Hepatic Deletion of X-Box Binding Protein 1 in FXR Null Mice Leads to Enhanced Liver Injury

Xiaoying Liu1, Mahmoud Khalafalla1, Chuhan Chung2, Yevgeniy Gindin2, Susan Hubchak1, Brian LeCuyer1, Alyssa Kriegermeier1, Danny Zhang3, Wei Qiu4, Xianzhong Ding5, Deyu Fang6, and Richard Green7

1Division of Gastroenterology and Hepatology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA; 2Gilead Sciences, Inc, Foster City, CA, USA; 3Division of Gastroenterology, Department of Pediatrics, Feinberg School of Medicine, Hepatology and Nutrition at Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University, Chicago, IL, USA; 4Department of Surgery & Department of Cancer Biology, Loyola University Chicago, Maywood, IL, USA; 5Department of Pathology, Stritch Medicine School of Loyola University Chicago, Maywood, IL, USA; 6Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Abstract FXR regulates bile acid metabolism, and FXR null (Fxr−/−) mice have elevated bile acid levels and progressive liver injury. The inositol-requiring enzyme 1α/X-box binding protein 1 (XBP1) pathway is a protective unfolded protein response pathway activated in response to endoplasmic reticulum stress. Here, we sought to determine the role of the inositol-requiring enzyme 1α/XBP1 pathway in hepatic bile acid toxicity using the Fxr−/− mouse model. Western blotting and quantitative PCR analysis demonstrated that hepatic XBP1 and other unfolded protein response pathways were activated in 24-week-old Fxr−/− compared with 10-week-old Fxr−/− mice but not in WT mice. To further determine the role of the liver XBP1 activation in older Fxr−/− mice, we generated mice with whole-body FXR and liver-specific XBP1 double KO (DKO, Fxr−/− Xbp1LKO) and Fxr−/− Xbp1LKO single KO (SKO) mice and characterized the role of hepatic XBP1 in cholestatic liver injury. Histologic staining demonstrated increased liver injury and fibrosis in DKO compared with SKO mice. RNA sequencing revealed increased gene expression in apoptosis, inflammation, and cell proliferation pathways in DKO mice. The proapoptotic C/EBP-homologous protein pathway and cell cycle marker cyclin D1 were also activated in DKO mice. Furthermore, we found that total hepatic bile acid levels were similar between the two genotypes. At age 60 weeks, all DKO mice and no SKO mice spontaneously developed liver tumors. In conclusion, the hepatic XBP1 pathway is activated in older Fxr−/− mice and has a protective role. The potential interaction between XBP1 and FXR signaling may be important in modulating the hepatocellular cholestatic stress responses.

Supplementary key words bile acids • bile salts • apoptosis • cell signaling • inflammation • unfolded protein response • nuclear receptor • cholestatic liver injury • liver tumor • inositol-requiring enzyme 1α

FXR is a bile acid-activated transcription factor that plays a central role in bile acid metabolism by regulating the expression of genes that are involved in bile acid synthesis and hepatocellular transport (1). Mice with FXR deletion have elevated serum bile acid levels and total bile acid pool size and are susceptible to bile acid-induced cholestatic liver injury because of an attenuated ability to downregulate bile acid synthesis and excrete bile acids (2, 3). FXR null mice develop progressive liver inflammation and fibrosis as they age and spontaneously develop liver tumors by 15 months of age (3–5). In contrast, FXR agonists may regulate bile acid homeostasis and provide benefit to patients with cholestatic liver diseases. The FXR agonist obeticholic acid has been approved by the Food and Drug Administration for primary biliary cholangitis (PBC), and FXR agonists are currently in clinical trials for the treatment of other cholestatic and metabolic liver diseases (6).

Endoplasmic reticulum (ER) stress occurs when ER homeostasis is perturbed because of excessive accumulation of unfolded/misfolded proteins. The liver is prone to ER stress given its large requirement for protein synthesis and to support its many metabolic and secretory functions. The unfolded protein response (UPR), comprising inositol-requiring enzyme 1α (IRE1α), protein kinase R-like ER kinase, and activating transcription factor 6 signaling pathways, is a protective cellular pathway activated in response to ER stress. The hepatic UPR is important in the pathogenesis of several liver diseases, including viral hepatitis, nonalcoholic fatty liver disease, alpha-1 antitrypsin deficiency, alcohol-induced liver disease, and ischemia-reperfusion injury (7, 8). The IRE1α/X-box binding protein 1 (XBP1) pathway is evolutionarily conserved, being present in...
both yeast and mammals. The active phosphorylated form of IRE1α causes an atypical splicing of XBP1 mRNA, resulting in the production of transcriptionally active XBP1 spliced (XBPs) that regulates downstream target genes such as the chaperone ERdj4 to assist protein folding and EDEM, which is involved in ER-associated degradation of accumulated proteins (9). Whole-body XBPI null mice are embryonically lethal (10), and liver-specific XBPI-deficient mice are unable to adequately resolve pharmacologically induced hepatic ER stress (11). Mice with hepatic XBPI deletion also demonstrate decreased serum cholesterol, triglycerides, and total bile acid pool size (12, 13).

