Deficiency of Cholesterol 7α-Hydroxylase in Bile Acid Synthesis Exacerbates Alcohol-Induced Liver Injury in Mice

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Alcoholic fatty liver disease (AFLD) is a major risk factor for cirrhosis-associated liver diseases. Studies demonstrate that alcohol increases serum bile acids in humans and rodents. AFLD has been linked to cholestasis, although the physiologic relevance of increased bile acids in AFLD and the underlying mechanism of increasing the bile acid pool by alcohol feeding are still unclear. In this study, we used mouse models either deficient of or overexpressing cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting and key regulatory enzyme in bile acid synthesis, to study the effect of alcohol drinking on liver metabolism and inflammation. Mice were challenged with chronic ethanol feeding (10 days) plus a binge dose of alcohol by oral gavage (5 g/kg body weight). Alcohol feeding reduced bile acid synthesis gene expression but increased the bile acid pool size, hepatic triglycerides and cholesterol, and inflammation and injury in wild-type mice and aggravated liver inflammation and injury in Cyp7a1-deficient mice. Interestingly, alcohol-induced hepatic inflammation and injury were ameliorated in Cyp7a1 transgenic mice. Conclusion: Alcohol feeding alters hepatic bile acid and cholesterol metabolism to cause liver inflammation and injury, while maintenance of bile acid and cholesterol homeostasis protect against alcohol-induced hepatic inflammation and injury. Our findings indicate that CYP7A1 plays a key role in protection against alcohol-induced steatohepatitis. (Hepatology Communications 2018;2:99-112)

Introduction

Alcoholic fatty liver disease (AFLD) is a major cause of chronic liver disease. In the United States, AFLD accounts for ~48% of deaths from liver cirrhosis1,2 and comprises a spectrum of conditions, including steatosis, alcoholic hepatitis, fibrosis, and cirrhosis. Several factors, such as sex, obesity, drinking patterns, diet, and genetics, increase the risk of AFLD.3,4 Female individuals have an increased risk of AFLD due to lower levels of gastric alcohol dehydrogenase (ADH), a higher proportion of body fat, and the presence of estrogens.1 The pathogenesis of AFLD includes alcohol-induced oxidative stress, hepatic glutathione depletion, abnormal nutrient metabolism, and gut leakage of endotoxins with subsequent activation of innate immune cells.1 In humans, AFLD is associated with cholestasis,4,5 a pathologic condition caused by increased hepatic bile acids due to the obstruction of bile flow. The physiologic relevance of increased bile acid levels in patients with alcoholism is unclear. Bile acids are steroid molecules derived from

Abbreviations: Abcg, adenosine triphosphate-binding cassette G; ADH, alcohol dehydrogenase; AFLD, alcoholic fatty liver disease; Aldh, aldehyde dehydrogenase; ALT, alanine aminotransferase; CA, cholic acid; Casp1, caspase 1; CDCA, chenodeoxycholic acid; cDNA, complementary DNA; Cyp27a1, steroid 27-hydroxylase; Cyp7a1, cholesterol 7α-hydroxylase; Cyp7a1-Tg, cholesterol 7α-hydroxylase transgenic; Cyp8b1, oxysterol 7α-hydroxylase; Cyp8b1, sterol 12α-hydroxylase; FGF15, fibroblast growth factor 15; FXR, farnesoid X receptor; LPS, lipopolysaccharide; MCA, muricholic acid; mRNA, messenger RNA; Nlhp3, NACHT, LRR, and PYD domains-containing protein 3; OCA, obeticholic acid; Srebp1c, sterol regulatory element binding protein 1c; TCA, trichloroacetic acid; TGR5, Takeda G-protein receptor 5; Tnfα, tumor necrosis factor alpha; vol, volume.

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cholesterol in the liver and are signaling molecules that activate the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor Takeda G-protein receptor 5 (TGR5) in the liver and intestine to exert diverse effects on metabolism and inflammation.\(^\text{(6,7)}\)

Cholesterol 7α-hydroxylase (Cyp7a1) is the rate-limiting enzyme in the classic bile acid synthesis pathway, which plays a critical role in maintaining cholesterol and bile acid homeostasis,\(^\text{(6)}\) while sterol 12α-hydroxylation (Cyp8b1) is involved in cholic acid (CA) synthesis. Cyp7a1 transcription is inhibited by bile acids returning to the liver by enterohepatic circulation. CA and chenodeoxycholic acid (CDCA) are the primary bile acids in humans. CDCA is converted to highly soluble α-muricholic acid (α-MCA) and β-MCA in mouse liver.

