A chronic high-cholesterol diet paradoxically suppresses hepatic CYP7A1 expression in FVB/NJ mice[S]

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Abstract **Cholesterol 7α-hydroxylase (CYP7A1) encodes for the rate-limiting step in the conversion of cholesterol to bile acids in the liver. In response to acute cholesterol feeding, mice upregulate CYP7A1 via stimulation of the liver X receptor (LXR) α. However, the effect of a chronic high-cholesterol diet on hepatic CYP7A1 expression in mice is unknown. We demonstrate that chronic cholesterol feeding (0.2% or 1.25% w/w cholesterol for 12 weeks) in FVB/NJ mice results in a >60% suppression of hepatic CYP7A1 expression associated with a >2-fold increase in hepatic cholesterol content. In contrast, acute cholesterol feeding induces a >3-fold upregulation of hepatic CYP7A1 expression. We show that chronic, but not acute, cholesterol feeding increases the expression of hepatic inflammatory cytokines, tumor necrosis factor (TNF)α, and interleukin (IL)-1β, which are known to suppress hepatic CYP7A1 expression. Chronic cholesterol feeding also results in activation of the mitogen activated protein (MAP) kinases, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Furthermore, we demonstrate in vitro that suppression of CYP7A1 by TNFα and IL-1β is dependent on JNK and ERK signaling. We conclude that chronic high-cholesterol feeding suppresses CYP7A1 expression in mice. We propose that chronic cholesterol feeding induces inflammatory cytokine activation and liver damage, which leads to suppression of CYP7A1 via activation of JNK and ERK signaling pathways.—** Henkel, A. S., K. A. Anderson, A. M. Dewey, M. H. Kavesh, and R. M. Green. **A chronic high-cholesterol diet paradoxically suppresses hepatic CYP7A1 expression in FVB/NJ mice.** ***J. Lipid Res.*** **2011.** 52: **289–298.**

Supplementary key words bile acids • tumor necrosis factor α • hepatic inflammation

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States (1). Induction of hepatic inflammation marks the progression from simple steatosis to nonalcoholic steatohepatitis (NASH); however, the factors that initiate the inflammatory response remain largely unknown (2, 3). Studies on the pathogenesis of NASH have primarily emphasized the role of hepatic triglycerides; however, emerging data question the importance of hepatic triglycerides in the progression to NASH (4, 5). In fact, it has been proposed that hepatic triglyceride accumulation may actually serve a protective role to prevent progressive liver damage (4). Recent studies indicate that excess cellular cholesterol induces inflammation and release of inflammatory cytokines (5–7). Hepatic cholesterol accumulation and the resultant hepatic inflammatory response may contribute to the progression from simple steatosis to NASH (5, 6).

Hepatic cholesterol is metabolized to bile acids in the liver, which serves as the major means of cholesterol elimination from the human body. The rate-limiting step in this pathway is controlled by the hepatic enzyme cholesterol 7-α hydroxylase (CYP7A1). The CYP7A1 gene is highly regulated via numerous signaling pathways (8). Bile acids negatively regulate CYP7A1 via farnesoid X receptor (FXR)-dependent signaling (9). Two distinct FXR-dependent pathways exist in the liver and in the intestine. In the liver, bile acids bind FXR, stimulating transcription of the short heterodimer partner (SHP), which inhibits liver receptor homolog 1 and hepatocyte nuclear factor 4α (HNF4α) transactivation of CYP7A1 (10–12). In the ileum, bile acid binding to FXR stimulates release of fibroblast growth factor (FGF) 15/19, which binds to its

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Abbreviations: CYP7A1, cholesterol 7α-hydroxylase; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FXR, farnesoid X receptor; IBABP, ileal bile acid binding protein; IL, interleukin, JNK, c-Jun N-terminal kinase; LXR, liver X receptor; MAP, mitogen activated protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OST, organic solute transporter; PGC, peroxisome proliferator-activated receptor gamma coactivator; SHP, short heterodimer partner; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TMCA, tauromuricholic acid; TNF, tumor necrosis factor; TUDCA, tauroursodeoxycholic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one table.

receptor FGFR4 in the liver and inhibits hepatic CYP7A1 expression. Recent data indicate that activation of FXR in the intestine, and the resultant production of FGF15, is the primary means of bile acid feedback inhibition of hepatic CYP7A1 in mice (13, 14).

It has been shown that rodents upregulate hepatic CYP7A1 expression in response to short-term cholesterol feeding (15, 16). As a result, the conversion of cholesterol to bile acids is enhanced and cholesterol homeostasis is maintained. The upregulation of hepatic CYP7A1 in response to dietary cholesterol is mediated by the oxysterol sensor, liver X receptor α (LXRα). Mice that lack LXRα fail to upregulate hepatic CYP7A1 in response to dietary cholesterol and, hence, develop massive hepatic cholesterol accumulation (17). There is an LXR-response element within the promoter of the CYP7A1 gene of rodents but not humans (15, 16, 18). Accordingly, humans may not upregulate hepatic CYP7A1 in response to dietary cholesterol (19). Supporting this hypothesis is the observation that genetically altered mice expressing the human CYP7A1 gene and promoter, rather than the murine gene, lack induction of CYP7A1 when fed a high-cholesterol diet (19, 20). Several other species, including rabbits, hamsters, and some primates, also lack the ability to upregulate, and in fact, downregulate CYP7A1 in response to short-term cholesterol feeding (21, 22). The regulation of hepatic CYP7A1 in rodents in response to acute cholesterol administration is well characterized; however, a chronic high-cholesterol diet represents a more physiologic model of cholesterol exposure and may be more relevant to the modern human diet. Whether rodents upregulate CYP7A1 and maintain cholesterol homeostasis in response to chronic cholesterol feeding is unknown.

Hepatic inflammation and the acute phase response have been implicated in the regulation of lipid metabolism and hepatic CYP7A1 expression. Inflammatory cytokines such as tumor necrosis factor (TNF)α and interleukin (IL)-1β suppress hepatic CYP7A1 expression in vitro and in vivo (23–25). Additionally, TNFα has been shown to suppress peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α), a direct activator of CYP7A1, in human hepatoma cells and mice (26). The mitogen activated protein kinases (MAPKs), JNK and ERK, have been implicated in the inhibition of CYP7A1 and are known to be activated by IL-1β and TNFα (25, 27–31). The physiologic relevance of the regulation of CYP7A1 by inflammatory cytokines is not clear, but it has been proposed that suppression of bile acid synthesis may be cytoprotective in the setting of hepatic inflammation (8).

