LITHOCHOLIC ACID DECREASES EXPRESSION OF UGT2B7 IN CACO-2 CELLS: A

POTENTIAL ROLE FOR A NEGATIVE FARNESOID X RESEPTOR RESPONSE ELEMENT

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1

Running title: Lithocholic Acid Suppresses UGT2B7 Expression

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The abbreviations used are: UGT, UDP-glucuronosyltransferase; I-BABP, Ileal-Bile Acid Binding Protein; hFXR, human farnesoid X receptor; PPAR, peroxisome proliferator-activated receptors; BARE, bile acid response element; HepG2, human hepatocellular carcinoma cell line; Caco-2, human colorectal adenocarcinoma cells line; CREB, cyclic AMP response element-binding protein; hRXR, human retinoid X receptor; EMSA, electrophoretic mobility shift assay; hPXR, human pregnane X receptor; OATP2, organic anion transport protein 2; Mrp3, multidrug-resistance associated protein 3; HNF_4, hepatic nuclear factor 4; NFRE, negative farnesoid x receptor response element, hVDR, human vitamin D receptor

ABSTRACT

Human UDP-glucuronosyltransferase (UGT) 2B7 is the major isoform catalyzing the glucuronidation of a variety of endogenous compounds including bile acids. To determine the role of bile acids in the regulation of UGT2B7 expression, Caco-2 cells were incubated with the natural human farnesoid X receptor (hFXR) ligand, chenodeoxycholic acid, as well as the secondary bile acid, lithocholic acid, derived from chenodeoxycholic acid. Incubation of Caco-2 cells with lithocholic acid in the absence of exogenous hFXR resulted in a dose-dependent down-regulation of *UGT2B7* mRNA levels, with an IC_{50} of 13 µM. Similar down-regulation was also observed with chenodeoxycholic acid; however, much higher concentrations were required. Transient transfection of Caco-2 cells with hFXR or the hFXR-DNA binding domain suppressed *UGT2B7* mRNA expression both in the absence and the presence of ligand. *UGT2B7* promoter transfection experiments and deletion/mutation analysis showed that lithocholic acid-activated hFXR decreased *UGT2B7* promoter activity via a negative hFXR response element (NFRE) located between nucleotides -148 and -134. Co-transfection with hFXR and/or hretinoid orphan receptor (hRXR) further enhanced the repression. Electrophoretic mobility shift assays additionally confirmed the role of NFRE in *UGT2B7* down-regulation by lithocholic acid. These findings suggest that lithocholic acid, an activator of nuclear hFXR, acts as a negative regulator of UGT2B7 expression, indicating that hFXR may play an essential role in lithocholic acid homeostasis through negative regulation of this UGT that is involved in lithocholic acid biotransformation. Therefore, it is postulated that lithocholic acid toxicity may be due to down-regulation of genes involved in its detoxification, including UGT2B7, leading to limited excretion of lithocholic acid from the body.

INTRODUCTION

Bile acids, in addition to their role in the emulsfication of dietary lipids (Borgstrom et al., 1985),

have been recognized as important signaling molecules and physiological ligands for several nuclear

receptors (Chiang, 2002). The nuclear receptors that have been recognized as being involved in the

regulation of biosynthesis, metabolism, and transport of bile acids, are the human farnesoid orphan

receptor (hFXR), the liver X receptor, the peroxisome proliferator-activated receptors (PPARs), the

human pregnane orphan receptor (hPXR) (Chiang, 2002), and the human vitamin D receptor (hVDR)

(Makishima et al., 2002). hFXR is the major bile acid receptor and primary bile acids are its

physiological ligands (Takeshita et al., 2002).

Growing evidence has been collected that bile acids are strongly involved in the regulation of their

own synthesis and transport, as well as cholesterol homeostasis and lipid metabolism. It has been

shown that bile acids activate hFXR, which regulates several target genes, including drug metabolizing

enzymes. Several examples of gene regulation via bile acid activation of nuclear receptors include

cytochrome P450 7A1, which has been identified as a target gene of the bile acid-specific receptor,

hFXR (Russell and Setchell, 1992). hFXR negatively regulates bile acid synthesis by decreasing

transcription of *cytochrome P450 7A1* and cholesterol 12α-hydroxylase, the enzyme responsible for

cholic acid synthesis, is also under negative control by bile acids (Vlahcevic et al., 2000). In addition,

hFXR activates transcription of ileal-bile acid binding protein (I-BABP), which is involved in the

enterohepatic circulation of bile acid (Princen et al., 1997). Moreover, hFXR mediates bile acid

activation of phospholipid transfer protein gene expression (Urizar et al., 2000), which suggests that

bile acids and hFXR my play a role in high-density lipoprotein metabolism via the regulation of this

gene. Several more bile acid transporters, including organic anion transport protein 2 (OATP2) and the

4

bile salt export pump, which promotes bile acid efflux from the liver (Bjorkhem, 1985), have been recognized as hFXR target genes activated by bile acids (Ananthanarayanan et al., 2001).

It has been shown that hFXR also controls the metabolism of triglycerides. bile acid-activated hFXR suppresses apolipoprotein A-I and apolipoprotein C-III transcription (Claudel et al., 2003; Claudel et al., 2002). It was shown that the suppression of apolipoprotein A-I occurs *via* a novel negative hFXR response element (NFRE) (Claudel et al., 2002). Also, enzymes involved in bile acid detoxification, especially of lithocholic acid, have been identified as being under the control of bile acid-mediated regulation. Very recently, direct involvement of hFXR in the regulation of human UDP-glucuronosyltransferase (UGT) 2B4 has been demonstrated in human primary hepatocyte cultures (Barbier et al., 2003b).

Recent findings indicate that three additional nuclear receptors, PXR, PPARα and hVDR, accept bile acids as ligands and regulate bile acid synthesis and transport. It has been demonstrated that PPARα may modulate metabolic pathways common to hFXR. The first molecular evidence for cross-talk between hFXR and PPARα pathways in humans has been found (Torra et al., 2003). Bile acid-mediated activation of PXR has been postulated in the regulation of multidrug resistance-associated protein 3 (Mrp3) (Staudinger et al., 2003) and has been shown to regulate cytochrome P450 3A4 expression (Takeshita et al., 2002). Bile acids seem to be involved in the regulation of several biologically important metabolic pathways by the induction and/or suppression of hFXR, PPARα, and PXR target genes. hVDR is also activated by bile acids (Makishima et al. 2003). It has been demonstrated that lithocholic acid is a higher affinity ligand for hVDR than for either hFXR or PXR. The identification of hVDR as an intestinal bile acid sensor may indicate an additional mechanism for the protective effects of vitamin D and its receptor against toxic lithocholic acid. It has

been demonstrated that activation of hVDR by lithocholic acid or vitamin D induces expression *in vivo* of cytochrome P450 3A enzymes that detoxifies lithocholic acid in the liver and intestine.

Bile acids have been identified as excellent substrates for human UGTs (Gall et al., 1999). UGTs are phase II biotransformation enzymes localized to the endoplasmic reticulum (Chowdhury et al., 1985) that catalyze the conjugation of glucuronic acid to a broad spectrum of endobiotic and xenobiotic substrates with diverse chemical structures. Endogenous substrates for UGTs include bilirubin, bile acids, fatty acids, prostaglandins, serotonin, thyroid hormones, biogenic amines and steroid hormones (Radominska-Pandya et al., 1999). Additionally, UGTs have a large number of xenobiotic substrates, including fat-soluble vitamins, carcinogens, plant metabolites, environmental pollutants, and drugs such as acetaminophen, chloramphenicol, diethylstilbestrol, morphine, and salicylic acid (Burchell et al., 1998; Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000). UGT2B7 is one of two major human isoform involved in the glucuronidation of both the hydroxyl and carboxyl functions of bile acids.

