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**Cholesterol feeding prevents hepatic accumulation of bile acids in cholic acid-fed
Fxr-null mice: FXR-independent suppression of intestinal bile acid absorption**

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Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; ASBT, apical sodium-dependent bile salt transporter; BBMV, brush border membrane vesicle; BSEP, bile salt export pump; CA, cholic acid; Chol, cholesterol; FGF, fibroblast growth factor; FXR, farnesoid X receptor; IBABP, ileal bile acid binding protein; LXR, liver X receptor; NTCP, Na⁺-dependent taurocholate cotransporting polypeptide; OST α , organic solute transporter α ; PMSP, phenylmethanesulfonyl fluoride; PXR, pregnane X

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receptor; SHP, small heterodimer partner; SREBP, sterol regulatory element binding protein; TCDCA, taurochenodeoxycholic acid.

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Abstract

Cholic acid (CA) feeding of farnesoid X receptor (*Fxr*)-null mice results in markedly elevated hepatic bile acid levels and liver injury. In contrast, *Fxr*-null mice fed cholesterol plus cholic acid (CA+Chol) do not exhibit liver injury, and hepatic bile acid levels and bile acid pool size are reduced 51% and 40%, respectively, as compared to CA-treated *Fxr*-null mice. These decreases were not observed in wild-type mice. Despite a reduced bile acid pool size, hepatic Cyp7a1 mRNA expression was increased in *Fxr*-null mice fed the CA+Chol diet, and biliary bile acid output was not changed. Analysis of other potential protective mechanisms revealed significant decreases in portal blood bile acid concentrations and a reduced ileal bile acid absorption capacity, as estimated using an in situ loop method. Fecal bile acid excretion was also increased in *Fxr*-null mice fed the CA+Chol versus CA diet. The decreased ileal bile acid absorption correlated with decreased ileal apical sodium-dependent bile salt transporter (ASBT) protein expression in brush border membranes. These results suggest a critical role for ileal bile acid absorption in regulation of hepatic bile acid levels in *Fxr*-null mice fed cholic acid and cholesterol. Furthermore, experiments with *Fxr*-null mice suggest that cholesterol feeding can down-regulate ASBT expression through a pathway independent of FXR.

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Introduction

Bile acids are synthesized from cholesterol in the liver and play a key role in the intestinal absorption of dietary lipids and fat-soluble vitamins. However pathophysiological accumulation of bile acids can elicit cytotoxicity, and elevated concentrations are associated with liver injury (Hofmann, 1994). Hepatic bile acid levels are tightly regulated by balancing hepatic uptake, biosynthesis, and efflux (Russell, 2003; Trauner et al., 2005; Pauli-Magnus and Meier, 2006). Hepatic bile acid synthesis and uptake are suppressed and biliary bile acid excretion is enhanced under conditions of hepatic bile acid accumulation. These functional changes are dependent in part on the hepatic levels of CYP7A1, Na⁺-dependent taurocholate cotransporting polypeptide (NTCP) and bile salt export pump (BSEP) (Kullak-Ublick et al., 2004; Eloranta et al., 2006; Suchy and Ananthanarayanan, 2006; Norlin and Wikvall, 2007). Hepatic bile acid-activated FXR signaling plays a critical role in these functional changes by directly up-regulating BSEP expression (Ananthanarayanan et al., 2001) and down-regulating CYP7A1 and NTCP expression (Goodwin et al., 2000) (Lu et al., 2000; Denson et al., 2001).

In the body, more than 95% of bile acids are reabsorbed at the distal ileum and are carried back to the liver. As such, ileal bile acid absorption mediated by transporters such as ASBT (gene name *Slc10a2*) and organic solute transporter α (OST α)-OST β is also an important potential factor regulating hepatic bile acid levels (Xu et al., 2000; Dawson et al., 2005) (Rao et al., 2008). In some strains of mice, bile acids acting via FXR/small heterodimer partner (SHP) signaling down-regulate ileal ASBT expression (Chen et al., 2003) (Neimark et al., 2004) (Li et al., 2005). The ileal uptake of bile acids

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also plays a critical role in regulating hepatic bile acid content by acting via FXR to induce ileal enterocyte expression of fibroblast growth factor 15 (FGF15). The ileal derived FGF15 is released into the portal circulation and carried to the liver where it signals to down-regulate CYP7A1 expression (Inagaki et al., 2005). Results confirming a central role for ileal derived FGF15 in the regulation of hepatic CYP7A1 expression were obtained using the intestine-specific *Fxr*-null mouse (Kim et al., 2007). Hepatic bile acid levels are thus, coordinately modulated by FXR signaling pathways in both liver and small intestine. Furthermore, signaling via pathways not involving FXR are also likely to contribute to the maintenance of hepatic bile acid levels, although the exact mechanisms remain unclear.

Cholesterol can be directly excreted by the liver into bile or converted into bile acids. Approximately one-half of the biliary and dietary cholesterol is reabsorbed in the proximal small intestine (Altmann et al., 2004) and carried back in chylomicrons to the liver. Intestinal cholesterol absorption is facilitated by forming mixed micelles with bile acids (Woollett et al., 2004; Hui and Howles, 2005; Woollett et al., 2006). The bile acid pool size thus can affect intestinal cholesterol absorption and hepatic cholesterol catabolism (Ponz de Leon et al., 1981; Dawson et al., 2003). Dietary cholesterol increases the bile acid pool size and fecal bile acid excretion in mice (Tiemann et al., 2004). Thus, it is possible that cholesterol feeding increases hepatic bile acid and cholesterol levels.

Cholic acid (CA) feeding markedly increases hepatic bile acid levels and causes liver injury in *Fxr*-null mice (Sinal et al., 2000), which exhibit impaired regulation of bile acid homeostasis. Hepatic bile acid concentrations are positively correlated with markers of liver injury in *Fxr*-null mice fed a CA diet (Miyata et al., 2005). Hepatic

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cholesterol levels are also elevated in CA-fed *Fxr*-null mice, raising the possibility that accumulation of hepatic cholesterol contributes to the CA-induced hepatotoxicity in this model. In preliminary studies, the effect of cholesterol co-administration on CA-induced hepatotoxicity was tested in *Fxr*-null mice. Unexpectedly, the addition of cholesterol to the diet ameliorated the hepatotoxicity induced by CA feeding of the *Fxr*-null mice.

To understand the mechanisms underlying the protection against bile acid-induced toxicity in the cholesterol plus CA-fed *Fxr*-null mice, the effects of cholesterol co-administration on bile acid synthesis and transport were investigated. The present study reveals a role for cholesterol-mediated suppression of ileal bile acid absorption as an important FXR-independent mechanism for reducing hepatic bile acid content and bile acid-induced toxicity.

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Materials and Methods

Materials.

Cholic acid (CA), taurocholic acid (TCA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), tauroolithocholic acid (TLCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA) and taurodeoxycholic acid (TDCA) were purchased from Sigma-Aldrich (St. Louis, MO). β -Muricholic acid (β MCA), tauro- β -muricholic acid (T β MCA), tauroursodeoxycholic acid (TUDCA), ursodeoxycholic acid (UDCA), and 5 β -cholan-3 α , 6 β -diol (Internal standard for HPLC assay) were purchased from Steraloids (Newport, RI). L-column ODS (2.1 \times 150 mm) was obtained from Kagaku Ikenkyo (Tokyo, Japan). Enzymepak 3 α -HSD column was purchased from Jasco (Tokyo, Japan). Monoclonal anti- β -actin antibody (clone AC-15) was purchased from Sigma-Aldrich (St. Louis, MO).

