Cholangiocyte bile salt transporters in cholesterol gallstone–susceptible and resistant inbred mouse strains

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Abstract

Background and Aim—We investigated the dietary and gender influences on the expression and functionality of cholangiocyte bile salt transporters and development of biliary hyperplasia in cholesterol gallstone-susceptible C57L/J and resistant AKR/J mice.

Methods—C57L and AKR mice were fed chow, a lithogenic diet, or a cholic acid-containing diet for 14 days. Expression of cholangiocyte bile salt transporter proteins ASBT (SLC10A2), ILBP (FABP6), and MRP3 (ABCC3) were studied by Western blot analysis. Taurocholate uptake studies were performed using microperfusion of isolated bile duct units. The pre- and post-perfusion taurocholate concentrations were analyzed by high performance liquid chromatography. Biliary proliferation in liver sections was scored.

Results—The lithogenic diet induced ductular proliferation in C57L mice. On chow, SLC10A2 and ABCC3 were overexpressed in male and female C57L compared to AKR mice. A lithogenic diet reduced the expressions of FABP6 in both male and female C57L mice, SLC10A2 in female C57L mice, and ABCC3 in male C57L mice. These alterations in transporter expressions were not associated with changes in taurocholate uptake. The lithogenic diet induced biliary hyperplasia and reduced bile salt transporter expressions in C57L mice.

Conclusions—Although bile salt uptake was not increased in the bile duct unit, we speculate that the biliary hyperplasia on the lithogenic diet may lead to an increase in intrahepatic bile salt recycling during cholesterol cholelithogenesis.

Keywords

bile salt transporters; cholangiocytes; cholehepatic shunting; cholelithiasis; cholesterol; proliferation

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Introduction

Cholelithiasis affects an estimated 20–30% of Western populations and hence is a significant and costly health problem.[1] Two inbred mouse strains C57L (cholesterol gallstone–susceptible) and AKR (cholesterol gallstone–resistant) are important animal models for studying the genetics and pathophysiology of cholesterol gallstone disease.[2] C57L mice are characterized phenotypically by higher bile flow, higher bile salt, phospholipid, and especially cholesterol secretion rates, and larger bile salt pool sizes compared to AKR mice.[3] On a lithogenic diet (containing 0.5% cholic acid, 1% cholesterol, and 15% dairy fat), C57L mice develop sustained hypersecretion of cholesterol compared to bile salt plus phospholipid, which leads to lithogenic supersaturation of gallbladder bile and rapid cholesterol crystal nucleation, and gallstone formation with C57L males displaying a higher prevalence compared to females.[3] There were no gender differences in the prevalence of cholesterol gallstones in the AKR strain; therefore, the bile salt transport system in the female AKR mice were not studied. The possibility that intrahepatic recycling of bile salts might augment differences in cholesterol contents of bile between the two strains is an appealing supposition.

Several bile salt transporters have been identified on large cholangiocytes. These include the apical sodium dependent bile salt transporter (SLC10A2) also known as apical or ileal sodium-dependent bile acid transporter (ASBT or IBAT) located on the apical membrane[4,5] and mapped to mouse chromosome 8;[6] fatty-acid- binding protein subclass 6 (FABP6) also known as ileal lipid (bile acid) binding protein (ILBP or IBABP) in the cytosol[7,8] and mapped to mouse chromosome 11;[9] and putatively the multiple-drug-resistance-related protein isoform 3 (ABCC3 also known as MRP3), expressed on the basolateral membrane domain as evidenced by human[10] and rat[11,12] studies. On the basolateral and apical domains of hepatocytes, the two principal bile salt transporters are the sodium taurocholate cotransporter (SLC10A1 also known as NTCP) and the bile salt export pump (ABCB11 also known as BSEP).[13,14] In the normal intrahepatic biliary tree, cholangiocytes are morphologically and functionally heterogeneous with water and solute transport occurring primarily in large cholangiocytes (16).

Based on previous observations of inducible biliary hyperplasia and increased circulating bile salt pool size,[15,16] we hypothesized that increases in bile salt secretion rate in C57L compared to AKR mice might be promoted, in part, by augmented intrahepatic bile salt recycling via increased transporter expression or an increase in absorptive surface area of large cholangiocytes. A putative increase in bile salt secretion rate may in turn lead to enhanced cholesterol secretion, which would augment cholesterol supersaturation of bile. Here we examined the influence of a standard lithogenic diet, an identical diet containing cholic acid alone without cholesterol, plus gender and mouse strain on the induction of biliary hyperplasia as well as the expression and functionality of bile salt transporters on large cholangiocytes.

