

Lithocholic Acid Decreases Expression of Bile Salt Export Pump through Farnesoid X Receptor Antagonist Activity*

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Bile salt export pump (BSEP) is a major bile acid transporter in the liver. Mutations in BSEP result in progressive intrahepatic cholestasis, a severe liver disease that impairs bile flow and causes irreversible liver damage. BSEP is a target for inhibition and down-regulation by drugs and abnormal bile salt metabolites, and such inhibition and down-regulation may result in bile acid retention and intrahepatic cholestasis. In this study, we quantitatively analyzed the regulation of BSEP expression by FXR ligands in primary human hepatocytes and HepG2 cells. We demonstrate that BSEP expression is dramatically regulated by ligands of the nuclear receptor farnesoid X receptor (FXR). Both the endogenous FXR agonist chenodeoxycholate (CDCA) and synthetic FXR ligand GW4064 effectively increased BSEP mRNA in both cell types. This up-regulation was readily detectable at as early as 3 h, and the ligand potency for BSEP regulation correlates with the intrinsic activity on FXR. These results suggest BSEP as a direct target of FXR and support the recent report that the BSEP promoter is transactivated by FXR. In contrast to CDCA and GW4064, lithocholate (LCA), a hydrophobic bile acid and a potent inducer of cholestasis, strongly decreased BSEP expression. Previous studies did not identify LCA as an FXR antagonist ligand in cells, but we show here that LCA is an FXR antagonist with partial agonist activity in cells. In an *in vitro* co-activator association assay, LCA decreased CDCA- and GW4064-induced FXR activation with an IC_{50} of 1 μ M. In HepG2 cells, LCA also effectively antagonized GW4064-enhanced FXR transactivation. These data suggest that the toxic and cholestatic effect of LCA in animals may result from its down-regulation of BSEP through FXR. Taken together, these observations indicate that FXR plays an important role in BSEP gene expression and that FXR ligands may be potential therapeutic drugs for intrahepatic cholestasis.

Bile salt export pump (BSEP)¹ mediates the rate-limiting step in overall hepatocellular bile salt excretion, the transport

of bile acids across the canalicular membrane (1–3). Mutations in BSEP result in progressive familial intrahepatic cholestasis type 2, a severe liver disease characterized by impaired bile flow and irreversible liver damage (4). Progressive familial intrahepatic cholestasis type 2 patients secrete less than 1% of biliary bile salts compared with normal individuals (5). Inhibition or down-regulation of BSEP by drugs and abnormal bile salt metabolites results in hepatic bile acid retention and subsequent intrahepatic cholestasis (6).

Lithocholate (LCA) is a hydrophobic secondary bile acid that is formed in the intestines by bacterial 7 α -dehydroxylation of chenodeoxycholate (CDCA). LCA comprises 2–4% of total bile acids in humans (3). Its level is elevated in patients with chronic cholestasis and other liver diseases (7). Administration of LCA or its conjugates to animals is known to cause intrahepatic cholestasis (8, 9). The mechanism for LCA-induced cholestasis remains unknown. In this study, we show that LCA down-regulates BSEP expression through its antagonist activity on the nuclear hormone receptor FXR. This down-regulation may explain the cholestatic effect of LCA.

FXR is a bile acid receptor (10–13). Bile acids such as CDCA, deoxycholate, cholate, and their conjugates bind to and activate FXR, which consequently regulates transcription of FXR targets. FXR controls expression of critical genes in bile acid and cholesterol homeostasis. It has been shown that FXR inhibits expression of cholesterol 7 α -hydroxylase (Cyp 7 α) (14–17), the enzyme catalyzing the first and rate-limiting step of bile acid synthesis (18), and activates expression of intestinal bile acid-binding protein (11), phospholipid transfer protein (19), BSEP (20), and dehydroepiandrosterone sulfotransferase (21). In this study, we demonstrate that CDCA and a synthetic FXR agonist, GW4064 (22), dramatically up-regulate expression of BSEP mRNA in primary human hepatocytes and HepG2. The BSEP up-regulation by CDCA and GW4064 was readily detectable within 3 h of ligand treatment. The ligand potency for BSEP regulation in cells correlates with several measures of agonist activities on FXR *in vitro*. In addition, the FXR antagonist LCA effectively down-regulates BSEP gene expression. Taken together, these data support the notion of BSEP as a direct target of FXR.

MATERIALS AND METHODS

Reagents—The following reagents were obtained from Invitrogen: tissue culture media of DME, M199, and Opti-MEM I; regular and charcoal-stripped fetal bovine serum (FBS); TRIZOL reagents; PCR

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¹ The abbreviations used are: BSEP, bile salt export pump; FXR, farnesoid X receptor; LCA, lithocholate; CDCA, chenodeoxycholate; Cyp 7 α , cholesterol 7 α -hydroxylase; RXR α , retinoid X receptor α ; SRC-1, steroid receptor coactivator protein-1; PXR, pregnane X receptor; FBS, fetal bovine serum; LBD, ligand-binding domain; DBD, DNA-binding domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; Cyp 3A4, cytochrome P450 monooxygenase 3A4; 6FAM, 6-carboxyfluorescein; TAMRA, N,N,N,N-tetramethyl-6-carboxyrhodamine; SXR, steroid and xenobiotic receptor.

