Mice Lacking FXR Are Susceptible to Liver Ischemia-Reperfusion Injury

Running Title: FXR and liver ischemia-reperfusion injury

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### Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| BA           | bile acid   |
| CDCA         | chenodeoxycholic acid |
| EDEM         | ERAD-enhancing α-mannosidase-like protein |
| ER           | Endoplasmic reticulum |
| ERAD         | ER associated degradation |
| FXR          | farnesoid X receptor |
| GRP94        | glucose-regulated protein 94 |
| IRI          | ischemia reperfusion injury |
| SHP          | small heterodimer partner |
| TUNEL        | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| XBP1         | X box binding protein 1. |
Abstract

Activation of bile acid (BA) receptor, farnesoid X receptor (FXR) has been shown to inhibit inflammatory responses and improve tissue ischemia-reperfusion injury (IRI). This study investigated the effect of FXR deficiency on liver IRI, using a liver warm IRI mouse model. We demonstrate that liver IRI resulted in decreased FXR expression in the liver of WT mice. FXR−/− mice displayed greater liver damage and inflammatory responses than WT mice, characterized by significant increases in liver weight, serum AST and ALT, hepatocyte apoptosis and liver inflammatory cytokines. Liver IRI increased expression of X box binding protein 1 (XBP1) and FGF21 in WT liver, but not in FXR−/− liver, which conversely increased CHOP expression, suggesting a loss of ER stress protection in the absence of FXR. FXR deficiency increased circulating total BAs and altered BA composition with reduced TUDCA and hepatic BA synthesis markers. FXR deficiency also reshaped gut microbiota composition with increased Bacteroidetes and Proteobacteria and decreased Firmicutes. Curiously, Bacteroidetes were positively and Firmicutes were negatively correlated with serum ALT levels. Administration of FXR agonist CDCA inhibited NF-κB activity and TNFα expression in vitro and improved liver IRI in vivo. Our findings demonstrate that FXR signaling plays an important role in the modulation of liver IRI.

Key words: Liver ischemia-reperfusion injury; Bile acids; FXR; Gut microbiota
Introduction

Recent improvements in surgical techniques, liver preservation and immunosuppression continue to improve liver operations and increase survival of liver grafts in transplantation (1, 2). However, liver ischemia and reperfusion injury (IRI) remains an important problem in the clinical scenario of liver surgery (3). In major liver resection, a primary side effect is liver warm IRI, i.e. ischemia of remnant liver following temporary vascular occlusion, and additional reperfusion injury added to the damage sustained during ischemia (4). In liver transplantation, liver warm and cold IRI may occur in situ during recipient surgery or donor liver harvest, which may cause cellular injury, organ dysfunction, or even complete graft failure (5).

Tissue ischemia and reperfusion initiates a defensive process known as the unfolded protein response (UPR) for adaptation and safeguard of cell survival. Activation of the inositol-requiring enzyme (IRE)/X-box binding protein 1 (XBP1) pathway in response to the endoplasmic reticulum (ER) stress protects hepatocytes from apoptosis. However, sustained activation of ER triggers proapoptotic signals via C/EBP homologous protein (CHOP), which is responsible for cellular dysfunction (6, 7). Evidence for the role of BAs and their receptors in the regulation of inflammation and tissue IRI already exists in experimental and animal settings (8, 9). Activation of the BA membrane receptor, G protein-coupled BA receptor 1 (TGR5), by TGR5 agonists inhibits inflammatory responses and attenuates liver IRI by suppressing the Toll like receptor 4 (TLR4)-NF-κB mediated pathway (10). Recent experimental data suggest that activation of BA nuclear receptor, farnesoid X receptor (FXR), stimulates the IRE/XBP1 pathway and may be protective during liver injury (11). Similarly, administration of the FXR-agonist obeticholic acid (OCA) improves survival in a rodent model of intestinal IRI, through gut barrier preservation and mitigated inflammation (12). However, in the setting of liver IRI, the role and fundamental mechanism of FXR signaling in the modulation of ER stress and inflammatory responses remain unexplored.

There is growing evidence of bidirectional interactions between BAs and the gut microbiota, i.e. BAs in the distal intestine influence the composition of the gut microbiota that in turn modify primary BAs to generate secondary BAs (13). FXR deficiency significantly reduces the abundance of Firmicutes and increased Bacteroidetes (14). Dysbiosis directly impairs intestinal barrier function and increases gut permeability. Bacteria and their components, such as
lipopolysaccharide (LPS), pass through the intestinal barrier to the liver, causing inflammatory responses. Modulation of the gut microbiota may inhibit inflammatory responses and represent a novel therapeutic approach for the prevention of tissue IRI (15).

