Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein

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Abstract Mice in which the gene for mdr2 P-glycoprotein has been disrupted have a severe deficiency in biliary phospholipid and cholesterol secretion. We studied the relation between *mdr2* gene expression and biliary lipid secretion with emphasis on the role of bile salt hydrophobicity. Control mice (+/+), and mice with a homozygous (-/-) or heterozygous (+/-) disruption of the *mdr2* gene, were infused with taurodeoxycholate (TDC) or tauroursodeoxycholate (TUDC). In *mdr2* (-/-) mice, virtually no phospholipids were secreted into bile, irrespective of the type of bile salt infused. In contrast, cholesterol secretion in (-/-) mice increased upon TDC infusion from less than 0.1 to more than 2 nmol/min · 100 g, which was similar to controls under the same conditions. After infusion of TUDC in (-/-) mice, cholesterol secretion also rose (to 1.8 nmol/min · 100 g) but remained much lower than in controls (8 nmol/min · 100 g). In (+/-) mice, cholesterol secretion was equal to (+/+) mice during secretion of endogenous bile salts and during TDC infusion, but was 50% of control levels during maximal TUDC infusion. We conclude that biliary phospholipid secretion completely depends on *mdr2* gene expression but cholesterol can, at least partially, be secreted in an mdr2 Pgp-independent mechanism. The extent to which cholesterol is secreted via this mechanism may depend on the hydrophobicity (i.e., cholesterol-solubilizing capacity) of the secreted bile salt.—**Oude Elferink, R. P. J., R. Ottenhoff, M. van Wijland, C. M. G. Frijters, C. van Nieuwkerk, and A. K. Groen.** Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein. *J. Lipid Res.* 1996. **37:** 1065–1075.

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Biliary lipid secretion is a complex process that is poorly understood. A clear-cut relation between lipid and bile salt secretion has been observed in several species (1) including mice (2) and this has led to the hypothesis that bile salts in the canalicular lumen are necessary for the release of phosphatidylcholine (PC) from the canalicular membrane (3, 4). The physicochemical mechanism of this release is, however, unknown. It was demonstrated recently, that the function of mdr2 P-glycoprotein, present in the canalicular membrane, is essential for phospholipid secretion (5). Mice in which the *mdr2* gene was disrupted had virtually no phospholipid in their bile. We proposed that mdr2 Pgp functions as a flippase translocating PC from the inner to the outer leaflet of the canalicular membrane. Two recent studies with model systems seem to confirm this hypothesis (6, 7). In addition, an ATP-independent flippase activity has been reported to reside in the canalicular membrane (8). So far this activity has not been further characterized. The role of a flippase in secretion of PC severely alters the possible hypotheses concerning the mechanism of biliary lipid secretion. Clearly, budding and pinching of complete bilayer stretches of membrane (3, 4) are no longer en vogue. The activity of a flippase also constitutes a problem for the proposed role of intracellular lipid vesicles in transport of lipid to the canalicular membrane. The asymmetric character of a flippase function makes the insertion of PC into the cytoplasmic leaflet of the membrane by phosphatidylcholine-transfer protein, as suggested by Cohen, Leonard, and Carey (9), more plausible.

In the past, changes in phospholipid secretion were found to be accompanied by similar changes in cholesterol secretion under a variety of experimental conditions, suggesting that the two lipids are released as intact units (1, 10). In accordance with this hypothesis we

Abbreviations: Pgp, P-glycoprotein; TDC, taurodeoxycholate; UDC, ursodeoxycholate; TUDC, tauroursodeoxycholate; TC, taurocholate; MC, muricholate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; CHOL, cholesterol; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography.

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observed that the absence of phospholipid in bile of *mdr2* knockout mice was accompanied by a strong decrease in cholesterol. However, it was also observed that mice, in which *mdr2* gene expression was reduced but not absent (heterozygous *mdr2* knockouts), had a reduced phospholipid secretion but normal cholesterol secretion, suggesting that cholesterol and phospholipid secretion are not fully coupled (2, 5). This anomalous behavior of cholesterol prompted us to further study biliary cholesterol secretion in mice with different levels of *mdr2* gene expression. In the present report we have investigated this relation with special emphasis on bile salt hydrophobicity. These experiments suggest that cholesterol can be secreted via two mechanisms, one that is coupled to phospholipid secretion and therefore to *mdr2* gene expression and another that is independent of phospholipid secretion. The extent to which the second mechanism is operable appears to depend on the hydrophobicity (i.e., cholesterol solubilizing capacity) of the secreted bile salts.

These experiments were carried out with mice in which the *mdr2* gene disruption was bred into another genetic background (the FVB strain) than the original 129/OLA mice which yield very few offspring (5). The bile secretion parameters of these FVB (+/+) and (-/-) mice were therefore first characterized.

MATERIALS AND METHODS

Animals

The generation of mice that are homozygous (-/-) for the *mdr2* gene disruption with the 129/Ola strain (+/+) as genetic background has been described by Smit et al. (5). These mice have a very poor breeding efficiency. Therefore, they were crossbred with the FVB strain which breeds very well. Crossbreeding was continued until a 94% FVB background was obtained for (+/+), (+/-), and (-/-) mice. All mice were bred in our own colony and were used for experiments at 2–4 months of age. In all experiments we used male mice. The animals were anesthetized by i.p. injection of 1 ml/kg Hypnorm (fentanyl/fluanisone) and 10 mg/kg diazepam. The abdomen was opened and the gallbladder was cannulated after distal ligation of the common bile duct. Bile salts (dissolved in phosphate-buffered saline) were infused into the tail vein at the indicated rate. Directly after cannulation bile sampling was started and after the indicated time bile salt infusion was started. The body temperature was maintained by placing the animals on a thermostatted heating pad and by covering them with a piece of tin foil. Bile samples were collected and immediately frozen at -20°C. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile.