ER stress and an inadequate compensatory UPR activation have been implicated in many liver diseases, including cholestatic liver disorders (14, 15). ER stress downregulates FXR expression in the liver, and FXR inhibits ER stress-induced NLRP3 inflammasome activation (16). Thus, an inadequate UPR may lead to sustained ER stress, reduced FXR expression, and accentuated inflammatory responses. We have previously shown that FXR agonists induce hepatic XBPIs activation, and hepatic XBPIs is induced in bile acid feeding and bile duct ligation models of cholestasis (17). However, the crosstalk between liver UPR and FXR signaling, especially in the setting of cholestatic liver injury, remains unclear. In this study, we developed novel FXR null mice with liver-specific deletion of XBPI and utilized these mice to investigate the role of the UPR in bile acid injury.

MATERIALS AND METHODS

Animal use and treatment

C57BL/6j WT and whole-body FXR KO (Fxr−/−) mice were obtained from Jackson Laboratory, and colonies were established. C57BL/6j–Xbp1fl/fl mice with loxP sites flanking exon 2 of the Xbp1 gene were kindly provided by Dr Laurie H. Glumcher (Cornell University, Ithaca, NY) (12). These mice were bred with C57BL/6–Albumin-Cre mice (Jackson Laboratory, Bar Harbor, ME) to generate liver-specific XBP1 KO (Xbp1−/−/LKO) mice. Fxr−/−/Xbp1fl/fl double KO (DKO) and Fxr−/−/Xbp1fl/fl single KO (SKO) mice were generated by crossing Fxr−/−/ mice with Xbp1fl/fl and Xbp1fl/fl mice. All the mice were house-housed with aspen woodchip bedding on a 14-h light, 10-h dark cycle with free access to standard mouse Chow diet (Teklad 7912) and water. Male mice were fasted for 14-h light, 10-h dark cycle with free access to standard mouse

Histology

Livers were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned. Immunohistological staining with H&E, Sirius red, TUNEL, and Ki67 was performed by Northwestern University Mouse Histology and Phenotyping Laboratory. The quantification of Sirius red was performed using ImageJ (National Institutes of Health). The number of TUNEL stain-positive cells and Ki67-positive cells was counted from 9 to 10 random fields per slide. The Ishak inflammation and fibrosis scores (18) were assessed by two investigators (R.G. and A.K.) blinded to the study groups. The H&E staining of liver slides of 60-week-old mice was blindly assessed by a pathologist (X.D.).

Bile acid analysis

Serum bile acid levels were measured colorimetrically according to the manufacturer’s protocol (GenWay Biotech, Inc, San Diego, CA). Hepatic bile acids were extracted and quantified by LC-MS/MS analysis using the Biocrates Bile Acids Kit. The extraction and quantification analysis were performed using the Duke University Proteomics Core (Durham, NC). Hydrophobicity index was calculated using the method of Heuman (19).

RNA extraction and quantitative PCR

Total RNA was extracted from frozen livers using Trizol according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, CA), and complementary DNA (cDNA) was made with qScript cDNA Synthesis Kit (Quanta Bioscience, Gaithersburg, MD). Quantitative real-time PCR was then performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) with the Applied Biosystems Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative expression of the gene of interest was calculated by the ΔΔCt method using 18s as a reference gene. All primers were synthesized by Sigma (St. Louis, MO). Primer sequences are included in Supplemental Table S1.

RNA sequencing

Stranded mRNA sequencing (mRNA-Seq) was conducted in the Northwestern University NUSeq Core Facility. Briefly, total RNA samples were checked for quality using RINs generated from Agilent Bioanalyzer 2100. RNA quantity was determined with Qubit fluorometer. The Illumina TruSeq Stranded mRNA Library Preparation Kit was used to prepare sequencing libraries from 1 μg of high-quality RNA samples (RIN >7) using the methodology of manufacturer. This procedure includes mRNA purification and fragmentation, cDNA synthesis, 3' end adenylation, Illumina adapter ligation, and library PCR amplification and validation. Illumina HiSeq 4000 sequencer was used to sequence the libraries with the production of single-end 50 bp reads at the depth of 20–25 M reads per sample. Gene expression was quantified from RNA sequencing (RNA-Seq) reads with Salmon (20) using mouse reference genome version GRCh38 obtained from GENCODE (21). Read counts were converted to counts-per-million using edgeR (22). Gene set enrichment analysis was accomplished by calculating a normalized enrichment score, as implemented in clusterProfiler (23), against Molecular Signatures Database hallmark gene set collection (24) with a ranked gene list constructed with all the genes. A P-adj less than 0.05 was considered significant. The list of genes in each

Serum biochemical analysis

Serum alanine aminotransferase (ALT) was measured by using a spectrophotometric assay according to the manufacturer’s protocol (Teco Diagnostics, Anaheim, CA).
Western blotting

Protein extraction was made from frozen liver tissues using T-Per protein extraction reagent (Thermo Fisher Scientific, Waltham, MA) or nuclear extraction kit (Cayman Chemical, Ann Arbor, Michigan) with protease inhibitor cocktail (MilliporeSigma, Burlington, MA) and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). Protein quantification and immunoblotting were performed as described previously (25). The antibodies used in this study are listed in supplemental Table S3.