In the present study, we demonstrate that chronic high-cholesterol feeding to mice suppresses hepatic CYP7A1 expression and is associated with significant hepatic cholesterol accumulation. Chronic, but not acute, cholesterol feeding results in induction of hepatic inflammatory cytokines and activation of JNK and ERK signaling, which we propose serves as a major mechanism of CYP7A1 suppression.

METHODS

Animals and diets

Male FVB/NJ mice (8–10 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed a control, 0.2% cholesterol (w/w), or 1.25% cholesterol (w/w) diet for 7 days or 12 weeks (Research Diets, New Brunswick, NJ). Feeding experiments were performed twice with a sample size of five mice for each cohort. Mice underwent 12 h light/dark cycling and were given free access to food and water. Mice were fasted for 4 h prior to euthanasia and were euthanized by CO_2 inhalation. In animals used for bile salt analysis, the liver, gallbladder, and small intestine were removed and minced in 100% ethanol. In the remaining mice, the livers were rapidly excised, weighed, and flushed with ice-cold saline. An aliquot was fixed in 10% formalin for histologic analysis, which was performed at the Northwestern University Pathology Core (Chicago, IL). The remainder of the liver was sectioned and snap-frozen in liquid nitrogen. The small intestine was removed, flushed with ice-cold saline, sectioned into 5 cm segments, and snap-frozen in liquid nitrogen. The livers and small intestine were stored at −80°C until analyzed. All animal protocols were approved by the Northwestern University Animal Care and Use Committee (ACUC).

Liver chemistries

Liver samples were homogenized in Dulbecco's phosphate buffered saline for hepatic lipid analysis (100 mg liver tissue/1ml). Triglyceride and cholesterol levels were measured in liver homogenate using an Infinity spectrophotometric assay per the manufacturer's protocol (Thermo Electron Corporation, Melbourne, Australia).

Cell culture

HepG2 cells (ATCC, Mannasas, VA) were cultured in DMEM with 10% fetal bovine serum and maintained at 37°C in 5% CO_2. Cells were grown to 80% confluence in 6-well plates and treated with either a JNK inhibitor, SP600125 (Sigma-Aldrich, St. Louis, MO) at 25 μM, an ERK inhibitor, PD184352 (Santa Cruz Biotechnology) at 5 μM, or vehicle (DMSO/saline). At one h, TNFα (50ng/ml) or IL-1β (10ng/ml) (Sigma-Aldrich) in serum-free DMEM was added and cells were incubated for an additional 6 h. In a second set of experiments, cells were transfected with small interfering RNA targeting JNK1 or cotransfected with siRNA targeting ERK1 and ERK2 per protocol (Santa Cruz Biotechnology). Control cells were transfected with control siRNA (Santa Cruz Biotechnology). Eighteen hours after siRNA transfection, TNFα (50ng/ml) or IL-1β (10ng/ml) in serum-free DMEM was added and cells were incubated for an additional 6 h. RNA isolation was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA) per protocol. Successful knockdown of the genes of interest was confirmed by Western blot.

Analysis of gene expression by real-time quantitative PCR

Total RNA from frozen liver samples was isolated using TRIzol reagent. Two micrograms of total RNA was used for reverse transcription PCR using a qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). Real-time quantitative PCR was performed using 2 μL of cDNA from each sample in a 25 μL reaction mixture containing Quantitect SYBR Green PCR Mastermix (Qiagen, Valencia, CA) along with primers specific for the gene of interest. GAPDH was employed as a housekeeping gene. The primer sequences are shown in supplementary Table I. Amplification was performed on an ABI 7300 sequence detector (Applied Biosystems, Foster City, CA). Gene expression was calculated relative to respective age and gender matched controls using the comparative threshold cycle method as described in the Applied Biosystems Sequence Detection Systems instruction guide.

Analysis of protein expression by Western blot

Liver samples were homogenized in T-Per (Thermo Scientific, Rockford, IL) containing Halt phosphatase inhibitor (Thermo Scientific) and protease cocktail inhibitor (Calbiochem, San Diego, CA). Protein concentrations of homogenates were determined by the Bradford assay using Coomassie Blue reagent (Thermo Scientific) and subsequently diluted with Laemmli buffer (BioRad, Hercules, CA) containing β-mercaptoethanol to a standard concentration of 2 μg/uL and heated at 95°C for 5 min. Samples containing 50 μg of protein were separated on a 12% SDS polyacrylamide gel by electrophoresis. Protein was then transferred to a nitrocellulose membrane by electrophoresis. Protein detection was performed using polyclonal rabbit antibodies to total and phosphorylated ERK, JNK, and AKT (Cell Signaling Technology, Danvers, MA) and monoclonal mouse antibodies to PGC-1α (Calbiochem) and β-actin (Sigma-Aldrich). Bound antibody was detected using goat anti-rabbit or goat anti-mouse polyclonal HRP antibody (Santa Cruz Biotechnology) and developed using ECL Western Blotting Substrate (Thermo Scientific). Representative Western blots of pooled samples are shown. Quantitation of changes in protein expression was performed on Western blots of nonpooled samples by densitometry using Adobe Photoshop CS5.

HPLC assay

Total bile acid pool size and composition were measured by HPLC as previously described (32). Samples were spiked with glycocholic acid as an internal standard to control for extraction efficiency. The bile acid pool size and composition was calculated from a standard curve and bile acid content is reported as μmol/100 g mouse.

Statistical analysis

Data are presented as mean ± SD. Comparisons between groups were performed using Student's *t*-test analysis.