Supermix; and oligonucleotide primers for gene cloning. FuGENE6 transfection reagent was obtained from Roche Diagnostics. Reagents for β -galactosidase and luciferase assays were purchased from Promega (Madison, WI). Bile acids were obtained from Steraloids, Inc. (Newport, RI). GW4064 was synthesized at Merck. TaqMan reagents for cDNA synthesis and real time PCR and TaqMan oligonucleotide primers and probes for human BSEP, Cyp 7a, and Cyp 3A4 were purchased from Applied Biosystems (Foster City, CA). SA/XL665 and (Eu)K were from CIS Biointernational (Bagnols-sur-Ceze, France) and Packard Instrument Co. The goat anti-GST antibody and glutathione-Sepharose were from Amersham Biosciences. Dry milk was from Bio-Rad.

Plasmid Constructs—pGST-hFXR (LBD) was constructed by inserting the cDNA encoding the ligand-binding domain (LBD) of human FXR (amino acids Leu-193 to Gln-472) into pGEX-KG vector (23) at *Bam*HI/*Xho*I. The expression vector pcDNA3.1-GAL4-hFXR (LBD) was constructed by inserting the cDNA fragment (also encoding amino acids Leu-193 to Gln-472) of human FXR into pcDNA3.1GAL4 (24), which contains the GAL4 DNA-binding domain (DBD). In both constructs, the N terminus of hFXR (LBD) was fused to the C terminus of GST or GAL4 (DBD). The integrity of the sequence was confirmed by DNA sequencing. The expression vectors of pUAS(5X)-tk-LUC and pCMV-lacZ were described previously (24). pcDNA3.1-RXR α was constructed by inserting the cDNA encoding the full-length of RXR α into pcDNA3.1.

Preparation of GST-FXR (LBD) Fusion Protein—*Escherichia coli* strain BL21 (Stratagene) harboring pGST-hFXR (LBD) was cultured in LB medium to a density of A_{600} 0.7–1.0 and induced for overexpression by the addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.2 mM. The isopropyl- β -D-thiogalactopyranoside-induced cultures were grown at room temperature to an additional 2–5 h. The cells were harvested by centrifugation for 10 min at 5000 $\times g$. The cell pellet was used for GST fusion protein purification according to the recommended procedure from Amersham Biosciences using glutathione-Sepharose beads.

FXR Co-activator Association Assays—A homogeneous time-resolved fluorescence-based FXR and co-activator SRC-1 interaction assay was used to examine the interaction of FXR with various ligands according to methods previously described for other nuclear receptors (23, 25) with minor modifications. Briefly, 198 μ l of reaction mixture (100 mM HEPES, 125 mM KF, 0.125% (w/v) CHAPS, 0.05% dry milk, 4 nM GST-FXR LBD, 2 nM anti-GST-(Eu)K, 10 nM biotin-SRC-(568–780), 20 nM SA/XL665) were added to each well, followed by the addition of 2 μ l of Me₂SO or ligands (in Me₂SO) into appropriate wells in the presence or absence of 9 μ M CDCA or 100 nM GW4064. Plates were incubated overnight at 4 °C, followed by measurement of fluorescence reading on a Packard Discovery instrument. Data were expressed as the ratio of the emission intensity at 665 nm to that at 620 nm multiplied by a factor of 10⁴.

Cell Treatment—HepG2 cells, a human hepatoma cell line, were obtained from ATCC. HepG2 cells were maintained in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 5 mM HEPES. For determination of gene-specific expression by TaqMan analysis, cells were seeded in six-well plates at a density of 1.2 million cells/well in M199 medium containing 10% FBS, 1% penicillin/streptomycin, and 25 mM HEPES. 24 h after seeding, cells were treated with various concentrations of compounds in M199 medium containing 0.5% charcoal-stripped FBS, 1% penicillin/streptomycin, and 25 mM HEPES. Unless specified, cells were treated for 24 h.

Transient Transfection Assay—HepG2 cells were seeded at a density of 3.2×10^4 cells/well of 96-well plates in DMEM containing 10% FBS 24 h prior to transfection. Cells were transfected with transfection mixes in serum-free Opti-MEM I medium using the FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Typically, transfection mixes for each well contained 0.405 μ l of FuGENE6, 3 ng of pcDNA3.1-GAL4-hFXR (LBD) expression vector, 3 ng of pcDNA3.1-hRXR α expression construct, and 60 ng of pUAS(5X)-tk-LUC reporter vector and 60 ng of pCMV-lacZ as an internal control for transfection efficiency. Cells were incubated in the transfection mixture for 4 h at 37 °C in an atmosphere of 10% CO₂. The cells were then incubated for ~40–48 h in fresh DMEM containing 5% charcoal stripped FBS with or without various concentrations of ligands. Cell lysates were produced using reporter lysis buffer (Promega, Madison, WI) according to the manufacturer's directions. Luciferase activity in cell extracts was determined using luciferase assay buffer (Promega) in an ML3000 luminometer (Dynatech Laboratories). β -Galactosidase activity was determined using β -D-galactopyranoside (Calbiochem) as described previously (24). Luciferase activities were normalized to β -galactosidase activities individually for each well. When assayed for antagonist activity of LCA, cells were incubated with in-

creasing concentrations of LCA in the presence of 100 nM GW4064.