Assays

Total choline content of phosphatidylcholine and sphingomyelin was determined enzymatically with phospholipase D and choline oxidase (11). Cholesterol was assayed enzymatically with cholesterol oxidase (12). Total bile salt concentration was measured spectrophotometrically with 3α-hydroxysteroid dehydrogenase (13). Lipid composition of selected bile samples was assessed by thin-layer chromatography. Samples were chromatographed on silica gel DC plates (Merck) with chloroform–methanol–acetic acid–H_2O 100:20:12:5 (by vol) as eluent. The lipids were visualized by spraying with phosphomolybdic acid–ethanol 10:90 (w/v) followed by methanol–sulfuric acid 1:1 (v/v) and heating at 120°C.

Bile salt species from selected bile samples were analyzed by HPLC as described by Ruben and Van Berge-Henegouwen (14), modified as follows. Samples were diluted 1:200 in eluens (methanol 60%, 3 mM K_2HPO_4, pH 3.75; 40%). Twenty μl of this sample was injected on the HPLC with a C_{18} Chromspher 5 μm (Chrompack) with a flow of 800 μl/min. Peaks were detected using an UV detector at 205 nm.

In order to study the effect of chronic oral bile salt administration, (+/+) and (-/-) mice were fed a purified diet directly after weaning for a period of 3 weeks. This diet was either supplemented or not with 0.1% taurocholate. After 3 weeks on the diet bile samples were collected and analyzed as described above.

Chemicals

All chemicals used were from Sigma (St. Louis, MO). The phospholipid assay kit was from Wako Chemicals GmbH (Neuss, Germany). Cholesterol assay kit was from Boehringer (Mannheim, Germany). 3-OH-steroid dehydrogenase was from Worthington Biochem. Corp. (Freehold, NJ).

Statistics

All values represent means ± standard deviation from at least three animals of each strain. Differences between the groups were analyzed by unpaired, two-tailed Student's t-test.

RESULTS

Phenotype of *mdr2* gene disruption in FVB genetic background

Table 1 gives the bile composition of male FVB (+/+), (+/-), and (-/-) mice. Endogenous bile salt secretion in all strains is lower than the earlier reported values for the 129/OLA strain (2). Similar to our previous observations (2, 5), phospholipids are extremely low in bile of (-/-) mice, although very small amounts were detectable.

Also, cholesterol secretion in FVB (-/-) mice was considerably lower than in 129/OLA (-/-) animals. In accordance with our previous observations with the 129/OLA genetic background, the phospholipid secretion in heterozygotes (+/-) is intermediate between (+/+) and (-/-) mice while cholesterol secretion is normal. The endogenous bile salt composition (**Table 2**) is comparable to that reported for 129/OLA mice. The proportion of muricholate was significantly higher in (-/-) mice than in (+/+) mice. This may relate to a higher rate of bile salt synthesis in (-/-) mice due to the elevated level of cholesterol in the liver (2). It can be concluded that bile salt, phospholipid, and cholesterol secretion in FVB mice is somewhat lower than in 129/OLA, but the essential observations made in 129/OLA (-/-) and (+/-) also hold for the FVB background.

Manipulation of bile salt hydrophobicity by intravenous infusion of taurodeoxycholate

The effect of biliary secretion of a very hydrophobic bile salt in (-/-) mice was tested by intravenous infusion of taurodeoxycholate (TDC). In order to reach a maximal replacement of endogenous bile salts by TDC, bile was first collected for 90 min after bile duct cannulation. **Figure 1** shows that during this period the endogenous bile salt pool was largely depleted; after 90 min bile salt secretion had decreased to 30% of the initial value, both in (+/+) and in (-/-) mice. Subsequently, TDC was infused at 100 nmol/min · 100 g and the rate of infusion was increased every 30 min to reach a maximum of 800 nmol/min. This protocol led to a constant level of total bile salt secretion between 100 and 200 nmol/min · 100 g. This level is considerably lower than the infusion rate suggesting that the secretory maximum for TDC was reached. At the end of the infusion period the average bile flow and bile salt secretion tended to decrease, especially in the (-/-) animals. The variation in the data within each experimental group is large, due to the development of cholestasis in some but not all animals. At the end of the infusion period, 2 out of 5 (+/+) mice and 2 out of the 5 (-/-) mice had developed cholestasis (bile flow <10% of the original level). At the end of the TDC infusion period, the total bile salt concentration in all strains varied between 20 and 50 mM. In (+/+) mice the secreted bile salts consisted of 67 ± 7% TDC at the end of infusion, while this was 47 ± 8% in (-/-) mice (Table 2). This difference may result from (-/-) mice having a higher endogenous rate of bile salt synthesis due to increased intrahepatic cholesterol levels (2).

Upon infusion of TDC, phospholipid secretion increased in (+/+) mice but only traces of phospholipid could be measured in bile of (-/-) animals. In (-/-) mice cholesterol secretion was initially very low, but increased to substantial values during TDC infusion. During maximal TDC secretion there was no longer a difference in cholesterol secretion between (+/+) and (-/-) mice.

Identical experiments were also carried out with (+/-) mice. According to Fig. 1 there was no difference in phospholipid secretion between (+/+) and (+/-) animals, which would be unexpected in view of our earlier observations (2, 5). The bile salt secretion in this particular set of (+/-) animals was, however, higher than in (+/+) mice. In order to compare the extent of bile salt-induced phospholipid secretion in (+/+) and (+/-) mice, we therefore plotted these two parameters against each other (**Fig. 2**). It is clear from this figure that the slope of this relation obtained during the depletion phase of the experiment is decreased in (+/-) compared to (+/+) mice (Fig. 2A). Upon infusion of TDC the slope of this relation increased in both (+/+) and (+/-) mice but the difference between the two strains remained (Fig. 2B). This indicates that even during infusion of a hydropho-

TABLE 1. Endogenous secretion rates of the main bile components in FVB (+/+), (+/-) and (-/-) mice

Strain	Bile Flow	Bile Salts	Phospholipid	Cholesterol
	μl/min · 100 g		*nmol/min · 100 g*	
+/+, n = 7	9.8 ± 1.5	309 ± 88	26.8 ± 4.5	2.7 ± 0.7
+/-, n = 6	9.5 ± 0.5	373 ± 84	20.9 ± 2.7[a]	3.1 ± 1.0
-/-, n = 8	16.9 ± 3.3[b]	394 ± 88	0.3 ± 0.5[b]	0.08 ± 0.07[b]

Bile flow and the bile components were determined in bile samples that were obtained during the first 10 min directly after cannulation of the gallbladder.
[a] $P < 0.05$.
[b] $P < 0.001$.