In this study, we examined FXR deficiency in the aggravation of liver IRI using a mouse model of liver warm IRI. We hypothesized that a lack of FXR exaggerates hepatocyte ER stress and apoptosis during liver IRI. FXR deficiency may alter BA and gut microbiota homeostasis, which may also contribute to the pathogenesis of liver IRI. Our results demonstrate that mice lacking FXR are more susceptible to liver IRI than wild type (WT) mice.
Materials and Methods

Mice and surgical procedures

FXR knockout (FXR\(^{-/-}\), B6.129X1-Nr1h4tm1Gonz/J) mice (8-weeks of age) were purchased from Jackson Laboratory. Male homozygous FXR\(^{-/-}\) and FXR wild type (WT) littermate mice (5 mice per group) were used. All mice were housed in a specific pathogen-free, temperature-controlled and 12-hour light-dark cycle environment in the Animal Resources Center (ARC) at the University of Chicago.

The liver warm IRI model was conducted under general anesthesia with isoflurane and equal O\(_2\) through a cone placed around the mouse’s nose. A small clip (Roboz, RS-5424) was used to interrupt arterial/portal venous blood supply to the median and left lateral lobes, in which 70% of liver blood flow was blocked (16). To test whether a lack of FXR exaggerates liver damage, after 90 minutes of ischemia, the clip was removed followed by 6 hours of reperfusion in FXR\(^{-/-}\) mice compared to WT mice. To test whether FXR activation improved liver IRI, FXR agonist chenodeoxycholic acid (CDCA) was administered to WT mice at a dose of 150 mg/kg (dissolved in 100μl 1% carboxymethylcellulose or CMC) by oral gavage daily for a week prior to IRI challenge. The same volume of CMC (Vehicle) was used in control mice. The animal’s vital signs were monitored throughout surgery, including respiratory rate, response to pedal or palpebral stimulus and assessment of spontaneous movements. All animals received humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the NIH. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) analysis

Blood samples were collected at 6 hours (WT vs. FXR\(^{-/-}\)) or 24 hours (WT with and without FXR agonist CDCA) after ischemia and were centrifuged to obtain serum. ALT and AST were measured to assess the extent of hepatocyte damage using an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan).

Bile acid analysis

Total BAs and BA composition were analyzed as described in our previous reports (17, 18). Briefly, stock solutions of individual BAs and NDCA were prepared in methanol at a concentration of...
5μg/mL. Calibration standards were prepared by adding individual bile acids at a concentration range of 12ng/mL to 1.5μg/mL to charcoal stripped human serum. 1.6μL of NDCA was added to 40μL of standards and samples. Deproteinization was carried out by adding 15X ice-cold methanol to 40μL of standards and samples. The supernatant was transferred to a new tube, evaporated under vacuum and dissolved in 100μL of 50% methanol. The tubes were centrifuged at 11,000xg for 1 min before transfer in to specific vials for injection in to an LC-MS/MS system. Data were acquired on AB Sciex triple quadrupole mass spectrometer in negative ion mode coupled to Shimadzu Nexera XR HPLC system. Chromatographic separation was carried out on Thermo Scientific Accucore XLC8 column (4μm, 100 x 3mm I.D.). Quantitation of bile acids was carried out on MultiQuant software v3.0.2 (AB Sciex). BAs, including CA, CDCA, DCA, LCA, TCA, TDCA, TCDCA, UDCA and TUDCA, were purchased from Sigma-Aldrich. bMCA, TbMCA and internal standard 23-nordeoxycholic acid (NDCA) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Cecal content microbiota analysis

Gut microbiota in the cecal content was analyzed as described in our previous report (17, 18). Briefly, primers specific for 16S rRNA V4-V5 region (Forward: 515F: 5’-GTGYCAGCMGCCGCGGTAA -3’ and Reverse: 806R: 5’- GGACTACHVGGGTWTCTAAT-3’) that contained Illumina 3' adapter sequences, as well as a 12-bp barcode were used. Sequences were generated by an Illumina MiSeq DNA platform at Argonne National Laboratory and analyzed by the program Quantitative Insights Into Microbial Ecology (QIIME) (19). Operational Taxonomic Units (OTUs) were picked at 97% sequence identity using open reference OTU picking against the Greengenes database (20). OTUs generated in QIIME were then analyzed using linear discriminant analysis (LDA) effect size (LEfSe) where non-parametric factorial Kruskal-Wallis sum-rank testing ($p < 0.05$) identified significantly abundant taxa followed by unpaired Wilcoxon rank-sum test to determine LDA scores > 2 (21).

