Disrupted Bile Acid Homeostasis Reveals an Unexpected Interaction among Nuclear Hormone Receptors, Transporters, and Cytochrome P450*

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Erin G. Schuetz‡, Stephen Strom§, Kazuto Yasuda‡, Valerie Lecureur‡, Mahfoud Assem‡, Cynthia Brimer‡, Jatinder Lamba‡, Richard B. Kim¶, Vinod Ramachandran‡, Bernard J. Komoroski‡, Raman Venkataramanan‡, Hongbo Cai‡, Christopher J. Sinaí**, Frank J. Gonzalez**, and John D. Schuetz‡ ‡‡

From the ‡Department of Pharmaceutical Sciences, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105, the Departments of §Pathology and ¶Pharmaceutical Sciences, the University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the ‡Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37203, and the **Laboratory of Medicine, National Institutes of Health, Bethesda, Maryland 20892

Sister of P-glycoprotein (SPGP) is the major hepatic bile salt export pump (BSEP). BSEP/SPGP expression varies dramatically among human livers. The potency and hierarchy of bile acids as ligands for the farnesyl/bile acid receptor (FXR/BAR) paralleled their ability to induce BSEP in human hepatocyte cultures. FXR:RXR heterodimers bound to IR1 elements and enhanced bile acid transcriptional activation of the mouse and human BSEP/SPGP promoters. In FXR/BAR nullizygous mice, which have dramatically reduced BSEP/SPGP levels, hepatic CYP3A11 and CYP2B10 were strongly but unexpectedly induced. Notably, the rank order of bile acids as CYP3A4 inducers and activators of pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) closely paralleled each other but was markedly different from their hierarchy and potency as inducers of BSEP in human hepatocytes. Moreover, the hepatoprotective bile acid ursodeoxycholic acid, which reverses hydrophobic bile acid hepatotoxicity, activates PXR and efficaciously induces CYP3A4 (a bile-metabolizing enzyme) in primary human hepatocytes thus providing one mechanism for its hepatoprotection. Because serum and urinary bile acids increased in FXR/BAR −/− mice, we evaluated hepatic transporters for compensatory changes that might circumvent the profound decrease in BSEP/SPGP. We found weak MRP3 up-regulation. In contrast, MRP4 was substantially increased in the FXR/BAR nullizygous mice and was further elevated by cholic acid. Thus, enhanced hepatocellular concentrations of bile acids, due to the down-regulation of BSEP/SPGP-mediated efflux in FXR nullizygous mice, result in an alternate but apparent compensatory up-regulation of CYP3A, CYP2B, and some ABC transporters that is consistent with activation of PXR/SXR by bile acids.

Bile acids are synthesized from cholesterol in the liver and secreted into the bile duct via an active process in the canaliculus. Canalicular secretion of bile acids from the liver in the form of bile facilitates the emulsification of dietary lipids and fat-soluble vitamins. Bile acids are found in high concentrations in hepatic nuclei (1), where they regulate gene expression through the farnesol X-activated receptor (FXR)‡ (also known as bile acid receptor (BAR)) (2, 3). Recent studies in mice lacking FXR/BAR indicate that fecal excretion of bile acids is markedly impaired in these mice, but serum concentrations and urinary excretion of bile acids are increased (4). The decreased hepatic bile acid secretion correlated with decreased expression of the major canalicular bile salt transporter, sister of P-glycoprotein (SPGP) (5) also known as the bile salt export pump (BSEP) (6). This finding is consistent with results demonstrating genetic mutations in BSEP that are associated with a severe genetic disease, type 2 progressive familial intrahepatic cholestasis, in which total bile salt secretion decreases to about 1% of normal (7, 8). However, despite the decreased expression of BSEP/SPGP in the absence of FXR, it is unknown if the murine BSEP/SPGP gene is transcriptionally regulated by bile acids. Moreover, it is unknown if any other hepatic-ABC transporters compensate for decreased SPGP, leading to increased serum and urinary bile acids.

In the absence of appropriate bile acid secretion, activation of other compensatory metabolic pathways might occur. This possibility was suggested by the recent studies in the SPGP nullizygous mouse (9). In the absence of SPGP the liver accumulates large amounts of tetra-hydroxylated bile acids. Typically, such poly-hydroxylated bile acids are not seen in the liver, and this suggests an increase in metabolizing enzymes. Notably, one of the major human cytochromes, CYP3A4, is capable of mono-oxygenation of bile acids leading to formation of more hydrophilic bile acids, such as 6β-hydroxylated lithocholic acid.