In this work, we have identified the gene encoding human UGT2B7 as a new target of lithocholic acid-mediated suppression. The transcription of UGT2B7 is down-regulated by lithocholic acid, and we have collected reliable evidence that hFXR is involved in this process. This down-regulation could result in a dramatic accumulation of toxic lithocholic acid in cells and the development of severe cholestasis. It is anticipated that identification of the mechanism of lithocholic acid toxicity will result in the successful development of drugs that will prevent cholestasis and other lithocholic acid-mediated toxicities.

METHODS

Cell Culture and Transient Transfections—The human hepatocellular carcinoma cell line, HepG2

(ATCC HB-8065) and human colorectal adenocarcinoma cells line, Caco-2 (ATCC HTB-37), were

obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained

at 37ºC, 5% CO_2 in high glucose Dulbecco's Modified Eagle Medium (DMEM) with Earle's salts and

L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 1% non-essential amino acids, 1mM

sodium pyruvate and 10% fetal bovine serum (Invitrogen). The culture medium was changed twice

weekly during maintenance and untransfected cells used for RNA isolation were harvested when they

neared confluence. In experiments with transcription inhibitor, 1 µg/ml of actinomycin-D was added to

the culture medium together with vehicle or lithocholic acid, and mRNA was collected at various time

points, as shown in the figure.

For transient transfection, HepG2 or Caco-2 cells were seeded in 6-well plates at $1x10^6$ and $2.5x10^5$

cells per well, respectively. Transfections were performed in triplicate using the indicated amount of

expression plasmid or empty parent vector and 10 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA)

according to the manufacturer's instructions. 5 hours after transfection, the cells were washed in

DMEM and replacement medium containing bile acids (Sigma-Aldrich, St. Louis, MO) at the

concentration given in the figures or vehicle (0.1% DMSO) was added. The sodium salts of lithocholic

acid. Chenodeoxycholic acid, hyodeoxycholic acid, and ursodeoxycholic acid were solubilized in

100% DMSO, and during experiments control cells were exposed to the same DMSO concentration as

the cells treated with bile acids. At 24 hours post-transfection, the cells were treated again with bile

acids or solvent in fresh DMEM. After a total of 48 hours, all cells were washed in phosphate buffered

saline and harvested for RNA extraction.

Cell Viability Assay - Cell viability was determined by trypan blue exclusion. Cells were plated at a density of 5×10^5 cells per well in 6-well plates and cultured for 24 h. Lithocholic acid, at the concentrations indicated in the figures, was then added. After an additional 48 h incubation, the cells were released from attachment to the plates with trypsin/EDTA (Invitrogen), incubated with 0.4% trypan blue (Mediatech, Herndon, VA) for 5 minutes, and counted using a hemocytometer. Since the monolayers were not washed prior to trypsin-EDTA treatment or scraping of adherent cells, cells that detached during incubation with lithocholic acid were detected.

RNA Isolation - Total RNA was isolated from cell cultures using a phenol and guanidine isothiocyanate RNA extraction method (Trizol; Invitrogen), following the instructions of the supplier. To avoid any contamination of the RNA by genomic DNA, DNase treatment was performed using RQ1 RNase-Free DNase (Promega, Madison, WI).

cDNA was synthesized by mixing 1 μg of total RNA from each sample with 100 pmol random hexamer primers in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, containing 100 U M-MLV reverse transcriptase, 20 U RNase inhibitor, and 1 mM each dNTP (Promega, Madison, WI) in a total volume of 20 μl. The samples were incubated at 37°C for 60 min and then heated at 95°C for 5 min to inactivate the reverse transcriptase. The reaction mixture was diluted to 100 μl with sterile diethylpyrocarbonate-treated H_2O.

Semi-Quantitative RT-PCR of hFXR, UGT2B7, PXR, and I-BABP—Primers for UGT2B7, hFXR, PXR, I-BABP, and GAPDH are described in Table 1. hFXR, PXR, UGT2B7, and I-BABP primers were designed in this study. PCR reactions were performed as follows: a 10 μl aliquot of cDNA was added to a reaction mixture containing 10 mM Tris-HCl buffer (pH 8), 20 mM KCl, 0.1% Triton X-100; 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer and 2 units of Taq DNA polymerase

(Promega), in a total volume of 50 µl. Amplification of the ubiquitously expressed GAPDH cDNA was performed under the same conditions in separate experiments. Table 1 details the annealing temperature and cycle number required for each template. Primer pairs were designed to specifically amplify across exon boundaries in mRNA from GAPDH, I-BABP, and UGT2B7 (Table 1). The specificity of all primer pairs was confirmed through sequencing of the PCR products. The PCR products were resolved by 2% agarose electrophoresis and detected by ethidium bromide. The bands were visualized under UV light and photographed by a computed-assisted camera. Quantification of each band was performed by densitometric analysis by using NIH Image software (NIH, Bethesda, MD).

For each primer pair, we performed PCR with different cycle numbers and plotted these data to form a standard curve. We chose the cycle that was found to be within the non-saturable range of amplification for use in further experiments. All other conditions were kept consistent unless significant changes in mRNA level were observed.

Real-time PCR assay - Real-time PCR analyses were performed with lithocholic acid-treated Caco-2 cells for comparison with the data obtained from semi-quantitative PCR. Real-time PCR primer sequences for UGT2B7 and GAPDH were obtained from Congiu (Congiu et al., 2002) and Knerr (Knerr et al., 2002), respectively.

Inhibition of *UGT2B7* transcription in the presence of varying concentrations of lithocholic acid was evaluated by real-time RT-PCR and used to find the IC_{50} value, which was determined using Prisim4 software (GraphPad, San Diego, CA). The real-time PCR reaction mixture contained 5 µl of the sample and 20 µl of a master mix containing forward and reverse primers (300 nM each), IQ SYBR Green Supermix (2x, Bio-Rad, Hercules, CA) and the appropriate volume of sterile water. PCR

conditions were 3 min at 95°C, followed by 40 cycles of 95 °c for 10 s and 60°C for 45 s. Optical data

were collected during the 60 °C step. PCR reactions were carried out in 96-well thin-wall PCR plates

covered with optically clear sealing film (Bio-Rad). Amplification, detection and data analysis were

performed with an iCycler IQ real-time detection system (Bio-Rad).

A standard curve was generated from a cDNA dilution series (1 ng, 5 ng, 25 ng) constructed as

reference samples from the DMSO control sample. Real-time PCR of UGT2B7, I-BABP and GAPDH

were performed in duplicate on both the experimental cDNA samples (10 ng) and reference standards.

Values for target abundance in each experimental sample were extrapolated from the standard curve

generated with the reference standards. The final data were expressed as the ratio of the values for the

genes of interest and the corresponding values for GAPDH.

Isolation of UGT2B7 promoter fragments - Clones containing UGT2B7 promoters were isolated

from a human placenta lambda genomic library (Clontech, Palo Alto, CA) after several rounds of

screening. Briefly, DNA fragments from the 5'-ends of cDNAs encoding the UGTs of interest were

labeled with ^{32}P-dATP by random priming using the DECAprime II DNA labeling kit (Ambion, Austin,

TX). Hybond-N membranes (Amersham Biosciences, Piscataway, NJ) from plaque lifts were

denatured, neutralized and UV cross-linked according to the manufacturer's protocol (PT1010-1).