Methods

Animals

Male and female C57L and male AKR mice, 9 to 11 weeks of age, were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Animals were housed in a temperature-controlled environment of 20 to 22°C, with normal light–dark cycles of 12 h each. Animals were permitted free access to water, and were fed standard chow, or the lithogenic diet as described below. For histologic studies, male/female C57L mice and male AKR mice, were fed either regular chow or the lithogenic diet containing 0.5% cholic acid, 1% cholesterol, and 15% dairy fat for 14 days. For transporter protein expression and functionality studies,

male/female C57L mice and male AKR mice were fed regular chow or the lithogenic diet for 14 days.

Isolation of cholangiocytes

Cholangiocyte isolation followed the method of Alpini *et al.*[17] Briefly, fasting mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal [i.p.]). A laparotomy was performed and the peritoneal cavity was entered, and the portal vein was cannulated with a 20-gauge intravenous (i.v.) catheter. To remove blood cells prior to harvesting, livers were perfused via the portal vein with a preoxygenated sterile solution of 0.9% NaCl. The livers were then placed in a preoxygenated Hepes-buffered saline (HBS) buffer (20 mM Hepes, 150 mM NaCl [pH 7.4]); hepatocytes were removed first by gentle mechanical disruption of Glisson's capsule. The portal tract segments were then digested with an enzyme solution containing RPMI-1640 supplemented with 0.03% collagenase XI, 0.02% DNAase, and 0.03% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C. The remaining portal tract segments were washed with preoxygenated HBS buffer solution, and further microdissection was performed at higher magnification to remove residual hepatocytes. The biliary tree segments were redigested with 0.02% DNAase, 0.03% collagenase type XI, and 0.05% hyaluronidase for 10 min at 37°C, and further microdissected under higher magnification.

Western blot analysis

Freshly isolated portal tract units were homogenized on ice in a Teflon homogenizer in buffer solution containing protease-inhibitors (pepstatin A 0.2 ug/uL and phenylmethylsulphonyl fluoride 0.4 uM; leupeptin 0.5 ug/uL, Tris 2.5 mM, and EDTA 0.13 mM [pH 7.4]), then sedimented at a relative centrifugal force (RCF) of 2000 *g* for 10 min. The resulting pellet was discarded and the supernatant was sedimented again at an RCF of 2000 *g* for 10 min. Protein concentrations were determined using the Bradford method (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as the reference standard.

For Western blot analysis of SLC10A2, FABP6, and ABCC3 transporters, 100 µg of protein homogenate were heated to 95°C for 5 min, and cooled on ice before the samples were loaded onto NuPage 4–12% Bis-Tris mini-gel (Invitrogen, Carlsbad, CA, USA) for electrophoresis. The gel was then transferred overnight to nitrocellulose membranes. The blots were then washed at room temperature with phosphate-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h, followed by incubation with primary antibodies for SLC10A2, FABP6, and ABCC3 for 2 h. The SLC10A2 antibody was obtained from Research Genetics (Invitrogen) using immunization of rabbits with peptide synthesized according to the published sequence of Schneider *et al.*[4] The FABP6 antibody was monoclonal generously donated by Dr. Luis Agellon (University of Alberta, Edmonton, AB, Canada). The ABCC3 antibody was a polyclonal antibody used in previous studies,[18] kindly provided by Dr. Hiroshi Suzuki (University of Tokyo, Tokyo, Japan). The primary SLC10A2 antibody was incubated at 1 : 100 dilution, FABP6 antibody at 1 : 14 000 dilution, and ABCC3 antibody at 1 : 1000 dilution. Following these procedures, the membrane was incubated with 0.2-µg/mL goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (Biosource International, Camarillo, CA, USA).

Protein size was estimated using prestained molecular weight standards (Superscript II RT protein ladder; Invitrogen). Immunoreactive bands were revealed with enhanced chemiluminescence light substrate (Amersham Biosciences, Piscataway, NJ, USA). The density of each band was read with a densitometeter (Biorad Life Sciences, Hercules, CA, USA) and quantified using Geldoc densitometer software (Biorad).

Histopathologic examination

Immediately after sacrifice, livers or fragments of liver tissue from experimental and control mice were placed in 10% buffered formalin. The formalin fixed tissue was embedded in paraffin, and 5-µm thick sections were stained with hematoxylin–eosin. Sections were reviewed by a hepatopathologist (J.N.G.) who was blinded to the mouse strain, gender, and diet. Bile ducts were quantified by counting the number of bile duct images per 100 portal tracts. Bile duct density greater than a normal of 1 per portal tract was considered to be in a proliferative phase.

