

Hepatic deletion of X-box binding protein 1 impairs bile acid metabolism in mice

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Abstract The unfolded protein response (UPR) is an adaptive response to endoplasmic reticulum stress and the inositol-requiring enzyme 1 α /X-box binding protein 1 (IRE1 α /XBP1) pathway of the UPR is important in lipid metabolism. However, its role in bile acid metabolism remains unknown. We demonstrate that liver-specific *Xbp1* knockout (LS-*Xbp1*^{-/-}) mice had a 45% reduction in total bile acid pool. LS-*Xbp1*^{-/-} mice had lower serum 7 α -hydroxy-4-cholesten-3-one (C4) levels compared with *Xbp1*^{fl/fl} mice, indicating reduced cholesterol 7 α -hydroxylase (CYP7A1) synthetic activity. This occurred without reductions of hepatic CYP7A1 protein expression. Feeding LS-*Xbp1*^{-/-} mice cholestyramine increased hepatic CYP7A1 protein expression to levels 2-fold and 8-fold greater than cholestyramine-fed and chow-fed *Xbp1*^{fl/fl} mice, respectively. However, serum C4 levels remained unchanged and were lower than both groups of *Xbp1*^{fl/fl} mice. In contrast, although feeding LS-*Xbp1*^{-/-} mice cholesterol did not increase CYP7A1 expression, serum C4 levels increased significantly up to levels similar to chow-fed *Xbp1*^{fl/fl} mice and the total bile acid pool normalized. In conclusion, loss of hepatic XBP1 decreased the bile acid pool and CYP7A1 synthetic activity. Cholesterol feeding, but not induction of CYP7A1 with cholestyramine, increased CYP7A1 synthetic activity and corrected the genotype-specific total bile acid pools. These data demonstrate a novel role of IRE1 α /XBP1 regulating bile acid metabolism.—Liu, X., A. S. Henkel, B. E. LeCuyer, S. C. Hubchak, M. J. Schipma, E. Zhang, and R. M. Green. Hepatic deletion of X-box binding protein 1 impairs bile acid metabolism in mice. *J. Lipid Res.* 2017. 58: 504–511.

Supplementary key words cholesterol • cholesterol 7- α hydroxylase • liver • gene expression • endoplasmic reticulum • unfolded protein response • serum 7 α -hydroxy-4-cholesten-3-one

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK093807, the George Lockerie Liver Cancer Foundation, and the Max Goldenberg Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Manuscript received 2 August 2016 and in revised form 21 December 2016.

Published, JLR Papers in Press, December 30, 2016

DOI 10.1194/jlr.M071266

Bile acids are amphipathic compounds that are synthesized in the liver from cholesterol. Bile acids facilitate hepatobiliary secretion and intestinal lipid absorption, regulate glucose and lipid metabolism in the liver, and regulate energy expenditure in the peripheral tissues (1, 2). Bile acids are synthesized from cholesterol through a classic (neutral) pathway and an alternative (acidic) pathway. These bile acid biosynthetic pathways involve a number of enzymes and the rate-limiting enzyme of the classic pathway is cholesterol 7 α -hydroxylase (CYP7A1). Bile acids in the liver are secreted into bile, released into the intestine, reabsorbed in the ileum, and transported via the portal circulation back to the liver. Over 95% of the bile acid pool is conserved through this enterohepatic circulation, with the small amount of fecal and urinary bile acid loss compensated by de novo biosynthesis in the liver (2–4).

The unfolded protein response (UPR) is an adaptive cellular response to endoplasmic reticulum (ER) stress that maintains homeostasis by increasing protein processing capacity and attenuating protein translation. When the inositol-requiring enzyme 1 α /X-box binding protein 1 (IRE1 α /XBP1) pathway of the UPR is activated in the presence of ER stress, *XBP1* mRNA undergoes unconventional splicing by phosphorylated IRE1 α to remove a 26-nucleotide sequence, causing a translational frameshift and producing the transcriptionally active XBP1 spliced form. While this pathway of the UPR has been implicated in the pathogenesis of and as a protective response to liver injury (5–8), the IRE1 α /XBP1 pathway is also important in hepatic lipid metabolism. In fact, liver-specific deletion of *Xbp1* has been shown to reduce hepatic lipogenic gene expression, fatty acid synthesis, and VLDL secretion (9–11). Although bile

Abbreviations: C4, 7 α -hydroxy-4-cholesten-3-one; CYP7A1, cholesterol 7 α -hydroxylase; ER, endoplasmic reticulum; GO, Gene Ontology; IRE1 α , inositol-requiring enzyme 1 α ; KEGG, Kyoto Encyclopedia of Genes and Genomes; LS-*Xbp1*^{-/-}, liver-specific X-box binding protein 1 knockout; LXR, liver X receptor; TC, taurocholic acid; TMCA, tauro-muricholic acid; UPR, unfolded protein response; XBP1, X-box binding protein 1.

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acids are important hepatobiliary lipids that regulate metabolism, the role of XBP1 in bile acid metabolism remains unexplored.

MATERIALS AND METHODS

Materials

Cholestyramine resin and cholesterol were purchased from Sigma (St. Louis, MO). Antibodies against CYP7A1 and GAPDH were purchased from Proteintech (Rosemont, IL). GRP78 antibody was purchased from Cell Signaling Technology (Danvers, MA).

