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CHAPTER 5
FXR-activating ligands inhibit rabbit ASBT expression via FXR-SHP-FTF cascade

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Li, Hai, Frank Chen, Quan Shang, Luxing Pan, Benjamin L. Shneider, John Y. L. Chiang, Barry M. Forman, M. Ananthanarayanan, G. Stephen Tint, Gerald Salen, and Guorong Xu. FXR-activating ligands inhibit rabbit ASBT expression via FXR-SHP-FTF cascade. Am J Physiol Gastrointest Liver Physiol 288: G60-G66, 2005; doi:10.1152/ajpgi.00170.2004.—The regulation of the rabbit apical sodium-dependent bile acid transporter (ASBT) was studied both in vivo and in vitro. New Zealand White rabbits were fed 0.5% deoxycholic acid (DCA) or SC-435, a competitive ASBT inhibitor, for 1 wk. In DCA-fed rabbits, ASBT expression was repressed, associated with activated FXR, and evidenced by increased ileal short heterodimer partner (SHP) mRNA. Feeding SC-435 to the rabbits blocked bile acid absorption, decreased SHP mRNA, and increased ASBT expression. A 1.9-kb rabbit ASBT 5′-flanking region (promoter) was cloned, and a cis-acting element for α-fetoprotein transcription factor (FTF) was identified (-1166 to -1158). The effects of transcriptional factors and different bile acids on the rabbit ASBT promoter were studied in Caco-2 cells. FTF stimulated the rabbit ASBT promoter activity fourfold but not after the FTF binding site was deleted from the promoter. Increasing the SHP protein notably inhibited FTF-dependent trans-activation of rabbit ASBT. Adding hydrophobic bile acids deoxycholic acid, chenodeoxycholic acid, and cholic acid, activating ligands for FXR, inhibited rabbit ASBT promoter activity in Caco-2 cells, but this inhibitory effect was abolished after the FTF binding site was deleted. Ursodeoxycholic acid and ursolic acid, nonactivating ligands for FXR, did not repress ASBT promoter activity. Thus the rabbit ASBT promoter is negative-feedback regulated by bile acids via a functional FTF binding site. Only FXR-activating ligands can downregulate rabbit ASBT expression through the regulatory cascade FXR-SHP-FTF.

The apical sodium-dependent bile acid cotransporter (ASBT/SLC10A2) is the primary bile salt uptake protein in the intestine. It is mainly located on the apical surface of the terminal ileal enterocytes and is also expressed on renal proximal tubular cells and large cholangiocytes (12, 19). ASBT is an efficient transporter for conjugated and unconjugated bile salts. Bile salt reabsorption by ASBT in the ileum is sodium dependent and can be saturated (7). ASBT has been cloned from the human (26), rabbit (11), rat (19), mouse (17), and hamster (25).

Regulation of ASBT expression by intestinal bile acid flux has been studied in guinea pigs (13), rats (2, 8, 10, 18, 20), and mice (21). However, whether ASBT expression is positively or negatively regulated by increasing bile acid flux remains controversial. Observations in guinea pigs (13) and mice (21) showed that ASBT was negatively regulated by the intestinal bile acid flux, whereas in rats, ASBT was positively regulated by bile acids (8, 10, 18, 20). Nevertheless, the results reported by Arrese et al. (2) showed that in rats, no regulatory response to changes in the intestinal bile acid flux occurred. Recently, Chen et al. (5) identified a physiologically functional liver receptor homolog-1 (LRH-1; also called FTF, α-fetoprotein transcription factor in other species) transcriptional binding site in the mouse ASBT promoter that was not present in the rat. As a result, chenodeoxycholic acid (CDCA), an activating ligand of nuclear receptor farnesoid X receptor (FXR), repressed mouse ASBT expression through the FXR/short heterodimer partner (SHP)/LRH-1 [α-fetoprotein transcription factor (FTF)] cascade. This negative feedback regulation did not occur in rat ASBT because of lack of the FTF binding site. It has been proposed that bile acids downregulate CYP7A1, the rate-limiting enzyme for bile acid synthesis, by activating the FXR/SHP/FTF cascade (9, 14). Our recent study (27) showed that in rabbits, hydrophobic deoxycholic acid (DCA), but not hydrophilic ursolic acid (UCA), activated FXR and then repressed CYP7A1 expression through the FXR/SHP/FTF cascade.