Isolation of bile duct units and bile salt uptake studies

Bile salt uptake by cholangiocytes was studied using microperfusion of freshly isolated bile duct units (IBDU) employing previously published methods.[19] IBDU are formed by large cholangiocytes; bile ductules are formed by small cholangiocytes. In brief, mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). A laparotomy was performed and the liver was perfused with sterile 0.9% NaCl solution through the portal vein at 4°C. Subsequently, liquid Trypan blue agar (2 to 3 mL) was injected into the portal vein. The liver was then removed and immersed in preoxygenated HBS buffer solution at 4°C. After the hepatic capsule and surface hepatocytes were removed, intrahepatic bile ducts were dissociated under a dissecting microscope using the portal vein (now filled with Trypan blue agar) as reference marker. The dissociated bile duct was digested by shaking at 37°C for 10 min in an enzyme solution as used for cholangiocyte isolation (vide supra). Further microdissection was performed at higher magnification to remove residual hepatocytes, fragments of portal veins and hepatic arteries, and excess connective tissue

The IBDUs were then perfused using a microperfusion apparatus previously described[19] with perfusate containing Krebs–Ringer bicarbonate buffer (KRB) of the following composition: 120 mM NaCl, 5.9 mM KCl, 1.2 mM Na_2HPO_4, 5 mM glucose, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM $NaHCO_3$. Purified sodium taurocholate was then added to the KRB solution to achieve a final concentration of 50 mM at 7.4 pH. The perfusion rate was 114 nL/min for 60 min in the microperfusion apparatus.[20] Pre- and post-perfusates from the perfusion studies were collected and analyzed for taurocholate concentration using high performance liquid chromatography.

Statistical analysis

Data were analyzed using Student's *t*-test. All results were expressed as mean ± standard error. *P*-values of less than 0.05 were considered to be significant.

Results

Histologic studies

Figure 1 displays representative liver histology of AKR and C57L on chow (Fig. 1a,b) and lithogenic diets (Fig. 1c,d), respectively. In the C57L strain, ductular proliferation became significantly more obvious than on the lithogenic diet with 31 ± 3 ductules per 100 portal tracts compared to 6 ± 2 ductules per 100 portal tracts on the chow diet ($P < 0.01$). The ductular proliferation on the cholic acid diet was comparable to the lithogenic diet with 26 ± 4 ductules per 100 portal tracts compared to the same control ($P < 0.01$). In the AKR strain, ductular proliferation, although increased on the lithogenic and cholic acid diets, 21 ± 5 and 12 ± 2 respectively, was not significantly different compared to chow-fed animals (10 ± 1).

Bile salt transporter protein expression

Figure 2 shows representative Western blots of SLC10A2, FABP6, and ABCC3. Densitometric analysis for SLC10A2, FABP6, and ABCC3 expressions were then normalized to actin, which was highly variable as displayed. Figure 3 illustrates that the actin-normalized relative protein expression of apical membrane transporter SLC10A2 was higher for male C57L mice on chow (3.6 ± 1.2, $P = 0.05$) compared to the AKR strain. On the lithogenic diet, the relative SLC10A2 expression in the AKR strain and C57L males (0.63 ± 0.1, $P = 0.06$) did not change.

As shown in Figure 3, the expression of the cytosolic transporter FABP6 was similar for male and female C57L mice compared to male AKR mice on chow. While ingesting the lithogenic diet, the expression of FABP6 was downregulated in both male C57L mice (0.47 ± 0.05 *vs* 1, $P = 0.04$), and in female C57L mice (0.66 ± 0.02, $P < 0.01$). On the lithogenic diet, the relative FABP6 expression in male AKR mice remained constant.

As displayed in Figure 3, the relative expression of basolateral membrane transporter ABCC3 was higher in male C57L mice (1.9 ± 0.3, $P = 0.03$) and female C57L mice (1.8 ± 0.3, $P = 0.04$) compared to AKR mice on chow. In the case of the C57L strain, although expression was downregulated in male mice on the lithogenic diet (1.1 ± 0.2, $P = 0.03$), it remained unchanged in the female mice (1.8 ± 0.4). In the AKR strain, there were no changes in ABCC3 expression on the lithogenic diet. The relative expression levels of all three transporters in male AKR mice on the lithogenic diet, and male/female C57L mice on chow or lithogenic diet are summarized in Table 1.

Bile salt uptake by bile duct units

Taurocholate uptake studies using IBDUs were performed for male AKR and C57L mice on chow and after 2 weeks of lithogenic diet feeding. As shown in Fig. 4, taurocholate uptake by IBDUs from mice on chow was 2.0 ± 0.4 nmol/min/mm length of bile duct unit for the AKR strain ($n = 3$) and 3.2 ± 1.2 nmol/min/mm for the C57L strain ($n = 6$). After 2 weeks of lithogenic diet feeding, sodium taurocholate uptake remained unchanged in both strains at 2.2 ± 1.1 nmol/min/mm for AKR ($n = 4$), and at 1.5 ± 0.4 nmol/min/mm for C57L ($n = 3$), all displaying nonsignificant differences between strains and diet.