Animal use and treatment

Liver-specific *Xbp1* knockout (LS-*Xbp1*^{-/-}) mice were generated by breeding C57BL/6-*Xbp1*^{fl/fl} mice (kindly provided by Dr. Laurie J. Glimcher, Harvard University, MA) with C57BL/6-albumin-Cre mice (Jackson Laboratory, ME) as previously described (6). LS-*Xbp1*^{-/-} mice and control littermate *Xbp1*^{fl/fl} mice were cohoused on a 14 h light/10 h dark cycle with free access to food and water. Male *Xbp1*^{fl/fl} and LS-*Xbp1*^{-/-} mice (8–10 weeks old) were randomly assigned to receive standard chow, chow supplemented with 2% (w/w) cholestyramine, or chow supplemented with 2% (w/w) cholesterol for 1 week. The mice were fasted for 4 h prior to euthanasia, blood was obtained using cardiac puncture, and the liver and ileum were removed and rinsed with ice-cold saline, sectioned, and snap-frozen in liquid nitrogen. For mice used in the total or organ-specific bile acid pool analysis experiments, the liver, gallbladder, and small intestine were collected from nonfasted mice and immediately minced in 100% methanol either separately for organ-specific bile acid analysis, or together for measurement of the total bile acid pool. In bile analysis experiments, bile was aspirated from the gallbladders of mice fasted for 4 h. All protocols and procedures were performed in conformity with the Public Health Service policy on the Humane Care and Use of Laboratory Animals and approved by the Northwestern University Institutional Animal Care and Use Committee guidelines.

Bile acid analysis

Total bile acid pool size, estimated as the total amount of bile acids circulating in the enterohepatic circulation, and bile acid contents and composition were measured by high-performance liquid chromatography as previously described (12). Samples were spiked with glycocholic acid as an internal standard to control for extraction efficiency. Individual bile acid species were identified by their characteristic retention times and by using bile acid standards. The fecal bile acid content was measured spectrophotometrically after a 72 h collection using a colorimetric assay kit according to the manufacturer's instructions (GenWay Biotech, San Diego, CA) as previously described (12).

Serum biochemistries

Serum cholesterol was determined using an Infinity spectrophotometric assay according to the instructions of the manufacturer (Thermo Scientific). Serum 7 α -hydroxy-4-cholesten-3-one (C4) measurement was performed at the Mayo Clinic Immunochemical Core Lab (Rochester, MN). Serum bile acids were measured colorimetrically.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from frozen liver and ileum using TRIZOL reagent according to the manufacturer's protocol

(Invitrogen Life Technologies, Carlsbad, CA). One microgram of total RNA was reverse-transcribed to cDNA with the qScript cDNA synthesis kit (Quanta Bioscience, Gaithersburg, MD). Quantitative (q)PCR was then performed using Power SYBR[®] Green PCR Master Mix (Thermo Scientific, Waltham, MA) with the Applied Biosystems Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). Real-time data were collected for 40 cycles of 95°C, 10 s; 60°C, 1 min. Relative expression of the gene of interest was estimated by the $\Delta\Delta C_t$ method using 18s as a reference gene. Samples were analyzed in duplicate, and experiments were repeated a minimum of three times. All primers were synthesized by Integrated DNA Technology (Coralville, IA). RNA-Seq, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed as previously reported (6). The GEO accession number for the data set is GSE64824.

Preparation of whole liver homogenates and microsomal protein

Protein homogenates from frozen liver were isolated using T-Per protein extraction reagent (Thermo Scientific) containing protease inhibitor cocktails (EMD Millipore, Billerica, MA) and Halt[™] phosphatase inhibitor (Thermo Scientific). Microsomal protein was isolated from liver homogenate via differential centrifugation. Livers were homogenized in microsome buffer (50 mM KH₂PO₄, 50 mM KCl, 100 mM sucrose, 30 mM EDTA, 50 mM NaCl, 2 mM DTT) and centrifuged at 8,600 *g* for 15 min. Supernatants were further centrifuged at 81,000 *g* for 1 h, washed, and resuspended in microsome buffer.

Western blotting

After protein quantification with Coomassie Plus protein assay reagent (Thermo Scientific), equal amounts of protein samples were subjected to immunoblotting for target proteins, and immunoreactive bands were visualized using Amersham ECL Western blotting detection reagents according to the manufacturer's protocol (GE Healthcare, Piscataway, NJ). Densitometry was performed with ImageJ. CYP7A1 protein expression was normalized to GAPDH for liver homogenates. GRP78 was used to verify microsomal enrichment and protein loading. The results were expressed as a relative amount to chow-fed *Xbp1*^{fl/fl} mice.

Free cholesterol assay

Hepatic lipids were extracted using a modified Folch method. Briefly, liver tissues were homogenized in 2 ml of chloroform:methanol (2:1). The homogenates were incubated at room temperature for 20 h, followed by centrifugation at 1,000 *g* for 5 min. Supernatants were washed with saline and solvents were removed by evaporation under a N₂ stream. Dried lipids were resuspended in assay buffer, followed by free cholesterol measurement using a cholesterol quantitation kit (Sigma) according to manufacturer's instructions.

Statistics

Data are shown as mean \pm SEM. Comparison between two groups was performed using two-tailed Student's *t*-test. Statistical significance was defined as *P* \leq 0.05.

