-Cpr-null mice were 59.6% lower than in WT mice. The concentrations of unconjugated, glycine-conjugated, and taurine-conjugated BAs were 71.6, 65.1, and 58.5% lower, respectively, in livers of H-Cpr-null mice (Fig. 3a). The concentrations of unconjugated α-MCA, β-MCA, and CA were lower, but CDCA and LCA were higher in livers of H-Cpr-null mice (Fig. 3b). The total concentration of glycine-conjugated CA was lower, but glycine-conjugated DCA and LCA were higher in livers of H-Cpr-null mice (Fig. 3c). The concentrations of taurine-conjugated MCA, CA, and UDCA were markedly lower, but taurine-conjugated CDCA (0.5-fold) and LCA (25-fold) were higher in livers of H-Cpr-null mice (Fig. 3d).

Total amount of BAs in livers of H-Cpr-null and WT mice. Hepatomegaly occurs in H-Cpr-null mice (1.2 ± 0.1 g/WT mouse liver; 1.9 ± 0.1 g/H-Cpr-null mouse liver), similar to that previously reported (Gu et al., 2003; Henderson et al., 2003), but the average body weight was not different (23.6 ± 0.3 g/ WT mice; 24.0 ± 0.5 g/H-Cpr-null mice) leading to a 50% increase in liver-to-body weight ratio in the H-Cpr-null mice (Fig. 4a). Total BA concentrations were 59.6% lower in livers of H-Cpr-null than WT mice (Fig. 4b). In contrast, total amount of BAs per liver were only 26% lower in H-Cpr-null than WT mice (Fig. 4c).

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Percentage of individual BAs in serum and livers of H-Cpr-null and WT mice. The percentage of individual BAs in serum is shown in Fig. 5 (*upper panel*). Serum MCA and CA accounted for 55.4 and 34.7%, respectively, of the total serum BAs in WT mice, but only 26 and 21.2% in H-Cpr-null mice. The percentage of serum CDCA was 9-fold higher in H-Cpr-null than WT mice. UDCA was nearly non-detectable in serum of WT mice, but accounted for 7.7% of total serum BAs in H-Cpr-null mice. DCA contributed 5% to the total BAs in serum of WT mice, but 25.5% in H-Cpr-null mice. Taken together, CDCA, UDCA, and DCA only accounted for 8.5% of total serum BAs in WT mice, but 51.7% in H-Cpr-null mice.

The percentage of individual BAs in liver is shown in the *lower panel* of Fig. 5. The percentage of MCA in livers was 34% in WT mice, but only 20.7% in H-Cpr-null mice. CA accounts for 51% of the total BAs in liver of WT mice, but only 14% in H-Cpr-null mice. The percentages of CDCA, DCA, and LCA were 6.6-, 3-, and 66-fold higher, respectively, in livers of H-Cpr-null than WT mice. CDCA, DCA, and LCA account for only 13.3% of total liver BAs in WT mice, but 62.2% of that in H-Cpr-null mice.

Concentrations of primary and secondary BAs in serum and livers of H-Cpr-null and WT mice. BAs can be divided into primary BAs that are synthesized in liver (including CA and CDCA), and secondary BAs (including DCA, LCA, and UDCA) that are converted from primary BAs by intestinal microflora. In livers, CDCA can be further converted to α/β-MCA in rodents (through hepatic 6α/β-hydroxylation). Thus, MCA is also a primary BA.

As shown in Fig. 6 (*upper panel*), the concentrations of primary BAs were similar in serum of WT and H-Cpr-null mice, but secondary BAs were increased 5.9-fold in serum of H-Cpr-null mice. Thus, the percentage of secondary BAs in serum increased from 6.5% in WT mice to 27% in H-Cpr-null mice. Primary BAs account for 93.5% of total serum BAs in WT mice, but 73% in H-Cpr-null mice.

In livers, the concentrations of primary BAs were about 70% lower in H-Cpr-null than WT mice (Fig. 6, the *lower panel*). The concentrations of secondary BAs were similar in livers of H-Cpr-null and WT mice. However, the percentage of primary BAs in livers was 91% in WT mice, but 70% in H-Cpr-null mice. In contrast, the percentage of secondary BAs in livers was 9% in WT mice and 30% in H-Cpr-null mice. This suggests an increased microbial metabolism of BAs in the intestine.

BA biosynthesis genes in livers of H-Cpr-Null and WT mice. Messenger RNA expression of Cyp7a1 and 8b1, two critical microsomal enzymes for BA biosynthesis, were 3 to 4-fold higher in livers of H-Cpr-null than WT mice. However, these enzymes are non-functional in the absence of Cpr. The enzymes in the alternative BA biosynthesis pathway were differently regulated: Cyp27a1 was increased 30%, whereas Cyp39a1 and Cyp7b1 were decreased 63% and 87%, respectively, in livers of H-Cpr-null mice (Fig. 7).

The last step in BA biosynthesis is conjugation of BAs with an amino acid, such as taurine and glycine, by BA CoA ligase (BAL) and BA CoA: amino acid *N*-acyltransferase (BAT) (Russell, 2003; Thomas et al., 2008). As shown in Fig. 7, mRNA expression of BAL was not altered, but BAT was increased (21%) in livers of H-Cpr-null mice.

Small heterodimer partner (Shp), the transcriptional repressor of Cyp7a1 gene expression, decreased 55% in livers of H-Cpr-null mice. However, mRNA of FXR (farnesoid X receptor), the BA sensor, was not altered in livers of H-Cpr-null mice (Fig. 7).

Messenger RNA expression of transporters in livers of H-Cpr-null and WT mice*.* Messenger RNA expression of the two main hepatic uptake transporters, Ntcp and Oatp1b2, was similar in H-Cpr-null and WT mice (Fig. 8). Oatp1a4 mRNA expression was markedly higher, but Oatp1a1 was lower in livers of the H-Cpr-null mice (Fig. 8). While it was previously thought that Oatp1a1 and 1a4 might be important for the uptake of BAs into livers of rodents, more recent studies have shown that neither Oatp1a1 (Zhang et al., 2012) nor Oatp1a4 (Zhang et al., 2013) is important for BA uptake. Messenger RNA of the steroidogenic acute regulatory protein (StAR) that transports cholesterol into mitochondria was 90% higher in livers of H-Cpr-null than WT mice.

Canalicular Bsep mRNA was not altered in livers of H-Cpr-null mice. In contrast, Mrp2, which is located in the canalicular membrane, and Mrp3 and 4 transporters, which are located in the sinusoidal membrane, were higher in livers of H-Cpr-null mice (Fig. 8).