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†† To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, St. Jude Children’s Research Hospital, 332 N. Lauderdale Ave., Memphis, TN 38105. Tel.: 901-495-2174; Fax: 901-525-6689; E-mail: John.schuetz@stjude.org.

** The abbreviations used are: FXR, farnesol X-activated receptor; ABC, ATP-binding cassette; BAR, bile acid receptor; PXR, pregnane X receptor; SXR, steroid and xenobiotic receptor; IRX, retinoid X receptor; BSEP, bile salt export pump; SPGP, sister of P-glycoprotein; PCR, polymerase chain reaction; NTCP, sodium taurocholate cotransporter protein; bp, base pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (5β-cholan-3α,7α,12α-triol); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); TUDCA, tauroursodeoxycholic; UDCA, ursodeoxycholic acid.
and H11002 acid (10) may protect from bile acid-mediated hepatotoxicity. In reduced allograft transplantation. Donor livers were flushed, and receptor/steroid and xenobiotic receptor (PXR/SXR), was unex-
hormone receptor that regulates CYP3A, the pregnane X re-
(10). Indeed, recent studies demonstrated that one nuclear

in Modified Williams E for 48 h and then treated with bile acids or
by Dr. Barry Forman. Gal4-PM2-SXR and TK-(MH100)4-LUC (tkUAS-
Kliewer) was subcloned into pcDNA3. CMX-FXR was kindly provided

essentially as described by Strom

reasons for not using tissues for transplan-

prepared by Dr. Rommel Tirona as described previously (14).
create mBSEP/SPGP-LUC.
was digested with

the PCR product

AAGCACTGAACAGAATTCAA-3

cloned into pCR2.1-TOPO and the sequence verified. The insert was

BSEP/SPGP or MRP3 (12)) compensatorily increase. Such

interaction included traumatic damage, errors in organ harvest, brief anoxic

Within 24 h of cross-clamp. Reasons for not using tissues for transplan-
'm UW solution. Hepatocytes were isolated

Human livers were procured from donor organs that were not suit-

s UW solution. Hepatocytes were isolated

Preparation of Primary Cultures of Human Hepatocytes

Human livers were procured from donor organs that were not suit-
able for whole organ transplantation or from remaining tissue after
reduced allograft transplantation. Donor livers were flushed, in situ, with
Belgar’s UW solution. Hepatocytes were isolated within 24 h of cross-clamp. Reasons for not using tissues for transplan-
tion included traumatic damage, organs in error harvest, brief anoxic
periods, or macro- or microsteatosis. Human hepatocytes were isolated
essentially as described by Strom et al. (15, 16). Cells were plated on
collagen-coated 6-well plates or 80-mm culture dishes and maintained in
Modified Williams E for 48 h and then treated with bile acids or
drugs for 48 h. The activity of CYP3A4 was determined by measuring the testosterone metabolism in cultured hepatocytes as described by Kostrubsky et al. (17). Cells were then scraped from the plates and proteins analyzed on immunoblots.

For membrane transport proteins, crude membranes were prepared from human liver or mouse as described previously (18). Protein was estimated by the Bio-Rad protein assay using bovine serum albumin as standard. The crude membrane proteins (300 μg) were analyzed on 7.5% polyacrylamide gels followed by immunoblotting with rabbit anti-
SPGP IgG (19) appropriate secondary antibody and developed with the Amersham ECL detection system (Amersham Pharmacia Biotech). The same blot was stripped of antibodies and redeveloped with polyclonal rabbit anti-P-glycoprotein (18). Proteins on the blot were stained with Ponceau Reagent (Sigma), and the SPGP signal was normalized to Ponceau staining signal in 20 region of comparable molecular weight. Mouse liver microsomes were prepared (20), and 10 or 20 μg of protein was analyzed on 10% slab polyacrylamide gels electrophoresed and immunoblotted using the following antibodies: polyclonal goat anti-rat CYP3A1 antibodies (21) or a monoclonal antibody against rat CYP2B1 (mAb BE239.2) from Dr. Paul Thomas (Reuter’s University, Piscataway, NJ). The primary antibodies were followed by secondary antibodies coupled with peroxidase and developed with the ECL detec-
tion system (Amersham Pharmacia Biotech) (22).

Northern Blots
Total RNA was extracted and 10 μg analyzed by Northern blot with probes to CYP3A11 and β-actin as described previously (23).