Real-time RT-PCR for quantification of mRNA expression

Liver tissue was collected at the time of animal euthanization and immediately placed in Trizol reagent (Ambion, Austin, TX) (22, 23). Total RNA was reverse-transcribed to complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN). RT-PCR amplification was consisted of an initial denaturation step (95°C for 10 min), 45 cycles
of denaturation (95°C for 10s), annealing (55°C for 20s) and extension (60°C for 30s), followed by a final incubation at 55°C for 30s and cooling at 40°C for 30s. All measurements were normalized by the expression of GAPDH gene, considered as a stable housekeeping gene. Gene expression was determined using the delta-delta Ct method: 2-ΔΔCT (ΔΔCT = [Ct(target gene) – Ct(GAPDH)] tested - [Ct(target gene) -Ct(GAPDH)]control) and displayed as relative mRNA levels.

Cell culture and tissue luciferase assay

To test whether the FXR agonist CDCA inhibited NF-κB activity and TNFa expression in macrophages, bone marrow-derived macrophages (BMDMs) transfected with a luciferase gene under the control of the NF-κB promoter (kindly provided by Dr. Wei Han, Vanderbilt University) (24) were incubated with lipopolysaccharide (LPS, 0.025μg/ml, Sigma-Aldrich, St. Louise, MO). BMDMs were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS at 37°C in a 5% CO₂ incubator for 4 hours. Luciferase activity in BMDMs was determined by the tissue luciferase assay using the Bradford Luciferase Reporter Assay kit (Promega, Madison, WI, USA) following the manufacturer’s instructions as described in our previous report (25). TNFa mRNA was analyzed by qRT-PCR as described above.

Histological examination

Liver tissue was collected 6 hours following ischemia and subjected to H&E staining. Hepatocyte apoptosis was analyzed by TUNEL assay. TUNEL labeling was performed using a TUNEL kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions. Quantitation of apoptotic cells was accomplished by calculating of the labeling index, which was defined as the ratio between the number of labeled cells and the total cells counted in triplicate field by two blinded investigators.

Statistical analysis

Data from the current studies were analyzed by ANOVA tests (StatView 4.5, Abacus Concepts, Berkeley, CA) with Tukey post-hoc multiple comparisons, or by 2-tailed Student’s t test when appropriate, where p-value < 0.05 was considered significant. The results are presented as mean ± SEM.
Results

Mice lacking FXR were more susceptible to liver IRI

A lack of FXR increased TGR5 expression in naïve FXR\(^{-/-}\) mice, indicating a compensation of TGR5 for the deficiency of FXR. Liver IRI reduced hepatic expression of FXR in WT mice, and conversely, the same procedure increased expression of TGR5 in WT and FXR\(^{-/-}\) mice (Fig. 1, A and B). FXR\(^{-/-}\) mice also revealed an increase in liver weight (liver/body weight) in untreated FXR\(^{-/-}\) (FXR\(^{-/-}\) Control) mice, compared with untreated WT (WT Control) mice. Liver IRI further increased liver weight (Fig. 1C) and aggravated liver injury in the deficiency of FXR evidenced by increased circulating AST (Fig. 1D) and ALT (Fig. 1E) levels in FXR\(^{-/-}\) IRI mice, compared with WT mice. The gross examination suggested evidence of liver steatosis and cholestasis in FXR\(^{-/-}\) controls and FXR\(^{-/-}\) IRI mice, which was confirmed histologically (H&E) (Fig. 1F), and suggested possible steatosis, which was previously identified as a susceptibility factor under IRI (26).

A lack of FXR resulted in increased TNF\(\alpha\), IL-1\(\beta\) and IL-6 transcript in FXR\(^{-/-}\) control mice (FXR\(^{-/-}\) Control vs. WT Control, \(p < 0.05\), Fig. 2, A to C). As expected, liver IRI also increased TNF\(\alpha\), IL-1\(\beta\) and IL-6 transcripts in both WT and FXR\(^{-/-}\) mice \((p < 0.01)\), and TNF\(\alpha\) was significantly increased in FXR\(^{-/-}\) IRI mice, compared to WT IRI mice (Fig. 2A). Superoxide dismutase 2 (SOD2) binds to the superoxide to improve tissue injury. Reduced SOD2 expression was observed in WT mice following IRI, but more dramatically in FXR\(^{-/-}\) mice \((p = 0.05\), Fig. 2D).