Primary Human Hepatocytes—Plated primary human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD). Cells were seeded at a density of 2×10^6 cells/well of six-well plates in DMEM with 5% FBS. Upon arrival, cells were switched in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 25 mM HEPES and cultured at 37 °C in an atmosphere of 5% CO₂ for 24 h. Cells were then incubated with various concentrations of CDCA and GW4064 in phenol red-free DMEM containing 0.5% charcoal-stripped FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 25 mM HEPES for various periods of time as indicated in the legend to Fig. 3.

RNA Isolation—Total RNA was extracted from the cultured cells using the TRIZOL reagent according to the manufacturer's instructions. RNA was then extracted with phenol/chloroform/isoamyl alcohol and dissolved in water. RNA concentration was determined by a spectrophotometer.

TaqMan Primers and Probes—Oligonucleotide primers and probes for human BSEP and Cyp 7a were designed using the Primer Express program and were synthesized by Applied Biosystems (Foster City, CA). These sequences (5' to 3') are as follows: human BSEP, forward primer (GGGCCATTGTACGAGATCCTAA), probe (6FAM-TCTTGCTA-CTAGATGAAGCCACTTCTGCCTTAGA-TAMRA), and reverse primer (TGCACCGTCTTTTTCACCTTCTG); human Cyp 7a, forward primer (GAGAAGGCAAACGGGTGAAC), probe (6FAM-TGGATTAATTCCAT-ACCTGGCTGTGTGCTCT-TAMRA), and reverse primer (GGTATG-ACAAGGGATTGTGTGATGA); human Cyp 3A4, forward primer (GCAGGAGGAAATTGATGCAGTT), probe (6FAM-ACCCAATAAGGC-ACCACCCACCTATGA-TAMRA), and reverse primer (GTCAAGATAC-TCCATCTGTAGCACAGT). Primers and probe for human 18 S RNA were also purchased from Applied Biosystems.

Real Time Quantitative PCR—Reverse transcription reactions and TaqMan-PCRs were performed according to the manufacturer's instructions (Applied Biosystems). Sequence-specific amplification was detected with an increased fluorescent signal of FAM (reporter dye) during the amplification cycles. Amplification of human 18 S RNA was used in the same reaction of all samples as an internal control. Gene-specific mRNA was subsequently normalized to 18 S mRNA. Levels of BSEP, Cyp 7a, and Cyp 3A4 mRNA were expressed as -fold difference of ligand-treated cells against Me₂SO-treated cells.

RESULTS

CDCA Up-regulates Expression of BSEP—To determine whether BSEP is a target of FXR, HepG2 cells were treated with various doses of CDCA for 3, 6, 12, 24, and 48 h. BSEP mRNA levels were analyzed at each time point by TaqMan-PCR. Consistent with previous reports, HepG2 cells expressed very low levels of BSEP with an average threshold cycle of 40 in untreated cells (data not shown). CDCA treatment greatly increased BSEP mRNA production in a dose-dependent manner with a half-maximum (EC₅₀) of 10–30 μ M and a maximum induction of 500–600-fold (Fig. 1, A–E). Up-regulation of BSEP was readily detectable within 3 h with a maximum induction of 20-fold (Fig. 1A), which increased to 120-fold at 6 h and 250-fold at 12 h (Fig. 1, B and C). 24-h treatment yielded a 400-fold induction (Fig. 1D), which increased slightly at 48 h (Fig. 1E). The EC₅₀ at each of the time points was around 10–30 μ M, which correlates with the EC₅₀ of CDCA in the FXR transactivation assay (Fig. 6B). This correlation, together with the observation that BSEP up-regulation by CDCA was readily detectable at 3 h, suggests that BSEP is a direct target of FXR.

GW4064 Up-regulates Expression of BSEP—GW4064 was reported to be a potent and selective synthetic agonist of FXR (26). To further confirm that BSEP up-regulation was mediated by FXR, HepG2 cells were treated with GW4064 for 24 h and assayed for BSEP expression. Similar to the results of CDCA treatment, GW4064 also robustly increased BSEP mRNA in a dose-dependent manner with an EC₅₀ of about 0.1 μ M (Fig. 2). This value also correlates with its potency in FXR transactivation (Fig. 6C). This result, together with the result of CDCA treatment, suggests that BSEP up-regulation is mediated by FXR and that BSEP is a direct FXR target.

FIG. 1. Time course for induction of BSEP mRNA by CDCA. HepG2 cells at a density of 1.2 million cells/well in six-well plates were treated with various concentrations of CDCA for 3 (A), 6 (B), 12 (C), 24 (D), and 48 h (E) in M199 medium containing 0.5% charcoal-stripped FBS. Total RNA was prepared and BSEP mRNA was analyzed by TaqMan-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells *versus* vehicle), and data are the mean \pm S.D. of four determinations.