RESULTS

LS-*Xbp1*^{-/-} mice have reduced hepatic fatty acid synthesis and altered lipoprotein metabolism (9, 10). Because bile acids are hepatobiliary lipids that regulate hepatic metabolism, we hypothesized that the IRE1 α /XBP1 signaling

pathway is also important in hepatic bile acid metabolism. Therefore, we initially examined the total bile acid pool and bile acid species in chow-fed LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. The total bile acid pool size was reduced by 45% in LS-*Xbp1*^{-/-} mice, with total bile acids of 10.7 ± 1.3 μmol/100 g mouse and 19.4 ± 2.7 μmol/100 g mouse in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice, respectively (*P* < 0.05) (Fig. 1A). Tauromuricholic acid (TMCA) and taurocholic acid (TC) contents were also reduced by 40% and 56%, respectively (*P* < 0.05) in LS-*Xbp1*^{-/-} mice. Bile acid contents were similarly reduced in the liver and small intestine in LS-*Xbp1*^{-/-} mice, but not in the gallbladder (Fig. 1B). We also measured the bile acid composition of gallbladder bile. As shown in Fig. 1C, the bile in *Xbp1*^{fl/fl} mice contained 67.5 ± 2.3% TMCA and 32.5 ± 2.3% TC; and the bile in LS-*Xbp1*^{-/-} mice contained a slightly higher percentage of TMCA (78.2 ± 1.9%, *P* < 0.05) with a correspondingly lower percentage of TC (21.8 ± 1.9%, *P* < 0.05). Serum bile acid levels were also reduced in LS-*Xbp1*^{-/-} mice, being 13.9 ± 0.7 μmol/l and 18.4 ± 1.8 μmol/l in LS-*Xbp1*^{-/-} mice and *Xbp1*^{fl/fl} mice, respectively (*P* < 0.05) (Fig. 1D). There were no differences in fecal bile acid output between the two genotypes (0.87 ± 0.11 μmol/day/100 g mouse vs. 0.86 ± 0.05 μmol/day/100 g in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice, respectively) (Fig. 1E). Urine bile acid excretion was less than 0.1% of the bile acid pool and also did not differ between the two genotypes. Therefore, enhanced bile acid excretion cannot account for the reduced bile acid pool in LS-*Xbp1*^{-/-} mice.

We next performed RNA-Seq on hepatic mRNA isolated from LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice to identify bile acid metabolic genes that were altered in mice lacking hepatic *Xbp1*. KEGG pathway analysis of differentially expressed hepatic genes revealed that the primary bile acid biosynthesis and bile secretion pathways were downregulated in LS-*Xbp1*^{-/-} mice (Fig. 2A). We subsequently performed qPCR on several of these genes to confirm the differences in hepatic gene expression (Fig. 2B). Because the ileum has an important role in bile acid metabolism, we analyzed

ileal bile acid metabolic genes and determined that there were no differences between LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (Fig. 2C).

Because the total bile acid pool is reduced in LS-*Xbp1*^{-/-} mice without changes in bile acid excretion, we next measured gene expression of the major bile acid synthetic genes, *Cyp7a1*, *Cyp27a1*, and *Cyp8b1*. Figure 3 demonstrates that hepatic gene expression of *Cyp7a1* was higher in LS-*Xbp1*^{-/-} mice compared with *Xbp1*^{fl/fl} mice (*P* < 0.05), while gene expression of *Cyp27a1* and *Cyp8b1* was similar to the controls. Hepatic gene expression of bile acid synthetic pathway genes, *Akr1d1* and *Slc27a5*, was reduced by 36% (*P* < 0.01) and 60% (*P* < 0.001), respectively (Fig. 2B). Hepatic *Shp* expression did not change in LS-*Xbp1*^{-/-} mice compared with *Xbp1*^{fl/fl} mice (1.30 ± 0.26 vs. 1.06 ± 0.13, respectively; *n* = 9).

C4 is an intermediate in the classic pathway of bile acid synthesis and the measurement of serum C4 levels is an accurate method for detecting in vivo CYP7A1 synthetic activity (13–17). Figure 4A demonstrates that serum C4 levels were significantly lower in LS-*Xbp1*^{-/-} mice, being 9.8 ± 2.6 ng/ml compared with 20.5 ± 1.5 ng/ml in *Xbp1*^{fl/fl} mice (*P* < 0.05), indicating reduced CYP7A1 synthetic activity in LS-*Xbp1*^{-/-} mice. Because the gene expression of *Cyp7a1* was actually higher in LS-*Xbp1*^{-/-} mice, we performed Western blotting to confirm CYP7A1 protein expression levels. CYP7A1 protein expression did not change in either the liver homogenate or the liver microsomal fraction from LS-*Xbp1*^{-/-} mice (Fig. 4B–E). Therefore, the reduced hepatic CYP7A1 synthetic activity in LS-*Xbp1*^{-/-} mice was not due to changes in CYP7A1 expression.