Statistics

Data are shown as means ± SEM and graphed with Prism (GraphPad Software, Inc, San Diego, CA). Comparison between two groups was performed by two-tailed Student’s t-test. Comparison between more than two groups was performed by one-way ANOVA or two-way ANOVA with a Tukey or Bonferroni post hoc test, respectively. Statistical significance was defined as P values of less than 0.05.

RESULTS

Hepatic IRE1α/XBP1 pathway was activated in 24-week-old Fxr−/− mice

Fxr−/− mice develop spontaneous and progressive liver injury and fibrosis (4). To establish whether there was UPR activation indicative of ER stress in Fxr−/− mice as they age, we first examined the activation of the UPR pathways in the livers of WT and Fxr−/− mice at 10 weeks and 24 weeks of age. As shown in Fig. 1A, the nuclear protein expression of XBP1s was significantly upregulated in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (7.6-fold, P < 0.05). Although there was a variability in XBP1s protein expression in 24-week-old Fxr−/− mice, no phenotypic differences were observed within the group. The hepatic mRNA expression of Xbp1s and its downstream target genes E Rodj4 and Edem was significantly higher in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (3-fold, P < 0.001; 21-fold, P < 0.001; and 1.4-fold, P < 0.05, respectively, Fig. 1B). The protein expression of the XBP1s upstream activator phosphor-ylated IRE1α was also increased in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (26-fold, P < 0.05; Fig. 1A). In contrast, there was no significant change in hepatic nuclear XBP1s protein level, mRNA expression of Xbp1s, E Rodj4, and Edem, and IRE1α phosphorylation when comparing 10-week-old versus 24-week-old WT mice (Fig. 1A, B). The nuclear XBP1s protein expression was 7-fold higher in 24-week-old Fxr−/− mice compared with 24-week-old WT mice (P < 0.005; Fig. 1C). These data demonstrate that the hepatic IRE1α/XBP1 pathway was activated in 24-week-old compared with 10-week-old Fxr−/− mice but not in WT mice of similar ages. This increase of nuclear XBP1s protein expression was maintained in 60-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (4-fold increase, P < 0.05; Fig. 1D), suggesting that the activation of XBP1s was sustained in Fxr−/− mice as they age.

C/EBP-homologous protein (CHOP) is a transcription factor induced by ER stress and plays an important role in ER stress-induced apoptosis (26). The nuclear protein expression of CHOP was increased in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (11.8-fold, P < 0.01) but not in WT mice (Fig. 2A). The gene expression of the CHOP downstream target death receptor 5 (DR5) significantly increased in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (1.9-fold, P < 0.01) but not in WT mice (Fig. 2B). Gene expression of the CHOP upstream activator, activating transcription factor 4 (ATF4), was similarly increased in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (1.5-fold, P < 0.01) but not in WT mice (Fig. 2B). The hepatic gene expression of Atp6 and its downstream targets, Bip and Hyoul, was also significantly higher in 24-week-old Fxr−/− mice compared with 10-week-old Fxr−/− mice (1.3-fold, P < 0.05; 2.3-fold, P < 0.05; and 2.7-fold, P < 0.01, respectively) but not in WT mice (Fig. 2B). Together, these data indicate that ER stress and UPR activation occurred in older Fxr−/− mice but not WT mice.

Hepatic deficiency of XBP1 promoted liver injury and fibrosis in Fxr−/− mice at 10 weeks of age

To determine the role of hepatic XBP1 activation in Fxr−/− mice, we developed colonies of Fxr−/−/liver-specific XBP1-KO DKO mice and control Fxr−/−/XBP1-flox SKO mice (supplemental Fig. S1) and characterized their phenotypes at 10 weeks of age. DKO mice had 2.5-fold higher serum ALT levels compared with SKO mice, being 209.1 ± 16.6 versus 83.1 ± 17.2 U/l, P < 0.001 (Fig. 3A). H&E staining showed mild inflammation with a strong trend toward higher Ishak inflammation scores in DKO mice (P = 0.06) (Fig. 3B, C). DKO mice also had significantly greater liver fibrosis compared with SKO mice as evidenced by increased Sirius red staining and higher Ishak fibrosis scores (Fig 3B, D, E). In addition, more TUNEL stain-positive cells were observed in DKO mice livers compared with SKO mice (Fig. 3B, F), consistent with greater liver injury in DKO mice. These data indicate that Fxr−/− mice lacking liver XBP1 have more liver injury and increased fibrosis.