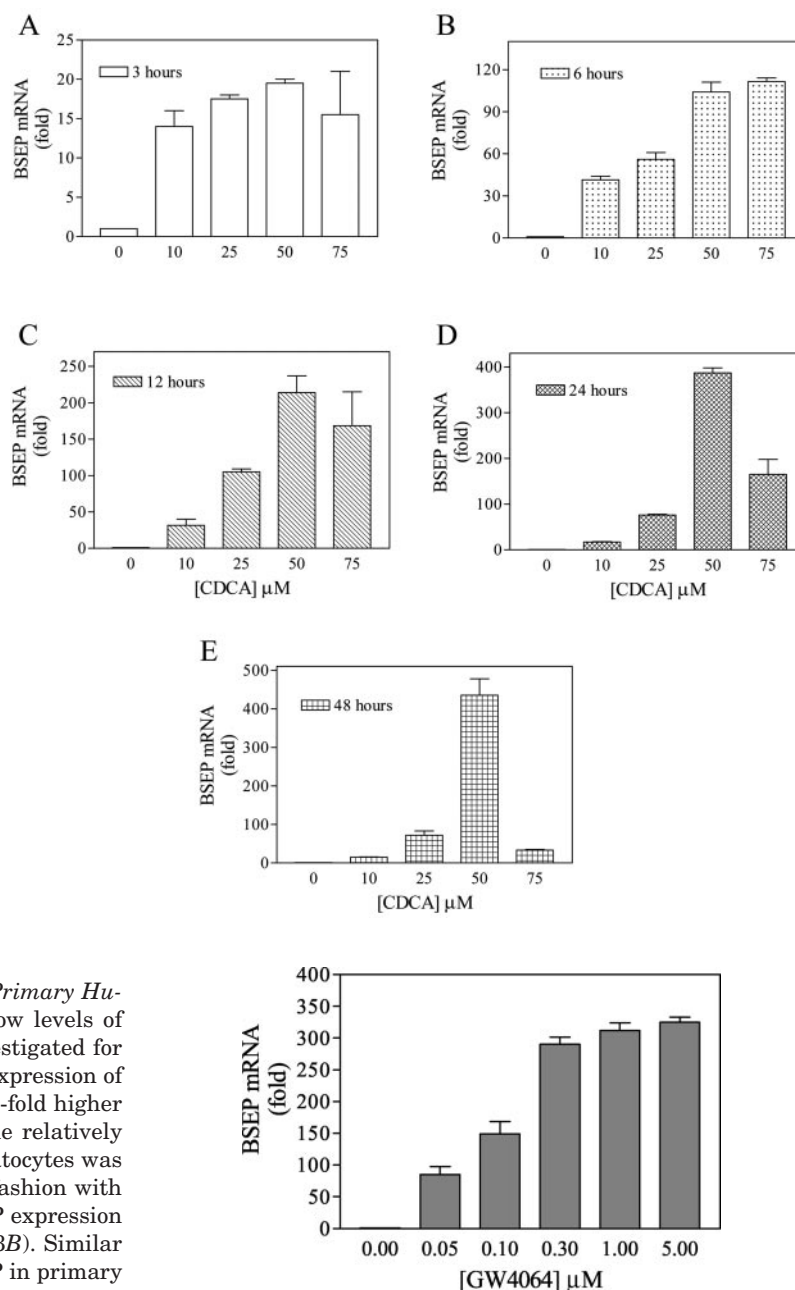


FIG. 2. Induction of BSEP mRNA by GW4064. HepG2 cells at a density of 1.2 million cells/well in six-well plates were treated with various concentrations of GW4064 for 24 h in M199 medium containing 0.5% charcoal-stripped FBS. Total RNA was prepared and BSEP mRNA was analyzed by TaqMan-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells *versus* vehicle), and data are the mean \pm S.D. of four determinations.

BSEP Up-regulation by CDCA and GW4064 in Primary Human Hepatocytes—Since HepG2 cells expressed low levels of BSEP, primary human hepatocytes were also investigated for BSEP gene regulation by FXR ligands. The basal expression of BSEP in primary hepatocytes was more than 1000-fold higher than that in HepG2 (data not shown). Despite the relatively high basal level, BSEP expression in primary hepatocytes was further induced by GW4064 in a dose-dependent fashion with an EC_{50} of 0.1 μ M (Fig. 3A). The induction of BSEP expression by CDCA and GW4064 was time-dependent (Fig. 3B). Similar to the results in HepG2, the up-regulation of BSEP in primary hepatocytes was readily detectable at 3 h with a 1.5–2-fold induction (Fig. 3B). During the period of 3–48 h, BSEP induction showed a linear increase with time. The induction reached 8–9-fold for CDCA and 10–12-fold for GW4064 (Fig. 3B) at 48 h.

LCA Decreases GW4064-stimulated BSEP Expression—LCA, a hydrophobic bile acid with a single hydroxy group at position 3, is a potent inducer of cholestasis when administered to animals (8, 9). We hypothesized that the cholestatic effect of LCA could result from down-regulation of BSEP expression. To test this hypothesis, HepG2 cells were treated with LCA in the presence or absence of GW4064, and BSEP expression was analyzed by TaqMan-PCR. In the absence of GW4064, LCA alone slightly increased BSEP mRNA to a maximum of 16-fold (Fig. 4A). This is about 5% of the maximal stimulation of BSEP mRNA by CDCA (Fig. 1D). This partial stimulation is consistent with the partial agonist activity of LCA in FXR transactivation (Fig. 6A).