Cholesterol is the precursor for bile acid synthesis by CYP7A1 and serum cholesterol levels were reduced by 70% in LS-*Xbp1*^{-/-} mice compared with *Xbp1*^{fl/fl} mice (*P* < 0.001) (Fig. 5A). GO biological process analysis of differentially expressed hepatic genes identified by RNA-Seq revealed that the cholesterol metabolic process, cholesterol homeostasis, and cholesterol biosynthetic process pathways were downregulated in LS-*Xbp1*^{-/-} mice (Fig. 5B). Figure 5C

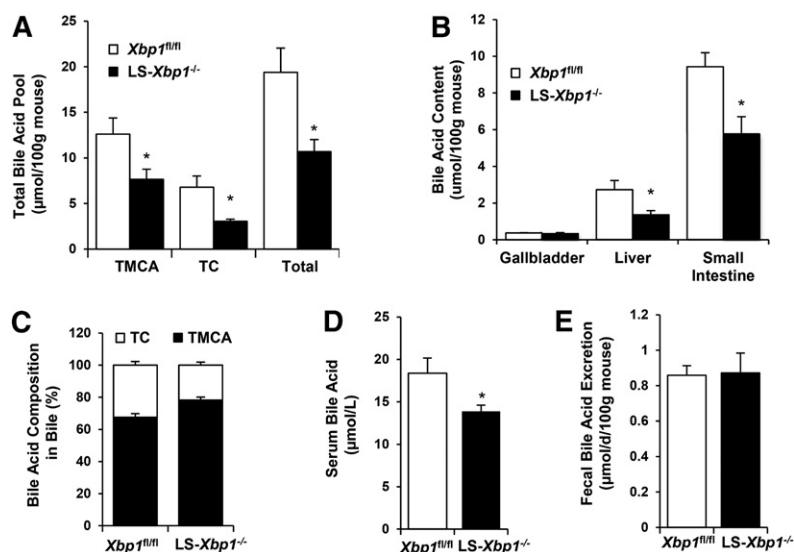


Fig. 1. Bile acid levels in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. Total bile acid pool and bile acid species (A); bile acid content in gallbladder, liver, and small intestine (B); bile acid composition in bile (C); serum bile acid concentration (D); and fecal bile acid excretion (E) in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (*n* = 3–5). The total bile acid pool, TMCA content, TC content, and serum bile acid level were reduced in LS-*Xbp1*^{-/-} mice, while fecal bile acid excretion was unchanged. **P* < 0.05 compared with *Xbp1*^{fl/fl} mice.

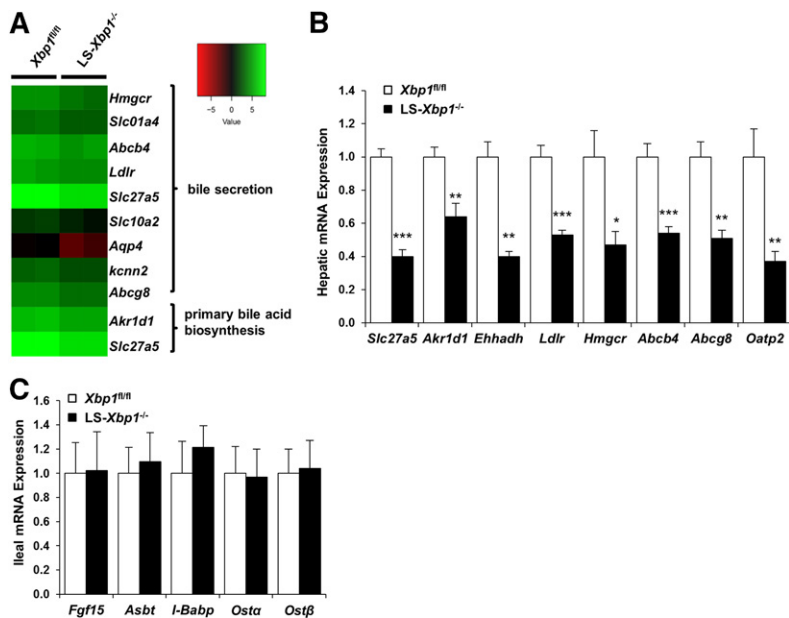


Fig. 2. Hepatic and ileal gene expression of bile acid metabolism genes in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. A: RNA-Seq and KEGG pathway analysis of bile acid metabolism genes. B: Hepatic bile acid metabolism gene expression in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 5). C: Ileal bile acid metabolism gene expression in LS-*Xbp1*^{-/-} mice compared with *Xbp1*^{fl/fl} mice (n = 4–5). Expression of multiple hepatic bile acid metabolism genes was reduced in LS-*Xbp1*^{-/-} mice, while ileal gene expression was unchanged. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with *Xbp1*^{fl/fl} mice.

shows the qPCR measurement performed to confirm the RNA-Seq expression differences of some of these genes. Of note, there is no difference in the hepatic free cholesterol level between *Xbp1*^{fl/fl} mice and LS-*Xbp1*^{-/-} mice (2.33 ± 0.08 mg/g liver vs. 2.38 ± 0.11 mg/g liver, respectively; n = 5).

Cholestyramine is a bile acid binder that induces hepatic CYP7A1 expression and increases hepatic CYP7A1 activity in wild-type mice (18, 19). When LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice were fed chow with 2% cholestyramine for 7 days, hepatic CYP7A1 protein expression increased in LS-*Xbp1*^{-/-} mice, but not in *Xbp1*^{fl/fl} mice, and the CYP7A1 protein expression levels were higher in LS-*Xbp1*^{-/-} mice (Fig. 6A, B). Despite the increased hepatic CYP7A1 protein expression in LS-*Xbp1*^{-/-} mice, serum C4 levels were 63% lower in cholestyramine-fed LS-*Xbp1*^{-/-} mice compared with the cholestyramine-fed *Xbp1*^{fl/fl} mice (*P* < 0.05) (Fig. 6C). In fact, although CYP7A1 protein expression was approximately 8-fold higher in the cholestyramine-fed LS-*Xbp1*^{-/-} mice compared with the chow-fed *Xbp1*^{fl/fl} mice, serum C4 levels still remained 34% lower than the baseline C4 levels in chow-fed *Xbp1*^{fl/fl} mice (*P* < 0.05).