TABLE 2. Bile salt composition before and after infusion of TDC and TUDC

	Before Infusion	TDC Infusion	TUDC Infusion
		%	
+/+			
Muricholate	59 ± 6	12 ± 4	4 ± 1
Cholate	41 ± 6	21 ± 4	2 ± 1
Ursodeoxycholate	n.d	n.d	93 ± 2
Deoxycholate	n.d	67 ± 7	1 ± 1
n =	5	5	3
-/-			
Muricholate	75 ± 4[b]	21 ± 4[a]	6 ± 3
Cholate	23 ± 6[b]	33 ± 8[a]	4 ± 3
Ursodeoxycholate	n.d.	n.d.	88 ± 4
Deoxycholate	n.d.	47 ± 8[b]	1 ± 1
n =	4	4	4

Bile salt species were determined by HPLC in samples obtained directly after cannulation (before infusion) and at the end of the infusion period. Data represent percentages of total and are expressed as means ± SD from the indicated number of bile samples. Differences in composition between (+/+) and (-/-) were tested by Student's *t*-test; n.d., not detected.
[a] $P < 0.05$.
[b] $P < 0.01$.

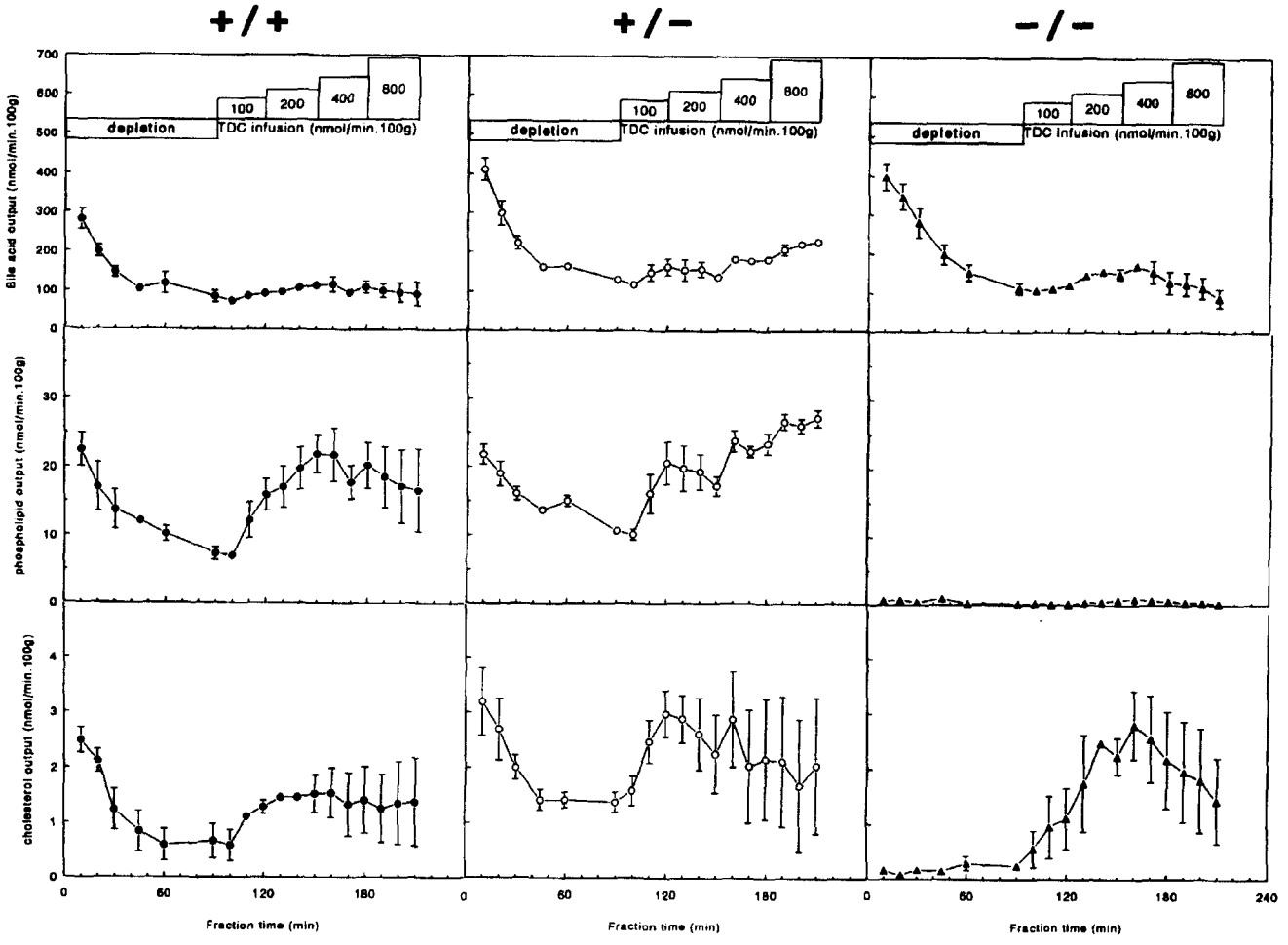

Fig. 1. Bile formation and lipid secretion during i.v. infusion of TDC. Mice of the various genotypes were cannulated in the gallbladder; bile was sampled for 90 min in order to deplete the endogenous bile salt pool and subsequently taurodeoxycholate was infused via the tail vein at the indicated, increasing, rates. Data represent means ± SD for 5 animals of each strain. Upper three figures: bile salt secretion. Middle three figures: phospholipid secretion. Lower three figures: cholesterol secretion.

bic bile salt like TDC, mdr2 Pgp remains rate-controlling for phospholipid secretion. In contrast, cholesterol secretion was not significantly reduced during TDC infusion in (+/-) as compared to (+/+) mice (Fig. 1). Replacement of the endogenous bile salt pool by the infused TDC in (+/-) mice was identical to that in (+/+) mice (not shown).

