Knockdown of ATP8B1 expression leads to specific downregulation of the bile acid sensor FXR in HepG2 cells: effect of the FXR agonist GW4064

Pilar Martínez-Fernández,1 Loreto Hierro,2 Paloma Jara,2 and Luis Alvarez1

1Research Unit, La Paz University Hospital-Fundación para la Investigación Biomédica del Hospital Universitario La Paz (FIBHULP) and 2Pediatric Liver Service, La Paz Children’s University Hospital, Madrid, Spain

Submitted 10 June 2008; accepted in final form 16 February 2009

Farnesoid X receptor (FXR) is a bile acid-sensing nuclear receptor that controls bile acid homeostasis. It has been suggested that downregulation of FXR contributes to the pathogenesis of an inherited disorder of bile secretion caused by mutations in ATP8B1. We have investigated the relationship between ATP8B1 knockdown and FXR downregulation in the human hepatoblastoma cell line HepG2. Transfection of HepG2 cells with ATP8B1 small interfering RNA (siRNA) duplexes led to a 60% reduction in the endogenous levels of ATP8B1 mRNA and protein and a concomitant decrease in FXR mRNA and protein content, as well as in FXR phosphorylation. This decrease was accompanied by a marked reduction in mRNA levels of a subset of FXR targets, such as bile salt export pump (ABCB11), small heterodimer partner, and uridine 5′-diphosphate-glucuronosyltransferase. ATP8B1 inhibition specifically targeted FXR since mRNA expression of other prominent nuclear receptors, such as pregnane X receptor and constitutive androstane receptor, or liver-enriched transcription factors, such as hepatic nuclear factor 1α (HNF-1α) and HNF-4α, was not altered. The expression of other key genes involved in bile acid synthesis, detoxification, and transport also remained unchanged upon ATP8B1 knockdown. Supporting the specificity of the effect, siRNA-mediated silencing of ABCB11, whose defect is associated with another inherited disorder of bile secretion, did not affect FXR expression. Treatment with the synthetic FXR agonist GW4064 was able to partially neutralize ATP8B1 siRNA-mediated FXR downregulation and fully counteract inhibition of FXR target genes. Collectively these findings indicate that ATP8B1 knockdown specifically downregulates FXR, and this action can be circumvented by treatment with FXR agonists.

farnesoid X receptor; ABCB11; nuclear receptors; progressive familial intrahepatic cholestasis

FARNESOID X RECEPTOR (FXR; NR1H4) is a nuclear receptor that functions as a ligand-activated transcription factor. Bile salts such as chenodeoxycholate, cholate, deoxycholate, and their conjugates are its physiological ligands (39). To modulate target gene transcription, it also usually requires heterodimerization with retinoid X receptor (RXR; NR2B1) (12). FXR accounts for pleiotropic effects in the enterohepatic system, but, so far, its most well-defined action is the regulation of bile acid homeostasis (18, 21). In the liver, and in response to elevated concentrations of bile acids, FXR coordinately induces the expression of genes involved in bile acid detoxification (SULT2A1, UGT2B4), sinusoidal efflux (organic solute transporters, OST-α-OST-β), and biliary excretion (ABCB11, ABCC2) and represses those involved in bile acid biosynthesis (CYP7A1, CYP8B1) and sinusoidal uptake (SLC10A1, SLCO1B1) (7, and references therein). It ultimately protects the hepatocyte from excessive intracellular bile acid accumulation.

To date, FXR has not been directly linked to any human disease although it has been implicated in the development of two disorders of bile secretion, intrahepatic cholestasis of pregnancy and a form of progressive familial intrahepatic cholestasis (PFIC) associated with mutations in ATP8B1 (ATPase, Class I, type 8B, member 1). In the former, the occurrence of FXR variants with decreased transcriptional activity has been detected in affected women (38); in the latter, reduced expression of FXR has been found in intestine and liver of affected children (2, 9).

PFIC attributable to ATP8B1 deficiency is an inherited disorder with systemic manifestations, including a severe form of cholestasis, or impaired secretion of bile, that presents early in infancy and progresses to end-stage liver disease (1, 17, 37). It arises from mutations in ATP8B1 (6, 19), also referred to as FIC1, which encodes a P-type ATPase that flips phosphatidylserine from the outer to the inner leaflet of the plasma membrane (27, 36). Various mechanisms have been proposed for explaining impaired bile acid excretion in this disorder. Compelling data from a mouse model indicate that Atp8b1 deficiency disturbs canalicular membrane phospholipid asymmetry, which in turn results in defective biliary excretion of hydrophobic bile salts (28). Other putative pathological mechanisms deal with indirect effects of ATP8B1 dysfunction on downregulation of the expression of cystic fibrosis transmembrane conductance regulator (11) or, as mentioned, FXR.