RESULTS

Chronic high-cholesterol feeding induces significant hepatic cholesterol accumulation

Mice fed either a 0.2% or 1.25% cholesterol diet for 12 weeks exhibited significant hepatic cholesterol accumulation (114 ± 37 and 99 ± 31 mg/g protein in 0.2% and 1.25% cholesterol diets versus 47 ± 10 mg/g protein

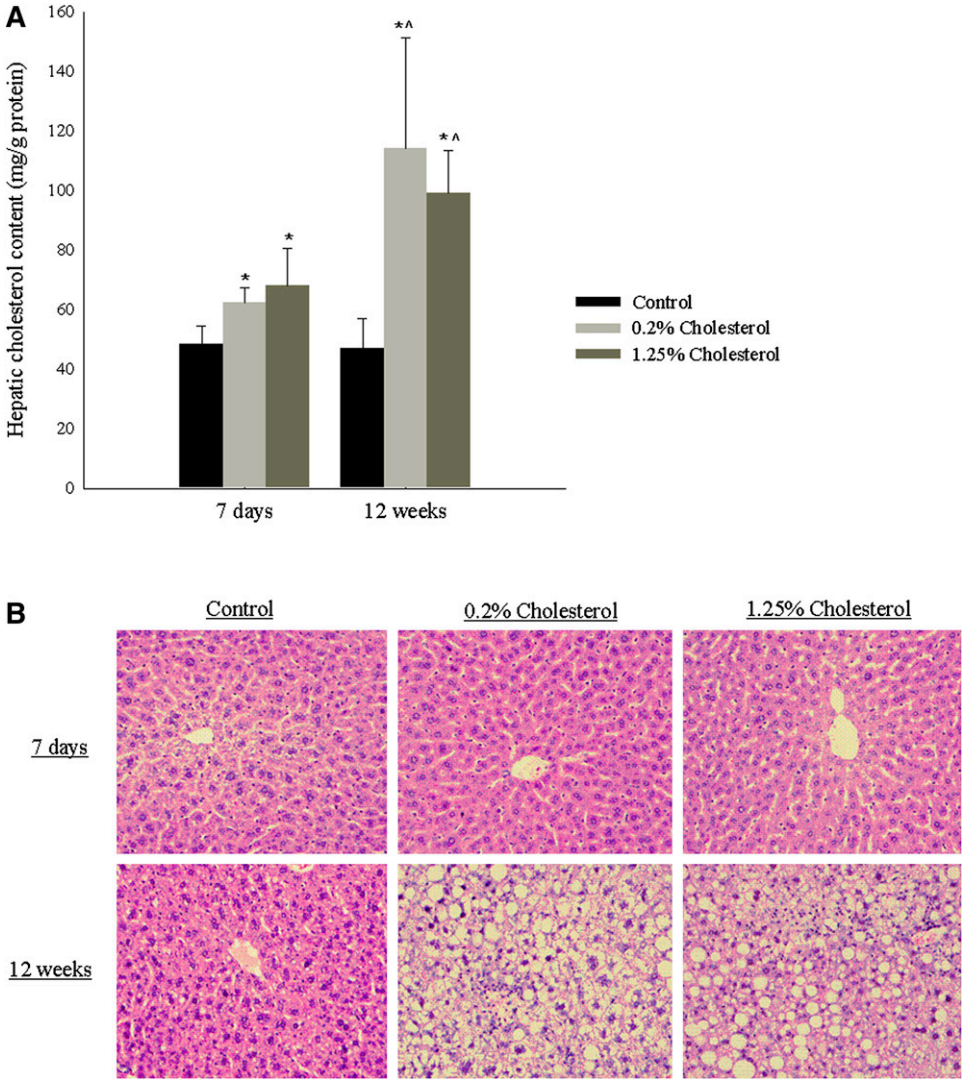

Fig. 1. A: Hepatic cholesterol content (mg/g protein); B: Histology of liver samples (hematoxylin and eosin stain, 20× original magnification) in mice fed control, 0.2% cholesterol, or 1.25% cholesterol diet for 7 days or 12 weeks. Values expressed as mean (N = 5) ± SD. *$p < 0.05$ versus control. ^$p < 0.05$ versus matched diet at 7 days.

in control, $P < 0.01$) (**Fig. 1A**). The hepatic cholesterol content in mice fed a high-cholesterol diet for 7 days was minimally increased compared with controls, but was significantly less than in the 12 week high-cholesterol cohorts (62 ± 5 and 68 ± 12 mg/g protein in 0.2% and 1.25% cholesterol diets, vs. 48 ± 6 mg/g protein in control, $P < 0.05$). Liver histology demonstrated >75% macrovesicular steatosis with a lymphocytic inflammatory infiltrate in the 12 week high-cholesterol cohorts, but no significant steatosis or inflammation in the 7 day high-cholesterol cohorts (Fig. 1B). There was no significant increase in hepatic triglyceride content in mice fed a high-cholesterol diet for 7 days or 12 weeks.

Chronic high-cholesterol feeding results in suppression of hepatic CYP7A1 expression

Mice fed a high-cholesterol diet for 12 weeks demonstrated significant suppression of hepatic CYP7A1 expression (0.27 ± 0.14 and 0.44 ± 0.26 in 0.2% and 1.25% cholesterol diets vs. 1.11 ± 0.51 in control, $P < 0.05$) (**Fig. 2A**). Consistent with previous findings, mice fed a high-cholesterol diet for 7 days exhibited upregulation of hepatic CYP7A1 expression (3.59 ± 1.77 and 4.75 ± 2.72 in 0.2% and 1.25% cholesterol diets vs. 1.12 ± 0.52 in control, $P < 0.05$).

Hepatic PGC-1α induces hepatic CYP7A1 expression (33, 34). Mice fed a high-cholesterol diet for 12 weeks exhibited a 78% suppression of hepatic PGC-1α mRNA (Fig. 2B). In contrast, expression of PGC-1α mRNA was unchanged in mice fed a high-cholesterol diet for 7 days. Protein expression of NT-PGC-1α, the active splice variant of PGC-1α (35), was suppressed in mice fed a high-cholesterol diet for 12 weeks, but was unchanged by 7 days of cholesterol feeding, paralleling the changes of PGC-1α mRNA (Fig. 2C).

There was decreased expression of CYP27A1, the presumed rate-limiting enzyme in the alternative pathway of bile acid synthesis, in mice fed a 0.2% cholesterol diet for 12 weeks but no significant suppression in the 1.25% cholesterol cohort at 12 weeks. There was no significant change in CYP27A1 expression in mice fed a high-cholesterol diet for 7 days, and there was no change in the expression of the bile acid synthetic enzyme, CYP8B1, with either acute or chronic high cholesterol feeding (**Table 1**).