Animal treatment and sample collection.

Fxr-null (Sinal et al., 2000) and wild-type mice were housed under a standard 12 hr light (9 a.m.-9 p.m.)-12 hr dark cycle. Prior to administration of the special diets, mice were fed standard rodent chow (CE-2, Clea Japan) and water ad libitum. CE-2 originally contains 0.1% cholesterol. The experimental diets used for these studies included standard control diet (CE-2), or control diet supplemented with the following: 0.5% (w/w) cholic acid (CA), 1.25% cholesterol (Chol), 0.5% cholic acid + 1.25% cholesterol (CA+Chol) and 0.5% cholic acid + 0.5% cholesterol (CA+Chol 0.5%). Age matched groups of 8- to 9-week-old male mice were used for all experiments. Bile, blood and tissue samples were taken at 9 a.m. for biochemical assays after feeding the

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special diets for six days. In the present study, the short period (6 days) of feeding was chosen because we originally thought that cholesterol co-administration would enhance CA-induced hepatotoxicity in *Fxr*-null mice. Total RNA was prepared from livers and ileum using the ULTRASPEC II RNA isolation system (Biotecx Lab., Houston, TX). Biliary excretion was monitored in mice anaesthetized with ethyl ether. After ligating the common bile duct, bile samples were collected by cannulating the gall bladder using polyethylene tubing (PE-10; internal diameter of 0.28 mm). After a 5 min equilibration period, bile was collected for 30 min. Bile acid pool size was determined as bile acid content of the small intestine, liver, and gallbladder. These tissues were removed and extracted in ethanol as described (Schwarz et al., 1998). All experiments were performed in accordance with Guidelines for Animal Experiments of Tohoku University.

Plasma diagnosis parameters and plasma, hepatic and biliary lipid parameters.

Plasma alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were measured using a commercial kit, Transaminase CII-B-test Wako for ALT and Alkali-phospha B-test Wako for ALP (Wako Pure Chemicals, Osaka, Japan). Plasma, hepatic and biliary total cholesterol concentrations were measured using the cholesterol E-test Wako (Wako Pure Chemicals, Osaka, Japan). Bile acid pool size and hepatic, biliary and fecal 3 α -hydroxy bile acid concentrations were measured by HPLC as described previously (Kitada et al., 2003; Miyata et al., 2006). The content of β MCA, T β MCA, UDCA, TUDCA, CA, TCA, CDCA, TCDCA, DCA TDCA, LCA and TLCA was measured.

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Ileal bile acid absorption (in situ loop method).

The mice were anesthetized with ethyl ether. An ileal loop of approximately 10 cm in length was isolated using ligatures at both ends, and 500 μ l of a dosing solution (5 mM TCDCA in phosphate buffer saline) was injected into the loop with a syringe. The portal blood was collected once from each mouse at three or ten min after the injection.

Western blot analysis.

Ileal brush border membranes were prepared as described (Kessler et al., 1978). Ileal segments (5 cm) were opened and the ileal mucosa was obtained by scraping. The ileal mucosa was suspended in 2 mM Tris-HCl (pH 7.1) buffer containing 50 mM mannitol, 0.1 mM PMSF and was homogenized using a Teflon glass homogenizer. After addition of CaCl_2 to a final concentration of 10 mM, the homogenate was incubated on ice for 20 min. The homogenate was centrifuged at 3,000 \times g for 15 min and the supernatant was centrifuged at 27,000 \times g for 30 min. The pellet was suspended in 1 mM Hepes (pH7.5) buffer containing 100 mM mannitol and 10 μ M MgSO_4 .

Ileal brush border membranes (15 μ g/lane) were subjected to SDS-PAGE on 8% or 10% polyacrylamide gels, and transferred to nitrocellulose filters. The ileal brush border membrane filters were probed with a polyclonal anti-rodent ASBT antibody (Shneider et al., 1995) (Wong et al., 1995) or monoclonal β -actin antibody (Sigma-Aldrich). The filters were washed five times with Tween 20 containing phosphate buffer, and incubated with phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution). Immunoreactive bands were detected using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazonium. The stained membranes were scanned with an Epson GT-8700 scanner.

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Analysis of mRNA levels.

Hepatic and ileal total RNA were isolated using RNeasy Total RNA Isolation System (Promega, Madison, WI). Single strand cDNA were synthesized using an oligo(dT) primer and the Ready-to-Go You-Prime First-strand Beads kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). These cDNA templates were used for real-time PCR using SYBR Green 1 with ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification was performed by the comparative $\Delta\Delta C_t$ method. The following specific forward and reverse primers were used for real-time quantitative PCR:

CYP7A1 sense, 5'-AGCAACTAAACAACCTGCCAGTACTA-3'

antisense, 5'-GTCCGGATATTCAAGGATGCA-3';

ASBT sense, 5'- TGGGTTTCTTCCTGGCTAGACT-3'

antisense, 5'- TGGTCTGCATTCCAGTTTCCAA-3';

GAPDH sense, 5'-TGTGTCCGTCGTGGATCTGA-3',

antisense, 5'- CCTGCTTCACCACCTTCTTGAT-3'

β -actin sense, 5'-ACCCTGTGCTGCTACCGA-3';

antisense, 5'-CTGGATGGCTACGTACATGGCT

OST α sense, 5'-GCCAGGCAGGACTCATATCAAA-3'

antisense, 5'-GGCAACTGAGCCAGTGGTAAGA-3'

Ileal bile acid binding protein (IBABP) sense, 5'-AGATCATCACAGAGGTCCAGC-3'

antisense, 5'-GGTAGCCTTGAACCTTCTTGCC-3'

Statistical analysis.

All values are expressed as the means \pm S.D. Data were analyzed by unpaired

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Student's t test or by ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 4 for significant differences between the mean values of each group. Probability values of less than 0.05 were considered to be statistically significant.

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Results

Liver damage diagnostic markers and bile acid content in CA and/or cholesterol-fed mice.

Feeding cholic acid resulted in elevated hepatic bile acid levels, and plasma ALT but not ALP activities were slightly increased in wild-type mice fed the CA or CA+Chol diets (Table 1), with levels less than 200 IU/L. However there was no significant difference between wild-type mice fed the CA and CA+Chol diets with regard to plasma ALT or hepatic bile acid content. In contrast to wild-type mice, the CA-fed *Fxr*-null mice had markedly elevated plasma ALT and ALP activities (13.5-fold and 10.0-fold, respectively, relative to the control diet)(Table 1). Plasma ALT and ALP activities were also elevated in *Fxr*-null mice fed the CA+Chol diet, however the increases were significantly blunted compared to the CA-fed *Fxr*-null (20% and 48% of the CA-diet levels, respectively). The hepatic bile acid content was also markedly increased in *Fxr*-null mice fed the CA diet (4.6-fold relative to the control diet) (Table 1). However as with the liver damage diagnostic markers, feeding CA+Chol diet significantly blunted the increase in hepatic bile acid levels; in the *Fxr*-null mice fed CA+Chol, hepatic bile acid content was decreased 51% as compared to the CA-fed *Fxr*-null mice. Significant increases in hepatic cholesterol levels were observed in *Fxr*-null mice fed the Chol, CA, and CA+Chol diets, and in wild-type mice fed the CA+Chol diet (Table 1). In contrast to liver, plasma cholesterol levels were significantly increased only in *Fxr*-null mice fed the CA+Chol diet. The effect of cholesterol on CA-induced toxicity and hepatic bile acid content was further examined in *Fxr*-null mice fed diets containing CA and increasing amounts (0%, 0.5% and 1.25%) of cholesterol. Feeding increasing amounts of dietary cholesterol plus cholic acid to *Fxr*-null mice reversed the elevation

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of hepatic bile acid content, plasma ALT and ALP activities in a dose-dependent manner (Fig. 1A, B). An inverse relationship was found between hepatic content of bile acids and cholesterol (Fig. 1C).