The present work was undertaken to further investigate the diminution in hepatic FXR expression found in humans with ATP8B1 deficiency. By using small interfering RNA (siRNA) technology in the polarized human hepatoblastoma cell line HepG2, we demonstrate that ATP8B1 inhibition results in a consistent downregulation of FXR and major FXR target genes, without affecting the expression of other ligand-activated nuclear receptors or liver-enriched transcription factors involved in the handling of bile acids. We also show that the inhibitory effect of ATP8B1 knockdown can be neutralized by treatment with an FXR agonist.

MATERIALS AND METHODS

Cell culture and human liver samples. The human hepatoblastoma cell line HepG2 was obtained from the European Collection Cell Culture (Salisbury, UK). Cells were cultured at 37°C in a humidified 5% CO2 atmosphere in DMEM (Invitrogen, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum, 2 mM t-glutamine, and 1% penicillin/streptomycin. For activating FXR, cells were cultured in...
serum-depleted medium in the presence of 1 μM GW4064 (kindly provided by Dr. Timothy M. Willson, GlaxoSmithKline, Research Triangle Park, NC). Specimens of normal human liver (n = 3) used for comparative analysis of ATP8B1 and FXR expression and for Western blotting came from unused donor segments. Informed consent and La Paz University Hospital ethics committee approval were obtained before tissue collection.

Antibodies. Two polyclonal antibodies against human ATP8B1 were raised in rabbits on demand (Affinity BioReagents, Golden, CO) by coinjection of two synthetic peptides: EEPNNRLDKFTGTLF (peptide I, amino acid residues 266-280) and HARMENQRNRGGV (peptide II, amino acid residues 776-778). Antibodies produced from each of the peptides were purified out separately by immunoaffinity chromatography. Both antibodies recognized a protein of ~140 kDa in immunoblots of crude extracts from HepG2 cells and human liver. This protein was not recognized by preimmune sera and was not detected when antibodies were preincubated with 20 μg of the peptides used for immunization. Immunofluorescent costaining assays in HepG2 also showed that both antibodies reacted with a protein that colocalizes with the canalicular marker protein multidrug resistance-associated protein 2 (MRP2) in pseudocanaliculi. Specificity of antibody raised against peptide II was further assessed upon expression in bacteria of a CDNA fragment encoding amino acid residues 653-924 of human ATP8B1. This cDNA was subcloned into the expression vector pT7-7 and the resulting plasmid transformed into Escherichia coli (E. coli) BL21(DE3) following the procedures described in Ref. 3. After induction with 0.5 mM isopropyl β-D-thiogalactopyranoside, the antibody specifically reacted with a bacterially expressed peptide of the expected molecular mass (~30 kDa). This protein was not detected in lysates from noninduced E. coli cells. Antibody raised against peptide II was used in Western blotting assays described below. Other primary antibodies used were as follows: a goat polyclonal anti-FXR (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal anti-pregnane X receptor (PXR) (H-160; Santa Cruz Biotechnology), a rabbit polyclonal anti-cAMP-responsive element-binding protein (CREB) (Upstate Biotechnology, Lake Placid, NY), and a mouse monoclonal anti-MRP2 (clone M2III-6; Chemicon International, Temecula, CA).

Gene silencing with siRNA. Double-stranded siRNAs targeting human ATP8B1 (GeneBank accession no. NM_005603) and ABCB11 (GeneBank accession no. NM_003742) were synthesized by Ambion (Austin, TX). All siRNAs were 21 nucleotides long and contained symmetric 3′ overhangs of two deoxynucleotides. Sequences are as follows (top strand shown): ATP8B1 siRNA1, 5′-GGCAGUUCCUCAAAAUCUCUTTT-3′ (targeted exons: 3, 4); ATP8B1 siRNA2, 5′-GGAGAGUAAUAUGCGAAATT-3′ (targeted exons: 2, 3); ABCB11 siRNA, 5′-CCAUUGUAUUGGAGCUACAGTTT-3′ (targeted exon: 5). Scrambled versions of ATP8B1 and ABCB11 siRNAs (four changes of nucleotide positions) were used as controls. HepG2 cells were transfected by the NeoFX method, according to the supplier’s protocol (Ambion). All siRNAs were used at a concentration of 1 nM, except in dose-dependence experiments in which concentrations ranged from 0.025 nM to 50 nM. Unless indicated, cells were harvested 48 h after the beginning of transfection for mRNA and/or protein analysis. Transfection efficiency was assessed by fluorescence microscopy using 6-FAM-labeled ATP8B1 siRNA2 (Ambion). Briefly, HepG2