RNA-Seq demonstrated increased gene expression in inflammation, apoptosis, and proliferation pathways in DKO mice

In order to delineate the mechanisms by which hepatic Xbp1 deletion promotes liver injury and fibrosis, we performed RNA-Seq on 10-week-old DKO and SKO mice livers. The first principal component of the RNA-Seq data accounted for 76% of variance in hepatic gene expression (Fig. 4A). Gene set enrichment analysis using Hallmark gene set collection revealed that multiple
immune-related pathways (inflammatory_response, TNFα_signaling_via_NFκb, IL6_JAK_STAT3_signaling, and IL2_STAT5_signaling) were upregulated in DKO mice compared with SKO mice (Fig. 4B). The apoptosis pathway was also upregulated in DKO mice. Cell proliferation related (G2M_checkpoint, E2F_targets, p53_pathway, and Myc_target_v2) and angiogenesis pathways were similarly upregulated in DKO mice compared with SKO mice. These results were consistent with the more injurious phenotype of DKO mice. Protein secretion, bile acid metabolism, fatty acid metabolism, and xenobiotic metabolism pathways were downregulated in DKO mice, which is consistent with previous reports in hepatic-specific XBPI KO mice (13, 25). Hepatic expression of genes in the adipogenesis pathway was also downregulated in the DKO mice,
although the gene expression of \textit{Pparg}, a key regulator of adipogenesis, was similar between SKO and DKO mice livers (Fig. 4C). Therefore, the transcriptome profile of the DKO mice is consistent with the enhanced tissue injury that occurs in the absence of a functional liver XBP1 pathway in \textit{Fxr} \textsuperscript{−/−} mice.

The proapoptotic CHOP protein was induced in DKO mice

Since both TUNEL staining and RNA-Seq data demonstrated increased apoptosis in DKO mice compared with SKO mice, and CHOP mediates ER stress-induced cell apoptosis, we examined the hepatic expression of CHOP in 10-week-old SKO and DKO mice. As shown in Fig. 5A, hepatic gene expression of \textit{Chop} and its downstream target \textit{Dr5} was 7.7-fold ($P < 0.001$) and 3.8-fold ($P < 0.001$) higher in DKO compared with SKO mice, respectively. The proapoptotic protein BCL2-associated X (BAX) was also upregulated in DKO compared with SKO mice (1.9-fold, $P < 0.001$; Fig. 5B). To evaluate if these changes were due, at least in part, to hepatic XBPI deletion, we examined the activation of the CHOP pathway in \textit{Xbp1} \textsuperscript{LKO} and \textit{Xbp1} \textsuperscript{fl/fl} mice. \textit{Xbp1} \textsuperscript{LKO} mice had higher hepatic \textit{Chop} and \textit{Dr5} gene expression as well as higher BAX protein expression compared with \textit{Xbp1} \textsuperscript{fl/fl} mice (Fig. 5A, B), similar to the findings in DKO mice compared with SKO mice.

Hepatic bile acid concentration and species in SKO and DKO mice

We have previously shown that hepatic XBPI deficiency modulates bile acid metabolism (13); therefore, we investigated the serum and hepatic bile acid content in 10-, 24-, and 40-week-old DKO and SKO mice. Serum bile acid levels were similar between SKO and DKO at all ages (Fig. 6A). Using LC-MS/MS, we determined that there was no difference in total hepatic bile acid levels between SKO and DKO at 10, 24, or 40 weeks of age (Fig. 6B). The hydrophobicity index was similar in SKO and DKO mice (Fig. 6C). The majority of the hepatic bile acids was taurine-conjugated bile acids, including taurocholic acid, taurodeoxycholic acid, and...
tauromuricholic acid (Fig. 6D). The concentration of hepatic unconjugated \( \beta \)-muricholic acid and deoxycholic acid was significantly higher in 10-week-old DKO compared with 10-week-old SKO mice \((8.18 \pm 1.98 \text{ vs. } 2.70 \pm 0.91 \text{ nmol/g liver, } P < 0.05; 0.38 \pm 0.07 \text{ vs. } 0.09 \pm 0.07 \text{ nmol/g liver, } P < 0.05, \text{ respectively; Table 1})\). There was no difference in the concentration of other measured bile acids between SKO and DKO mice at any ages. The hepatic mRNA expression of the bile acid transporter \( \text{Bsep} \) was lower in DKO compared with SKO mice at 10 and 40 weeks of age but not at 24 weeks. Hepatic \( \text{Ntcp} \) gene expression was also lower in DKO mice but only at 10 weeks of age (Fig. 6E). Hepatic expression of the bile acid synthesis genes \( \text{Cyp7a1}, \text{Cyp8b1}, \) and \( \text{Cyp2c70} \) was similar in 10-week-old SKO and DKO mice, whereas \( \text{Cyp27a1} \) gene expression was slightly deceased in DKO compared with SKO mice (Fig. 6F). Hepatic CYP7A1 protein expression was significantly higher in DKO compared with SKO mice despite similar gene expression (Fig. 6G).