Influence of cholesterol on bile acid pool size.

To determine whether the decreased hepatic bile acid levels in *Fxr*-null mice fed the CA+Chol diet is reflected in whole body bile acid levels, bile acid pool sizes were also measured (Table 2). There were no significant differences in bile acid pool size and composition between wild-type mice fed the CA and CA+Chol diets. Despite significantly higher hepatic levels of bile acids in the CA-fed *Fxr*-null mice as compared to CA-fed wild type mice, the total bile acid pool tended to be lower in the *Fxr*-null mice. Thus, ratio of the hepatic bile acid content to total bile acid pool size in *Fxr*-null mice fed a CA diet was 6.5 times higher than that in wild-type mice. However consistent with changes in hepatic bile acid levels, the bile acid pool size in CA+Chol diet-fed *Fxr*-null mice was decreased 40% as compared to CA-fed *Fxr*-null mice. Analysis of the bile acid pool's composition revealed a significant decrease in total CA (TCA + CA) contents in *Fxr*-null mice fed the CA+Chol diet versus CA diet (Table 2). Significant decreases in total DCA (TDCA + DCA) contents were observed in *Fxr*-null mice, compared to wild-type mice.

Hepatic bile acid synthesis and secretion in *Fxr*-null mice fed cholic acid and/or cholesterol.

Cholesterol feeding may protect the liver from bile acid accumulation by inducing hepatic bile acid secretion. Initially, the hepatic expression of CYP7A1 was examined in

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Fxr-null and wild-type mice fed the control, CA, and CA+Chol diets. CYP7A1 mRNA levels were significantly lower in *Fxr*-null and wild-type mice fed the CA diet as compared to the control diet (Fig. 2), whereas CYP7A1 mRNA expression was significantly higher in those mice fed the CA+Chol diet as compared to the CA diet. To determine the influence of cholesterol co-administration on biliary bile acid excretion in *Fxr*-null mice fed the CA diet, biliary bile acid output rates were determined. No differences in biliary bile acid output rates were observed between *Fxr*-null mice fed the control, CA and CA + Chol diets (Fig. 3).

Ileal absorption and fecal excretion of bile acids.

Another potential mechanism for protecting the liver from dietary cholic acid-induced bile acid accumulation is reduced intestinal absorption. To estimate ileal bile acid absorption capacity, bile acid concentrations of portal blood were measured in *Fxr*-null and wild-type mice fed the control, CA, Chol, or CA+Chol diets for six days. The portal blood bile acid concentration was approximately 11-fold higher in *Fxr*-null mice fed the CA diet as compared to *Fxr*-null mice fed the control diet (Fig. 4). The portal blood bile acid concentration in *Fxr*-null mice fed the CA+Chol diet was reduced ~70% compared to the CA diet fed group. In wild-type mice, no significant differences in the portal blood bile acid concentrations were observed between CA diet and CA+Chol diet.

Next, the ileal bile acid absorption was measured using the *in situ* loop method. TCDCA is efficiently transported by the mouse ASBT, but is not typically a component of the mouse bile acid pool. TCDCA concentrations in portal blood were measured after TCDCA was infused into an *in situ* ileal loop taken from *Fxr*-null and wild-type mice

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fed the control, Chol, CA or CA+Chol diets for six days. TCDCA concentrations in the portal blood depend on the time after TCDCA injection. The ileal bile acid absorption rate was estimated as TCDCA concentration of portal blood at ten min after the injection in the following experiments. The ileal bile acid absorption rate was significantly higher in *Fxr*-null mice than that in the wild-type mice. The rate was reduced to 49% of control in the wild-type mice fed the CA diet (Fig. 5A). No significant changes in the rates were found in *Fxr*-null mice fed the control diet, Chol diet and CA diet. The rate in *Fxr*-null mice fed the CA + Chol diet was reduced to 38% of the mice fed the CA diet. The rates did not differ in the wild-type mice fed the CA diet and the CA + Chol diet. To confirm the differences in the influence of cholesterol co-administration on ileal bile acid absorption rates between *Fxr*-null and wild-type mice, the ileal bile acid absorption rate was also estimated as TCDCA concentration of portal blood at three min after the injection. Consistent with the results of TCDCA concentration at ten min after injection, the concentrations at three min after injection were significantly lower in *Fxr*-null mice fed the CA+Chol diet as compared to CA diet-fed *Fxr*-null mice (Fig. 5B). In wild-type mice, no significant differences in the concentration were observed between CA diet and CA+Chol diet. These results suggest that cholesterol co-administration decreases ileal bile acid absorption capacity in *Fxr*-null mice fed the CA diet, but not the wild-type mice.

Fecal bile acid excretion in *Fxr*-null mice fed the control, CA and CA+Chol diets were 9.4 ± 1.1 , 14.2 ± 3.2 and 55.7 ± 15.5 ($\mu\text{mol/day/100 g body weight}$) (mean \pm S.D. (n=5)), respectively. The fecal bile acid excretion was significantly increased in *Fxr*-null mice fed CA and CA+Chol diets, compared to *Fxr*-null mice fed the control diet. Furthermore, concomitant with decreases in ileal bile acid absorption, fecal bile acid

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excretion was significantly increased in *Fxr*-null mice fed the CA+Chol versus CA-diet. In wild-type mice, no significant differences in fecal bile acid excretion were observed between CA diet (50.2 ± 7.5 $\mu\text{mol/day/100 g body weight}$) and CA+Chol diet (52.1 ± 4.5 $\mu\text{mol/day/100 g body weight}$).

Ileal ASBT protein and mRNA levels.

To identify the mechanisms responsible for reduced ileal bile acid absorption in *Fxr*-null mice fed the CA+Chol diet, the expression of ASBT protein in ileal brush border membranes (BBMs) were analyzed by Western blot analyses. Proteins recognized by Asbt antibody were higher in ileal BBMs than those in duodenum BBMs from wild-type mice fed CA diet (data not shown). Marked decreases in the contents were found in both wild-type and *Fxr*-null mice fed the CA+Chol diet, compared to those mice fed the CA diet (Fig. 6A). Ileal ASBT mRNA levels were not significantly lower in *Fxr*-null and wild-type mice fed CA+Chol diet compared to those mice fed CA diet (Fig. 6B) although marked differences in the protein contents were observed. Similar levels of ileal bile acid binding protein (IBABP) and organic solute transporter α (OST α) mRNAs were observed between *Fxr*-null mice fed the CA diet and CA+Chol diet (data not shown).

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Discussion

The present study revealed that cholesterol co-administration decreases bile acid pool size and hepatic bile acid concentrations in CA-fed *Fxr*-null mice. Among the potential mechanisms responsible for the effects of cholesterol co-administration are decreased hepatic bile acid synthesis and decreased intestinal absorption. However, mRNA expression for CYP7A1, the rate-limiting enzyme for hepatic bile acid synthesis was significantly increased in *Fxr*-null mice fed the CA+Chol diet, as compared to the CA diet. Conversely, several lines of evidence suggest that ileal bile acid absorption is decreased in *Fxr*-null mice fed the CA+Chol diet. This includes the findings that the portal blood bile acid concentration was significantly decreased, ileal bile acid absorption was significantly reduced as measured using an in situ loop model, and fecal bile acid excretion was increased. Thus, ileal bile acid absorption is likely responsible in part for the reduced bile acid hepatic accumulation and bile acid pool size in CA+Chol-fed *Fxr*-null mice.