![Fig. 1. Effect of small interfering RNA (siRNA) transfection on endogenous ATP8B1 mRNA levels in HepG2 cells. HepG2 cells were transfected with 1 nM of 2 different double-stranded siRNAs targeting ATP8B1 or with their scrambled versions (control). RNA was isolated 48 h after the beginning of transfection and analyzed by SYBR Green real-time quantitative RT-PCR (A) and Northern blotting (B). ATP8B1 mRNA values were normalized to β-actin mRNA. A: data represent the means ± SD of 4 independent experiments with triplicate determinations. Values in untransfected cells were arbitrarily set to 1. B: Northern blotting was carried out with 30 μg of total RNA. Blots were sequentially hybridized with radiolabeled ATP8B1 and β-actin cDNA probes. 28S and 18S refer to the ribosomal RNA markers. C: fluorescence analysis of HepG2 cells transfected with 6-FAM-labeled siRNA2. Top: left, phase contrast; top, right: same field of cells showing distribution of FAM-labeled siRNA (green); bottom, left: DAPI staining; bottom, right: merged green and blue images; a predominant perinuclear localization of siRNA complexes is observed. *P < 0.05.](image-url)
cells were cultured in a chamber slide at ~60% confluence and transfected with 1 nM of labeled siRNA. After 48 h, cells were washed with PBS and mounted in Vectashield/DAPI (Vector Laboratories, Burlingame, CA). Immunostained cells were analyzed by fluorescence microscopy.

Analysis of RNA expression. Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and treated with Ambion's Turbo DNA-free kit. First-strand cDNA was synthesized from 0.5 µg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Simultaneous real-time PCR reactions of 32 selected genes were performed by using TaqMan Low-Density Arrays in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Predesigned TaqMan assays for target genes are summarized in Supplementary Table S1. Supplemental material for this article is available at the American Journal of Physiology Gastrointestinal and Liver Physiology website. Three replicates were run for each gene and each sample. Relative changes in mRNA expression were calculated by the \( \Delta \Delta C_t \) method (23), using the Sequence Detection System 2.1 software (Applied Biosystems). Expression levels of target genes were normalized to expression of 18S RNA and β-actin. Results were referred to β-actin values since they were found to be more relatively constant in different control samples. Relative mRNA levels of \( ABCB11 \), \( ATP8B1 \), and \( FXR \) were also quantified separately by SYBR Green fluorescence-based real-time quantitative PCR, following conditions previously described (2). For reliable determination of \( ABCB11 \) mRNA levels, cDNA preparations from 2.5 µg of total RNA were required. The detection procedure of \( ABCB11 \) mRNA in conventional reverse-transcriptase PCR (RT-PCR) assays consisted of a denaturing step at 95°C for 5 min followed by 40 cycles of 30 s at 95°C, 45 s at 60°C, and 60 s at 72°C. Forward and reverse primers used in RT-PCR analyses were as follows (the predicted lengths of amplified products are shown in parentheses): \( ATP8B1 \), 5′-GCTACAGGATGGAGTTCCAG-3′ and 5′-ATTCACAGGC-CAATGTGGGC-3′ (603 bp); \( ABCB11 \), 5′-TGCGCAATGACATTCGCTTC-3′ and 5′-GTGTTCTGACCACCACTCATC-3′ (248 bp); \( FXR \), 5′-CGACAAAGTTGTCACTCAACAC-3′ and 5′-TCAACCGCAGACCCCTTCAG-3′ (290 bp); \( FXR-1 \), 5′-AGACCACATAAAGAAGTG-3′ and 5′-CAGTTAACAAGCCTGTATAC-3′ (648 bp); \( FXR-2 \), 5′-AGACCACATCTTACCCAACT-3′ and 5′-ACAAGCATTACACGAGGAAAATG-3′ (631 bp); \( FXR-3 \), 5′-TCAACCGCAGACCCCTTCAG-3′ and 5′-CAGTTAACAAGCCTGTATAC-3′ (604 bp);

![Fig. 2. Characterization of ATP8B1 antibody and effect of siRNA transfection on ATP8B1 protein levels in HepG2 cells. A: total proteins from HepG2 cells (30 µg) or normal human liver (10 µg) were fractionated by 10% SDS-PAGE and subjected to immunoblotting with a rabbit antibody raised against peptide II of human ATP8B1 (see MATERIALS AND METHODS for details) at a 1:8,000 dilution, or with preimmune serum at a dilution of 1:300. B: subconfluent HepG2 cells were incubated simultaneously with the rabbit anti-ATP8B1 antibody (1:8,000) and a mouse monoclonal anti-multidrug resistance-associated protein 2 (MRP2) antibody (1:100). Anti-ATP8B1 antibody (green) reacts with a protein that colocalizes with the canalicular marker protein MRP2 (red) in a pseudocanicular structure formed between adjacent cells (arrow). Top, left: phase contrast. Blue, DAPI staining. C: Western blot analysis of ATP8B1 in siRNA-transfected cells. Equal amounts of total proteins (10 µg) from aliquots of the same cell cultures used for real-time PCR were resolved in SDS-PAGE and subjected to immunoblot analysis with the antibody raised against human ATP8B1 (dilution 1:8,000) or with an anti-cAMP-responsive element-binding protein (CREB) (dilution 1:4,000), as a loading control. Corrected densitometric quantitation is shown on the right. *P < 0.05.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00787.2008)
FXR-α1, 5’-TCAATTTACGCTCCTAAGGCA-3’ and 5’-AACCATCTCATGAGGCGG-3’ (587 bp); β-actin, 5’-GAGGGCAATACTGGGTCGTC-3’ and 5’-GAAAGTAGTTCGCTGATGCC-3’ (210 bp). Northern blot analysis was performed as previously described (15). Total RNA (30 μg) was size fractionated on a 0.9% agarose denaturing gel and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized with a 32P-labeled cDNA probe of human \textit{ATP8B1} (a 1,021-bp cDNA fragment obtained by using primers corresponding to nucleotides 681–701 and 1681–1702) and then reprobed with a 32P-labeled cDNA probe of human β-actin (obtained with primers used in real-time quantitative PCR).

