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Hepatic Canalicular Membrane Transport of Bile Salt in C57L/J and AKR/J Mice: Implications for Cholesterol Gallstone Formation

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Abstract. C57L/J (gallstone-susceptible) and AKR/J (gallstone-resistant) mice have been utilized for quantitative trait loci (QTL) analysis to identify the *Lith 1* locus for cholelithiasis. *Abcb11* encodes for the liver canalicular membrane bile salt export pump (BSEP), which maps to this QTL and is a candidate gene for *Lith 1*. We investigated the transmembrane transport of taurocholate in canalicular liver membrane vesicles isolated from these murine strains. Canalicular liver plasma membranes (cLPM) and RNA were isolated from C57L/J and AKR/J mice livers, and were utilized for Northern and Western blot analysis and functional ^{3}H-taurocholate uptake studies. ATP-dependent ^{3}H-taurocholate uptake was significantly higher in AKR/J, compared to C57L/J mice. V_{max} was 127 *vs.* 42 pmol TC/mg/s in the murine strains, respectively, while K_m was unchanged. In contrast, gene and protein expression of hepatic *Abcb11* was increased three-fold in C57L/J, compared to AKR/J mice. Thus, *Abcb11* bile salt transport activity per unit protein was reduced nine-fold in the C57L/J, compared to AKR/J mice. In contrast, canalicular membrane cholesterol:phospholipid content was also significantly higher in the C57L/J mice. We conclude that gallstone-susceptible C57L/J mice demonstrate increased gene and canalicular membrane expression of *Abcb11*, however, taurocholate transport is functionally diminished. The latter may be due to the increased cholesterol membrane content of the cLPM in C57L/J mice. These findings may be important for the pathogenesis of gallstone formation.

Key words: Gallstone — Mice — Bile salt export pump — ABCB11

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Introduction

Cholesterol gallstone disease affects 40 million Americans and several studies indicate that there is an inheritable component to cholelithiasis (van der Linden & Simonson, 1973; Gilat et al., 1983; Johnston & Kaplan, 1993; Sarin, 1995; Kratzer et al., 1998). When mice are fed a lithogenic diet, they develop gallstones with a strain-specific prevalence. The differing prevalence has been exploited using quantitative trait loci (QTL) analysis in order to determine the polygenic nature of gallstone formation in this murine model (Khanuja et al., 1995; Lammert et al., 2001). Gallstone-susceptible (C57L/J) and gallstone-resistant (AKR/J) mice have been effectively employed for QTL analysis in order to identify the *Lith 1* locus on mouse chromosome 2 (Paigen et al., 2000; Lammert et al., 2001). Although the identity of the *Lith 1* gene or genes remains elusive, the liver canalicular bile salt export pump (BSEP, *Abcb11*, Spgp) has been identified to be a candidate gene for *Lith 1*. *Abcb11* is the major and potentially sole canalicular transport protein mediating ATP-dependent canalicular bile salt transport, a process which is rate-limiting for hepatobiliary bile salt secretion (Nathanson & Boyer, 1991; Gerloff et al., 1998; Green, Hoda & Ward, 2000).

Physical chemistry studies have demonstrated the importance of bile salts in preventing cholesterol nucleation and gallstone formation (Hay & Carey, 1990). In addition, ursodeoxycholic acid and chenodeoxycholic acid are effective therapies for the dissolution of cholesterol gallstones (Fromm & Malavolti, 1992). Therefore, a liver bile salt transporter is likely to be important for the development of gallstone formation, further supporting the role of *Abcb11* as the putative *Lith 1* gene. Gene mapping data also localize the mouse *Abcb11* to the *Lith 1* loci (Khanuja et al., 1995; Paigen et al., 2000;

Lammert et al., 2001). Thus, genetic mapping data, physiologic function and physical chemistry investigations all support the hypothesis that *Abcb11* is the *Lith 1* gene.