Pre-hybridization was carried out in a solution of 5x SSC (0.375 M NaCl, 0.0375 M sodium citrate), 5x

Denhardt's (Ficoll, polyvinylpyrrolidone, and BSA, 1 mg/ml each) and 0.5% SDS for 4 hours at 42°C

before hybridization with 1×10^6 cpm/µl of probe overnight. Membranes were washed twice in 2x SSC,

0.1% SDS for 5 minutes at room temperature, and twice in 0.1x SSC, 0.1% SDS for 30 minutes at 65°C

followed by exposure to autoradiography film with intensification screens at -70°C overnight. Positive

plaques were picked and screened by restriction digestion and sequencing for appropriate UGT

promoter inserts. A UGT2B7 1.3 kb proximal promoter was amplified from these clones by PCR, using

primers designed with information from the sequencing of the genomic clones and/or from the human

genome databases. The primers contained Kpn1 and Mlu1 sites for directional cloning into the KpnI

and MluI sites of the pGL3-Basic luciferase reporter vector (Promega, Madison, WI).

Generation of the UGT2B7 promoter-luciferase reporter constructs - A series of 5' deletion

constructs containing different promoter fragments were generated from the 1.3 kb proximal UGT2B7

promoter construct [2B7(p1328)] using the Erase-a-Base® system (Promega). The plasmid containing

the insertion was double-digested by *Mlu*I and *Kpn*I. The linear fragment was further digested by

Exonuclease III and aliquots were removed at different times to obtain a series of 5'-deletions. S1

nuclease was added to remove the remaining 5'-overhang and the resulting blunt ends were linked by

adding Klenow fragment, dNTPs and T4 DNA ligase. Plasmids were transformed into the *E.coli*

DH10B strain and incubated at 37°C overnight. Positive clones containing the UGT2B7 promoter were

verified by direct DNA sequencing.

Deletions and site-directed mutagenesis – For deletion of the NFRE sequence in 2B7(p1328)-Luc

to create 2B7(p1315)NFREΔ-Luc, two oligonucleotides were designed by Oligo Lite software and

synthesized by Integrated DNA Technologies (Coralville, IA):

Sense: 5'-GGCATGTCCATACAATAGCTGAAGGATAGCAC-3'

Antisense: 5'-GTGCTATCCTTCAGCTATTGTATGGACATGCC-3'.

The NFRE was in the middle of the oligonucleotides with 16 bases of correct sequence on either side.

Constructs with deletions within the NFRE, 2B7(p1328)mut-Luc and 2B7(p428)mut-Luc, were

prepared using the following primers:

Sense: 5'-CCATACAA<u>GATCCTT**ATAG**TGT</u>GCTGAAGGATAGC-3'

Antisense: 5'-GCTATCCTTCAGC<u>**ACACTAT**AAGGATC</u>TTGTATGG-3'

(bases within the NFRE are underlined and the mutated bases are shown in bold). The deletions were

done using the Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by direct DNA

sequencing.

Dual-Luciferase Assay - Caco-2 cells were seeded in 12-well plates at 0.5 x 10^5 cells per well the

day before transfection. Transfections were performed in triplicate using 0.5 µg of UGT2B7

promoter-driven luciferase plasmid in the absence/presence 100 ng of the hFXR and/or hRXR

expression vectors, pCMX-hFXR and pCMX-hRXR. The total amount of DNA was supplemented by

pCMX-null vector to 1.5 µg per well. Five hours after transfection, the cells were washed in DMEM

and DMEM containing 1% fetal bovine serum and either bile acid at the concentrations shown in the

figures or vehicle (0.1% DMSO) was added. After a total of 48 h, all cells were washed in

phosphate-buffered saline and lysed with Passive Lysis buffer (Promega), centrifuged at 16,000 x g for

5 min at 4 °C, and 20 µl of each supernatant was used to determine firefly luciferase activity. To

normalize transfection efficiency, cells were co-transfected with 0.5 µg of the *Renilla* luciferase reporter

plasmid pRL-TK (Promega) and dual luciferase activity was recorded using TD-20/20 luminometer

(Turner Designs, Sunnyvale, CA). Results are expressed as relative luciferase units (RLU) after

normalization to the *Renilla* luciferase activity.

Electrophoretic Mobility Shift Assay (EMSA) - For EMSAs, nuclear extracts were prepared from

Caco-2 cells using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentrations were

determined by Bio-Rad Protein Assay (Bio-Rad). The following single-stranded oligonucleotides and

their antisense complements were synthesized by Integrated DNA Technologies:

IR–1: (5'- GATCTCAAGAGGTCATTGACCTTTTTG-3')

apolipoprotein A-I: (5'-CAGAGCTGATCCTTGAACTCTTAAGTT-3')

NFRE: (5'-CATACAAGATCCTTGATATTAGCTGAA-3')

mutant IR-1: (5'-CAAATGAGCAAATTAACAATTCCAGTG-3')

CREB: (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3')

Sense and antisense strands of the same element were annealed by heating at 65°C for 10 minutes and cooling to room temperature. Double strand probes were labeled by γ-[^{32}P]ATP by T4 polynucleotide kinase (Promega, Madison, WI). Binding reactions contained 5 µg of crude nuclear extract and 2 µg of poly-(dI-dC)·(dI-dC) in a buffer containing 10 mM HEPES, pH 7.9, 1 mM MgCl$_2$, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10 % glycerol, and, when appropriate, various molar excesses of unlabeled double-stranded oligonucleotides. Reactions were carried out at room temperature for 20 minutes and protein-DNA complexes were separated by electrophoresis on 6% polyacrylamide gels in 0.25x Tris-borate-EDTA buffer (TBE) at 4°C. The gel was dried and exposed to blue sensitive film (Midwest Scientific, Valley Park, MO) and analyzed by NIH Image software (NIH, Bethesda, MD). Unlabeled double-stranded oligonucleotides corresponding to inverted repeat (IR)-1, apolipoprotein A-I, and NFRE mutant IR-1 and cyclic AMP response element-binding protein (CREB) were used as competitor DNAs.

RESULTS

lithocholic acid down-regulates the expression of UGT2B7-To determine whether UGT2B7 is a target of hFXR, Caco-2 and HepG2 cells were treated with various concentrations of lithocholic acid and chenodeoxycholic acid for 48 h. UGT2B7 mRNA levels at each concentration were analyzed by semi-quantitative RT-PCR, and the final data were obtained with real-time RT-PCR. lithocholic acid treatment of Caco-2 cells, which contain significant endogenous levels of both human hFXR and UGT2B7 mRNA (data not shown), resulted in a significant dose-dependent decrease in UGT2B7 mRNA levels as compared with vehicle-treated cells (Fig. 1A). Incubation of Caco-2 cells with increasing concentrations of chenodeoxycholic acid, a known hFXR activator, for 48 hours had no significant effect on UGT2B7 mRNA levels until the concentration reached 50 μM (Figure. 1B). To confirm these results quantitatively, real time RT-PCR experiments were performed (data not shown). An IC_{50} value of 13.7 μM for lithocholic acid-mediated inhibition of UGT2B7 mRNA expression was calculated from these real-time RT-PCR experiments.

To test the response of UGT2B7 gene expression to other, non-toxic bile acids, hyodeoxycholic acid and ursodeoxycholic acid were tested in Caco-2 cells, and RT-PCR showed that neither hyodeoxycholic acid nor ursodeoxycholic acid induced or suppressed UGT2B7 mRNA levels (data not shown). Experiments carried out with HepG2 cells showed that neither lithocholic acid nor chenodeoxycholic acid, up to a concentration of 75 μM, affected UGT2B7 mRNA levels (data not shown). The lack of effect of chenodeoxycholic acid on UGT2B7 mRNA in HepG2 cells was in an agreement with recently published data by (Barbier et al., 2003a).