**DKO mice had greater liver injury and fibrosis compared with SKO mice at 24 and 40 weeks of age**

In order to determine whether the enhanced liver injury in DKO mice persists at older ages, we next compared DKO and SKO mice at 24 and 40 weeks of age. H&E staining and Ishak scores indicated increased liver inflammation in DKO mice compared with SKO at 40 weeks old, without a genotype-specific difference at 24 weeks (Fig. 7A–C). Serum ALT levels remained significantly higher in DKO compared with SKO mice at 24 weeks of age \((127.2 \pm 13.6 \text{ vs. } 92.8 \pm 9.9 \text{ U/l, } P = 0.08)\). At age 24 and 40 weeks, DKO mice had more fibrosis than SKO mice, evident by both Sirius red staining quantification and Ishak fibrosis scores (Fig. 7A–C). Thus, the absence of hepaticXBPI leads to persistent liver injury in the DKO mice.

**Hepatic deficiency of XBPI promoted liver tumorigenesis in 60-week-old \( \text{Fxr}^{-/-} \) mice**

Since RNA-Seq studies revealed that multiple cell proliferation pathways were upregulated in DKO mice, we aged cohorts of DKO and SKO mice to 60 weeks old to assess the effect of hepatic \( \text{Xbp1} \) deletion on tumor development in \( \text{Fxr}^{-/-} \) mice. We did not observe any liver tumor formation in \( \text{Fxr}^{-/-} \) mice \((n = 4), \text{SKO mice} \) \((n = 10), \) or \( \text{Xbp1} \) \( \text{LKO} \) mice \((n = 7) \) at age 58–60 weeks. In contrast, six of six \((100\%) \) DKO mice spontaneously developed multiple liver tumors at 60 weeks of age (Fig. 8A). Tumor nodules showed diffuse hepatocellular proliferation with increased hepatocyte density, loss of normal hepatic architecture, and lack of portal tracts. Tumors exhibited marked nuclear crowding, increased nuclear-to-cytoplasmic ratio, dense nuclear chromatin, and increased proliferation index. The H&E histologic findings were consistent with hepatocellular adenomas. The liver to body weight ratio was significantly higher in DKO mice compared with SKO mice (Fig. 8B). Ki67 staining showed greater than 30-fold increase in DKO mice compared with SKO mice (Fig. 8A, C), whereas serum ALT was similar between the two groups (Fig. 8D). No hepatic tumors were observed in DKO or SKO mice at 10, 24, or 40 weeks of age. The hepatic expression of the cell cycle protein cyclin D1 was
upregulated in DKO mice compared with SKO mice at 10 and 24 weeks of ages but was not significantly different at age 40 weeks (Figs. 8E and S2). The hepatic cyclin D1 protein expression was similar between Xbp1LKO mice and Xbp1fl/fl mice (Fig. 8F).

**DISCUSSION**

The nuclear receptor FXR plays a central role in hepatocellular physiology and pathophysiology by regulating several important hepatic metabolic and transporter processes as well as eliciting cellular protective functions in immunologic and nonparenchymal cells. FXR null mice are a well-characterized murine model of bile acid toxicity and cholestasis since intrahepatic bile acid concentrations are elevated. Decreased expression of FXR has been associated with aging, and diseases such as PBC, progressive familial intrahepatic cholestasis, and hepatocellular carcinoma (HCC) (27–30). It has been previously reported that FXR-deficient mice develop spontaneous progressive inflammation, steatosis, fibrosis, and HCC, which mimics human HCC progression (4). In this study, we...
Fig. 6. The hepatic bile acid profiles in Foxo1/−Xbp1fl/fl and Foxo1/−Xbp1LKO mice at 10, 24, and 40 weeks of age. A: Serum bile acid (n = 4–6 in each group). B–D: Hepatic bile acid analysis was performed with LC-MS/MS (n = 4 in each group). B: Total hepatic bile acid concentration. C: Hepatic bile acid hydrophobicity index. D: Hepatic bile acid composition. E: Hepatic mRNA expression of bile acid transporter, Bsep and Ntcp, was measured in SKO and DKO mice. *P < 0.05. F: Hepatic mRNA expression of bile acid synthesis genes was measured in 10-week-old SKO (n = 10) and DKO (n = 12) mice. *P < 0.05. G: Western blotting and densitometry analysis demonstrating hepatic CYP7A1 protein expression in 10-week-old male SKO (n = 10) and DKO (n = 12) mice. Each lane represents a pool of two mice. GAPDH was used as a loading control. ***P < 0.001.
TABLE 1. Hepatic bile acid species and concentration (nmol/g liver) in FXR+/−Xbp1fl/fl (SKO) and FXR−/−Xbp1ΔKO (DKO) mice at 10, 24, and 40 weeks of age