BSEP-null (*Abcb11*-null) mice have been developed and their biliary secretion has been analyzed (Wang et al., 2001). These mice demonstrate an increased cholesterol:phospholipid ratio in bile, consistent with the hypothesis that diminished *Abcb11* function would result in a more lithogenic bile. However, Wang et al. (1999) found that gallstone-susceptible C57L/J mice have an increased, rather than diminished bile salt secretory rate. Since bile salts inhibit cholesterol stone formation, it is seemingly paradoxical that enhanced bile salt secretion should increase the prevalence of gallstone formation. However, this can potentially be explained by concomitant coupled changes in biliary cholesterol and phosphatidylcholine secretion, which may reduce the time required for cholesterol supersaturation of bile and gallstone formation. (Wang et al., 1999). In order to resolve these seemingly conflicting observations, we sought to isolate liver canalicular membranes from AKR/J and C57L/J mice and assay the expression and function of *Abcb11*.

In the present study, we have characterized the expression and transmembrane bile salt transport function of *Abcb11* in the liver canalicular membrane. These studies demonstrate that *Abcb11* transport function is diminished in the C57L/J mice, potentially due to an increased canalicular membrane cholesterol:phospholipid content. These studies may be important in our understanding of the pathogenesis of cholesterol gallstone formation.

Materials and Methods

Animals

C57L/J and AKR/J mice (6 to 8 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were maintained in a temperature-controlled room with light–dark cycling, and had free access to water and a normal chow diet. All animals were sacrificed without prior fasting using carbon dioxide narcosis between 9:00 and 10:00 a.m., and the livers were immediately utilized or snap-frozen in liquid nitrogen and stored at −70°C for RNA isolation. Protocols were approved by the Institutional Animal Care and Use Committee and euthanasia was consistent with recommendations of the American Veterinary Medical Association.

Isolation and Analysis of Canalicular Liver Plasma Membranes (cLPM)

Canalicular liver plasma membranes (cLPM) were isolated as previously described (Green et al., 2000). Two mouse livers were pooled and washed with 50 mL of ice-cold PBS with PMSF and homogenized with 15 strokes in buffer. The suspension was centrifuged at 1500 × *g* for ten minutes, the supernatant removed and resuspended at 5.5 volumes of 56% sucrose solutions (w/w). A sucrose gradient was layered below the suspension using a 42% (w/w) and 38% (w/w) sucrose layered gradient, and centrifugation for one hour was performed at 4°C at 66,000 × *g*. The upper interface layers were removed and resuspended in ten volumes of PBS. All proteins were quantitated using the method of Bradford (Biorad, CA). Cholesterol content was assayed spectrophotometrically using the instructions of the manufacturer (Sigma, St. Louis, MO) and phospholipid content was measured using the Bartlett phosphorus assay (Bartlett, 1959). The canalicular enzyme enrichment was verified using LAP enzymatic activity, and α-Na^+/K^+ ATPase protein expression.

^{3}H-Taurocholate Uptake Studies

^{3}H-Taurocholate uptake by cLPM vesicles was performed at 37°C using a rapid filtration technique (Green et al., 1997, 2000). ^{3}H-Taurocholic acid (2.1 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Taurocholate acid was dried under argon and resuspended in 3 mL of 250 mM sucrose/100 mM NaCl/10 mM HEPES buffer containing taurocholate acid, pH 7.4. Uptake was performed using 100 μg of plasma membrane vesicles in a total volume of 250 μL. Uptake was performed in duplicate at varying taurocholate concentrations (2.5–25 μM) for 20 s, and was terminated by rapid filtration through nitrocellulose filters, which were presoaked in taurocholate acid buffer solution. The membranes were immediately washed twice with one mL of ice-cold 100 μM taurocholate buffer solution. All experiments were performed both in the presence and absence of 10 mM ATP. Additional experiments were also performed in the presence of phosphoinositol (PI) 3,4-bisphosphate (20 μM) or wortmannin (100 nM). Preliminary studies documented that uptake was linear with respect to time, bile salt concentration and protein content for all experiments.