In the next series of experiments, we investigated and compared the effect of lithocholic acid and chenodeoxycholic acid on I-BABP expression. We selected I-BABP because it was the first gene

identified as a target of chenodeoxycholic acid-activated hFXR, representing hFXR-mediated induction

(Grober et al., 1999). As shown in Figure 1C, lithocholic acid acted as an activator of I-BABP

expression up to a concentration of 10 μM, but at 20 μM, mRNA levels began to decrease and by 30 μM,

mRNA expression had returned to basal levels. In contrast, chenodeoxycholic acid showed a strong

activating effect on I-BABP up to 75 μM (Fig. 1D), in agreement with previous reports (Grober et al.,

1999). These experiments with I-BABP confirmed that both lithocholic acid and chenodeoxycholic

acid up-regulate the expression of I-BABP genes. Strong negative regulation by bile acids, especially

lithocholic acid, was observed exclusively with UGT2B7. Moreover UGT2B7 was also more sensitive

to the higher concentrations of chenodeoxycholic acid. The direct comparison of the effect of

chenodeoxycholic acid on UGT2B7 and I-BABP can be seen in Figure 1B and D. The experiments

with I-BABP serve as important positive controls for our studies with lithocholic acid and

chenodeoxycholic acid, proving that the effects of these two ligands are in fact gene dependant.

To exclude the possibility that toxic lithocholic acid affects the integrity of Caco-2 cells, we

checked their viability by Trypan Blue staining. All concentrations of lithocholic acid, including those

that significantly decreased UGT2B7 mRNA expression, were found to be well tolerated by the cells,

and no obvious cytotoxicity was observed. (Data not shown).

Finally, to assure that UGT2B7 mRNA suppression was not due to increased instability of mRNA in the

presence of lithocholic acid, we used actinomycin-D, an inhibitor of transcription, to evaluate the stability of

UGT2B7 mRNA in the presence of lithocholic acid. As shown in Figure 2, the half-life of UGT2B7 mRNA

was calculated by plotting the percentage of RNA remaining in actinomycin-D treated cells versus time and

fitting that plot using GraphPad Prisim4 software. The half lives of mRNA decay in the presence and

absence of lithocholic acid were 1.68 and 1.72 hours, respectively. Thus, treatment with lithocholic acid did

not affect the stability of UGT2B7 mRNA, indicating that the suppression of UGT2B7 mRNA is at the transcriptional level.

lithocholic acid down-regulation of UGT2B7 expression does not involve PXR – In our previously published experiments in Caco-2 cells (Gardner-Stephen et al., 2004), three UGT1A isoforms, which have been proven to be PXR target genes, were induced by rifampicin (Rif) treatment. Since lithocholic acid is also a ligand for PXR (Xie et al., 2001), we investigated whether UGT2B7 is also a target gene of PXR. To eliminate the possibility that negative regulation of UGT2B7 by lithocholic acid could be mediated via PXR, Caco-2 cells were treated with Rif, a PXR-specific ligand (Kliewer and Willson, 2002), and it was demonstrated that Rif had no effect on UGT2B7 transcription (data not shown). In HepG2 cells, Rif did not change UGT2B7 mRNA expression. Additional experiments involving the transfection of Caco-2 cells with activated PXR in the presence and absence of lithocholic acid, and it was demonstrated that PXR transfection had no effect on UGT2B7 transcription (data not shown). The outcome of both sets of experiments suggests that PXR is not involved in UGT2B7 down regulation.

The effect of chenodeoxycholic acid and lithocholic acid on the 1.3 kb UGT2B7 promoter fragment - In the first series of experiments, we compared the effects of lithocholic acid and chenodeoxycholic acid on the 1.3 kb UGT2B7 promoter construct in Caco-2 cells co-transfected with hFXR and/or hRXR (Fig 3). As observed previously, hFXR co-transfected into Caco-2 cells with 2B7(p1328)-Luc caused a decrease in luciferase activity even in the absence of ligand, and co transfection with hRXR additionally decreased the luciferase activity. However, the two bile acids had different effects on luciferase activity in these co-transfected cells. In the absence of receptors, 25 µM chenodeoxycholic acid did not suppress the luciferase activity, whereas 15 µM lithocholic acid resulted in a suppression of approximately 50%. In cells co-transfected with hFXR and then exposed to ligand, additional

16

suppression was observed in cells exposed to lithocholic acid, but chenodeoxycholic acid seemingly

protected the cells from some or all of the suppressive effects of hFXR and hRXR on luciferase activity.

Mapping of an hFXR response element in the human UGT2B7 promoter – We next investigated

whether the observed lithocholic acid-mediated changes in UGT2B7 gene expression were the due to

response elements located in the gene promoter. To localize the region within the UGT2B7 promoter

that contains transcriptional responsiveness to lithocholic acid, serial deletion constructs of the

promoter, from 2B7(p1328)-Luc to 2B7(p428)-Luc, were generated, cloned in the pGL3-luciferase

reporter plasmid, and transfected into Caco-2 cells, which were then treated with lithocholic acid (Fig.

4A). A significant decrease in reporter activity in response to lithocholic acid was observed for each

deletion constructs except p-128, indicating that the region controlling the suppressive effect of

lithocholic acid on the UGT2B7 promoter was between -428 bp and -128 bp.

To further demonstrate that the -428 to -128 bp region of the UGT2B7 promoter was responsible for

the hFXR-mediated lithocholic acid suppression, we measured the effect of lithocholic acid treatment

and co-transfection with hFXR and/or hRXR on these promoter constructs. Caco-2 cells were

transfected with the luciferase reporter plasmids driven by these constructs and co-transfected with the

expression vectors for hFXR and hRXR in the presence and absence of lithocholic acid (Fig 4B). While

the activity of the -428 bp promoter was suppressed by lithocholic acid, hFXR, and hRXR, this effect

was completely abolished in the -128 bp promoter fragment. These results demonstrate the importance

of the region between -428 to -128 bp in the regulation of the UGT2B7 promoter.

Identification and mutation of a NFRE in the -428 to -128 promoter region – Computer analysis of

the -428 to -128 bp region of the UGT2B7 promoter revealed the presence of a GATCCTTGATATTA

sequence between nucleotides -148 to -134, which resembles the NFRE, 3'-GATCCTTGAACTCT-5',

17

previously identified in apolipoprotein A-I (Claudel et al., 2002). To determine whether this sequence could be a NFRE, this 14 bp region was deleted from the 1.3 kb promoter luciferase construct, resulting in the 2B7(p1315)NFREΔ-Luc construct. We also mutated this response element in the luciferase construct to create 2B7(p1329)mut-Luc. Both of these new constructs were then analyzed for lithocholic acid suppression, and compared to that of 2B7(p1328)-Luc. As shown in Figure 5, these mutations abolished the suppression of UGT2B7 promoter activity by hFXR and hRXR. However, the effect of lithocholic acid alone was not completely reversed. Two conclusions can be drawn from these data. First, deletion of the NFRE eliminates the suppression of UGT2B7 mRNA by hFXR and hRXR suggesting that this is a true NFRE. Second, lithocholic acid suppression of UGT2B7 mRNA is only partially related to the presence of this NFRE; thus, a significant portion of the suppression is the result of another signaling pathway.

