

Liver Receptor Homolog-1 Regulates Bile Acid Homeostasis but Is Not Essential for Feedback Regulation of Bile Acid Synthesis

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Liver receptor homolog 1 (LRH-1), an orphan nuclear receptor, is highly expressed in liver and intestine, where it is implicated in the regulation of cholesterol, bile acid, and steroid hormone homeostasis. Among the proposed LRH-1 target genes in liver are those encoding cholesterol 7 α -hydroxylase (CYP7A1) and sterol 12 α -hydroxylase (CYP8B1), which catalyze key steps in bile acid synthesis. *In vitro* studies suggest that LRH-1 may be involved both in stimulating basal CYP7A1 and CYP8B1 transcription and in repressing their expression as part of the nuclear bile acid receptor [farnesoid X receptor (FXR)]-small heterodimer partner signaling cascade, which culminates in small heterodimer partner binding to LRH-1 to repress gene transcription. However, *in vivo* analysis of LRH-1 actions has been hampered by the embryonic lethality of *Lrh-1* knockout mice. To over-

come this obstacle, mice were generated in which *Lrh-1* was selectively disrupted in either hepatocytes or intestinal epithelium. LRH-1 deficiency in either tissue changed mRNA levels of genes involved in cholesterol and bile acid homeostasis. Surprisingly, LRH-1 deficiency in hepatocytes had no significant effect on basal *Cyp7a1* expression or its repression by FXR. Whereas *Cyp8b1* repression by FXR was also intact in mice deficient for LRH-1 in hepatocytes, basal CYP8B1 mRNA levels were significantly decreased, and there were corresponding changes in the composition of the bile acid pool. Taken together, these data reveal a broad role for LRH-1 in regulating bile acid homeostasis but demonstrate that LRH-1 is either not involved in the feedback regulation of bile acid synthesis or is compensated for by other factors. (*Molecular Endocrinology* 22: 1345–1356, 2008)

LIVER RECEPTOR HOMOLOG-1 (LRH-1; also called NR5A2, α -fetoprotein transcription factor, FTZ-F1-related factor, and CYP7A1 promoter binding factor) is an orphan member of the nuclear receptor superfamily that is highly expressed in liver, exocrine pancreas, intestine, and ovary (1–3; reviewed in 4). LRH-1 belongs to a nuclear receptor subfamily that includes steroidogenic factor 1 and fushi tarazu factor 1. Members of this subfamily regulate target gene transcription by binding as monomers to DNA re-

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Abbreviations: ABC, ATP-binding cassette; ASBT, apical sodium-dependent bile acid transporter; BrdU, bromodeoxyuridine; BSEP, bile salt export pump; CYP7A1, cholesterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; ES, embryonic stem; FGF, fibroblast growth factor; FXR, farnesoid X receptor; hepKO, hepatocyte knockout; HNF4 α , hepatocyte nuclear factor 4 α ; IBABP, ileal bile acid-binding protein; iekO, intestinal epithelium knockout; LRH-1, liver receptor homolog-1; MCA, muricholic acid; MRP, multidrug resistance protein; OST, organic solute transporter; RT-qPCR, real-time quantitative PCR; SCARB1, scavenger receptor B1; SHP, small heterodimer partner.

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sponse elements with consensus sequence 5'-Py-CAAGGPyCPu-3' (4). LRH-1 has high constitutive transcriptional activity that can be regulated by phosphorylation (5). Although various phospholipids have been shown to bind in the ligand-binding pocket of human LRH-1 (6–8), the physiological relevance of these interactions remains uncertain.

In the intestine, LRH-1 binding sites have been identified in the regulatory regions of a number of genes involved in cholesterol and bile acid homeostasis, including those encoding the ATP-binding cassette (ABC) transporters ABCG5 and ABCG8, the organic solute transporters (OST) α and β , and the ileal apical sodium-dependent bile acid transporter (ASBT; also known as solute carrier family 10, member A2) (9–11). LRH-1 is also implicated in the regulation of other intestinal processes including epithelial cell renewal and the synthesis of glucocorticoids (12, 13). Heterozygous *Lrh-1*^{+/-} mice have decreased epithelial cell proliferation and are susceptible to chemical-induced intestinal inflammation (13, 14).

LRH-1 binding sites have also been identified in the regulatory regions of many genes involved in cholesterol and bile acid homeostasis in liver (4). Among these are the genes encoding cholesterol 7 α -hydrox-

ylase (CYP7A1), which catalyzes the first and rate-limiting step in bile acid synthesis, and cholesterol 12 α -hydroxylase (CYP8B1), which catalyzes a step in the conversion of chenodeoxycholic acid to cholic acid (15). *In vitro* studies have suggested that LRH-1 has dual effects on CYP7A1 and CYP8B1 expression (16, 17). First, it enhances basal transcription of these genes. Second, it is involved in the feedback repression of CYP7A1 and CYP8B1 through a signaling cascade involving the farnesoid X receptor (FXR), a nuclear bile acid receptor, and small heterodimer partner (SHP) (16, 17). Activation of FXR by bile acids induces expression of SHP, an atypical orphan nuclear receptor lacking a DNA-binding domain that functions as a strong transcriptional repressor through interactions with other nuclear receptors and transcription factors (18; reviewed in Ref. 19). Because SHP binds efficiently to LRH-1 and inhibits its transcriptional activity *in vitro*, it is proposed that this interaction causes CYP7A1 and CYP8B1 repression (16, 17). Whereas the roles of FXR and SHP in this signaling cascade have been demonstrated *in vivo* using gene knockout mice (20–22), *Lrh-1*^{-/-} mice die during early embryogenesis, precluding their use in the analysis of LRH-1 function in adult tissues (23). Interestingly, heterozygous *Lrh-1*^{+/-} mice have elevated CYP7A1 and CYP8B1 mRNA levels (23, 24), suggesting that the dominant effect of LRH-1 on *Cyp7a1* and *Cyp8b1* transcription is the recruitment of SHP, not the induction of their basal activity. LRH-1 overexpression studies in mice have yielded contrasting outcomes: whereas CYP7A1 mRNA levels were increased in LRH-1 transgenic mice (23), overexpression of LRH-1 using an adenoviral delivery system caused a strong decrease in CYP7A1 and CYP8B1 mRNA levels (24). Given these mixed results, the precise role of LRH-1 in the regulation of bile acid synthesis remains unclear.

