Farnesoid X receptor represses hepatic lipase gene expression

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Abstract The farnesoid X receptor (FXR) is a nuclear receptor that regulates gene expression in response to bile acids (BAs). FXR plays a central role in BA, cholesterol, and lipoprotein metabolism. Here, we identify HL, an enzyme involved in the metabolism of remnant and high density lipoproteins, as a novel FXR-regulated gene. The natural FXR ligand, chenodeoxycholic acid (CDCA), downregulates HL gene expression in a dose- and time-dependent manner in human hepatoma HepG2 cells. The nonsteroidal synthetic FXR agonist GW4064 also decreases HL mRNA levels in HepG2 cells and in primary human hepatocytes. Moreover, the decrease of HL mRNA levels after treatment with FXR agonists was associated with a significant decrease in secreted enzymatic activity. In addition, FXR-specific gene silencing using small interfering RNAs demonstrated that CDCA- and GW4064-mediated downregulation of HL transcript levels occurs via an FXR-dependent mechanism. Finally, using transient transfection experiments, it is shown that FXR represses transcriptional activity of a reporter driven by the −698/+13 bp human HL promoter. Taken together, these results identify HL as a new FXR-regulated gene in human liver cells. In view of the role of HL in plasma lipoprotein metabolism, our results further emphasize the central role of FXR in lipid homeostasis.—Sirvent, A., A. J. M. Verhoeven, H. Jansen, V. Kosykh, R. J. Darteil, D. W. Hum, J-C. Fruchart, and B. Staels. **Farnesoid X receptor represses hepatic lipase gene expression. *J. Lipid Res.* 2004.** 45: **2110–2115.**

Supplementary key words hepatocytes • small interfering ribonucleic acids • lipid metabolism

Hepatic lipase is a glycoprotein synthesized by hepatocytes that exhibits phospholipase A1 and triglyceride hydrolase activities (1, 2). It is extracellularly present in liver, ovaries, and adrenals bound via heparan sulfate proteoglycans and can be released into the circulation by the infusion of heparan sulfate (3, 4). The HL enzyme hydrolyzes triglycerides of intermediate density lipoproteins to produce LDLs, triglycerides, and phospholipids of HDL. Several lines of evidence demonstrate the important role of HL in HDL metabolism. Patients with a genetic deficiency of HL have increased plasma levels of HDL cholesterol and phospholipids (5, 6). Through its function as a lipolytic enzyme, HL thus plays a major role in the metabolism of circulating plasma lipoproteins. However, controversial evidence exists concerning whether HL is proatherogenic or antiatherogenic (7).

The farnesoid X receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily that is highly expressed in liver, kidney, adrenals, and intestine (8). FXR is activated by bile acids (BA), such as the primary BA chenodeoxycholic acid (CDCA) (9, 10). In addition to BAs, synthetic FXR agonists have also been identified (11, 12). In response to ligand binding, FXR regulates a variety of genes involved in BA and cholesterol metabolism. FXR negatively regulates BA production by inhibiting transcription of the cholesterol-7α-hydroxylase gene (13), which encodes the rate-limiting enzyme catalyzing the conversion of cholesterol into BAs. In addition, FXR regulates the expression of various proteins involved in the uptake, intracellular transport, and export of BAs, such as intestinal BA binding protein (14), the bile salt export pump (BSEP) (15), and the Na^+-taurocholate-cotransporting polypeptide (16). Recent findings have shown that FXR also regulates lipid and lipoprotein metabolism. Genetic evidence came from FXR-deficient mice that display increased serum cholesterol and triglyceride levels (17). Moreover, FXR positively regulates the phospholipid transfer protein gene (18), which encodes a secreted protein that

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Abbreviations: apoA-I, apolipoprotein A-I; BA, bile acid; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; FXR, farnesoid X receptor; FXRE, FXR response element; IR1, inverted repeat spaced by one nucleotide; SHP, small heterodimer partner; siRNA, small interfering RNA.

