# The mechanism of ABCG5/ABCG8 in biliary cholesterol secretion in mice[1]

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**Abstract** **The main player in biliary cholesterol secretion is the heterodimeric transporter complex, ABCG5/ABCG8, the function of which is necessary for the majority of sterols secreted into bile. It is not clear whether the primary step in this process is flopping of cholesterol from the inner to the outer leaflet of the canalicular membrane, with desorption by mixed micelles, or decreasing of the activation energy required for cholesterol desorption from the outer membrane leaflet. In this study, we investigated these mechanisms by infusing *Abcg8*$^{+/+}$, *Abcg8*$^{+/-}$, and *Abcg8*$^{-/-}$ mice with hydrophilic and hydrophobic bile salts. In *Abcg8*$^{-/-}$ mice, this failed to substantially stimulate biliary cholesterol secretion. Infusion of the hydrophobic bile salt taurodeoxycholate also resulted in cholestasis, which was induced in *Abcg8*$^{-/-}$ mice at a much lower infusion rate compared with *Abc8*$^{-/-}$ and *Abcg8*$^{+/-}$ mice, suggesting a reduced cholesterol content in the outer leaflet of the canalicular membrane. Indeed, isolation of canalicular membranes revealed a reduction of 45% in cholesterol content under these conditions in *Abcg8*$^{-/-}$ mice. Our data support the model that ABCG5/ABCG8 primarily play a role in flopping cholesterol (and sterols) from the inner leaflet to the outer leaflet of the canalicular membrane.**—Kosters, A., C. Kunne, N. Looije, S. B. Patel, R. P. J. Oude Elferink, and A. K. Groen. **The mechanism of ABCG5/ABCG8 in biliary cholesterol secretion in mice.** ***J. Lipid Res.*** **2006.** 47: **1959–1966.**

**Supplementary key words** liver • canalicular membrane • bile salt infusion • diosgenin

The ABC half-transporters ABCG5 and ABCG8 play a major role in biliary cholesterol secretion and intestinal absorption of sterols. Mutations in either of these genes underlie the genetic disease sitosterolemia (1–3). Patients with this disorder show increased intestinal absorption of plant and noncholesterol sterols and fail to excrete sterols into bile, resulting in high plasma sterol levels and accumulation in tissues (4). In mice overexpressing the human *ABCG5/ABCG8* genes or in mice lacking either one or both of the endogenous genes, a role for these transporters in intestinal absorption and biliary secretion of sterols was confirmed (5–8). Hepatic expression levels of Abcg5/Abcg8 have been shown to correlate with cholesterol secretion in most rodent models studied to date (9–12), although exceptions have been reported as well (12–14). In particular, in mice of the FVB strain fed a diosgenin-containing diet, a 16-fold increase in cholesterol secretion was shown, whereas no change in expression of either gene was observed (12). In C57Bl/6 mice, the stimulatory effect of diosgenin was less prominent but proved to be almost fully dependent on the presence of the Abcg5/Abcg8 heterodimer (13, 14).

The mechanism by which cholesterol is secreted in bile has long been an enigma. The discovery of ABCG5/ABCG8 has changed this situation and led to detailed hypotheses for cholesterol transport. Wittenburg and Carey (15) suggested that the ABCG5/ABCG8 heterodimer acts as a floppase, defined as the translocation of cholesterol from the inner to the outer leaflet of the canalicular membrane, in a manner similar to the floppase mechanism shown for the translocation and secretion of phosphatidylcholine (PC) into bile mediated by ABCB4 (16–18). After translocation of cholesterol, ABCG5/ABCG8 would then mediate the extrusion of cholesterol monomers from the extracellular leaflet of the canalicular membrane into the canalicular space, where mixed micelles and phospholipid vesicles act as acceptors. The necessity of a floppase function can be questioned, as cholesterol has been shown to flip-flop between membrane leaflets spontaneously. Several studies have reported fast flip-flop rates for cholesterol in PC and phosphatidylcholine:cholesterol vesicles and in erythrocyte membranes with half-

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Abbreviations: PC, phosphatidylcholine; TC, taurocholic acid; TDC, taurodeoxycholic acid; TUDC, tauroursodeoxycholic acid.

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times ranging from 50 min (19) to <1 s (20), depending on the method used to quantify the flip-flop rates. This spontaneous translocation activity of cholesterol led Small (21) to propose another role for ABCG5/ABCG8 in cholesterol secretion. He suggested that the heterodimer is involved in decreasing the energy required to release cholesterol from the outer leaflet of the canalicular membrane (21). According to this proposal, ABCG5/ABCG8 would form a cholesterol binding complex "pushing" cholesterol partially into the lumen, using ATP hydrolysis as the driving force. Under these circumstances, the accessibility of cholesterol for extraction by bile salt/phospholipid micelles would be enhanced. However, micellar extraction of cholesterol is not necessarily the only mechanism for cholesterol secretion. Vesicles consisting of cholesterol and phospholipids have been observed in bile (22–24), and vesiculation of the outer leaflet as a mechanism for lipid secretion has been proposed as well (25). Vesicle secretion as a mechanism for lipid secretion is not taken into account in the proposal of Small (21). Indeed, when ABCG5/ABCG8 primarily function to lift cholesterol partially out of the membrane, the absence of these proteins would result in the accumulation of the sterol in the outer leaflet of the canalicular membrane. On the other hand, when the heterodimer is a floppase, the outer leaflet of the canalicular membrane should be low in cholesterol in the absence of *Abcg5/Abcg8* and sensitive to the detergent action of hydrophobic bile salts.