Phospholipid species in bile during taurodeoxycholate infusion

It has been demonstrated previously in the rat that upon infusion of hydrophobic bile salts like TDC, the phospholipid species secreted into bile changes from almost exclusively PC to a mixture of phospholipid species that resembles more the membrane composition (15). **Figure 3** shows TLC analysis of bile samples from (+/+) and (-/-) mice before and after TDC infusion. During secretion of endogenous bile salts, bile in (+/+) mice contained almost exclusively PC with a small amount of PE (lane 1). Surprisingly, this composition hardly changed upon TDC infusion (lane 2); there was a slight increase in phosphetidylethanolamine (PE) but no sphingomyelin (SM) could be detected. Bile samples from (-/-) mice under the same conditions revealed a virtual absence of phospholipids during endogenous bile salt secretion (Fig. 3, lane 3) and a very small amount of PC and PE during maximal TDC secretion (Fig. 3, lane 4); qualitatively the ratio of PC to PE was the same in (-/-) as in (+/+) bile. In order to further substantiate this finding, we directly compared bile samples from a (+/+) mouse that became cholestatic upon TDC infusion with samples from a mouse that displayed no reduction in bile flow. These two samples (lanes 5 and 6) had an identical composition which again was not different from the phospholipid composition of samples obtained during secretion of endogenous bile salts. At a strongly reduced level the same pattern was observed in bile samples from (-/-) mice that had either or not

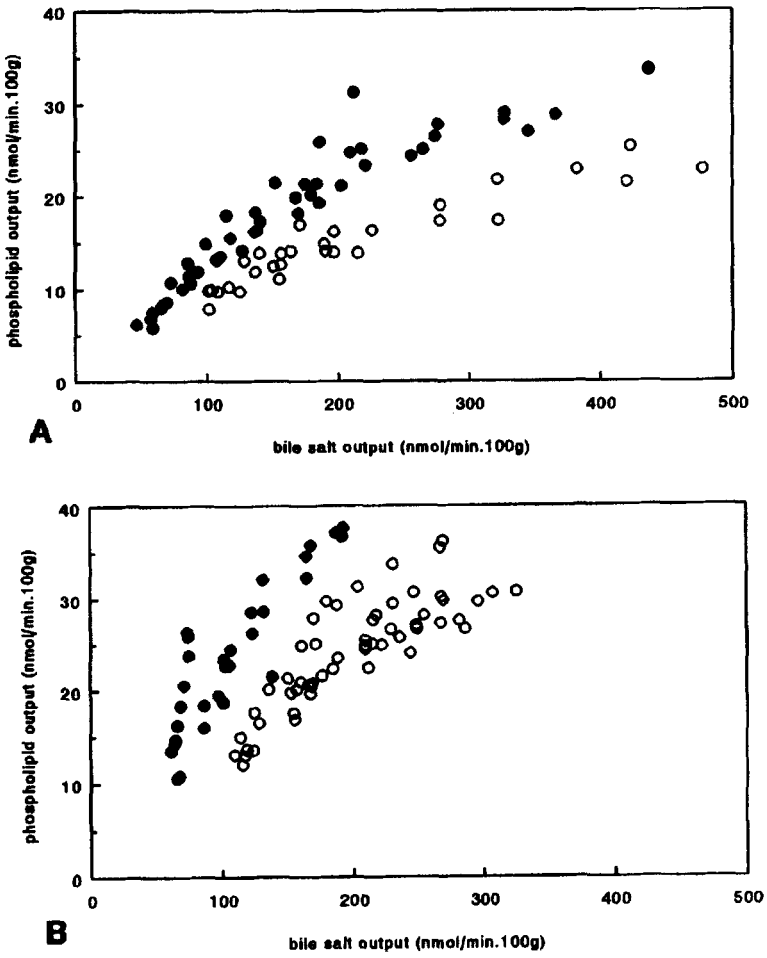

Fig. 2. Relation between phospholipid and bile salt secretion in *mdr2* (+/+) and (+/-) mice before and during infusion of TDC. The separate data from each bile sample obtained in the experiment of Fig. 1 for bile salt and phospholipid secretion rates were plotted against each other. Panel A: endogenous bile salts (bile samples obtained during the first 90 min of the experiment). Panel B: samples obtained during taurodeoxycholate infusion; ●–●: (+/+) mice; ○–○: (+/-) mice.

developed cholestasis (lanes 7 and 8). TLC analysis of (-/-) samples before and after TDC infusion demonstrated that cholesterol output increased from very low to levels comparable with (+/+) mice (lanes 3 and 4, respectively), which is in line with the enzymatic determination (Fig. 1).

Effect of intravenous TUDC infusion on biliary lipid secretion

In order to assess the importance of bile salt hydrophobicity we also infused tauroursodeoxycholate (TUDC). This bile salt is very hydrophilic and non-cytotoxic. Again, to obtain maximal replacement of endogenous bile salts by TUDC the mice were first depleted for 90 min and then infused with TUDC at increasing rates. As expected and in contrast to the experiment with TDC, infusion of TUDC led to a very high bile salt output without development of cholestasis (**Fig. 4**). In (+/+) mice this was accompanied by an increase in phospholipid and cholesterol secretion. In (-/-) mice phospholipid secretion remained absent, but at high rates of TUDC secretion there was an increase in cholesterol output to a maximum of 1.8 nmol/min · 100 g which is 21% of the control under the same conditions. As with TDC infusion, phospholipid secretion was reduced in (+/-) mice compared to controls. However, in contrast to what was found for TDC infusion, cholesterol secretion in (+/-) mice was also reduced during TUDC infusion compared to (+/+) animals. During maximal TUDC secretion, the cholesterol output in (+/-) mice was approximately 50% of that in (+/+) mice. In both (+/+) and (-/-) mice the applied TUDC infusion protocol led to a 88–93% replacement of the endogenous bile salts by TUDC (Table 2) and the same replacement was observed in (+/-) animals. These results suggest that when TUDC is used as the primary bile salt to drive lipid secretion, phospholipid and cholesterol remain tightly coupled; except at very high TUDC secretion a small phospholipid-independent level of cholesterol output in (-/-) mice can be obtained. **Figure 5** gives the relation between cholesterol and phospholipid secretion in this experiment. A tight relation between the two lipids was observed which was equal in (+/+) and (+/-) animals; only at the highest bile salt secretion rates