Discussion

Our study aimed at determining indirectly the contribution, if any, of intrahepatic recycling (cholehepatic shunting) of bile salt via the transport system on large cholangiocytes to cholesterol gallstone formation using quantitative histologic assessment of the biliary tree as well as molecular and functional studies of bile salt transporters in *ex vivo* cholangiocyte units. We hypothesized that the differences in cholesterol contents of bile between the cholesterol gallstone–susceptible C57L mice and the cholesterol gallstone–resistant AKR mice is due to increased intrahepatic recycling of bile salts in the C57L strain. Our data showed (i) enhanced biliary hyperplasia in the cholesterol gallstone–susceptible C57L mice on the lithogenic diet; (ii) higher expression levels of membrane bile salt transport proteins SLC10A2 and the exchanger ABCC3 in C57L mice compared to AKR mice on chow; (iii) the lithogenic diet decreased expression levels of FABP6 and ABCC3 in male C57L mice, and SLC10A2 and FABP6 proteins in female C57L mice; and (iv) similar taurocholate uptake levels per length of isolated duct units in male C57L and AKR mice on chow and the lithogenic diet. These data strongly support the concept that the lithogenic diet–induced biliary hyperplasia but decreased expression of bile salt transporters all without significantly influencing their functionality per unit length as assayed in *ex vivo* sodium taurocholate uptake studies. The increase in the absorptive surface area of biliary epithelia resulting from

the marked proliferation of bile ducts in C57L mice on the lithogenic diet may predict increases in cholangiocytic bile salt absorption; hence an increase in intrahepatic recycling of bile salts.

Previous studies have shown that bile acid feeding induced biliary hyperplasia in rats and increased cholehepatic recycling of the bile salt pool due to increases in SLC10A2 protein expression and bile salt transport activity.[15,16] The increase in the density of intrahepatic bile ducts was attributed to increased concentration and secretion of bile salts in hepatic bile induced by bile acid feeding.[20] In addition, in rats fed bile acids, Alpini *et al.*[15] demonstrated by DNA synthetic rates and ductual secretory activities that the increased proliferative activity of cholangiocytes involved large and not small cholangiocytes. The results of the present study confirm previous findings in mice that a cholic acid–enriched diet (i.e. same diet as the lithogenic diet minus cholesterol) induces increased bile flow and biliary lipid secretion rates,[21] and in our study, enhanced biliary ductular proliferation. The ductular proliferation observed with cholic acid feeding as well as the lithogenic diet strongly suggest that the cholesterol component is not responsible for this effect.

Bile salts are the natural ligands for the nuclear farnesoid X receptor (FXR). Bile salt responsiveness of the mouse *SLC10A2* gene has been shown to be mediated indirectly by FXR-dependent activation of the short heterodimer partner SHP1 and subsequent inhibition of liver receptor homologue-1 (LRH-1) activity.[22] Prior to this study, there were conflicting reports of positive, negative, and no feedback regulation of SLC10A2 expression by bile salt, with variable experimental methodologies and animal species.[7,23–31] ASBT expression has been quantified at the level of protein[7,27–31] and mRNA,[7,26,27,31] and demonstrates how the contradictory nature of the literature reflects the difficulties of *in vivo* assessment of a transport system that is part of a highly integrated and tightly regulated metabolic pathway.

The bile salt transport system of large cholangiocytes responsible for cholehepatic shunting of bile salts consists of confirmed transporters—the apical transporter SLC10A2 and cytosolic transporter FABP6—and when this study was completed, ABCC3 was believed to be the putative basolateral transporter exchanger.[11–13] Recently, an heteromeric organic solute transporter (OST) α/β was characterized and found to be a more likely basolateral true transporter for bile salts in ileocytes and cholangiocytes.[32,33] The expression of OSTα-OSTβ proteins were not investigated in the present study since it was only described in the little skate and not in mammals[32] when the present work was performed. The basolateral ABCC3 transporter could, of course, be an ancillary anion exchanger for bile salt, with OSTα-OSTβ as the principal transporter. Future investigations are needed to assess the lithogenic diet responsiveness of the OSTα-OSTβ basolateral transporter to fully define its perturbations in cholangiocyte bile salt transport. The gallstone susceptible C57L strain appeared to have higher levels of membrane transporters on chow compared to the gallstone-resistant AKR mice. Feeding the lithogenic diet, which contains both cholic acid and cholesterol, resulted in an overall reduction in the expression of all three transport proteins in cholangiocytes from C57L mice, but no net change in cholangiocytes from AKR mice. Our study of the cholangiocyte bile salt transporters in the C57L mice has clearly demonstrated exquisite sensitivity to the lithogenic diet in the absence of any significant alteration in normalized bile salt uptake in the microperfused bile duct system.