In contrast, when we treated LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice with 2% cholesterol for 7 days, hepatic CYP7A1 protein expression did not change in either genotype of mice (Fig. 6D, E), even though the mRNA expression of the liver

X receptor (LXR) target genes, *Abcg5* and *Abcg8*, was up-regulated in both genotypes (Fig. 6F). However, serum C4 levels increased in response to cholesterol feeding in both genotypes (Fig. 6C, *P* < 0.05). Although, hepatic CYP7A1 protein expression did not change in LS-*Xbp1*^{-/-} mice, cholesterol feeding raised the serum C4 levels up to the baseline levels present in the chow-fed *Xbp1*^{fl/fl} mice. Cholesterol feeding, but not induction of hepatic CYP7A1 protein levels with cholestyramine, increased the serum C4 levels of LS-*Xbp1*^{-/-} mice. Consistent with these findings, cholesterol feeding increased the total bile acid pool size in LS-*Xbp1*^{-/-} mice and corrected the genotypic differences in total bile acid pool size between *Xbp1*^{fl/fl} mice and LS-*Xbp1*^{-/-} mice (Fig. 7).

DISCUSSION

Bile acids are synthesized in the liver and regulate metabolism in both normal physiology and pathophysiologic states. Cholestasis and other hepatic disorders can induce ER stress and activate the UPR (5, 20–22). Although UPR activation is a compensatory and protective response to ER stress, the hepatic IRE1α/XBP1 pathway of the UPR also regulates hepatic fatty acid and lipoprotein metabolism and secretion (9, 10, 23). Bile acids are biliary lipids that

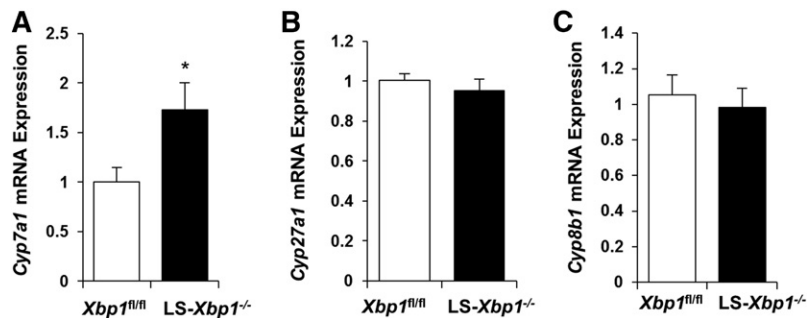


Fig. 3. Hepatic bile acid synthesis gene expression in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. Hepatic gene expression of *Cyp7a1* (A), *Cyp27a1* (B), and *Cyp8b1* (C) was measured in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 9). *Cyp7a1* expression was increased in LS-*Xbp1*^{-/-} mice, while expression of *Cyp27a1* and *Cyp8b1* was similar. **P* < 0.05 compared with *Xbp1*^{fl/fl} mice.

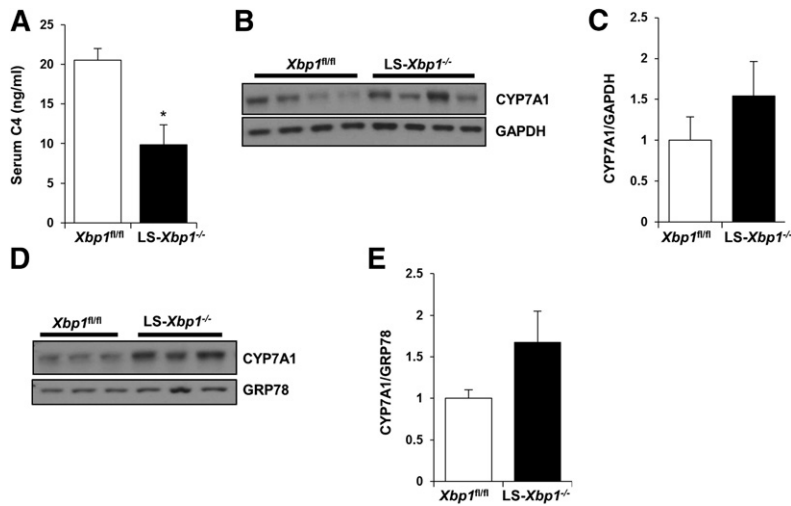


Fig. 4. Hepatic CYP7A1 protein expression and serum C4 levels in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. A: Serum C4 levels were measured to determine CYP7A1 synthetic activity in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 3–4). Representative Western blot (B) and densitometry quantification (C) of CYP7A1 protein expression in liver homogenates from LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 4). GAPDH was used as a loading control. Representative Western blot (D) and densitometry quantification (E) of CYP7A1 protein expression in microsomes from LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 3). GRP78 was used to confirm microsome enrichment and as a loading control. Although CYP7A1 protein expression in whole liver homogenates and microsomes was similar in both genotypes, CYP7A1 synthetic activity was reduced in LS-*Xbp1*^{-/-} mice. **P* < 0.05 compared with *Xbp1*^{fl/fl} mice.

regulate many hepatic lipid metabolic and transport processes. Therefore, we investigated the role of the IRE1α/XBP1 pathway in bile acid metabolism.