FXR deficiency triggered hepatocyte apoptosis via activation of ER stress

ER stress activates a number of proteins that straddle ER membranes. Activated IRE1\(\alpha\) functions as an endoribonuclease splicing a 26 base pair intron from XBP1 mRNA. Spliced XBP1 (sXBP1) mRNA is translated into a stable and active UPR transcription factor. Therefore, measuring XBP-1 splicing represents a reliable indirect method of determining IRE1\(\alpha\) activation (27). Liver IRI promoted expression of total XBP1 (TXBP1, Fig. 3A), sXBP1 (Fig. 3B) and unconventional splicing XBP1 (usXBP1, Fig. 3C) in WT mice. However, mice deficient in FXR showed no change in these genes (Fig. 3, A to C). Liver IRI decreased expression of ATF4 and GFP94 in the absence of FXR (WT IRI vs. FXR\(^{-/-}\) IR, \(p < 0.05\), Fig. 3, D and E). Furthermore, FXR deficiency resulted in decreased ERAD-enhancing \(\alpha\)-mannosidase-like protein (EDEM, Fig. 3F), ultimately resulting in hepatocyte susceptibility to apoptosis. Consistently, we observed increased CHOP with FXR
deficiency (Fig. 3G). TUNEL tests showed increased apoptotic cells in untreated FXR\textsuperscript{+/−} control mice, but without statistical difference. Liver IRI increased apoptotic cells in all groups; however, apoptotic cells were significantly increased in FXR\textsuperscript{+/−} mice compared with WT IRI mice (Fig. 3H).

**Both the lack of FXR and liver IRI altered BA homeostasis**

FXR represses transcription of the gene encoding CYP7A1 that is the rate-limiting enzyme in BA synthesis, and thus, the lack of FXR thus resulted in increased circulating total BAs (Fig. 4A). BA composition analysis demonstrated that the lack of FXR specifically increased circulating TbMCA (Fig. 4B), TCA (Fig. 4D) and DCA (Fig. 4F) when compared to WT control mice. Liver IRI increased circulating total BAs (Fig. 4A), TbMCA (Fig. 4B), bMCA (Fig. 4C), TCA (Fig. 4D) and TUDCA (Fig. 4G) in WT mice. Although CA and DCA were increased in WT liver IRI mice, there were no statistical differences ($p > 0.05$) (Fig. 4, E and F). There were no changes to circulating LCA in any groups (Fig. 4H). Given that the mutation of FXR affects BA synthesis and transport (28), and IRI may also impacts liver BA homeostasis, we analyzed total BAs in the liver. As expected, the absence of FXR causes BA accumulation of total BAs in the liver; however, IRI results in decreases of liver total BAs in both WT and FXR\textsuperscript{+/−} mice (Fig. 4I).

Enzyme CYP7A1 initiates the classic pathway of BA synthesis, followed by CYP8B1 that produces the majority of the BA pool. The alternative pathway involves the BA synthetic enzyme CYP27A1 followed by BA hydroxylation by CYP7B1. BA synthesis is under the regulation of FXR, which enhances expression of small heterodimer partner (SHP) and stimulates ileal fibroblast growth factor 15 (FGF15) in mice (FGF19 in humans). FGF15/19 travels to the liver where the membrane receptor, FGFR4, triggers a signaling cascade that results in BA synthesis inactivation. ER stress and inflammatory responses suppress BA synthesis and enhance BA removal from hepatocytes (29). Our results showed that liver IRI inhibited expression of SHP (Fig. 5A). Liver IRI and the lack of FXR reduced FGFR4 gene, but no statistical difference (Fig. 5B). BA synthesis enzymes, including CYP7A1 (Fig. 5C), CYP8B1 (Fig. 5D), CYP27A1 (Fig. 5E) and CYP7B1 (Fig. 5F), were reduced in WT and FXR\textsuperscript{+/−} mice. Naïve FXR\textsuperscript{+/−} animals demonstrated decreased mean expression levels of CYP27A1 and CYP7B1 mRNAs, but not significantly (Fig. 5, E and F, $p > 0.05$).

Fibroblast growth factor 21 (FGF21) is identified as one of tissue protective proteins (30). To test whether FGF21 is altered under liver IRI, liver FGF21 expression was analyzed. Results
showed that liver IRI increased FGF21 transcript in the WT liver. However, IRI failed to enhance liver FGF21 expression in the absence of FXR (Fig. 5G), suggesting FGF21 expression may be, at least in part, regulated by FXR (31).