Changes in the ileal brush border membrane content of ASBT protein correlated with ileal bile acid absorption rates in *Fxr*-null, but not wild-type mice fed the CA and CA+Chol diets. ASBT-mediated uptake across the brush border membrane is thought to be one of the rate-limiting step in bile acid transport from the gut lumen into the portal circulation. As such, in *Fxr*-null mice, the reduced ileal ASBT protein expression likely accounts for the decreased ileal bile acid absorption capacity under the conditions of bile acid and cholesterol feeding, although other mechanisms for the reduction of ileal bile acid absorption cannot be excluded. Previous studies have shown that the bile acid pool size is decreased in *Asbt*-null mice or ASBT inhibitor-treated rabbits, despite

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increased CYP7A1 expression (Dawson et al., 2003; Li et al., 2004). Conversely, cholesterol-fed rabbits exhibit increased ileal ASBT expression and an increased bile acid pool size, despite reduced cholesterol 7 α -hydroxylase activity (Xu et al., 2000). These reports support the hypothesis that ileal ASBT protein expression is at least partially responsible for controlling the bile acid pool size and hepatic bile acid levels in *Fxr*-null mice fed cholic acid plus cholesterol.

Previous studies have reported that feeding a diet containing 2% cholesterol to C57BL/6J mice for 14 days decreased ileal expression of ASBT protein and mRNA (Thomas et al., 2006). The present results found a similar decrease in ASBT protein expression in *Fxr*-null and wild-type mice fed cholesterol in addition to CA. However, the decrease in ileal ASBT mRNA expression in *Fxr*-null and wild-type mice fed the CA+Chol diet was not significant in the present study. The differences in regulation of ASBT mRNA expression between the 2 studies may reflect the inclusion of cholic acid and different amounts of cholesterol (0.02% versus 0.1% in the control diet and 2% versus 1.25% in the cholesterol supplement diet), and the length of feeding (14 days versus 6 days). Although long-term cholesterol feeding might be necessary for the cholesterol-mediated reduction of ileal ASBT mRNA level in mice, the present studies were limited to 6 days because of increased toxicity after this time. Marked decrease (more than 30%) in body weight and severe physical debilitation were observed in some of *Fxr*-null mice fed CA diet for 10 days. It should be noted that both studies found that the decreases in ileal ASBT protein expression was greater than the change in ASBT mRNA expression. These results raise the possibility that cholesterol can down-regulate ASBT protein expression in the ileal brush border membrane by posttranslational mechanisms such as protein stability or membrane translocation. Posttranslational

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regulation of ASBT has previously been reported. For example, it has been shown in tissue culture cells that interleukin-1 β (IL-1 β) can down-regulate ASBT expression by inducing the ubiquitylation and proteasomal degradation of ASBT protein (Xia et al., 2004). Very recently, it was shown that ASBT protein is localized in cholesterol-containing rafts and modulation of the cholesterol content regulated ASBT activity. However in those studies, depletion of membrane cholesterol by using methyl-beta-cyclodextrin inhibited ASBT activity in tissue culture cells (Annaba et al., 2008). While our studies indicate that FXR is not required for this cholesterol regulation of ASBT protein expression, the underlying mechanisms responsible remain to be identified. It has been reported that human ASBT expression is directly down-regulated by 25-hydroxycholesterol (Alrefai et al., 2005). The suppressive effect of cholesterol and 25-hydroxycholesterol on ASBT expression is mediated through SREBP-2 and HNF1 α in Caco-2 cell (Thomas et al., 2006). ASBT expression is likely down-regulated at least by two distinct mechanisms, cholesterol signaling (SREBP) and bile acid signaling (FXR/SHP). Cholesterol-mediated signaling seems to play a crucial role in the down-regulation of ileal ASBT expression under the conditions of lacking negative feedback regulation by FXR signaling such as found in the *Fxr*-null mice. The cholesterol-mediated suppression of ileal ASBT expression might be a second defense system against hepatic bile acid accumulation.

Cholesterol metabolites and intermediates in the bile acid biosynthetic pathway can activate LXR and PXR (Goodwin et al., 2003; Sonoda et al., 2005). Feeding the 1.25% cholesterol-containing diet might lead to an accumulation of these intermediates, which activate LXR and PXR. Under conditions of absence of FXR, activated LXR and PXR might suppress hepatic bile acid accumulation to protect liver against cholic

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acid-induced injury (Guo et al., 2003; Teng and Piquette-Miller, 2007).

No significant differences in bile acid pool size were observed between *Fxr*-null and wild-type mice fed a CA diet whereas marked differences in hepatic bile acid content were found. These results raise a possibility that under the CA feeding condition, FXR signaling plays a crucial role in the suppression of accumulating hepatic bile acid content, but not that of bile acid pool size. On the other hand, cholesterol-mediated signaling is likely involved in the suppression of accumulating bile acid pool size and hepatic bile acid content under the high bile acid condition of lacking FXR.

In the present study, we showed that cholesterol feeding prevents hepatic bile acid accumulation in CA-fed *Fxr*-null mice via down-regulation of ileal bile acid absorption. Furthermore, using *Fxr*-null mice co-treated with cholesterol and CA, we demonstrated a crucial role of ileal bile acid absorption in regulation of hepatic bile acid levels. Because CA-fed *Fxr*-null mice markedly increase hepatic bile acid levels, the mice seem to be a good model to identify bile acid regulatory mechanisms independent of FXR signaling. The cholesterol-mediated negative regulation of ileal bile acid absorption may be involved in the maintenance of not only bile acid, but also cholesterol homeostasis. Further studies are necessary to identify the mechanism for the suppression of ileal bile acid absorption through cholesterol signaling.

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Footnotes

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Figure legends

Fig. 1. Dose-dependent effect of cholesterol feeding on liver injury diagnostic markers, hepatic bile acid and cholesterol levels in *Fxr*-null mice. (A) Plasma ALT activity. (B) Plasma ALP activity. (C) Hepatic bile acid (black bar) and cholesterol (white bar) levels. *Fxr*-null mice were fed a diet supplemented with 0.5% cholic acid (CA), 0.5% cholic acid and 0.5% cholesterol (CA + Chol 0.5%) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. Data are shown as the mean \pm S.D. (n=4). The data are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs CA diet group.

Fig. 2. Hepatic levels of CYP7A1 mRNA. Hepatic CYP7A1 mRNA levels were measured by real-time quantitative PCR. Hepatic RNAs were prepared from *Fxr*-null and the wild-type mice fed a control diet (Cont), or diet supplemented with 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. The mRNA expression levels were normalized to GAPDH mRNA. Ct values for CYP7A1 and GAPDH of wild-type mice fed control diet were 29.1 and 22.0, respectively. Data are shown as the mean \pm S.D. (n=4). The data are representative of two independent experiments. *, $p < 0.05$; ***, $p < 0.001$ vs control diet group. #, $p < 0.05$ vs CA diet group.

Fig. 3 Changes in biliary bile acid excretion in *Fxr*-null mice. *Fxr*-null mice were fed a control diet (Cont), or diet supplemented with 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. Bile was collected for 30 min by

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bile duct cannulation. Data are shown as the mean \pm S.D. (n=5). The data are representative of two independent experiments.