Treatment of HepG2 with 100 nM GW4064 alone resulted in an induction of BSEP expression by 350-fold (Fig. 4B). This induction was effectively decreased by LCA in a dose-depend-

ent manner with a half-maximum inhibition (IC_{50}) of 10–20 μ M (Fig. 4B). LCA inhibited GW4064-induced BSEP expression by 90% at 30 μ M (Fig. 4B). At concentrations above 30 μ M, LCA treatment caused cell toxicity (data not shown).

LCA Is an FXR Antagonist—Although previous studies did not identify LCA as an FXR ligand, we reasoned that inhibition of BSEP expression by LCA was mediated through FXR. A homogeneous time-resolved fluorescence-based FXR co-activator association assay was set up and used to determine LCA activities on FXR *in vitro*. This assay measures ligand-dependent association of FXR with the co-activator SRC-1 (see "Materials and Methods"). In the agonist mode, LCA alone failed to activate FXR (Fig. 5A). As a control, CDCA

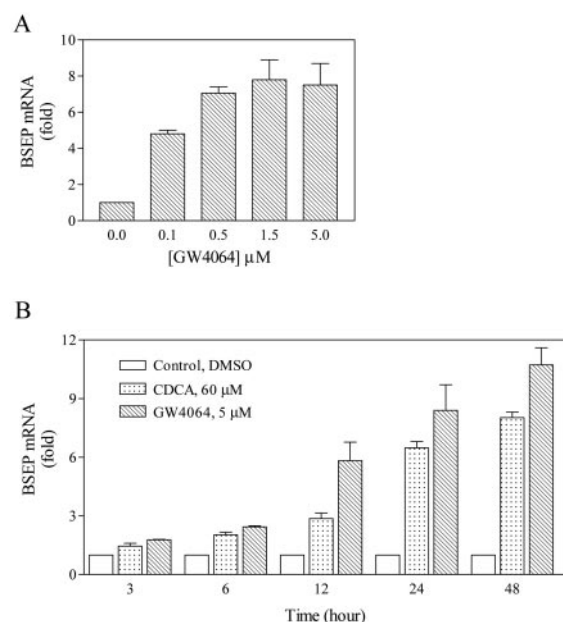


FIG. 3. Time course for induction of BSEP mRNA by CDCA and GW4064 in primary human hepatocytes. Primary human hepatocytes at a density of 2 million cells/well in six-well plates were treated with various concentrations of GW4064 for 24 h (A) or with 60 μM CDCA or 5 μM GW4064 at various time points (B) in DMEM containing 0.5% charcoal-stripped FBS. Total RNA was prepared, and BSEP mRNA was analyzed by TaqMan-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells *versus* vehicle), and data are the mean \pm S.D. of four determinations. DMSO, Me_2SO .

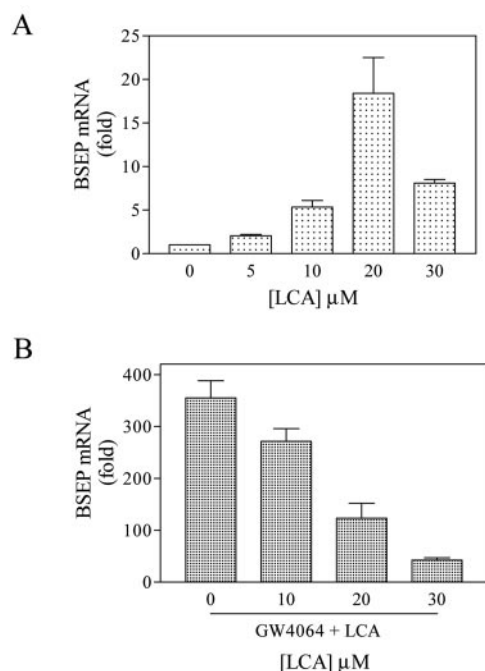


FIG. 4. Effect of LCA on BSEP mRNA. HepG2 cells at a density of 1.2 million cells/well in six-well plates were treated with various concentrations of LCA in the absence (A) or presence of 100 nM GW4064 (B) for 24 h in M199 medium containing 0.5% charcoal-stripped FBS. Total RNA was prepared, and BSEP mRNA was analyzed by TaqMan-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells *versus* vehicle), and data are the mean \pm S.D. of three determinations.

activated FXR with an EC_{50} of 8 μM (Fig. 5A). In the antagonist mode, LCA decreased CDCA- or GW4064-induced FXR activation with an IC_{50} of 0.7 and 1.4 μM , respectively (Fig.