**FXR deficiency altered gut microbiota composition**

Taxonomic analysis of gut microbiota composition showed changes at the phylum level, where Bacteroidetes was increased and Firmicutes decreased - with increased Bacteroidetes/Firmicutes ratios - in FXR<sup>-/-</sup> IRI mice with and without liver IRI (Fig. 6, A and B) consistent with a previous report (14). Our data showed that Proteobacteria was increased in untreated FXR<sup>-/-</sup> and FXR<sup>-/-</sup> IRI mice (Fig. 6C). More interestingly, altered Bacteroidetes and Firmicutes were correlated with markers of liver function; Bacteroidetes was positively and Firmicutes was negatively correlated with serum ALT levels (Fig. 6, D and E) following liver IRI. Increasing data identify Proteobacteria as lipopolysaccharide (LPS) producers that act as possible microbial signature of host disease (32). Within the phylum Bacteroidetes, the relative abundance of *Bacteroides* was increased in naïve and FXR<sup>-/-</sup> IRI mice (Fig. 6, F and G). Within the phylum Firmicutes, the relative abundances of *Turicibacter* and *Clostridiales* were decreased in FXR<sup>-/-</sup> mice with and without liver IRI (Fig. 6, H and I). Finally, within the phylum Proteobacteria, the relative abundance of *Desulfovibrio* was increased in FXR<sup>-/-</sup> mice with and without liver IRI (Fig. 6J).

**Activation of FXR inhibited inflammatory reaction in vitro and improved liver IRI in vivo**

Macrophage-induced inflammatory responses are mediated through inflammatory signaling pathways, such as NF-κB, causing TNFα expression. In the bone marrow-derived macrophage (BMDM) culture, LPS increased luciferase activity, indicating increased NF-κB activity, which was inhibited by administration of chenodeoxycholic acid (CDCA, Supplemental Fig. 1S.A). TNFα mRNA was increased by the stimulation of LPS but also inhibited by CDCA (Supplemental Fig. 1S.B).

To test whether FXR activation improved liver IRI, FXR agonist CDCA was administered to WT mice undergone 90 minutes of ischemia and 24 hours of reperfusion. The results showed that serum ALT levels were significantly decreased in CDCA-treated WT mice compared to Vehicle-treated WT mice (Supplemental Fig. 1S.C). Although CDCA also decreased AST levels in the same animals, differences between CDCA-treated and control mice did not reach significance (p > 0.05, Supplemental Fig. 1S.D).
Discussion

We employed animals lacking FXR compared with WT to investigate critical signaling pathways in liver IRI. The results herein demonstrated that liver IRI decreased hepatic FXR expression in WT mice in line with elevated IL-6 levels, a known FXR repressor (33), indicating an inverse relationship between FXR and liver IRI. Under FXR deficiency, more profound increases in pro-inflammatory cytokines may directly contribute to elevated susceptibility to liver IRI, including elevated circulating AST and ALT, and more pronounced hepatocyte apoptosis compared with WT controls.

ER stress triggers the adaptive response that acts in concert to mitigate the load of new proteins entering the ER through increasing ER content, such as chaperone capacity, thereby degrading misfolded proteins and inhibiting cell apoptosis. Chaperone GRP94 and ATF4 are the most abundant glycoprotein in the ER and participate in protein folding and assist in the targeting of misfolded proteins for ER associated degradation (ERAD) (34). Promotion of ERAD through upregulation of ERAD-enhancing α-mannosidase-like protein (EDEM) promotes cell recovery by accelerating ERAD of terminally misfolded polypeptides and protects against cell apoptosis (35). A recent report suggests that FXR signaling modulates ER stress via activation of the IRE/XBP1 pathway (11). Our findings show that mice lacking FXR resulted in reduced expression of GRP94, ATF4, EDEM, XBP1 (including total, spliced and unconventional spliced XBP1) in the liver when suffering from IRI, suggesting that the absence of FXR forfeits protection of hepatocytes from apoptosis, contributing to the susceptibility to liver IRI.

ROS generation inflicts tissue damage and initiates a cascade of deleterious cellular responses leading to inflammation, cell death and ultimately liver failure. SOD2 transforms toxic superoxide and clears mitochondrial ROS and therefore confers protection against hepatocyte apoptosis (36). In the absence of FXR, liver IRI fails to promote production of SOD2, and accumulation of ROS may initiate the inflammatory reaction and liver damage.

