Inhibition of Ileal Bile Acid Transport Lowers Plasma Cholesterol Levels by Inactivating Hepatic Farnesoid X Receptor and Stimulating Cholesterol 7α-Hydroxylase

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We investigated the effect of SC-435, a competitive inhibitor of ileal apical sodium-dependent bile acid cotransporter (ASBT) on ileal bile acid absorption and the hepatic nuclear receptor FXR (farnesoid X receptor), which regulates cholesterol 7α-hydroxylase (CYP7A1) activity and mRNA levels. Eighteen New Zealand White (NZW) rabbits were divided into 2 groups: controls (n = 10) and fed SC-435 125 mg/kg/d for 1 week (n = 8). In rabbits treated with SC-435, fecal bile acid outputs increased by more than 8 times, reflecting substantial bile acid malabsorption. Plasma cholesterol levels decreased 26%, while bile acid pool sizes and biliary bile acid outputs did not change after treatment. CYP7A1 activity increased 64% and mRNA rose by 4 times after treatment. The expression of FXR target genes in the liver, short heterodimer partner (SHP) and bile salt export pump (BSEP), decreased 11.6 and 2.6 times, respectively, after treatment, which indicates inactivation of hepatic FXR. However, the mRNA levels of ileal bile acid binding protein (IBABP) did not change significantly, while ileal ASBT mRNA expression increased by 2.4 times after treatment. Rabbits treated with SC-435 developed ileal bile acid malabsorption, which decreased the return of bile acids (FXR ligands) to the liver to inactivate hepatic FXR, which upregulated CYP7A1 and lowered plasma cholesterol levels. Although fecal bile acid malabsorption was substantial, increased bile acid production from hepatic cholesterol kept biliary bile acid outputs intact. Thus, a new balance was reached in the liver, where increased bile acid synthesis compensated for diminished ileal bile acid absorption to maintain the circulating enterohepatic bile acid pool.

WE REPORTED PREVIOUSLY[1] that in hypercholesterolemic Watanabe rabbits and cholesterol-fed New Zealand White (NZW) rabbits, cholesterol 7α-hydroxylase (CYP7A1), the rate-controlling enzyme of bile acid synthesis, mRNA and activity was inhibited. We believe this effect played a major role in the development of hypercholesterolemia in these rabbits and reported[2] that stimulating CYP7A1 by bile fistula drainage (bile acid depletion) reduced plasma cholesterol levels significantly. Further, we demonstrated that in cholesterol-fed rabbits, the bile acid pool actually doubled and hypothesized that the enlarged bile acid pool was responsible for the feedback inhibition of CYP7A1.[3] In addition, in these rabbits fed cholesterol where the bile acid pool size doubled, increased protein levels of ileal apical sodium-dependent bile acid transporter (ASBT), which is responsible for active absorption of bile acids from the ileum, was present.[4] We hypothesized that the increased absorption of bile acids from the ileum together with increased activity of cholesterol 27-hydroxylase (alternative bile acid synthesis) were responsible for maintaining the expanded bile acid pool in the cholesterol-fed rabbits.[3]

The mechanism by which an enlarged bile acid pool inhibits CYP7A1 is now better understood since recent studies demonstrated that farnesoid X receptor (FXR) serves as a negative regulator of CYP7A1 transcription. FXR is located in the nucleus of hepatocytes. As a bile acids sensor, FXR is activated by the flux of bile acids through the liver.[5-7] Thus, in cholesterol-fed rabbits, expansion of the circulating bile acid pool, which contains 85% deoxycholic acid—a potent high-affinity ligand, activates FXR, which in turn downregulates CYP7A1. Short heterodimer partner (SHP) is a positively regulated target gene of FXR. Currently, it is thought that activated FXR induces transcription of SHP that in turn inactivates liver receptor homolog-1 (LRH-1), an essential transcriptional factor for CYP7A1 expression,[8,9] to inhibit CYP7A1 transcription. Bile salt export pump (BSEP) is also a target gene positively regulated by FXR.[10] BSEP is responsible for canalicular bile acid excretion into the bile.[12]

SC-435 is a competitive inhibitor of ASBT that produces ileal bile acid malabsorption.[13] Plasma low-density lipoprotein (LDL) cholesterol and apolipoprotein B (apoB) levels decreased in miniature pigs treated with SC-435.[14]

In this study, we investigated effect of SC-435 on FXR activation, which regulates CYP7A1 expression and, in turn, reduces plasma cholesterol levels. We showed that the SC-435 treatment increased fecal bile acid outputs that inactivated FXR in the liver and stimulated CYP7A1 activity and mRNA levels. As a result of increased bile acid synthesis, plasma cholesterol concentrations decreased.