Both cDNAs were synthesized from 3 μg of human liver Poly (A)+ by reverse transcription coupled to PCR and sequenced to confirm its authenticity. Hybridization signals were detected by a Cyclone Storage Phosphor System (Packard, Meriden, CT) and analyzed by OptiQuant Image Analysis software (Packard).

**Western blot analysis.** Samples from HepG2 cells or human liver were homogenized in ice-cold homogenization buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1% protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentration was determined by the Bradford assay, SDS-PAGE and Western blotting were performed as previously described (15) with the use of 10 μg of total proteins. Antibody against human ATP8B1 was used at a 1:8,000 dilution, anti-FXR and anti-CREB at 1:4,000, and anti-β-actin at 1:1,000. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies from DakoCytomation (Glostrup, Denmark) and Affinity BioReagents (Golden, CO). The signal was developed by the enhanced chemiluminescence method, using the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK).

**EMSA.** Nuclear extracts from HepG2 cells were prepared as described (4), 48 h after transfection with scrambled or \textit{ATP8B1} siRNA. Double-stranded oligonucleotides containing a consensus FXR-responsive element (FXRE, GATCAAGAGGTCATTGACCTT), or its mutant sequence (lowercase) (GATCAAGAcagaATaatgCTTT), with 4-bp 5’ overhangs were labeled with [α-32P]dATP by the Klenow fill-in reaction. EMSAs were carried out in a 20 μl binding reaction containing 5 μg of crude nuclear extracts, 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, 20% glycerol, 2 μg poly(dI-dC)poly(dI-dC), and, when indicated, a 100-fold molar excess of competitor oligonucleotides. The reaction mixtures were incubated on ice for 10 min, and then 10^5 counts per minute of double-stranded oligonucleotides were added. Incubation was continued for an additional 30 min. The DNA-protein complexes were resolved in a 5% nondenaturing acrylamide gel in 1× Tris-borate-EDTA (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA). Gels were vacuum dried and autoradiographed. Densitometric analysis of the shifted complexes was performed using OptiQuant Image software.

![Fig. 3. Knockdown of \textit{ATP8B1} expression leads to downregulation of farnesoid X receptor (FXR). RNA from \textit{ATP8B1} siRNA- or scrambled \textit{ATP8B1} (control) siRNA-transfected cells was assayed for mRNA expression of FXR (A, B). A: conventional RT-PCR analyses carried out with sets of primers specific for each expressed isoform (FXR-α1 and FXR-α2) or common to all isoforms (FXR). B: SYBR Green real-time quantitative RT-PCR. Values were normalized to β-actin mRNA. Data are expressed as means ± SD of 4 independent experiments, each sample being assayed in triplicate. Open bars, control siRNA; filled bars, \textit{ATP8B1} siRNA. C: aliquots from the same cell cultures were used for analysis of FXR protein expression. To assess equivalent loading of the samples, the same blots were reprobed with an antibody against the transcription factor CREB (dilution, 1:4,000). Corrected densitometric quantitation is shown below. D: gel mobility shift assays. Nuclear extracts from untreated HepG2 cells or cells transfected with scrambled (control) or \textit{ATP8B1} siRNA were incubated with a labeled FXR responsive element (FXRE) oligonucleotide as described under MATERIALS AND METHODS. Competition was carried out with a 100-fold excess of unlabeled probe (cold FXRE). Specificity of the binding was also verified by incubating nuclear extracts with a labeled FXRE mutant probe (MtFXRE). DNA-protein complex is indicated by an arrowhead. *P < 0.05.
Immunoprecipitation and fluorescence detection of phosphoprotein. Extracts of total proteins from HepG2 cells transfected with scrambled ATP8B1 siRNA or ATP8B1 siRNA (50 μg and 100 μg, respectively) were incubated overnight at 4°C in the presence of 1.4 μg of anti-FXR antibody. Protein G agarose beads were then added, and incubation was continued for an additional 7 h. The beads were washed four times with cold lysis buffer with protease and phosphatase inhibitors, resuspended in Laemmli, and heated for 5 min at 95°C. The Immunocomplexes were fractionated by SDS-PAGE. The resulting gel was fixed in 50% methanol, 10% acetic acid overnight, thoroughly rinsed with deionized water, and incubated in Pro-Q Diamond phosphospecific fluorescent dye (Molecular Probes, Eugene, OR) for 90 min. Destaining was accomplished with three successive washes of 20% acetonitrile in 50 mM sodium acetate, pH 4.0. Following image acquisition, the gel was rinsed and stained with SYPRO Ruby protein dye (Molecular Probes) to determine total protein content. Images were acquired on an Etan DIGE Image (GE Healthcare, Barcelona, Spain) with 540 nm excitation and 595 nm bandpass emission filter for Pro-Q Diamond dye detection and with 480 nm excitation and 595 nm bandpass emission filter for SYPRO Ruby dye detection. Quantitation was carried out with ImageQuant 5.2 software (GE Healthcare). Immunoprecipitated proteins were also analyzed by Western blotting, as detailed in Western blot analysis.