This study was designed to determine whether in rabbits, ASBT expression is negatively regulated by bile acids, in particular, FXR-activating ligands; whether there is a functional FTF binding site in rabbit ASBT promoter region, and rabbit ASBT is also regulated by bile acids via FXR/SHP/FTF cascade; and whether rabbit ASBT should be downregulated only by FXR-activating ligands.

MATERIALS AND METHODS

Animal Studies

Male New Zealand White (NZW) (n = 18) rabbits weighing 2.5–2.75 kg (Convance, Denver, PA) were used in this study. Six rabbits were fed regular rabbit chow, six rabbits were fed regular chow containing 0.5% DCA (Purina Mills, St. Louis, MO), and six rabbits were fed a competitive inhibitor of ASBT, SC-435 (24) (125 mg·kg⁻¹·day⁻¹), with regular chow for 7 days. Feces were collected

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during the last 2 days of the feeding experiment for measurement of fecal bile acid outputs. One-half of the rabbits in each study group was killed to collect distal one-third ileal mucosa specimens, which were immediately frozen for measurement of mRNA levels of ASBT and SHP. Bile fistulas were constructed with the remaining half of the rabbits (3/6 in each group). The bile drainage continued for 5 days to recover the DCA pool to calculate the total bile acid pool as previously described (28).

Animal protocol was approved by the Institutional Animal Care and Use Committee at Veterans Affairs Medical Center, East Orange, NJ and the Institutional Animal Care and Use Committee at University of Medicine and Dentistry-New Jersey Medical School, Newark, NJ.

Cell Culture

Human Caco-2 colon epithelial cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM, containing 10% fetal calf serum. Plasmid-transfected cells were cultured for 40 h in DMEM containing 0.5% charcoal-treated fetal calf serum before harvesting for reporter gene assays. The change in medium was designed to minimize the effect of bile salts found in fetal calf serum, as previously reported (5).

Cloning and Construction of Rabbit ASBT Promoter

The rabbit ASBTS' flanker 3.4-kb fragment was amplified by PCR using the following primers: sense: 5'-gatattagctgcctttactae-3' and antisense: 5'-aaccttagctagagaaatc-3' according to GeneBank accession no. AJ002005. Then its fragments were subcloned into a mammalian expression vector pGL3-Basic (Promega, Madison, WI) upstream of a firefly luciferase gene to form rabbit pGL3-ASBTS'/3.4 kb. With the use of this 3.4-kb ASBTS' sequence as a template, another two fragments were PCR synthesized and subcloned into pGL3 basic vector forming the rabbit ASBTS' construct pGL3-ASBTS'/1.9 kb (P2) and pGL3-ASBTS'/1.1 kb (P1). P1 corresponds to pGL3-ASBTS'/1-1082/+79, and P2 corresponds to pGL3-ASBTS'/1-1816/+79.

DNA Oligonucleotides and Site-Directed Mutagenesis

To prepare ASBTS', FTI binding site-deleted mutant construct, complimentary strands of DNA oligonucleotides containing the appropriate sequences and the desired nine nucleotides-deleted mutation for whole FTI element were synthesized (Integrated DNA Technologies, Coralville, IA): 5'-frac2ttataacacacacctgttagactccggggttcca-3'; 5'-aaccttagctagagaaatc-3'; 5'-aaccttagctagagaaatc-3' and 5'-aaccttagctagagaaatc-3'; and 5'-aaccttagctagagaaatc-3'. The site-directed point mutagenesis of rabbit ASBTS' sequence was performed by a Quick Change site-directed mutagenesis kit (Stratagene, Coralville, LA) targeted to the potential FTI cis-acting element (positioned from -1166 to -1158). The resulting construct P2m was the FTI binding site-deleted P2. The mutation (deletion of FTI cis-acting element) was confirmed by DNA sequencing.