MATERIALS AND METHODS

Animal Experiment

Male NZW (n = 18) rabbits weighing 2.5 to 3.0 kg (Convance, Denver, PA) were used in this study. The rabbits were divided into (1)

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controls (n = 10) and (2) fed SC-435 125 mg/kg/d for 1 week (n = 8). SC-435 was provided by Pharmacia Discovery Research (St Louis, MO) and was suspended in 20% Liposyn (Abbott Laboratories, North Chicago, IL) to make a concentration of 187.5 mg/mL. The average body weight of the rabbits was approximately 3 kg, such that 2 mL of 187.5 mg/mL SC-435 solution provided (125 mg/kg/d) administered by gastric gavage through a 16-French tube. To measure bile acid pool sizes, bile fistulas were constructed in half of the rabbits from each group after completion of the treatment and bile drainage was continued for 5 days to recover total deoxycholic acid in the bile acid pool during treatments. The bile acid pool sizes were calculated from the total recovered deoxycholic acid divided by the percentage of deoxycholic acid in the initial bile collected during the first 30 minutes after the construction of the bile fistula. After completion of the experiment, the rabbits that were not operated upon were killed in the morning at a similar time (between 9 AM and 10 AM) to collect liver specimens, which were immediately frozen for measurements of hepatic SHP, BSEP, and FXR mRNA levels, and CYP7A1 activity and mRNA levels. Ileal mucosa was also collected and immediately frozen for measurements of ASBT and ileal bile acid binding protein (IBABP) mRNA levels. Blood samples were taken for measurement of plasma cholesterol levels. The biliary bile acid outputs (mg/h) were determined by measuring the excretion of bile acids in the bile collected during the first 30 minutes immediately after construction of the bile fistula. Feces were collected during the last 3 days of the experiment for measurement of fecal bile acid outputs (mg/d).

The animal protocol was approved by the Subcommittee on Animal Studies at VA Medical Center, East Orange, NJ and Institutional Animal Care and Use Committee at UMD-New Jersey Medical School, Newark, NJ.

Biochemical Analyses

Northern blotting analyses. Total RNA was isolated from frozen rabbit liver tissue using single-step RNA isolation method with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA) as described by Chomozynski et al.[15] PolyA$^+$ RNA was isolated from 2 mg total RNA by oligo dT cellulose using the FastTrack 2.0 mRNA isolation Kit (Invitrogen Life Technologies) described by Biesecker et al.[16] Northern blot hybridizations were performed as previously described by Thomas.[17] Briefly, 10 μg polyA$^+$ RNA was electrophoresed on a formaldehyde-agarose (1.0%) gel, and transferred to a nylon membrane (Nytran supercharge nylon transfer membrane; Schleicher & Schuell, Keene, NH). The membrane was baked for 2 hours at 80°C and hybridized to a ^{32}P-labeled RNA probe for 16 hours at 42°C. The membrane was washed at 68°C in 0.1x NaCl and Na citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS) for 30 minutes. Relative expression levels were quantified using a phosphor Imager (Molecular Dynamics, Piscataway, NJ) and standardized against cyclophilin controls.