Reverse Transcription-PCR
5 μg of total RNA from mouse liver was reverse-transcribed according to the manufacturer’s instructions (Life Technologies, Inc.). Transporter cDNAs were amplified from first-strand cDNA by using the following oligonucleotides: MDR1 sense, aac aat gtc ttc gag gat tgg g, and antisense, cat tgc cta gag ccc gaa gata t; MDR1b sense, cag tgt ttc cag taa ttt cca gga ttt g, and antisense, ccc ttc act aga atc ac ac; MDR2 sense, cat ccc cta cgg cgg gaa gga, and antisense, atc ggg cct cta cca cga tgg; MRP 1 (bp 2072–2573 in AF029268) sense, 5’-TTCGTG-
GCTGAGATGGACAAG-3’, and antisense, 5’-CGGTCCTACAGCTCCT-
GATA-3’; MRP 2 (bp 112–446 in AA027420) sense, 5’-GTCTACCTAC-
TATCCGACAACAG-3’, and antisense, 5’-TTCCTACAGGGTTGGTGA-
GA-3’; MRP 3 (bp 42–352 in AI391389) sense, 5’-GGCTCTACTGCAC-
CATG-3’, and antisense, 5’-GGTACTCTGGTCTCCAACTG-3’; MDR3 sense, 5’-CGGATGACTGACTGAC-3’, and antisense, 5’-GGGCCCCCACTGATTC-3’; MRP4 (bp 161–382 in W54702) sense, 5’-GGTTGGAAATGTGGGCAAGA-3’, and antisense, 5’-TTCGTCCGTTGCTCATTGAA-3’. The control GAPDH cDNA was amplified with the following: sense, 5’-aac aca gtc cat cgc atc-3’, and antisense, 5’-tcc acc acc tct tgg tct-3’.

PCR products were amplified 1–5 μl of cDNA, 5 μl of buffer, 3 μl of 25 mM MgCl2, 1 μl of each 10 mM dNTP, 1–2 μl of 10 μM primers, 0.4 μl of high fidelity Taq polymerase, and 50 μl of water. The reactions were initially denatured at 95 °C (5 min) and then cycled in a MJ Research Tetrad (Waltham, MA) at 95 °C for 30 min; 55, 58, or 60 °C for 30 min; and 68 °C for 1 min for 23 cycles (GAPDH) or 35 cycles (ABC transport-
ners) with a final 68 °C extension step performed for 10 min at the end of all the cycles. PCR products were analyzed on 0.5% NuSieve, 0.5% agarose (w/v) containing ethidium bromide for visualization.

Electrophoretic Mobility Shift Assay
Human FXR and RXR-α were each in vitro transcribed and translated using a TNT Kit (Promega, Madison, WI) according to the manu-
facturer’s instructions. Double-stranded, 32P-labeled oligonucleotides representing the human SPGP FXR DNA-binding sequence, containing a potential inverted repeat with 1-base pair spacer (IR-1, underlined) (hereafter referred to as hBSEP) 5’-GGTGCGCTTGGGGAATTGGCAGAC-CTTAGGGAAATAGATAAT-3’ or an oligonucleotide of the mouse BSEP promoter (mBSEP) containing an IR-1 (underlined) 5’-TCTGGAGCTTTGAGGATCTAAAGGAAATAGATAAT-3’; or an oligonucleotide of the mouse BSEP promoter (mBSEP) containing an IR-1 (underlined) 5’-TCTGGAGCTTTGAGGATCTAAAGGAAATAGATAAT-3’; or an oligonucleotide of the mouse BSEP promoter (mBSEP) containing an IR-1 (underlined) 5’-TCTGGAGCTTTGAGGATCTAAAGGAAATAGATAAT-3’. were incubated with 10 nm Tris (pH 8.0), 40 mM KCl, 0.05% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.2 μg of poly(dI-dC), and 2.5 μl of RXR and FXR in vitro transcribed and translated protein (24). Reactions were set up in the absence or presence of 50–, 100-, or 200-fold molar excess unlabeled hBSEP, 200 × excess mBSEP or hBSEP-5 for competition.
were resolved by electrophoresis through a nondenaturing 4% polyacrylamide gel and analyzed on PhosphorImager.

**Transient Transfection Assays**

HepG2 cells were plated in 24-well dishes at 0.3 × 10^6 cells per well. Twenty four hours later cells were transfected with 1000 ng of human or mouse Sister-P-glycoprotein-LUC, 200 ng of mFXR, and 500 ng of β-galactosidase plasmid (pSV-β-galactosidase; Promega Corp., Madison, WI) by calcium phosphate overnight. HepG2-PXR cells stably expressing FXR (created by calcium phosphate co-precipitation of hPXR-pcDNA3 and clonal selection in G418) were co-transfected with 300 ng of CYP3A4-PXRE-LUC and 30 ng of an NTCP expression plasmid. COS-7 cells were plated in 12-well dishes at 0.3 × 10^6 cells per well. Twenty four hours later, cells were transfected with 400 ng of Gal4-PM2-SXR, 600 ng of TK-MH100), LUC (tkUAS-LUC), and 60 ng of NTCP expression plasmid by LipofectAMINE (Life Technologies, Inc.). The next day, all cells were washed once with medium and fresh medium containing 10% charcoal-stripped dilipidated calf serum (Sigma) with or without xenobiotics or bile acids. Twenty four hours later, cells were harvested, lysed, and centrifuged at 10,000×g,