In this report, we describe the generation and characterization of mice deficient for LRH-1 in either hepatocytes or the intestinal epithelium. These studies demonstrate roles for LRH-1 in regulating bile acid homeostasis in both tissues. However, they also unexpectedly show that LRH-1 is not essential for FXR-mediated feedback repression of bile acid synthesis.

RESULTS

Generation of Tissue-Specific LRH-1-Deficient Mice

To circumvent the early embryonic lethality caused by complete LRH-1 deficiency, mice were generated in which exon 5 of *Lrh-1*, which encodes the second zinc finger of the DNA-binding domain, was flanked by loxP sites (Fig. 1, A–C). The resulting *Lrh-1*^{lox/lox} mice were crossed against either albumin-cre or villin-cre mice to generate knockout mice lacking an intact *Lrh-1* gene in either hepatocytes (hepKO) or intestinal epithelium (ieKO), respectively (Fig. 1B). As predicted, real-time

quantitative PCR (RT-qPCR) assays performed with primers directed against the floxed region revealed a marked decrease in LRH-1 mRNA in liver and intestinal epithelium of hepKO and ieKO mice, respectively (Fig. 1, D and E). The residual LRH-1 mRNA containing exon 5 in liver of hepKO mice (Fig. 1D) is likely due to *Lrh-1* expression in nonhepatocytes. Whereas an LRH-1 transcript was detected by RT-PCR in liver of hepKO mice and intestine of ieKO mice using primers flanking exon 5, cloning and sequencing of this transcript from both tissues revealed a stop codon created by deletion of the floxed region that precludes production of functional protein (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Consistent with the mRNA data, there was a marked decrease in LRH-1 protein in liver extracts prepared from hepKO mice (Fig. 1F). For unknown reasons, we were unable to detect LRH-1 protein in intestinal extracts prepared from either control or ieKO mice (data not shown). Based on these data and the gene expression data shown below, we conclude that LRH-1 is efficiently eliminated in hepatocytes and intestinal epithelium of hepKO and ieKO mice, respectively.

There were no overt abnormalities in the hepKO and ieKO mice nor were there changes in tissue morphology seen in hematoxylin and eosin-stained sections of liver or small intestine (data not shown). Analysis of plasma parameters did not reveal significant changes in triglyceride, free fatty acid, glucose, bile acid or alanine aminotransferase concentrations in hepKO or ieKO mice (Table 1). Plasma cholesterol and aspartate aminotransferase levels were modestly but significantly decreased in hepKO and ieKO mice, respectively (Table 1). Hepatic cholesterol and triglyceride concentrations were not significantly decreased in either the hepKO and ieKO mice (Table 1).

Effects of LRH-1 Deficiency in Hepatocytes

The consequences of eliminating LRH-1 in hepatocytes on the expression of a panel of genes involved in bile acid and cholesterol homeostasis were examined by RT-qPCR using mRNA prepared from livers of hepKO and control *Lrh-1*^{lox/lox} mice. Although CYP7A1 mRNA levels trended upward in hepKO mice, this difference was not statistically significant (Table 2). In contrast, CYP8B1 mRNA levels were decreased approximately 10-fold in the hepKO mice, demonstrating differential effects of LRH-1 on *Cyp7a1* and *Cyp8b1* expression. Significant decreases were also seen in the mRNAs encoding SHP, FXR, the bile acid biosynthetic enzyme CYP27A1, the scavenger receptor B1 (SCARB1), and the transporters ABCG5, ABCG8, bile salt export pump (BSEP), multidrug resistance transporter 2 (also known as ABCB4), multidrug resistance protein (MRP2; also known as ABCC2), MRP3 (also known as ABCC3), and the sodium-dependent bile acid cotransporter (NTCP; also