Real-time polymerase chain reaction (PCR) for measuring gene expression. In this study, mRNA levels were quantitatively measured by real-time PCR and mRNA levels shown as data in the text are relative fold. Total RNA was isolated and treated with DNase I by the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA). One microgram of DNase I-treated total RNA was reverse-transcripted by Omniscript Reverse Transcriptase (Qiagen, Valencia, CA) using oligo dT primer. Real-time PCR was performed with the ABI PRISM 7700 sequence detection system using one fiftieth of reverse transcription (RT) reaction and was analyzed with the 1.7 software (Applied Biosystems, Forest City, CA). Primers and minor groove binder (MGB) probes were synthesized by Applied Biosystems Assays-by-Design Service (Forest City, CA). PCR was performed in a 50-μL reaction volume containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 20 ng cDNA templates, 0.9 μmol/L of each forward and reverse primer, and 0.25 μmol/L MGB probe. Cyclophilin was set as an endogenous control. Because validation experiments showed that amplification efficiencies of the target and cyclophilin were approximately equal, quantitation was performed with the comparative $\Delta\Delta C_t$ method.[18,19] The primers and probe for detecting are as follows: ASBT (sense: 5′-cgttatgccctttatgctacaccaa-3′/antisens: 5′-aagagcaaccaaggaagtacctatc-3′/probe: tggtcccagagtcc); IBABP (sense: 5′-gagacctccaccagcttgtc-3′/antisense: 5′-ggcgaactttcccaactatcag-3′/probe: cacacctcggagatc); cyclophilin (sense: 5′-ccttcgagctatttgcagacaa- 3′/antisense: 5′-gaacccttataaccgaatccttttct-3′/probe: cagcagaaacttc); CYP7A1 (sense: 5′-aggagaaggcgaatgggtgc-3′/antisense: 5′-gcacagcccagatatggaatc-3′/probe: ccacctctggagaaag); SHP (sense: 5′-tggcccaagacatggtgac-3′/antisense: 5′-gctcctccagcaggatcttct-3′/probe: ctgaagcccccggtgc).

Assay for CYP7A1 activity. Hepatic microsomes were prepared by differential ultracentrifugation,[20] and the protein determined according to Lowry et al.[21] CYP7A1 activity was measured in hepatic microsomes by the isotope incorporation method of Shefer et al.[20]

Assay for bile acids. Biliary bile acids were analyzed using capillary gas–liquid chromatography (GLC) method as previously described.[22] Fecal bile acids were determined by a simplified method proposed by Batta et al.[23] Briefly, internal standard (nor-cholic acid, 20 μg in 200 μL n-butanol) 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 hours followed by evaporation of the solvents. Trimethylsilylethers were prepared and taken up in 200 μL n-hexane and a 2-μL aliquot was chromatographed on a 25-mol/L CP5 SIL capillary column. The retention times for deoxycholic acid methylesters are 16.83 minutes, lithocholic acid methylesters are 15.30 minutes, and nor-deoxycholic acid methylesters are 14.05 minutes.

Statistical Method

Data are shown as means ± SD and were compared statistically by Student's *t* test (unpaired). The BMDP Statistical Software (BMDP Statistical Software, Inc, Los Angeles, CA) was used for statistical evaluations.

RESULTS

Fecal bile acid outputs measured by GLC increased 8.2 times (1.9 ± 1.5 to 17.0 ± 10.1 mg/d, $P < .001$) in rabbits treated with SC-435 for 1 week, reflecting substantial bile acid malabsorption (Fig 1A). However, there was no diarrhea found in the rabbits after the treatment with SC-435. Plasma cholesterol levels decreased 26%, from 34 ± 5 to 25 ± 5 mg/dL ($P < .05$) after treatment with SC-435 (Fig 1B). However, the bile acid pool size did not change significantly in rabbits treated with SC-435 (239 ± 52mg) as compared with untreated baseline (287 ± 23 mg). Similarly, there was no significant difference in biliary bile acid outputs after the treatment (28 ± 3 mg/h *v* control 32 ± 14 mg/h). (Fig 1C)

CYP7A1 activity increased 64% (from 28.3 ± 8.3 to 46.4 ± 11.6 pmol/mg/min, $P < .05$) in the rabbits treated with SC-435 (Fig. 2). After treatment with SC-435, CYP7A1 mRNA levels rose 4 times (from 0.8 ± 0.2 relative fold to 3.2 ± 0.8 relative fold, $P < .01$) as quantitatively measured by real-time PCR shown as relative fold. Figure 3 shows mRNA abundance of CYP7A1 by Northern blotting.