Nuclear Protein Extraction and Gel-Shifting

Nuclear proteins were extracted from frozen rabbit ileal mucosa using NE-PER nuclear extraction reagents (Pierce, Rockford, IL). The protein concentration of nuclear extracts was determined by the protein assay kits (Bio-Rad, Hercules, CA), with bovine serum albumin used as a standard. The DNA protein binding reaction was carried out as described previously (27), with 5 μg of nuclear protein extract and 5,000 counts/min of end-labeling DNA oligos. Competition experiments were performed using unlabelled cis-element oligo probes and unlabelled mutant cis-element oligo probes. Samples were analyzed by 7% native polyacrylamide gel. The cis-element probe, used as a rabbit FTI-specific probe, was a double-stranded oligonucleotide containing the sequence sense (strand): 5'-gtttggttagagaaatc-3', and antisense: 5'-ccggagttggttagatgtttgcctaaac-3'. The sequence of the FTI mutant probes were 5'-gtttggttagagaaatcctcagactgac-3' and 5'-ccggagttggttagatgtttgcctaaac-3'.

Transient Transfection and Firefly Luciferase Assay

Confluent Caco-2 cells (~5 million cells/plate) were harvested and resuspended in 700 μL of phosphate-buffered saline containing 20 μg of rabbit ASBTS'/luciferase hybrid plasmid construct and 0.1 μg of control plasmid containing a thymidine kinase promoter-driven Renilla luciferase gene (Promega). Transfection was accomplished by electroporation (6) at 0.22 kV and 9.5 μF × 1,000 (Bio-Rad). After electroporation, the cells were cultured for an additional 40 h before performing dual luciferase assays (Promega) as described by manufacturer.

Expression plasmid constructs

pCDNA3-mLRH-1 (a generous gift from Dr. Alan R. Tall, Columbia University, New York, New York) directs expression of the mouse LRH-1 gene, which is a homolog of the orphan nuclear receptor fushi tarazu F1 from Drosophila and is called a fett protein transcription factor (FTF) in other species. This mouse LRH-1 plasmid construct was used to express FTF in the present study. pCMX-mutant SHP, pCDNA3-human FXR (hFXR), and PCMX-human retinoid X receptor (hRXR); generous gift from Dr. David Mangelsdorf, University of Texas, Dallas, TX) harbor a copy of the mouse SHP, hFXR, and human RXR genes, respectively. PCMX-hFXR-W469A (from Dr. David Mangelsdorf), a mutated hFXR, contains a point mutation within the AF-2 domain of the gene (1). The resulting gene product contains bile acid and DNA binding domains but lacks a functional trans-activation motif. The effect of the orphan nuclear receptors on basal activity and bile acid-mediated responses was assessed by cotransfection of these constructs with pGL3-rabbit ASBTS' (the cloned rabbit ASBTS' promoter) in luciferase-transfected Caco-2 cells.