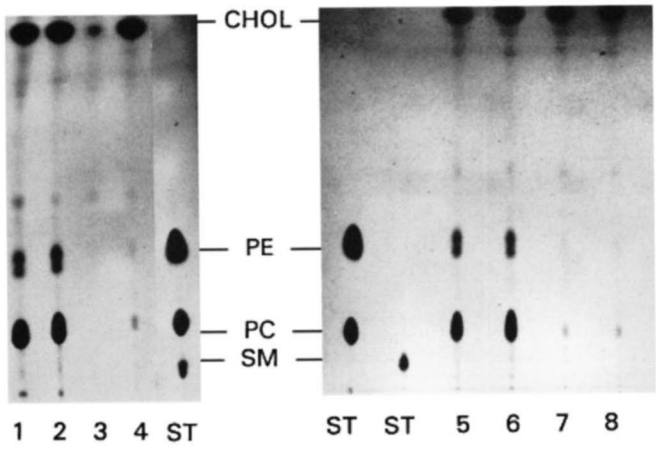

Fig. 3. Analysis of the phospholipid species before and after infusion of TDC. Representative bile samples obtained in the experiment of Fig. 1 were analyzed by thin-layer chromatography. Lanes 1, 2, 5, 6: samples from (+/+) mice. In these lanes equal amounts of phospholipid were applied (40 nmol). Lanes 3, 4, 7, 8: samples from (-/-) mice. In these samples a maximal volume of (-/-) bile (10 μl), which corresponds with < 1 nmol, was extracted and applied to the TLC plate. Bile samples were obtained directly after cannulation of the gallbladder (lanes 1 and 3) or at the end of the TDC infusion period (t = 210 min, lanes 2 and 4). Bile samples obtained during the 20 min prior to the onset of TDC-induced cholestasis (lanes 6 and 8). Bile samples from mice that did not develop cholestasis during the entire TDC infusion period (lanes 5 and 7, samples were taken at the same time point as those in lane 6 and 8, respectively).

(i.e., high phospholipid secretion rates) did the relation in (+/-) deviate slightly from that in (+/+).

The data from the experiments in Figs. 1 and 4 demonstrate that cholesterol secretion is completely dependent on phospholipid secretion when TUDC is the secreted bile salt while this is not the case with endogenous bile salts and with TDC. This is illustrated in **Fig. 6** where the maximal rates of cholesterol secretion are depicted under the different conditions. The cholesterol secretion rate during TDC infusion was taken from a time point (160 min) at which cholestasis had not yet developed in any of the animals. With endogenous bile salts and with TDC infusion there is no significant difference in maximal cholesterol secretion between (+/+) and (+/-) animals while this is not the case with TUDC secretion.

Cholesterol secretion upon feeding cholate-supplemented diet

The increased cholesterol secretion in (-/-) mice could be the consequence of acute infusion of high amounts of bile salts. In order to test this possibility we also chronically fed mice a purified diet either supplemented or not with 0.1% cholate. Bile composition of these mice is shown in **Table 3**. Both (+/+) and (-/-) mice on the purified control diet had a much lower endogenous bile salt secretion rate than on lab chow (compare Tables 1 and 3). The reason for this is unclear but may be related to the relative cholesterol contents of these diets. Addition of cholate to the diet led to an increase of total bile salt output and to an almost complete replacement of the endogenous bile salts by taurocholate. Bile salt composition in both strains was 87 ± 10% taurocholate, the remainder being muricholate. It is clear that also in this experimental set-up of chronic bile salt feeding, (-/-) mice secrete a substantial amount of cholesterol into bile as opposed to (-/-) mice on a control diet. In **Fig.**