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facilitates the transfer of phospholipids between lipoproteins and modulates plasma HDL metabolism. Furthermore, apolipoprotein A-I (apoA-I), the major apolipoprotein component of HDL, is downregulated by FXR (19). In addition, FXR controls plasma triglyceride levels by activating the apoC-II gene (20), an obligate cofactor for lipoprotein lipase responsible for the hydrolysis of triglycerides in chylomicrons, and by inhibiting apoC-III (21), which inhibits intravascular triglyceride metabolism. Taken together, these findings identify FXR as a key player in lipid and lipoprotein metabolism.

We further explored the influence of FXR on HDL metabolism using a microarray approach aimed at identifying new FXR target genes in liver. In this study, we identify HL as a FXR-regulated gene in human liver cells. In view of the role of HL in the metabolism of circulating plasma lipoproteins, our data further emphasize the central role of FXR in lipid homeostasis.

MATERIALS AND METHODS

Materials

CDCA was obtained from Sigma (Saint-Quentin, France). Human hepatoblastoma HepG2 and HuH7 cells were purchased from the American Type Culture Collection (Rockville, MD). JETPEI transfection reagent was from PolyPlus Transfection (Illkirch, France). GW4064 was synthesized in the chemistry department of GENFIT (Loos, France) according to Maloney et al. (11).

Cell culture and treatments

HepG2 and HuH7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, streptomycin/penicillin, sodium pyruvate, glutamine, and nonessential amino acids (Life Technologies, Cergy-Pontoise, France) at 37°C in a humidified 5% CO_2 atmosphere. Human primary hepatocytes were isolated as described (22) and incubated for 48 h with CDCA (75 μM) or GW4064 (1 μM). For mRNA analysis, HepG2 cells were treated on six-well plates at 60% confluence with either CDCA or GW4064 at the indicated concentration.

Transient transfection assays

HepG2 cells were transfected using JETPEI reagent on 24-well plates with 70×10^3 cells/well. One hundred nanograms of luciferase reporter plasmids were cotransfected with 20 ng of PRL-null, a renilla reporter internal control plasmid, with or without 20 ng of pcDNA3 hFXR and 20 ng of pSG5 human retinoid X receptor α or pSG5 (Stratagene, La Jolla, CA) and pcDNA3 (Invitrogen, Leek, The Netherlands) as empty control vectors. All samples were complemented with pBSK+ plasmid (Stratagene) to an identical total amount of DNA (500 ng/well). Five hours later, cells were incubated with or without FXR agonists in medium containing 2% Ultroser SF serum (Life Technologies). Luciferase activity was assayed 48 h later using a TR717 microplate luminometer (Applied Biosystems).

RNA analysis

Total RNA was isolated using TRI Reagent according to the manufacturer's instructions (Sigma). Total RNA was reverse transcribed using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, Scotland). Real-time quantitative PCR analysis was performed using the LightCycler Technology (Roche Diagnostic). cDNA (0.5 μl) was added in a 20 μl final volume to 2 μl of LightCycler DNA Master SYBR Green I mix (Roche Diagnostic), $MgCl_2$ solution (3 mM), and forward and reverse oligonucleotide primers (100 nM). The PCR program consisted of a denaturing step at 95°C for 8 min followed by 40 cycles of 10 s at 55°C, 10 s at 95°C, and 15 s at 72°C. Primers used were as follows: HL forward, 5′-GGAGGAATCTGTTCAACTCTCTCG-3′; HL reverse, 5′-AGAAAGACGATTGCTGGGGG-3′; BSEP forward, 5′-AGTTGCTCATCGCTTGTCTACG-3′; BSEP reverse 5′-GCTTGATTTCCCTGGCTTTG-3′; GAPDH forward, 5′-GACATCAAGAAGGTGGTGAA-3′; GAPDH reverse, 5′-CCACATACCAGGAAATGAGC-3′; FXR forward, 5′-CCGTGAATGAAGACAGTGAAGGTCG-3′; FXR reverse, 5′-ACCCTTTCAGCAAAGCAATCTGGTC-3′; small heterodimer partner (SHP) forward, 5′-GGCTGGCAGTGCTGATTCAG-3′; SHP reverse, 5′-TGGGGTGTGGCTGAGTGAAG-3′; 36B4 forward, 5′-CATGCTCAACATCTCCCCCTTCTCC-3′; 36B4 reverse, 5′-GGGAAGGTGTAATCCGTCTCCACAG-3′. mRNA levels were normalized to 36B4 mRNA.