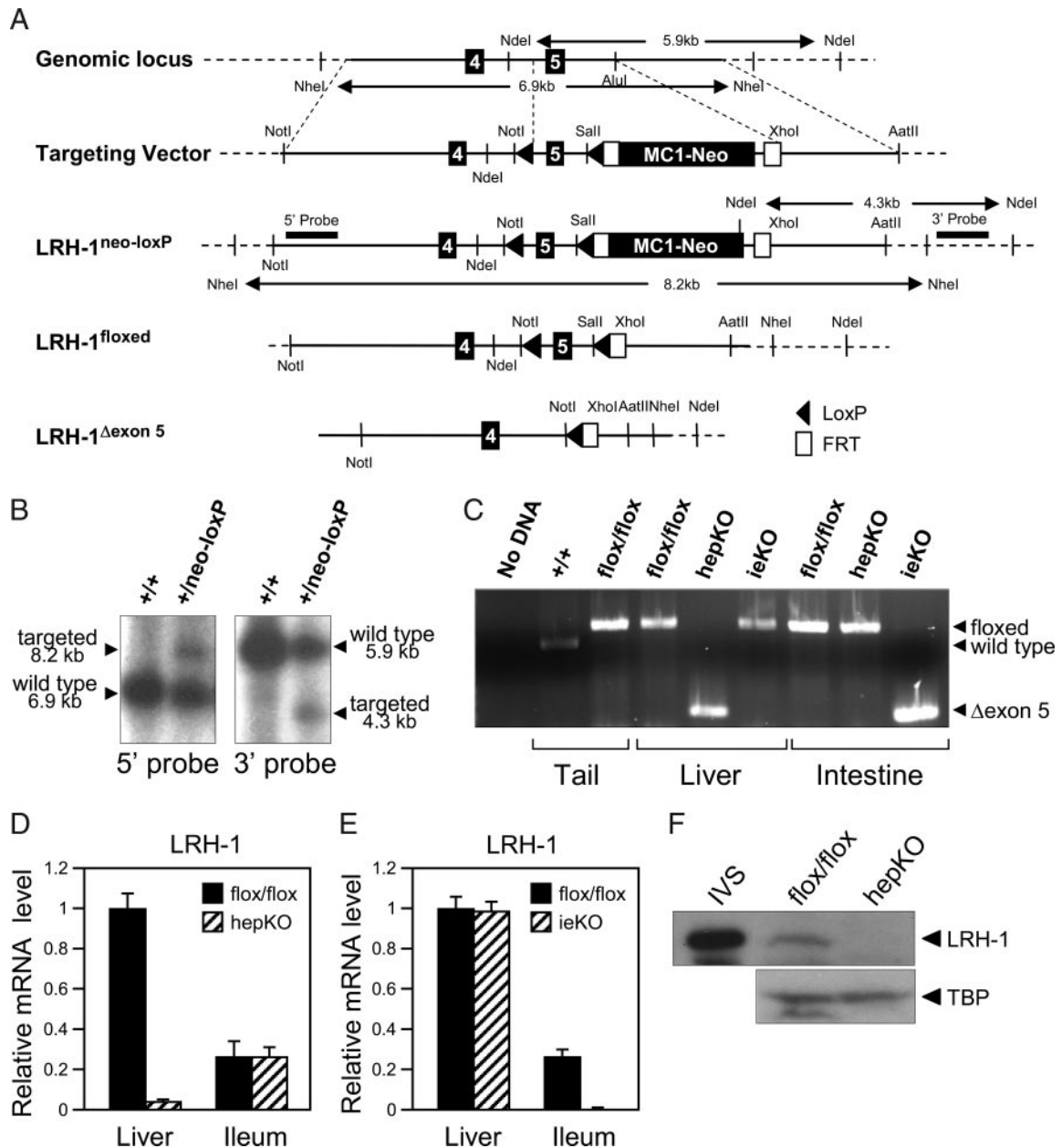


Fig. 1. Generation of Mice Deficient for LRH-1 in Liver or Intestine

A, Schematic representation of the targeting strategy to disrupt *Lrh-1*. The schematic shows exons 4 and 5 of *Lrh-1*, the targeting vector, and the targeted *Lrh-1* allele both before and after successive removal of the MC1-neocassette with FLPe recombinase and exon 5 with cre recombinase. LoxP sites (solid arrowheads), FLPe recombinase target sites (Frt; open boxes), selected restriction enzyme sites and the 5'- and 3'-probes used for Southern blots are indicated. B, Southern blot analysis of DNA derived from wild-type (+/+) or targeted (+/neo-loxP) ES cells digested with *NdeI* or *NheI* and probed with 5'- and 3'-probes. Positions of wild-type and targeted alleles and their sizes are shown. C, PCR analysis of *Lrh-1* alleles using DNA prepared from tail, liver, and intestine of *Lrh-1*^{+/+}, *Lrh-1*^{flox/flox}, hepKO, or ieKO mice as indicated. A control PCR reaction performed with no DNA is shown on the left. The positions of the PCR products generated by the wild-type *Lrh-1* allele and the *Lrh-1*^{flox/flox} (floxed) and deleted exon 5 *Lrh-1* (Δ exon 5) alleles are indicated by arrows on the left. D and E, RT-qPCR analysis done using a primer set that recognizes *Lrh-1* exon 5 and cDNA from liver and ileum of either *Lrh-1*^{flox/flox} and hepKO mice (D) or *Lrh-1*^{flox/flox} and ieKO mice (E). F, Western blot analysis using *in vitro* synthesized LRH-1 (IVS) or nuclear extracts prepared from livers of *Lrh-1*^{flox/flox} and hepKO mice and antibodies against LRH-1 or TATA-binding protein (TBP). The LRH-1 band migrates at approximately 62 kDa.

known as solute carrier family 10, member A1) (Table 2). Thus, LRH-1 deficiency affects expression of a number of genes involved in cholesterol and bile acid

homeostasis in liver. There were no significant changes in the levels of mRNAs encoding apolipoprotein A1, CYP7B1, 3-hydroxy-3-methylglutaryl-coen-

Table 1. Plasma and Liver Parameters in LRH-1-Deficient Mice

Parameter	flox/flox	hepKO	P Value	flox/flox	ieKO	P Value
Cholesterol (mg/dl)	156 ± 4.0	125 ± 2.4	0.033	109 ± 3.5	115 ± 6.3	0.70
Triglycerides (mg/dl)	140 ± 6.8	122 ± 4.4	0.42	90.8 ± 2.4	97.8 ± 5.2	0.57
Free fatty acids (mmol/liter)	0.71 ± 0.01	0.74 ± 0.02	0.64	0.58 ± 0.01	0.57 ± 0.02	0.81
Bile acids (μmol/liter)	5.5 ± 0.5	5.3 ± 0.1	0.88	4.2 ± 0.2	4.5 ± 0.2	0.61
Glucose (mmol/liter)	17.1 ± 0.5	16.1 ± 0.4	0.57	17.6 ± 0.3	19.7 ± 0.8	0.27
Aspartate aminotransferase (U/liter)	43 ± 0.8	37.8 ± 0.5	0.055	44 ± 0.2	41.3 ± 0.4	0.033
Alanine aminotransferase (U/liter)	40.3 ± 1.5	38.4 ± 0.9	0.69	34.6 ± 0.4	33.3 ± 0.7	0.46
Hepatic cholesterol (mg/g)	2.8 ± 0.1	2.7 ± 0.1	0.34	2.1 ± 0.15	2.5 ± 0.15	0.080
Hepatic triglyceride (mg/g)	10.3 ± 1.7	7.0 ± 0.6	0.092	9.8 ± 1.5	9.0 ± 1.5	0.73

Plasma parameters were measured in mice fasted for 4 h (n = 4–6 mice per group). Data are shown ± SEM

zyme A synthase 1, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and hepatocyte nuclear factor 4α (HNF4α).

We also examined the effect of LRH-1 deficiency in hepatocytes on gene expression in ileum. There was a significant increase in FXR mRNA and a decrease in fibroblast growth factor 15 (FGF15) mRNA, which encodes a hormone secreted from the small intestine that is required for FXR-mediated feedback repression of *Cyp7a1* (25, 26) (Table 3).

Effects of LRH-1 Deficiency in Intestinal Epithelium

We next examined the effect of eliminating LRH-1 in the intestinal epithelium on the expression of genes involved in bile acid and cholesterol homeostasis. mRNA was prepared from the ileal epithelium of ieKO and control *Lrh-1*^{flox/flox} mice. SHP mRNA was reduced 10-fold in ileum of ieKO mice (Table 4). The

absence of LRH-1 also caused significant reductions in ileal bile acid-binding protein (IBABP) and FGF15 mRNA levels (Table 4). These genes have not been previously described as regulated by LRH-1. There were also trends toward decreased expression of *Asbt*, *Ostα*, and *Ostβ*, which have been shown to be regulated directly by LRH-1 *in vitro* (10, 11).

No significant changes were seen in hepatic gene expression in ieKO mice although there was a strong trend toward decreased FXR mRNA (Table 5). Although there was a 2-fold decrease in FGF15 mRNA in the intestine of ieKO mice, there was no corresponding increase in *Cyp7a1* expression (Table 5).