Immunofluorescence. HepG2 cells were cultured in a chamber slide until ~80% confluence, fixed with 4% paraformaldehyde, and incubated simultaneously with the antibody raised against peptide II of human ATP8B1 (1:8,000) and a mouse anti-MRP2 antibody (1:100) for 2 h at 4°C. Cells were further incubated with both AlexaFluor 488-conjugated goat anti-rabbit and AlexaFluor 594-conjugated goat anti-mouse immunoglobulins (Molecular Probes), mounted in Vectashield/DAPI, and analyzed by fluorescence microscopy.

Statistical analysis. Differences between specific groups were determined using the unpaired Student’s t-test and the unpaired Mann-Whitney test. A P value <0.05 was considered significant.

RESULTS

Knockdown of ATP8B1 expression leads to downregulation of FXR in HepG2 cells. We initially tested the ability of two specific siRNA duplexes to reduce the endogenous levels of ATP8B1 mRNA in the polarized human hepatoblastoma cell line HepG2. Of note, the degree of expression of ATP8B1 in these cells, relative to that of β-actin, was found to be threefold lower than in normal human liver, as determined by real-time quantitative PCR (Ct averages for β-actin and ATP8B1 in human liver, 19.092 and 27.968, respectively; Ct averages for β-actin and ATP8B1 in HepG2, 19.440 and 29.863, respectively; n = 3). HepG2 cells were transfected with 1 nM each of either ATP8B1 siRNA or their scrambled versions, and ATP8B1 mRNA content assessed by real-time quantitative PCR. The siRNA2 was found to be more efficient in reducing the expression of ATP8B1 mRNA, leading to a 60% decrease 48 h after transfection (Fig. 1A). This effect was confirmed by Northern blot analysis, which showed an approximately twofold decrease in endogenous ATP8B1 mRNA levels following ATP8B1 siRNA treatment (Fig. 1B). No significant differences in ATP8B1 expression were detected between untransfected cells and cells transfected with scrambled siRNAs (Fig. 1, A and B). Transfections with concentrations of siRNA from 0.1 to 5 nM yielded similar reductions in ATP8B1 mRNA, but decreasing (0.025 and 0.05 nM) or elevating the concentration (10, 20, and 50 nM) diminished the inhibitory effect (data not shown). A weaker reduction in ATP8B1 mRNA levels was also obtained when cells were transfected for 16 or 24 h with 0.1 or 5 nM of siRNAs. For subsequent experiments, cells were transfected for 48 h with 1 nM of siRNA2.

In parallel assays, transfection efficiency was evaluated by using a fluorescein-labeled siRNA2 (Fig. 1C). On average, it was estimated to be higher than 80%. Thus the silencing effect of ATP8B1 siRNA did not fully correlate with its cellular uptake.

To evaluate whether the decrease in ATP8B1 mRNA levels was accompanied by relative changes in the amount of the corresponding protein, extracts of total proteins derived from aliquots of the cell cultures used for real-time PCR analysis were subjected to immunoblotting with the use of an antibody raised against human ATP8B1 (Fig. 2, A and B). As shown in Fig. 2C, a significant decrease in immunoreactive protein, similar to that for ATP8B1 mRNA, was detected in ATP8B1 siRNA-transfected cells (P < 0.05).