Secreted hepatic lipase activity measurement

HepG2 and HuH7 cells were cultured on six-well plates (0.5×10^6 cells/well) in DMEM containing 25 units/ml heparin and treated with or without FXR agonists. Medium was collected after 30 h, and HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol [^{14}C]trioleate emulsion as substrate (23). Assays were performed for 30 min at 30°C, and activities were expressed as milliunits (nanomoles of free fatty acids released per minute).

Gene silencing with small interfering RNAs

The small interfering RNAs (siRNAs) targeting human FXR (cDNA sequence 5′-GTCGTGACTTGCGACAAG-3′) were synthesized by Eurogentec (Seraing, Belgium) and annealed according to the manufacturer's instructions. siRNA negative control and siRNAs specific for GAPDH from Ambion (Austin, TX) were used to test nonspecific effects on gene expression. HepG2 cells were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions on 12-well plates containing 150×10^3 cells/well with 125 nM siRNA/well. Twelve hours after transfection, cells were treated for 36 h with FXR agonists at the indicated concentrations before RNA analysis.

RESULTS

FXR agonists repress hepatic lipase gene expression

To investigate the effect of FXR agonists on HL gene expression, HepG2 cells were treated with different concentrations of the natural FXR ligand CDCA. Analysis of HL mRNA levels by real-time quantitative PCR revealed a significant dose- and time-dependent decrease of HL transcript levels in CDCA-treated compared with vehicle-treated HepG2 cells (**Fig. 1A**, **B**). Because BAs may exert FXR-independent effects by activating other signal transduction pathways (24, 25), the influence of the synthetic nonsteroidal FXR-specific agonist GW4064 was tested on HL gene expression. Treatment of HepG2 cells with GW4064 also resulted in a significant dose-and time-dependent decrease of HL mRNA levels compared with control vehicle-treated cells (Fig. 1A, B). Moreover, downregulation of HL gene expression was also observed in HuH7 cells (data not shown) and primary human hepatocytes treated with CDCA and GW4064 (**Fig. 2**). To determine if the FXR agonist-mediated downregulation of HL gene expression ob-

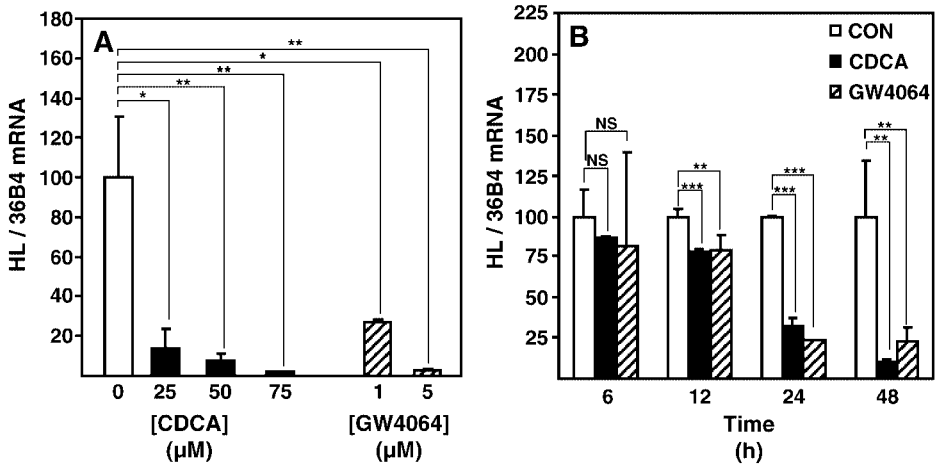

Fig. 1. Farnesoid X receptor (FXR) agonists downregulate HL gene expression in a time- and dose-dependent manner in HepG2 cells. HL mRNA levels were measured by real-time quantitative PCR. A: HepG2 cells were treated with increasing concentrations (as indicated) of chenodeoxycholic acid (CDCA) or GW4064 for 48 h. B: HepG2 cells were treated with DMSO [control (CON)], CDCA (75 μM), or GW4064 (5 μM) for 6, 12, 24, and 48 h. Values represent means ± SD (n = 3). Statistically significant differences between control and FXR agonist-treated samples are indicated by asterisks (Student's *t*-test): *** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$.