The expression of the hepatic FXR target genes, SHP and BSEP, mRNA levels decreased 11.6 times (from 0.78 ± 0.32 relative fold to 0.05 ± 0.03 relative fold, $P < .01$) and 2.6 times (from 0.79 ± 0.31 relative fold to 0.31 ± 0.13 relative fold,

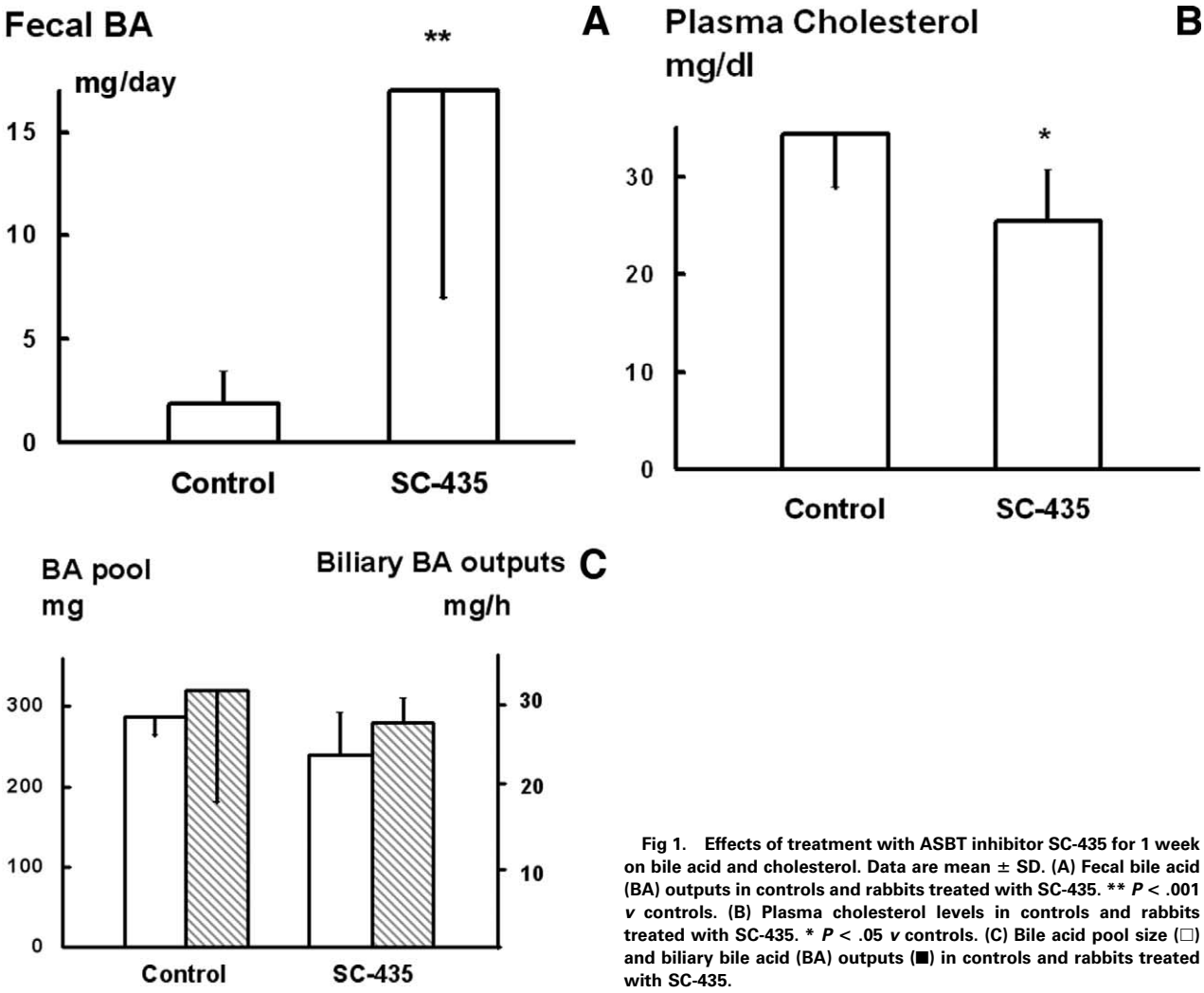

Fig 1. Effects of treatment with ASBT inhibitor SC-435 for 1 week on bile acid and cholesterol. Data are mean ± SD. (A) Fecal bile acid (BA) outputs in controls and rabbits treated with SC-435. ** $P < .001$ *v* controls. (B) Plasma cholesterol levels in controls and rabbits treated with SC-435. * $P < .05$ *v* controls. (C) Bile acid pool size (□) and biliary bile acid (BA) outputs (■) in controls and rabbits treated with SC-435.

$P < .01$) respectively, after SC-435 treatment (Figs 4 and 5). However, hepatic FXR mRNA abundance shown by Northern blotting did not change significantly after SC-435 treatment as compared to untreated controls (Fig 4).

Ileal apical bile acid transporter ASBT mRNA levels increased 2.4 times in rabbits fed SC-435, whereas IBABP mRNA levels remained unchanged after the treatment (Fig 6). Figure 7 shows mRNA abundance of both ileal ASBT and IBABP measured by Northern blotting.

DISCUSSION