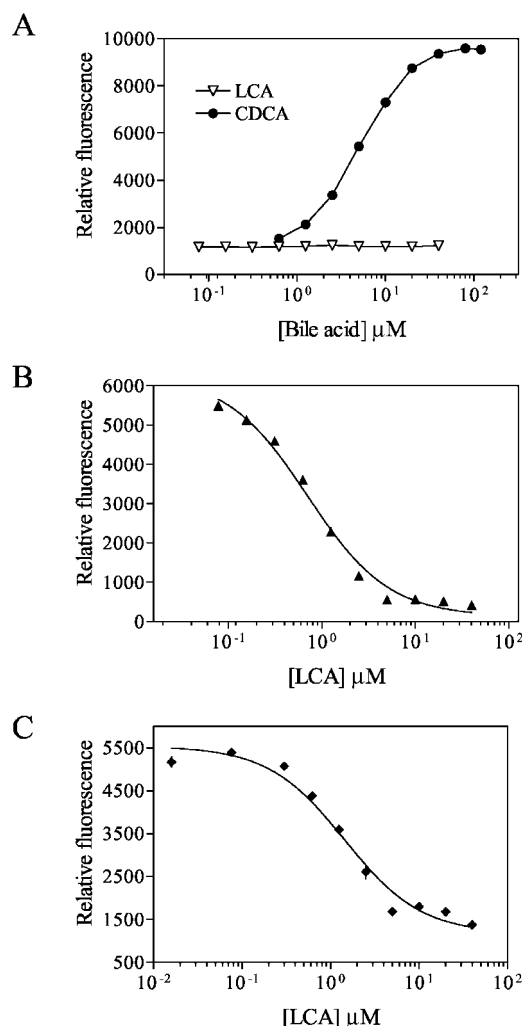


FIG. 5. Effect of LCA on interaction of FXR with co-activator SRC-1. 4 nM purified GST-FXR was incubated with 2 nM anti-GST-(Eu)K, 10 nM biotin-SRC-1-(568–780), 20 nM SA/XL665, and various concentrations of LCA or CDCA in the absence (A) or presence of 9 μM CDCA (B) or 10 nM GW4064 (C). The mixture was incubated at 4 $^{\circ}\text{C}$ overnight. The fluorescent signal was measured, and results were calculated as described under "Materials and Methods." Each value represents the mean \pm S.D. of six determinations.

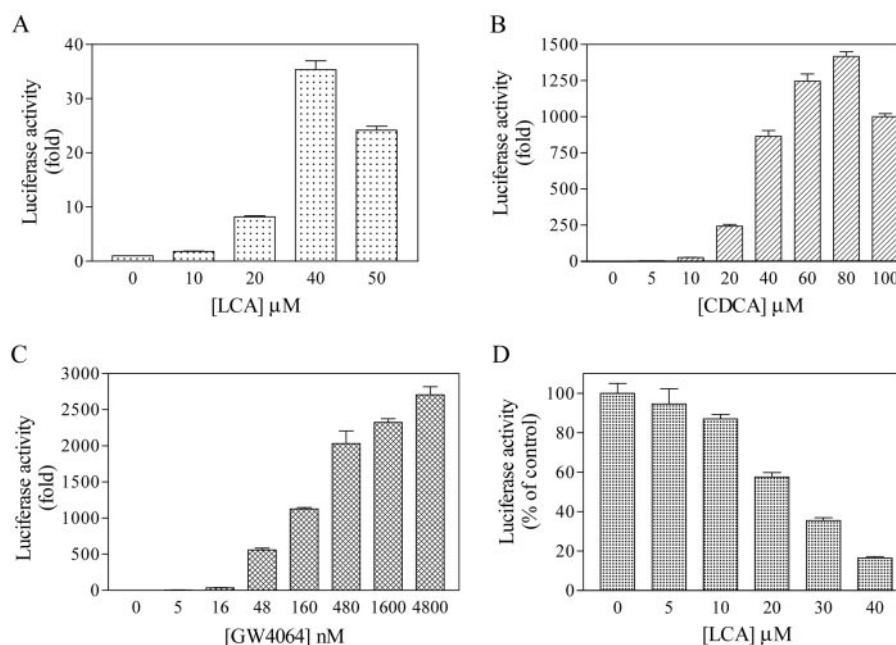
5, B and C). These results demonstrate that LCA is indeed a *bona fide* antagonist ligand of FXR.

LCA Weakly Activates but Strongly Antagonizes FXR Transactivation—The FXR antagonist activity of LCA was also confirmed in HepG2 cells using the Gal4-based FXR transactivation assay. LCA alone partially activated FXR with a maximal activation of 35-fold at 40 μM (Fig. 6A). In parallel experiments, CDCA activated FXR with a maximum of 1300-fold (Fig. 6B). Thus, LCA has less than 5% of the agonist activity of CDCA. This partial agonist activity of LCA may explain the partial induction of BSEP by LCA in HepG2 (Fig. 3A). Compared with CDCA, GW4064 was a superagonist of FXR, with a maximum induction of 2800-fold and an EC_{50} of 100–200 nM (Fig. 6C).

In the antagonist assay, consistent with the *in vitro* results, LCA effectively antagonized GW4064-induced FXR transactivation in a dose-dependent manner with an IC_{50} of 20–30 μM (Fig. 6D). These results indicate again that LCA is an antagonist ligand of FXR.