Chronic high-cholesterol feeding results in enhanced upregulation of ileal FGF15

We next examined the expression of ileal FGF15, the primary mediator of bile acid feedback inhibition of CYP7A1. Mice fed a high-cholesterol diet for 12 weeks demonstrated significant upregulation of ileal FGF15 mRNA (7.81 ± 4.21 and 10.38 ± 5.44 in 0.2% and 1.25% cholesterol diets vs. 1.10 ± 0.45 in control, $P < 0.01$). Ileal FGF15 mRNA was also upregulated at 7 days of cholesterol feeding, but to a lesser magnitude (2.42 ± 0.55 and 4.71 ±

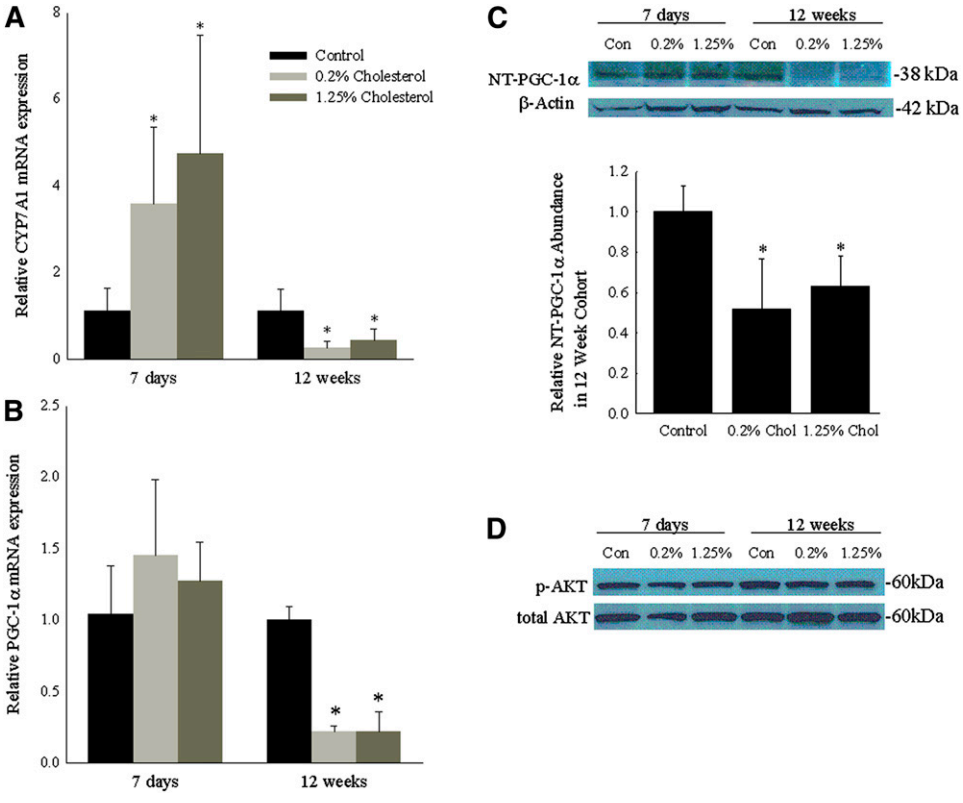

Fig. 2. Hepatic mRNA expression of (A) CYP7A1 and (B) PGC-1α, and protein expression of (C) NT-PGC-1α with densitometric analysis and (D) total and phosphorylated AKT in mice fed control, 0.2% cholesterol, or 1.25% cholesterol diet for 7 days or 12 weeks. Relative mRNA expression, mean ± SD, *$p < 0.05$ versus control. Representative Western blots of pooled samples (N = 5). Densitometric analysis of NT-PGC-1α is derived from a Western blot of nonpooled samples (N = 5).

TABLE 1. Hepatic and intestinal gene expression in mice fed a 0.2% or 1.25% cholesterol diet for 7 days or 12 weeks

	7 Days			12 Weeks		
	Control	0.2% Cholesterol	1.25% Cholesterol	Control	0.2% Cholesterol	1.25% Cholesterol
Hepatic						
ABCG5	1.01 ± 0.16	4.11 ± 0.61[a]	3.17 ± 0.70[a]	1.09 ± 0.40	3.45 ± 0.84[a]	2.48 ± 0.76[a]
ABCG8	1.01 ± 0.11	2.21 ± 0.39[a]	1.59 ± 0.73	1.04 ± 0.30	2.50 ± 0.88[a]	1.70 ± 0.60[a]
ABCA1	1.01 ± 0.13	1.26 ± 0.43	1.08 ± 0.37	1.05 ± 0.36	1.78 ± 0.48[a]	1.82 ± 0.36[a]
SHP	1.09 ± 0.39	1.69 ± 0.80	1.75 ± 0.54	1.16 ± 0.53	2.52 ± 1.56[a]	3.79 ± 2.20[a]
CYP8B1	1.00 ± 0.11	1.13 ± 0.21	0.74 ± 0.22	1.05 ± 0.35	1.32 ± 0.17	0.67 ± 0.18
CYP27A1	1.02 ± 0.21	1.20 ± 0.29	0.96 ± 0.31	1.02 ± 0.23	0.68 ± 0.06[a]	0.87 ± 0.29
Ileal						
FGF15	1.22 ± 0.99	2.42 ± 0.55[a]	4.71 ± 1.33[a]	1.10 ± 0.45	7.81 ± 4.21[a]	10.38 ± 5.44[a]
SHP	1.18 ± 0.76	3.58 ± 1.20[a]	7.62 ± 2.70[a]	1.06 ± 0.42	8.96 ± 5.10[a]	10.84 ± 5.55[a]
OSTα	1.01 ± 0.19	1.02 ± 0.28	1.43 ± 0.51	1.09 ± 0.41	2.52 ± 0.75[a]	2.55 ± 0.64[a]
IBABP	1.03 ± 0.25	1.43 ± 0.32	1.22 ± 0.32	1.03 ± 0.27	0.72 ± 0.17	0.68 ± 0.14[a]

Relative expression, mean (n = 6) ± SD. CYP7A1, cholesterol 7α-hydroxylase; FGF, fibroblast growth factor; IBABP, ileal bile acid binding protein; OST, organic solute transporter; SHP, short heterodimer partner.
[a] $p < 0.05$ versus control.

1.33 in 0.2% and 1.25% cholesterol diets vs. 1.22 ± 0.99 in control, $P < 0.05$). Similarly, the downstream FXR targets hepatic and ileal SHP and ileal organic solute transporter (OST)α were upregulated to a greater degree with chronic cholesterol feeding. Ileal mRNA expression of ileal bile acid binding protein (IBABP) was suppressed in mice fed a 1.25% cholesterol diet for 12 weeks (Table 1).

Recently it has been proposed that ileal FGF15 may regulate CYP7A1 in part via activation of forkhead box 01 by the AKT signaling pathway. Given the finding of increased FGF15 expression in our model, we assayed for activation of the AKT signaling pathway. There was no significant increase in phosphorylated hepatic AKT in mice fed a high-cholesterol diet chronically (Fig. 2D).