We next examined the effect of ATP8B1 inhibition on FXR mRNA expression by conventional RT-PCR and real-time quantitative PCR (Fig. 3, A and B). These analyses were carried out with sets of primers specific for each of the four human FXR isoforms, FXR-α1, FXR-α2, FXR-α3, and FXR-α4 (reviewed in Ref. 21), and with primers corresponding to sequences common to all isoforms. The expression of FXR transcripts, relative to that of β-actin, was also found to be lower (4.5-fold) in HepG2 cells than in normal human liver, as determined by real-time quantitative PCR using the set of primers common to all FXR isoforms (Ct averages for β-actin
and FXR in human liver, 19.092 and 22.543, respectively; Ct averages for β-actin and FXR in HepG2, 19.440 and 24.998, respectively; n = 3). Only FXR-α1 and FXR-α2, whose encoded products differ in four amino acid residues in the hinge domain, were found to be expressed in HepG2 cells (Fig. 3A); FXR-α3 and FXR-α4 mRNAs were undetectable. The expression of FXR-α2 was higher than that of FXR-α1, the ratio FXR-α1:FXR-α2 being ~1:3 (data not shown). SYBR Green real-time quantitative RT-PCR assays performed with the set of primers common to all FXR isoforms showed that the levels of FXR transcripts were significantly reduced in ATP8B1 siRNA-transfected cells (60% compared with scrambled ATP8B1 siRNA-transfected cells; P < 0.05) (Fig. 3B). When specific primers for FXR-α1 and FXR-α2 were used, the relative mRNA levels of each isoform were found to be reduced to a similar extent in ATP8B1 siRNA-transfected cells.

The content of FXR protein was then examined using an antibody raised against the COOH terminus of human FXR, which should recognize all FXR isoforms. As shown in Fig. 3C, the levels of FXR protein were decreased by 60% in ATP8B1 siRNA-transfected cells. To assess whether this effect correlated with a reduction in functional FXR, we performed electrophoretic mobility shift assays with a consensus FXRE probe. The radiolabeled FXRE probe formed a major DNA-protein complex with nuclear extracts from HepG2 cells (Fig. 3D). The specificity of DNA binding was established by the ability of an excess of the same unlabeled probe to compete for protein binding and by the fact that nuclear extracts incubated with a labeled FXRE mutant sequence failed to form this complex. When nuclear extracts from cells transfected with ATP8B1 siRNA were used, a twofold reduction in FXRE binding was observed compared with extracts from scrambled ATP8B1 siRNA-transfected cells. Thus ATP8B1 knockdown also results in decreased levels of functional FXR protein in nuclei.

The phosphorylation status of FXR was also assessed by using Pro-Q Diamond phosphoprotein-selective dye, which detects phosphorylated proteins in polyacrylamide gels (30).
FXR was immunoprecipitated from HepG2 cells transfected with scrambled ATP8B1 siRNA or ATP8B1 siRNA and immunocomplexes resolved on SDS-PAGE. The gel was sequentially incubated with Pro-Q Diamond dye and SYPRO Ruby to estimate FXR phosphorylation and total protein content, respectively. The amount of immunoprecipitated FXR protein was also analyzed by Western blotting in a parallel gel. Both Western blotting and SYPRO Ruby staining revealed that similar levels of FXR were immunoprecipitated (Fig. 4A). The ratio of the phosphate signal to total protein signal indicated...
that the phosphate content of FXR was reduced by 40% in ATP8B1 siRNA-transfected cells compared with scrambled-transfected controls (Fig. 4B).

**SiRNA-mediated silencing of ABCB11 does not affect FXR expression.** Reduced hepatic expression of FXR has been reported not only in children with ATP8B1 deficiency (2, 11, 26) but also in a patient with severe deficiency of the canalicular bile salt export pump (11), another form of PFIC that results from mutations in ABCB11 (33, 34). To determine whether FXR expression is also influenced by ABCB11 inhibition, siRNA-mediated silencing of ABCB11 was achieved. Although HepG2 cells express low levels of ABCB11 mRNA (29, 40), under conditions described in MATERIALS AND METHODS, they could be readily detected by conventional RT-PCR analysis in cells transfected either with an ABCB11 siRNA duplex or with its scrambled version (Fig. 5A). Real-time quantitative RT-PCR determinations showed that ABCB11 mRNA levels were decreased by more than 50% 48 h after transfection with ABCB11 siRNA (Fig. 5B). Aliquots from the same cell cultures were then assayed for FXR expression. As shown in Fig. 5, A–C, siRNA-mediated reduction of ABCB11 expression did not result in any significant changes in FXR mRNA or protein levels, indicating that ABCB11 knockdown does not directly influence FXR expression in HepG2 cells.

**Specificity of the effect of ATP8B1 inhibition.** To evaluate the specificity and impact of ATP8B1 inhibition, the expression of a number of genes encoding other nuclear receptors and liver-enriched transcription factors (PXR/NR1I2, CAR/NR1H3, SHP/NR0B2, HNF-1a/TCF1, HNF-4a/NR2A1, VDR/NR1I1, FXR/NR2B1), canalicular and basolateral transporters (MDR3/ABCB4, MRP2/ABCC2, MRP3/ABCC3, MRP4/ABCC4, ABCG5, ABCG8, OST-α, OST-β, OATP1B1/SLCO1B1, OATP1B3/SLCO1B3, NTCP/SLC10A1), and products involved in bile acid synthesis and metabolism (CYP7A1, CYP8B1, CYP27A1, BAAT, SULT2A1, UGT2B4, FGF19) was analyzed simultaneously by using TaqMan Low-Density Arrays, following transfection with either ATP8B1 or ABCB11 siRNAs. The relative mRNA levels of ATP8B1 and FXR were also examined in this assay and, as controls, those of ATP8B2 (a close homologue of ATP8B1) and ATP8B3 (whose expression is restricted to the testis) (16). A comparison of the expression degree of all these genes in HepG2 cells is shown in Fig. 6A.