Northern Blot Analysis and Nucleotide Sequencing

Total RNA was isolated by phenol:chloroform extraction (Ultraspec, Biotex, TX) using the instructions of the manufacturer. Northern blotting was performed as previously described (Green et al., 1997). Briefly, 15 μg of total RNA was isolated from mouse livers and loaded per lane. Electrophoresis through 1% agarose gels containing 2.2 M formaldehyde in MOPS buffer at constant voltage (100 V) was performed for three hours. Ethidium bromide staining of the 18S and 28S bands was performed to ensure equal RNA loading. RNA was transferred to nylon membranes by capillary action and fixed by UV cross-linking. Hybridization was performed for 15 hours at 42°C, and the blots were washed under high-stringency conditions. Following autoradiography, all blots were stripped and reprobed, using an ubiquitin cDNA to verify RNA integrity and loading. Results were quantitated using laser densitometry and normalized for ubiquitin. Sequencing was performed on hepatic RNA using high-fidelity reverse transcriptase polymerase chain reaction (RT-PCR) employing Thermoscript RT-PCR System Plus Platinum Taq DNA polymerase High Fidelity (Gibco, MD), with automated sequencing performed at the Northwestern University Biotechnology Laboratory Core Sequencing Facility.

Western Blot Analysis

Western blot analysis was performed as previously described, using a polyclonal serum directed against the carboxy terminus (18 amino acid) of murine *Abcb11* (Green et al., 2000) or α-P-glyco-

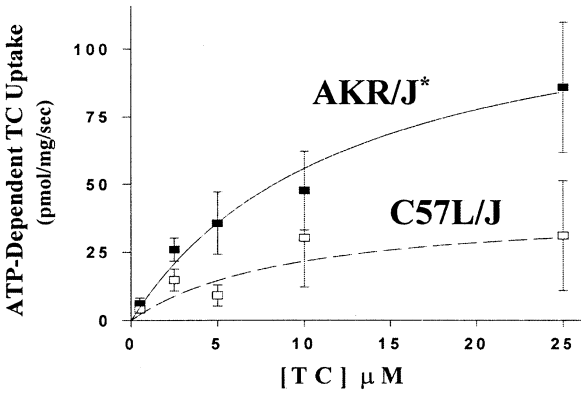

Fig. 1. ATP-Dependent taurocholate uptake in cLPM isolated from AKR/J and C57L/J mice. cLPM vesicles were isolated from livers obtained from C57L/J and AKR/J mice. [^{3}H]-taurocholate (TC) uptake (1–2.5 μM) was assayed for 20 s at 37°C in the presence or absence of 10 mM ATP. V_{max} was 127 *vs.* 42 pmol TC/mg/s in AKR/J and C57L/J mice, respectively ($p < 0.01$), while K_m was similar (12 μM *vs.* 9 μM) in the respective strains. Each point represents data from 4–6 membrane preparations performed in triplicate. Data represents mean ± SD. (*:$p < 0.01$ compared to C57L/J).

protein C219 antibody (Zymed, Menlo Park, CA). Briefly, 20 μg of cLPM was loaded per lane, and SDS-PAGE electrophoresis was performed using a 10% gel. The proteins were electrophoretically transferred to a nitrocellulose membrane, and equal protein loading and electrophoretic transfer were confirmed using Ponceau S staining. Anti-*Abcb11* or α-P-glycoprotein C219 antibodies (1:200) were employed as primary antibodies, and an anti-rabbit HRP-conjugated antibody was used as the secondary antibody (1:5,000). Enhanced chemiluminescence was employed for protein detection, using the instructions of the manufacturer (Amersham, IL). The data was quantitated using densitometry and normalized for LAP enzymatic values.