Chronic cholesterol feeding increases the content of FXR ligands in the bile acid pool

Hydrophobic bile acid species are potent FXR ligands and thus, we evaluated whether bile acid pool size and composition were affected by cholesterol feeding (**Table 2**). Mice fed a high-cholesterol diet for 7 days demonstrated expansion of the total bile acid pool, primarily attributable to an increased content of taurocholic acid (TCA). There was an increased content of the FXR ligand taurodeoxycholic acid (TDCA) in mice fed a 1.25% cholesterol diet for 7 days but no other significant increases in FXR ligands. At 12 weeks of high-cholesterol feeding, there was a significant increase in the concentrations of the FXR ligands, TDCA and taurochenodeoxycholic acid (TCDCA), relative to controls. The bile acid pool size after 12 weeks of cholesterol feeding was similar to controls and reduced compared with mice fed a high-cholesterol diet for 7 days.

LXRα target genes are activated by both acute and chronic cholesterol feeding

LXRα is activated by acute cholesterol feeding in mice (15, 16). We found that the hepatic LXRα downstream target genes, ABCG5 and ABCG8, were increased in mice fed a high cholesterol diet for 7 days (Table 1). ABCG5 and ABCG8 were upregulated to a similar degree in mice fed a high-cholesterol diet for 12 weeks. However, the LXRα target gene, ABCA1, was increased only in mice fed a high-cholesterol diet for 12 weeks.

Chronic high-cholesterol feeding induces hepatic inflammatory cytokine and MAPK expression

Inflammatory cytokines, such as TNFα and IL-1β, have been shown to suppress hepatic CYP7A1 expression (23, 24). Mice fed a high-cholesterol diet for 12 weeks showed significant upregulation of hepatic TNFα mRNA (5.79 ± 1.94 and 6.55 ± 1.20 in 0.2% and 1.25% cholesterol diets vs. 1.22 ± 0.68 in controls, $P < 0.001$) (**Fig. 3A**). The TNFα downstream target gene, ICAM-1, was also markedly upregulated with chronic cholesterol feeding (10.00 ± 7.49 and 22.82 ± 15.95 in 0.2% and 1.25% cholesterol diets vs. 1.35 ± 1.01 in controls, $P < 0.01$) (Fig. 3B). There was increased expression of hepatic IL-1β in mice fed a 1.25% cholesterol diet for 12 weeks (8.08 ± 4.02 vs. 1.05 ± 0.34 in controls, $P < 0.01$) and a strong trend toward increased expression in the 0.2% cholesterol cohort (2.90 ± 1.84 vs. 1.05 ± 0.34 in controls, $P = 0.06$) (Fig. 3C). Conversely, there was no activation of TNFα and IL-1β in mice fed either a 0.2% or 1.25% cholesterol diet for 7 days. Plasma levels of TNFα and IL-1β were unchanged with acute or chronic cholesterol feeding.

Hepatic inflammatory cytokines have been shown to activate the MAPK signaling pathways including ERK and JNK (29–31). Phosphorylated ERK and JNK have been shown to negatively regulate CYP7A1 expression (25, 27). Thus, we next explored whether dietary cholesterol induces JNK or ERK signaling. Chronic cholesterol feeding increased hepatic expression of phosphorylated JNK1 by 32% and 38% in mice fed a 0.2% and 1.25% cholesterol diet, respectively, for 12 weeks. JNK2 was not activated by high-cholesterol feeding (**Fig. 4A**). Phosphorylated ERK1 and ERK2 were increased over 2-fold by high-cholesterol feeding for 12 weeks (Fig. 4B). Conversely, there was no activation of JNK or ERK in response to acute cholesterol feeding.

TABLE 2. Bile acid pool size and composition in mice fed a 0.2% or 1.25% cholesterol diet for 7 days or 12 weeks

		7 Days			12 Weeks		
		Control	0.2% Cholesterol	1.25% Cholesterol	Control	0.2% Cholesterol	1.25% Cholesterol
TMCA	Content (μmol/100g)	31.1 ± 13.9	39.5 ± 3.6	39.0 ± 6.3	31.5 ± 2.5	28.9 ± 1.6	29.0 ± 2.8
	% of total pool	66.8 ± 15.4%	61.9 ± 4.6%	58.0 ± 5.4%	72.5 ± 4.8%	66.1 ± 0.3%	68.8 ± 5.1%
TCA	Content (μmol/100g)	11.8 ± 3.9	21.9 ± 6.2[a]	24.8 ± 5.7[a]	10.6 ± 2.4	12.7 ± 4.0	10.8 ± 2.3
	% of total pool	28.2 ± 12.2%	33.5 ± 5.2%	36.5 ± 4.7%	24.4 ± 4.6%	28.3 ± 5.7%	25.6 ± 4.6%
TUDCA	Content (μmol/100g)	1.3 ± 0.7	1.9 ± 0.9	1.6 ± 0.6	1.1 ± 0.1	1.6 ± 0.1[a]	1.4 ± 0.1[a]
	% of total pool	3.5 ± 2.9%	3.1 ± 1.4%	2.5 ± 1.2%	2.5 ± 0.1%	3.7 ± 0.5%[a]	3.4 ± 0.1%[a]
TCDCA	Content (μmol/100g)	0.3 ± 0.2	0.5 ± 0.4	0.6 ± 0.4	0.1 ± 0.0	1.6 ± 0.2[a]	0.7 ± 0.2[a]
	% of total pool	0.7 ± 0.5%	0.8 ± 0.6%	0.9 ± 0.8%	0.3 ± 0.1%	1.3 ± 0.3%[a]	1.6 ± 0.4%[a]
TDCA	Content (μmol/100g)	0.3 ± 0.3	0.5 ± 0.4	1.4 ± 0.9[ab]	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1[a]
	% of total pool	0.8 ± 0.9%	0.7 ± 0.6%	2.1 ± 1.2%[ab]	0.3 ± 0.3%	0.6 ± 0.4%	0.9 ± 0.2%[a]
Total pool	Content (μmol/100g)	44.8 ± 11.4	64.4 ± 9.4[a]	67.4 ± 10.0[a]	43.5 ± 2.7	44.1 ± 5.5	42.3 ± 3.4

Values expressed as mean (n = 6) ± SD. TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TMCA, tauromuricholic acid; TUDCA, tauroursodeoxycholic acid.