These results taken together revealed that the lithogenic diet induced more biliary hyperplasia in the cholesterol gallstone–susceptible C57L mice compared to gallstone-resistant AKR mice.[3] Decreased expression levels or little change of bile salt transport proteins on the lithogenic diet in both male and female C57L mice, but their relative constancy in the AKR mice suggests more efficient enterohepatic cycling and FXR activation in C57L mice. The changes in expression levels of bile salt transporters did not

translate into alterations in bile salt uptake, that is, normalized per mm bile duct length, as measured by microperfusion of freshly isolated bile duct units. This observation may in part relate to the concept that the function of membrane transporters is not merely regulated by the total number of transporters but also their functional state and cellular localization. Moreover, the functional state of other bile salt transporters in the transport system may also be crucial. Furthermore, as in the ileum, the cytosolic transporter FABP6 of cholangiocytes appears to be critical in preventing the direct exposure of intracellular organelles to potential bile salt toxicity. Therefore, the functional state of FABP6, which was uniformly decreased in expression in both mouse strains by the lithogenic diet, may also influence the uptake of bile salt by cholangiocytes.

The lithogenic mechanisms in gallstone-susceptible C57L mice are believed to be a combination of imbalance of lipid hemostasis and inflammatory responses to environmental stimuli. Our study focuses on the role of lipid hemostasis imbalance, principally, bile salt hypersecretion in C57L mice, and its role in cholesterol gallstone formation. Our results suggest that there may be increased overall cholehepatic bile salt recycling, thereby contributing to cholesterol supersaturation.

Finally, taking all our results together, we argue that an increase in the absorptive surface area of IBDUs due to the marked proliferation of bile ducts in C57L mice on the lithogenic diet may predict increases in cholangiocytic bile salt absorption. As found in this work, although equivalent levels of bile salt uptake per mm by individual bile duct units occurred, the proliferation density of transporters on the lithogenic diet, especially in C57L mice, should be capable of augmenting cholehepatic shunting. Therefore we can tentatively conclude from our studies that the lithogenic diet, which induces more biliary hyperplasia in C57L mice compared with AKR mice, should support increased overall cholehepatic bile salt shunting within the intrahepatic biliary tree despite constant bile salt absorption per unit length of ductules. This may contribute to enhanced bile salt shunting and hepatic resecretion, and possibly may augment cholesterol supersaturation of bile in the cholesterol gallstone–susceptible C57L murine strain.