Real-Time PCR Assay

In this study, mRNA levels were also quantitatively measured by real-time PCR, and the data shown in the text are relative fold. Total RNA was isolated and treated with DNase I by Absolutely RNA RT-PCR mini-prep kit (Stratagene). One microgram of DNase I of treated total RNA was reverse transcribed by Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA) using the oligo-dT primer. Real-time PCR was performed with the ABI PRISM 7700 sequence detection system using one fiftieth of the reverse transcription (RT) reaction, and was analyzed with the 1.7 software (Applied Biosystems, Forest City, CA). Rabbit SHP, ASBT, cyclophilin primers, minor groove binder (MGB) probes, human SHP, FTI, cyclophilin primers, and MGB probes were synthesized by Applied Biosystems with Assays-by-Design service (Applied Biosystems, Forest City, CA). PCR was carried out in a 50-μL reaction volume containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 20-ng cDNA templates, 0.9 μM of each forward and reverse primer, and a 0.25 μM MGB probe. Cyclophilin was set as loading control. Because validation experiments showed that amplification efficiency of the target and the cyclophilin were approximately equal, accumulation was performed using the comparative ΔΔC method (4, 22). The detecting primers and probes were rabbit ASBT (sense: 5'-ctgtgccctttacctcatacaca-3'; antisense: 5'-agagaaaaactgatggacatct-3'; probe: ttgctctgacagctgac-3'; antisense: 5'-gctctctgcaccttcct-3'; probe: ctgagaaacctggctgac), and rabbit cyclophilin (sense: 5'-ccctgtgtttctgctgttttttgctct-3'; antisense: 5'-gcctgtttctgctgttttttgctct-3'; probe: cagcagacatccagctgac). The primers and the assay ID of human SHP, FTI, and cyclophilin in Assay-by-Demand (for detecting Caco-2 cell's SHP, and FTI mRNA expression after P2 cotransfection and DCA treatment) were
Biliary bile acid analysis. Bile acids in the bile were measured by the capillary gas-liquid chromatograph (GLC) method as previously described (28).

Fecal bile acid analysis. Internal standard (nor-cholesterol, 20 μg) in 200 μl n-butanol (10–15 mg) was added to the freeze-dried feces. Concentrated hydrochloric acid (20 μl) was then added, and the suspension was heated at 55°C for 4 h. The solvents were evaporated, and the residue was subjected to trimethylsilylation, taken in 200 μl n-hexane, and an aliquot was subjected to GLC (3).

Statistical methods. Data are shown as means ± SD and were compared statistically by ANOVA followed by the Bonferroni multiple comparisons test. BMDP statistical software (BMDP Statistical Software, Los Angeles, CA) was used for statistical evaluations.

RESULTS

In Vivo Studies in Rabbits Fed 0.5% DCA or the Ileal ASBT Inhibitor SC-435

In NZW rabbits fed 0.5% DCA for 1 wk, the circulating bile acid pool size, which consisted of 89% DCA and 10% cholic acid (CA), increased greater than twofold from 274 ± 24 to 587 ± 68 mg (P < 0.01). Ileal mucosa ASBT mRNA expression decreased 40% (1.03 ± 0.07 vs. 0.62 ± 0.02-fold; P < 0.05), whereas SHP mRNA levels rose 50% (1.00 ± 0.09 vs. 1.54 ± 0.26-fold; P < 0.05) compared with baseline values measured by real-time PCR (Fig. 1, A and B).

In NZW rabbits treated with SC-435 (125 mg·kg−1·day−1), a competitive inhibitor of ASBT (19) for 1 wk, the reabsorbed bile acid flux containing 85% DCA and 14% CA through the ileum decreased as the fecal bile acid outputs increased ninefold (1.9 ± 1.5 to 17.0 ± 10.1 mg/day; P < 0.001). Ileal mucosa ASBT mRNA expression rose 50% (1.53 ± 0.24-fold; P > 0.05), whereas SHP mRNA declined 34% (0.66 ± 0.13-fold; P < 0.05; Fig. 1, A and B).

Identification of FTF Element Involved in Rabbit ASBT Expression

Sequence analysis of 5'-flanking regions of rat (19), human (26), rabbit (11), and mouse (17) ASBT genes revealed several basic transcription factor elements, hepatic nuclear factor α-1, u-AP1, and dAP1, which are located at the proximal region of the rabbit ASBT promoter. At 5'-flanking region −1166/−1158, a nine-nucleotide putative FTF binding element (TCAAAAGCCT) was identified (Fig. 2). It was highly conserved to the FTF binding site in the human and rat CYP7A1 promoter. Two rabbit ASBT 5'-flanking region-pGL3 luciferase reporter vectors were constructed: a 1.9-kb 5'-flanking region-pGL3 (P2), which contained the putative FTF binding site, and a 1.1-kb ASBT 5'-flanking region-pGL3 (P1), which did not contain the FTF binding site. We found both constructs expressed luciferase activities in Caco-2 cells. The activity of P2 was twofold (P < 0.001) stronger than P1 (4,013 ± 267 vs. 1,670 ± 89 U; Fig. 3).