BA transporters, Fgf15, and FXR in small intestine of H-Cpr-null and WT mice. The apical sodium-dependent BA transporter (Asbt), intestinal BA binding protein (Ibabp), and Ostα/β are important in the transfer of BAs from the intestinal lumen into the portal blood, and the fibroblast growth factor (Fgf) 15 in the ilea is important for regulating BA synthesis. Therefore, the mRNAs of these genes were quantified in the

ileum. BAs are primarily taken up into enterocytes by Asbt, and possibly Oatp2b1. As shown in Fig. 9, Oatp2b1 mRNA expression was not different in small intestine of H-Cpr-null and WT mice, but Asbt mRNA level is higher in ileum of H-Cpr-null mice. Once in the enterocytes, BAs bind to Ibabp and translocate from the apical to the basolateral region of enterocytes. As shown in Fig. 9, Ibabp is not altered in ileum of H-Cpr-null mice. mRNA levels of organic solute transporters (Ost) α/β, which is the major BA efflux transporter located in the basolateral membrane of enterocytes (Dawson et al., 2005; Rao et al., 2008), were more than 2.5-fold higher in small intestine of H-Cpr-null than WT mice (Fig. 9). Mrp3, which might play a secondary role in transporting BAs out of enterocytes into the circulation (Inokuchi et al., 2001; Shoji et al., 2004; Thomas et al., 2008), was similar in H-Cpr-null and WT mice (Fig. 9).

Fgf15 plays important roles controlling BA biosynthesis in liver, as well as gallbladder filling (Choi et al., 2006; Inagaki et al., 2005; Kim et al., 2007). As shown in Fig. 9, Fgf15 mRNA decreased approximately 90% in the ileum of H-Cpr-null mice, compared with WT mice. Messenger RNA expression of the BA sensor FXR was not altered in the intestine of H-Cpr-null mice.

Discussion

H-Cpr-null mice have a 65% reduction in serum cholesterol and a 90% decrease in gallbladder bile volume (Henderson et al., 2003), leading to a reasonable hypothesis that the amount of BAs would be severely reduced in H-Cpr-null mice due to the loss of function of the Cpr-dependent BA biosynthetic enzymes, in particular, Cyp7a1, which is the rate-limiting enzyme in the classical BA biosynthesis pathway. Surprisingly, the total amount of BAs in livers of H-Cpr-null mice is decreased only about 25% compared to WT mice. The considerable presence of CA and its microbiota product DCA suggests that previously synthesized CA in H-Cpr-null mice at an earlier age still remain in the enterohepatic circulation at the time of analysis (approximately 2 months of age). This is not surprising because the enzyme activity of Cpr is not completely diminished in livers of H-Cpr-null mice until 2 months after birth (Gu et al., 2003). Thus, the microsomal Cyp8b1 enzyme, which is essential for the biosynthesis of CA, might remain active until 2 months of age. Interestingly, the concentrations of CDCA and its microbiota product LCA were increased in livers of H-Cpr-null mice. CDCA can be produced by both the classical and alternative BA synthesis pathways. The reason why there is an increase in CDCA is likely due to an increase in the alternative pathway of BA synthesis, as evidenced by an increase in mRNA of Cyp27a1, a mitochondrial enzyme that does not require Cpr to function, as well as a doubling of StAR, which transports steroids into the mitochondria and is the rate-limiting step in the alternative pathway of BA synthesis (Pandak et al., 2002). A prominent increase in the mRNA of both StAR and Cyp27a1 suggests that livers of H-Cpr-null mice tend to compensate for the decreased BA concentrations by enhancing the alternative pathway of BA synthesis. However, the entire alternative BA synthesis pathway is not likely enhanced in livers of H-Cpr-null mice because of the decreased mRNA of Cyp7b1, another enzyme in the alternative pathway of BA synthesis that requires Cpr. Therefore, the increased concentrations CDCA and its metabolites in livers of H-Cpr-null mice could be due to an enhanced alternative BA synthesis pathway in extrahepatic tissues. Taken together, the severity of impaired BA synthesis in livers of H-Cpr-null mice appears to be compensated by an enhanced alternative pathway of BA synthesis.

Various transcription factors mediate the feedback regulation of BA biosynthesis to ensure the homeostasis of BAs. Cyp7a1 is the rate-limiting enzyme in the classical BA synthesis pathway. Cyp8b1 is the sterol 12α-hydroxylase and thus controls the amount of CA in the BA pool, and represents a critical regulation point for BA diversity in the body. Both Cyp7a1 and 8b1 are negatively regulated by the nuclear receptor FXR, the BA sensor that is important for the down-regulation of BA synthesis and the regulation of some BA transporters (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). It appears that the induced expression of hepatic Cyp7a1 and 8b1 in H-Cpr-null mice is mainly due to the deactivation of FXR, as reflected by a decrease in mRNAs of Fgf15 in intestines (Fig. 9) and Shp in livers. However, due to lack of Cpr in the livers, these microsomal Cyps are not able to synthesize BAs.

One might suspect that the concentrations of BAs in the serum of the H-Cpr-null mice might resemble that observed in liver. But surprisingly, the present study shows that liver-specific elimination of Cpr, which is necessary for hepatic BA biosynthesis, did not markedly decrease total BA concentrations in serum. Instead, H-Cpr-null mice have almost twice the serum concentrations of total BAs, mainly due to a 10-20 fold increase in T-CDCA and T-DCA. The exact mechanisms by which T-CDCA and T-DCA increase in serum of H-Cpr-null mice is not known. In fact, little is known about the regulation of BA concentrations in serum. Theoretically, concentration and composition of BAs in serum are dependent on many factors, including: 1) synthesis of primary BAs in liver, 2) excretion of BAs into bile, 3) expulsion of bile from gallbladder into intestine, 4) conversion of primary BAs to secondary BAs by intestinal flora, 5) reabsorption of BAs from the intestine, 6) transport of BAs into liver, 7) conversion of secondary BAs back to primary BAs in the liver, 8) transport of BAs from liver back into blood, and 9) BA synthesis in extrahepatic tissues. The relative importance of each of these nine processes in determining the composition and concentration of individual BAs in serum is not known.

In H-Cpr-null mice, reduced BA biosynthesis resulted in a decrease in liver BA concentrations, which may send a feedback signal to the intestine to enhance intestinal BA absorption into the circulation. This is reflected by the induction of intestinal BA transporters Asbt and Ostα/β that function in concert to transport BAs from intestinal lumen to blood. Similarly, it is expected that the reduced hepatic BA concentrations in H-Cpr-null mice may result in the liver to take up more BAs and efflux less BAs. However, no alteration was

observed in the mRNA expression of the three major hepatic BA transporters, namely Ntcp, Oatp1b2, and Bsep in H-Cpr-null mice. Recently, Oatp1a1 and 1a4 have been shown to be important for secondary BA metabolism in mice (Zhang et al., 2013; Zhang et al., 2012). The decreased Oatp1a1 and increased Oatp1a4 could be due to activation of some transcriptional factors, such as constitutive androstane receptor and pregnane-X receptor in the H-Cpr-null mice (Cheng and Klaassen, 2006; Wang et al., 2005; Weng et al., 2005). Further studies are required to elucidate the regulatory mechanisms of Oatp1a1 and 1a4 in livers of H-Cpr-null mice. Mrp2, 3, and 4 play an important role in effluxing both xenobiotics and endogenous compounds, such as BAs, out of the liver. The induction of these Mrps in livers of H-Cpr-null mice would enhance the excretion of toxic compounds, such as BAs, and thus represent a compensary response to the loss of Cpr-dependent detoxification. BAs in the intestine possess the ability to inhibit the growth of bacteria in the intestine. The reduced hepatic synthesis of BAs in H-Cpr-null mice likely results in an overgrowth of intestinal bacteria, which may contribute to the increase of secondary BAs in the circulation. For instance, despite the reduced hepatic production of CA, its microbiota product DCA is increased in both serum and livers of H-Cpr-null mice. Therefore, it appears that the alterations of BA concentration and composition in serum of H-Cpr-null mice could be attributed to a combined effect from enhanced intestinal BA absorption, hepatic BA excretion, extrahepatic BA synthesis, and increased secondary BA synthesis.