LCA Down-regulates Cyp 7a Expression—It is well known that FXR agonists inhibit transcription of Cyp 7a. LCA, although an FXR antagonist, significantly inhibited Cyp 7a ex-

FIG. 6. Effect of LCA on FXR transactivation. HepG2 cells (3.2×10^4 cells/well of 96-well plates) were transfected with 0.405 μ l of FuGENE6, 3 ng of pcDNA3.1-GAL4-hFXR (LBD) expression vector, 3 ng of pcDNA3.1-hRXR α expression construct, 60 ng of pUAS(5X)-tk-LUC reporter vector, and 60 ng of pCMV-lacZ in serum-free Opti-MEM 1 medium using the FuGENE6 transfection reagent according to the manufacturer's instructions. The transfected cells were treated with various concentrations of LCA (A), CDCA (B), or GW4064 (C). LCA treatment was also carried out in the presence of 100 nM GW4064 (D) to determine the antagonist activity. After 24 h of treatment, cells were harvested, and the cell lysate was used for determination of luciferase and β -galactosidase activities as described under "Materials and Methods." Luciferase activities were normalized to β -galactosidase activities individually for each well. Each value represents the mean \pm S.D. of six determinations.



pression in HepG2 cells in a dose-dependent manner with an IC_{50} of 20 μ M (Fig. 7A). This inhibition reached 90% at 30 μ M. As a control, CDCA effectively decreased Cyp 7a mRNA production (Fig. 7B). The extent of Cyp 7a inhibition by LCA cannot fully be explained by its partial agonist activity (less than 5% of CDCA) on FXR. Indeed, two recent publications identified LCA as a PXR/SXR agonist, and PXR activation also results in repression of Cyp 7a expression (27, 28). Thus, it is likely that the observed Cyp 7a down-regulation by LCA involves pathways in addition to FXR activation.

Rifampicin Does Not Regulate BSEP Expression—To eliminate the possibility that BSEP regulation by LCA could be mediated through PXR, rifampicin, a PXR-specific ligand (29, 30), was used to treat HepG2 cells and was examined for its effects on BSEP transcription. Rifampicin had no activities on FXR in co-activator association assay and FXR transactivation (data not shown). As predicted, rifampicin alone did not change BSEP expression (Fig. 8A). In contrast to LCA, rifampicin did not decrease GW4064-induced BSEP mRNA (Fig. 8B). In the same experiment, rifampicin increased Cyp 3A4 expression in a dose-dependent manner (Fig. 8C), indicating activation of PXR by rifampicin, since Cyp 3A4 is a direct target of PXR (31, 32). These results suggest that PXR is not involved in BSEP regulation and further support the conclusion that the down-regulation of BSEP by LCA is mediated through the antagonist activity on FXR.

DISCUSSION

BSEP is a major bile acid transporter. BSEP expression is critically important for maintenance of bile flow and protecting the liver from bile acid toxicity. It has been reported that BSEP is up-regulated by dexamethasone and hypoosmolarity and down-regulated by endotoxin (33). In this study, we quantitatively analyzed the regulation of endogenous BSEP expression by various FXR ligands in primary human hepatocytes and HepG2 by TaqMan-PCR. We demonstrate that BSEP expression in both cell types is up-regulated by the FXR agonists CDCA and GW4064 in a time- and dose-dependent fashion. BSEP up-regulation was readily detectable at as early as 3 h in both HepG2 and primary hepatocytes. The ligand potency for BSEP regulation correlates well with the ligand *in vitro* potencies on FXR. All of these data suggest BSEP as a direct target of FXR. Consistent with our results, Ananthanarayanan *et al.*

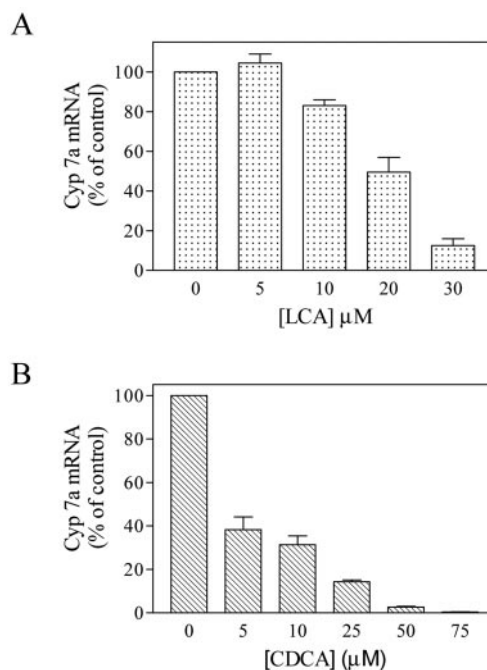


FIG. 7. Effect of LCA on Cyp 7a mRNA. HepG2 cells at a density of 1.2 million cells/well in six-well plates were treated with various concentrations of LCA and CDCA (B) for 24 h in M199 medium containing 0.5% charcoal-stripped FBS. Total RNA was prepared, and BSEP mRNA was analyzed by TaqMan-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells versus vehicle), and data are the mean \pm S.D. of four determinations.

(20) recently reported that the BSEP promoter contains an FXR response element (IR-1) and that FXR directly binds to the BSEP promoter. Indeed, BSEP expression is greatly decreased in FXR knockout mice (34).