[a] $p < 0.05$ versus control.

[b] $p < 0.05$ versus 0.2% cholesterol diet.

Inflammatory cytokine-mediated CYP7A1 suppression is dependent on JNK and ERK signaling in HepG2 cells

We have shown that chronic cholesterol feeding activates inflammatory cytokines and induces JNK1 and ERK1/2 signaling. We next explored whether cytokine-mediated suppression of CYP7A1 is dependent on JNK1 and ERK1/2 signaling in vitro. HepG2 cells, a human hepatoma cell line, were treated with TNFα or IL-1β in the presence or absence of inhibitors of ERK (PD184352) or JNK (SP600125). Treatment with TNFα and IL-1β alone resulted in suppression of CYP7A1 mRNA expression by 57% and 94% respectively (**Fig. 5**). In cells pretreated

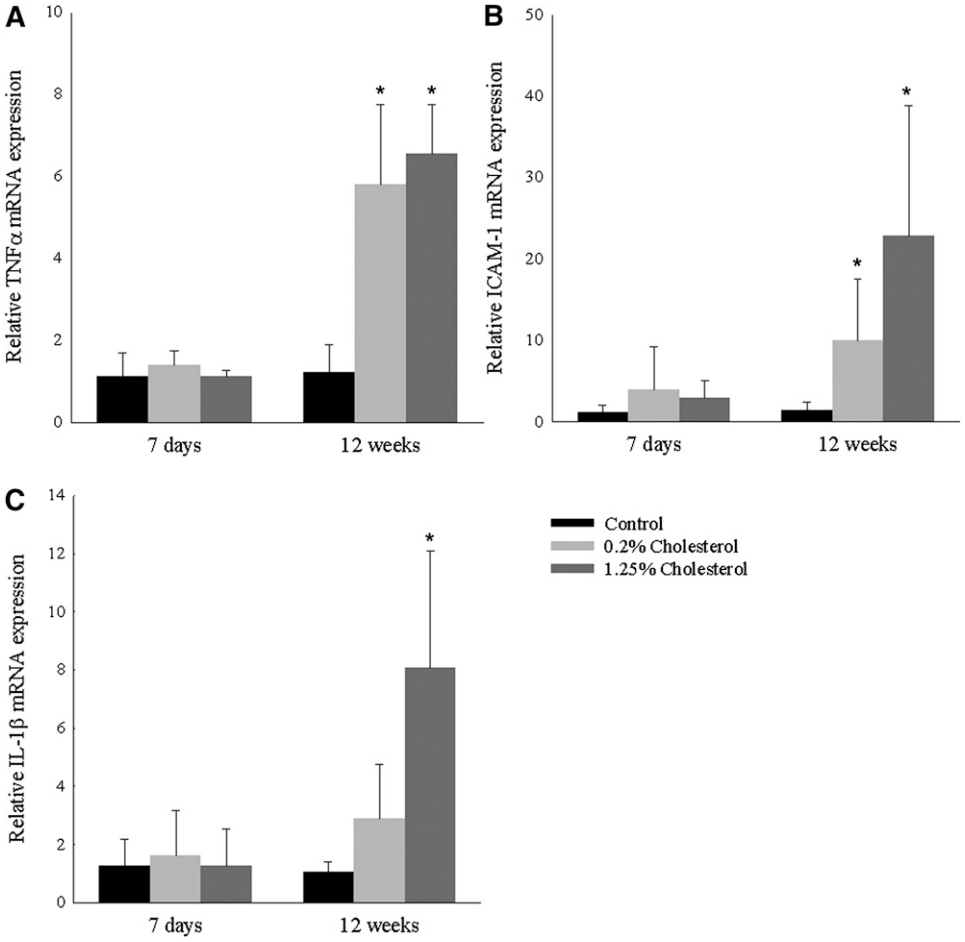

Fig. 3. Hepatic mRNA expression of (A) TNFα, (B) ICAM-1, (C) IL-1β in mice fed control, 0.2% cholesterol, or 1.25% cholesterol diet for 7 days or 12 weeks. Relative mRNA expression, mean (n = 5) ± SD, *$p < 0.05$.

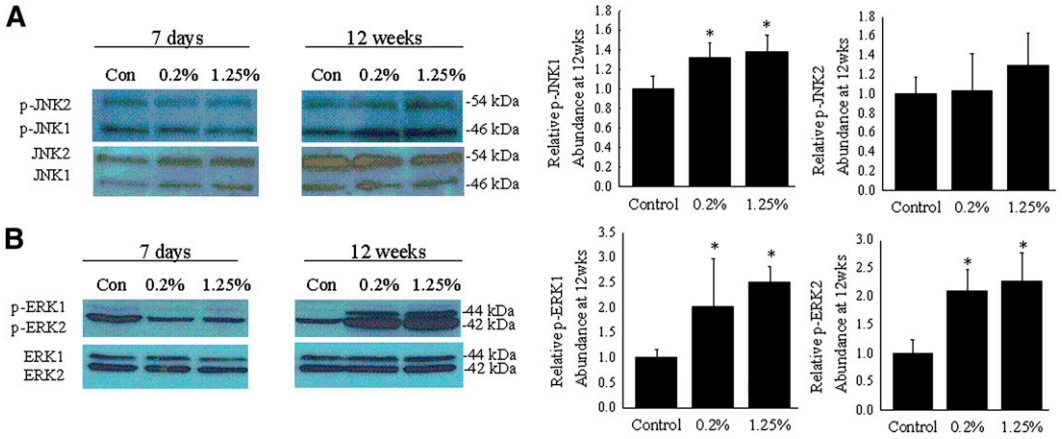

Fig. 4. Hepatic protein expression of (A) total and phosphorylated JNK1 and JNK2 and (B) total and phosphorylated ERK1 and ERK2. Representative Western blots of pooled samples (N = 5). Densitometric analysis is derived from Western blots of nonpooled samples (N = 5).

with SP600125, TNFα-mediated CYP7A1 suppression was abolished (Fig. 5A). Suppression of CYP7A1 by IL-1 β was unaffected by pretreatment with SP600125 (Fig. 5B). Pretreatment of cells with PD184352 abolished the suppressive effects of TNFα on CYP7A1 expression (Fig. 5C) and attenuated the suppressive effects of IL-1β (Fig. 5D).