In the intestine, bile acids activate FXR to induce fibroblast growth factor 15 (FGF15), which activates hepatic FGF receptor 4/β-Klotho signaling to inhibit Cyp7a1 and Cyp8b1 gene transcription. When the classic bile acid synthesis pathway is impaired, bile acids can be synthesized through the alternative pathway, which is initiated by sterol 27-hydroxylase (Cyp27a1) and oxysterol 7α-hydroxylase (Cyp7b1).\(^\text{(6)}\)

In humans, the bile acid pool is highly hydrophobic and toxic, and accumulation of bile acids in the liver can cause cholestatic liver disease.

Activation of FXR by bile acids and agonists protects against alcohol-induced liver injury, whereas deficiency of FXR aggravates alcoholic steatohepatitis in mice.\(^\text{(8,9)}\)

Alcohol increases hepatic esterified cholesterol, indicating increased conversion of cholesterol to bile acids, decreased biliary bile acids and cholesterol excretion in rats.\(^\text{(10)}\) However, recent studies reported that chronic or binge feeding of alcohol increased Cyp7a1 expression in mice.\(^\text{(11,12)}\)

Thus, the effects of alcohol on Cyp7a1 expression and bile acid synthesis are controversial, and the underlying mechanism is complicated, poorly understood, and requires further study.

In this study, we used the mouse chronic plus binge ethanol-feeding model (NIAAA model)\(^\text{(13)}\) to study the role of Cyp7a1 and bile acids in liver inflammation and injury in Cyp7a1-deficient (Cyp7a1\(^\text{−/−}\)) and Cyp7a1 transgenic (Cyp7a1-Tg) mouse models that we developed recently. This chronic plus binge ethanol-feeding model resembles the drinking patterns of people with alcoholism. The Cyp7a1\(^\text{−/−}\) mice are in a pure C57BL/6J genetic background and have increased hepatic cholesterol, reduced bile acid pool size (about 60% of the wild type), and more hydrophilic bile acid composition with decreased taurocholic acid (TCA) and increased Tauro-α-MCA content.\(^\text{(14,15)}\) The Tauro-α and β-MCA are FXR antagonists that reduce FXR signaling in the intestine.\(^\text{(16)}\)

Cyp7a1\(^\text{−/−}\) mice are prone to lipopolysaccharide (LPS)-induced hepatic inflammation and methionine-choline diet-induced liver fibrosis, while overexpression of Cyp7a1 in Cyp7a1\(^\text{−/−}\) mice reduced hepatic cholesterol and attenuated LPS-induced hepatic inflammation and fibrosis.\(^\text{(15)}\)

On the other hand, Cyp7a1-Tg mice overexpressing rat Cyp7a1 complementary DNA (cDNA) have a bile acid pool size ~2.5-fold higher than wild-type mice, more hydrophobic bile acid composition with reduced TCA and increased tauroursodeoxycholic acid, Tauro-α-MCA and Tauroursodeoxycholic acid, and are resistant to high-fat diet-induced obesity and insulin resistance.\(^\text{(17)}\)

Results from the current study show that chronic plus binge alcohol feeding decreased bile acid synthesis gene expression but increased intestinal bile acid content and total bile acid pool size. Lack of Cyp7a1 exacerbated alcohol-induced hepatic inflammation and hepatic injury by increasing hepatic cholesterol, inflammatory cytokines, and inflammasomes, whereas overexpression of Cyp7a1 reduced hepatic cholesterol, triglycerides, and inflammation and protected mice from alcohol-induced liver injury. Our current findings indicate that CYP7A1 plays a key role in maintaining bile acid and cholesterol homeostasis and protecting against alcohol-induced steatohepatitis.

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Materials and Methods

MOUSE MODELS

Cyp7a1 knockout mice (Cyp7a1–/–) (Jackson Laboratory) were backcrossed with C57BL/6J mice as described. Cyp7a1-Tg overexpressing rat Cyp7a1-coding cDNA were backcrossed with C57BL/6J mice as described. Mice were first maintained on a standard chow diet (LabDiet #5008; St. Louis, MO) with water ad libitum and housed in a temperature-controlled facility with a 12-hour light and 12-hour dark cycle. Female wild-type and Cyp7a1–/– mice were used for most experiments. Male Cyp7a1-Tg mice were used due to insufficient mice available for experiments. All mouse experiments were performed in the animal facility at Northeast Ohio Medical University and were approved by the Institutional Animal Care and Use Committee.