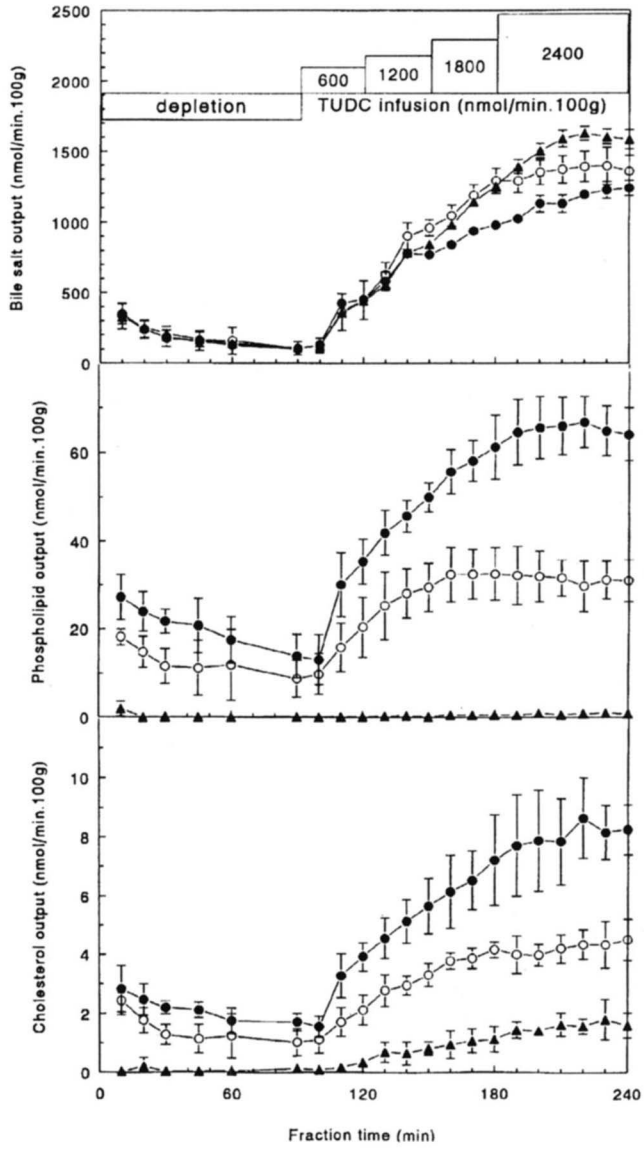

Fig. 4. Bile formation and lipid secretion during i.v. infusion of TUDC. Mice of the various genotypes were cannulated in the gallbladder; bile was sampled for 90 min in order to deplete the endogenous bile salt pool and subsequently tauroursodeoxycholate was infused via the tail vein at the indicated, increasing, rates. Data represent means ± SD for 5 animals of each strain. Upper figure: bile salt secretion; middle figure: phospholipid secretion; lower figure: cholesterol secretion; ●–●: (+/+) mice; ○–○: (+/-) mice; ▲–▲: (-/-) mice.

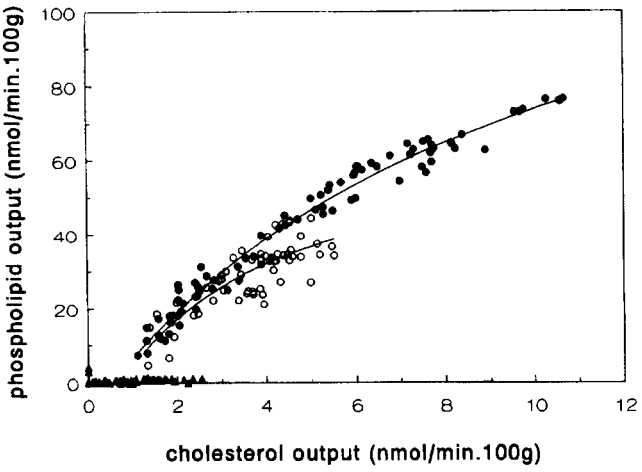

Fig. 5. The relation between phospholipid and cholesterol secretion during TUDC infusion in the various mouse strains. The individual data for phospholipid and cholesterol secretion obtained in the experiment of Fig. 4 were plotted against each other; ●—●: (+/+) mice; ○—○: (+/-) mice; ▲—▲: (-/-) mice.

7A the relation between phospholipid and bile salt secretion during interruption of the enterohepatic circulation is given for these animals. In (+/+) animals a steep hyperbolic relation is observed which is in line with a more efficient phospholipid secretion when taurocholate is the main secreted bile salt. It is clear that (-/-) mice fed a cholate-rich diet still have a very low phospholipid secretion, although Table 3 shows that it is significantly increased compared to the same strain on a control diet. In contrast, substantial amounts of cholesterol were secreted by (-/-) mice on the cholate-supplemented diet. Maximal cholesterol secretion in (-/-) mice was about 30% of that in (+/+) animals. As a consequence these animals secrete more cholesterol than phospholipid, which is highly unfavorable in terms of cholesterol solubilization. Indeed, microscopic examination of gallbladder bile of these animals revealed massive amounts of cholesterol crystals (not shown). The relation between cholesterol and bile salt secretion turned out to be linear in both (+/+) and (-/-) animals (Fig. 7B).

DISCUSSION

We have previously proposed that mdr2 Pgp plays a crucial role in the hepatobiliary secretion of phosphatidylcholine. Our present results substantiate this claim. We infused *mdr2* (+/+) and (-/-) mice with the hydrophobic bile salt TDC. This bile salt was chosen for two reasons. First, it was important to see whether TDC could elicit phospholipid secretion in the knockout mice; i.e., whether a very hydrophobic bile salt is able to extract phospholipids directly from the membrane by micellization in the absence of mdr2 P-glycoprotein. Second, we wanted to observe whether the *mdr2* (-/-) mice would be more sensitive to the cholestatic action of TDC that has been reported in studies with rats and other species (15–17). In the absence of mdr2 Pgp expression, the hydrophobic bile salt TDC at concentrations as high as 20 mM is unable to elicit significant PC secretion. The extremely small amount of phospholipid that is found in bile of (-/-) mice under these conditions has the same composition as in (+/+) mice: most is PC with a small amount of PE. Surprisingly, even during maximal TDC secretion no sphingomyelin was observed. Many studies have shown that hydrophobic bile salts are extremely cytotoxic in the absence of phospholipid and cholesterol (18–20). The similar sensitivity of (+/+) and (-/-) mice towards the cholestatic action of TDC (cholestasis in 2 out of 5 animals in both groups) therefore came as a surprise. It must be taken into account that analysis of the bile salt composition under these conditions revealed that the content of TDC in (+/+) mice was slightly, but significantly, higher than in (-/-) mice. This may be caused by the higher endogenous bile salt synthetic rate in (-/-) mice due to the higher availability of cholesterol (2). We can, therefore, not exclude that at an equal rate of TDC secretion the (-/-) mice would become cholestatic somewhat more rapidly. Nevertheless our results clearly indicate that the canalicular membrane of (-/-) is highly resistant against the lytic activity of hydrophobic bile salts. This resistance may also hold for the membrane of (+/+) mice as we did not observe a decrease in the bile phospholipid selectivity during maximal TDC secretion rates in (+/+) mice.