Fig. 4 Portal blood bile acid levels. *Fxr*-null and wild-type mice were fed a control diet (Cont), or diet supplemented with 1.25% cholesterol (Chol), 0.5% cholic acid (CA), or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. Portal blood was collected from mice anesthetized with ethyl ether. Bile acid concentration was measured by HPLC as described in Materials and Methods. Data are shown as the mean \pm S.D. (n=3). The data are representative of two independent experiments. The data are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs control diet group. #, $p < 0.05$ vs CA diet group.

Fig. 5 Changes in ileal bile acid absorption rate. *Fxr*-null and wild-type mice were fed a control diet (Cont) or the diet supplemented with 1.25% cholesterol (Chol), 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. Portal blood was collected at ten min (A) or three min (B) after TCDCA injection. Data are shown as the mean \pm S.D. (n=5). The data are representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs control group; ##, $p < 0.01$ vs CA group.

Fig. 6 Changes in ileal ASBT protein and mRNA levels. (A) Ileal ASBT protein level. Ileal brush border membranes were isolated from wild-type and *Fxr*-null mice fed a control diet supplemented with 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA+Chol) for six days. Pooled brush border membrane extracts (15 μ g) from four mice were subjected to immunoblot analysis using a polyclonal anti-rodent

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Asbt antibody or monoclonal anti β -actin antibody. (B) Ileal ASBT mRNA level. Total RNA was isolated from ileum of wild-type and *Fxr*-null mice fed a control diet supplemented with 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA+Chol) for six days. ASBT mRNA levels were evaluated by real-time quantitative PCR. The mRNA expression was normalized to β -actin mRNA. Ct values for ASBT and β -actin of wild-type mice fed CA diet were 21.5 and 15.5, respectively. Data are shown as the mean \pm S.D. (n=4). The data are representative of two independent experiments.

Table1 Changes in bile acid and cholesterol levels and liver damage diagnostic markers

The data are shown as the mean \pm S.D. from four to six mice. *Fxr*-null and wild-type mice were fed a control diet (Cont) or the diet containing 1.25% cholesterol (Chol), 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days.

	Wild-type				<i>Fxr</i> -null			
	Cont	Chol	CA	CA + Chol	Cont	Chol	CA	CA + Chol
Liver bile acid (nmol/g liver)	157 \pm 42	72 \pm 68	424 \pm 98 ^a	490 \pm 38 ^a	580 \pm 350	485 \pm 305	2687 \pm 646 ^a	1313 \pm 374 ^{a,b}
Liver cholesterol (mg/g liver)	2.72 \pm 0.32	2.66 \pm 0.46	3.86 \pm 0.95	9.10 \pm 2.16 ^{a,b}	2.83 \pm 0.41	6.14 \pm 0.71 ^a	4.97 \pm 0.90 ^a	14.1 \pm 5.10 ^{a,b}
Plasma cholesterol (mg/dl)	75 \pm 5	57 \pm 6	69 \pm 18	58 \pm 20	111 \pm 9	97 \pm 14	110 \pm 14	160 \pm 16 ^{a,b}
ALT (IU/L)	8 \pm 1	12 \pm 2	103 \pm 53 ^a	183 \pm 63 ^a	38 \pm 7	30 \pm 15	509 \pm 159 ^a	103 \pm 63 ^{a,b}
ALP (IU/L)	29 \pm 4	30 \pm 5	40 \pm 9	33 \pm 10	60 \pm 11	52 \pm 2	602 \pm 103 ^a	291 \pm 60 ^{a,b}

^a, $p < 0.05$ versus control group.

^b, $p < 0.05$ versus CA group.

Table 2 Bile acid composition of bile acid pool.

Fxr-null and wild-type mice were fed a control diet containing 0.5% cholic acid (CA) or 0.5% cholic acid and 1.25% cholesterol (CA + Chol) for six days. Liver, gallbladder and small intestine were isolated and bile acids were extracted. Bile acid pool size was analyzed by HPLC. The data are shown as the mean \pm S.D. from five mice. TLCA, LCA, β MCA, UDCA, TCDCA and CDCA were below detection limits (0.1 μ mol/ 100 g BW).

	TCA + CA	TDCA + DCA	T β MCA + β MCA (μ mol/ 100 g BW)	TUDCA + UDCA	Total
Wild-type (CA)	95.9 \pm 11.0	27.9 \pm 16.0	1.1 \pm 1.5	4.0 \pm 2.9	128.9 \pm 15.1
Wild-type (CA + Chol)	79.7 \pm 12.8	29.7 \pm 4.9	0.3 \pm 0.5	4.1 \pm 1.4	113.9 \pm 13.3
<i>Fxr</i> -null (CA)	96.4 \pm 26.1	6.1 \pm 3.4	1.3 \pm 1.0	5.8 \pm 1.8	109.6 \pm 26.2
<i>Fxr</i> -null (CA + Chol)	55.5 \pm 12.6 ^a	5.8 \pm 2.8	0.2 \pm 0.2	3.9 \pm 1.8	65.4 \pm 16.6 ^a