CHRONIC PLUS BINGE ALCOHOL FEEDING

The NIAAA model was used for alcohol feeding studies in 15- to 17-week-old male and female wild-type (C57BL/6J) and Cyp7a1–/– mice and in male Cyp7a1-Tg mice. Mice were acclimated to control Lieber-DeCarli liquid diet (Catalog #F1259; BioServ, Flemington, NJ; protein, 151 kcal/L; fat, 359 kcal/L; carbohydrate, 490 kcal/L) for 5 days. Mice were then divided into groups (n = 5-6) and were pair fed either control Lieber-DeCarli liquid diet or Lieber-DeCarli ethanol diet (5% volume [vol]/vol) (Catalog #F1258; BioServ; protein, 151 kcal/L; fat, 359 kcal/L; carbohydrate, 135 kcal/L; ethanol, 355 kcal/L) for 10 days. At 9 AM on day 11, mice were orally gavaged with a single binge dose of maltose dextrin (9 g/kg body weight, control mice) or isocaloric ethanol (5 g/kg body weight, ethanol mice). Mice were euthanized by isoflurane inhalation and cervical dislocation 9 hours postgavage (5 pm). Blood was clotted on ice for 20 minutes and was spun at 700 x g to collect serum, while liver and intestine tissues were collected and stored at –80 °C until analysis.

SERUM AND TISSUE ANALYSES

Blood alcohol content was measured using nicotinamide adenine dinucleotide-ADH reagent for alcohol determination (#N7160; Sigma-Aldrich, St. Louis, MO). Serum alanine aminotransferase (ALT) levels were analyzed using an Infinity ALT Liquid Stable Reagent kit (ThermoFisher Scientific Inc., Waltham, MA). Tissue lipids were extracted by homogenizing 100 mg frozen liver in 500 μL phosphate-buffered saline; 700 μL of a chloroform:isopropanol:Nonidet P40 (7:11:0.1 vol/vol) solution was added, and the samples were vortexed and incubated in a sonicating water bath for 1 hour. Samples were centrifuged at 18,000 x g for 10 minutes, and the organic layer was collected and air dried. The lipids were resuspended in 200 μL distilled H2O + 2% Triton. Triglyceride content was determined using Triglyceride–SL reagent (Sekisui Diagnostics, Lexington, MA), free fatty acids were quantified using a nonesterified fatty acids (NEFA) kit (Wako Diagnostic, Richmond, VA), and total and free cholesterol levels were quantified using a Total Cholesterol Assay kit (BioVison Inc., Milpitas, CA).

HISTOLOGY

Tissues were fixed in 10% buffered formalin and were processed overnight for paraffin embedding. Paraffin sections (5 μm) were stained with hematoxylin and eosin. For free cholesterol detection, frozen sections (5 μm) were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.05 mg/mL filipin working solution for 2 hours at room temperature. The slides were visualized by fluorescence microscopy using an ultraviolet filter set (340-380 nm excitation, 430 nm emission). Images were visualized using an Olympus IX73 microscope and captured by an Olympus DP80 color camera and Olympus CellSens software.

BILE ACID ANALYSIS

Bile acids were extracted from 100 mg frozen liver, whole intestine, and whole gallbladder by a series of ethanol extractions (95%, followed by 85%) and a chloroform:methanol extraction (2:1 vol/vol), each overnight in a 65 °C water bath. Bile acid content in tissue and serum was quantified using a Total Bile Acid Assay kit (Catalog #DZ042A-K; Diazyme Laboratories, Poway, CA). Total liver bile acid content was determined by back calculation using whole liver weight. Bile acid pool size was determined by totaling bile acid content in whole liver, gallbladder, and intestine.
QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS OF MESSENGER RNA

Total RNA was isolated from frozen tissue using Trizol (Sigma-Aldrich), followed by chloroform extraction and isopropanol precipitation. Reverse transcription was performed using RETROscript Reverse Transcription kits (Catalog #AM1710) and 2 μg hepatic or intestine RNA. Real-time polymerase chain reaction was performed for quantitative messenger RNA (mRNA) assay using Taqman primer/probe sets (Applied Biosystems, Foster City, CA). Relative mRNA expression was quantified using the delta-delta threshold cycle (ΔΔCt) method, and glyceraldehyde 3-phosphate dehydrogenase or 36B4 ribosomal RNA was used as an internal standard.