| Bile acid | 10 weeks | 24 weeks | 40 weeks |
|-----------|----------|----------|----------|
|            | SKO      | DKO      | SKO      | DKO      |
| CA         | 1.65 ± 1.07 | 5.53 ± 2.66 | 1.43 ± 0.75 | 3.15 ± 1.11 |
| DCA        | 0.09 ± 0.07 | 0.38 ± 0.07 | 0.09 ± 0.07 | 0.09 ± 0.07 |
| GCA        | 0.60 ± 0.41 | 0.68 ± 0.14 | 0.08 ± 0.07 | 0.60 ± 0.27 |
| MCA(a)     | 0.09 ± 0.07 | 0.16 ± 0.05 | 0.53 ± 0.23 | 0.31 ± 0.25 |
| MCA(b)     | 2.70 ± 0.91 | 8.18 ± 1.98 | 9.60 ± 4.59 | 17.33 ± 7.23 |
| MCA(o)     | 0.60 ± 0.21 | 1.05 ± 0.26 | 0.08 ± 0.08 | 1.20 ± 0.41 |
| TCA        | 102.30 ± 35.6 | 151.20 ± 28.58 | 102.15 ± 22.29 | 180.23 ± 28.63 |
| TCDCA      | 3.68 ± 1.68 | 1.93 ± 0.31 | 4.65 ± 1.98 | 3.45 ± 1.50 |
| TDGA       | 28.58 ± 17.07 | 15.15 ± 3.47 | 1.50 ± 1.10 | 8.93 ± 3.15 |
| TMCA(a + b) | 46.73 ± 15.00 | 42.75 ± 10.54 | 66.38 ± 15.52 | 66.30 ± 16.03 |
| TUDCA      | 2.48 ± 1.17 | 0.68 ± 0.19 | 4.20 ± 1.67 | 3.08 ± 1.64 |
| Total      | 189.48 ± 71.53 | 225.69 ± 45.34 | 191.27 ± 47.56 | 242.65 ± 52.76 |

CA, cholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; MCA(a), α-muricholic acid; MCA(b), β-muricholic acid; MCA(o), Ω-muricholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TMCA (a + b), taurotaurursodeoxycholic acid.

*P < 0.05 compared with 10-week-old SKO via Student’s t-test.

Initially demonstrated that the liver IRE1α/XBP1 and other UPR pathways were activated in 24-week-old FXR+/− mice, and the nuclear XBP1s remained elevated in FXR−/− mice at 60 weeks of age, indicative of sustained ER stress through aging in FXR−/− mice. Increased hepatic bile acid concentrations are known to induce ER stress and activate the UPR (31, 32), and the ER stress pathways that were downregulated in DKO compared with SKO mice, such as xenobiotic metabolism, protein secretion, adipogenesis, fatty acid metabolism, and bile acid metabolism. These pathways were similarly downregulated in DKO compared with SKO mice (13, 25, 35), which suggests that these changes are attributed to the loss of XBP1 in the liver. Studies have shown that mice lacking liver XBP1 were resistant to acetaminophen-induced liver injury (36, 37), although we have not challenged the SKO and DKO mice with acetaminophen or toxins. We did not observe any difference in liver fat accumulation between SKO and DKO mice at 10 weeks of age.

Prolonged and unresolved ER stress can lead to cell death. Mice with hepatic XBP1 deletion that are treated with the ER stress inducer tunicamycin fail to adequately resolve ER stress and have resultant increased apoptosis (11). Consistent with these data, our hepatic TUNEL staining showed more apoptosis and RNA-Seq demonstrated enhanced apoptosis pathway expression in DKO mice. CHOP and its downstream activation of DR5 induce apoptosis during ER stress (26), and the hepatic gene expression of Chop and Dr5 was consistently higher in the DKO mice. This was likely because of, at least in part, to the increased liver ATF4 expression in DKO mice. The hepatic expression of another proapoptotic protein, BAX, was also higher in DKO mice, suggesting that multiple apoptotic pathways were activated in DKO mice. Although similar
increases of Chop, Dr5, and BAX were observed in Xbp1LKO mice, the increased expression of inflammatory, apoptosis, and proliferation pathways, and increased hepatic cyclin D1 protein expression, occurred only in DKO mice compared with SKO mice, when FXR signaling was deficient. These changes seen in DKO mice may be attributed to further increases of ER stress because of elevated bile acid levels or potential crosstalk between liver XBP1 and FXR signaling.