LRH-1 is abundant in the crypts of both the small and large intestine, and *Lrh-1*^{+/-} mice have reduced intestinal proliferation, decreased crypt depth, and decreased mRNA levels of cyclin D1, cyclin E1, and *c-myc* (12). Consistent with this previous study, *c-myc* mRNA levels were decreased in duodenum of ieKO mice, and cyclin E1 mRNA levels trended lower (Fig. 2A). However, there were no significant changes in villus length, crypt depth, bromodeoxyuridine (BrdU) labeling index, or mRNA levels of cyclin D1 in the duodenum of ieKO mice (Fig. 2, A–D). These data raise the possibility that LRH-1 deficiency in both the epithelial and stromal compartments is required to blunt

Table 2. Liver Gene Expression in Mice Deficient for LRH-1 in Hepatocytes

Gene	hepKO /flox/flox	P Value	Change
ABCG5	0.41 ± 0.05	0.001	↓
ABCG8	0.38 ± 0.03	0.001	↓
APOA1	0.88 ± 0.07	0.21	
BSEP	0.71 ± 0.08	0.037	↓
CYP27A1	0.82 ± 0.03	0.030	↓
CYP7A1	1.6 ± 0.2	0.14	
CYP7B1	1.6 ± 0.4	0.15	
CYP8B1	0.090 ± 0.012	0.002	↓
FXR	0.53 ± 0.10	0.031	↓
HMGCR	0.80 ± 0.16	0.38	
HMGCS1	0.71 ± 0.09	0.25	
HNF4α	0.92 ± 0.07	0.61	
MDR2	0.46 ± 0.03	<0.001	↓
MRP2	0.60 ± 0.05	<0.001	↓
MRP3	0.15 ± 0.03	<0.001	↓
NTCP	0.42 ± 0.03	<0.001	↓
SHP	0.033 ± 0.006	<0.001	↓
SCARB1	0.50 ± 0.02	<0.001	↓

Data are shown ± SEM (n = 6–7 mice per group). Significant changes are indicated by arrows. HMGCR, HMG-CoA reductase; HMGCS1, HMG-CoA synthase 1.

Table 3. Intestinal Gene Expression In Mice Deficient for LRH-1 in Hepatocytes

Gene	hepKO /flox/flox	P Value	Change
ABCG5	1.0 ± 0.03	0.63	
ABCG8	1.5 ± 0.2	0.080	
ASBT	2.3 ± 0.7	0.17	
FGF15	0.49 ± 0.13	0.047	↓
FXR	2.0 ± 0.2	0.002	↑
HNF4α	1.2 ± 0.1	0.18	
IBABP	1.0 ± 0.1	0.84	
OSTα	1.6 ± 0.2	0.058	
OSTβ	0.74 ± 0.02	0.15	
SHP	0.65 ± 0.26	0.52	
SCARB1	1.8 ± 0.3	0.077	

Data are shown ± SEM (n = 6–7 mice per group). Significant changes are indicated by arrows.

Table 4. Intestinal Gene Expression in Mice Deficient for LRH-1 in Intestinal Epithelium

Gene	ieKO /flox/flox	P Value	Change
ABCG5	1.2 ± 0.1	0.39	
ABCG8	1.2 ± 0.5	0.49	
ASBT	0.55 ± 0.14	0.073	
FGF15	0.49 ± 0.08	0.041	↓
FXR	1.2 ± 0.1	0.30	
HNF4 α	1.2 ± 0.1	0.16	
IBABP	0.19 ± 0.05	0.010	↓
OST α	0.56 ± 0.09	0.098	
OST β	0.60 ± 0.09	0.058	
SHP	0.10 ± 0.04	0.002	↓
SCARB1	0.78 ± 0.18	0.37	

Data are shown \pm SEM (n = 7–8 mice per group). Significant changes are indicated by arrows.

proliferation in the intestine. Alternatively, there may be a compensatory proliferative response in the intestinal epithelium of ieKO mice.

Effects of LRH-1 Deficiency on the Bile Acid Pool

The effect of LRH-1 deficiency on bile acid pool size and composition was analyzed. In hepKO mice, there was no change in the bile acid pool size but a marked change in its composition (Fig. 3, A and B). Compared with control mice, bile from hepKO mice contained significantly higher concentrations of tauromuricholic acids, taurochenodeoxycholic acid, α/ω -muricholic acid, and β -muricholic acid and lower concentrations of taurocholic acid and taurodeoxycholic acid (Fig. 3B). This altered profile is consistent with the decreased CYP8B1 mRNA levels in hepKO mice (Table

Table 5. Liver Gene Expression in Mice Deficient for LRH-1 in Intestinal Epithelium

Gene	ieKO/flox/flox	P Value
ABCG5	1.1 ± 0.2	0.75
ABCG8	1.0 ± 0.2	0.99
APOA1	1.0 ± 0.2	0.87
BSEP	1.3 ± 0.1	0.062
CYP27A1	0.90 ± 0.17	0.60
CYP7A1	0.75 ± 0.21	0.56
CYP7B1	1.2 ± 0.4	0.69
CYP8B1	1.0 ± 0.1	0.88
FXR	0.79 ± 0.11	0.053
HMGCR	1.4 ± 0.5	0.48
HMGCS1	0.85 ± 0.14	0.61
HNF4 α	1.2 ± 0.1	0.21
MDR2	1.1 ± 0.2	0.41
MRP2	1.1 ± 0.2	0.75
MRP3	0.84 ± 0.11	0.37
NTCP	1.1 ± 0.1	0.64
SHP	0.81 ± 0.12	0.20
SCARB1	1.2 ± 0.2	0.38

Data are shown \pm SEM (n = 7–8 mice per group). HMGCR, HMG-CoA reductase; HMGCS1, HMG-CoA synthase 1.

2). ieKO mice had no significant changes in either bile acid pool size or composition (Fig. 3, C and D). No change was seen in fecal bile acid excretion in either the hepKO or ieKO mice (Fig. 3, E and F).

Effects of Activating FXR on Gene Expression in hepKO and ieKO Mice

Hepatic LRH-1 is proposed to play a central role in FXR-mediated repression of *Cyp7a1* and *Cyp8b1* (16, 17). To address this hypothesis directly, hepKO and control *Lrh-1*^{flox/flox} mice were administered the potent, selective FXR agonist GW4064 for 14 h, at which point the mice were killed and CYP7A1 and CYP8B1 mRNA levels were measured by RT-qPCR. Surprisingly, *Cyp7a1* was repressed as efficiently in hepKO mice as in control mice (Fig. 4A). *Cyp8b1* was also repressed by GW4064 in hepKO mice, although the fold repression was reduced relative to that seen in control mice due to the decrease in basal mRNA levels in the hepKO mice (Fig. 4B). GW4064 also efficiently repressed *Cyp7a1* and *Cyp8b1* expression in livers of ieKO mice (Fig. 4D and data not shown).