EMSA – Suppressive regulation of UGT2B7 by the NFRE indicated that this motif might be a binding site for hFXR. Binding of the hFXR complex to the NFRE in the UGT2B7 promoter was examined by EMSA (Fig. 6). As a control, a known NFRE from apolipoprotein A-I was also used as a probe. When nuclear extract prepared from Caco-2 cells treated with lithocholic acid was incubated with a ^{32}P-labeled UGT2B7-NFRE probe, induced DNA–protein complexes were detected (Fig. 6A). Binding of hFXR was inhibited in a concentration dependent manner by increasing concentrations of unlabeled oligonucleotides. A similar experiment was done with a ^{32}P-labeled IR-1 probe (Fig. 6B). Competition for the labeled probe was observed when a 50-fold excess of unlabeled IR-1, UGT2B7-NFRE, or apolipoprotein A-I-NFRE was included in the reaction. In order to eliminate unspecific binding, experiments were done using mutated IR-1 and CREB oligonucleotides as non-specific inhibitors (Fig. 6C). In contrast to the IR-1 and UGT2B7-NFRE probes, binding of the

mutated IR-1 and CREB oligonucleotides to Caco-2 nuclear extract was not observed under these

experimental conditions. These results indicated that hFXR binds specifically to the UGT2B7-NFRE.

DISCUSSION

In these studies, we have identified human UGT2B7 as a target gene of lithocholic acid-activated hFXR. However, UGT2B7 transcription is very strongly suppressed, rather than induced, by lithocholic acid. We have collected evidence that this lithocholic acid suppression occurs *via* hFXR binding to a novel NFRE in a 1.3 kb UGT2B7 promoter fragment. Our results also show that this suppression by hFXR is increased upon co-transfection with hRXR, indicating that hFXR binds to the NFRE as a heterodimer with hRXR. Thus, we have demonstrated that UGT2B7 is an hRXR-dependent, negatively regulated target gene of hFXR. This is in contrast to the bile acid-activated hFXR suppression of apolipoprotein A-I transcription mediated by a similar NFRE, but in a manner independent of hRXR (Claudel et al., 2002). Recently, it has been reported that another human UGT isoform, UGT2B4, is a target gene for hFXR and induction of this UGT increases bile acid glucuronidation in HepG2 cells (Barbier et al., 2003). In this case, hFXR binds as a monomer to the BARE in the UGT2B4 gene promoter. In addition, activation of hRXR represses the UGT2B4 gene expression induced by chenodeoxycholic acid and over-expression of UGT2B4 in HepG2 cells results in reduced hFXR-mediated induction of the short heterodimeric partner.

Our results reveal that, in Caco-2 cells, lithocholic acid dramatically decreased expression of UGT2B7 mRNA levels (Figs. 1) and also decreased the luciferase activity of the UGT2B7 promoter. In contrast, this effect was not observed in HepG2 cells. We carried out a series of experiments to examine the effect of transfection of hFXR on UGT2B7 mRNA expression in Caco-2 cells. Transfection of cells with hFXR resulted in a very significant suppression of UGT2B7 expression in the absence of lithocholic acid and additional suppression was observed when the cells were treated with lithocholic acid. The observation that hFXR, and hFXR/hRXR suppress UGT2B7 transcription in the absence of

ligand is not surprising. Many orphan receptors, such as human liver X receptor, are able to stimulate

gene transcription without ligand binding (Peet et al., 1998). Another example has been presented for

cytochromes P450 where, without bile acid activation, hFXR binds and stimulates rat cytochrome P450

7A1 mRNA expression in HepG2 cells; on the other hand, in the absence of hFXR, bile acids suppress

the transcription of the cytochrome P450 7A1 gene (Chiang et al., 2000). Our data demonstrate that

hFXR can act as a UGT2B7 transcriptional suppressor either when overexpressed and presumably

unliganded as well as in the presence of one of its natural ligands, lithocholic acid.

hFXR may compete with other nuclear receptors for limiting co-activators, such as steroid receptor

coactivator-1. For example, hepatic nuclear factor 4 (HNF_4) binds to the direct repeat (DR)-1 motif in

BARE-II and plays a role in the transactivation of cytochrome P450 7A1 transcription (Crestani et al.,

1998). Evidence has also been presented showing that the suppression of apolipoprotein A-I by bile

acid, mediated by hFXR, is carried out in a manner independent of the SHP regulatory pathway, but *via*

a NFRE (Claudel et al., 2002). It has been documented that PXR is a bile acid receptor that plays a

critical role in bile acid detoxification by the liver; specifically, lithocholic acid has been identified as a

PXR activator ligand (Xie et al., 2001). In the present studies, we found no evidence that lithocholic

acid activation of PXR suppresses the transcription of UGT2B7. Specifically, the PXR specific ligand,

Rif, had no effect on the repression of UGT2B7 transcription in Caco-2 cells, indicating that PXR is not

involved in this phenomenon. The exclusion of SHP and PXR involvement suggests that the

down-regulation of UGT2B7 by lithocholic acid is mediated though the supressor activity of hFXR.

In order to understand the mechanism of this negative regulation, we cloned a 1.3 kb fragment of

UGT2B7 promoter and carried out deletion analysis in order to identify the region of the promoter

involved in the repression of UGT2B7 transcription. Promoter deletion identified a promoter fragment

21

between -428 and -128 bp as being involved in the bile acid-activated hFXR-mediated decrease in

UGT2B7 promoter activity. Computer-assisted analysis of this fragment revealed nucleotide sequences

relevant to the binding of hFXR and regulation *via* lithocholic acid. It is recognized that hFXR REs are

inverted repeats in which consensus receptor-binding hexamers are separated by one nucleotide (IR-1).

hFXR/hRXR heterodimers bind to these specific DNA motifs. However, hFXR/hRXR can also bind to

direct repeats of the AGGTCA hexamer separated by 0, 3, or 4, nucleotides (Laffitte et al., 2000).

Moreover, hFXR also binds as a monomer to a negative regulation site in the apolipoprotein A-1 gene

promoter (Claudel et al., 2002). In this study, we have demonstrated that hFXR can bind to the slightly

modified NFRE in the human UGT2B7 promoter in a manner similar to that described for the

apolipoprotein A-1 promoter Mutations of this element in the UGT2B7 -1328 bp promoter fragment

abolished the repression of UGT2B7 promoter activity by hFXR and/or hRXR, indicating an absolute

requirement of this fragment for the hFXR-induced repression.

The physiological role of the UGT2B7 suppressive regulation pathway remains to be clearly

delineated. We have demonstrated experimentally that cholestatic lithocholic acid is converted to the

hydrophilic hyodeoxycholic acid in a reaction catalyzed by cytochrome P450 3A4 (Radominska et al.,

1991; Xie et al., 2001). Since, in humans, hyodeoxycholic acid-6-0-glucuronide, rather than

hyodeoxycholic acid itself, is excreted in bile and urine, we postulated that coupling between

cytochrome P450 3A4 and UGT2B7 occurs as an indispensable pathway for lithocholic acid

detoxification (Radominska et al., 1991; Zimniak et al., 1991). We have also demonstrated that, in rats,

lithocholic acid can be directly glucuronidated at the 3-OH position, forming the 3-O-glucuronide of

lithocholic acid one of the most cholestatic compounds identified thus far (Oelberg et al., 1984). The

sequential detoxification of lithocholic acid by cytochrome P450 3A4 and UGT2B7 is highly specific to

humans. We propose the pathway depicted in Figure 12 as a putative mechanism for hFXR/PXR-mediated bile acid regulation of the transcription of several genes involved in the synthesis and detoxification of lithocholic acid. In this figure, lithocholic acid is produced from chenodeoxycholic acid by the action of bacterial 7α-dehydroxylase. Subsequently, lithocholic acid is specifically hydroxylated at carbon 6 by cytochrome P450 3A4 and then conjugated at this hydroxyl group to produce a water soluble glucuronide. We have shown previously that the 6-hydroxyl group of hyodeoxycholic acid and both the carboxyl and hydroxyl group of lithocholic acid can be glucuronidated by human UGT2B7 (Gall et al., 1999).