We have shown previously that the bile salt tauroursodeoxycholic acid (TUDC) was minimally effective in stimulating cholesterol secretion in *Abcg8*$^{-/-}$ mice (13). TUDC is a very hydrophilic bile salt with a low cholesterol-solubilizing capacity, which may explain the lack of efficacy of solubilizing cholesterol despite the very high bile salt concentrations reached in bile during secretion. In this study, we infused *Abcg8*$^{-/-}$ mice with more hydrophobic bile salts such as taurocholic acid (TC) and taurodeoxycholic acid (TDC). Particularly, TDC readily dissolves cholesterol from lipid bilayers in model systems and should allow us to explore whether Abcg8 is involved in the translocation of cholesterol from the inner to the outer leaflets of the membranes or whether it acts by decreasing the activation energy needed to extrude the cholesterol from the outer leaflet into the lumen of the bile ducts. If Abcg5/Abcg8 act as floppase, in the absence of the heterodimer the presence of TDC in the canalicular space should decrease cholesterol in the outer leaflet of the canalicular membrane, leading to cholestasis.

## MATERIALS AND METHODS

### Animals and diets

*Abcg8*$^{-/-}$ mice (C57Bl/6 × 129Sv) were generated and bred as described (7). In feeding experiments, *Abcg8*$^{+/+}$, *Abcg8*$^{+/-}$, and *Abcg8*$^{-/-}$ mice were fed either a control diet (AM-II; Hope Farms, Woerden, The Netherlands) or the control diet supplemented with 1% (w/w) diosgenin (Sigma Chemical, St. Louis, MO) for 18 days. All studies were approved by the Animal Ethics Committee of the Academic Medical Center.

### Bile sampling

Mouse surgery, cannulation of the gallbladder, and bile collection were performed as described previously (7). Directly after cannulation, bile was collected for 10 min. Bile samples were immediately frozen at −20°C. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile. To determine maximal rates of biliary lipid secretion, bile fractions were collected for 90 min to deplete the endogenous bile salt pool. Subsequently, TUDC (45 mM stock solution), TC (30 mM stock solution), or TDC (10 mM stock solution) (all from Sigma), dissolved in phosphate-buffered saline, pH 7.4, was infused into the jugular vein at stepwise increasing concentrations as indicated in the figures. During infusion, bile was collected in 10 min fractions and analyzed for bile salt and lipid content as described previously (26). Maximal biliary secretion and bile flow rates were determined for each mouse as the average of four consecutive time points with highest secretion rates. For each group, these values were averaged for the number of mice.

### Isolation of liver plasma membrane fractions after TDC infusion

Liver plasma membrane fractions were isolated in two different sets of experiments. *1*) Livers were harvested from four *Abcg8*$^{+/+}$ and four *Abcg8*$^{-/-}$ mice after decapitation of the animals followed by fractionation of the pooled livers as described below. *2*) The gallbladders of six *Abcg8*$^{+/+}$ and six *Abcg8*$^{-/-}$ mice were cannulated as described above and bile was collected for a period of 30 min, after which mice were infused with 400 nmol/min/100 g TDC via the jugular vein. Bile was collected for 30 min in 10 min periods, and livers were harvested subsequently. Pooled livers were homogenized in 1 mM $NaHCO_3^-$ with 0.1 mM PMSF, pH 7.4, and liver plasma membrane fractions were isolated at 4°C by sucrose density ultracentrifugation according to the method of Meier et al. (27), with some modifications as described below. After centrifugation for 120 min at 200,000 *g*, total plasma membrane fractions were recovered from the 36.5–44% sucrose interface. The plasma membranes were homogenized in 8.1% sucrose and recentrifuged at 200,000 *g* for 3 h, followed by recovery of canalicular-enriched liver plasma membranes from the 8.1–29.7% sucrose interface and basolateral-enriched liver plasma membranes from the 34–38% sucrose interface. Membrane subfractions were washed in 8.1% sucrose and centrifuged for 1 h at 100,000 *g*. The membrane pellets were resuspended and revesiculated through a 25 gauge needle and stored until further analysis at −80°C in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl, and 0.1 mM PMSF, pH 7.4. Purity of the membrane fractions was judged by measuring the enrichment of the canalicular marker enzyme aminopeptidase N. In all fractionation procedures, the enrichment was >100-fold.