The efficient repression of *Cyp7a1* by GW4064 in hepKO mice was particularly surprising in light of their low hepatic SHP mRNA levels (Table 2). However, GW4064 administration caused marked increases in hepatic SHP mRNA in hepKO mice (Fig. 4C). *Fgf15* and *Ibabp* were also efficiently induced in ileum of ieKO mice despite their reduced basal expression (Fig. 4, E and F). Thus, although LRH-1 contributes to the basal expression of *Shp*, *Ibabp*, and *Fgf15*, it is not required for their efficient induction by FXR.

DISCUSSION

LRH-1 is implicated in the regulation of many genes involved in cholesterol and bile acid homeostasis (4). On the *CYP7A1* gene, LRH-1 is proposed to serve both as a positive-acting basal transcription factor and as a docking site for the transcriptional repressor SHP, which is induced by bile acids through an FXR-dependent mechanism (16, 17). The role of SHP in repressing *Cyp7a1* has been validated *in vivo* using *Shp* knockout mice (21, 22). Although LRH-1 was identified in an unbiased screen as a major transcription factor bound to the *CYP7A1* promoter (2) and several studies have shown that LRH-1 can regulate *CYP7A1* either *in vitro* or *in vivo* (16, 17, 23, 24, 27), a comprehensive analysis of LRH-1 function *in vivo* has been hampered by the lack of *Lrh-1* knockout mice. In this report, we describe the generation and characterization of mice deficient for LRH-1 in hepatocytes and intestinal epithelium.

A surprising outcome of our studies was that LRH-1 deficiency in hepatocytes had no significant effect on either basal *Cyp7a1* expression or its repression by FXR. This was unexpected given that LRH-1 binds to

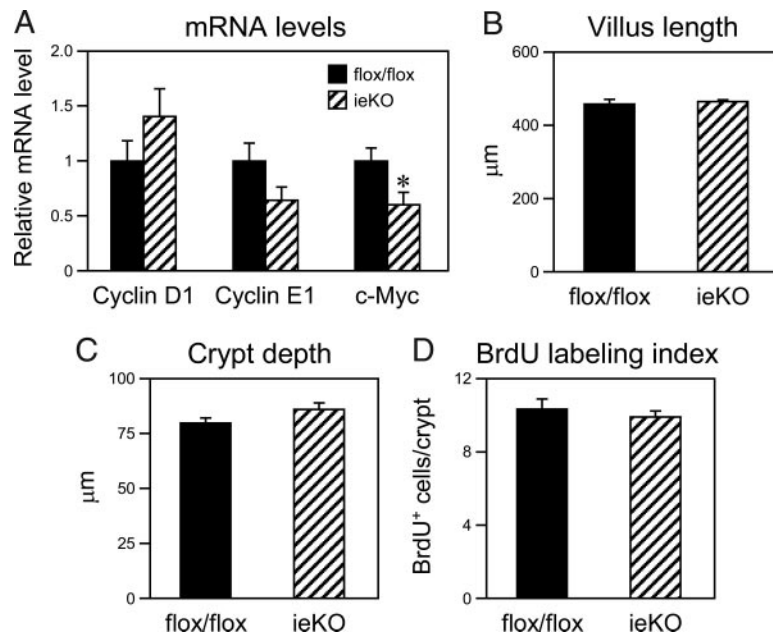


Fig. 2. LRH-1 Deficiency in Intestinal Epithelium Does Not Affect Proliferation in Duodenum

A, mRNA levels of the indicated genes were analyzed by RT-qPCR using intestinal epithelium derived from *Lrh-1*^{flox/flox} and ieKO mice (n = 7–8 mice per group). *, $P < 0.05$. B–D, Villus length (B), crypt depth (C), and BrdU labeling index (D) were measured in duodenum of *Lrh-1*^{flox/flox} and ieKO mice.

the murine *Cyp7a1* promoter as measured by chromatin immunoprecipitation assays (28), and LRH-1 haploinsufficiency or overexpression affects hepatic CYP7A1 mRNA levels in mice (23, 24). There are at least two possible explanations for these data. First, LRH-1 may not regulate *Cyp7a1* or play a relatively minor role. Alternatively, there may be a redundant factor or compensatory response in the hepKO mice that maintains *Cyp7a1* regulation in the absence of LRH-1. One possible candidate for mediating a compensatory response is HNF4 α , which binds to the same composite response element in the *CYP7A1* promoter as LRH-1 and, like LRH-1, is repressed by SHP *in vitro* (29, 30). *In vitro* studies have shown that bile acids can down-regulate *CYP7A1* transcription by reducing HNF4 α activity (31). Moreover, mice deficient for HNF4 α in liver have decreased CYP7A1 mRNA and protein levels during the dark cycle (32). Although no significant changes in HNF4 α mRNA levels were seen in hepKO mice, additional studies will be needed to determine the role of HNF4 α and its relationship with LRH-1 in regulating both basal expression and FXR-mediated repression of *CYP7A1*. Additional studies will also be required to determine whether our findings in mice are relevant in other species, including humans. There are well-established species differences in the regulation of *CYP7A1*, especially with regard to its modulation by cholesterol (33–40). Thus, LRH-1 may be important for the regulation of *CYP7A1* in other species.

In contrast to *Cyp7a1*, basal *Cyp8b1* expression was significantly reduced in mice deficient for LRH-1 in liver. As predicted, the decrease in *Cyp8b1* expres-

sion was accompanied by decreased concentrations of taurocholic acid and increased levels of tauromuri-cholic acids in the bile acid pool. As in the case of *Cyp7a1*, FXR-mediated repression of *Cyp8b1* still occurred in the hepKO mice, although the magnitude of the decrease was reduced. These data demonstrate an important role for LRH-1 in determining the composition of the bile acid pool through effects on *Cyp8b1* expression. They also reveal marked differences in the regulation of *Cyp7a1* and *Cyp8b1*. The differential regulation of *Cyp7a1* and *Cyp8b1* was also recently highlighted by Kim et al. (26), who showed that *Cyp7a1* is repressed more efficiently than *Cyp8b1* by FGF15.