FGF21 is a member of the fibroblast growth factor family that regulates cell growth, differentiation, and glucose and lipid metabolism. FXR activation promotes FGF21 expression (31), which alleviates hepatic ER stress under the physiological condition (37). Increased FGF21 protects against acetaminophen-induced hepatotoxicity by enhancing antioxidant capacity (38). Clinical findings show that serum FGF21 increases following liver or cardiac ischemia and is
associated with protective responses (39). We observed that liver IRI promoted expression of liver FGF21 in WT mice, but deficiency of FXR failed to increase FGF21 expression, thereby losing liver protection.

The lack of FXR stimulates BA synthesis, resulting in increased circulating total BAs, TbMCA, MCA, TCA and DCA. Due to the damage of BA transports in the absence of FXR (28), BAs are accumulated in the liver as evidenced by increased total BAs in the liver of FXR\(^{-/-}\) mice. Liver IRI causes immediate reduction of synthesis as confirmed by decreased BA synthetic enzymes and reduced total BAs in the liver in both WT and FXR\(^{-/-}\) mice. However, liver IRI induces rapid increases of circulating total BAs and alterations of BA composition in both WT and FXR\(^{-/-}\) mice. Among the notable changes in circulating BAs was TUDCA, a hydrophilic BA that regulates ER stress and mediates cytoprotective responses during liver IRI (40); however, FXR\(^{-/-}\) mice failed to demonstrate elevated TUDCA. Nevertheless, whether the alteration of BA composition mediates liver IRI needs further study.

A biochemical link between the gut microbiota and FXR signaling has been demonstrated (41). Taxonomic tree analysis displaying differential taxa in hierarchical layers supports the concept that FXR deficiency is associated with distinct microbiota and liver IRI may generate selection pressure for certain gut bacterial taxa. Recent work shows that FXR\(^{-/-}\) mice exhibit reduced levels of the phylum Firmicutes and destabilizes the gut microbiota when compared with WT animals (42). Our findings demonstrate that liver IRI decreased the relative composition of the bacterial phylum Bacteroidetes in WT animals, consistent with previous work (43). FXR deficiency leads to increased Bacteroidetes and reduced Firmicutes. Increased Bacteroidetes and reduced Firmicutes were correlated with higher levels of circulating ALT after liver IRI across all animals. This analysis also showed increased cecal Proteobacteria in the absence of FXR. Proteobacteria are a major phylum of gram-negative bacteria that include a wide variety of pathogens, such as *Escherichia Coli*, capable of producing LPS (44). However, Proteobacteria are not increased in WT IRI mice, suggesting that the lack of FXR, but not stress (IRI) contributes to increased Proteobacteria. Within the Proteobacteria, the genus *Desulfovibrio* is a Gram-negative obligate anaerobic that was elevated by IRI. Increased *Desulfovibrio* is associated with inflammation of gut tissues (45) and may mediate inflammatory responses following liver IRI. Due to the presence of LPS in the outer membrane, increased Proteobacteria may induce inflammatory responses in the liver, where LPS and other danger associated molecular patterns
reach the liver via the portal vein. Consequently, a lack of FXR results in dysbiosis of the microbiome that are empirically associated with hepatocyte injury and therefore may contribute to the liver IRI pathogenesis.

BMDMs functionally resemble Kupffer cells and are used to study immune responses that mediate liver injury (46). Here we show that FXR agonist CDCA attenuated NF-κB activity and TNFα expression in the BMDM cell culture consistent with a previous report (47). When CDCA is administered to WT animals, it improves liver IRI supported by decreased serum ALT levels. However, a major limitation to our work relates to the evidence that mice and humans are inherently different in the homeostasis of BAs, i.e. the human liver synthesize CA and CDCA as FXR agonists, whereas mice synthesize TbMCA that are considered antagonistic towards FXR.

In conclusion, these data illustrate fundamental roles for FXR in the modulation of liver IRI. FXR expression is decreased during liver IRI in WT mice. Mice lacking FXR reduce baseline chaperone expression and exhibit significant changes of BA composition, which potentially triggers IRI-induced hepatocyte apoptosis. The absence of FXR results in dysbiosis of the gut microbiota, which may further exacerbate liver inflammatory reactions during liver IRI. Our findings suggest that hepatocyte protective mechanisms are impaired in the absence of FXR signaling. As proof of this concept, our work shows that activation of FXR with exogenous CDCA inhibits inflammatory responses \textit{in vitro} and improves liver IRI markers \textit{in vivo}. 