Next, HepG2 cells were treated with TNFα or IL-1β in the presence or absence of siRNA directed against JNK1 or ERK1 and ERK2. In cells pretreated with JNK1 siRNA, TNFα-mediated CYP7A1 suppression was abolished (**Fig. 6A**), whereas IL-1β-mediated CYP7A1 suppression was unaffected (Fig. 6B). Pretreatment of cells with ERK1 and

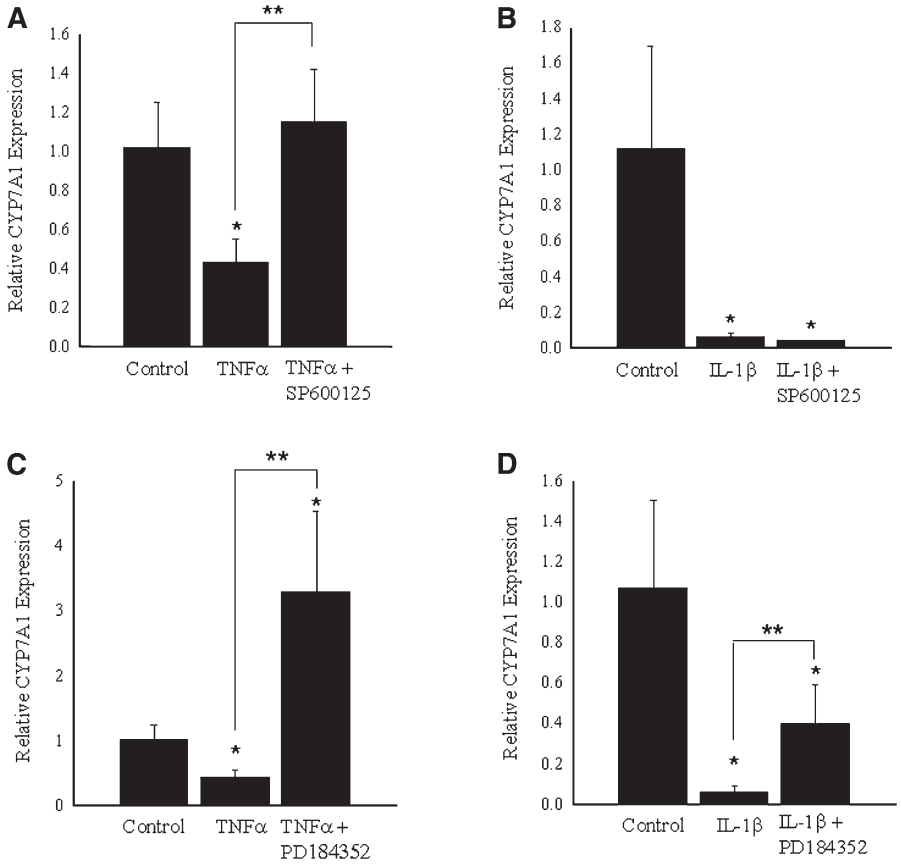

Fig. 5. CYP7A1 mRNA expression in HepG2 cells treated for 6 h with (A) TNFα (50ng/ml) or (B) IL-1β (10ng/ml) ± JNK inhibitor, SP600125 (25μM), (C) TNFα (50ng/ml) or (D) IL-1β (10ng/ml) ± ERK inhibitor, PD184352 (5μM). Values expressed as mean (N = 6) ± SD. *$p < 0.05$ versus control, **$P < 0.05$.

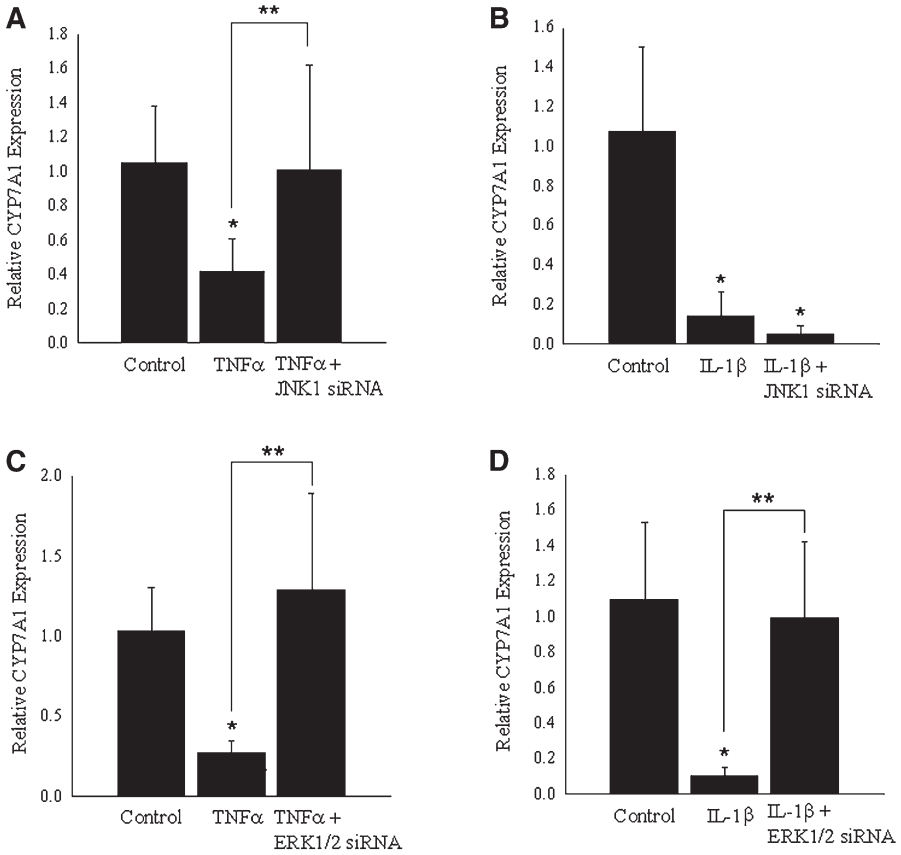

Fig. 6. CYP7A1 mRNA expression in HepG2 cells treated for 6 h with (A) TNFα (50ng/ml) or (B) IL-1β (10ng/ml) ± JNK1 siRNA, (C) TNFα (50ng/ml) or (D) IL-1β (10ng/ml) ± ERK1/2 siRNA. Values expressed as mean (N = 6) ± SD. *$p < 0.05$ versus control, **$P < 0.05$.

ERK2 siRNA abolished the suppressive effects of TNFα and IL-1β on CYP7A1 expression (Fig. 6C, D).