This study demonstrated that SC-435 is a potent inhibitor of ileal bile acid absorption in rabbits. After 1 week of treatment with SC-435, fecal bile acid outputs increased 8.3-fold. As a result, the flux of bile acids (activating ligands for FXR) returned to the liver diminished and hepatic FXR was inactivated. This change was indicated by reduced hepatic expression of 2 FXR target genes, SHP and BSEP, after treatment with SC-435. Since FXR is a powerful negative regulator of CYP7A1 transcription, inactivation of hepatic FXR removed the inhibitory effects from CYP7A1[24] such that CYP7A1 mRNA rose and

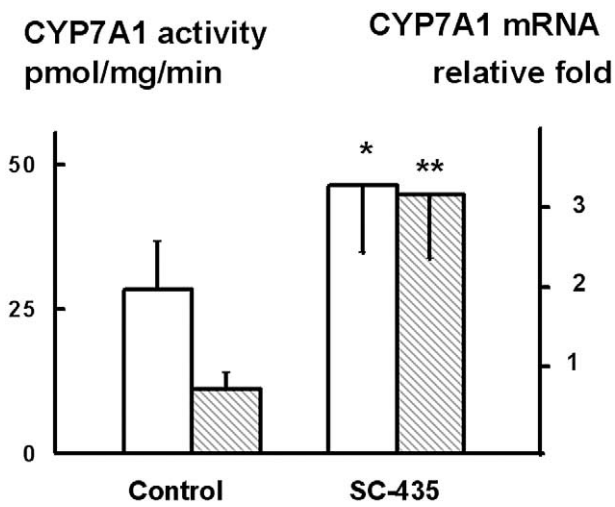

Fig 2. CYP7A1 activity (□) and mRNA (■) in controls and rabbits treated with SC-435 for 1 week. mRNA levels of CYP7A1 were determined by real-time PCR and quantified with TaqMan comparative $\Delta\Delta C_t$ method. Data are shown as relative fold (mean ± SD). * $P < .05$, ** $P < .01$ *v* controls.

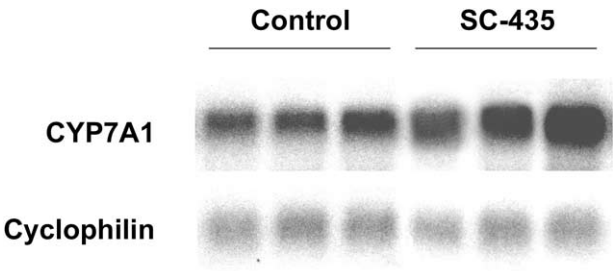

Fig 3. Northern blot for hepatic CYP7A1 mRNA abundance in controls and rabbits treated with SC-435 for 1 week. PolyA$^+$ RNA was applied and cyclophilin was used as internal standard.

activity was markedly upregulated after SC-435 treatment. Therefore, bile acid synthesis increased as evidenced by enhanced fecal bile acid outputs (mg/d), which should be equivalent to the amount of bile acids synthesized daily.