Acknowledgments

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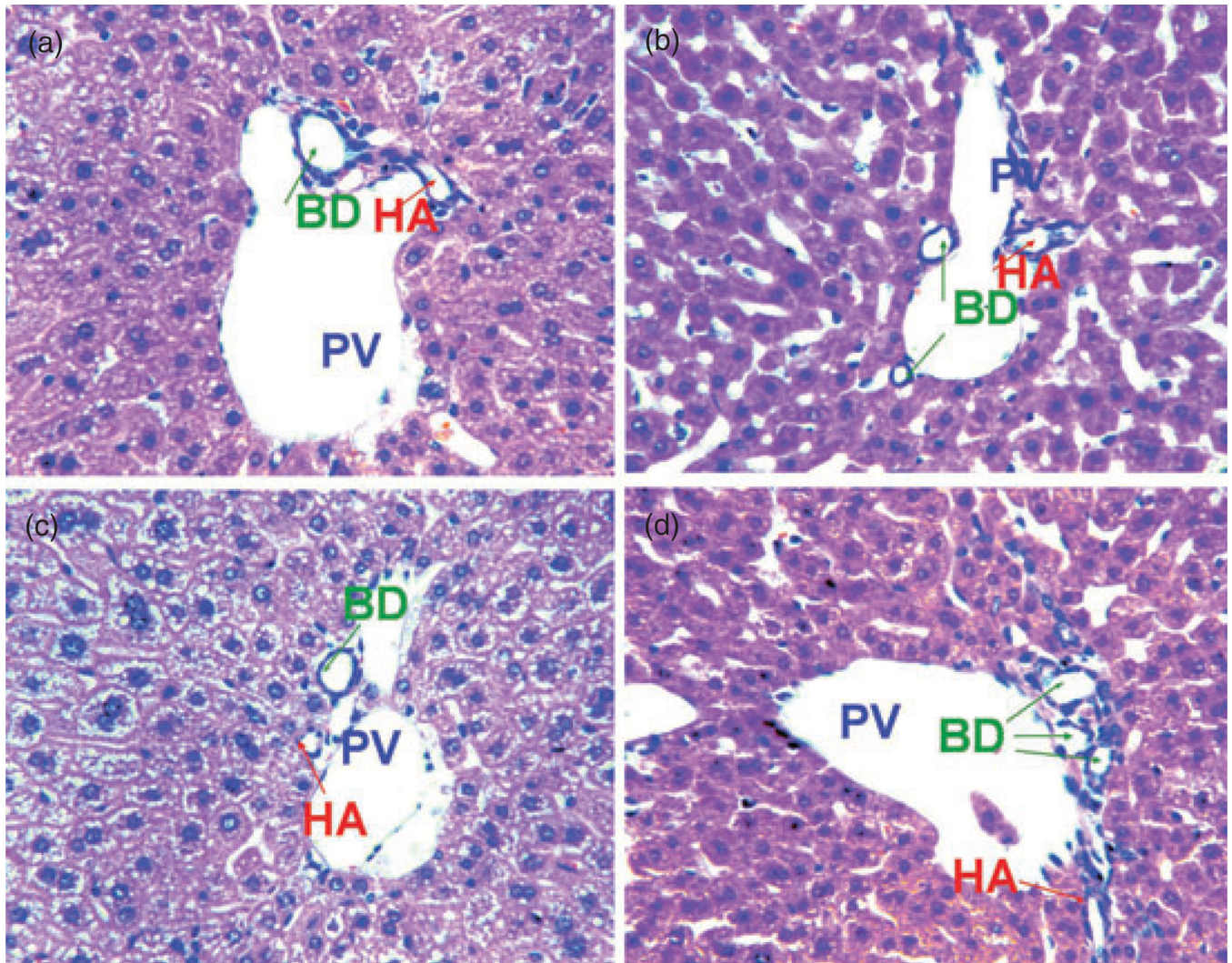
Figure 1.
Representative histopathologic sections of mouse livers. (a) Liver of an AKR mouse on chow; (b) liver of an AKR mouse on the lithogenic diet; (c) liver of an C57L mouse on chow; (d) liver of an C57L mouse on the lithogenic diet. Bile ducts (BD) and hepatic arteries (HA) are highlighted by means of arrows, and the portal vein (PV) is labeled intralumenally.
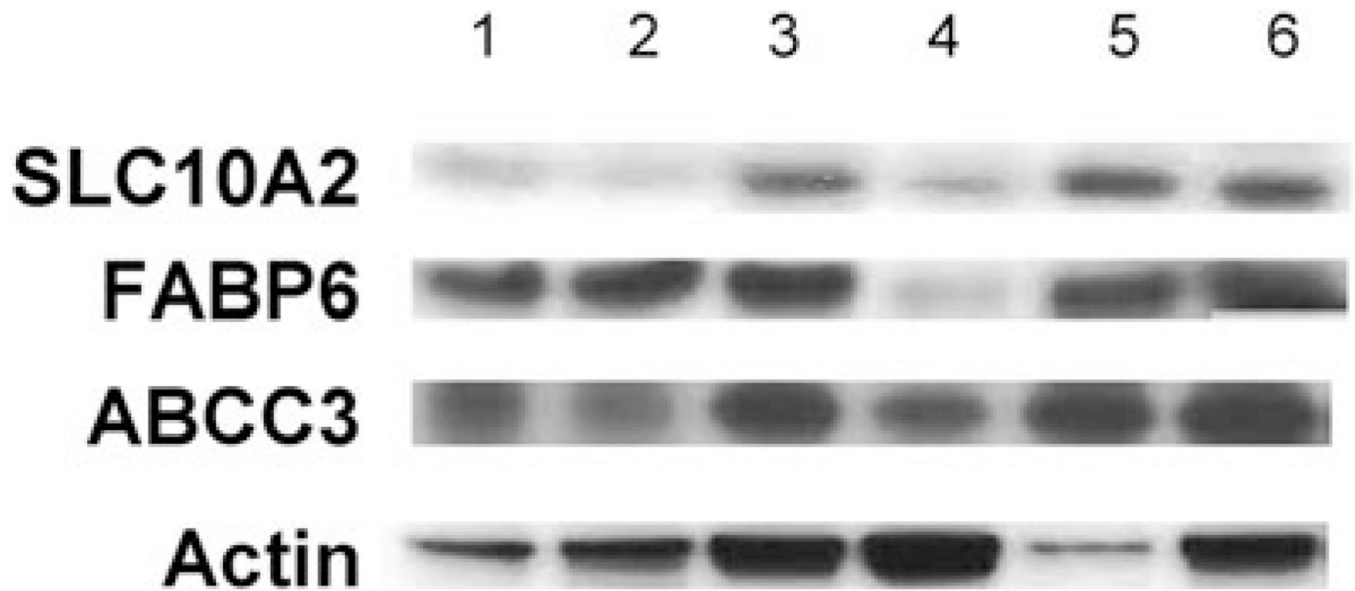
Figure 2.
Western blot analysis of bile salt transporters apical sodium dependent bile salt transporter (SLC10A2), fatty-acid-binding protein subclass 6 (FABP6), and multiple-drug-resistance-related protein isoform 3 (ABCC3) expression with actin levels as controls. Lane 1, AKR male mouse on chow ($n = 21$); lane 2, AKR male mouse on the lithogenic diet ($n = 21$); lane 3, C57L male mouse on chow ($n = 25$); lane 4, C57L male mouse on the lithogenic diet ($n = 25$); lane 5, C57L female mouse on chow ($n = 28$); lane 6, C57L female mouse on the lithogenic diet ($n = 28$).
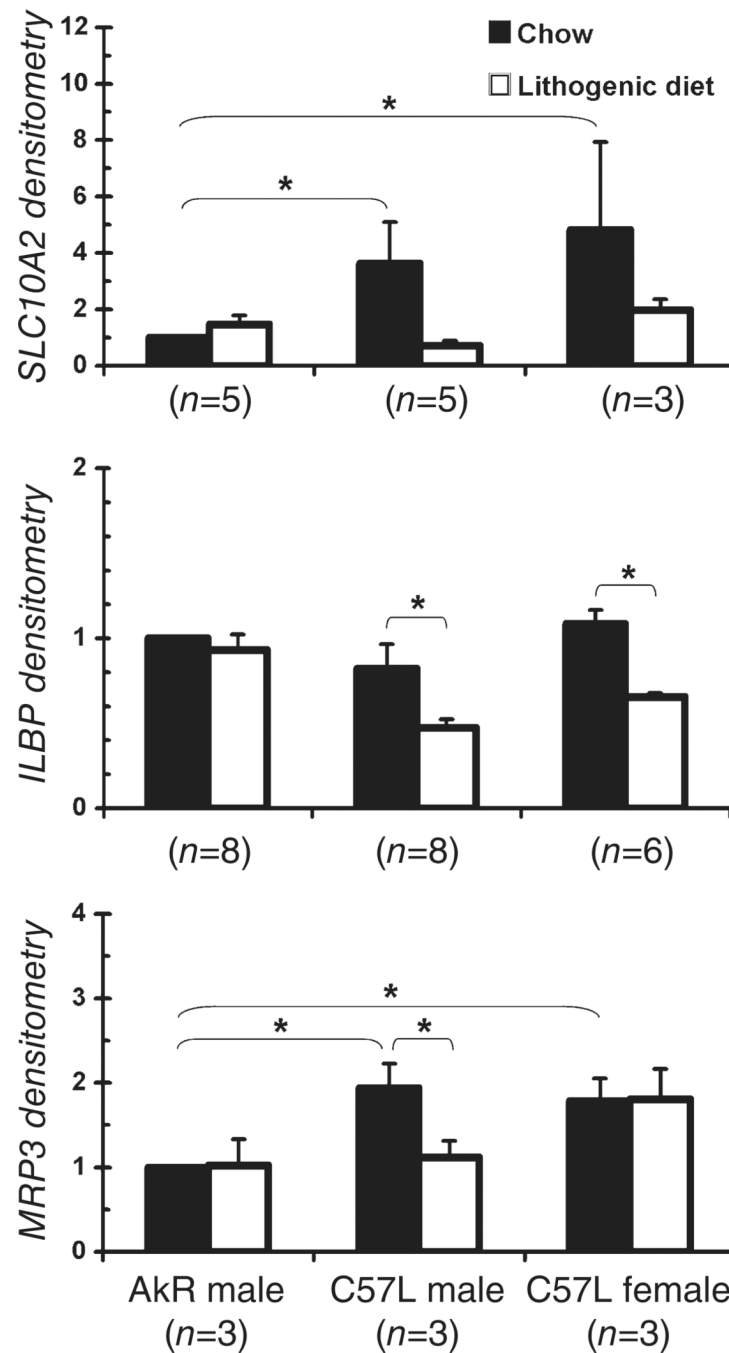
Figure 3.
Densitometric analysis of apical sodium dependent bile salt transporter (SLC10A2), fatty-acid-binding protein subclass 6 (FABP6), and multiple-drug-resistance-related protein isoform 3 (ABCC3) expression in AKR male mice, C57L male mice, and C57L female mice on chow and the lithogenic diet, normalized for actin. Asterisk indicates significant differences between bars ($P < 0.05$).