To further determine whether the FTF cis-element in the rabbit ASBT promoter was functional, a pair of oligonucleotide probes was synthesized according to the putative FTF cis-element sequence in the rabbit ASBT promoter region. Protein binding for FTF was seen after using EMSA using nuclear extracts from NZW rabbit ileal mucosa. FTF binding could be competitively blocked by unlabeled (cold) cis-element oligonucleotide probe (Fig. 4A, lanes 3 and 4), but was not competitively blocked by a cold mutant oligonucleotide probe (Fig. 4A, lanes 7 and 8). A shift of the binding band of FTF protein/FTF cis-element was seen after adding the FTF antibody (goat polyclonal antibody from Santa Cruz Biotechnology; Fig. 4A, lane 9). However, the shift did not occur when a control antibody (anti-histone H1 antibody) was applied instead (Fig. 4A, lane 10).

To determine the relationship between FTF cis-element and its binding protein, P2 with the deleted FTF binding site (P2m) was constructed. After cotransfection into Caco-2 cells, P2m had less than one-half the activity (1,620 ± 81 U; P < 0.01) than P2 with the intact FTF binding site (3,451 ± 191 U). FTF protein increased the activity fourfold (14,263 ± 950 U; P < 0.001) in P2 with the intact FTF binding site but did not stimulate the activity of P2m (1,809 ± 108 U; Fig. 4B).

SHP is a Potent Repressor of Rabbit ASBT by Deactivating FTF

The effect of SHP on the potential transcriptional activity of the rabbit ASBT-luciferase reporter gene was determined using transfection assays in Caco-2 cells. Cotransfection with mouse SHP expression plasmid resulted in a 58% decrease (1,443 ± 32 vs. 3,451 ± 191 U, P < 0.001) in the activity of rabbit P2. This inhibitory effect of SHP disappeared in rabbit P2m, where
the binding element was deleted (Fig. 5). In addition, increasing the concentration of the cotransfected SHP expression plasmid resulted in a dose-dependent decrease in the FTI-induced transactivation of rabbit P2 (Fig. 5). The data showed when the ratio of SHP to FTI reached 1:1, FTI-induced P2 activity was totally inhibited.

**FXR and DCA Repressed Rabbit ASBT Promoter Activity**

Adding 50 μM DCA, an activating ligand for FXR, to Caco-2 cells cotransfected with P2 decreased the promoter activity by 76% (969 ± 39 U; P < 0.001) compared with the baseline value of P2 (4,013 ± 267 U). When human FXR (hFXR) and human RXR (hRXR) expression vectors were cotransfected into the cells, rabbit P2 activity was repressed by 46% (2,175 ± 126 U; P < 0.001). Adding 50 μM DCA to Caco-2 cells cotransfected with hFXR and hRXR expression vectors inhibited rabbit P2 activity by 79% (840 ± 60 U; P < 0.001). After being cotransfected with RXR and mutated FXR W469A (FXRm), which could bind bile acids (ligands) but cannot transactivate promoter of its target genes (SHP), P2 activity (7,382 ± 153 U) was 84% (P < 0.001) higher than baseline. After 50 μM DCA was added to the Caco-2 cells cotransfected with FXRm and RXR, P2 activity still remained 34% (P < 0.01) higher than the baseline level (Fig. 6).

**Dose-Dependent Inhibition of Rabbit ASBT Promoter by FXR-Activating Ligands**

Rabbit P2 hybrid construct transfected Caco-2 cells were treated with different concentrations of DCA (0.25, 1, 4, 20, and 50 μM). Rabbit P2 promoter activity, represented by luciferase activity, showed an inverse relationship with increasing concentrations of DCA in the medium (Fig. 7). Because there were no gross changes in cell viability and no effect on Renilla luciferase activity (as a control construct under the TK promoter), the dose-dependent reduction of P2 activity in this experiment was not due to a nonspecific effect of cellular toxicity but to a biological effect of DCA.