**Animals**

Mice were housed in a pathogen-free animal facility under standard 12 h light/12 h dark cycle. Prior to the administration of special diets, mice were fed standard rodent chow and water ad libitum. All diets were prepared by BioServ (Frenchtown, NJ) and were based upon a standard AIN-93G rodent diet containing 56.6% carbohydrate, 18.1% protein, 7.2% fat, 5.1% fiber, 3.4% ash, 10% moisture (control diet). The cholic acid diet was identical to the control diet but supplemented with 1% (w/w) cholic acid. 8–12-Week-old male mice were used for all experiments and were allowed water ad libitum. After 5 days of feeding the indicated diets, animals were euthanized by carbon dioxide asphyxiation at the mid-light phase period. Tissues were weighed, sexioned, and snap-frozen in liquid nitrogen and stored at −80 °C until use. All protocols and procedures were approved by the NCI Division of Basic Sciences Animal Care and Use Committee and are in accordance with the National Institutes of Health guidelines.

**RESULTS**

**Expression of BSEP in Human Liver and in Primary Human Hepatocytes**—We found over 7-fold variation in BSEP/SPGP expression in normal human liver (Fig. 1a). Because some of the normal human liver samples were from donors who received drugs that can induce drug detoxification genes due to activation of PXR/SXR and CAR nuclear hormone receptors, we reasoned that some of the interindividual variation in BSEP/SPGP expression was due to drug induction of this protein. We compared the expression of BSEP/SPGP in primary human hepatocytes treated with prototypical ligands for PXR and CAR receptors (24, 26, 27). However, treatment with rifampin or phenobarbital or troglitazone (Fig. 1, b and c) caused no induction of BSEP expression.

Because the hepatic concentration of bile acids varies among individuals, we tested whether any bile acids affected BSEP expression in primary human hepatocytes. The major human bile acids chenodeoxycholic acid (CDCA); all bile acids are according to accepted nomenclature [47]) and deoxycholic acid (DCA) (1) (Fig. 1, b and c) each induced BSEP/SPGP at 10 μM and further induced it at 50 μM. Although there was interindividual variation in BSEP/SPGP induction between preparations of human hepatocytes, the bile acids consistently induced BSEP/SPGP with an apparent rank order of potency of CDCA > DCA with LCA the least potent (Fig. 1c). The hydrophilic DKCA also modestly induced BSEP/SPGP. This rank order of BSEP/SPGP induction is similar to the potency of these same bile acids as ligands for the FXR receptor and is consistent with a role for FXR in the induction of BSEP/SPGP.

**Bile Acids Transcriptionally Activate the Human and Mouse BSEP Promoters and Require FXR**—To determine whether bile acids utilize FXR to activate transcriptionally the murine and human BSEP promoters, we isolated the murine BSEP and subcloned them into promoterless luciferase (model 3550, Bio-Rad) set at a wavelength of 415 nm. Luciferase activities were normalized to β-galactosidase activity or cellular protein. All experimental values were averaged from triplicate determinations in individual experiments, and the experiment was repeated at least three times.

**Interactions between Nuclear Receptors and Bile Acids**

Because the hepatic concentration of bile acids varies among individuals, we tested whether any bile acids affected BSEP expression in primary human hepatocytes. The major human bile acids chenodeoxycholic acid (CDCA); all bile acids are according to accepted nomenclature [47]) and deoxycholic acid (DCA) (1) (Fig. 1, b and c) each induced BSEP/SPGP at 10 μM and further induced it at 50 μM. Although there was interindividual variation in BSEP/SPGP induction between preparations of human hepatocytes, the bile acids consistently induced BSEP/SPGP with an apparent rank order of potency of CDCA > DCA with LCA the least potent (Fig. 1c). The hydrophilic DKCA also modestly induced BSEP/SPGP. This rank order of BSEP/SPGP induction is similar to the potency of these same bile acids as ligands for the FXR receptor and is consistent with a role for FXR in the induction of BSEP/SPGP.