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Author Contribution

JFP and YL contributed to most in vitro and in vivo experiments; PR and CG contributed to lab analysis; EBC and DPY designed the experiment, and DPY wrote the manuscript. All authors approved the final version.
Figure Legends

**Fig. 1.** The lack of FXR was more susceptible to liver IRI (n = 5 per group). (A) Liver IRI decreased FXR expression in WT mice. WT IRI vs. WT Control, *p* < 0.05. (B) Liver IRI increased TGR5 expression in WT and FXR<sup>-/-</sup> mice. FXR<sup>-/-</sup> IRI vs. FXR<sup>-/-</sup> Control, *p* < 0.05; WT IRI vs. WT Control, *p* < 0.01, and FXR<sup>-/-</sup> Control vs. WT Control, *p* < 0.05. (C) Liver IRI increased liver weight in FXR<sup>-/-</sup> mice. FXR<sup>-/-</sup> Control vs. WT Control, *p* < 0.01; FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.001. (D) The lack of FXR resulted in increase of serum AST. WT IRI vs. WT Control, *p* < 0.001; FXR<sup>-/-</sup> IRI vs. FXR<sup>-/-</sup> Control, *p* < 0.01, and FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.05. (E) The lack of FXR aggravated liver damage. Images for left panels are WT and FXR<sup>-/-</sup> livers, and right two panels showed histological changes (H&E staining) before and after IRI.

**Fig. 2.** The lack of FXR increased expression of inflammatory cytokines (n = 5 per group). (A) Liver IRI and lack of FXR increased TNFα mRNA. WT Control vs. WT IRI, *p* < 0.01; WT Control vs. FXR<sup>-/-</sup> Control, *p* < 0.05; FXR<sup>-/-</sup> IRI vs. FXR<sup>-/-</sup> Control, *p* < 0.01 and FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.01. (B) Liver IRI and lack of FXR increased IL-1β expression. WT Control vs. FXR<sup>-/-</sup> Control, *p* < 0.05; WT Control vs. WT IRI, *p* < 0.01, and FXR<sup>-/-</sup> Control vs. FXR<sup>-/-</sup> IRI, *p* < 0.01. (C) Liver IRI and lack of FXR increased IL-6 expression. WT Control vs. WT IRI, *p* < 0.01; WT Control vs. FXR<sup>-/-</sup> Control, *p* < 0.05, and FXR<sup>-/-</sup> Control vs. FXR<sup>-/-</sup> IRI, *p* < 0.01. (D) The lack of FXR decreased SOD expression. FXR<sup>-/-</sup> IRI vs. FXR<sup>-/-</sup> Control, *p* < 0.01.

**Fig. 3.** The lack of FXR altered gene expression of ER stress markers (n = 5 per group). (A) FXR deficiency failed to enhance total XBP1 (TXBP1) expression. WT Control vs. WT IRI, *p* < 0.05; FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.05. (B) FXR deficiency failed to enhance spliced XBP1 (sXBP1) expression. WT IRI vs. WT Control, *p* < 0.01; and FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.05. (C) FXR deficiency failed to enhance unconventional spliced XBP1 (usXBP1) expression. WT IRI vs. WT Control, *p* < 0.01; FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.05. (D) FXR deficiency failed to increase ATF4 expression. FXR<sup>-/-</sup> IRI vs. WT IRI, *p* < 0.05. (E) FXR deficiency failed to increase GRP94 expression. FXR<sup>-/-</sup> IRI vs. FXR<sup>-/-</sup> Control, *p* < 0.05. (F) Liver IRI reduced EDEM expression in the deficiency of FXR. FXR<sup>-/-</sup> Control vs. FXR<sup>-/-</sup> IRI, *p* < 0.05. (G) FXR deficiency increased CHOP expression. WT Control vs. other groups, *p* < 0.05. (H) The lack of FXR enhanced...
hepatocyte apoptosis. WT Control vs. WT IRI, \( p < 0.05 \); FXR\(^{-/-}\) Control vs. FXR\(^{-/-}\) IRI, \( p < 0.001 \), and WT IRI vs. FXR\(^{-/-}\) IRI, \( p < 0.001 \).

**Fig. 4.** Liver IRI and the lack of FXR altered total BAs and BA composition (n = 5 per group). (A) Liver IRI and the lack of FXR increased circulating total BAs. WT IRI vs. WT Control, \( p < 0.05 \); FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.05 \). (B) Liver IRI and the lack of FXR increased circulating TbMCA. WT IRI vs. WT Control, \( p < 0.01 \); FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.05 \). (C) Liver IRI increased circulating bMCA. WT IRI vs. WT Control, \( p < 0.05 \); FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.05 \). (D) Liver IRI and the lack of FXR increased circulating TCA. WT IRI vs. WT Control, \( p < 0.01 \); and FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.05 \). (E) Liver IRI and the lack of FXR increased circulating CA, but no statistical differences (\( p > 0.05 \)). (F) The lack of FXR increased circulating DCA. FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.05 \). (G) Liver IRI increased circulating TUDCA. WT IRI vs. WT Control, \( p < 0.05 \). (H) Liver IRI increased circulating UDCA in WT mice, but not statistically significant (\( p > 0.05 \)). (I) There were no significant changes of circulating LCA (\( p > 0.05 \)).