**FXR-Activating Ligands Repressed ASBT Promoter Activity via FTI**

Caco-2 cells were treated with 10 μM hydrophobic bile acids DCA, CDCA, and CA, which activate FXR, and 10 μM UCA and UDCA, which almost do not activate FXR, for 40 h. Rabbit P2 activity decreased in Caco-2 cells treated with DCA (–47%; P < 0.001), CDCA (–42%; P < 0.001), and CA (–36%; P < 0.01; Fig. 8A). However, the inhibitory effects of

**Fig. 3.** Measurement of baseline activity of the 1.1 (P1)- and 1.9-kb cloned ASBT promoter (P2). A dual-luciferase reporter assay system (Promega) was employed to examine transcriptional activities of the rabbit ASBT promoter. All activities were normalized by thymidine kinase promoter-driven Renilla luciferase. The results showed that in Caco-2 cells transfected with P2, which contained the FTI binding site, ASBT expression indicated by luciferase activity was 2-fold higher (P < 0.001) than that with P1 without the FTI binding site. The high expression level in the Caco-2 cells transfected with a known positive promoter SV40 demonstrated good performance of this experimental system.
Fig. 5. SHP represses FTF-dependent rabbit ASBT promoter expression. After P2 and 4 \( \mu \)g of mouse SHP plasmid were cotransfected, P2 expression indicated that luciferase activity decreased 58% (\( p < 0.001 \)). The change of P2 luciferase activity was examined after cotransfection of 2 \( \mu \)g mouse LRH-1 (FTF) plasmid and increasing cotransfected SHP plasmid (20 ng, 200 ng, and 2 \( \mu \)g). When the ratio of FTF to SHP (FTF:SHP) transfected into Caco-2 cells reached 1:1, FTF-induced P2 activity was totally abolished. Ctrl, control.

Fig. 7. Dose-dependent inhibition of rabbit ASBT promoter by DCA. The Caco-2 cells were cotransfected with P2, which was connected to a reporter gene, luciferase. The cells were treated with DCA for 40 h at different doses as indicated in the figure. P2 luciferase activity decreased, whereas the concentration of DCA was gradually increased in the medium.

**DISCUSSION**

In the present study, after rabbits were fed 0.5% DCA, an FXR-activating ligand, for 1 wk, the influx of bile acid increased through the ileal enterocytes. This was demonstrated by a twofold enlargement of the circulating bile acid pool size, whereas ileal ASBT mRNA decreased by 40%. This in vivo study demonstrated feedback regulation of ASBT expression in rabbits by ileal bile acid influx. To confirm this observation, NZW rabbits were treated with SC-435, a competitive inhibitor of ASBT, for 1 wk. The influx of bile acids into the ileal apical epithelium was reduced, as evidenced by a ninefold increase in fecal bile acid outputs, whereas ASBT mRNA expression increased 50%. These results further demonstrated the negative feedback regulation of ASBT expression by the FXR-activating ligand influx into ileal epithelium in the in vivo rabbit studies.

We know that FXR played a crucial role in the feedback regulation of ASBT in mice via the FXR/SHP/LRH-1 (FTF homolog in mice) cascade (5). CA inhibited ASBT expression in wild-type (FXR+/+) mice but not in mice in which FXR was knockedout. Furthermore, the feedback regulation did not occur in rats because there was no functional FTF binding element in the rat ASBT promoter. To clarify the mechanisms involved in the feedback regulation of rabbit ASBT expression by bile acids, we cloned a 1.1 (P1) - and a 1.9-kb (P2) rabbit ASBT 5'-flanking region-PGL3 construct. We identified that P2 contained a putative FTF binding site at 5'-flanking region \(-1166/-1158\) (TCAAAAGCCT). Although both P2 and P1 showed baseline expression indicated by luciferase activities in Caco-2 cells, the activity of P2 was twice as strong as P1 (Fig. 4). With the use of this putative rabbit FTF binding site
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(TCAAGGCCCT) as a probe, gel shift assays showed that the binding of FFT protein with this probe could be competed by nonlabeled cold probe but not the mutant probe and was shifted by adding the FFT antibody. Thus, this putative FFT binding site can specifically bind to rabbit FFT protein extracted from rabbit ileal mucosa (Fig. 5A). Furthermore, FFT protein increased ASBT activity fourfold in the Caco-2 cells cotransfected with P2 but not in those cells with P2m, where FFT binding site was deleted (Fig. 5B). These results confirmed that the proposed element in P2 was a functional rabbit FFT binding site.