### Analytical methods

Cholesterol- and choline-containing phospholipids were determined in liver membrane fractions after extraction according to Bligh and Dyer (28). Phospholipid was measured as described previously. Cholesterol was determined by gas chromatography as described previously (8). Protein was determined with the BCA method (29). Alkaline phosphatase was assayed as described (30).

### Statistical analysis

Data are shown as means ± SD or as means ± SEM as indicated in the figures. A minimum of four animals per group was used. Statistical significance of differences was evaluated by Student's *t*-test or the Mann-Whitney *U*-test. Significance was set at $P < 0.05$.

## RESULTS

To investigate whether biliary cholesterol secretion could be induced by hydrophobic bile salts in the absence of *Abcg8*, experiments were performed in which $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice were infused with increasing concentrations of the hydrophobic bile salt TC or TDC. TC is a less hydrophobic bile salt than TDC and has less capacity to solubilize cholesterol (31). The results were compared with experiments in which $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice were infused with TUDC (7, 13).

As reported previously (7, 13), no significant changes in endogenous bile flow, bile salt, and phospholipid secretion rates were observed between $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice (**Table 1**). Cholesterol secretion was reduced on average by 70% in $Abcg8^{-/-}$ mice (Table 1). **Figure 1A** shows that the maximal bile salt secretion rates observed after infusion of TUDC, TC, and TDC were not significantly different between $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice for each of the infused bile salts. Note that the maximal bile salt secretion rates reached after TC and TUDC infusion were considerably higher compared with those reached after TDC infusion for all three genotypes. Maximal bile flow rates were significantly increased in $Abcg8^{-/-}$ mice after TUDC infusion (Fig. 1B), whereas no significant differences in bile flow were observed between genotypes after TDC and TC infusion. In general, significantly lower bile flow rates were measured after infusion with TDC compared with TC and TUDC. In $Abcg8^{+/+}$ mice, infusion of TDC, TC, and TUDC did not result in significant differences in maximal cholesterol secretion rates (3.9 ± 1.5, 4.8 ± 1.7, and 4.6 ± 1.5 nmol/min/100 g, respectively) (Fig. 1C). Infusion of the bile salts TUDC, TDC, and TC in $Abg8^{-/-}$ mice all resulted in a slight increase of cholesterol secretion (Fig. 1C) compared with endogenous secretion rates (Table 1). Maximal cholesterol secretion rates in $Abcg8^{-/-}$ mice remained significantly lower compared with $Abcg8^{+/+}$ mice ($P < 0.05$). The effects of TUDC, TC, or TDC infusion on cholesterol secretion rates in $Abcg8^{-/-}$ mice were similar, indicating that even hydrophobic bile salts are not able to extract cholesterol from canalicular membranes of $Abcg8^{-/-}$ mice, suggesting that bile salt hydrophobicity is not the primary determinant for the rate of cholesterol secretion in the absence of Abcg5/Abcg8. Furthermore, as shown in Fig. 1C, no differences in maximal biliary cholesterol secretion rates in $Abcg8^{+/-}$ mice were found after infusion of TUDC, TC, or TDC.

Previous experiments have shown that feeding mice a diet containing the plant sterol diosgenin induced biliary cholesterol secretion and that *Abcg8* was required for this increase (13, 14), although diosgenin feeding did not alter the expression levels of either Abcg5 or Abcg8 (12). These studies also suggested that diosgenin may affect a step in cholesterol trafficking before Abcg5/Abcg8 (13). One option is that diosgenin may increase the flux of cholesterol into the canalicular membrane. If so, more cholesterol may then be available for extraction by the more hydrophobic bile salts and may lead to increased biliary cholesterol, even in the absence of *Abcg8*. Therefore, $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice were fed a 1% diosgenin-supplemented diet for 18 days and subsequently infused with increasing rates of TDC or TUDC. Endogenous bile flow, bile salt, and phospholipid secretion were not affected by diosgenin (Table 1). Endogenous cholesterol secretion rates were induced by 2.9-fold ($P < 0.0001$) in $Abcg8^{+/+}$ mice fed the diosgenin diet but not in $Abcg8^{-/-}$ mice fed the diosgenin diet (Table 1). As expected, diosgenin feeding significantly increased the maximal biliary cholesterol measured in $Abcg8^{+/+}$ mice; however, this was independent of whether TUDC (2.8-fold) or TDC (2.9-fold) was infused (compare **Fig. 2C** with Fig. 1C). No significant increase in biliary cholesterol was noted in $Abcg8^{-/-}$ mice (control diet, 1.2 ± 0.3 nmol/min/100 g; 1% diosgenin diet, 1.5 ± 0.1 nmol/min/100 g). Note that $Abcg8^{+/-}$ mice showed an increase (2.4-fold, $P = 0.003$) in maximal biliary cholesterol secretion when TDC was infused, but no increase in cholesterol secretion was observed when TUDC was used.