As presented in Figure 7, there are three enzymes involved in the biosynthesis (7α-dehydroxylase) and detoxification (cytochrome P450 3A and UGT2B7) of lithocholic acid. Detoxification of lithocholic acid by cytochrome P450 3A4 is up-regulated by activation of cytochrome P450 3A4 expression by lithocholic acid-activated PXR. cytochrome P450 3A4 converts lithocholic acid to hyodeoxycholic acid, which, as a substrate for UGT2B7, is effectively glucuronidated and then excreted in bile and/or urine. Up-regulation of cytochrome P450 3A4 via lithocholic acid is a significant hepatoprotective mechanism. However, we have unexpectedly discovered that UGT2B7, the final enzyme involved in this scheme, is down regulated in Caco-2 cells by the presence of high levels of lithocholic acid. The down regulation of this last enzyme involved in the lithocholic acid detoxification process, could be highly deleterious both in the intestine and the liver.

We would like to emphasize that suppression of UGT2B7 mRNA levels might occur only in high concentrations of lithocholic acid. It has been demonstrated that low, micromolar concentrations of lithocholic acid are effectively cleared *via* sulfation (Halvorsen et al., 1999; Radominska et al., 1993); whereas at high concentrations, glucuronidation takes over the lithocholic acid detoxification process.

Therefore, we postulate that the suppression of UGT2B7 expression might only occur under pathological conditions, such as liver damage and/or cholestasis, which are known to produce significant levels of lithocholic acid.

All this work in contrast to work done by Barbier et al. (Barbier et al., 2003a) in human hepatocytes, where hFXR activated *via* chenodeoxycholic acid induces UGT2B4 and may contribute to a reduction of bile acid toxicity, positively affecting bile acid metabolism and cholesterol homeostasis in human hepatocytes. The effect of lithocholic acid on UGT2B7 expression was not investigated in this hepatocyte model. In our experiments with UGT2B7 in Caco-2 or HepG2 cells, chenodeoxycholic acid did not show any activating effect. Nevertheless, these two human UGTs, both of which are involved in the glucuronidation of bile acid, despite their differing repressive/inductive mechanisms, are significantly regulated by their bile acid substrates.

In this study, we have demonstrated that hFXR negatively regulates UGT2B7 gene expression by binding to a NFRE. This is the first, novel demonstration of suppression of UGT genes in general and, specifically, in response to their bile acid substrates. In addition, we cannot exclude that other, as yet unknown, mechanisms distinct from hFXR may contribute to the observed repression of UGT2B7 transcription by lithocholic acid. Several mechanisms could underlie the distinct behavior of hFXR with differing hFXR REs. These alternatives are currently under investigation.

ACKNOWLEDGEMENTS

REFERENCES

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangeldorf DJ and Suchy FJ (2001) Human
bile salt export pump is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* **276**:
28857-28865.

Barbier O, Peneda Torra I, Sirvent A, Claudel T, Blanquart C, Duran-Sandoval D, Kuipers F, Kosykh V,
Fruchart J-C and Staels B (2003a) FXR induces the UGT2B4 enzyme in hepatocytes: a potential
mechanism of negative feedback control of FXR activity. *Gastroenterololg* **124**: 1926-1940.

Bjorkhem I (1985) Mechanism of bile acid biosynthesis in mammalian liver. in *Sterols and bile acids*
New comprehensive biochemistry"(Danielsson H and Sjovall J, eds., ed.) pp 231-278, Elsevier,
Amsterdam, New York.

Borgstrom B, Barrowman JR and Lindstrom M (1985) Roles of bile acids in intestinal lipid digestion
and absorption. in *Sterols and Bile Acids"*(Danielsson H and Sjovall J, ed.) pp 405-425, Elsevier,
Amsterdam and New York.

Burchell B, Brierley CH, Monaghan G and Clarke DJ (1998) The structure and function of the
UDP-glucuronosyltransferase gene family. *Adv Pharmacol* **42**: 335-338.

Chiang JY, Kimmel R, Weinberger C and Stroup D (2000) Farnesoid X receptor responds to bile acids
and represses cholesterol 7α-hydroxylase gene (CYP7A1) transcription. *J Biol Chem* **275**:
10918-10924.

Chiang JYL (2002) Bile acid regulation of gene expression: Roles of nuclear hormone receptors.
Endocrine Reviews **23**: 443-463.

Chowdhury JR, Novikoff PM, Chowdhury NR and Novikoff AB (1985) Distribution of
UDPglucuronosyltransferase in rat tissue. *Proc Natl Acad Sci USA* **82**: 2990-2994.

Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, Fruchart J-C, Gonzalez FJ and Staels B (2003) Farnesoid X receptor agnoists suppress hepatic apolipoprotein CIII expression. *Gastroenterology* **125**: 544-555.

Claudel T, Sturm E, Duez H, Torra IP, Sirvent A, Kosykh V, Fruchart J-C, Dallongeville J, Hum DW, Kuipers F and Staels B (2002) Bile acid-acivated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. *J Clin Invest* **109**: 961-971.

Congiu M, Mashford ML, Slavin JL and Desmond PV (2002) UDP-glucuronosyltransferase mRNA levels in human liver disease. *Drug Metab Dispos* **30**: 129-134.

Crestani M, Sadeghpour A, Stroup D, Galli G and Chiang JY (1998) Transcriptional activation of the cholesterol 7α-hydroxylase gene (CYP7A) by nuclear hormone receptors. *J Lipid Res* **39**: 2192-2200.

Gall WE, Zawada G, Mojarrabi B, Tephly TR, Green MD, Coffman BL, Mackenzie PI and Radominska-Pandya A (1999) Differential glucuronidation of bile acids, androgens and estrogens by the human recombinant UDP-glucuronosyltransferases UGT1A3 and 2B7. *J Steroid Biochem Mol Biol* **70**: 101-108.

Gardner-Stephen DA, Heydel J-M, Goyal A, Lu Y, Xie W, Lindblom T, Mackenzie P and Radominska-Pandya A (2004) Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. *Drug Metab Dispos* **32**: 340-347.

Grober J, Zaghini I, Fujii H, Jones SA, Kliewer SA, Willson TM, Ono T and Besnard P (1999) Identification of a bile acid=responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/0-cis-retinoic acid receptor heterodimer. *J Biol Chem* **274**: 29749-29754.

Halvorsen B, Kase BF, Prydz K, Garagozlian S, Andrresen MS and Kolset SO (1999) Sulphation of

lithochlic acid in the colon-carcinoma cell line Caco-2. *Biochem J* **343**: 533-539.

Kliewer SA and Willson TM (2002) Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res* **43**: 359-364.

Knerr I, Dachert C, Beinder E, Metzler M, Dotsch J, Repp R and Rascher W (2002) Adrenomedullin, calcitonin gene-related peptide and their receptors: evidence for a decreased placental mRNA content in preeclampsia and HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol* **101**: 47-53.

Laffitte B, Kast H, Nguyen C, Zavacki A, Moore D and Edwards P (2000) Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor. *J Biol Chem* **725**: 10638-10647.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR and Mangelsdorf DJ (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science* **296**: 1313-1316.