Not much consideration has been given to BA concentrations in serum. Some research has been conducted to determine whether BA concentrations might be useful to differentiate various liver diseases (Bathena et al., 2013). It has generally been thought that an increase in BAs in serum is secondary to high BA concentrations in liver, due to a decrease in uptake of BAs into liver, or an increased retro-transport of BAs from liver to serum to protect the liver from BA-induced toxicity. However, the present study demonstrates that in the H-Cpr-null mouse model, there is an increase in BAs in serum when there is a decrease in BA concentrations in the liver. Thus, the body might purposely regulate BA concentrations in the serum. In the present study, it appears that the increase in serum BA concentration is primarily due to both an increase in transport of BAs from the intestine to the serum, and an increase in retro-transport of BAs from the liver to the serum. If the only purpose of BAs was to aid in the absorption of fats and fat-

soluble proteins, there would be no reason for regulating serum concentrations of BAs. However, it is now known that there is a BA receptor (TGR5) that regulates triglyceride, cholesterol, glucose, and energy metabolism, and thus the regulation of BA concentrations in the serum might be physiologically important.

To conclude, decreasing the activity of all microsomal Cyps in the liver by Cre-Lox targeting of Cpr in hepatocytes of mice exerts differential effects on BA concentrations in liver and serum. Reduced BA concentration in livers of Cpr-null mice is attributed to an impaired classic pathway of BA synthesis, which is likely compensated by enhancing the alternative BA synthesis pathway. The increased serum BA concentrations in H-Cpr-null mice appear to be the result of enhanced intestinal BA absorption, hepatic BA retro-transport into the serum, and increased secondary BA synthesis. Overall, the present study suggests the importance of the alternative BA synthesis pathway in regulating BA concentrations in livers and serum of H-Cpr-null mice.

Acknowledgements:

The authors would like to thank Dr. Xinxin Ding (Wadsworth Center, State University of New York at Albany, NY) for providing H-Cpr-null mice, and Lucy Lei for help with sample processing.

Authorship Contributions:

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Wrote or contributed to the writing of the manuscript: Xingguo Cheng, Youcai Zhang, and Curtis D. Klaassen.

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Footnotes:

The authors (X.C. and Y.Z.) contributed equally to this work. This work was supported by National Institute of Health grant (ES-09649).

JPET Fast Forward. Published on July 17, 2014 as DOI: 10.1124/jpet.114.216796
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Legends for Figures

Fig. 1. Enterohepatic circulation and metabolism of bile acids in mice. a, bile acid synthesis pathways, transporters, and metabolism in mice. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are two major primary bile acids synthesized in livers of both humans and mice. Cyp8b1 is essential for the biosynthesis of CA. CDCA can be synthesized through both classic and alternative pathways. In mice, CDCA can be further metabolized to form alpha- and beta-muricholic acids (MCAs). Two enzymes, namely bile acid CoA ligase (BAL) and bile acid CoA: amino acid *N*-acyltransferase (BAT), mediate the conjugation of bile acids with taurine and glycine. Bsep transports conjugated bile acids into bile and enter intestine where bacterial enzymes metabolize primary bile acids to form secondary bile acids. In enterocytes, Asbt and Ostα/β work in concert to transport bile acids from intestinal lumen to the circulation. In hepatocytes, Ntcp and Oatp transport bile acids from blood to liver to complete one cycle of enterohepatic circulation of bile acids. **b**, structures and abbreviations of major primary and secondary bile acids. T-BA and G-BA stand for taurine- and glycine-conjugated bile acids, respectively.

Fig. 2. Bile-acid concentrations in serum of H-Cpr-null and wild-type mice. Total bile acids were extracted from serum of H-Cpr-null and WT mice. The concentrations of serum total BAs, serum unconjugated BAs, serum glycine-conjugated BAs, and serum taurine-conjugated BAs were determined (**a**). Specifically, the concentrations of unconjugated (**b**), glycine-conjugated (**c**), and taurine-conjugated (**d**) α-MCA, β-MCA, CA, UDCA, CDCA, DCA, and LCA in serum from adult male H-Cpr-null and wild-type mice (n=5/genotype) were quantified by LC-MS/MS method described in **Materials and Methods** section. Black bars represent data from WT mice; gray bars represent data from H-Cpr-null mice. The data are presented as mean ± SEM (n = 5/genotype). Asterisks (*) represent a statistical difference ($p < 0.05$) between WT and H-Cpr-null mice.

Fig. 3. Bile-acid concentrations in liver of H-Cpr-null and WT mice. Total bile acids were extracted from livers of H-Cpr-null and WT mice. The concentrations of liver total BAs, liver unconjugated BAs, liver glycine-conjugated BAs, and liver taurine-conjugated BAs were determined (**a**). Specifically, the concentrations of unconjugated (**b**), glycine-conjugated (**c**), and taurine-conjugated (**d**) α-MCA, β-MCA, CA, UDCA, CDCA, DCA, and LCA in livers from adult male H-Cpr-null and wild-type mice (n=5/genotype) were quantified by LC-MS/MS method described in **Materials and Methods** section. Black bars represent data from WT mice; gray bars represent data from H-Cpr-null mice. The data are presented as mean ± SEM (n = 5/genotype). Asterisks (*) represent a statistical difference ($p < 0.05$) between WT and H-Cpr-null mice.

Fig. 4. Liver-to-body weight ratio and total bile-acid levels in liver of H-Cpr-null and wild-type mice. (**a**) Weight ratio of liver-to-body of adult male H-Cpr-null and wild-type mice (n=5/genotype); (**b**) The alteration of concentration of total bile acids in livers of adult male H-Cpr-null and wild-type mice

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(n=5/genotype); and (**c**) Average levels of total bile acids in one liver of adult male H-Cpr-null and adult male wild-type mice (n=5/genotype). Black bars represent data from WT mice; gray bars represent data from H-Cpr-null mice. The data are presented as mean ± SEM (*n* = 5/genotype). Asterisks (*) represent a statistical difference ($p < 0.05$) between WT and H-Cpr-null mice.