^a, $p < 0.05$ versus corresponding CA group

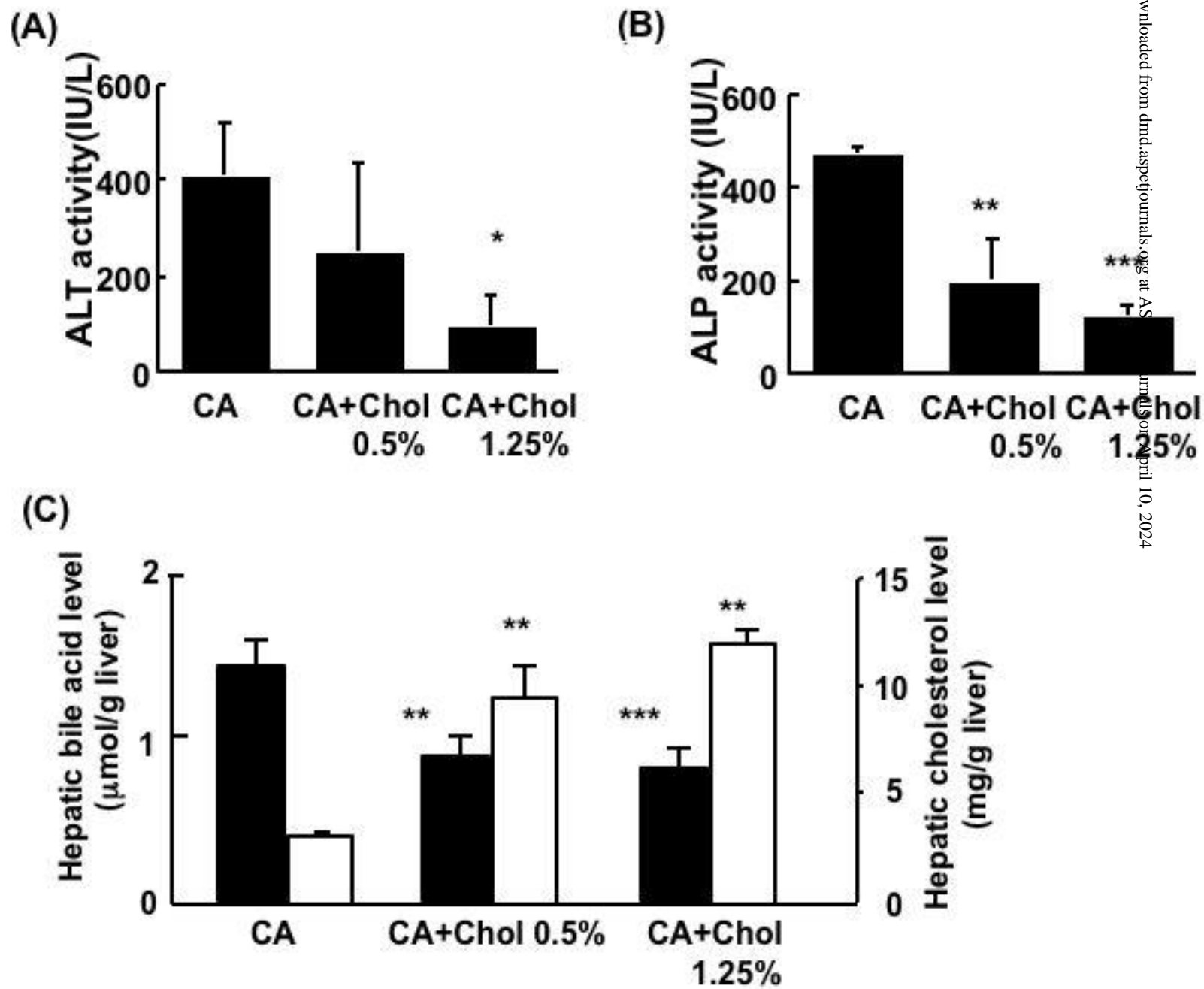


Fig. 1

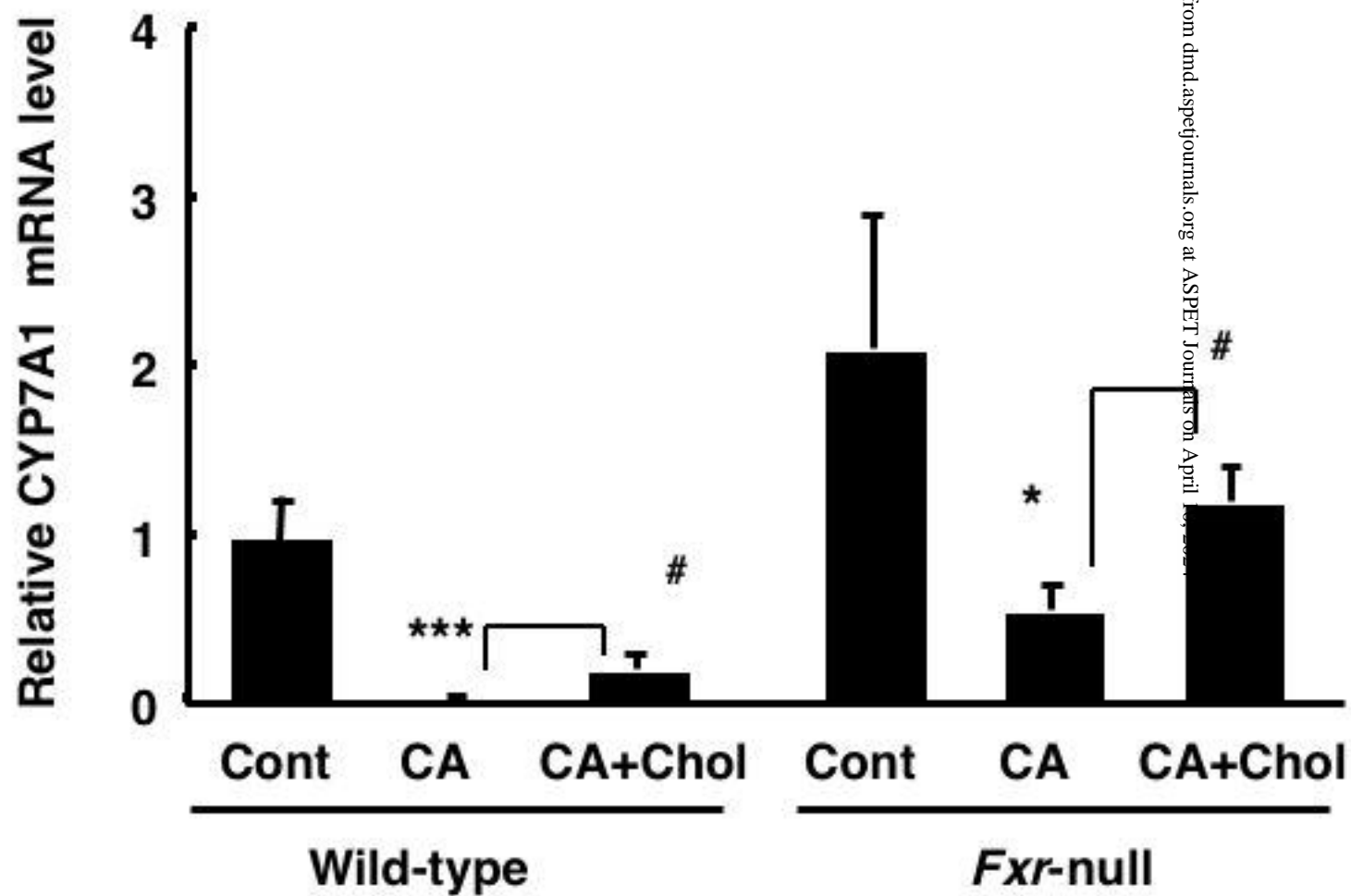


Fig. 2