**Fig. 7. Ligand-induced upregulation of FXR.** HepG2 cells were transfected with siRNA duplexes targeting ATP8B1 or ABCB11 or with the respective scrambled versions (control) and treated with 1 μM GW4064 or vehicle (dimethyl sulfoxide). After 48 h, cells were harvested and total proteins isolated for immunoblot analysis. A: representative bands of FXR, PXR, and CREB in lysates of ATP8B1 siRNA-transfected cells. B: representative bands of FXR and CREB in lysates of ABCB11 siRNA-transfected cells. Densitometric analyses of immunoreactive FXR protein are shown in bottom panels. Statistical significance is as follows: *P < 0.05, ATP8B1 siRNA-transfected cells vs. control siRNA-transfected cells; #P < 0.05, GW4064-treated cells vs. vehicle-treated cells.
No signal was detected for ATP8B3, SLC01B1, SLC10A1, vitamin D receptor (VDR), and ABCB11. The latter was analyzed separately by SYBR Green real-time quantitative RT-PCR as already detailed. We did not consider the determinations for BAAT and CYP7A1 because variability among control samples was too high to draw firm conclusions (Fig. 6A).

Quantification of ATP8B1 and FXR mRNA levels by this approach was largely consistent with determinations carried out by SYBR Green real-time RT-PCR, showing a decrease of about 60% upon transfection of cells with ATP8B1 siRNA (data not shown). Following ATP8B1 inhibition, no significant changes in the expression of various nuclear receptors and liver-enriched transcription factors were observed. Only a twofold decrease in mRNA levels of short heterodimer partner (SHP) (a primary FXR target) was detected (Fig. 6B). The expression of a majority of genes involved in bile acid synthesis, metabolism, and transport also remained unchanged, but mRNA levels of ABCB11 and UGT2B4, two other targets of FXR, were reduced by 50% in cells transfected with ATP8B1 siRNA (Fig. 6C). Results of ABCB11 mRNA expression are consistent with the previous finding that knockdown of ATP8B1 expression in HepG2 cells leads to diminished human ABCB11 promoter activity (9). The decrease in FXR expression did not affect other known FXR targets, such as ABCB4, ABCC2, SULT2A1, OST-α, or OST-β. This suggests that FXR is not involved in basal expression of these genes in HepG2 cells, which is in agreement with data reported for Fxr-null mice (20, 31, 41). Interestingly, ATP8B2 mRNA content was increased in ATP8B1 siRNA-transfected cells (Fig. 6C), probably reflecting a compensatory mechanism. On the other hand, and consistent with data on FXR expression shown in Fig. 5, no changes in mRNA levels of SHP or UGT2B4 were detected in cells transfected with ABCB11 siRNA (Fig. 6, B and C).

Effect of the FXR-specific agonist GW4064. The occurrence of a ligand-mediated autoregulation of FXR has been evidenced (7). Particularly, in HepG2 cells, FXR protein expression can be effectively induced upon treatment with either natural or synthetic FXR agonists (10, 22). Therefore, we also examined whether a selective and strong agonist of FXR, GW4064 (24), could counteract ATP8B1 siRNA-mediated downregulation of FXR and FXR target genes. HepG2 cells were transfected with scrambled or ATP8B1 siRNA and then treated with either 1 μM GW4064 or vehicle (dimethyl sulfoxide). After 48 h, FXR expression was assessed by Western blotting. As expected, ATP8B1 inhibition resulted in decreased levels of FXR protein in vehicle-treated cells (Fig. 7A). In the presence of GW4064, FXR protein was upregulated in both control siRNA- and ATP8B1 siRNA-transfected cells, whereas the levels of PXR, another bile acid receptor, remained unchanged. In cells transfected with ATP8B1 siRNA, the GW4064-induced increase in FXR was slightly but significantly attenuated (P < 0.05). Nevertheless, the amount of FXR protein in these cells was still twofold and fourfold higher than in vehicle-treated cells transfected with control siRNA and ATP8B1 siRNA, respectively. Of note, ABCB11 inhibition did not influence agonist-mediated induction of FXR (Fig. 7B).