Fig. 5. Percentage of individual bile acids in serum or liver of H-Cpr-null and wild-type mice. Total bile acids were extracted from serum and livers of H-Cpr-null and WT mice, and quantified using LC-MS/MS method described in **Materials and Methods** section. The u*pper panel*, percentage of individual bile acid in serum total bile acids of H-Cpr-null or WT mice. The *lower panel*, percentage of individual bile acid in liver total bile acids of H-Cpr-null or WT mice. Black bars represent data from WT mice; gray bars represent data from H-Cpr-null mice. The data are presented as mean ± SEM (*n* = 5/genotype). Asterisks (*) represent a statistical difference ($p < 0.05$) between WT and H-Cpr-null mice.

Fig. 6. Primary and secondary bile-acid concentrations in serum and liver of H-Cpr-null and WT mice. Total bile acids were extracted from serum and liver of H-Cpr-null and WT mice. The concentrations of primary bile acids (including unconjugated and conjugated CA, CDCA, and MCA) and secondary bile acids (including unconjugated and conjugated DCA, LCA, and UDCA) in serum (*upper panel*) and liver (*lower panel*) of H-Cpr-null and WT mice. Black bars represent data from WT mice; gray bars represent data from H-Cpr-null mice. The data are presented as mean ± SEM (*n* = 5/genotype). Asterisks (*) represent a statistical difference ($p < 0.05$) between WT and H-Cpr-null mice.

Fig. 7. Messenger RNA expression of important bile-acid biosynthesis genes in liver of H-Cpr-null mice. Total RNA samples from livers of wild-type and H-Cpr-null mice (n=5/genotype) were analyzed for mRNA expression of each gene by bDNA assay. The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in wild-type mice per 10μg total RNA. The asterisk (*) indicates statistically significant differences of mRNA expression between H-Cpr-null mice and wild-type mice ($p < 0.05$).

Fig. 8. Messenger RNA expression of transporters in liver of H-Cpr-null mice. Total RNA samples from livers of wild-type and H-Cpr-null mice (n=5/genotype) were analyzed for mRNA expression of each transporter by bDNA assay. The data are represented as the ratio of hepatic mRNA expression in H-Cpr-null mice to that in wild-type mice per 10μg total RNA. The asterisk (*) indicates statistically significant differences of mRNA expression between H-Cpr-null mice and wild-type mice ($p < 0.05$).

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Fig. 9. Messenger RNA expression of intestinal genes in small intestine of H-Cpr-null mice. Total RNA samples from full-length small intestine or specifically ileum of wild-type and H-Cpr-null mice (n=5/genotype) were analyzed for mRNA expression of each gene by bDNA assay. Because Asbt, IBABP, and Fgf15 are specifically expressed in ileum of mice, regulation of these genes were only characterized in ileum of mice. The data are represented as the ratio of intestinal mRNA expression in H-Cpr-null mice to that in wild-type mice per 10μg total RNA. The asterisk (*) indicates statistically significant differences of mRNA expression between H-Cpr-null mice and wild-type mice ($p < 0.05$).

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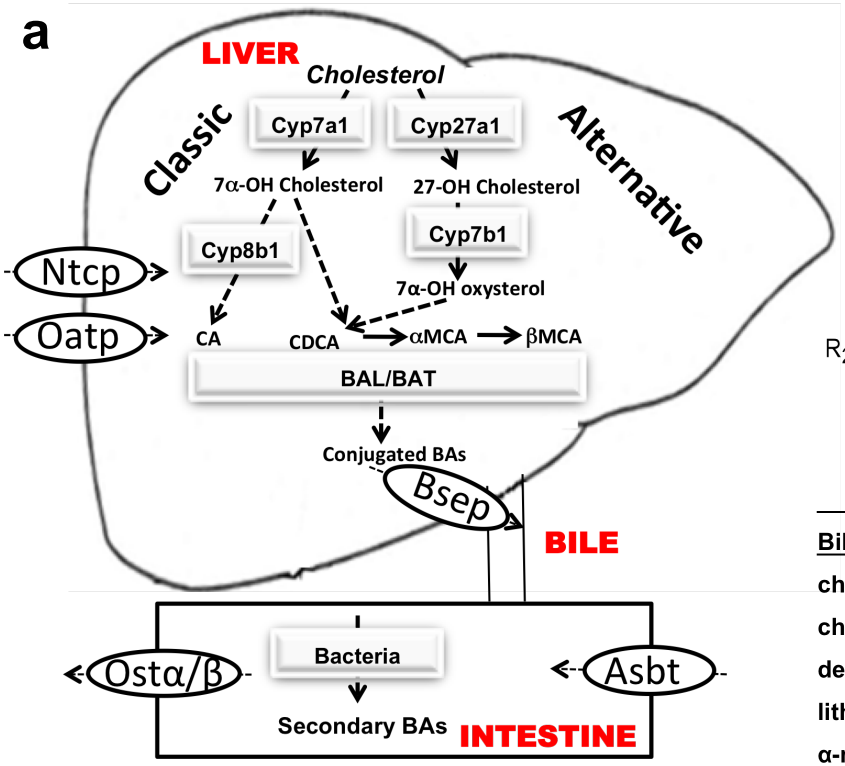
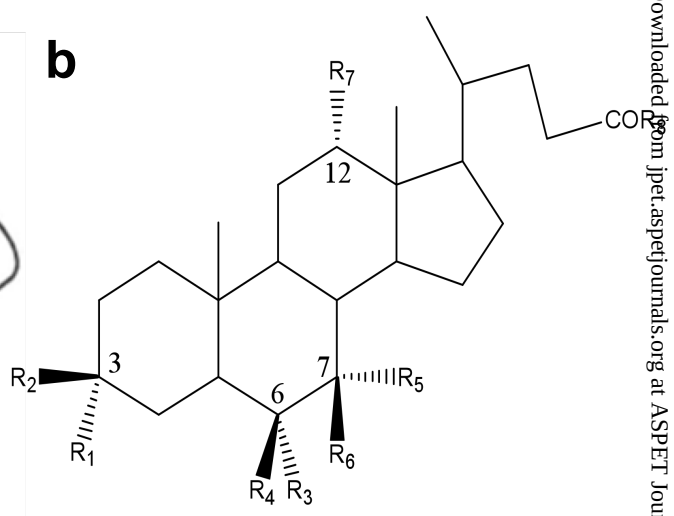
Table 1. Oligonucleotide probes generated for analysis of mouse StAR and Cyp7b1 mRNAs expression by Quantigene branched DNA signal amplification assay