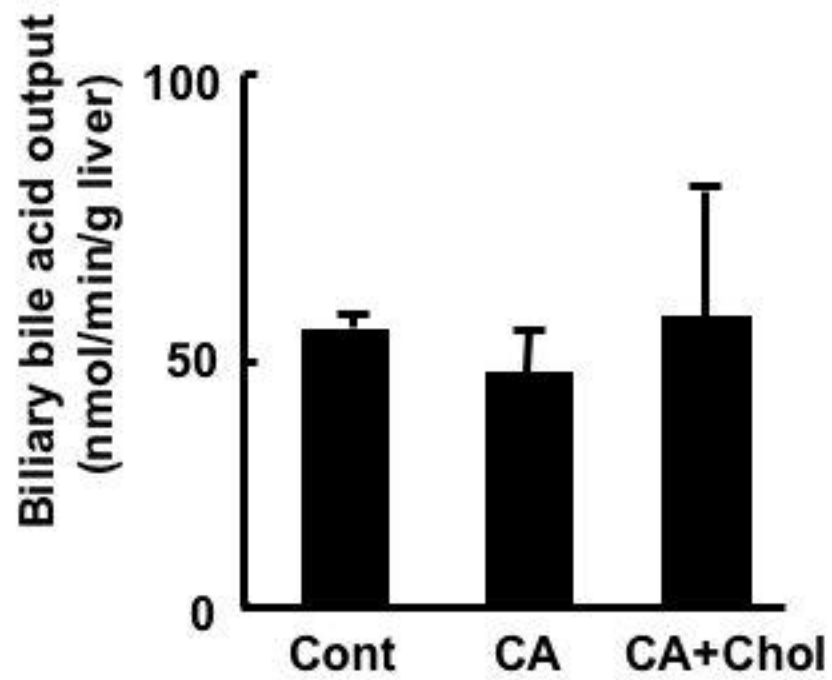


Fig. 3

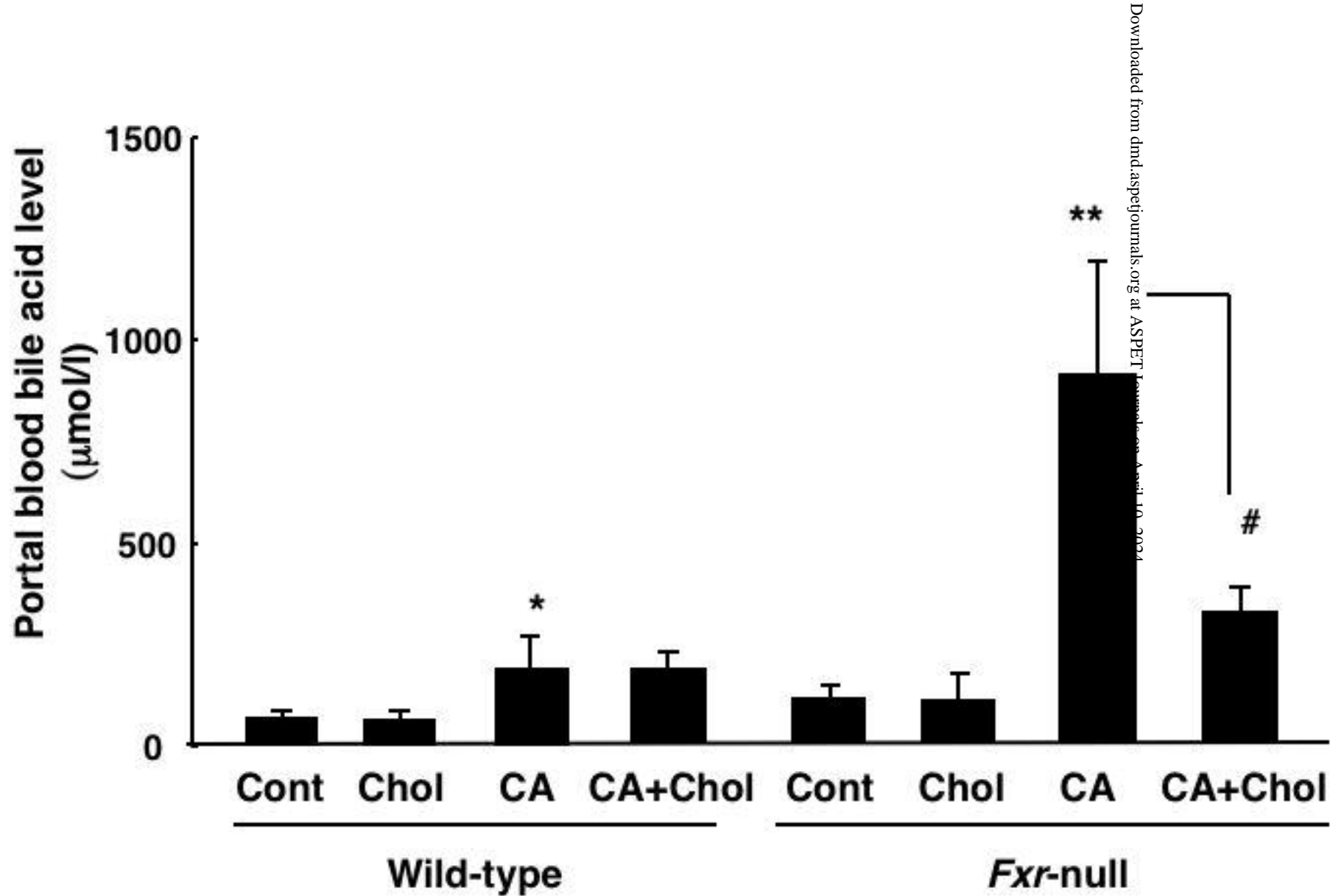


Fig. 4

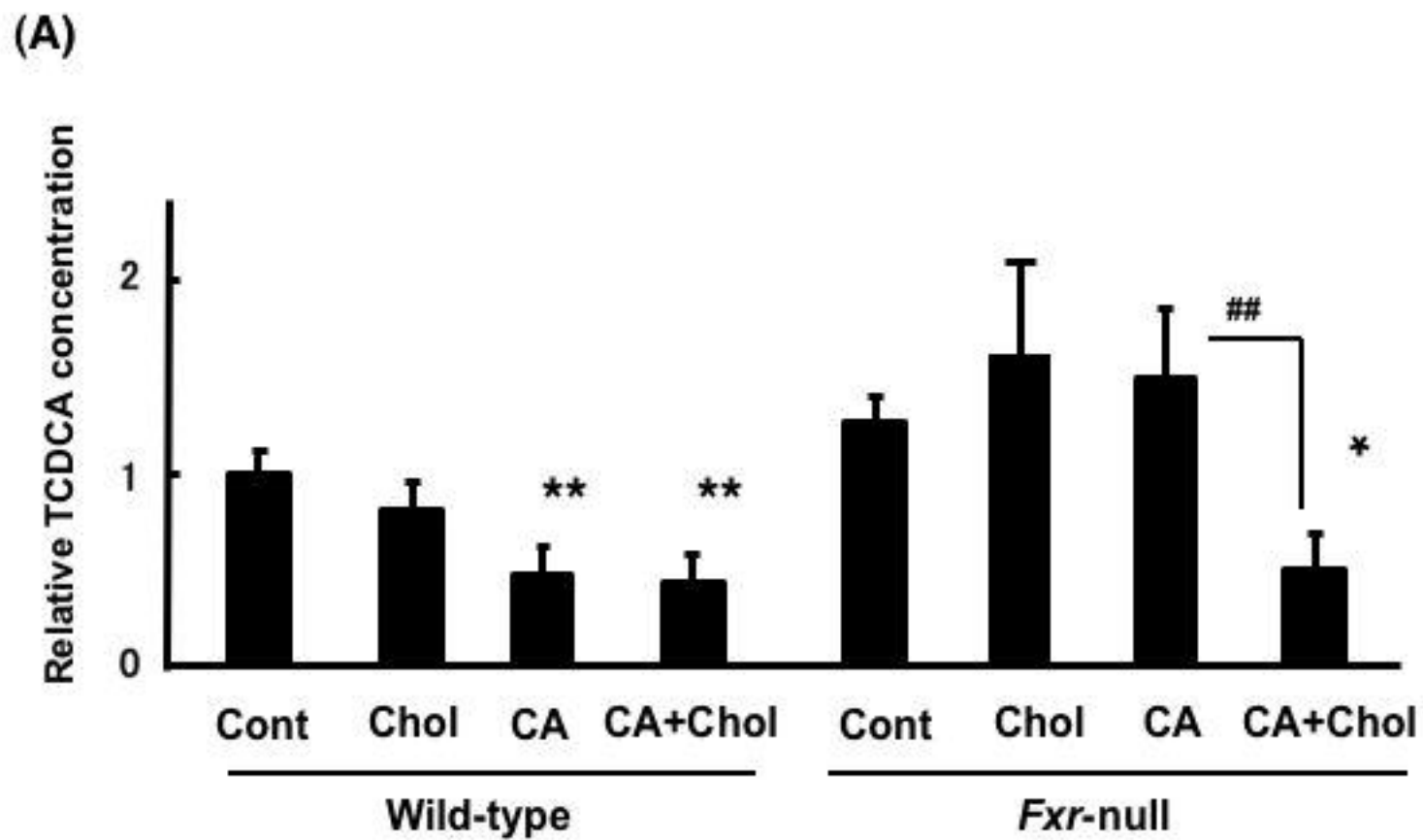


Fig. 5A