Finally, we observed that during the infusion of TC and TDC, nearly all mice showed a reduction in bile flow at higher concentrations of bile salt infusion. Interestingly, especially the $Abcg8^{-/-}$ mice were more susceptible to bile salt-induced cholestasis than the $Abcg8^{+/+}$ and $Abcg8^{+/-}$ mice (**Fig. 3**). Increased cholesterol content of the canalicular membrane has been reported to contribute to resistance against bile salt-induced cholestasis (32). Therefore, the increased susceptibility of $Abcg8^{-/-}$ mice to cholestasis

TABLE 1. Endogenous biliary output of $Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice fed a control diet or a 1% diosgenin diet

| Genotype and Diet | Body Weight | Bile Flow | Bile Salt Secretion | Cholesterol Secretion | Phospholipid Secretion |
|---|---|---|---|---|---|
| | *g* | *μl/min/100 g* | | *nmol/min/100 g* | |
| Control diet | | | | | |
| $Abcg8^{+/+}$ | 25.1 ± 4.0 | 6.1 ± 0.3 | 279 ± 28 | 3.1 ± 0.4 | 40.9 ± 6.2 |
| $Abcg8^{+/-}$ | 26.8 ± 5.5 | 6.3 ± 0.6 | 348 ± 78 | 3.1 ± 0.9 | 31.0 ± 5.2 |
| $Abcg8^{-/-}$ | 23.9 ± 2.1 | 8.2 ± 0.4[a] | 292 ± 25 | 0.9 ± 0.1[a] | 37.9 ± 3.2 |
| 1% diosgenin diet | | | | | |
| $Abcg8^{+/+}$ | 26.7 ± 3.6 | 7.2 ± 0.8 | 228 ± 40 | 8.9 ± 1.4[b] | 30.2 ± 5.5 |
| $Abcg8^{+/-}$ | 27.8 ± 3.6 | 6.1 ± 0.4 | 218 ± 26 | 5.1 ± 1.3[a] | 20.2 ± 3.8 |
| $Abcg8^{-/-}$ | 26.1 ± 6.3 | 9.2 ± 0.7 | 242 ± 33 | 1.3 ± 0.1[a] | 25.4 ± 4.2 |

$Abcg8^{+/+}$, $Abcg8^{+/-}$, and $Abcg8^{-/-}$ mice were fed either a control diet or a 1% diosgenin diet for 18 days. The gallbladder was cannulated, and bile was collected for 10 min. Lipids were determined as described in Materials and Methods.

[a] $P < 0.05$ versus wild-type mice fed the same diet.

[b] $P < 0.05$ versus the control diet.