In the hepKO mice, there were significant decreases in hepatic SHP, CYP27A1, SCARB1, ABCG5, ABCG8, BSEP, multidrug resistance transporter 2, MRP2, MRP3, and sodium-dependent bile acid cotransporter mRNA levels. Several different mechanisms may contribute to their decreased expression. First, LRH-1 may regulate these genes directly. In the case of *Shp*, *Mrp3*, *Abcg5*, *Abcg8*, *Scarb1*, and *Bsep*, sites that bind LRH-1 have been identified in the gene-regulatory regions (9, 16, 17, 28, 41, 42). The decreased expression of a subset of these genes may be due also to changes in the activity of FXR, which directly regulates the *Shp*, *Bsep*, and *Mrp2* genes (16, 17, 43, 44). FXR mRNA levels were decreased approximately 2-fold in livers of hepKO mice consistent with the previous finding that LRH-1 binds to the *Fxr* promoter (28). Furthermore, it was previously shown that cholic acid is an important endogenous agonist for FXR in mice (45). In hepKO mice, the reduction in CYP8B1

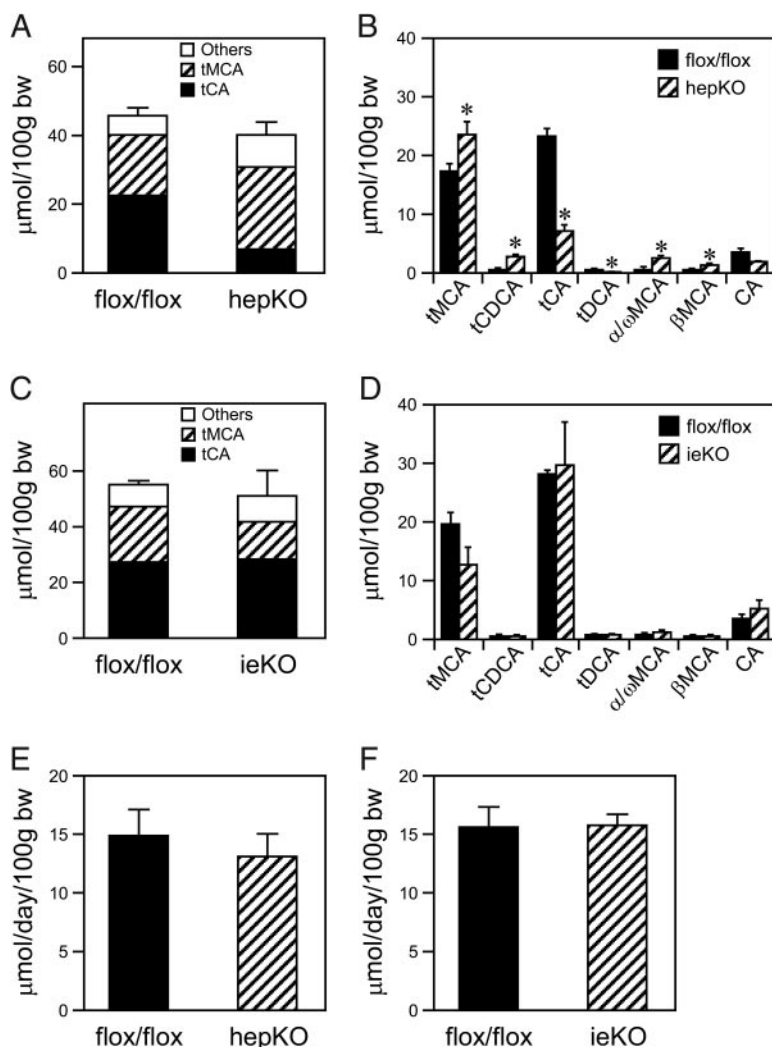


Fig. 3. Effects of LRH-1 Deficiency in Liver or Intestine on Bile Acid Pool Size, Composition, and Excretion

A–D, Bile acid pool size and composition were measured in either hepKO (A and B) or ieKO mice (C and D) and corresponding *Lrh-1*^{flox/flox} mice. tMCA, Tauromuricholic acid; tCDCA, taurochenodeoxycholic acid; tCA, taurocholic acid; tDCA, taurodeoxycholic acid; α/ω MCA, combined α/ω -muricholic acid; β MCA, β -muricholic acid; CA, cholic acid. All bile acid species shown in supplemental Table 1 were measured but only those at concentrations more than 1 $\mu\text{mol}/100\text{g}$ body weight (bw) are shown. *, $P < 0.05$. E and F, Fecal bile acid excretion was measured in either hepKO (E) or ieKO mice (F) and corresponding *Lrh-1*^{flox/flox} mice. Data are the mean \pm SEM ($n = 4$ –6 mice per group).

mRNA and resulting decrease in cholic acid may contribute to the reduced hepatic expression of genes that are regulated by FXR. Thus, decreases in both FXR protein levels and its endogenous ligands could contribute to the altered expression of FXR target genes in hepKO mice.

LRH-1 also appears to have both direct and indirect effects on gene expression in ileum. In ieKO mice, SHP, IBABP, and FGF15 mRNA levels were significantly decreased in ileum, and OST α , OST β , and ASBT mRNA levels trended lower. Whereas *Shp*, *Ost α* , *Ost β* , and *Asbt* have been shown previously to be regulated by LRH-1 *in vitro* (10, 11, 16, 17), *Ibabp* and *Fgf15* have not. It remains to be determined whether intestinal LRH-1 regulates *Ibabp* and *Fgf15* directly or indirectly. Notably, FGF15 mRNA was also

decreased in ileum of mice deficient for LRH-1 in liver. Because FGF15 is regulated strongly by FXR (25, 46), this decrease may be due to the aforementioned reduction in FXR agonist activity in the hepKO mice. However, because *Shp* and *Ibabp* are also FXR target genes (16, 17, 47), it is surprising that their mRNA levels were not also significantly decreased in the hepKO mice.