StAR (NM_011485[a])		Cyp7b1 (BC038810)	
CE[b]	tcctgcttttgtgagacggtcTTTTTctcttggaaagaaagt	CE	gctggaatggtgtttgctagagagTTTTTctcttggaaagaaagt
CE	gaagctgctagctctgagccaTTTTTctcttggaaagaaagt	CE	ccaggccctttctttttgaccTTTTTctcttggaaagaaagt
CE	tctagctatttgtcttcctagaaggtaTTTTTctcttggaaagaaagt	LE	tgcctatagtatcttttcagcagatcTTTTTaggcataggacccgtgtct
CE	cacctggctcccaatttataccTTTTTctcttggaaagaaagt	LE	gtgctcctatttcagaatcgtcaTTTTTaggcataggacccgtgtct
LE	aactaccaactcccaagattttgtTTTTTaggcataggacccgtgtct	LE	agaatataatacattgcccagaacataTTTTTaggcataggacccgtgtct
LE	ctgccaggatgctgttaatcttTTTTTaggcataggacccgtgtct	LE	ccatagcttcaggatgccgaTTTTTaggcataggacccgtgtct
LE	tggtcagtattatttgatcagtataggtaTTTTTaggcataggacccgtgtct	LE	tttcgtcacgcagggcTTTTTTTaggcataggacccgtgtct
LE	caatagaacagtcactttatcttaacattatTTTTTaggcataggacccgtgtct	LE	gcagaccaagctgtccaattgTTTTTaggcataggacccgtgtct
LE	ggctggcatctaatctgtctataaaTTTTTaggcataggacccgtgtct	LE	tggagtatgagcacagcctcagTTTTTaggcataggacccgtgtct
LE	acataacattttaacctttcaaataagTTTTTaggcataggacccgtgtct	LE	cctgcacttctcggatgatgcTTTTTaggcataggacccgtgtct
BL	tcactggggtccaagctgg	BL	gcccagagaaagccaagatgat
BL	aatgaaaatcccgtcataatcttc	BL	tgttgactgcaggaaactgtcaa
BL	gaacaggaatgttatagctctactgact	BL	tctctggtgaagtggactgaaatt
BL	cttaaagtgtaatatctccaaatcctg	BL	aacctcaagaatagtgctttccag
BL	attaaagtcgtgtgccaccgt		

[a] GenBank accession numbers for each transcript are given in parenthesis after the gene name.
[b] The type of Function of each bDNA oligonucleotide probe. CE, capture extender; LE, label extender; BL, blocker.





Bile acids	Abv	R1	R4	R5	R6	R7	R8
cholic acid	CA	OH	H	OH	H	OH	H
chenodeoxycholic acid	CDCA	OH	H	OH	H	H	H
deoxycholic acid	DCA	OH	H	H	H	OH	H
lithocholic acid	LCA	OH	H	H	H	H	H
α-muricholic acid	αMCA	OH	OH	OH	H	H	H
β-muricholic acid	βMCA	OH	OH	H	OH	H	H
ursodeoxycholic acid	UDCA	OH	H	H	OH	H	H
unconjugate	Unconj BA						OH
taurine conjugate	T-BA						NH(CH2)2SO3H
glycine conjugate	G-BA						NHCH2COOH