We initially determined that liver-specific deletion of *Xbp1* resulted in a reduced bile acid pool and decreased in vivo bile acid synthesis. Hepatic and ileal bile acid content was reduced, and TC and TMCA content in the bile acid pool was also lower in LS-*Xbp1*^{-/-} mice than in *Xbp1*^{fl/fl}

littermate controls. Fecal bile acid excretion was similar in both genotypes, despite the different bile acid pool size. The bile acid cycling time and fecal loss per cycle are unknown in LS-*Xbp1*^{-/-} mice, which could account for the observed fecal excretion. Fecal bile acid loss was low, and we cannot exclude the possibility that small differences in fecal loss of bile acid were not detected. Nonetheless, this does not detract from the significant findings of reduced

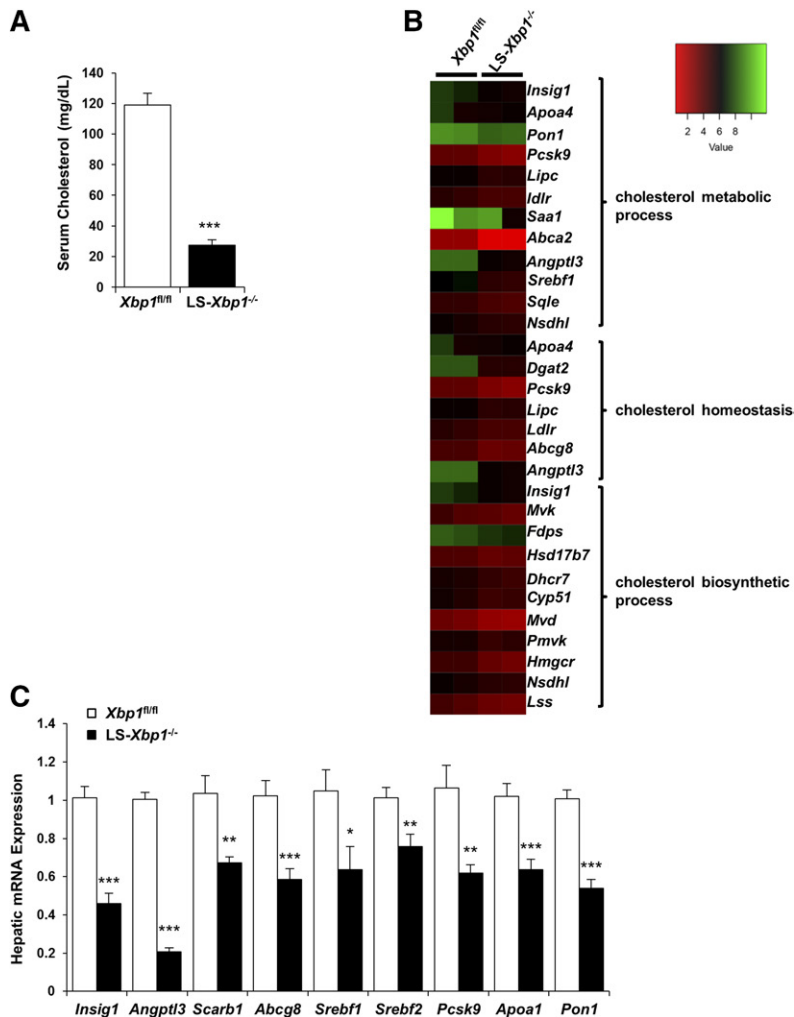


Fig. 5. Serum cholesterol and hepatic gene expression of cholesterol metabolic genes in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice. A: Serum cholesterol was measured in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 4). B: RNA-Seq and GO pathway analysis of cholesterol metabolism genes. C: qPCR validation of hepatic cholesterol metabolic gene expression in LS-*Xbp1*^{-/-} and *Xbp1*^{fl/fl} mice (n = 9). Serum cholesterol and hepatic expression of several cholesterol metabolic genes were reduced in LS-*Xbp1*^{-/-} mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with *Xbp1*^{fl/fl} mice.