**Fig. 1. BSEP/SPGP expression in human liver and up-regulation by bile acids in human hepatocyte culture. a**, 300 μg of crude membranes from human livers were analyzed by immunoblot for BSEP/SPGP and P-glycoprotein. The signal for BSEP was normalized to the amount of Ponceau staining. b and c, primary human hepatocytes from representative persons (HH786 and HH607) were cultured for 48 h and then treated from 48 to 96 h with CDCA, DCA, DRCA, and LCA at 10 or 50 μM (50 μM bile acids b, HH786), 10 μM rifampin (RIF) or troglitazone (TGZ), or 2 μM phenobarbital (PB), and lysates were prepared and 100 μg examined on immunoblots developed with anti-BSEP/SPGP IgG. 20 μg of LLC-PK1 cells stably expressing SPGP/BSEP served as a positive control.
vated by rifampin treatment regardless of whether FXR (Fig. 2, b and c) or PXR (not shown) was co-transfected. Electrophoretic mobility shift assay analysis was performed with in vitro translated FXR, RXR, and PXR on segments of both the mouse and human BSEP promoters that contained putative FXR response elements (Fig. 2d). Both human and mouse BSEP promoters contained regions that specifically bound FXR/RXR complexes, and FXR/RXR binding was competed dose-dependently from murine and human BSEP that bound FXR/RXR. PXR/RXR failed to form any complex with either the mouse or human BSEP/SPGP promoter fragments (not shown). In total, these studies indicate that the human and mouse BSEP/SPGP genes are transcriptionally activated by FXR and that their minimal promoters specifically bind FXR.

**BSEP/SPGP Expression Requires FXR in Vivo, whereas CYP3A, CYP2B10, MRP3, and MRP4 Are Induced in the Absence of FXR**—BSEP mRNA decreases in the absence of FXR (4). We compared expression of BSEP protein in normal mice and mice nullizygous for FXR, and in those same mice fed either normal chow or 1% cholic acid, a concentration that increases the amount of total hepatic nuclear bile acids (4) (Fig. 3a). Consistent with the expression of BSEP mRNA, basal levels of BSEP were dramatically reduced in the absence of FXR, and dietary cholic acid increased BSEP expression only in wild type mice (Fig. 3a).

Mice lacking BSEP/SPGP (9, 29) showed elevated concentrations of hydroxylated bile acids in the liver and serum suggesting compensatory changes in expression of bile acid-metabolizing enzymes and alternative transporters. Moreover, a recent report (11) found that although treatment with lithocholic acid induced CYP3A in mouse liver, this up-regulation occurred even in mice lacking the pregnane X receptor (PXR), demonstrating that some other receptor, such as FXR, must also

![Figure 2](image2.png)

**Fig. 2.** The human and mouse BSEP/SPGP promoters and their regulation by FXR/BAR. a, alignment of mouse and human BSEP/SPGP 5'-untranslated regions and promoter regions cloned into luciferase reporter constructs. b and c, HepG2 cells were co-transfected with SV40-β-galactosidase. The human (hBSEP/SPGP) promoter-luciferase reporter was co-transfected with the FXR expression plasmid (open bars) or not (closed bars) and treated with the indicated bile acids or rifampicin (RIF). (b) The mouse BSEP/SPGP promoter-luciferase promoter with or without the FXR expression plasmid were untreated controls (CT) or were treated with 20 μM rifampin (RIF) or 50 μM bile acids as indicated (c). Luciferase activities were normalized to β-galactosidase activity, and the fold increases in treated groups over untreated controls are shown. Values represent the mean ± S.D. from triplicate determinations of a single experiment that was performed at least three times. d, electrophoretic mobility shift assay for FXR-RXR-BSEP promoter complexes. 32P-Labeled oligonucleotides containing the conserved FXR binding sequence in mBSEP or hBSEP were incubated with in vitro transcribed and translated FXR and RXR. Reactions were incubated in the absence (no competitor) or presence of 50–200-fold molar excess of the indicated unlabeled oligonucleotides and electrophoresed and complex formation was assessed by a PhosphorImager.