Fig. 1

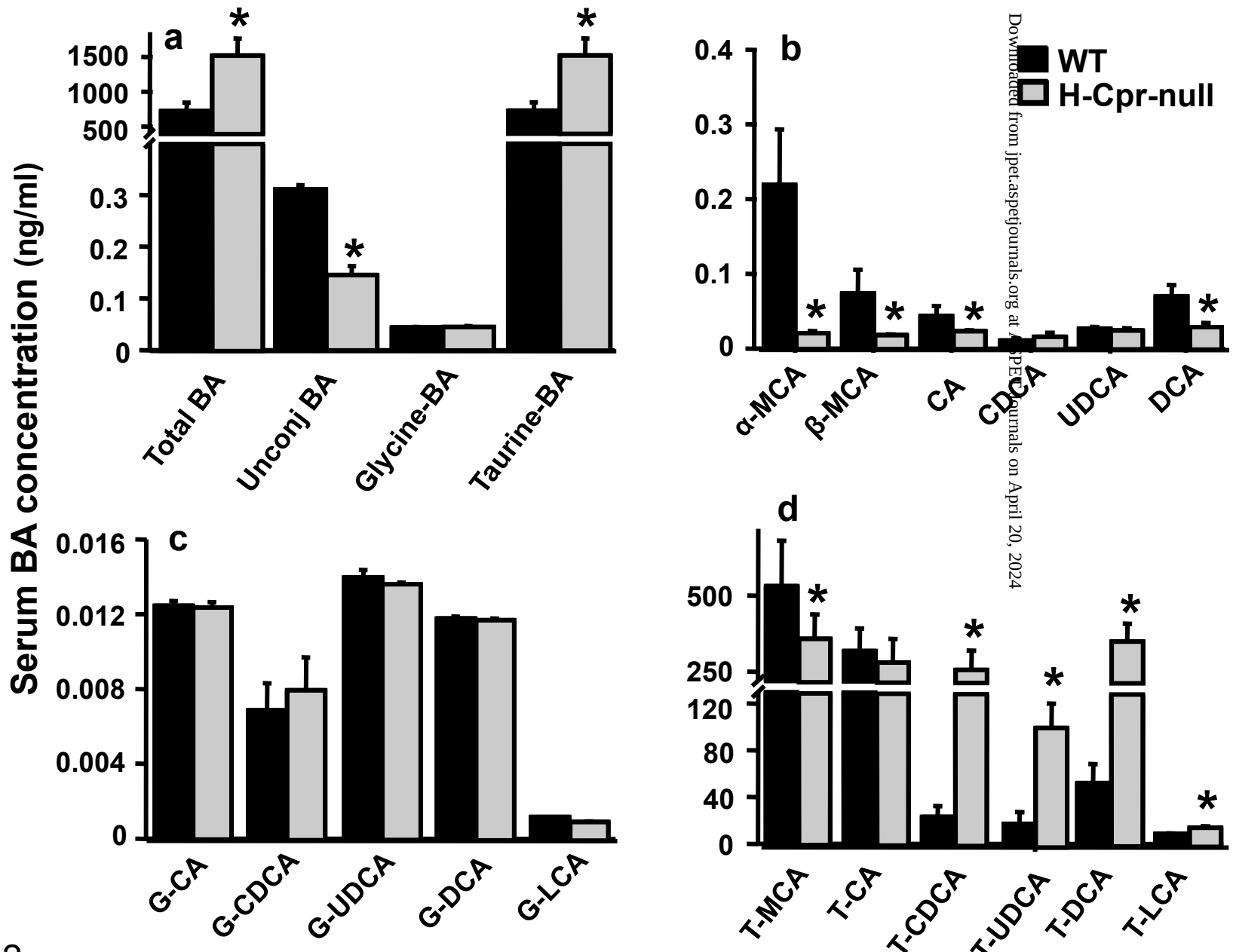


Fig. 2

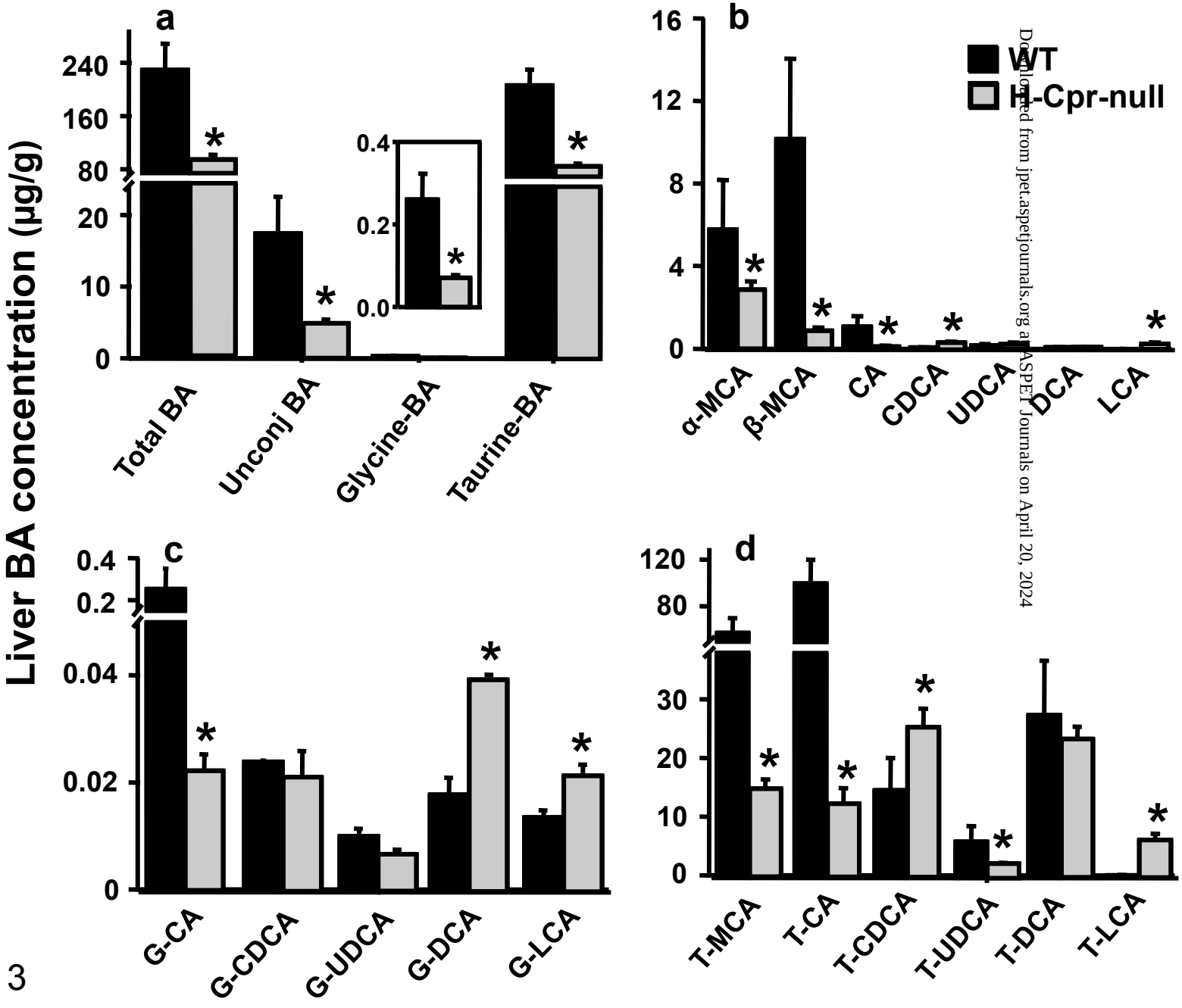


Fig. 3

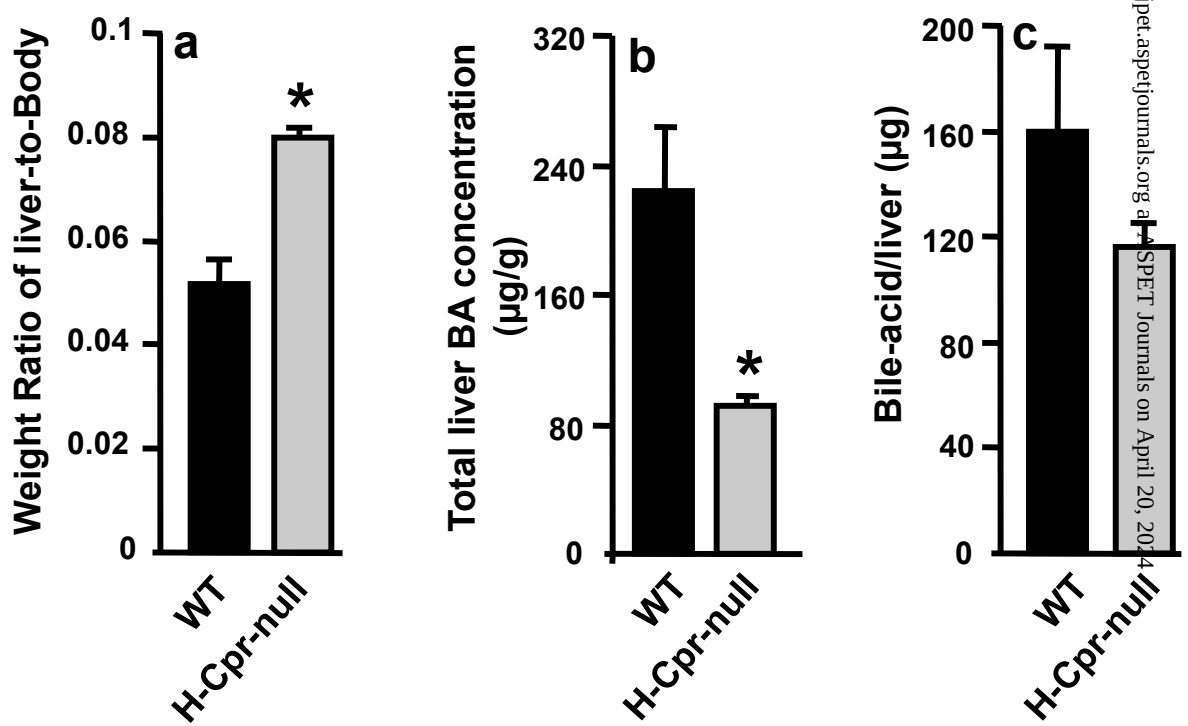


Fig. 4

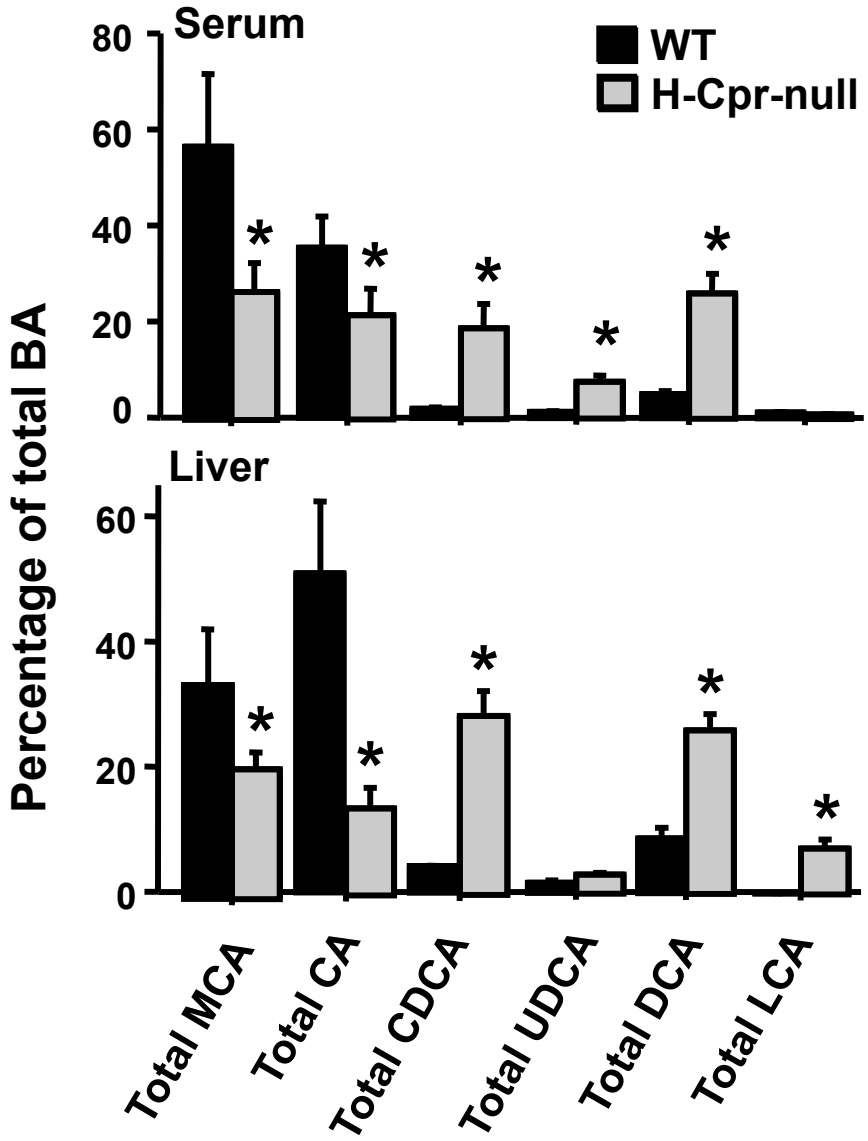
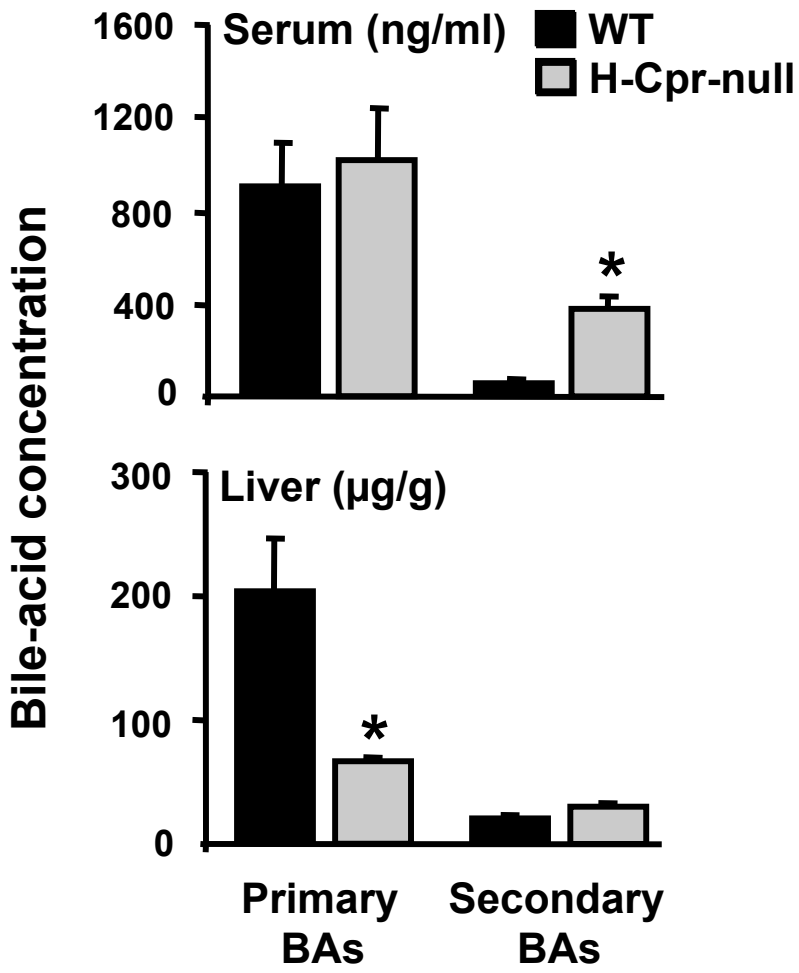


Fig. 5