We previously reported[2] that stimulating CYP7A1 activity reduced plasma cholesterol levels substantially in both NZW and hypercholesterolemic Watanabe rabbits by increasing bile acid synthesis. In the present study, we believe that reduced plasma cholesterol levels after SC-435 treatment were also due to enhanced CYP7A1 activity and new bile acid synthesis. The increased bile acid synthesis utilized cholesterol, which reduced hepatic cholesterol levels, and stimulated hepatic LDL receptor function, which increased hepatic uptake of cholesterol from plasma. In addition to SC-435,[14] other ASBT inhibitors[25,26] have also been reported to upregulate CYP7A1 and lower plasma cholesterol levels.

It is important to emphasize that although ileal bile acid absorption was significantly decreased, resulting in diminished bile acids returning to the liver, biliary bile acid outputs and the

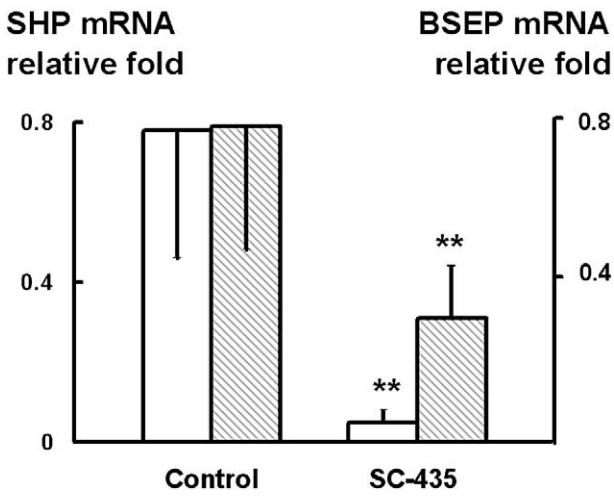

Fig 5. Real-time PCR determinations for quantitative mRNA levels of hepatic SHP (□) and BSEP (■) in controls and rabbits treated with SC-435 for 1 week. Data are shown as relative fold (mean ± SD). ** *P* < .01 *v* controls.

bile acid pool sizes were similar to baseline levels. The unchanged biliary bile acid outputs and hepatic pool sizes were associated with increased bile acid synthesis to compensate diminished bile acid influx from ileum and suggested that after treatment with SC-435, a new hepatic balance was reached. The reduced bile acids returning to the liver were compensated for by increased newly synthesized bile acids such that biliary bile acid outputs from the liver and pool size were maintained. That bile acid pool size was not changed due to a compensatory increase in bile acid synthesis has also been observed in pa-

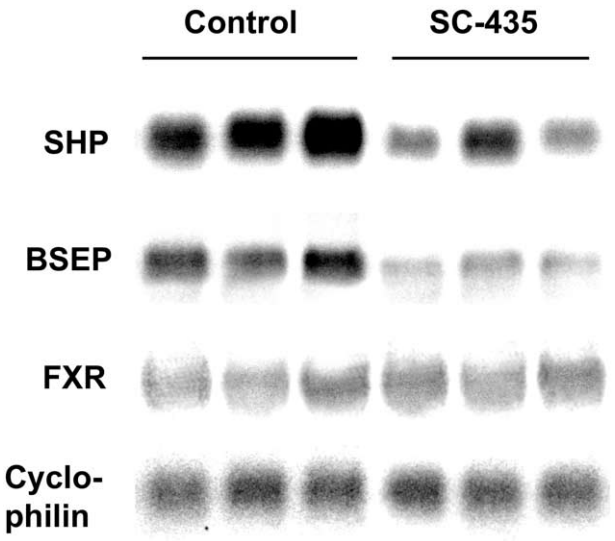

Fig 4. Northern blot for mRNA abundance of hepatic FXR and FXR target genes SHP and BSEP in controls and rabbits treated with SC-435 for 1 week. PolyA$^+$ RNA was applied and cyclophilin was used as internal standard.