![Figure 3](image3.png)

**Fig. 3.** Absence of FXR profoundly decreases BSEP/SPGP but induces CYPs and other ABC transporters. Livers of FXR (+/+) or (−/−) mice (half of each genotype ate a normal diet and half ate a diet containing 1% cholic) were analyzed. a, crude liver membranes or microsomes were analyzed on immunoblots developed with antibody to BSEP/SPGP, CYP3A, or CYP2B. b, Northern blot analysis of CYP3A11 and β-actin in total liver RNA. c, total liver RNA was analyzed by reverse transcription-PCR analysis using primers specific for the indicated mouse ABC transporters or GAPDH.
mediate CYP3A induction by bile acids. However, FXR is clearly not a positive regulator of CYP3A expression because CYP3A11 protein and mRNA were dramatically increased in the absence of FXR (Fig. 3, a and b). Concurrent analysis of CYP2B10 protein revealed a pattern of regulation identical to CYP3A11. These studies indicate that a strong decrease in BSEP/SPGP in the absence of FXR (Fig. 3, a and b) enhanced secretion of hydroxylated bile salts into bile. These findings suggest that in the absence of BSEP compensatory induction of hydroxylation is a mechanism that potentially involved induction of CYP3A4. Only UDCA and TUDCA were effective inducers of CYP3A4 protein in primary human hepatocytes. To confirm that each of the bile acids inducing CYP3A4 was a PXR/SXR ligand, we compared activation of a CYP3A4 PXRE-LUC reporter in HepG2 cells with and without stable expression of PXR. All of the cells were co-transfected with an NTCP expression plasmid to permit uptake of the conjugated bile acids. Robust transcriptional activation of PXRE-LUC by rifampin and dose-dependent induction by bile acids was observed in HepG2-PXR cells (Fig. 5) but not in cells not co-transfected with PXR (not shown). To determine if UDCA and the taurine-conjugated bile acids directly activated PXR, we utilized a chimeric receptor system employing a ligand of FXR (Fig. 1). Whereas CDCA was the most efficacious inducer of BSEP, UDCA and DKCA were the most effective bile acids inducing CYP3A4 protein (Fig. 4A) and associated testosterone 6β-hydroxylase activity (Fig. 4B). Although two recent reports in CV1 cells (10, 11) and our own studies in COS-7 cells (Fig. 5) found that 100 μM lithocholic acid, CDCA, and DCA could activate PXR/SXR and transcriptionally activate the CYP3A4 promoter, this concentration of bile acids was visibly toxic to the primary human hepatocytes. Total hepatic protein was decreased 50, 23, and 12% by 100 μM LCA, UDCA, and CDCA, respectively, and 38% by 50 μM LCA. In hepatocyte preparations from two other persons, only 50 μM DKCA (as directly compared with 50 μM LCA, DCA, or CDCA) induced CYP3A4 protein expression and associated testosterone 6β-hydroxylase activity (2.5- and 3.4-fold). Of the bile acids tested, only 100 μM LCA and tauroursoxycholic acid failed to induce testosterone 6β-hydroxylase activity (Fig. 4B). We also tested UDCA, TUDCA, TLCA, and TLDCA, because these more hydrophilic bile acids have been shown to decrease the toxicity of more hydrophobic bile acids in humans or rodents, a protective mechanism that potentially involved induction of CYP3A4. Only UDCA and TUDCA were effective inducers of CYP3A4 protein in primary human hepatocytes.

FIG. 4. Bile acids induce CYP3A in primary human hepatocytes. A, primary hepatocytes were cultured for 48 h and then treated with the indicated μM concentrations of bile acids, 10 μM rifampin, or were untreated controls (CT). 100 or 50 μg of total cell lysate was analyzed on immunoblots with antibodies to BSEP/SPGP and CYP3A (18), respectively. B, immediately before harvest, the hepatocytes were incubated with testosterone and the medium later analyzed for the formation of 6β-hydroxytestosterone.
50 and 100 μM LCA and 100 μM DCA and CDCA caused a decrease of 25–60% in total cellular protein in COS-7 cells but not HepG2 cells, resulting in a significant decrease in GAL-SXR activation in COS-7 cells and likely accounting for the discrepancy in their ability to activate SXR in the two assays. The data also shows that in the presence of NTCP, the more physiologically relevant taurine-conjugated bile acids can also activate PXR.