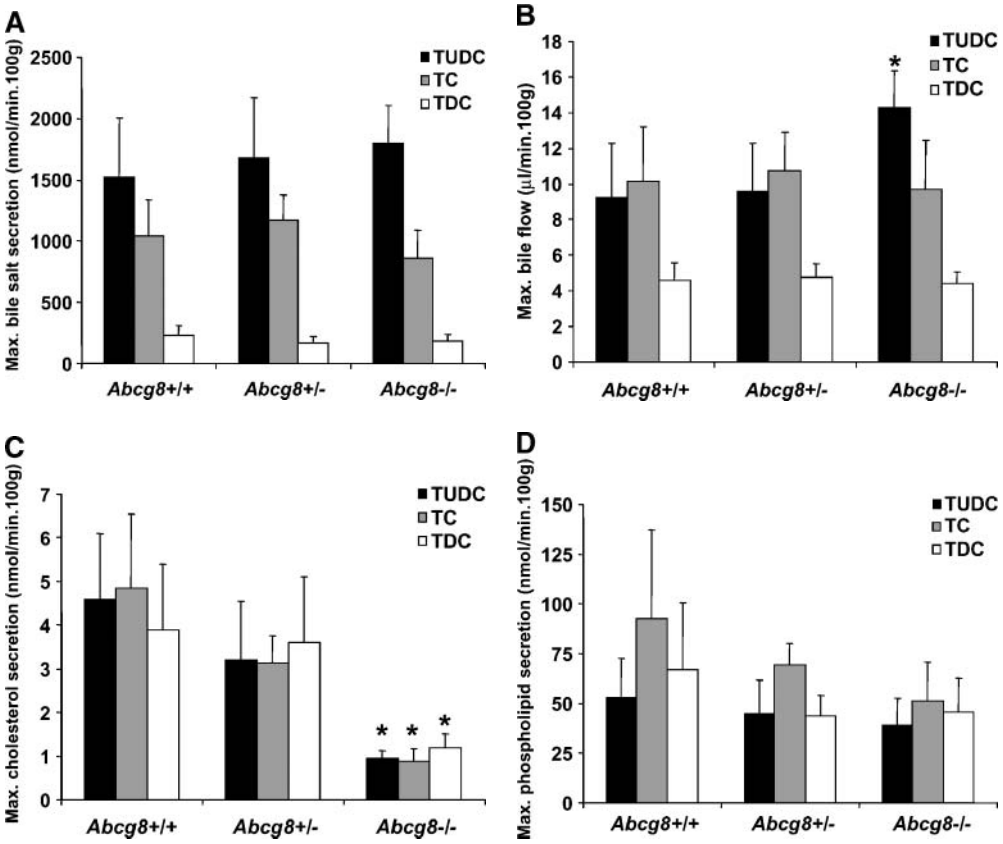


**Fig. 1.** Effect of tauroursodeoxycholic acid (TUDC), taurocholic acid (TC), and taurodeoxycholic acid (TDC) infusion on maximal biliary output in *Abcg8*$^{+/+}$, *Abcg8*$^{+/-}$, and *Abcg8*$^{-/-}$ mice. Bile duct ligation was performed, the gallbladder was cannulated, and after depletion of the endogenous bile salt pool for 90 min, mice were infused via the jugular vein with TUDC (600–2,400 nmol/min/100 g; black bars), TC (400–1,600 nmol/min/100 g; gray bars); or TDC (100–400 nmol/min/100 g; white bars) in stepwise increasing rates. Maximal secretion rates were determined as the average of four consecutive time points with highest secretion rates. The panels depict maximal bile salt secretion (A), maximal bile flow (B), maximal cholesterol secretion (C), and maximal phospholipid secretion (D). Data are given as means ± SEM (n = 4–7 for each experimental group). * $P < 0.05$ compared with *Abcg8*$^{+/+}$ mice.

suggested that the cholesterol content of the canalicular membrane may have decreased. To investigate this option, we isolated canalicular and basolateral membrane fractions of *Abcg8*$^{+/+}$ and *Abcg8*$^{-/-}$ mice before and after infusion with TDC up to the onset of cholestasis in the *Abcg8*$^{-/-}$ mice. **Table 2** shows that under control conditions, the cholesterol, as determined by gas chromatography, to (choline-containing) phospholipid ratios of canalicular membranes from *Abcg8*$^{+/+}$ and *Abcg8*$^{-/-}$ mice were similar. Infusion of TDC again induced cholestasis in *Abcg8*$^{-/-}$ but not in *Abcg8*$^{+/+}$ mice (Table 2). Although TDC infusion did not change the cholesterol/phospholipid ratio in *Abcg8*$^{+/+}$ mice (Table 2), it did reduce the cholesterol/phospholipid ratio by 45% in canalicular membranes of *Abcg8*$^{-/-}$ mice compared with *Abcg8*$^{-/-}$ mice that did not get TDC infusion. This reduction in cholesterol content in *Abcg8*$^{-/-}$ mice is specifically caused by infusion of TDC, because no effect on phospholipid content in the canalicular membranes from both *Abcg8*$^{+/+}$ and *Abcg8*$^{-/-}$ mice was observed after TDC infusion, nor was any reduction in cholesterol content observed in *Abcg8*$^{+/+}$ mice.

## DISCUSSION

Several studies (5–8, 13, 14) have shown that Abcg5/Abcg8 play an important role in the regulation of biliary sterol secretion. The molecular mechanism(s) by which cholesterol enters bile from the hepatocyte has not yet been resolved. A prerequisite is the vectorial delivery of cholesterol to the canalicular membranes of hepatocytes. Once there, two models for the appearance of cholesterol in bile have been proposed. The first model suggests that the increased cholesterol in the canalicular membrane is "lifted" just out of the outer leaflet of the membrane by Abcg5/Abcg8 to be extracted subsequently by the bile salt/phospholipid mixed micelles in bile (21). The second model (15) proposes that Abcg5/Abcg8 actively translocate cholesterol from the inner leaflet to the outer leaflet of the apical membrane. Note that this floppase activity does not exclude a subsequent lifting of cholesterol in the outer leaflet of the canalicular membrane.

Our studies showed that forced biliary secretion of very hydrophobic bile salts such as TDC failed to increase biliary cholesterol secretion in *Abcg8*$^{-/-}$ mice; infusion of TUDC,