IMMUNOBLOT ANALYSIS

Proteins were extracted from individual mouse liver tissue samples, and protein lysates were dissolved in radio immunoprecipitation assay buffer for immunoblot analysis. Protein concentration was determined by Pierce bicinchoninic acid (BCA) analysis (ThermoFisher Scientific), and 50 μg protein was loaded in each well. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4% stacking and 10% resolving gel) and transblotted onto polyvinylidene fluoride membranes. Blots were blocked for 1 hour in 5% nonfat dry milk in trishydroxymethylaminomethane-buffered saline Tween 20 followed by overnight incubation at 4°C with antibodies against Cyp7a1 (#sc-14423; Santa Cruz Biotechnology, Inc., Dallas, TX), Cyp7b1 (P-18, #sc-23515; Santa Cruz Biotechnology), Cyp7b1 (#ab138497; Abcam, Cambridge, MA), Cyp27a1 (#ab126785; Abcam), sterol regulatory element binding protein 1c (Srebp1c) (#NB100-2215; Novus Biologicals, Littleton, CO), Cyp2e1 (#ab28146; Abcam), or caspase 1 (Casp1) (#ab108362; Abcam), with glyceraldehyde 3-phosphate dehydrogenase (#5174; Cell Signaling) or actin (#4967; Cell Signaling) as the internal calibration control. After overnight incubation, blots were washed 3 times in trishydroxymethylaminomethane-buffered saline Tween 20 for 10 minutes each and were then incubated with respective secondary antibodies at room temperature for 60 minutes. Blots were visualized by an image scanner (ImageQuant LAS 4000; GE Healthcare Bio-Sciences, Pittsburgh, PA) and were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

STATISTICAL ANALYSIS

Statistical significance between two groups was determined using a two-tailed Student t test, and statistical significance between multiple groups was determined using one-way analysis of variance followed by a Tukey post-hoc test using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM, with P < 0.05 considered to be statistically significant.

Results

DEFICIENCY OF Cyp7a1 AGGRAVATED ALCOHOL-INDUCED LIVER INJURY AND HEPATIC LIPID METABOLISM

We used both male and female wild-type and Cyp7a1<sup>−/−</sup> mice for this study. It has been reported that female mice are more sensitive to developing AFLD.(13,20) Chronic plus binge alcohol feeding significantly suppressed hepatic Cyp7a1 mRNA expression in female mice but not in male mice compared to respective pair-fed control mice (Fig. 1, left panel). Blood alcohol content did not change significantly between female wild-type and Cyp7a1<sup>−/−</sup> mice (Fig. 1, right panel). Alcohol feeding increased the liver-to-body weight ratio but not adipose tissue-to-body ratio in female wild-type and Cyp7a1<sup>−/−</sup> mice (Fig. 1B) and increased serum ALT in both wild-type and Cyp7a1<sup>−/−</sup> mice (Fig. 1C). In wild-type mice, alcohol significantly increased serum cholesterol and triglycerides, whereas in Cyp7a1<sup>−/−</sup> mice only triglycerides increased; no change was observed in serum free fatty acid levels (Fig. 1D). Furthermore, alcohol feeding increased hepatic triglycerides in both wild-type and Cyp7a1<sup>−/−</sup> mice but did not affect hepatic total cholesterol or free cholesterol levels (Fig. 1E). However, total liver cholesterol was significantly higher in Cyp7a1<sup>−/−</sup> mice compared to wild-type mice in both alcohol-fed mice and pair-fed controls (Fig. 1E). Hematoxylin and eosin staining indicated alcohol feeding increased hepatic steatosis in both wild-type and Cyp7a1<sup>−/−</sup> mice (Fig. 1F, left panel). Filipin staining of free cholesterol confirmed that Cyp7a1<sup>−/−</sup> mice had increased hepatic free cholesterol compared to wild-type mice, and
FIG. 1. Cyp7a1 deficiency aggravated alcohol-induced hepatic injury and increased cholesterol levels. (A) Left panel, hepatic Cyp7a1 mRNA expression in alcohol-fed (n = 6) and pair-fed control (n = 5) female and male WT and Cyp7a1–/– mice. Right panel, blood alcohol levels in female WT and Cyp7a1–/– mice. In experiments B to F, female WT and Cyp7a1–/– mice were used for alcohol feeding (n = 6) and pair-fed controls (n = 5). (B) Liver-to-body weight and adipose tissue-to-body weight ratio of alcohol-fed and pair-fed control WT and Cyp7a1–/– female mice. (C) Serum ALT levels. (D) Serum cholesterol, triglyceride, and free fatty acid levels. (E) Hepatic triglyceride and cholesterol levels. (F) Hematoxylin and eosin staining of liver (left) and filipin (right) staining of hepatic free cholesterol in liver (magnification × 200). Results are mean ± SEM; * indicates significant difference between the groups indicated (P < 0.05). Abbreviations: B.W, body weight; chol, cholesterol; Con, control; EtOH, ethanol; FFA, free fatty acid; H&E, hematoxylin and eosin; LW, liver weight; TG, triglyceride; WT, wild-type.
alcohol increased free cholesterol in wild-type mice but not Cyp7a1−/− mice (Fig. 1F, right panel). These results indicate that lack of Cyp7a1 resulted in increased hepatic triglycerides and esterified cholesterol and exacerbated ethanol-induced liver injury and hepatic steatosis.