We have previously shown that hepatic XBP1 deletion decreases the total bile acid pool size and hepatic bile acid levels (13). In this study, serum and total hepatic bile acid concentrations were similar in the DKO and SKO mice indicating that both the FXR-dependent and XBP1-dependent changes in bile acid metabolism likely occur. Although there were small decreases of hepatic Bsep and Ntcp gene expression in DKO mice at 10 weeks of age, the functionality of hepatocyte bile acid secretion and uptake was not directly measured. While hepatic Cyp7a1 mRNA expression did not differ, CYP7A1 protein expression was elevated in DKO mice compared with SKO mice, which may contribute to the equalization of hepatic bile acid concentration. In a previous study, we have demonstrated a
similar increase of CYP7A1 protein expression in Xbp1LKO mice compared with Xbp1fl/fl mice (13). Therefore, deletion of hepatic XBP1 appears to cause posttranscriptional or posttranslational regulation of CYP7A1. Hepatic bile acid species concentrations were similar in SKO and DKO mice, with the exception of small changes in the hydrophilic bile acid unconjugated β-muricholic acid and hydrophobic bile acid deoxycholic acid only at 10 weeks of age and no resultant change of hydrophobicity.

Although it has been reported that Fxr−/− mice spontaneously develop HCC by 15 months of age (3, 5), neither the SKO mice nor Fxr−/− mice exhibited liver tumors at 60 weeks old. This could be due to possible differences in the intestinal microbiome in different animal facilities. Nonetheless, 100% of the DKO mice developed liver tumors by 60 weeks of age. Hepatic RNA-Seq at the age prior to tumor development demonstrated increased expression of tumorigenic myc-targets and IL6_JAK_STAT3_signaling pathways.
in DKO mice compared with SKO mice. Consistent with increased cell proliferation, DKO mice had more liver Ki67+ cells and higher cyclin D1 expression compared with SKO mice.

FXR is a master regulator of many genes both in the liver and in the intestine. It is important to note that this study utilized whole-body FXR KO; therefore, other genes or signaling pathways regulated by hepatic and intestinal FXR may have a role in determining the phenotypes of SKO and DKO mice. For instance, ileal absorption of bile acids in the enteropancreatic circulation may be altered. Ileal bile acid transporter inhibitors have recently been approved for pediatric patients with cholestatic liver disease, further emphasizing the importance of ileal bile acid absorption on the enteropancreatic circulation of bile acids. In addition, previous studies have demonstrated a role of intestinal FXR signaling in regulating the microbiome, bile acid composition, and thereby metabolic functions (38). Further investigations are needed to determine potential differences in gut microbiome in DKO and SKO mice.

FXR null mice are widely used to study bile acid toxicity and cholestasis. As Fxr−/− mice age to 24 weeks, hepatic XBP1 and other UPR pathways were activated. The inability to appropriately upregulate liver XBP1 resulted in increased liver injury, apoptosis, fibrosis, and promoted spontaneous liver tumor development in Fxr−/− mice. These data suggest that hepatic XBP1 expression is protective in Fxr−/− mice, which may be due to a reduction of ER stress and/or crosstalk between liver XBP1 and FXR signaling. This may have important implications on the pathogenesis and treatment of cholestatic liver diseases.

Data Availability

All the data described in this article are located within the article including supplemental data. RNA-Seq data are deposited to Gene Expression Omnibus (accession number GSE201902).

Supplemental Data

This article contains supplemental data.

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Conflict of interest

C. C. and Y. G. were employees of Gilead Sciences, Inc during the drafting of this article. All other authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

ALT, alanine aminotransferase; ATF4, activating transcription factor 4; BAX, BCL2-associated X; CHOP, C/EBP-homologous protein; DKO, double KO; DR5, death receptor 5; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; IRE1α, inositol-requiring enzyme 1α; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; RNA-Seq, RNA sequencing; SKO, single KO; UDCA, ursodeoxycholic acid; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1s, XBP1 spliced.

Supplemental Data

This article contains supplemental data.
Liver XBPI is protective in FXR null mice

Lee, A. H., Scapa, E. F., Cohen, D. E., and Glimcher, L. H. (2008) Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science*, 320, 1492–1496.

Liu, X., Henkel, A. S., LeCuyer, B. E., Hubach, S. C., Shipma, M. J., Zhang, E., et al. (2017) Hepatic deletion of X-box binding protein 1 impairs bile acid metabolism in mice. *J. Lipid Res.* 58, 504–511.

Puri, P., Mirshahi, F., Cheung, O., Natarajan, R., Maher, J. W., et al. (2020) Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., and Kingsford, C. (2019) Heuman, D. M. (1989) Quantitative estimation of theunfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology*, 134, 506–576.

Gindin, Y., Chung, C., Jiang, Z., Zhou, J. Z., Xu, J., Billin, A. N., et al. (2021) A fibrosis-independent hepatic transcriptomic signature identifies drivers of disease progression in primary sclerosing cholangitis. *Hepatology*, 73, 1105–1116.

Han, C. Y., Rho, H. S., Kim, A., Kim, T. H., Jang, K., Jun, D. W., et al. (2018) FXR inhibits endoplasmic reticulum stress-induced NLRP3 inflammasome in hepatocytes and ameliorates liver injury. *Cell Rep.* 24, 2985–2999.