Results

To determine whether there were differences in liver canalicular ATP-dependent bile salt transport between C57L/J and AKR/J mice, we performed taurocholate uptake experiments in cLPM vesicles isolated from these inbred mice. Membrane enrichment was verified using LAP enzyme activity, and was comparably increased ≈20-fold compared to homogenate; while Na^+/K^+ ATPase was similar to homogenate levels in cLPM isolated from both mouse strains. This confirmed the comparable purity of the cLPM. Bile salt transport was markedly diminished in C57L/J, compared to AKR/J mice. Figure 1 demonstrates the ATP-dependent taurocholate uptake in cLPM vesicles isolated from the respective murine strains. Both strains exhibited saturable uptake of taurocholate; however, taurocholate uptake was decreased in C57L/J mice at all bile salt concentrations (2.5–25 μM) tested. When modeled according to the Michaelis-Menten equation, the V_{max} was 127 pmol TC/mg/s in AKR/J mice, versus

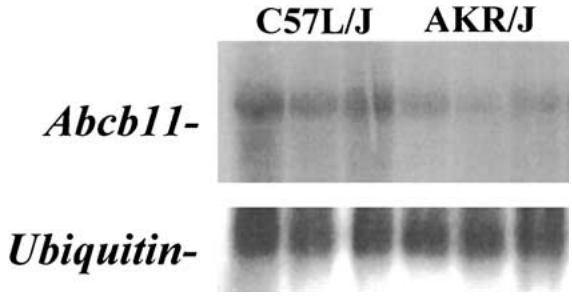

Fig. 2. Northern analysis of hepatic *Abcb11* gene expression in C57L/J and AKR/J mice. Representative Northern blot of mouse *BSEP* expression in hepatic RNA isolated from C57L/J and AKR/J mice. 15 μg of RNA was loaded per lane, and the blots were probed with a cDNA corresponding to the 3′ UT region of *Abcb11*. The blots were stripped and reprobed with a ubiquitin cDNA to confirm RNA integrity and equal loading ($n = 4$, $p < 0.01$).

42 pmol TC/mg/s in C57L/J mice ($p < 0.01$). The K_m was similar in both murine strains, being 12 μM and 9 μM in the respective strains. These K_m are similar to those previously reported in studies where mouse *Abcb11* was cloned and functionally expressed (Green et al., 2000).

We subsequently performed high-fidelity RT-PCR and sequenced the coding region of *Abcb11* cDNAs isolated from C57L/J and AKR/J mice, but failed to identify a polymorphism. Northern blotting was subsequently performed on hepatic RNA isolated from C57L/J and AKR/J mice. Figure 2 demonstrates that *Abcb11* gene expression was increased threefold in the C57L/J mice, compared to AKR/J mice ($n = 4$, $p < 0.01$). The blots were stripped and reprobed with ubiquitin to verify RNA integrity and loading.

In order to confirm that *Abcb11* protein expression was also increased and targeted to the canalicular plasma membranes, we performed Western blotting on cLPM vesicles isolated from C57L/J and AKR/J mice. Figure 3 demonstrates that the 160-kD *Abcb11* protein is also increased threefold in cLPM isolated from C57L/J mice, compared to AKR/J mice ($n = 4$, $p < 0.01$). Similar results were obtained when the blots were analyzed using densitometry and normalized for LAP enzyme activity. The smaller form of *Abcb11* previously identified in rat cLPM was not evident, consistent with our previously reported observation in mice (Gerloff et al., 1999; Green et al., 2000). When ATP-dependent taurocholate uptake was normalized for the level of *Abcb11* protein expression, it was increased approximately ninefold in AKR/J compared to C57L/J mice (Fig. 4).

We also utilized C219 antibody, which detects all of the P-glycoprotein class of transporters, in order to determine plasma canalicular liver plasma membrane expression of total P-glycoprotein. Total P-glycoprotein transporters were also increased in C57L/J mice compared to AKR/J mice (Fig. 5).