While this paper was in preparation, Matakai *et al.* (48) reported the characterization of mice in which exons 4 and 5 of *Lrh-1* were deleted in hepatocytes using a tamoxifen-inducible cre. Although many of their findings with respect to the effect of LRH-1 deficiency on hepatic gene expression and bile acid composition are similar to ours, they did not investigate the effect of LRH-1 deficiency on feedback reg-

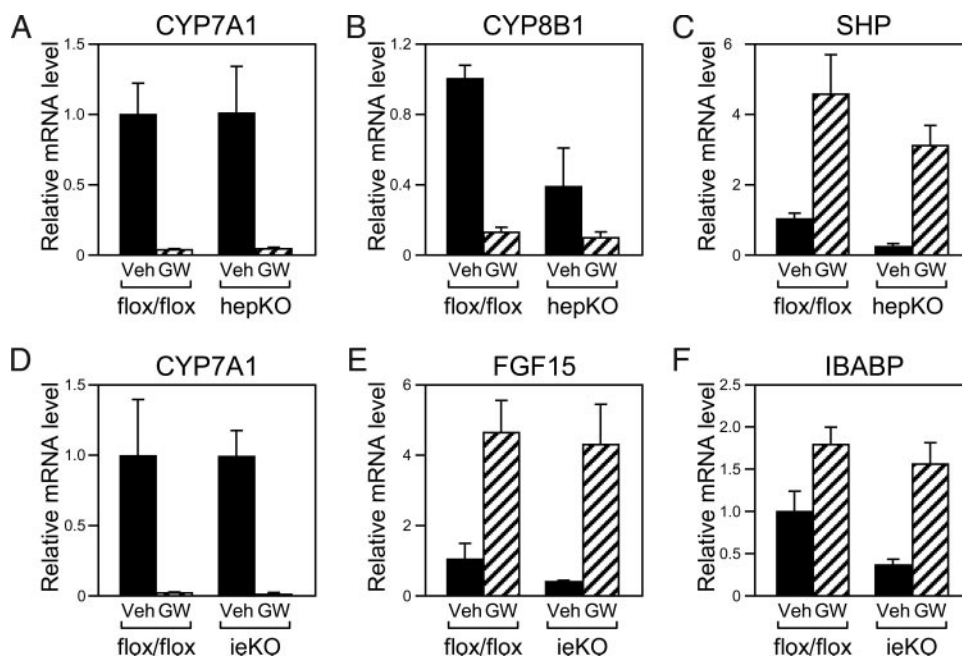


Fig. 4. Effect of FXR Activation on Gene Expression in Tissue-Specific LRH-1 Deficient Mice

hepKO, ieKO, and control *Lrh-1*^{flox/flox} mice were treated with vehicle (Veh) or GW4064 (GW) for 14 h and RNA prepared from liver and ileum. RT-qPCR was used to measure mRNA levels of CYP7A1 (A and D), CYP8B1 (B), and SHP (C) in liver and FGF15 (E) and IBABP (F) in ileum. Data are the mean \pm SEM ($n = 3-4$ mice per group).

ulation of bile acid synthesis nor did they generate mice lacking LRH-1 in the intestinal epithelium. Notably, they reported a significant decrease in the bile acid pool size and a significant increase in fecal bile acid excretion in their LRH-1-deficient mice that were not seen in the current study. Moreover, they did not see the modest decrease in plasma cholesterol that we observed in the hepKO mice. The basis for these differences is not clear but could relate to either the different strategies used to disrupt *Lrh-1* or the different background strains. Regarding the decreased plasma cholesterol levels in our hepKO mice, this is a surprising finding in light of the lack of change in CYP7A1 mRNA levels and decreased hepatic expression of ABCG5 and ABGG8, which promote cholesterol secretion into the bile. At present we do not have a molecular explanation for the effect of LRH-1 deficiency on cholesterol homeostasis.

In summary, we show that LRH-1 affects the expression of a number of genes in liver and intestine that are involved in cholesterol and bile acid homeostasis. Among these are several that were not previously linked to LRH-1, including *Cyp27a1* in liver and *Ibabp* and *Fgf15* in ileum. These findings reveal a broader role for LRH-1 in regulating bile acid homeostasis than was previously appreciated. We also demonstrate that LRH-1 plays a major role in determining the composition of the bile acid pool through effects on *Cyp8b1* expression. Surprisingly, however, LRH-1 is not essential for FXR-mediated feedback regulation of *Cyp7a1*. Additional studies will be required to determine whether LRH-1 has no role in the

feedback regulation of bile acid synthesis or whether there are redundant factors or a compensatory response that maintains *Cyp7a1* regulation in the absence of LRH-1.

MATERIALS AND METHODS

Animal Procedures

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Animals were housed in a pathogen-free and a temperature-controlled environment with 12-h light, 12-h dark cycles and fed standard irradiated rodent chow (TD7913; Harlan Teklad, Madison, WI) and water *ad libitum*. All animal experiments were done with 8- to 12-wk-old male mice killed between 1000 and 1200 h. For measurement of bile acid pool size and composition, mice were fasted 4 h before euthanasia. For the intestinal proliferation study, BrdU (100 mg/kg in PBS) was injected ip 2 h before euthanasia. For experiments with GW4064, mice were administered GW4064 (100 mg/kg, in 1% methylcellulose, 1% Tween 80) or vehicle by oral gavage and killed 14 h later.

Generation of LRH-1 Deficient Mice

High-fidelity PCR amplification of 129SvEv genomic DNA was used to generate an approximately 3.7-kb long arm including exon 4 and parts of introns 3 and 4, an approximately 0.8-kb targeting arm including exon 5 and parts of introns 4 and 5, and an approximately 1.6-kb short arm including intron 5. These fragments were assembled in the pMC1neo PolyA vector (Stratagene, La Jolla, CA) containing

a neomycin resistance cassette and loxP and FRT sites. AattII-linearized DNA was electroporated into 129SvEv-derived embryonic stem (ES) cells. ES cells were screened for targeted recombination by Southern blot analysis using 5'- and 3'-probes and DNA digested with *NheI* or *NdeI*, respectively. Probes were generated by PCR amplification using the following primers: 5'-probe forward primer, CCATGGTG-GATTGGTTCTC; reverse primer, TGTAGCATAAGTTGGTC-CGG; 3'-probe forward primer, GGCTGTTCTTGCTACTT-GAG; reverse primer, CAGGTGCACTGTATGTAGCT. Two independently derived ES cell clones were injected into C57BL/6J blastocysts to produce chimeric mice that transmitted the modified *Lrh-1* locus. The neomycin resistance cassette was removed by crossing *Lrh-1*^{lox/+} mice with R26::FLPe transgenic mice (49). Hepatocyte and intestinal epithelium-specific LRH-1 deficiency was achieved by breeding *Lrh-1*^{lox/lox} mice with albumin-cre (50) or villin-cre (51) transgenic mice. hepKO and iekO mice and their corresponding control *Lrh-1*^{lox/lox} mice were maintained on mixed C57BL/6/129 backgrounds. To confirm homologous recombination and tissue-specific deletion of exon 5 of *Lrh-1*, genomic DNA was extracted from tail, liver, and intestine, and PCR analysis was performed using the following primers: forward primer 1, CGATGTCCCTACTGTGCGA; reverse primer 1, CGCAGCATTCTTCGGCAG; forward primer 2, CATAAGGGCT-CAGTGGCAC; reverse primer 2, CTCTACTGGCTGCCAAGCTG.