The mechanism of resistance of the membrane against bile salts is unknown but may be caused by the specific composition of the outer leaflet of the canalicular membrane. Increased molar contents of cholesterol and sphingomyelin in biomembranes have been demon-

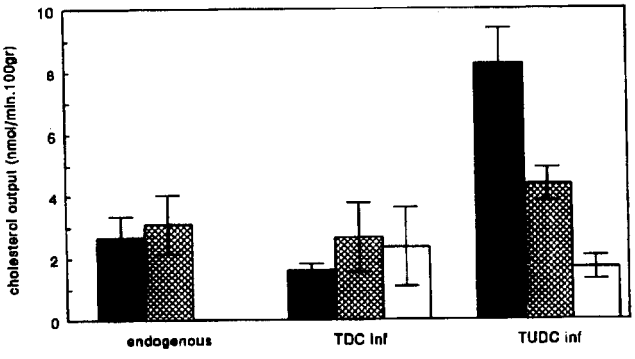

Fig. 6. Maximal cholesterol secretion rates during secretion of different bile salts. This figure summarizes the maximal rates of cholesterol secretion with endogenous bile salts (t = 0; combined data of Fig. 1 and 4), with TDC infusion (t = 160 min; Fig. 1) or TUDC infusion (t = 240 min; Fig. 4). Closed bars: (+/+) mice; cross-hatched bars: (+/-) mice; open bars: (-/-) mice.

TABLE 3. Bile composition of (+/+) and (-/-) mice after 3 weeks on a diet containing 0.1% cholate

Strain and Diet	Bile Flow	Bile Salts	Phospholipid	Cholesterol
	μl/min · 100 g		*nmol/min · 100 g*	
+/+				
Control diet	6.6 ± 1.2	188 ± 70	10.4 ± 2.7	1.28 ± 0.41
0.1% Cholate	9.6 ± 1.0	434 ± 93	65.1 ± 15.0	13.8 ± 2.6
-/-				
Control diet	7.8 ± 0.6	170 ± 40	0.15 ± 0.19	0.24 ± 0.15
0.1% Cholate	13.7 ± 2.3	539 ± 68	2.82 ± 1.16	5.03 ± 1.76

Mice of different genotype were fed with a synthetic diet either supplemented or not with 0.1% cholate directly after weaning (21 days after birth) for a period of 3 weeks. Subsequently the gallbladder was cannulated and bile flow and the indicated bile components were determined in samples obtained during 10 min directly after cannulation. Data represent means ± SD and are from 4 animals in each experimental group.

strated to confer resistance towards bile salts (21, 22). In addition, glycolipids in the outer leaflet of the canalicular membrane could further increase this resistance, although very little is known about the content of these lipids and their interaction with bile salts. It has been demonstrated, however, that glycolipid-rich microdomains display resistance towards detergent solubilization (23, 24).

A second unexpected finding of bile salt infusion into (-/-) mice was the appearance of cholesterol in bile. While cholesterol is virtually absent from bile under endogenous circumstances, infusion of TDC as well as TUDC led to substantial cholesterol output. Depending on the conditions, cholesterol secretion in (-/-) mice could be as high as 100% of normal (TDC infusion). As in normal animals, the ratio of cholesterol to bile salts was higher with TDC than with TUDC. In the past it has been shown in several species that both phospholipid and cholesterol secretion per mole of bile salt increases with increasing bile salt hydrophobicity (25–28). As phospholipid secretion remained extremely low under all conditions in (-/-) animals, these results demonstrate that cholesterol and phospholipid secretion can be partly uncoupled. These observations challenge the current paradigm that phospholipids and cholesterol are secreted as intact units; depending on the type of secreted bile salt, at least part of the cholesterol can be secreted via a mechanism that is independent from mdr2 Pgp function and therefore independent from phospholipid secretion.

What do the observations concerning mdr2 Pgp-dependent and -independent cholesterol secretion tell us about the mechanism of cholesterol secretion? As described above, it is our hypothesis that phospholipid is translocated to the outer leaflet where it resides in microdomains. Crawford et al. (29) recently provided evidence suggestive for direct vesiculation from the outer leaflet of the canalicular membrane. Cholesterol molecules in the outer leaflet of the membrane could laterally diffuse into these microdomains and then be secreted together with the phospholipid. In this model a close relation between cholesterol and phospholipid secretion rates is expected that will be determined by the cholesterol content of the canalicular membrane. This model is supported by the observation in many studies that cholesterol and phospholipid secretion are tightly coupled. It does not, however, explain the substantial cholesterol secretion that we observe in (-/-) mice in the virtual absence of phospholipid secretion nor does it explain the different PC/CHOL ratios observed during secretion of different bile salts.

An alternative mechanism could be that bile salt micelles extract cholesterol directly from the outer leaflet of the membrane. The efficiency of this extraction is determined by the cholesterol solubilizing capacity of bile salts, and the presence of phospholipids further increases this efficiency because mixed micelles of bile salts and phospholipids have a higher cholesterol solubilizing capacity (1). In this model cholesterol secretion would thus only be secondarily dependent on phospholipid secretion. The second model explains our observations with bile salt infusion/feeding in the (-/-) mouse. Cholesterol secretion in (-/-) mice could be increased to normal levels during TDC infusion, while it was much lower than normal during TUDC infusion; this is in line with the fact that TDC micelles have a much higher affinity for cholesterol than TUDC (30, 31). In line with this model, cholesterol secretion is negligible during secretion of endogenous bile salts (75% muricholate) because this bile salt has an even lower affinity for cholesterol than TUDC (32). Upon feeding cholate to (-/-) mice, an intermediate cholesterol secretion was observed suggesting that taurocholate has intermediate capacity for direct cholesterol micellization between TDC and TUDC. Similar results were obtained when we infused taurocholate into (-/-) mice (2).