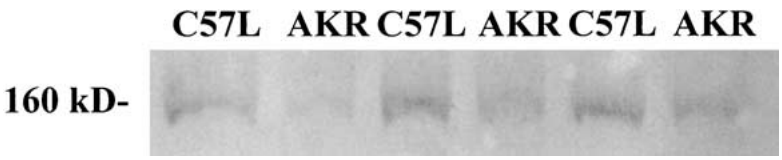

Fig. 3. Western analysis of hepatic *Abcb11* protein expression in cLPM isolated from C57L/J and AKR/J mice. Representative Western blots of mouse *Abcb11* expression in cLPM isolated from C57L/J and AKR/J mice. 20 μg of cLPM protein was loaded per lane, and the blots were probed with an antibody corresponding to the carboxy terminus of mouse *Abcb11* (160 kD). The 160 kD *Abcb11* protein, when quantitated and normalized for LAP activity, is increased threefold in cLPM isolated from C57L/J mice, compared to AKR/J mice ($n = 4$, $p < 0.01$).

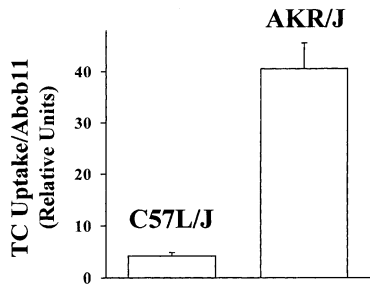

Fig. 4. Maximal uptake velocity (V_{max}) of ATP-dependent TC uptake normalized for cLPM BSEP expression in cLPM isolated from AKR/J and C57L/J mice, Maximal taurocholate uptake velocity in cLPM was normalized for *Abcb11* expression (in relative densitometry units), indicating that uptake per unit protein of *Abcb11* was increased approximately ninefold in AKR/J, compared to C57L/J mice, ($n = 4$, $p < 0.001$).

Uptake was subsequently performed in the presence of PI 3,4-bisphosphate (20 μM) or wortmannin (100 nM). These substances have been previously reported to alter the uptake of taurocholate in cLPM vesicles isolated from rat liver (Misra et al., 1999). Neither of these substances affected bile salt transport in cLPM isolated from either C57L/J or AKR/J mice.

Membrane cholesterol and phospholipid content has been reported to modify the functional activities of many hepatic transporters (Miura et al., 1997; Accatino et al., 1998; Hyogo Tazuma & Kajiyama, 2000; Kajihara et al., 2000; Verkade, 2000). Thus, we subsequently measured the cholesterol and phospholipid content of canalicular plasma membrane vesicles isolated from C57L/J and AKR/J mice. Table 1 shows the lipid content of murine cLPM isolated from C57L/J and AKR/J mice. The cholesterol:phospholipid content is significantly higher in the C57L/J group, compared to AKR/J ($p < 0.01$). Unfortunately, attempts to further study the effects of increased cLPM cholesterol content using pharmacologic or dietary (high cholesterol, lithogenic diet) manipulation resulted in a significant reduction in cLPM enzyme enrichment (relative to liver homogenate) and thus did not allow for vesicular transport studies.

Discussion

Cholesterol gallstone disease is a common disease that is highly prevalent in individuals with Paleo-Indian descent, further supporting the genetic nature of cholelithiasis (van der Linden & Simonson, 1973; Gilat et al., 1983; Johnston & Kalpan, 1993; Sarin, 1995; Kratzer et al., 1998). Although cholelithiasis is a polygenic disease, environmental factors, such as high fat and cholesterol diets typical of Western society, obesity and weight loss likely contribute to cholesterol gallstone formation (Hay & Carey, 1990, Fromm & Malavolti, 1992; Wang et al., 1999). Data utilizing inbred strains of mice fed a lithogenic (0.5% cholate, 1% cholesterol, 15% fat) diet demonstrates that there is a strain-specific difference in the prevalence for the development of cholesterol gallstones. Quantitative Trait Locus (QTL) analysis has been utilized to identify *Lith* gene loci on several murine chromosomes, however, the identity of the actual *Lith* genes remains elusive.