served in vitro in human liver cells also occurs in other species, such as the mouse, C57BL/6 mice (n = 4) were treated for 5 days with taurocholic acid. In livers from C57BL/6 wild-type mice, taurocholic acid treatment significantly increased the expression of the well-characterized FXR target gene SHP but did not influence HL transcript levels (data not shown), suggesting a human-specific regulation of HL gene expression by BAs. Taken together, these data show that both natural and synthetic FXR agonists downregulate HL mRNA levels in human but not mouse liver cells.

FXR agonists decrease hepatic lipase enzymatic activity

To further investigate whether FXR agonists functionally influence HL activity, HepG2 and HuH7 cells were treated for 30 h with CDCA or GW4064 and incubated in the presence of heparin to stabilize the secreted HL. A significant decrease in secreted HL enzymatic activity was observed in both CDCA- and GW4064-treated compared with nontreated control cells (**Fig. 3A**, **B**). These results demonstrate that FXR agonists are able to decrease HL enzymatic activity secreted by human hepatic cell lines.

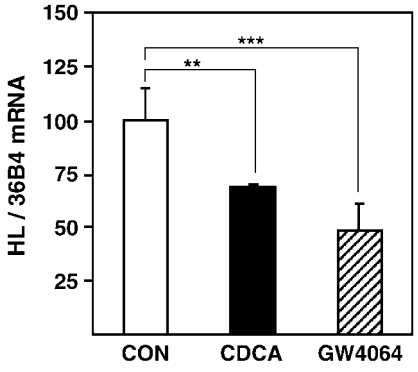

Fig. 2. FXR agonists downregulate HL gene expression in primary human hepatocytes. Primary human hepatocytes were treated with DMSO [control (CON)], CDCA (75 μM), or GW4064 (5 μM) for 48 h. HL mRNA levels were measured by real-time quantitative PCR. Values represent means ± SD (n = 5). Statistically significant differences between control and FXR agonist-treated samples are indicated by asterisks (Student's *t*-test): *** $P < 0.001$; ** $0.001 < P < 0.01$.

CDCA- and GW4064-mediated downregulation of HL gene expression occurs by an FXR-dependent mechanism

To establish whether the regulation of HL gene expression by FXR agonists observed in human liver cells occurs via FXR activation, a FXR gene-silencing experiment using specific siRNA duplexes to reduce the expression of FXR was performed. HepG2 cells were transfected with siRNAs to induce FXR or GAPDH gene silencing and then treated with FXR agonists. A negative control was also used to demonstrate that transfection does not induce non-specific effects on gene expression. Compared with control cells, transfection of HepG2 cells with GAPDH-specific siRNAs reduced GAPDH transcript levels by ~80% (100.0 ± 24.7% versus 23.3 ± 1.5%), whereas there was no significant effect on FXR expression levels (100.0 ± 24.7% versus 88.0 ± 12.4%). On the other hand, compared with control cells, transfection of HepG2 cells with FXR-specific siRNAs reduced FXR mRNA levels by ~80% (100.0 ± 15.1% versus 21.2 ± 1.3%), whereas there was no significant effect on GAPDH transcript levels (100.0 ± 15.1% versus 87.6 ± 6.0%). Interestingly, FXR silencing abolished the CDCA- and GW4064-mediated downregulation of HL mRNA levels (**Fig. 4A**). As positive controls, mRNA levels of the known BSEP and SHP FXR target genes were quantified in the same samples (Fig. 4B, C). As expected, FXR ligand treatment increased BSEP and SHP gene expression in control cells treated with FXR agonists but not in FXR silencing cells. Taken together, these results dem-