**Fig. 5.** Liver IRI and the lack of FXR altered expression of BA synthesis-related molecules (n = 5 per group). (A) Liver IRI and the lack of FXR decreased expression of SHP in the liver. WT Control vs. WT IRI, \( p < 0.001 \); FXR\(^{-/-}\) Control vs. WT Control, \( p < 0.001 \). (B) Liver IRI decreased expression of FGFR4, but not statistically significant (\( p > 0.05 \)). (C) Liver IRI decreased expression of CYP7A1. WT Control vs. WT IRI, \( p < 0.01 \); FXR\(^{-/-}\) IRI vs. FXR\(^{-/-}\) Control, \( p < 0.001 \). (D) Liver IRI decreased expression of CYP8B1. WT IRI and FXR\(^{-/-}\) IRI vs. WT Control and FXR\(^{-/-}\) Control, \( p < 0.01 \), respectively. (E) Liver IRI decreased expression of CYP27A1. WT IRI and FXR\(^{-/-}\) IRI vs. WT Control and FXR\(^{-/-}\) Control, \( p < 0.01 \), respectively. (F) Liver IRI decreased expression of CYP7B1. WT Control vs. WT IRI, and FXR\(^{-/-}\) Control vs. FXR\(^{-/-}\) IRI, \( p < 0.05 \), respectively. (G) Liver IRI increased FGF21 expression in WT mice, but not in FXR\(^{-/-}\) mice. WT Control vs. WT IRI, \( p < 0.05 \).

**Fig. 6.** The lack of FXR altered gut microbiota composition (n = 5 per group). (A) Reconfiguration of phyla. The lack of FXR reduced phylum Firmicutes and increased phylum Bacteroidetes. (B) Decreased Firmicutes and increased Bacteroidetes cause an increase in the ratio of Bacteroidetes/Firmicutes in FXR\(^{-/-}\) mice. (C) The lack of FXR increased phylum Proteobacteria. (D) Increased phylum Bacteroidetes was positively related with increased serum ALT levels in...
FXR± mice. (E) Increased phylum Firmicutes was negatively related with serum ALT levels in FXR± mice. (F) The relative abundance of cecal content genera of microbiome. (G) The lack of FXR led to increase in Bacteroides (under phylum Bacteroidetes). (H) The lack of FXR caused decrease of Turicibacter (under phylum Firmicutes). (I) The lack of FXR caused decrease of Clostridiales (under phylum Firmicutes). (J) The lack of FXR caused increase of Desulfovibrio (under phylum Proteobacteria).

Supplemental Fig. 1. FXR agonist chenodeoxycholic acid (CDCA) inhibited inflammatory responses in vitro and improved liver function in vivo. (A) Supplement of CDCA inhibited luciferase activity induced by LPS in the bone marrow-derived macrophage (BMDM) culture, indicating the inhibition of NF-κB activity. LPS CDCA vs. LPS, p < 0.001. (B) Supplement of CDCA inhibited TNFα expression induced by LPS in the BMDM culture. LPS CDCA vs. LPS, p < 0.001 (the cell culture was repeated twice). (C) Administration of CDCA decreased serum ALT levels in WT mice with liver IRI (CDCA vs. Vehicle, p < 0.05). (D) Serum AST levels in WT mice with liver IRI (CDCA vs. Vehicle, p > 0.05).
Figure 2

A

B

C

D

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Figure 3
Figure 5

A

B

C

D

E

F

G

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Figure 6

A

Relative Abundance

WT Control  WT RIL  FXR-/- Control  FXR-/- RIL

Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Proteobacteria
TM7
Tenericutes
Verrucomicrobia
Other

B

Bacteroidetes/Firmicutes

WT Control  WT RIL  FXR-/- Control  FXR-/- RIL

C

Relative abundance (%)

Proteobacteria

WT Control  WT RIL  FXR-/- Control  FXR-/- RIL

D

ALT Activity (mU/ml)

% Bacteroidetes

E

ALT Activity (mU/ml)

% Firmicutes