**DISCUSSION**

These studies were initiated by our observation that human liver BSEP/SPGP is subject to considerable interindividual variability. We reasoned that differences in hepatic BSEP/SPGP might be attributed, in part, to induction of BSEP/SPGP by co-administered drugs or variable activation of FXR/BAR by endogenous ligands such as bile acids. The current studies in human hepatocytes demonstrate that representative PXR/SXR and CAR ligands (rifampin and phenobarbital) did not increase BSEP/SPGP expression or activate its promoter. Thus, our studies indicate that the enhanced bile acid elimination in patients treated with rifampin (13) is unlikely due to transcriptional up-regulation of BSEP/SPGP. Nonetheless, we show that FXR/BAR transcriptionally activates both murine and human BSEP/SPGP promoters. Moreover, bile acids increased BSEP/SPGP expression in primary human hepatocytes with the potency being CDCA > DCA > LCA, a finding closely agreeing with their ability to activate the nuclear farnesyl/bile acid receptor, FXR/BAR (2, 3). FXR and RXR heterodimers bind to specific DNA motifs (e.g. consensus = AGGTCA (3)) arranged as an inverted repeat with a single nucleotide spacer (IR1). Such an element exists in a conserved region of both mouse and human BSEP/SPGP promoters (Fig. 2), and these DNA motifs specifically bound authentic FXR/RXR heterodimers and were activated in transient transfections by the same bile acids that increased expression of the endogenous BSEP/SPGP in human hepatocytes. It appears that coordinated induction of BSEP/SPGP is part of a pleiotropic response to regulate bile acids because hepatic canicular bile output decreases in the absence of FXR as does expression of BSEP/SPGP (4), and because BSEP/SPGP is positively up-regulated by the major bile acids. Therefore, interindividual differences in the expression of hepatic BSEP/SPGP could arise from varying levels of bile acid plus as well as functional differences in either BSEP/SPGP or FXR, perhaps due to mutations of other alterations. Hepatic variation in BSEP/SPGP expression could contribute to individual differences in bile flow, levels of bile acids, hepatic clearance of drugs (31), and also to gallstone susceptibility. Indeed, hepatic BSEP/SPGP expression is dramatically elevated in the mouse strain C57L, a strain highly susceptible to gallstones after ingesting a high fat diet, but BSEP expression is much lower in AHR-resistant mice that do not develop gallstones (32). Therefore, we imagine that high SPGP/BSEP expression could increase gallstone susceptibility in humans.

Our studies further demonstrate an intriguing relationship between the absence of the nuclear receptor, FXR, loss of SPGP expression, and up-regulation of CYP3A, CYP2B, and ABC transporters such as MRP3 and MRP4. These observations suggest the following sequelae: absence of FXR leads to strong SPGP/BSEP down-regulation that causes elevated hepatocellular bile acids, particularly SPGP/BSEP substrates (e.g. CDCA and DCA (9)), and these bile acids then bind PXR (this work and Ref. 10) and up-regulate CYP3A and CYP2B. This scenario is consistent with CYPs likely participating in the enhanced formation of tetrahydroxylated bile acids in mice lacking BSEP/SPGP (9). Notably, several bile acids, but particularly the more hydrophilic bile acids such as DKCA and UDCA, significantly up-regulated in primary human hepatocytes CYP3A4 protein and catalytic activity (testosterone 6β-hydroxylase). Furthermore, we provide evidence that these bile acids were potent ligands for SXR/PXR and provide a mechanistic basis for their induction of CYP3A4. In contrast, we unequivocally demonstrate that FXR does not mediate CYP3A induction by bile acids that seemed a formal possibility based upon a previous report (10). This finding coupled with a previous report demonstrating that lithocholic acid induces CYP3A in FXR nullizygous mice, and our finding of the parallel induction of CYP2B in FXR nullizygous mice, suggest that either CAR or another nuclear hormone receptor mediates this response.

Our results reveal that expression of ABC transporters compensatorily increase in FXR nullizygous mice. Although MRPI transports bile acids (12), MRP4 has not been linked to bile acid transport and has only been demonstrated to transport nucleotide monophosphate derivatives (33). Thus, it is a formal possibility that MRP4 plays a role in hepatic bile acid homeo-

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2 E. G. Schuetz, S. Strom, K. Yasuda, V. Lecureur, M. Assem, C. Brimer, J. Lamba, R. B. Kim, V. Ramachandran, B. J. Komoroski, R. Venkataramanan, H. Cai, C. J. Sinal, F. J. Gonzalez, and J. D. Schuetz, unpublished data.
stasis. The induction of these ABC transporters in the absence of FXR and a further increase after cholic acid feeding indicate regulation independent of FXR but nonetheless dependent upon hepatic bile acid accumulation. Such compensatory increases in ABC transporters are not without precedent as the absence of MRP2 due to a genetic deficiency leads to a compensatory increase in the expression of MRP1 to facilitate hepatic removal of anionic compounds by transport into the sinusoidal blood. Notably, MRP3 up-regulation by bile acids is also consistent with the recent report showing a 30-fold increase in hepatic MRP3 after ligation of the common bile duct, a model of cholestasis (34, 35). Finally, the pattern of MRP3 and MRP4 up-regulation in the FXR-null animals is remarkably similar to CYP3A and CYP2B and suggests a common regulator, such as PXR, in these animals.