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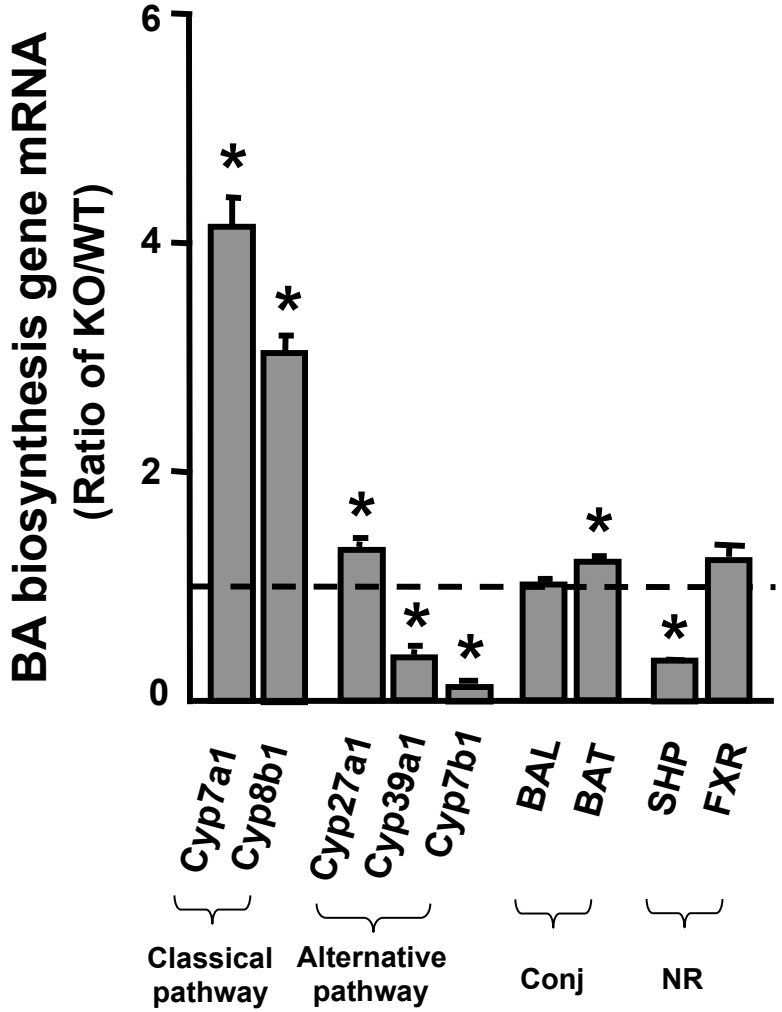
BA biosynthesis gene mRNA
(Ratio of KO/WT)
6
4
2
0
*
*
*
*
*
*
Cyp7a1
Cyp8b1
Cyp27a1
Cyp39a1
Cyp7b1
BAL
BAT
SHP
FXR
Classical pathway
Alternative pathway
Conj
NR