The present study demonstrated that the negative regulation of ASBT expression by bile acids in the rabbit is also through the FXR/SHP/FFT cascade. The data in Fig. 6 showed that in Caco-2 cells, SHP inhibited rabbit ASBT promoter (P2) baseline expression, whereas FFT induced the expression. This inhibitory effect of SHP was absent in P2m, where the FFT binding site was deleted. These results indicate that SHP has an inhibitory effect against the stimulatory effect of FFT on rabbit ASBT expression, and this inhibitory effect requires the presence of the FFT binding site in the ASBT promoter. Furthermore, the studies in Caco-2 cells showed that adding FXR, an FXR-activating ligand, resulted in a decrease of ASBT expression by 76%. However, when FXR in the Caco-2 cells was replaced by FXRm, which binds bile acids (ligands) but could not transactivate the promoter of its target genes, including SHP, not only the inhibitory effect of FXR was absent but the expression of ASBT was even higher than baseline value regardless of whether DCA was added (Fig. 7). We hypothesize that ASBT expression level at baseline (control in Fig. 7) resulted from the inhibitory effect of bile acid/FFT endogenously present in the Caco-2 cells. After FXRm was added, W469A FXR, which competitively replaced FXR, ASBT was released from the inhibitory effect of the DCA/FXR/SHP/FFT cascade such that ASBT expression was higher than baseline value. Without functioning FXR, DCA could not repress ASBT expression. These results demonstrated that the negative feedback regulation of rabbit ASBT by bile acids is FXR dependent.

In addition, not all bile acids activate the FXR protein (15, 16, 23). Our previous in vivo study in rabbits showed that DCA, but not UCA, activated FFT (27). In the present study, we found that FXR-activating ligands CDCA, DCA, and CA but not FXR nonactivating ligands, UDCA and UCA, repressed FFT-dependant transactivation of rabbit ASBT in Caco-2 cells. The inhibitory effects of FXR-activating ligands were absent when the FFT binding site was deleted. These data further demonstrated that in rabbits, downregulation of ASBT expression by bile acids is through the activation of FFT and is FFT dependent. More importantly, because only FXR-activating ligands downregulate ASBT expression, the composition of activating FXR ligands in the bile acid pool as well as the pool size will be crucial in the regulation of ASBT expression in vivo. This opinion is supported by the results observed in the in vivo studies (Figs. 1 and 2). ASBT mRNA decreased with increased SHP expression in rabbits fed DCA, where circulating bile acid pool with 89% DCA enlarged twofold, ASBT expression increased with reduced SHP mRNA in the rabbits treated with ASBT inhibitor SC-435. In those rabbits, the reabsorbed bile acid flux, containing 85% DCA, decreased significantly.

It should be emphasized that the expression of ASBT is not only regulated by the FXR-SHP-FFT cascade, because there are other binding elements for transcriptional factors in the rabbit promoter region. In this article, we only demonstrate the effect of bile acids on the regulation of ASBT through the FXR/SHP/FFT cascade using Caco-2 cells as the experimental model.

In summary, we identified a FFT functional binding element in the rabbit ASBT promoter. A functional FFT binding site as well as functioning FXR are required for the negative feedback regulation of rabbit ASBT by bile acids. Only FXR-activating ligands can downregulate rabbit ASBT expression via the regulatory cascade FXR-SHP-FFT.

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