**CHRONIC PLUS BINGE ALCOHOL FEEDING INCREASED BILE ACID POOL SIZE AND DECREASED BILE ACID SYNTHESIS GENE EXPRESSION**

Expression of the bile acid synthesis genes Cyp8b1, Cyp7b1, and Cyp27a1 mRNA levels was induced in isocaloric pair-fed control Cyp7a1−/− mice (Fig. 2A). Alcohol feeding significantly reduced Cyp7a1, Cyp8b1, and Cyp7b1 mRNA levels in wild-type mice and reduced Cyp8b1 mRNA levels in female Cyp7a1−/− mice (Fig. 2A). Immunoblot analysis indicated alcohol feeding reduced Cyp7a1 protein levels in pair-fed control wild-type mice (Fig. 2B), consistent with reduced Cyp7a1 mRNA levels. Hepatic protein levels of the CA synthesis enzyme Cyp8b1 were unchanged, while protein levels of Cyp7b1 were decreased by ethanol feeding in wild-type mice and increased by ethanol feeding in Cyp7a1−/− mice. Protein levels of Cyp27a1 were increased by ethanol feeding only in wild-type mice (Fig. 2C). In control mice, total bile acid pool size was reduced by ~30% in Cyp7a1−/− mice compared to wild-type mice (Fig. 2D). Alcohol feeding significantly increased bile acid content in the gallbladder and intestine, resulting in increased bile acid pool size in wild-type mice (Fig. 2D). Alcohol feeding insignificantly increased intestinal bile acid content and total bile acid pool size in Cyp7a1−/− mice without changing bile acid content in the liver or gallbladder (Fig. 2D). Serum bile acid levels were reduced in control-fed Cyp7a1−/− mice compared to wild-type mice, and alcohol increased serum bile acids only in Cyp7a1−/− mice. The data indicate that alcohol feeding reduced bile acid synthesis gene expression but increased bile acid pool size in both wild-type and Cyp7a1−/− mice. Gene expression analysis of the alcohol metabolism enzymes Adh and aldehyde dehydrogenase (Aldb2) showed that while pair-fed control Cyp7a1−/− mice had significantly increased Adh mRNA expression levels compared to wild-type mice, alcohol did not alter expression of this gene. Conversely, alcohol feeding significantly increased hepatic mRNA expression levels of Aldb2 in Cyp7a1−/− mice (Fig. 2E). Hepatic protein expression of the alcohol-inducible enzyme Cyp2e1 was increased after alcohol feeding in wild-type mice but not in Cyp7a1−/− mice (Fig. 2F). The data indicate that alcohol differentially affected expression levels of bile acid synthesis enzyme mRNA and protein in wild-type and Cyp7a1−/− mice and resulted in increased bile acid pool size without increasing bile acid synthesis in both wild-type and Cyp7a1−/− mouse liver.

**CHRONIC PLUS BINGE ALCOHOL FEEDING ALTERED CHOLESTEROL AND LIPID METABOLISM**

Chronic plus binge alcohol feeding did not significantly alter expression of hepatic genes involved in bile acid transport (Na+ -taurocholate cotransporting polypeptide [Ntcp], bile salt export pump [Bsep]) or bile acid regulation (Fxr, small heterodimer partner [Sbp], FGF15 receptor [Fgfr4]), although expression of many of these genes was higher in Cyp7a1−/− mice regardless of treatment (Fig. 3A). Alcohol feeding did not alter mRNA expression of apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (ApoB100) but did increase expression of microsomal triglyceride transfer protein (Mttp) (involved in lipoprotein assembly) mRNA in both wild-type and Cyp7a1−/− mice (Fig. 3A). Alcohol feeding had no effect on mRNA expression of the fatty acid synthesis genes hepatic acetyl-coenzyme A carboxylase 1 (Acac1), fatty acid synthase (Fasn), or Srebp1 mRNA (Fig. 3C) or protein (Fig. 3D). Expression of carnitine palmitoyltransferase 1 (Cpt1) mRNA, involved in fatty acid synthesis, was significantly reduced by alcohol feeding in Cyp7a1−/− mice (Fig. 3C). These data suggest that reduced fatty acid oxidation but not increased fatty acid synthesis may contribute to hepatic steatosis.