Fig. 7

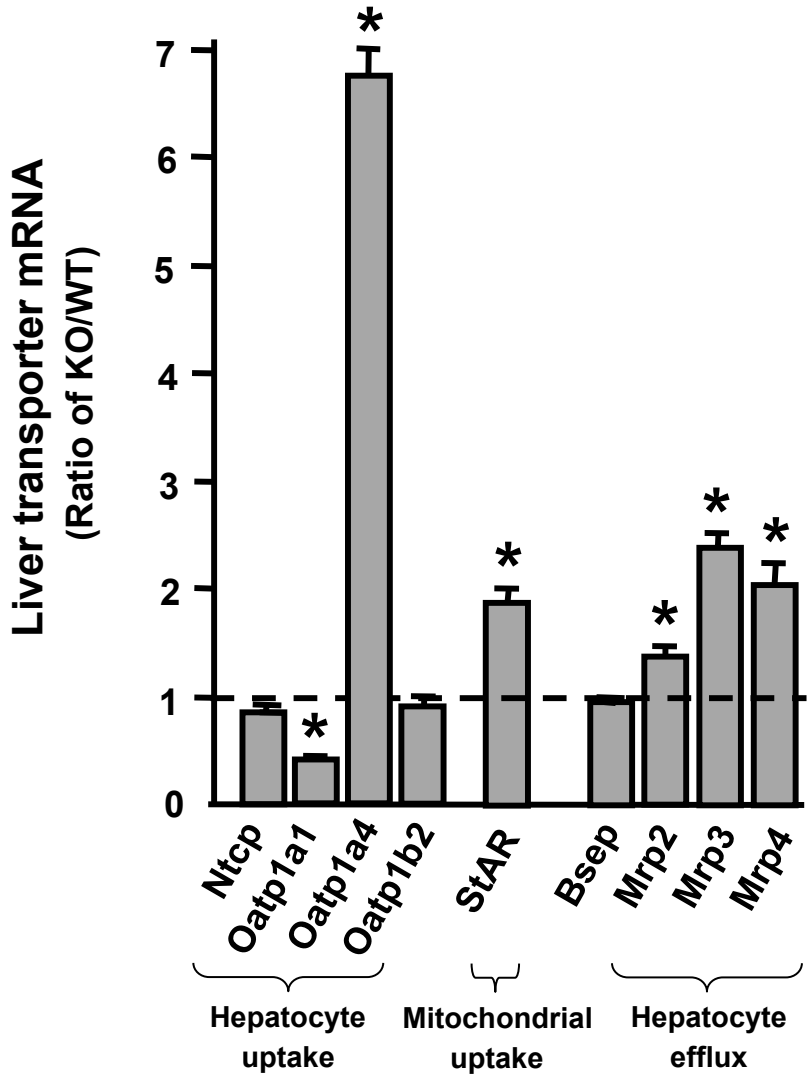
Liver transporter mRNA
(Ratio of KO/WT)
0
1
2
3
4
5
6
7
*
*
*
*
*
*
Ntcp
Oatp1a1
Oatp1a4
Oatp1b2
StAR
Bsep
Mrp2
Mrp3
Mrp4
Hepatocyte uptake
Mitochondrial uptake
Hepatocyte efflux

Fig. 8

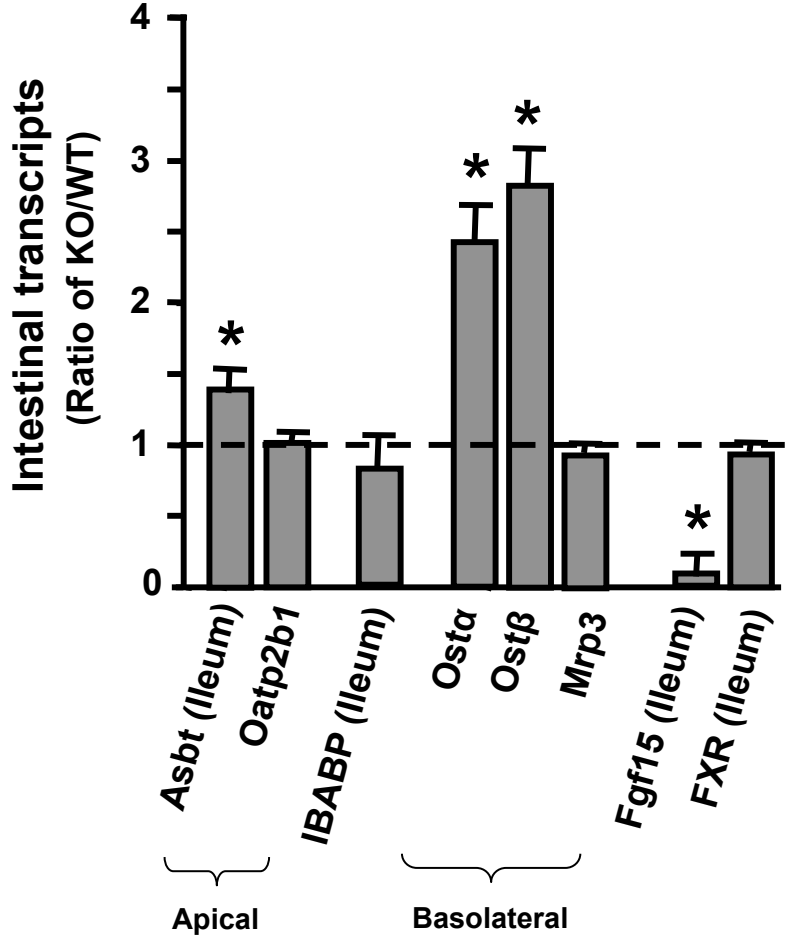
Intestinal transcripts
(Ratio of KO/WT)
4
3
2
1
0
*
*
*
*
Asbt (Ileum)
Oatp2b1
IBABP (Ileum)
Ostα
Ostβ
Mrp3
Fgf15 (Ileum)
FXR (Ileum)
Apical
Basolateral

Fig. 9