C57L/J (gallstone-susceptible) and AKR/J (gallstone-resistant) mice have been successfully utilized for QTL analysis to identify the *Lith 1* gene loci on mouse chromosome 2, coinciding with the map position of *Abcb11*. Although physical chemistry studies have demonstrated the importance of bile salts in reducing cholesterol nucleation and gallstone formation, and bile salts are a proven therapy for gallstone dissolution, a previous study has demonstrated that gallstone-susceptible C57L/J mice seemingly paradoxically hypersecrete bile salts, relative to AKR/J mice (Wang et al., 1999). In contrast, *Abcb11*-null mice have an increased cholesterol:phospholipid ratio in bile, which would result in a more lithogenic bile (Wang et al., 2001). This suggests that diminished or inactive *Abcb11* activity may predispose to cholesterol gallstone formation. *Abcb11* is the major liver canalicular bile salt transport protein. Therefore, it was the purpose of this investigation to resolve this apparent paradox by characterizing and determining the expression and activity of *Abcb11* and ATP-dependent canalicular bile salt transport in C57L/J and AKR/J mice.

Abcb11 gene expression was increased in C57L/J mice, and this difference in expression resulted in increased protein expression targeted to the liver

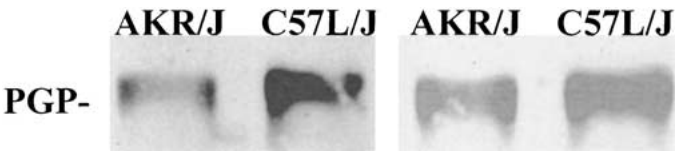

Fig. 5. Western analysis of hepatic P-glycoprotein expression in cLPM isolated from C57L/J and AKR/J mice. Representative Western blots of mouse *Abcb11* expression in cLPM isolated from C57L/J and AKR/J mice. 20 μg of cLPM protein was loaded per lane, and the blots were probed with a C219 antibody detecting P-glycoproteins. P-glycoprotein expression, when quantitated and normalized for LAP activity, is increased 2.5-fold in cLPM isolated from C57L/J mice, compared to AKR/J mice ($n = 3$, $p < 0.05$).

Table 1. Cholesterol and phospholipid content of cLPM isolated from C57L/6 and AKR/J mice

cLPM Lipid	C57L/6	AKR/J
Cholesterol (μmol/mg protein)	0.27 ± 0.05	0.18 ± 0.04
Phospholipid (μmol/mg protein)	0.32 ± 0.08	0.39 ± 0.08
Cholesterol/Phospholipid	0.80 ± 0.09*	0.48 ± 0.04

cLPM enrichment was 20-fold in membranes isolated from both strains of mice. *$p < 0.005$ compared to AKR/J

canalicular membrane. Western immunoblotting of cLPM isolated from C57L/J and AKR/J mice was consistent with the Northern analysis, and both gene and protein expression of *Abcb11* in isolated cLPM membranes were increased threefold. Canalicular marker enzyme enrichment was comparable, indicating that membrane enrichment and quality were similar in both murine strains.

Although it is highly likely that there are several genotypic and phenotypic differences between C57L/J and AKR/J mice, the physiologic differences in canalicular ATP-dependent bile salt transport are likely to significantly impact on cholesterol gallstone formation. Thus, the major focus of this study was to characterize bile salt transport in liver canalicular membranes isolated from C57L/J and AKR/J mice. ^{3}H-taurocholate uptake in membrane vesicles from both murine strains was saturable and, when modeled to the Michaelis-Menten equation, yielded virtually identical affinity constants (K_m) for bile salt transport. This study presents the first kinetic data on taurocholate transport in native mouse cLPM, so comparison with previous data or other murine strains is not possible. However, the K_m for ATP-dependent bile salt transport in cLPM from both strains of mice is similar to that reported when transport kinetics where defined in Balb/c-3T3 and Sf9 cells transfected with mouse *Abcb11*, consistent with the fact that *Abcb11* is the major liver canalicular bile salt transporter (Green et al., 2000).