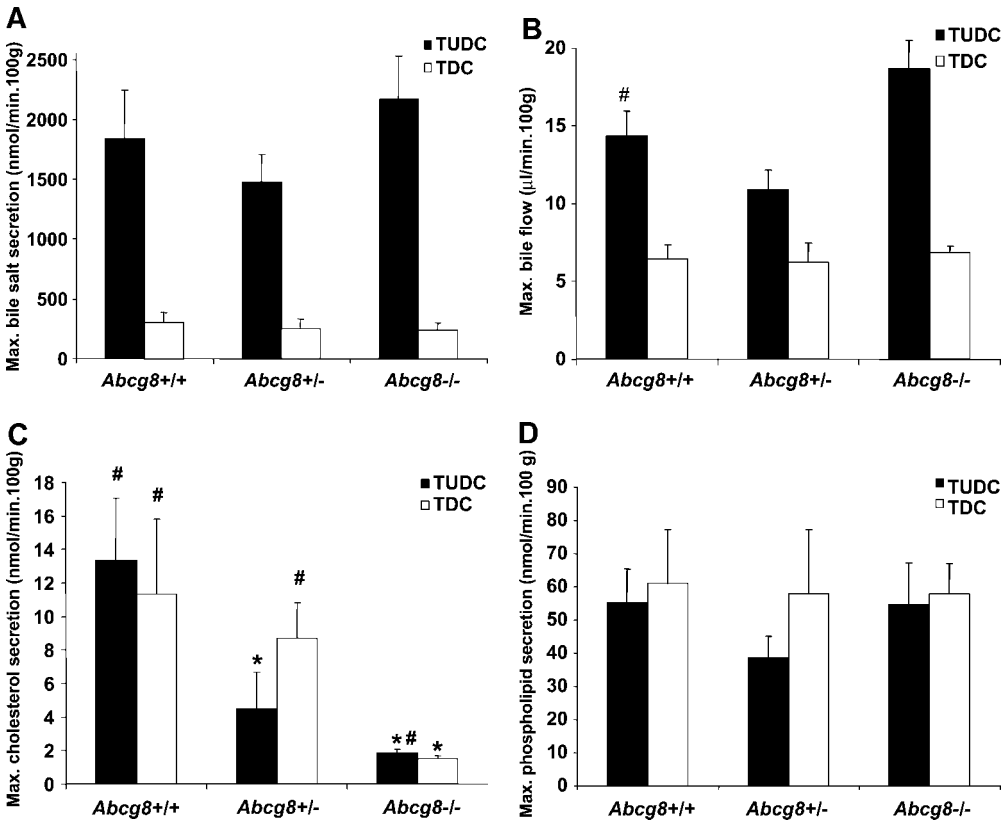


**Fig. 2.** Effect of dietary diosgenin on maximal biliary secretion output after TUDC or TDC infusion in *Abcg8*$^{+/+}$, *Abcg8*$^{+/-}$, and *Abcg8*$^{-/-}$ mice. Mice received a control diet or 1% diosgenin-supplemented diet for 18 days. The bile duct was ligated, and after depletion of the endogenous bile salt pool for 90 min, mice were infused with TUDC (600–2,400 nmol/min/100 g; black bars) or TDC (100–400 nmol/min/100 g; white bars), and maximal secretion rates were determined as described in the legend to Fig. 1. The panels depict maximal bile salt secretion (A), maximal bile flow (B), maximal cholesterol secretion (C), and maximal phospholipid secretion (D). Data are given as means ± SEM (n = 4–6 for each experimental group). * $P <$ 0.05 compared with *Abcg8*$^{+/+}$ mice fed the 1% diosgenin-supplemented diet; # $P < 0.05$ compared with the control diet.

TC, or TDC all led to a similar maximal cholesterol excretion of ~1 nmol/min/100 g, which was ~25% of that observed in *Abcg8*$^{+/+}$ mice under the same conditions. This may suggest that even a hydrophobic bile salt like TDC is not capable of extracting more cholesterol from the rigid canalicular membrane. Alternatively, these observations may also suggest that the cholesterol content of the outer leaflet (and thus spontaneous flip-flop) is rate-limiting under all of these conditions in the *Abcg8*$^{-/-}$ mice. In agreement with this suggestion, isolation of canalicular membrane fractions from *Abcg8*$^{-/-}$ mice revealed a 45% decrease in the cholesterol content after infusion of TDC, whereas the phospholipid content remained constant in these mice. A lower cholesterol content would increase the vulnerability of the canalicular membrane to the detergent action of bile salts. Although it is not possible to determine whether the observed reduction is attributable to a reduction in cholesterol of both membrane layers or to a reduction in only the outer membrane layer, we noted that the onset of bile salt-induced cholestasis occurred at a lower infusion rate of bile salts in the *Abcg8*$^{-/-}$ compared with *Abcg8*$^{+/-}$ and *Abcg8*$^{+/+}$ mice.

These results are in agreement with the hypothesis that Abcg5/Abcg8 facilitates (flops) cholesterol transfer from the inner leaflet to the outer leaflet of the canalicular membrane, because the absence of *Abcg8* would result in a reduction in cholesterol in the outer leaflet. Such an activity for the heterodimer is at variance with data from several studies demonstrating fast spontaneous flip-flop rates of cholesterol (33–36). Values for the half-life of cholesterol translocation of as low as 1 s have been reported, which is at least 2 orders of magnitude higher than required for the actual rate of biliary cholesterol secretion. The fast translocation of cholesterol in model systems led Small (21) to propose that Abcg5/Abcg8 might decrease the activation energy for the release of cholesterol from the outer leaflet of the canalicular membrane. This "liftase" hypothesis does not take into account the formation of cholesterol/phospholipid vesicles in the canalicular space, which has been suggested as a mechanism of cholesterol secretion as well (22, 23, 25, 37). Studies from Crawford et al. (25, 38) indicated that vesicles are secreted from the outer leaflet of the canalicular membrane, which requires the presence of *Abcb4*. Luminal bile salt/phospholipid micelles