Oelberg DG, Chari MV, Little JM, Adcock EW and Lester R (1984) Lithocholate glucuronide is a cholestatic agent. *J Clin Invest* **73**: 1507-1514.

Peet DJ, Janowski BA and Mangelsdorf DJ (1998) The LXRs: a new class of oxysterol receptors. *Curr OpinGenet Dev* **8**: 571-575.

Princen HMG, Post SM and Twisk J (1997) Regulation of bile acid biosynthesis. *Curr Pharm Design* **3**: 59-84.

Radominska A, Treat S and Little J (1993) Bile acid metabolism and the pathophysiology of cholestasis. *Sem in Liver Disease* **13**: 219-234.

Radominska A, Zimniak P and Lester R (1991) The detoxification of bile acids. in *Bile Acids as Therapeutic Agents From Basic Science to Clinical Practice"* (Paumgartner G, Stiehl A and Gerok W, ed.) pp 99-109, Kluwer Academic Publishers, Dordrecht, Boston, London.

Radominska-Pandya A, Czernik P, Little JM, Battaglia E and Mackenzie PI (1999) Structural and functional studies of UDP-glucuronsyltransferases. *Drug Metab Rev* **31**: 817-900.

Russell DW and Setchell KDR (1992) Bile acid biosynthesis. *Biochemistry* **31**: 4737-4749.

Schuetz EG, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, Lamba J, Kim RB, Ramachandran V, Komorski BJ, Venkataramanan R, Cai H, Sinal CJ, Gonzalez FJ and Schuetz JD (2001) Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J Biol Chem* **276**: 39411-39418.

Staudinger JL, Madan A, Carol KM and Parkinson A (2003) Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab Dispos* **31**: 523-527.

Stedman C, Robertson G, Coulter S and Liddle C (2004) Feed-forward regulation of bile acid detoxification by CYP3A4: studies in humanized transgenic mice. *J Biol Chem* **279**: 11336-11343.

Sumida A, Kinoshita K, Fukuda T, Matsuda H, Yamamoto I, Inaba T and Azuma J (1999) Relationship between mRNA levels quantified by reverse transcription-competitive PCR and metabolic activity of CYP3A4 and CYP2E1 in human liver. *Biochem Biophys Res Comm* **262**: 499-503.

Takeshita A, Taguchi M, Koibuchi N and Ozawa Y (2002) Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. *J Biol Chem* **277**: 32453-32458.

Torra IP, Claudel T, Duval C, Kosykh V, Fruchart J-C and Staels B (2003) Bile acids induce the expression of the human peroxisome proliferator-activated receptor α gene via activation of the farnesoid X receptor. *Mol Endocrinol* **17**: 259-272.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: metabolism, expression and disease. *Ann Rev Pharmacol Toxicol* **40**: 581-616.

Urizar NL, Dowhan DH and Moore DD (2000) The farnesoid X-activated receptor mediates bile acid

activation of phospholipid transfer protein gene expression. *J Biol Chem* **275**: 39313-39317.

Vlahcevic ZR, Eggersten G, Bjorkhem I, Hylemon PB, Redford K and Pandak WM (2000) Regulation

of sterol 12α-hydroxylase and cholic acid biosythesis in the rat. *Gastroenterology* **118**: 599-607.

Xie W, Barwick JL, Simon CM, Pierce A, Safe S, Blumberg B, Guzelian PS and Evans RM (2000)

Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes

Dev* **14**: 3014-3023.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ and Evans RM

(2001) An essential role for SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci*

98: 3375-3380.

Zimniak P, Radominska A and Lester R (1991) Phase I and II biotransformation of bile acids. in *Hepatic

Metabolism and Disposition of Endo- and Xenobiotics"*(Bock KW, Gerok W, Matern S and Smith R,

ed.) pp 183-192, Kluwer Academic Publishers, Dordrecht/Boston/London.

FOOTNOTES (Unnumbered footnote on Page 1.)

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LEGENDS FOR FIGURES

Figure 1.   The effect of lithocholic acid and chenodeoxycholic acid on UGT2B7 mRNA

expression. Caco-2 cells seeded in 6-well plates at a density of 0.5 x 10^6 cells per well were treated

with various concentrations (10, 20, 25, 30, 35 µM) lithocholic acid (Panel A and C) and (10, 25, 50, 75

µM) chenodeoxycholic acid (Panel B and D) for 48 hours.   Total RNA was prepared and UGT2B7

(Panel A and B) and I-BABP (Panel C and D) mRNA expression was analyzed by semi-quantitative

RT-PCR (as described in "Experimental Procedures"). Results are expressed as the ratio of treated cells

to the control cells treated with vehicle (fold change from control), and are the mean ± SEM of three

experiments.   The results were analyzed by one way ANOVA followed by a Dunnett's Multiple

Comparison Test. Measurements that vary significantly from the DMSO control are indicated. (*:

$p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$)

Figure 2. Analysis of stability of UGT2B7 mRNA in Caco-2 cells. Stability of UGT2B7 mRNA was

analyzed by addition of actinomycin-D (1 µg/ml). Total RNA was extracted at various time points and

RT-PCR of UGT2B7 mRNA was performed. Plotted data are expressed as "Percent mRNA remaining," and

are representative of 3 independent experiments. The half-life of UGT2B7 mRNA in actinomycin-D treated

cells was calculated by fitting the percent mRNA remaining vs. time data with a one phase exponential decay

equation using GraphPad Prism 4. ($Y = Span*e^{-K*X} + Plateau$; where X=time, and Y=% mRNA remaining.

Y=SPAN+PLATEAU at X=0 and decreases to PLATEAU with a rate constant K. The half-life of the decay

is 0.6932/K).

Figure 3.   Effect of chenodeoxycholic acid and lithocholic acid on the 1.3 kb UGT2B7

promoter.   Caco-2 cells were transfected with 2B7(p1328)-Luc and either pCMX-hFXR,

pCMX-hRXR and/or pRL-TK and treated with vehicle, 15 µM lithocholic acid (panel A), or 25 µM

chenodeoxycholic acid (panel B). Lysed cells were assayed for luciferase activity 48 hours later. The

results, normalized to *Renilla* luciferase activity, are expressed as fold change from control and are the

mean ± SD of triplicate determinations. The results were analyzed by one way ANOVA followed by a

Dunnett's Multiple Comparison Test. Measurements that vary significantly from the DMSO control are

indicated. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

Figure 4. Analysis of the proximal promoter region of human UGT2B7 gene and the

localization of a NFRE in the 428 kb UGT2B7 promoter fragment. In Panel A, a series of DNA

UGT2B7 promoter fragments were cloned into the pGL3-Basic luciferase reporter vector and tested for

suppression of UGT2B7 in the presence of 15 µM lithocholic acid (panel A). Constructs were

transiently transfected into Caco-2 cells together with pRL-TK. Cells were treated with vehicle or 15

µM lithocholic acid and lysed cells were assayed for luciferase activity 48 hours later. The results,

normalized to *Renilla* luciferase activity, are expressed as relative luciferase units (RLU) and are the

mean ± SD of triplicate determinations. The results were analyzed by one way ANOVA followed by a

Dunnett's Multiple Comparison Test. Measurements for lithocholic acid treatment that vary

significantly from the DMSO control are indicated. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

In Pannel B, Caco-2 cells were transfected with 2B7(p428)-Luc or 2B7(p128)-Luc and either

pCMX-hFXR, pCMX-hRXR or pRL-TK. Cells were then treated with vehicle or 15 µM lithocholic

acid and lysed cells were assayed for luciferase activity 48 hours later. The results were, normalized to