Alcohol feeding strongly reduced mRNA levels of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Hmgcr), low-density lipoprotein receptor (Ldlr), and proprotein convertase subtilisin/kexin type 9 (Pcks9, an inhibitor of Ldlr) but had no effect on angiopoietin-like 3 (Angptl3, lipoprotein lipase inhibitor) or Srebpl mRNA (cholesterol synthesis regulator) compared to wild-type mice. Lack of Cyp7a1 increased adenosine triphosphate-binding cassette G5 (Abcg5) and Abcg8 (cholesterol efflux transporters) in wild-type mice. Alcohol feeding reduced Hmgcr but induced Abcg5 and
FIG. 2. Alcohol feeding increased bile acid pool size and decreased bile acid synthesis gene expression in mice. WT and Cyp7a1−/− female mice were used for alcohol feeding (n = 6) and pair-fed controls (n = 5). (A) Hepatic mRNA expression of bile acid synthesis genes. (B) Hepatic Cyp7a1 protein expression (n = 3, each lane represents one mouse). (C) Hepatic bile acid synthesis protein expression (n = 3, each lane represents one mouse). (D) Bile acid pool and content in gallbladder, small intestine, liver, and serum. (E) Hepatic mRNA expression of alcohol metabolism genes. (F) Hepatic Cyp2e1 protein expression. Results are mean ± SEM; * indicates significant difference between the groups indicated (P < 0.05). Abbreviations: AU, arbitrary unit; Con, control; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GB, gallbladder; WT, wild-type.
Abcg8 in Cyp7at−/− mice (Fig. 3E). The data indicate that increases in hepatic total cholesterol and triglycerides by alcohol feeding are not caused by increased cholesterol synthesis but likely by reduced conversion of cholesterol to bile acids, indicated by reduced bile acid synthesis gene expression.
LACK OF Cyp7a1 INCREASED ALCOHOL-INDUCED HEPATIC INFLAMMATION

Hepatic inflammatory gene expression, including interleukin-1β (Iliβ), tumor necrosis factor alpha (Tnfα), and monocyte chemoattractant protein-1 (Mcp1) and the inflammasome protein NACHT, LRR, and PYD domains-containing protein 3 (Nlhp3), was induced in alcohol-fed Cyp7a1−/− mice compared to control-fed mice, and both Iliβ and Tnfα were significantly elevated over wild-type mice following alcohol treatment (Fig. 4A). Expression of the inflammasome marker Casp1 was significantly induced by alcohol in Cyp7a1−/− mice (Fig. 4B), which was confirmed with immunoblot analysis of Casp1 and the mature form of Casp1, Casp1 (p-20) (Fig. 4C).

Alcohol feeding significantly increased mRNA levels of the hepatic neutrophil marker lymphocyte antigen 6 complex, locus G (Ly6G) in wild-type mice, while Ly6G, chemokine (C-X-C motif) ligand 1 (Cxcl1), and C-X-C motif chemokine receptor 2 (Cxc2) were significantly increased in Cyp7a1−/− mice and expression of the cytokine-activated cell adhesion molecule E-selectin (Sele) was unchanged (Fig. 4D). Cxcl1 and Cxc2 play key roles in neutrophil infiltration in AFLD in mice.21 The effect of alcohol feeding on intestinal bile acid and FXR-regulated gene expression is shown in Fig. 4E. Alcohol did not alter mRNA expression of apical sodium-dependent bile acid transport (Asbt) or bile acid binding protein (Lbabhp) but significantly increased sinusoidal bile acid efflux transporter organic solute transporter β (Ostβ) mRNA levels in both wild-type and Cyp7a1−/− mice; alcohol feeding reduced Fgf15 expression in wild-type mice. Interestingly, expression of the intestinal barrier function genes occludin (Ocln), zona occludens 1 (also known as tight junction protein 1, Zoa1), junction adhesion molecule 1 (Jam1), and claudin-2 (Cldn2) were significantly reduced in control-fed Cyp7a1−/− mice compared to wild-type mice, and alcohol feeding reduced expression only in wild-type mice (Fig. 4F). The data indicate that alcohol feeding did not activate FXR signaling in the intestine in either wild-type or Cyp7a1−/− mice and suggest that alcohol feeding increased bile acid pool size by increasing intestinal bile acid reabsorption without increasing bile acid synthesis in the liver. Increased OStββ expression may be FXR independent and is consistent with increased intestinal bile acids in both wild-type and Cyp7a1−/− mice. Also, alcohol increased hepatic inflammasomes and neutrophil infiltration in Cyp7a1T− mice and may affect intestinal barrier function.