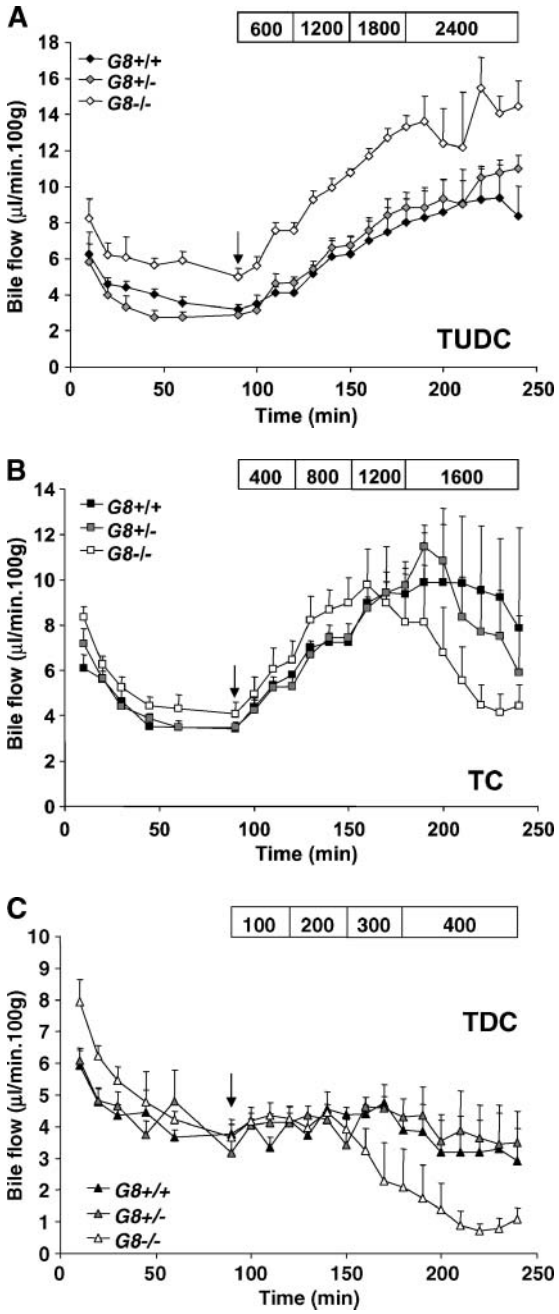


**Fig. 3.** Infusion of the hydrophobic bile salts TC and TDC induces cholestasis in *Abcg8*$^{+/+}$, *Abcg8*$^{+/-}$, and *Abcg8*$^{-/-}$ mice. After depletion of the endogenous bile salt pool for 90 min, mice were infused with TUDC (600–2,400 nmol/min/100 g) (A), TC (400–1,600 nmol/min/100 g) (B), or TDC (100–400 nmol/min/100 g) (C) in stepwise increasing rates as indicated. Bile was collected in fractions of 10 min. Data are given as means ± SEM (n = 4–7 for each experimental group). Arrows indicate the start of bile salt infusion.

would then extract cholesterol from these vesicles. Vesicular secretion is compatible with the floppase activity of Abcg5/Abcg8 but not with the liftase activity.

Yet, vesicular secretion of cholesterol is not the only mechanism by which sterols appear in bile. Earlier studies in *Abcb4*$^{-/-}$ mice demonstrated that although endogenous secretion of cholesterol was almost absent in these mice, infusion of TDC led to increased cholesterol secretion in the absence of vesicle formation (because phospholipid floppase activity is absent) (39). Whether Abcg5/Abcg8 are involved in this pathway is not clear. Overexpression of human *ABCG5/ABCG8* in the absence of *Abcb4* did not result in increased cholesterol secretion, as recently reported by Langheim et al. (40), who crossbred mice overexpressing *ABCG5/ABCG8* with *Abcb4*$^{-/-}$ mice. Unfortunately, they did not test the effect of hydrophobic bile salt infusion. In our study, TDC infusion in *Abcg8*$^{-/-}$ mice did not result in higher maximal cholesterol secretion rates compared with cholesterol secretion rates after TC or TUDC infusion, suggesting the cholesterol content in the outer leaflet of the canalicular membrane of these mice may be rate-limiting. This could be explained by a decrease of accessible cholesterol in the outer leaflet of the canalicular membrane of *Abcg8*$^{-/-}$ mice, which would happen in the case of Abcg5/Abcg8 acting as a floppase. Alternative possibilities are that the total cholesterol content is greatly reduced such that TDC is not able to extract it, or that the presence of plant sterols [mainly sitosterol and campesterol (9)] in the canalicular membrane interferes with the possible extraction of cholesterol by TDC. Treatment of *Abcg5/Abcg8* double knockout mice with the intestinal sterol uptake inhibitor ezetimibe normalized hepatic plant sterol and cholesterol levels but did not restore biliary cholesterol output (41), suggesting that reduction in cholesterol or the interference of plant sterols is not of major importance for cholesterol secretion under normal conditions.