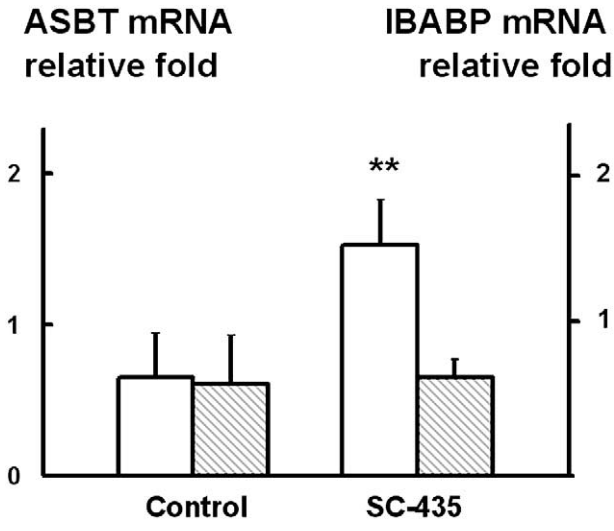

Fig 6. Real-time PCR determinations for quantitative mRNA levels of ileal ASBT (□) and IBABP (■) in controls and rabbits treated with SC-435 for 1 week. mRNA levels of each gene were determined by real-time PCR. Data are shown as relative fold (mean ± SD). ** *P* < .01 *v* controls.

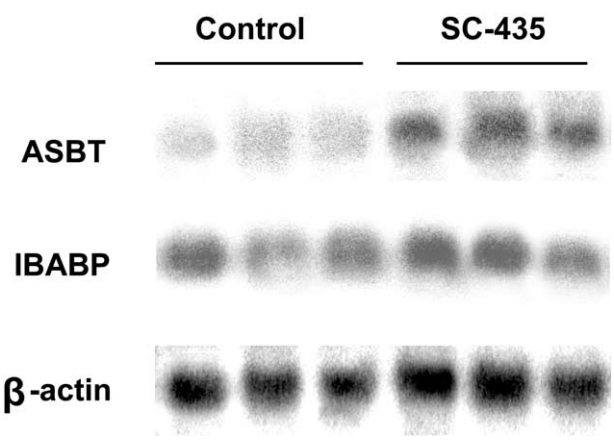

Fig 7. Northern blot for mRNA abundance of ileal ASBT and IBABP in controls and rabbits treated with SC-435 for 1 week. Total RNA was applied and β-actin was used as internal standard.

tients ingesting cholestyramine[27] and with small intestine resection.[28]

In this study, ASBT mRNA levels unexpectedly increased 2- to 3-fold after SC-435 treatment, where the absorbed bile acid flux through the ileal enterocytes decreased substantially. It is possible that ASBT transcription rose to produce more protein to compensate for reduced function of the ASBT protein that was competitively blocked with SC-435. However, bile acid absorption remained low. It has been reported in guinea pigs[29] and rats[30,31] that ASBT expression could be regulated by bile acids, although it remains controversy whether ASBT mRNA is negatively or positively regulated. The present study suggests that in rabbits, under this special condition (treatment with ASBT competitive inhibitor), ASBT expression seemed negatively regulated by the bile acid flux through the enterocyte.

It is surprising that mRNA levels of IBABP did not change significantly after treatment with SC-435, which diminished ileum bile acids absorption. Although FXR as a transcriptional factor and its binding site in the IBABP promoter region in both humans and mice have been identified,[6,11] its active binding site in the IBABP promoter in rabbits or rats has not been confined. In the meantime, the role of IBABP located in the ileum in the absorption of bile acid is not yet fully understood.

In summary, this study emphasized the important role of bile acids returning from the ileum to the liver in activation of hepatic FXR, which regulates CYP7A1 and bile acid synthesis. Diminished return of bile acids to the liver inactivated hepatic FXR, which upregulated CYP7A1 and increased bile acid synthesis to maintain constant biliary bile acid outputs and circulating pool size. Stimulating CYP7A1 and bile acid synthesis reduced plasma cholesterol levels substantially.

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