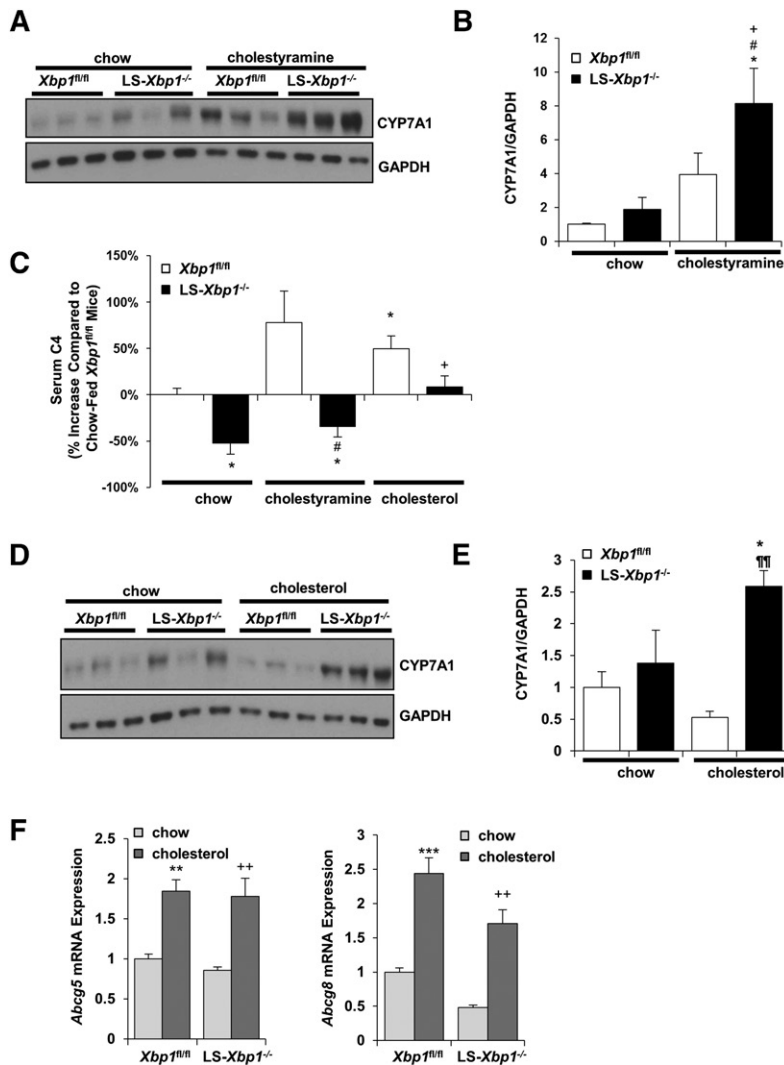


Fig. 6. Serum C4 levels and hepatic CYP7A1 protein expression in *LS-Xbp1^{-/-}* and *Xbp1^{fl/fl}* mice fed chow, cholestyramine, or cholesterol. *LS-Xbp1^{-/-}* and *Xbp1^{fl/fl}* mice were fed chow, chow with cholestyramine (2% w/w), or chow with cholesterol (2% w/w) for 7 days. Western blot (A) and densitometry quantification (B) of hepatic CYP7A1 protein expression in chow-fed and cholestyramine-fed mice. GAPDH was used as a loading control. C: Changes in serum C4 levels in *LS-Xbp1^{-/-}* and *Xbp1^{fl/fl}* mice fed chow, cholestyramine, and cholesterol. Data are expressed as percent increase compared with chow-fed *Xbp1^{fl/fl}* mice. Western blot (D) and densitometry quantification (E) of hepatic CYP7A1 protein expression in chow-fed mice and cholesterol-fed mice. GAPDH was used as a loading control. F: Hepatic gene expression of *Abcg5* and *Abcg8* was measured. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with chow-fed *Xbp1^{fl/fl}* mice. + $P < 0.05$, ++ $P < 0.01$ compared with chow-fed *LS-Xbp1^{-/-}* mice. # $P < 0.05$ compared with cholestyramine-fed *Xbp1^{fl/fl}* mice. ¶ $P < 0.01$ compared with cholesterol-fed *Xbp1^{fl/fl}* mice. $n = 3-5$.

serum C4 levels and decreased bile acid synthesis in *LS-Xbp1^{-/-}* mice.

RNA-Seq (with confirmatory qPCR) and KEGG pathway analysis demonstrate that *LS-Xbp1^{-/-}* mice had reduced expression of several hepatic genes involved in primary bile acid biosynthesis and bile secretion pathways. Many of these genes are regulated by the farnesoid X receptor, suggesting that their reduced expression may be either a primary effect of *Xbp1* deletion or a secondary effect due to the reduced bile acid pool. Hepatic mRNA expression of the major bile acid synthetic enzymes, *Cyp7a1*, *Cyp27a1*, and *Cyp8b1*, was not reduced and, therefore, was not the primary cause of the reduced bile acid pool. In fact, *Cyp7a1* gene expression increased, although protein expression was unchanged. Despite the changes in the bile acid pool, hepatic gene expression of *Shp* and ileal expression of *Fgf15* and other ileal bile acid metabolic genes remained unchanged. Hepatic gene expression of the bile acid synthetic pathway genes, *Slc27a5* and *Akr1d1*, was reduced in *LS-Xbp1^{-/-}* mice. Although human mutations of these genes have been reported to be associated with rare cholestatic liver diseases (24–26), these enzymes are not believed to be rate-limiting for bile acid synthesis. Moreover, *Slc27a5*

and *Akr1d1* are downstream to the production of C4 in the bile acid synthesis pathway. If the reduced bile acid synthesis was due to these enzymes, serum C4 levels should not be reduced and may in fact be elevated.

Serum C4 levels indicated that in vivo CYP7A1 synthetic activity was reduced in *LS-Xbp1^{-/-}* mice, while Western blotting demonstrated that both hepatic and microsomal CYP7A1 protein levels were unchanged. CYP7A1 is not known to be posttranslationally regulated by phosphorylation and endogenous physiologic inhibitors for this enzyme have not been described. Our data on microsomal CYP7A1 protein expression also do not suggest that CYP7A1 protein in the ER was reduced. When CYP7A1 protein expression was induced by cholestyramine feeding in *LS-Xbp1^{-/-}* mice, serum C4 levels did not increase and remained lower than those in *Xbp1^{fl/fl}* mice, even though CYP7A1 protein levels were 8-fold higher than the CYP7A1 levels in chow-fed *Xbp1^{fl/fl}* mice. Therefore, increasing CYP7A1 protein level by cholestyramine could not rescue CYP7A1 synthetic activity.