Liu, X., Guo, G. L., Kong, B., Hilburn, D. B., Hubach, S. C., Park, S., et al. (2018) Farnesoid X receptor signaling activates thehepatic X-box binding protein 1 pathway in vitro and in mice. *Hepatology*, 68, 304–316.

Ishak, K., Baptista, A., Bianchi, L., Callea, F., De Groote, J., Gudat, F., et al. (1995) Histological grading and staging of chronic hepatitis. *J. Hepatol.* 22, 696–699.

Heuman, D. M. (1999) Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. *J. Lipid Res.* 30, 719–730.

Patiro, K., Duggal, G., Love, M. I., Irizarry, R. A., and Kingsford, C. (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Met.* 14, 417–419.

Frankish, Á., Diekhans, M., Ferreira, A. M., Johnson, R., Jungreis, L., Loveland, J., et al. (2019) GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res.* 47, D760–D773.

McCarthy, D. J., Chen, Y., and Smyth, G. K. (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 40, 4288–4297.

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., et al. (2021) clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innovation (NY)*, 2, 100141.

Liberezon, A., Birger, C., Thorvaldsdottir, H., Ghandi, M., Mesirov, J. P., and Tamayo, P. (2015) The molecular signatures database (MSigDB) hallmark gene set collection. *Cell Syst.* 1, 417–425.

Liu, X., Taylor, S. A., Gromer, K. D., Zhang, D., Hubach, S. C., LeCuyer, B. E., et al. (2022) Mechanisms of liver injury in high fat sugar diet fed mice that lack hepatocyte X-box binding protein 1. *PLoS One* 17, e0261789.

Oyadomari, S., and Mori, M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* 11, 381–389.

Xiong, X., Wang, X., Liu, Y., Wang, E., Zhang, Z., Yang, J., et al. (2014) Hepatic steatosis exacerbated by endoplasmic reticulum stress-mediated downregulation of FXR in aging mice. *J. Hepatol.* 60, 847–854.

Jung, H., Chen, J., Hu, X., Sun, H., Wu, S. Y., Chiang, C. M., et al. (2020) BRD4 inhibition and FXR activation, individually beneficial in cholestasis, are antagonistic in combination. *JCI Insight* 6, e11640.

Su, H., Ma, C., Liu, J., Li, N., Gao, M., Huang, A., et al. (2012) Downregulation of nuclear receptor FXR is associated with multiple malignant clinicopathological characteristics in human hepatocellular carcinoma. *Am. J. Physiol. Gastrointest. Liver Physiol.* 303, G1245–G1253.

Gomez-Ospina, N., Potter, C. J., Xiao, R., Manickam, K., Kim, M. S., Kim, K. H., et al. (2016) Mutations in the nuclear bile acid receptor FXR cause progressive familial intrahepatic cholestasis. *Nat. Commun.* 7, 10713.

Bernstein, H., Payne, C. M., Bernstein, C., Schneider, J., Beard, S. E., and Crowley, C. L. (1999) Activation of the promoters of genes associated with DNA damage, oxidative stress, ER stress and protein malfolding by the bile salt, deoxycholate. *Toxicol. Lett.* 108, 57–46.

Cremers, C. M., Knoefler, D., Vitvitsky, V., Banerjee, R., and Jakob, U. (2014) Bile salts act as effective protein-unfolding agents and instigators of disulfide stress in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 111, E1610–E1619.

Bochkis, I. M., Rubins, N. E., White, P., Furth, E. E., Friedman, J. R., and Kaestner, K. H. (2008) Hepatocyte-specific ablation of Foxa2 alters bile acid homeostasis and results in endoplasmic reticulum stress. *Nat. Med.* 14, 828–836.

Kriegermeier, A., Hyon, A., Sommers, M., Hubach, S., LeCuyer, B., Liu, X., et al. (2021) Hepatic X-box binding protein 1 and unfolded protein response is impaired in weaning mice with resultant hepatic injury. *Hepatology*, 74, 3362–3375.

Liu, X., Henkel, A. S., LeCuyer, B. E., Shipma, M. J., Anderson, K. A., and Green, R. M. (2015) Hepatocyte X-box binding protein 1 deficiency increases liver injury in mice fed a high-fat/sugar diet. *Am. J. Physiol. Gastrointest. Liver Physiol.* 309, G965–G974.

Hur, K. Y., So, J. S., Ruda, V., Frank-Kamenetsky, M., Fitzgerald, K., Koteliansky, V., et al. (2012) IRE1alpha activation protects mice against acetaminophen-induced hepatotoxicity. *J. Exp. Med.* 209, 307–318.

Ye, H., Chen, C., Wu, H., Zheng, K., Martin-Adorados, B., Caparros, E., et al. (2022) Genetic and pharmacological inhibition of XBPI protects against APAP hepatotoxicity through the activation of autophagy. *Cell Death Dis.* 13, 143.

Pathak, P., Xie, C., Nichols, R. G., Ferrell, J. M., Boehme, S., Krausz, K. W., et al. (2018) Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism. *Hepatology* 68, 1574–1588.