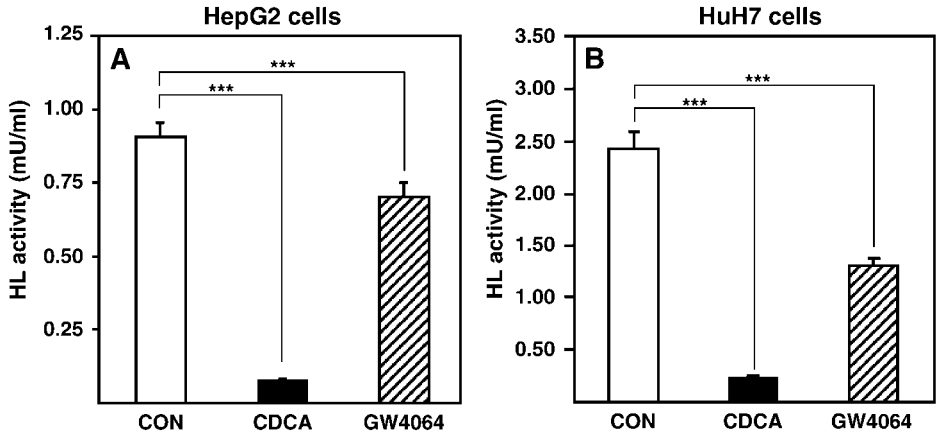

Fig. 3. FXR agonists downregulate HL activity. HepG2 (A) and HuH7 (B) cells were treated for 30 h with DMSO [control (CON)], CDCA (75 μM), or GW4064 (5 μM) in medium containing 25 units/ml heparin. Hepatic lipase activity was determined by a triacylglycerol hydrolase assay (23). Values represent means ± SD (n = 6). Statistically significant differences between control and FXR agonist-treated samples are indicated by asterisks (Student's *t*-test): *** $P < 0.001$.

onstrate that FXR mediates the downregulation of HL transcript levels by CDCA and GW4064 in HepG2 cells.

FXR agonist-mediated downregulation of HL gene expression occurs at the transcriptional level via the proximal human HL promoter

To test the hypothesis that FXR directly regulates HL transcription, HepG2 cells were transfected with various deletion constructs of the proximal human HL promoter (GenBank accession number L77731) in the pGL3-luciferase reporter plasmid in the presence or absence of FXR and/or GW4064. Overexpression of FXR decreased luciferase activity of the −698/+13 bp fragment of the human HL gene promoter even in the absence of FXR ligand (**Fig. 5**). This may be attributable to the presence of endogenous BA FXR ligands or, alternatively, indicate that FXR may also repress the promoter in a ligand-independent manner. This repression was further enhanced by GW4064. Interestingly, no repression was observed when the other 5′ deletion constructs were tested. Therefore, the regulation of human HL gene expression by FXR occurs, at least in part, at the transcriptional level via elements located in the proximal promoter between nucleotides −698 and −541.

DISCUSSION

This study identifies HL as a novel FXR-regulated gene in human liver cells. Our results demonstrate that treatment of various human hepatic cells, including primary hepatocytes, with FXR agonists is associated with the downregulation of HL transcript levels. By contrast, no significant change was observed in livers from mice upon BA