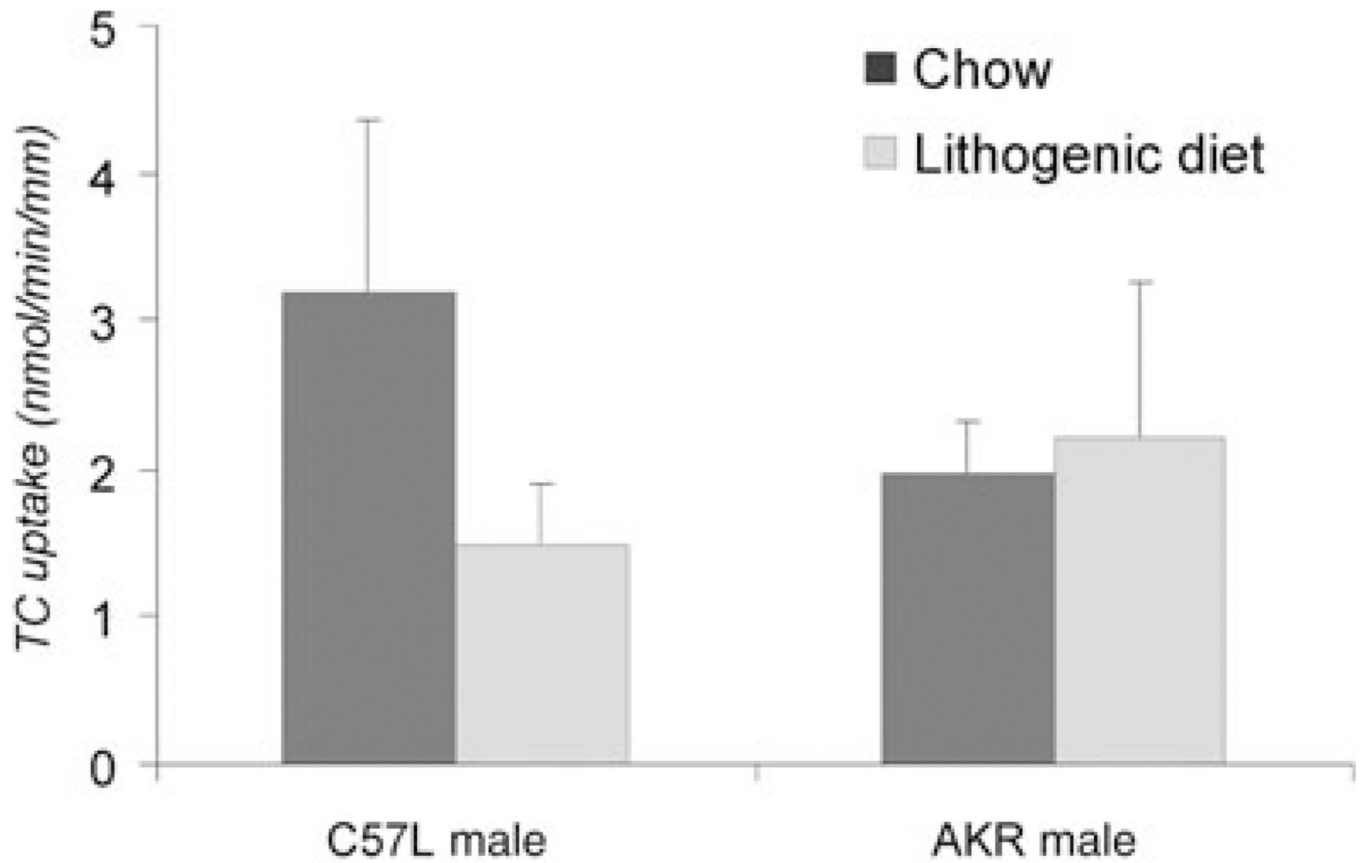
Figure 4.
Taurocholate uptake using microperfusion of freshly isolated bile duct unit (IBDU) in C57L male mice on chow ($n = 6$) and the lithogenic diet ($n = 6$) compared to AKR male mice on chow ($n = 6$) and the lithogenic diet ($n = 6$). No significant differences per mm length are evident for IBDUs isolated from mice on chow or lithogenic diet.

Table 1

Gender and dietary influences on cholangiocyte bile salt transporters in AKR and C57L mice

Bile salt transporter	Male AKR lithogenic diet	Male C57L chow	Male C57L lithogenic diet	Female C57L chow	Female C57L lithogenic diet
SCL10A2 (ASBT)	↔	←	↔	←	↔
FABP6 (ILBP)	↔	↔	→	↔	→
ABCC3 (MRP3)	↔	←	→	←	↔