Because rifampin can activate PXR/SXR to induce CYP3A which metabolizes lithocholic acid (10, 11), and because rifampin has been shown to induce remission of cholestasis in some patients (13), rifampin therapy has been proposed as a treatment for cholestatic liver disease (10, 11). Intriguingly, rifampin increases bile salt concentrations in serum (36) suggesting rifampin-activated PXR up-regulates alternative ABC transporters (perhaps such as MRP4 or MRP3) that contribute to the reported decrease in bile acid toxicity. However, it should also be noted that rifampin has been reported to decrease biliary secretion of bile acids (36), and high doses of rifampin have also been shown to induce reversible cholestasis in some patients (37), presumably mediated through inhibition of BSEP/SPGP (36) and canalicular secretion of bile acids. Therefore, alternative PXR agonists that do not inhibit BSEP/SPGP may be preferable to rifampin for treatment of human cholestasis.

Although a good correspondence exists between bile acids as inducers of either BSEP or CYP3A4 proteins in primary human hepatocytes and activation of the BSEP promoter and PXR, respectively, in HepG2 cells, some discrepancies exist. For instance, LCA is an efficacious PXR and Fxr activator in HepG2 and CV1 cells. In contrast, in human hepatocytes, LCA less effectively increases SPGP/BSEP and CYP3A4, a finding suggesting hepatic bile acid metabolism (e.g. CYP3A-mediated (10)) plays a role in the amount of ligand available for FXR and PXR in the liver. Discrepancies between LCA activation of FXR and PXR in CV1 cells and induction of target genes in human hepatocytes may also due to the much greater toxicity of LCA at 50 and 100 μM to primary hepatocytes. Another possibility is that LCA is differentially conjugated (e.g. sulfation) in primary cells compared with replicating cell lines, and this conjugation alters its efficacy as a nuclear receptor ligand.

Paradoxically, some bile acids can decrease liver toxicity. Leuschner et al. (38) found that ursodeoxycholic acid (UDCA) administration reversed hepatotoxicity, and UDCA treatment is used to ameliorate primary biliary cirrhosis (39, 40). Similarly, chenodeoxycholic acid- or deoxycholic acid-induced rodent liver hepatotoxicity can be reversed by co-administration of less detergent bile acids such as UDCA or tauurosodesoxycholic acid (TUDCA) (41). How does administration of one bile acid reverse the toxicity of another in rodents or humans? Whereas a number of mechanisms have been proposed (39, 42), one possibility was that hydroxylated bile acids such as UDCA (as opposed to the major hydrophobic bile acids) preferentially activated alternative bile acid signaling pathways. UDCA does not activate FXR (28), which is consistent with its poor induction of BSEP/SPGP in primary human hepatocytes. In contrast, UDCA activated PXR and was one of the most effective bile acids tested in inducing CYP3A4 in human hepatocytes. Thus the reversal of cholestasis in humans by UDCA may include PXR-mediated activation of CYP3A4 and perhaps drug transporter targets that lead to enhanced metabolism and efflux of hepatotoxic bile acids.

We have shown previously a functional relationship between the MR1 transporter, P-glycoprotein, CYP3A, and the nuclear hormone receptor PXR (18, 22, 43–45). Our results now extend this paradigm to include FXR, BSEP/SPGP, and CYP2B as well as MRP3 and MRP4 transporters. This interactive network of transporters and cytochromes P450 participate in hepatic bile acid homeostasis. Normal physiological concentrations of bile acids feed forward to activate FXR and induce BSEP/SPGP. In turn, BSEP/SPGP efflux of bile acids modulates the strength and duration of FXR activation. The intact FXR/BSEP pathway appears essential to keep the bile acid-mediated activation of PXR in check. Thus one can envision during abnormal physiological conditions where FXR signaling or BSEP/SPGP function is impaired or ablated, elevated concentrations of bile acids can activate nuclear hormone receptors such as PXR to induce CYPs and alternative ABC transporters to accelerate removal of bile acids. Although our studies reveal strong parallels between mouse and man and the genes regulating bile acid homeostasis, it should be noted that marked differences exist between the bile acid pool composition in mouse and man (29, 40). In man the primary bile acids are less hydrophilic (e.g. cholic and chenodeoxycholic acid), whereas in mouse the major and more hydrophilic bile acids are cholic and muricholic acids. Thus, accumulation of hepatic bile acids due to alteration or loss of BSEP/SPGP expression could lead to different phenotypes between mouse and man, and these may depend, in part, upon the ability to activate their respective nuclear receptors. This speculation seems likely when we consider that LCA is a much more potent activator of the human PXR/SXR compared with the murine form (11). Furthermore, it is tempting to postulate that species differences in bile acid composition are one physiological selective pressure driving the unexpectedly large differences in ligand binding pocket between rodent and human PXR/SXR (46).

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