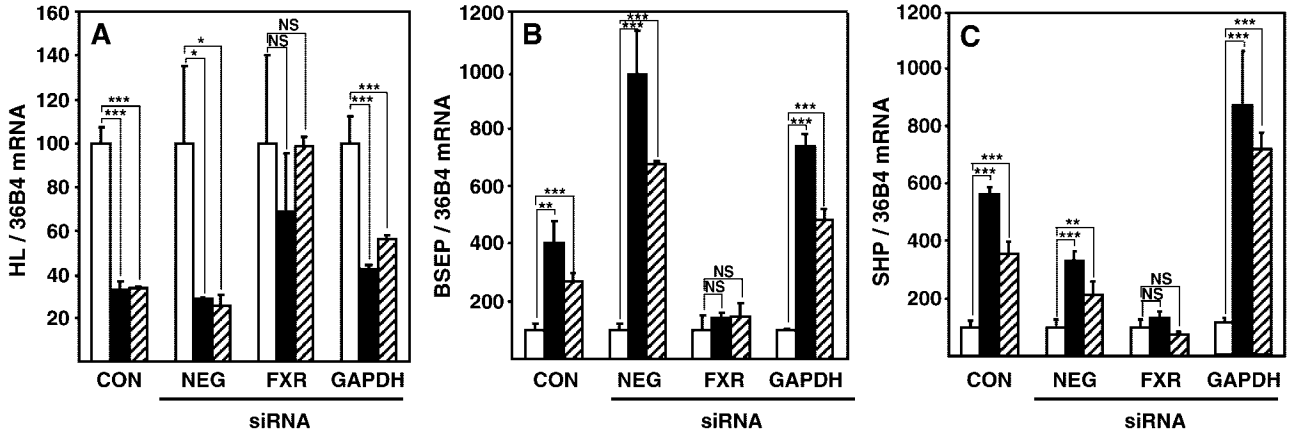

Fig. 4. Silencing of FXR mRNA levels abolishes the downregulation of HL gene expression by FXR agonists in HepG2 cells. HepG2 cells were transfected or not [control (CON)] with small interfering RNA (siRNA) duplexes specific for FXR or GAPDH, or with a siRNA negative control (NEG). After 12 h, cells were treated with DMSO (open bars), CDCA (75 μM; closed bars), or GW4064 (5 μM; hatched bars) for 36 h. HL (A), bile salt export pump (BSEP; B), and small heterodimer partner (SHP; C) mRNA levels were measured by real-time quantitative PCR. Values represent means ± SD (n = 3). Statistically significant differences between control and FXR agonist-treated samples are indicated by asterisks (Student's *t*-test): *** $P < 0.001$; ** $0.001 < P < 0.01$; * $0.01 < P < 0.05$.

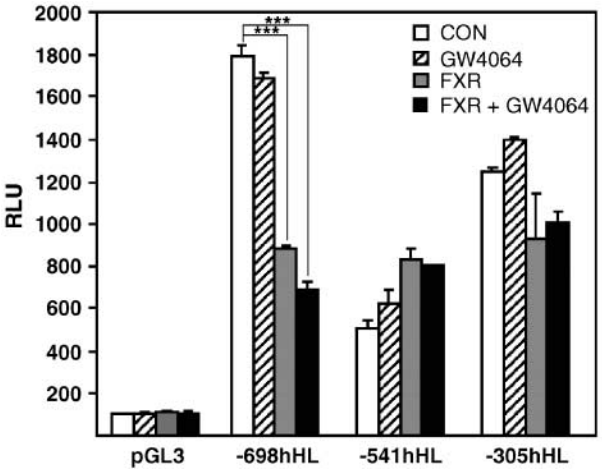

Fig. 5. FXR represses transcriptional activity of the −698/+13 bp fragment of the human HL promoter. HepG2 cells were cotransfected with 100 ng of luciferase reporter pGL3 plasmid driven by various deletion fragments of the proximal human HL promoter with or without pcDNA3-hFXR (20 ng) and pSG5-human retinoid X receptor α (20 ng). Cells were treated with DMSO [control (CON)] or GW4064 (1 μM) for 48 h. Results are expressed in relative luciferase units (RLUs) as fold induction of controls set at 1 and normalized to internal control renilla activity. Values represent means ± SD (n = 3). Statistically significant differences are indicated by asterisks (Student's *t*-test): *** $P < 0.001$.

treatment, suggesting a human-specific regulation of HL gene expression by FXR agonists. In agreement with our observation, it has been reported that cholic acid, the most potent FXR agonist in mice (18), does not significantly influence HL transcript levels in mouse liver (26) and that the postheparin HL activity is similar in FXR-deficient mice compared with wild-type mice (27). Our results show that the decrease of HL mRNA levels after treatment with FXR agonists is associated with a significant decrease in secreted enzymatic activity. Interestingly, compared with the effects on mRNA, CDCA appears more potent in reducing HL activity than the synthetic FXR agonist. Because BAs may exert FXR-independent effects by activating other signal transduction pathways (24, 25), it is tempting to speculate that modulations of HL activity may occur via posttranslational modifications in addition to the FXR-specific effect on HL transcript levels. In agreement with this, previous studies reported that human HL contains four glycosylation sites, at positions 20, 56, 340, and 375 (28), and that site-directed mutagenesis of Asn-56 is crucial for the secretion of active HL (29).