OVEREXPRESSION OF Cyp7a1 AMELIORATES ALCOHOL-INDUCED LIVER INJURY

Cyp7a1-Tg mice were generated by transgenic expression of rat Cyp7a1 cDNA to overexpress Cyp7a1, resulting in an enlarged bile acid pool with increased taurochenodesoxycholic acid and abolished TCA. This mouse model was used to test the hypothesis that increased bile acid pool and FXR signaling protect mice from alcohol-induced liver injury. In these experiments, male Cyp7a1-Tg mice were used due to a lack of sufficient number of female Cyp7a1−/− mice. Alcohol feeding increased serum ALT levels in wild-type mice but not in Cyp7a1−/− mice (Fig. 5A). Hepatic triglyceride and total cholesterol levels were significantly lower in Cyp7a1−/− mice compared to wild-type mice (Fig. 5B) and remained significantly reduced after alcohol feeding. Cyp7a1−/− mice had significantly reduced mouse Cyp7a1 and Cyp8b1 mRNA levels compared to wild-type mice (Fig. 5C) due to activation of FXR by CDCA.17 Alcohol feeding reduced Cyp7b1 and Cyp27a1 mRNA levels in both wild-type and Cyp7a1−/− mice (Fig. 5C). Immunoblotting analysis showed that alcohol feeding reduced Cyp7a1 protein in Cyp7a1−/− mice (Fig. 5D) as well as Cyp8b1 and Cyp7b1 protein, while Cyp27a1 protein levels were unchanged (Fig. 5D). Bile acid pool size in alcohol-fed Cyp7a1−/− mice was also determined. Gallbladder bile acid levels were unchanged in alcohol-fed wild-type and Cyp7a1−/− mice compared to respective pair-fed control mice, although alcohol feeding caused significantly increased bile acid content in the liver and intestine of Cyp7a1−/− mice compared to wild-type mice and resulted in a significant increase in bile acid pool size (Fig. 5F). However, alcohol feeding reduced bile acid content in the liver and serum of Cyp7a1−/− mice, consistent with decreased levels of Cyp7a1 protein in these mice (Fig. 5D).

Hepatic expression of Adh and Adh mRNA was unchanged by alcohol feeding in both wild-type and Cyp7a1−/− mice (Fig. 6A), but Cyp2e1 protein levels were significantly increased only in Cyp7a1−/− mice fed alcohol (Fig. 6B). Alcohol feeding did not alter hepatic mRNA expression of the transporters Ntcp or Bsep nor did it affect Fxr or Shp (Fig. 6C). Alcohol strongly induced Tnfα mRNA in wild-type mice but not in Cyp7a1−/− mice (Fig. 6D), and hepatic Nlhp3 mRNA levels decreased in control-fed mice.
and Casp1 mRNA levels were significantly lower in alcohol-fed Cyp7a1−/− female mice were used for alcohol feeding (n = 6) and pair-fed controls (n = 5). (A) Hepatic mRNA expression of proinflammatory genes. (B) Hepatic mRNA expression of the inflammasome genes Nlrp3 and Casp1. (C) Hepatic protein levels of pro-Casp1 and Casp1 (p20) isoforms (n = 3, each lane represents one mouse). (D) Hepatic mRNA expression of genes involved in neutrophil infiltration. (E) Ileum mRNA expression of bile acid transporter and FXR target genes. (F) Ileum mRNA expression of genes involved in cell adhesion and inflammation. Results are mean ± SEM; * indicates significant difference between the groups indicated (P < 0.05). Abbreviations: AU, arbitrary unit; Con, control; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; WT, wild-type.

and Casp1 mRNA levels were significantly lower in alcohol-fed Cyp7a1−/− female mice were used for alcohol feeding (n = 6) and pair-fed controls (n = 5). The data suggest that increased Cyp7a1 expression and bile acid pool size in Cyp7a1−/− mice ameliorated alcohol-induced hepatic inflammation.

**Discussion**

This study shows that chronic plus binge alcohol feeding significantly reduced bile acid synthesis gene expression but increased bile acid pool size, hepatic
triglycerides and cholesterol, inflammatory cytokines, and inflammasome and macrophage infiltration in wild-type mice. Cyp7a1−/− mice exhibited more severe liver inflammation and injury than wild-type mice. On the other hand, overexpression of Cyp7a1 resulted in increased bile acid pool size and FXR signaling and protected mice from alcohol-induced inflammation and injury. Alcohol may increase hepatic cholesterol by