The effect of GW4064 on the expression of FXR target genes was then assessed in RNA samples derived from aliquots of ATP8B1 siRNA-transfected cells by using TaqMan Low-Density Arrays. Consistent with upregulation of FXR, a concomitant increase in mRNA levels of various known FXR targets was detected in scrambled ATP8B1 siRNA-transfected cells when treated with GW4064 (Fig. 8). Interestingly, the ligand-induced expression of FXR target genes was not abrogated in cells transfected with ATP8B1 siRNA. On the contrary, in the presence of GW4064, these cells elicited a gene induction profile that was identical to that exhibited by control siRNA-transfected cells (Fig. 8). Thus, although agonist-mediated induction of FXR protein was lower upon ATP8B1 inhibition, a similar FXR transcriptional activity was achieved.

DISCUSSION

Bile acid homeostasis is maintained through a complex network of genes encoding enzymes involved in bile acid synthesis and metabolism as well as transporters that drive enterohepatic circulation. Most of these genes are subjected to the transcriptional control of an interacting array of nuclear receptors and liver-enriched transcription factors (7, 18, 21). FXR appears to be the most relevant bile acid-activated nuclear receptor, and its deregulation may compromise normal bile formation (14, 31). The present study shows that ATP8B1 knockdown decreases the steady state levels of FXR in HepG2 cells, without affecting the expression of other nuclear receptors or liver-enriched transcription factors that participate in bile acid handling, such as PXR, CAR, hepatocyte nuclear factor (HNF)-1α or HNF-4α. Also, it does not directly influence the expression of various key genes involved in the synthesis, detoxification, or basolateral and canalicular transport of bile salts. Only a subset of FXR targets, such as...
ABC2B1, SHP, and UGT2B4, displays decreased mRNA levels upon ATP8B1 inhibition. This can be attributed to the concomitant downregulation of FXR. A direct action of ATP8B1 on the expression of these genes seems unlikely, since the corresponding mRNA levels are not affected by ATP8B1 inhibition in the presence of the FXR agonist GW4064. We conclude that ATP8B1 knockdown specifically targets FXR.

Recent studies have revealed that ATP8B1 signals FXR via protein kinase C-ζ and have underscored the role of FXR phosphorylation in mediating ATP8B1 effect. Thus ATP8B1 overexpression has been shown to lead to an increase in phosphorylation, nuclear translocation, and transcriptional activity of FXR (13). In keeping with this, we have observed that ATP8B1 knockdown results in decreased FXR phosphorylation levels.

In contrast with the present findings, it has been reported very recently that FXR expression or function is not influenced by ATP8B1 knockdown in cultured human hepatocytes (8). This raises the possibility that a relationship between ATP8B1 deficiency and FXR downregulation takes place in dedifferentiated human hepatic cells, such as HepG2, but not in hepatocytes. The absence of an ATP8B1-FXR interplay in normal human liver cells would support the notion that decreased hepatic expression of FXR seen in tissue samples from children with mutations in ATP8B1 and affected with PFIC is not a primary event of ATP8B1 absence, but rather a secondary consequence of cholestasis (8, 11). The extent to which FXR downregulation contributes to the severe cholestasis of ATP8B1 disease remains to be elucidated.

The present results also indicate that potent FXR agonists can neutralize downregulation of FXR produced by ATP8B1 knockdown in HepG2 cells. In the presence of GW4064, upregulation of FXR protein was achieved either in cells transfected with control siRNA or with ATP8B1 siRNA. Although FXR protein levels were increased to a lesser extent following ATP8B1 inhibition, the GW4064-mediated induction of eight target genes was identical. This finding suggests that there is a threshold of FXR concentration above which it displays a similar transcriptional activity. It might also harbor potential therapeutic implications. FXR agonists are increasingly recognized as promising tools for the treatment of cholestatic disorders (5, 7, 25, 35) other than obstructive cholestasis (8, 11). The extent to which FXR protein levels were increased to a lesser extent though FXR protein levels were increased to a lesser extent following ATP8B1 knockdown in HepG2 cells. In the presence of GW4064, upregulation of FXR protein was achieved either in cells transfected with control siRNA or with ATP8B1 siRNA. Although FXR protein levels were increased to a lesser extent following ATP8B1 inhibition, the GW4064-mediated induction of eight target genes was identical. This finding suggests that there is a threshold of FXR concentration above which it displays a similar transcriptional activity. It might also harbor potential therapeutic implications. FXR agonists are increasingly recognized as promising tools for the treatment of cholestatic disorders (5, 7, 25, 35) other than obstructive cholestasis (8, 11). The extent to which FXR protein levels were increased to a lesser extent following ATP8B1 knockdown in HepG2 cells. In the presence of GW4064, upregulation of FXR protein was achieved either in cells transfected with control siRNA or with ATP8B1 siRNA. Although FXR protein levels were increased to a lesser extent following ATP8B1 inhibition, the GW4064-mediated induction of eight target genes was identical. This finding suggests that there is a threshold of FXR concentration above which it displays a similar transcriptional activity. It might also harbor potential therapeutic implications. FXR agonists are increasingly recognized as promising tools for the treatment of cholestasis (8, 11). The extent to which FXR expression and activity are maintained. Gastroenterology 136: 1060–1069, 2009.