In the present study, it is shown that BA-activated FXR mediates a time- and dose-dependent downregulation of HL transcript levels in human hepatic cells. In contrast with the SHP response to FXR agonists, which occurs very early (30), the downregulation of HL transcript levels is more delayed, as previously observed with other FXR target genes such as UGT2B4 and apoA-I (19, 30). Moreover, our data from transient transfection experiments indicate that the regulation of HL gene expression occurs at the transcriptional level via elements located in the proximal promoter. By using nonsteroidal FXR-selective agonist and FXR-specific siRNA, we have clearly demonstrated that CDCA downregulates HL gene expression via a FXR-dependent mechanism in human liver cells. Most previously characterized FXR response elements (FXREs) are inverted repeats spaced by one nucleotide (IR1) acting as positive sites to which FXR binds as a heterodimer with retinoid X receptor α. The proximal HL promoter does not contain IR1-type sequences that could function as FXREs. However, FXR was also shown to downregulate apoA-I gene transcription through a nonclassical mechanism by binding as a monomer to an atypical FXRE in the C site of the promoter (19). In electromobility shift assays, however, FXR did not bind in vitro to oligonucleotides covering the −698 and −541 region of the proximal HL promoter (data not shown). Thus, FXR could regulate HL gene transcription indirectly (31, 32).

HL is implicated in the intravascular remodeling of LDLs and HDLs. HL reduces HDL particle size by hydrolyzing its triglycerides and phospholipids. Although these studies were performed in isolated liver cells and therefore cannot be directly extrapolated to the in vivo situation, the downregulation of HL by FXR agonists suggests a novel mechanism that may contribute to the modulation of HDL and triglyceride metabolism by BA-activated FXR. FXR has already been reported to downregulate apoA-I, the major apolipoprotein component of HDL (19), and to positively regulate phospholipid transfer protein gene expression (18), which encodes a secreted protein that facilitates the transfer of phospholipids between lipoproteins and regulates plasma HDL levels. On the one hand, by inhibiting apoA-I synthesis, FXR should decrease HDL levels. On the other hand, by decreasing HL, FXR may also prevent HDL from being converted into smaller particles, which are more rapidly cleared in the kidney. So overall, in humans, FXR may not have a major quantitative impact on HDL but may modify its composition and hence its function. In agreement with this, it has been shown that CDCA does not affect HDL levels in humans (33). FXR also controls plasma triglyceride levels by inducing the expression of peroxisome proliferator-activated receptor α, another nuclear receptor controlling triglyceride metabolism (34), and apoC-II (20), an obligate cofactor for lipoprotein lipase responsible for the hydrolysis of triglycerides in chylomicrons, and by inhibiting apoC-III (21), which plays an important role in the control of triglyceride metabolism. In this way, FXR likely promotes peripheral triglyceride clearing. In this context, it is tempting to hypothesize that the repression of HL by FXR could be an additional mechanism to prevent lipid overloading in the liver. The repression of HL transcript levels by FXR agonists further emphasizes the key role of FXR in regulating lipid metabolism. However, further studies are required to determine the contribution of each mechanism to the triglyceride- and HDL cholesterol-decreasing activity of BAs. Because BA concentrations (25 μM) as low as those found in human liver cells in vivo (9, 10) are able to repress HL gene expression, it is tempting to speculate that the regulation of HL by FXR should be relevant not only in pathophysiological conditions of high intrahepatic BA con-

centrations, such as in progressive familial intrahepatic cholestasis, but also in normal physiological situations.

In conclusion, this study identifies HL as a novel FXR-regulated gene in human liver. These results suggest a novel mechanism by which BAs may modulate triglyceride and HDL metabolism in human liver cells and further emphasize the central role of FXR in lipid homeostasis.

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