RNA-Seq data demonstrate that genes involved in cholesterol metabolism are downregulated in *LS-Xbp1^{-/-}* mice relative to *Xbp1^{fl/fl}* mice, which could cause reductions of

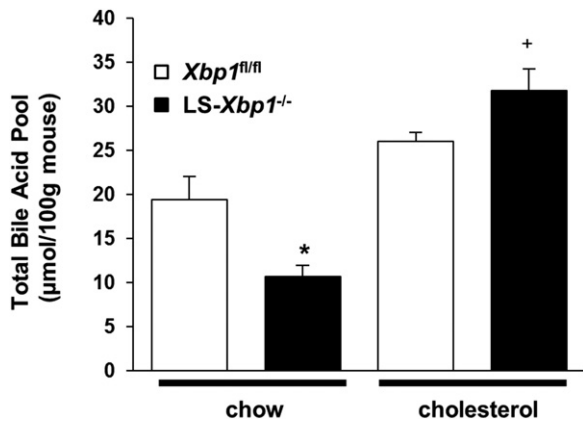


Fig. 7. Total bile acid pool in *Xbp1^{fl/fl}* and *LS-Xbp1^{-/-}* mice after cholesterol feeding. *LS-Xbp1^{-/-}* and *Xbp1^{fl/fl}* mice were fed chow or chow with cholesterol (2% w/w) for 1 week. The total bile acid pool was measured. Cholesterol feeding corrected the total bile acid pool in *LS-Xbp1^{-/-}* mice. * $P < 0.05$ compared with chow-fed *Xbp1^{fl/fl}* mice ($n = 4-5$). ⁺ $P < 0.05$ compared with chow-fed *LS-Xbp1^{-/-}* mice ($n = 4-5$).

cholesterol biosynthesis and intracellular trafficking. We observed decreases in serum cholesterol levels in the *LS-Xbp1^{-/-}* mice. Therefore, we verified the hypocholesterolemia in this albumin-Cre-driven XBP1 deletion model and it is consistent with a previous report of hepatic XBP1-deficient mice using Mx1-Cre with poly (I:C) administration (9). Mice lacking hepatic XBP1 have nearly absent LDL levels and a lesser reduction of serum HDL (9, 10). Because cholesterol is the precursor for bile acid synthesis, alterations in hepatic cholesterol metabolism in *LS-Xbp1^{-/-}* mice may affect bile acid synthesis and the bile acid pool.

Feeding rodents a diet supplemented with cholesterol increases the specific activity (picomoles per milligram protein per minute) of hepatic CYP7A1 and enriches hepatic microsomal cholesterol content (27, 28). When we fed *LS-Xbp1^{-/-}* mice diets supplemented with 2% cholesterol for 1 week, hepatic CYP7A1 protein expression did not change; however, CYP7A1 synthetic activity increased. In fact, serum C4 levels in the cholesterol-fed *LS-Xbp1^{-/-}* mice increased to the baseline levels present in chow-fed *Xbp1^{fl/fl}* mice. Thus, cholesterol feeding rescued the diminished CYP7A1 activity in *LS-Xbp1^{-/-}* mice, while the 8-fold increases of CYP7A1 protein induced by cholestyramine feeding had no effect.

Cholesterol is the substrate of CYP7A1 enzymatic activity. Gene expression of cholesterol biosynthesis and metabolic pathways are downregulated in *LS-Xbp1^{-/-}* mice; and dietary feeding of cholesterol restores CYP7A1 activity and completely corrects the bile acid pool size phenotype. Therefore, the reduction in CYP7A1 activity in *LS-Xbp1^{-/-}* mice may be due to decreased cholesterol availability to CYP7A1. Hepatic free cholesterol levels did not differ between *Xbp1^{fl/fl}* and *LS-Xbp1^{-/-}* mice, consistent with a previous report (9). However, the ER cholesterol content and the rate of cholesterol delivery to the ER in *LS-Xbp1^{-/-}* mice are not known. In rodents, cholesterol feeding can increase hepatic CYP7A1 expression through LXR signaling (29–31).

However, we did not observe increased expression of CYP7A1 after cholesterol feeding in *Xbp1^{fl/fl}* mice, even though other LXR target genes, such as *Abcg5* and *Abcg8*, were upregulated. Although this finding could be due to altered mouse genetics (wild-type vs. *Xbp1^{fl/fl}* in C57BL/6 background strains), CYP7A1 expression in response to chronic cholesterol feeding may not always be increased under certain cholesterol feeding conditions (32).

This study demonstrates a novel role for hepatic XBP1 in the regulation of bile acid metabolism. Mice lacking hepatic *Xbp1* have a reduced bile acid pool due to reduced CYP7A1 bile acid synthetic activity. In contrast to many studies on bile acid metabolism, the changes in hepatic CYP7A1 synthetic activity are not primarily due to changes of its level of expression, but are regulated by a cholesterol-responsive process. These data may have implications on hepatic and systemic lipid metabolism, as well as in the pathogenesis and treatment of cholestatic and fatty liver disorders. **BB**

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