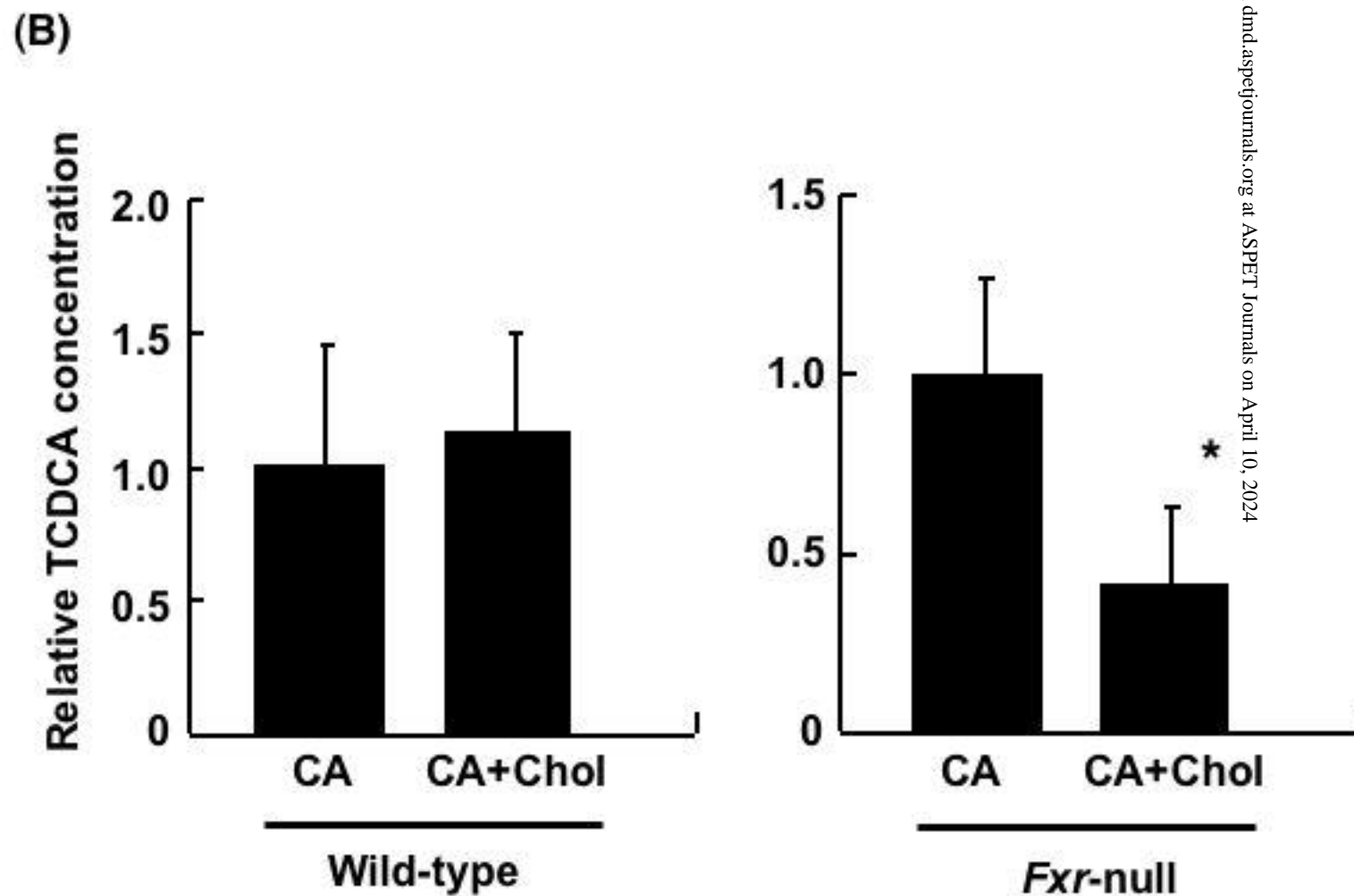


Fig. 5B

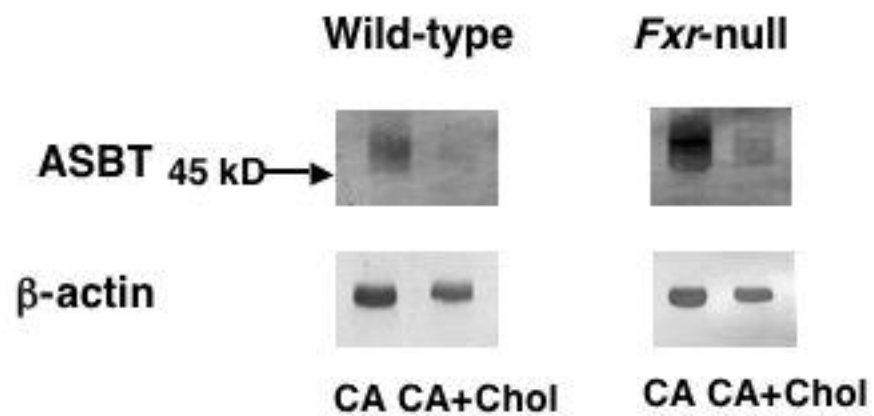


Fig. 6A

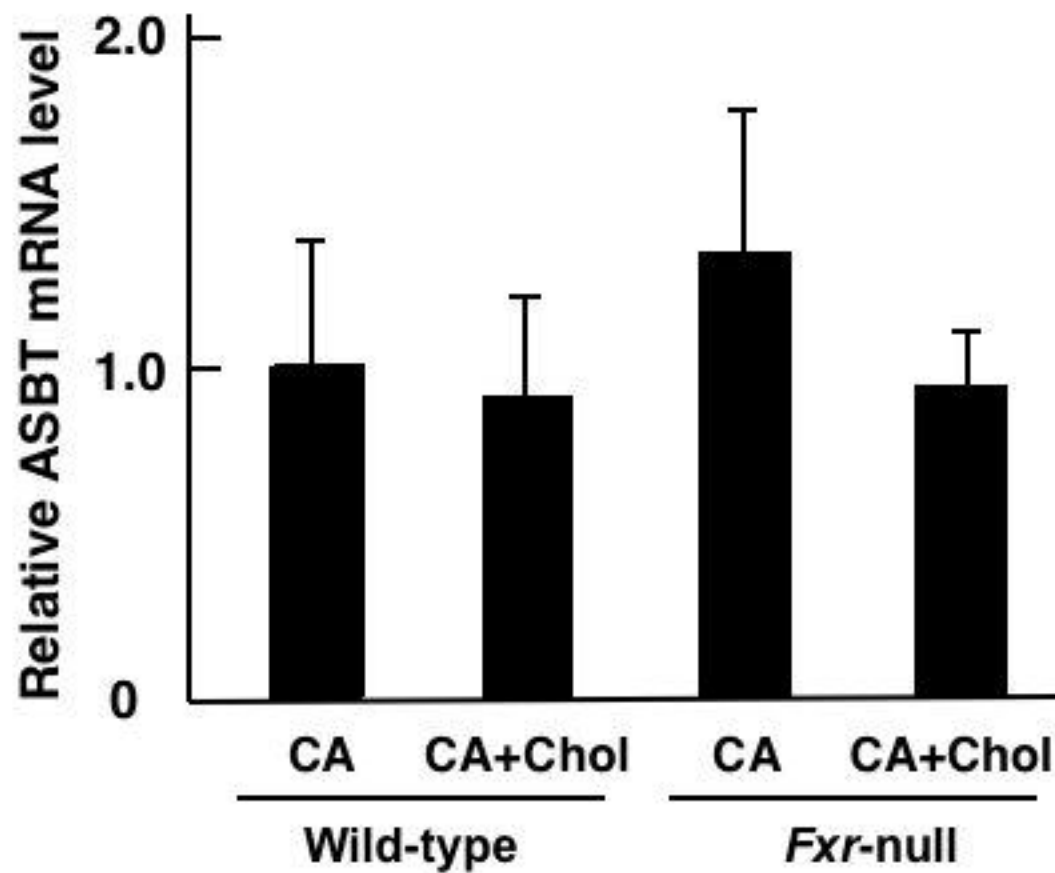


Fig. 6B