Renilla luciferase activity, are expressed as fold change from control, and are the mean ± SD of

triplicate determinations. The results were analyzed by two way ANOVA followed by a Bonferroni

post test. Measurements for the p-128 construct that vary significantly from the p-428 construct are

indicated. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

Figure 5. Mutation and Deletion of NFRE in the 1.3 kb UGT2B7 promoter. A deletion

construct, 2B7(p1315)NFREΔ-Luc, and a mutation construct, 2B7(p1315)mut-Luc, were prepared

from 2B7(p1328)-Luc by PCR-based site directed mutagenesis. Caco-2 cells were transfected with

2B7(p1315)NFREΔ-Luc,   2B7(p1315)mut-Luc,   or   2B7(p1328)-Luc,   and/or   pCMX-hFXR,

pCMX-hRXR and pRL-TK. Cells were then treated with vehicle or 15 µM lithocholic acid and lysed

cells were assayed for luciferase activity 48 hours later. The results, normalized to *Renilla* luciferase

activity, are expressed as fold of control, and are the mean ± SD of triplicate determinations. The results

were analyzed by two way ANOVA followed by a Bonferroni post test. Measurements for the

2B7(p1315)NFREΔ-Luc or 2B7(p1315)mut-Luc constructs that vary significantly from the

2B7(p1328)-Luc construct are indicated. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$)

Figure 6. A NFRE in the UGT2B7 promoter can bind to the hFXR/hRXR heterodimer in a

nuclear extract of Caco-2 cells. EMSA analysis was carried out by incubating ^{32}P -labeled NFRE and

IR-1 sites with 5 µg of Caco-2 nuclear extract. In addition to specific competitors, mutant IR-1 and

CREB oligonucleotides were used as non-specific competitors. Competition experiments were

performed by adding 0, 50, and 100-fold molar excesses of the indicated unlabeled probes. The specific

hFXR/hRXR complex in nuclear extract is indicated by an *arrow*.

Figure 7.   Putative hFXR/PXR-mediated mechanism of bile acid regulation of gene

transcription.

Table 1: Primers and conditions for semi-quantitative PCR analysis. Nucleotide sequences are reported for primer pairs used in RT-PCR analysis experiments in HepG2 and Caco-2 cells.

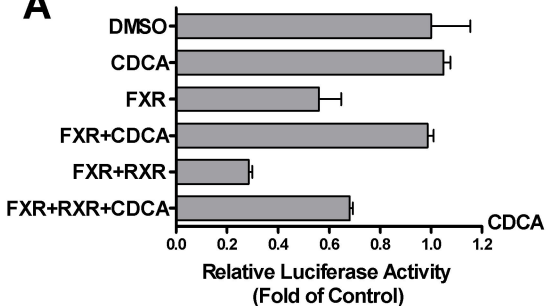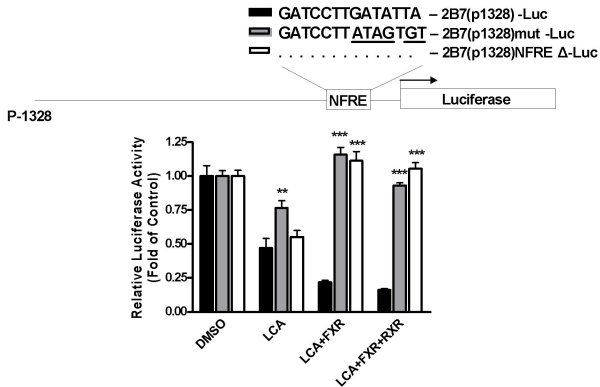| Target Transcript	Primer Set F: Forward primer 5'-3' R: Reverse primer 5'-3'	Anneal. Temp (°C)	Amplicon Size (bp)	No. of cycles on HepG2 cDNA	No. of cycles on Caco-2 cDNA
FXR	F: ggaaccatactcgcaatacagc R: tcgcatgtacatatccatcacac	56	402	31	32
PXR	F: caagccaagtgttcacagtgag R: caaagagcacagatcttccg	60	818	35	35
UGT2B7	F: agttggagaatttcatcatgcaacaga R: tcagcccagcagctcaccacaggg	58	232	26	30
I-BABP	F: gacttaggggctgagcctcagca R: ttgctcacgcgctcataggtcac	60	491	-	34
GAPDH	F: acccactcctccacctttg R: ctcttgtgctcttgctggg	64	178	25	25

Figure 1
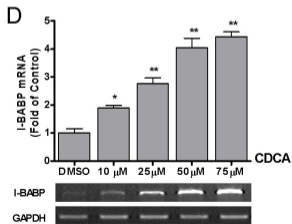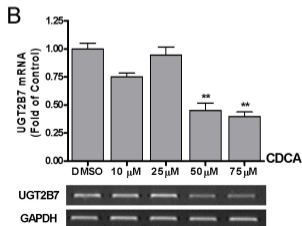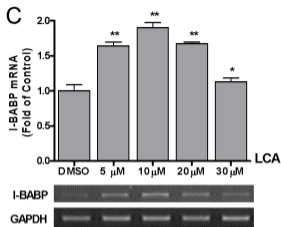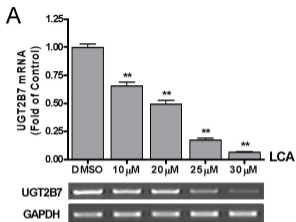
Figure 2
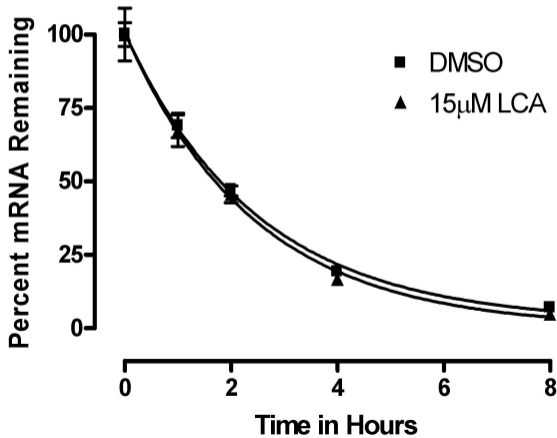
Figure 3
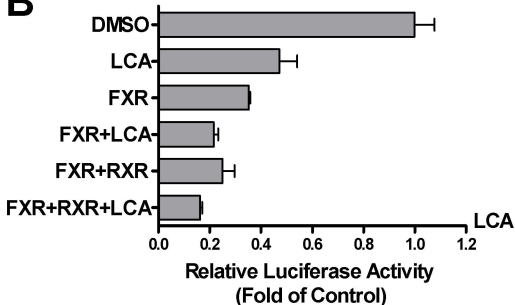
Figure 4

A

B

Figure 5

Figure 6

A. labeled NFRE probe

Caco-2 NE + + + + + +
Unlabeld Competitor: NFRE / Apo A-I

B. labeled IR-1 probe

Caco-2 NE + + + + + + + + +
Unlabeld Competitor: IR-1 / NFRE / Apo A-I

Free Probe →

C. labeled NFRE probe

Caco-2 NE + + + + + + + + + + + +
Unlabeld Competitor: IR-1 / NFRE / mut IR-1 / CREB

Figure 7

CDCA (3α, 7α) → *7α-dehydroxylase / Bacteria* → LCA (3α) → *CYP3A / Liver* → HDCA (3α, 6α) → *UGT2B7* → [glucuronide product]

+ (RXR–PXR with LCA, DNA Strands)

− (RXR–FXR with LCA, DNA Strands)