Morphometry and Proliferation Measurements

The small intestine was cut into three segments of equal length representing the duodenum, jejunum, and ileum. Each section was cut transversely, fixed with 10% formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. For villus and crypt measurements, duodenum sections were photographed using a Nikon DXM1200F camera and Nikon Eclipse 80i microscope at $\times X$ and $\times 20$ magnification, and 10 villi and crypts per mouse ($n = 11$ – 12 mice per group) were measured using MetaVue software (Meta Imaging Series 6.1; Lewisville, TX). To determine proliferation indices, BrdU incorporation was detected by immunohistochemistry. Sections were deparaffinized and rehydrated. DNA was denatured by 2 N HCl for 1 h at 37 C and neutralized by immersing sections two times in 0.1 M borate buffer (pH 8.5) for 5 min each. Sections were permeabilized by 0.3% Triton for 5 min, incubated in 1.5% horse serum for 30 min, and incubated with antimouse BrdU antibody (Roche Molecular Biochemicals, Indianapolis, IN) at a 1:25 dilution overnight at 4 C. Biotinylated horse antimouse IgG was added at a dilution 1:200 for 30 min at room temperature followed by fluorescein isothiocyanate-conjugated streptavidin at a 1:50 dilution for 30 min at room temperature. Ten crypts per mouse were analyzed for BrdU incorporation ($n = 4$ mice per group).

Liquid Chromatography/Mass Spectrometry Analysis of Bile Acids

Gall bladder, liver, and intestines were removed and placed into 50 ml of EtOH. The tissues in solution were spiked with 50 μ g of chenodeoxycholic acid- D_4 (C/D/N Isotopes; Pointe-Claire, Canada) and extracted. Samples were minced and boiled for 1 h to reduce the EtOH volume to approximately 30 ml. Samples were filtered through no. 2 Whatman paper, and the volume was brought to 50 ml using a volumetric flask. One milliliter of the extract was centrifuge filtered using a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) before analysis. Bile acids were quantified by liquid chromatography/mass spectrometry (Agilent Technologies, Palo Alto, CA) with electrospray ionization in negative ion mode by modification of a published procedure (52). Briefly, samples were loaded onto a precolumn (Zorbax C8, 4.6 \times 12.5 mm, 5 μ m; Agilent) at 1 ml/min for 1 min with water

containing 5 mM NH_4Ac and then backflushed onto the analytical column at 0.4 ml/min (Eclipse XDB-C18, 4.6 \times 50 mm, 5 μ m; Agilent). The mobile phase consisted of methanol/0.024% formic acid (A) and water/10 mM NH_4Ac /0.024% formic acid (B). The following gradient was run for a total run time of 30 min: 0–15 min, 70–80% (A); 15–17 min, 80% (A); 17–20 min, 80–95% (A); 20–26 min, 95% (A). MS parameters were set as follows: gas temperature, 350 C; nebulizer pressure, 30 pounds per square inch gauge; drying gas (nitrogen), 12 liters/min; capillary voltage, 4000 V; fragmentor voltage, 200 V. Under these conditions it is not possible to resolve the closely related taurine-conjugated trihydroxy bile acids, α -, β -, and ω -muricholic acid (MCA). Likewise, unconjugated α - and ω -MCA cannot be resolved. For this reason, the pair of unconjugated MCAs is reported as α/ω -MCA and the three taurine-conjugated bile acids are reported as taumuricholic acid. Selective ion monitoring was used to detect the conjugated and unconjugated bile acids (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Quantification was performed based on peak areas using external calibration curves of standards prepared in methanol. CDCA- D_4 was used to calculate the recovery of bile acids after extraction relative to a blank control.

Fecal Bile Acid Excretion

Fecal excrement was collected from individually housed mice over a continuous 72-h period. Bile acids were extracted and quantified as previously described (53).

RT-qPCR Analysis

Total RNA was extracted from liver and scraped intestine using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). After DNase I (Roche Molecular Biochemicals) treatment, RNA was reverse transcribed into cDNA with random hexamers using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers for each gene were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and were validated as previously described (54). Primer sequences are shown in supplemental Table 2. RT-qPCR reactions contained 25 ng of cDNA, 150 nM of each primer, and 5 μ l of SYBR GreenER PCR Master Mix (Invitrogen) and were carried out in triplicate using an Applied Biosystems Prism 7900HT instrument. Relative mRNA levels were calculated using either the comparative C_T or standard curve methods normalized to cyclophilin or 18S RNA, respectively.

Immunoblot Analysis

Nuclear extracts were prepared from liver using hypotonic buffer [10 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM dithiothreitol, Complete Protease Inhibitor Cocktail (Roche Diagnostic)] and nuclear lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Triton X-100; Complete Protease Inhibitor Cocktail). Nuclear extract (50 μ g) was resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was probed with guinea pig polyclonal anti-LRH-1 antibody against mouse LRH-1 residues 318–560. Recombinant LRH-1 protein was generated as previously described (55). The LRH-1 antibody was used at a dilution of 1:500 at 4 C overnight followed by a secondary horseradish peroxidase-conjugated antibody at a dilution of 1:10,000. The membrane was re-probed with antibody against TATA-binding protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a loading control. Proteins were detected by Super Signal West Femto chemiluminescence substrate (Pierce Chemical Co., Rockford, IL).

Plasma and Liver Parameters

Vena cava blood was collected and transferred into Li-heparin tubes (Sarstedt, Newton, NC). Samples were centrifuged at 4000 rpm at 4°C for 10 min, and total plasma cholesterol, triglycerides, glucose, aspartate aminotransferase, and alanine aminotransferase were measured using a Vitros 250 automated analyzer (Johnson & Johnson, New Brunswick, NJ). Plasma free fatty acids were measured with colorimetric assay kits (Roche). Total plasma bile acids were measured using enzymatic assay kits (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada). Hepatic cholesterol and triglyceride concentrations were measured as previously described (56) except that Triton X-100 was used in place of Triton X-114 and the kits used to measure cholesterol and triglyceride were from Roche and Trinity Biotech (Jamestown, NY), respectively.

Statistical Analyses

Minitab Release 14 software (Minitab, Inc., State College, PA) was used. Values are expressed as mean \pm SEM. Significant differences of two groups were evaluated using a two-tailed, unpaired Student's *t*-test. Multiple groups were analyzed by one-way ANOVA followed by Fisher's least significant difference test.

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