We previously showed (13) that diosgenin is involved in increasing the supply of cholesterol for Abcg5/Abcg8-mediated biliary secretion. It is not clear whether the step activated by diosgenin interacts directly with Abcg5/Abcg8 or facilitates cholesterol delivery to the inner leaflet of the canalicular membrane. Previous studies in rats showed that feeding diosgenin had no effect on the cholesterol content of canalicular membranes, nor did it result in more resistance of canalicular membrane fractions to Triton X-100 or TC (26), suggesting that the effect of diosgenin may involve increased flux of cholesterol through the canalicular membranes. Similar to mice fed the control diet, TDC infusion in diosgenin-fed *Abcg8*$^{-/-}$ mice failed to increase cholesterol secretion over TUDC infusion. Also in *Abcg8*$^{+/-}$ mice infused with TUDC, diosgenin was unsuccessful in inducing cholesterol secretion. In a previous study (13), we showed this to be attributable to the full rate control exerted by Abcg5/Abcg8 under these conditions. Interestingly, as shown in Fig. 2, diosgenin did stimulate cholesterol secretion in *Abcg8*$^{+/-}$ mice when TDC was infused. Apparently, when TDC is the cholesterol acceptor, Abcg5/Abcg8 is less rate-controlling and diosgenin stimulates the Abcg5/Abcg8-independent pathway of cholesterol secretion.

Infusion of TC and TDC induced cholestasis at higher infusion rates, and the onset of cholestasis correlated with the hydrophobicity of the infused bile salts in each genotype (Fig. 3). In addition, in *Abcg8*$^{-/-}$ mice, bile

TABLE 2. TDC infusion decreases cholesterol content of the canalicular membrane in *Abcg8*$^{-/-}$ mice

| Genotype and Treatment | Bile Flow | Cholesterol | Phospholipid | Cholesterol-Phospholipid Ratio | Aminopeptidase N |
|---|---|---|---|---|---|
| | *μl/min/100 g* | *μmol/mg protein* | | | *Fold enrichment vs. total homogenate* |
| Control | | | | | |
| *Abcg8*$^{+/+}$ | ND | 46.0 | 33.0 | 1.4 | 287 |
| *Abcg8*$^{-/-}$ | ND | 39.8 | 35.1 | 1.1 | 212 |
| After TDC | | | | | |
| *Abcg8*$^{+/+}$ | 4.99 ± 1.53 | 51.7 | 41.4 | 1.2 | 112 |
| *Abcg8*$^{-/-}$ | 0.99 ± 1.75 | 24.0 | 42.1 | 0.6 | 211 |

ND, not determined; TDC, taurodeoxycholic acid. *Abcg8*$^{+/+}$ and *Abcg8*$^{-/-}$ mice were infused with TDC as described in Materials and Methods. After 30 min of infusion with 400 nmol/min/100 g TDC, livers of six mice per group were pooled and liver membrane fractions were isolated. Data are presented for two groups of six mice and compared with two groups of four mice that did not undergo the TDC infusion procedure.

salt-induced cholestasis occurred at a lower concentration of infused bile salts compared with *Abcg8*$^{+/-}$ and *Abcg8*$^{+/+}$ mice. Cholestasis did not occur when the mice were fed a diosgenin-supplemented diet before the infusion protocol was applied, suggesting that increased supply of cholesterol may play a role in this.

The residual, Abcg5/Abcg8-independent cholesterol secretion observed in *Abcg8*$^{-/-}$ mice may reflect spontaneous flopping of cholesterol in the canalicular membrane. The fact that this rate is much lower than expected on the basis of experiments with model membranes or erythrocytes may be attributable to the specific composition of the canalicular membrane containing high amounts of sphingomyelin and probably tightly bound cholesterol in a highly ordered environment that strongly inhibits spontaneous flopping. The residual cholesterol secretion observed in *Abcg8*$^{-/-}$ mice remained constant under a variety of conditions and could not be stimulated by means of hydrophobic bile salts, indicating that the endogenous flop rate of cholesterol operated at maximal velocity. The slight increase of cholesterol secretion in diosgenin-fed *Abcg8*$^{-/-}$ mice can be explained by increased supply to the membrane in the absence of floppase activity, resulting in the accumulation of cholesterol in the inner leaflet of the membrane. An increased cholesterol gradient between the inner and outer leaflets will result in more spontaneous flop of cholesterol and increased availability in the outer leaflet of the membrane.

In conclusion, the data in this study confirm that the majority of biliary cholesterol secretion is primarily mediated by Abcg5 and Abcg8. Our data are more compatible with a model that involves Abcg5/Abcg8 acting primarily as a floppase and translocation of sterols to the outer leaflet of the canalicular membranes. Secondary to the floppase activity, the heterodimer may decrease activation energy for the mixed micelle-mediated uptake of cholesterol, particularly in the case of hydrophilic bile salts.

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