We also provide evidence to explain the molecular basis for a long standing issue of LCA-induced cholestasis. LCA effectively down-regulates agonist-dependent BSEP expression. This down-regulation would result in a decreased hepatic bile acid excretion and thus an increased liver bile acid concentration, which in turn could cause intrahepatic cholestasis and liver

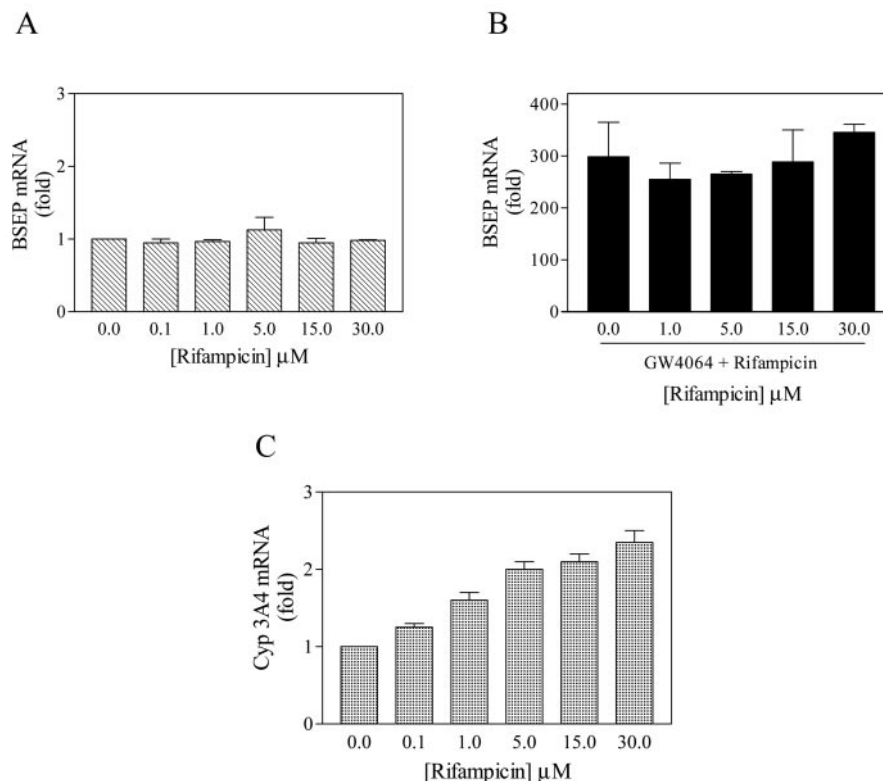


FIG. 8. Effect of rifampicin on BSEP and Cyp 3A4 mRNA. HepG2 cells at a density of 1.2 million cells/well in six-well plates were treated with various concentrations of rifampicin in the absence (A and C) or presence of 100 nM GW4064 (B) for 24 h in M199 medium containing 0.5% charcoal-stripped FBS. Total RNA was prepared, and BSEP mRNA (A and B) or Cyp 3A4 mRNA (C) was analyzed by Taq-Man-PCR (described under "Materials and Methods"). Results are normalized as -fold control value (treated cells *versus* vehicle), and data are the mean \pm S.D. of three determinations.

damage. Furthermore, we show that LCA is an FXR antagonist and that down-regulation of BSEP by LCA is mediated by FXR antagonist activity.

Bile acids such as CDCA, deoxycholate, and cholate were previously identified as FXR ligands (10, 12, 13). CDCA was thought to be the most potent agonist. Here, we show that LCA binds to FXR with a higher affinity than CDCA in the FXR co-activator association assay. In this assay, LCA acted as a pure antagonist with no detectable agonist activity (Fig. 5A). In the FXR transactivation assay performed in HepG2 cells, LCA acted as an antagonist with partial agonist activity (<5%) (Fig. 6, A and D). We believe that this partial agonist activity resulted in the partial induction of endogenous BSEP expression in HepG2 cells (see Fig. 4A). The lack of partial agonist activity in the co-activator association assay may be due to 1) co-activator specificity, since only the co-activator SRC-1 was included in the assay, or 2) assay sensitivity. The co-activator association assay had an approximate 10-fold window; however, both FXR transactivation and BSEP mRNA assay had a window of several hundredfold. Thus, co-activator association assay may not be sensitive enough to detect the less than 5% agonist activity of LCA.

LCA was recently identified as a PXR/SXR agonist ligand. PXR is thought to be the second bile acid receptor and plays a critical role in liver detoxification (27, 28). However, there is no evidence supporting the involvement of PXR in BSEP gene regulation. Indeed, the PXR-specific ligand rifampicin did not regulate BSEP expression, suggesting that PXR is not involved in BSEP regulation and further supporting the conclusion that the down-regulation of BSEP by LCA is mediated through the antagonist activity on FXR.

BSEP expression is critically important for liver protection. The identification of FXR as an important regulator of BSEP not only provides a molecular mechanism for FXR-mediated BSEP gene regulation but also suggests a potential for FXR ligands as therapeutic drugs for intrahepatic cholestasis and lipid disorders.

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