FIG. 5. Alcohol reduces bile acid synthesis gene and protein expression along with liver bile acid content in mice overexpressing Cyp7a1. WT and Cyp7a1-Tg male mice were used for alcohol feeding (n = 6) and pair-fed controls (n = 5). (A) Serum ALT levels. (B) Hepatic triglyceride and cholesterol levels. (C) Hepatic mRNA expression of bile acid synthesis genes. (D) Hepatic Cyp7a1 protein expression (n = 3, each lane represents one mouse). (E) Hepatic bile acid synthesis protein expression (n = 3, each lane represents one mouse). (F) Bile acid pool and content in gallbladder, small intestine, liver, and serum. Results are mean ± SEM; * indicates significant difference between the groups indicated (P < 0.05). Abbreviations: AU, arbitrary unit; chol, cholesterol; Con, control; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GB, gallbladder; TG, triglyceride; WT, wild-type.
decreasing conversion of cholesterol to bile acids rather than by increasing de novo cholesterol synthesis. These results are in contrast with previous reports\(^{11,12}\) but are consistent with a recent report that alcohol increased hepatic cholesterol by inhibiting Cyp7a1 mRNA expression in mice.\(^{22}\) Interestingly, the latter study shows that alcohol-induced hepatic iron overloading inhibited Cyp7a1 mRNA expression through an iron response element located in the 3'-untranslated region of Cyp7a1 mRNA. In Cyp7a1\(^{-/-}\) mice, the classic bile acid synthesis pathway is defective and bile acids are synthesized from the alternative pathway.\(^{14}\) Here, alcohol feeding also reduced Cyp7b1 expression in the alternative pathway. Studies have shown that a high-fat diet increased hepatic cholesterol and injury in Cyp7a1\(^{-/-}\) mice.\(^{14,15,23}\) The Lieber-DeCarli liquid diet used in this model is a high-fat and high-carbohydrate diet and thus may have increased hepatic total cholesterol levels as in Western high-fat diet-fed Cyp7a1\(^{-/-}\) mice.\(^{14}\) In Cyp7a1-Tg mice, alcohol did not increase hepatic cholesterol and triglycerides, and instead these mice were protected from alcohol-induced hepatic inflammation and activation of hepatic inflammasomes, suggesting that accumulation of cholesterol in hepatocytes contributes to hepatic inflammation and steatosis in nonalcoholic fatty liver disease.\(^{19,24}\) Our current study indicates that decreased bile acid synthesis exacerbates alcohol-induced liver injury by increasing hepatic free cholesterol levels to increase expression of Nlrp3 and Casp1 in Kupffer cells. It has been reported that Nlrp3 knockout mice are protected from alcohol-induced liver injury.\(^{25}\) Increased Nlrp3 and Casp1 along with hepatic free cholesterol may have increased natural killer T-cell recruitment in alcohol-fed Cyp7a1\(^{-/-}\) mice. Cyp7a1\(^{-/-}\) mice have decreased bile acid pool size but more tauro-MCA, which antagonizes FXR signaling to increase hepatic inflammation, whereas these effects were ameliorated in Cyp7a1-Tg mice. Alcohol binge drinking increases LPS production from gut bacteria. Our previous studies showed that LPS and cytokines suppressed Cyp7a1 and Cyp8b1 expression in hepatocytes by activating the c-Jun N-terminal kinase pathway.\(^{26,27}\) It has been reported that a high-fat diet plus binge drinking causes advanced stages of steatohepatitis and fibrosis in mice.\(^{28}\) In the current study, chronic plus binge drinking did not cause liver fibrosis in Cyp7a1\(^{-/-}\) mice.
mice. In Cyp7a1−/− mice, accumulation of hepatic cholesterol may exacerbate alcoholic liver injury by increasing hepatic injury. Overexpression of Cyp7a1 in Cyp7a1−Tg mice reduced hepatic cholesterol and protects against alcoholic liver injury. We demonstrated that bile acid composition rather than bile acid pool size regulated bile acid homeostasis and prevented cholestatic liver injury and inflammation. Many recent studies have shown that dietary cholesterol, not fat, causes liver injury by increasing oxidative stress and reactive oxidizing species to cause fatty liver as in Western diet-fed steatosis mouse models. It should be emphasized that accumulation of toxic bile acids can cause cholestatic liver injury, while activation of FXR signaling protects against inflammation. Some patients with alcoholic liver disease have cholestatic liver injury. The FXR agonist obeticholic acid (OCA) has been shown to reduce hepatic steatosis and oxidative stress induced by ethanol and a low-protein diet. OCA may reduce bile acid synthesis to reduce alcohol-induced cholestatic liver injury and hepatic inflammation and protect intestinal barrier function. OCA is in clinical trials for alcoholic liver disease (clinicaltrials.gov Identifier: NCT02654236).

In summary, this study demonstrated the importance of bile acid homeostasis in protection against AFLD, using chronic plus binge alcohol feeding. Decreasing bile acid synthesis increases hepatic free cholesterol accumulation and exacerbates alcohol-induced liver injury, whereas increasing bile acid synthesis reduces hepatic cholesterol and protects against AFLD. This study unveiled that bile acid composition and signaling play a central role in alcohol-induced liver injury and inflammation and that maintaining bile acid and cholesterol homeostasis is critical for protection against AFLD.

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