In sharp contrast to the K_m, the V_{max} was threefold lower in C57L/J mice, despite the fact that *Abcb11* protein expression in canalicular membranes was significantly greater in these mice. When normalized for the degree of *Abcb11* expression, ^{3}H-taurocholate transport in cLPM membranes was approximately ninefold lower in C57L/J compared to AKR/J mice. This finding of diminished canalicular ATP-dependent bile salt transport is consistent with data indicating that *Abcb11*-null mice have a more lithogenic bile (Wang et al., 2001). It has been well-recognized for decades that bile salt secretion and content are important factors affecting cholesterol nucleation and gallstone formation. The diminished specific activity of *Abcb11* transport function in the gallstone-susceptible C57L/J mouse and colocalization of *Abcb11* to the *Lith 1* gallstone QTL on mouse chromosome 2 support its candidacy as a *Lith* gene. (Lammert 2001, Paigen et al., 2000).

Previous physiologic data has seemingly paradoxically shown that the C57L/J mice hypersecrete bile salts relative to AKR/J mice. We believe that these findings may differ from our in vitro transport studies due to changes in bile salt species, hydrophobicity, and/or other in vivo compensatory mechanisms between these genetically diverse strains of mice. Our data of diminished *Abcb11* function in gallstone-susceptible C57L/J mice are in agreement with the increased lithogenic bile noted in the *Abcb11*-null mouse. The diminished V_{max} of cLPM bile transport in the C57L/J mouse suggests that these mice have a functional impairment of ATP-dependent bile salt transport, albeit to a lesser degree than the *Abcb11* null mice.

Canalicular membrane cholesterol content has been previously shown to modulate the function of hepatic transporters (Miura et al., 1997; Accatino, 1998; Hyogo et al., 2000; Kajihara et al., 2000; Verkade, 2000). We detected significant differences in the liver canalicular membrane cholesterol:phospholipid molar ratio, which is a potential mechanism responsible for the functional difference in transport function. In fact, the increased canalicular membrane cholesterol may be caused by increased hydrophobic bile acid content or flux, or other physiologic stimuli which may enhance cholesterol transport to the canalicular membrane. Thus, the apparent paradox between these data and Wang et al. (1999) may not, in fact, be contradictory. Cholesterol gallstone formation requires supersaturation of bile with cholesterol, and the biliary secretion of cholesterol requires the prior transport of cholesterol to the canalicular domain of the hepatocyte. This dynamic in vivo process can potentially re-

sult in an increased cLPM cholesterol content, which may also inhibit in vitro bile salt transport. This emphasizes the import of interpreting these data in light of both the in vivo and in vitro studies. Finally, the increased cholesterol in cLPM isolated from gallstone-susceptible C57L/J (compared to AKR/J) mice is consistent with human data indicating that patients with gallstones have increased membrane vesicle cholesterol content (Ahmed et al., 1995).

We were unable to document any effects on bile salt transport activity when vesicular uptake studies were performed in the presence of PI 3,4-bisphosphate or wortmannin. These substances have been reported to affect *Abcb11* activity in the rat, and this may potentially reflect species differences between mouse and rat. These pharmacologic agents, may, however, be effective for altering transporter trafficking into membranes if administered in vivo.

These studies demonstrate an impaired diminished functional activity of canalicular bile salt transport in gallstone-susceptible C57L/J mice, consistent with findings of a more lithogenic bile reported in the *Abcb11* null mice. The diminished activity of canalicular bile salt secretion may lead to a more lithogenic bile and an increased prevalence of gallstone formation, consistent with physical chemistry data obtained over the past several decades. These studies may have important implications for identification of the *Lith 1* gene and for enhancing our understanding of the pathogenesis of human cholesterol gallstone disease.

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