DISCUSSION

Hepatic cholesterol accumulation may be a key factor in the progression from simple steatosis to NASH via induction of hepatic inflammation (5, 6). Hepatic CYP7A1 controls the rate-limiting step in the conversion of cholesterol to bile acids, and the biliary secretion of bile salts serves as a major pathway of cholesterol elimination from the human body. Studies on the regulation of cholesterol metabolism in mice have typically relied on the short-term administration of dietary cholesterol (e.g., 1 to 2 weeks). Mice fed a short-term high-cholesterol diet are able to tightly maintain cholesterol homeostasis through feedforward upregulation of hepatic CYP7A1 via LXRα stimulation (15, 16). However, a chronic high-cholesterol diet is a more clinically relevant model given that many humans chronically consume a high-cholesterol diet. Whether this compensatory mechanism is maintained with chronic cholesterol feeding was previously unexplored. Although humans and rodents have differing mechanisms of CYP7A1 regulation in response to acute cholesterol feeding, the regulation of CYP7A1 may be similar with consumption of a high-cholesterol diet chronically.

We have now shown that mice fed a high-cholesterol diet for 12 weeks exhibit suppression, rather than upregulation, of hepatic CYP7A1 expression, which is associated with the development of severe hepatic cholesterol accumulation. Additionally, gene expression of the CYP7A1 coactivator, PGC-1α, is decreased with chronic cholesterol feeding but is normal with acute cholesterol feeding. We examined several signaling pathways in order to explain this seemingly paradoxical suppression of CYP7A1 with chronic cholesterol feeding.

The upregulation of hepatic CYP7A1 in response to acute cholesterol feeding in mice is likely due to stimulation of the oxysterol receptor LXRα (15, 16). The role of this compensatory feedforward response with chronic cholesterol feeding was previously unknown. We determined that the expression of hepatic LXRα downstream target genes is increased with chronic cholesterol feeding, similar to acute cholesterol feeding. This indicates that although LXRα is important for the regulation of CYP7A1 in response to acute cholesterol feeding, LXRα is not the primary regulator of CYP7A1 in response to chronic cholesterol feeding.

Excess cellular cholesterol has been shown to induce inflammation. Li et al. (7) have shown that cholesterol accumulation in the endoplasmic reticulum of macrophages stimulates release of TNFα, a finding which may have important implications for the pathogenesis of atherosclerosis.

We determined that chronic, but not acute, cholesterol feeding in mice results in increased hepatic expression of TNFα and IL-1β, which have been shown to downregulate hepatic CYP7A1 expression (23–25). We showed that a high-cholesterol diet activates hepatic JNK1 and ERK1/2, which are induced by TNFα and IL-1β (29–31). We demonstrated in vitro that cytokine-mediated suppression of CYP7A1 is dependent on JNK and ERK activation. Based on these data, we postulate that the primary mechanism of hepatic CYP7A1 suppression in mice fed a chronic high-cholesterol diet is via activation of hepatic inflammatory cytokines, leading to activation of JNK and ERK signaling pathways.

Ileal FGF15/19 is the primary mediator of bile acid feedback inhibition of hepatic CYP7A1 (13). We showed that mice fed a high-cholesterol diet for either 7 days or 12 weeks demonstrate upregulation of ileal FGF15 expression, however, the degree of upregulation is greater in the 12 week cohort. The ERK activation observed in mice fed a chronic high-cholesterol diet may be related, at least in part, to enhanced ileal FGF15 expression. Given that the expression of ileal FGF15 is increased by both acute and chronic cholesterol feeding, it is unlikely that expression of ileal FGF15 can completely explain the disparate expression of CYP7A1 in these feeding models. However, we cannot exclude the possibility that enhanced expression of FGF15 may contribute to the suppression of hepatic CYP7A1 expression with chronic cholesterol feeding.

We demonstrated that the total bile acid pool size is unchanged with chronic cholesterol feeding; however, there is an increase in the relative and absolute content of the hydrophobic bile acid species, TDCA and TCDCA, which have been shown to be potent ligands of FXR in vitro. The increased bile acid content of TCDCA and TDCA chronically may account for the enhanced upregulation of FGF15 expression with chronic cholesterol feeding. Maintenance of the bile acid pool size with chronic cholesterol feeding may not be explained by increased bile acid synthesis via the alternative pathway, as there is no significant upregulation of the rate-limiting enzyme, CYP27A1. However, the rate-limiting factor for the alternative pathway remains controversial (36). Studies in mice expressing the human CYP7A1 gene have shown that dietary cholesterol suppresses CYP7A1 expression, yet the bile acid pool size is expanded and fecal bile acid excretion is increased (19). This would suggest that dietary cholesterol may regulate bile acid pool size independently of CYP7A1 (19). The mechanism underlying this finding is yet unknown.

We chose to use two high-cholesterol diets for these experiments. The 1.25% cholesterol diet has the cholesterol content of a standard murine experimental high-cholesterol diet that is used to study atherosclerosis and cholelithiasis; however, this diet contains >50-fold more cholesterol than most standard rodent chow diets. Therefore, a moderately high-cholesterol diet (0.2%) was also employed, which contains a similar amount of cholesterol to that found in many standard rodent "Western" diets that are used to study diabetes, obesity, and cardiovascular disease. We found that the phenotype and gene expression patterns were similar in mice fed the 0.2% and 1.25% cholesterol diets. The fact that these effects were seen on a moderately high-cholesterol diet further supports the potential physiologic relevance of these observations.

These data may have important implications for human NASH. In the "two-hit" model of NASH, the first hit consists of hepatic lipid accumulation and the second hit involves the inflammatory response, including cytokine activation. This study supports the notion that the inflammatory response may, in turn, exacerbate hepatic lipid accumulation by downregulating hepatic CYP7A1. Ultimately, this feedforward loop may lead to significant hepatic cholesterol accumulation, as seen in our mouse model of chronic high-cholesterol feeding.

In summary, we demonstrated that chronic high-cholesterol feeding in mice leads to a >2-fold increase in hepatic cholesterol content and suppression of hepatic CYP7A1 expression. Chronic cholesterol feeding results in increased expression of hepatic inflammatory cytokines, including TNFα and IL-1β. This is associated with increased phosphorylation of ERK and JNK, which may serve as the primary mechanisms for hepatic CYP7A1 suppression. Enhanced upregulation of ileal FGF15 expression may further contribute to the suppression of hepatic CYP7A1 with chronic cholesterol feeding.

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