Although our data suggest that direct micellar extraction of cholesterol by bile salts is operative in the (-/-) mouse, it is difficult to predict whether it is also valid for normal mice. First, the (-/-) canalicular membrane may have a different composition, for example, by an accumulation of cholesterol in the membrane. Second, in the

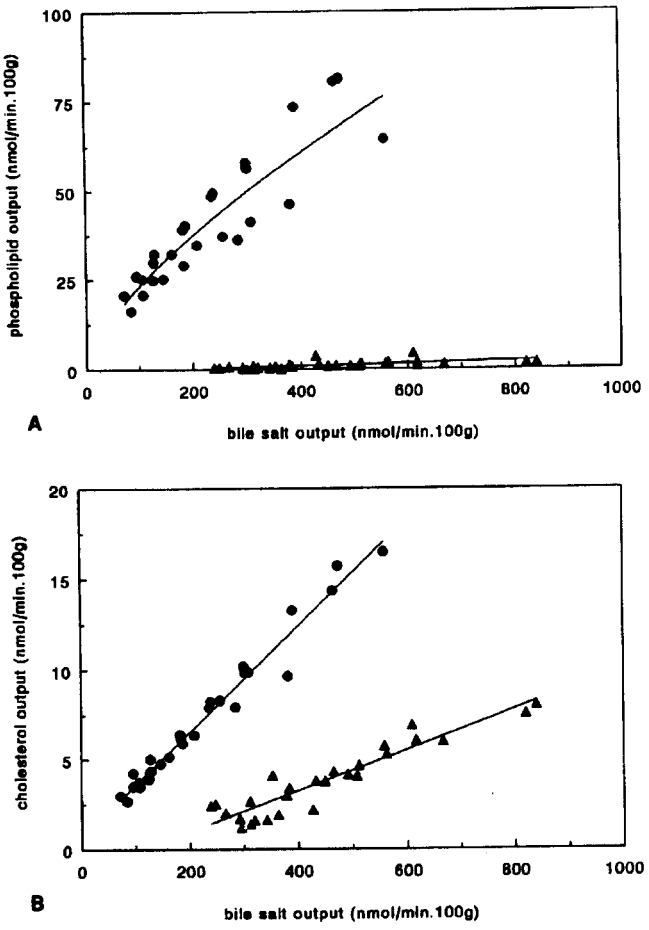

Fig. 7. Bile salt-induced cholesterol and phospholipid secretion in (+/+) and (-/-) mice on a diet with 0.1% cholate. Mice of different genotypes were fed a synthetic diet either supplemented or not with 0.1% cholate for 3 weeks directly after weaning (21 days after birth) and feeding was continued for a period of 3 weeks. Subsequently the gallbladder was cannulated and bile samples were collected for 120 min during which time interval the bile salt pool was gradually depleted. Data represent individual samples from 4 animals of each genotype. Panel A: phospholipid secretion; panel B: cholesterol secretion; ●—●: (+/+) mice; ▲—▲: (-/-) mice.

presence of normal phospholipid secretion, cholesterol secretion may proceed via the phospholipid-dependent mechanism without the need for direct extraction of cholesterol by bile salt micelles. In this respect, data from the (+/-) mice give important further information. We observed that under almost all conditions cholesterol secretion is normal in (+/-) mice except when a high rate of TUDC secretion is created. This implies that the cholesterol to phospholipid ratio may increase considerably with reduction of mdr2 Pgp activity and that the extent to which the ratio increases depends on the type of secreted bile salt. A possible explanation for this phenomenon could be that phospholipid-dependent vesicular cholesterol secretion as well as direct micellar extraction play a role in the total cholesterol secretion. As mentioned above, the efficiency of micellar extraction depends on the cholesterol solubilizing capacity of the bile salt. During taurodeoxycholate (and taurocholate (2)) infusion, the combined mechanisms apparently are sufficient to elicit normal cholesterol secretion rates in (+/-) mice. Even the endogenous bile salt composition (70% muricholate and 30% taurocholate) is capable of driving normal cholesterol secretion, in sharp contrast to the absence of cholesterol in (-/-) animals. This could be explained by the increase in affinity for cholesterol upon inclusion of phospholipid in the micelles. Carey and Small (33) demonstrated that the cholesterol-solubilizing capacity of pure taurocholate micelles at a total lipid concentration of 1–2 g/dl (which we find in mouse bile) increases about 5-fold when 10% lecithin (which is the approximate amount we find in mouse bile) is added. Although the system used by Carey and Small (33) (dissolution from cholesterol crystals) is completely different from the system under study (extraction from a membrane bilayer), it clearly demonstrates that the affinity of micelles for cholesterol strongly increases with the addition of phospholipid.

During TUDC infusion cholesterol secretion was very low in (-/-) and intermediate in (+/-) mice, suggesting that with this bile salt cholesterol secretion is closely coupled to phospholipid. At the applied high infusion rates the bile salt pool is almost completely replaced by TUDC and, in contrast to the endogenous situation, taurocholate is not present anymore. Pure TUDC is such a poor cholesterol solubilizer that direct extraction may not be possible and under this condition total secretion could be completely driven by the phospholipid-dependent mechanism. It was shown (32, 34) that the minimal number of UDC and TUDC molecules needed for solubilization of one cholesterol molecule is in the order of 300–700, UDC being somewhat better than TUDC. The ratio of cholesterol to bile salts in bile of (-/-) mice during maximal TUDC secretion is in this order of magnitude, suggesting that the micelles are saturated with cholesterol. It was also shown that addition of egg yolk phosphatidylcholine to a UDC solution in a ratio of 1:4 led to a more than 10-fold increase in the cholesterol-solubilizing capacity (32). During infusion of TUDC in (+/-) and (+/+) mice, the ratio of phosphatidylcholine to bile salts did not exceed 1:50 which may mean that in these animals also the amount of cholesterol secreted is close to the maximal solubilizing capacity of these mixed micelles.

In conclusion, our data suggest that there are two mechanisms of cholesterol secretion; one that is tightly coupled to phospholipid secretion and a second that depends on direct extraction by bile salts. The extent to which these two mechanisms contribute to total choles-

terol secretion may depend on the hydrophobicity of bile salt. The mechanism by which cholesterol reaches the outer leaflet of the membrane is presently unknown. It is often suggested that the spontaneous flip-flop of cholesterol is fast enough to account for this phenomenon. However, much controversy exists about flip-flop rates of cholesterol in model membranes and no studies have been carried out in systems of phospholipid composition comparable to the canalicular membrane. One can question, therefore, whether under conditions of high cholesterol flux, as occurs in humans, spontaneous flip-flop of cholesterol is sufficient or whether an active transporter must be present for this molecule as well.

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