Regulation of *mdr2* P-glycoprotein expression by bile salts

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The phosphatidyl translocating activity of the *mdr2* P-glycoprotein (Pgp) in the canalicular membrane of the mouse hepatocyte is a rate-controlling step in the biliary secretion of phospholipid. Since bile salts also regulate the secretion of biliary lipids, we investigated the influence of the type of bile salt in the circulation on *mdr2* Pgp expression and activity. Male mice were fed a purified diet to which either 0.1 % (w/w) cholate or 0.5 % (w/w) ursodeoxycholate was added. This led to a near-complete replacement of the endogenous bile salt pool (mainly tauro-muricholate) by taurocholate or tauroursodeoxycholate respectively. The phospholipid secretion capacity was then determined by infusion of increasing amounts of tauroursodeoxycholate.

Cholate feeding resulted in a 55 % increase in maximal phospholipid secretion compared with that in mice on the control diet. Northern blotting revealed that cholate feeding increased *mdr2* Pgp mRNA levels by 42 %. Feeding with ursodeoxycholate did not influence the maximum rate of phospholipid output or the *mdr2* mRNA content. Female mice had a higher basal *mdr2* Pgp mRNA level than male mice, and this was also correlated with a higher phospholipid secretion capacity. This could be explained by the 4-fold higher basal cholate content in the bile of female compared with male mice. Our results suggest that the type of bile salts in the circulation influences the expression of the *mdr2* gene.

INTRODUCTION

Biliary lipid secretion serves several important functions. On the one hand, it provides a route by which cholesterol can leave the body and, on the other, it decreases the detergent action of the bile salts that are present in high concentrations in the biliary tree (reviewed in [1]). In model systems the presence of phospholipids and cholesterol greatly decreases the cytotoxic action of bile salts [2,3].

We have demonstrated that the *mdr2* P-glycoprotein (Pgp) in the canalicular membrane of the mouse plays an important role in the secretion of phospholipid into bile [4]. Mice with a homozygous disruption of the *mdr2* gene (*mdr2* $-/-$) showed a complete absence of phospholipid in bile, and their cholesterol secretion rate was greatly decreased. Mice with a heterozygous disruption (*mdr2* $+/-$) showed intermediate levels of phospholipid secretion; levels of cholesterol secretion were normal. These results strongly suggest that the activity of *mdr2* Pgp is a rate-controlling step in the secretion of phospholipids. Quantitative analysis of lipid secretion in the different mouse genotypes confirmed this contention [5]. We have previously hypothesized that *mdr2* Pgp transports phospholipids from the inner leaflet to the outer leaflet of the canalicular membrane, and that the interaction of bile salts with the outer leaflet of the membrane leads to the secretion of phospholipids into canalicular bile [4,6]. This hypothesis has been confirmed in several model systems [7–9].

Bile salts also represent a major regulatory factor in the secretion of biliary lipids. Several studies describe a hyperbolic relationship between bile salt secretion and phospholipid or cholesterol secretion [6,10]. An increase in bile salt output leads to an increase in phospholipid and cholesterol secretion until a certain plateau is reached [11,12]. Furthermore, it has become

generally accepted that the secretion of biliary phospholipids is positively related to the hydrophobicity of the bile salt species secreted (reviewed in [1,13]).

Because both *mdr2* Pgp and bile salts seem to be major factors regulating the secretion of biliary phospholipid, we investigated the influence of bile salt composition on *mdr2* Pgp expression and phospholipid secretion capacity. Our results show that an increased cholate content of the bile salt pool, which increases the hydrophobicity of the pool, leads to increased *mdr2* Pgp expression and a concomitant increase in the maximal phospholipid secretion capacity.

EXPERIMENTAL

Bile sampling

Mice of the FVB strain were bred in our own colony and were used for experiments at 2–5 months of age. Animals were anaesthetized by intraperitoneal injection of 1 mg/kg Hypnorm (fentanyl/fluanisone) and 10 mg/kg Diazepam. The abdomen was opened and, after distal ligation of the ductus choledochus, the gall bladder was cannulated. Bile sampling began directly after cannulation and continued for up to 90 min. In some experiments, mice then received an infusion of tauroursodeoxycholate (TUDC) (45 mM in PBS, pH 7.4) into the tail vein at the indicated rate. Bile samples were collected and immediately frozen at -20°C . Bile flow was determined gravimetrically, assuming a density of 1 g/ml for bile.

Diets and materials

To determine the relationship between bile salt and lipid secretion in more detail, bile samples were collected from male and female

Abbreviations used: GADPH, glyceraldehyde-3-phosphate dehydrogenase; Pgp, P-glycoprotein; TUDC, tauroursodeoxycholate; UDC, ursodeoxycholate.

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mice maintained on three different diets. Control mice were fed a commercial purified diet containing 20% (w/w) casein (K4068.02; Hope Farms, Woerden, The Netherlands). The experimental diets consisted of purified diet supplemented with sodium cholate (0.1%, w/w) or ursodeoxycholate (UDC) (0.5%, w/w). Sodium cholate was obtained from Merck (Darmstadt, Germany); tauroursodeoxycholic acid and ursodeoxycholic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Food and water were allowed *ad libitum*. After 21 days on the selected diet, bile was collected during consecutive time periods for 2 h after cannulation. This interruption of the enterohepatic circulation leads to a time-dependent depletion of the bile salt pool to a stable level of approx. 20% of the initial output within 60–90 min [6]. Phospholipid and cholesterol output levels showed a similar time-dependent decrease.

RNA isolation

For isolation of total RNA, livers were excised, immediately freeze-clamped and stored at -80°C until further use. Total RNA was isolated by an acidic guanidinium isothiocyanate/phenol/chloroform extraction procedure as described by Chomczynski and Sacchi [14]. Samples of $10\text{ }\mu\text{g}$ of RNA were loaded on a 0.8% agarose gel containing 7% formaldehyde. Standard ethidium bromide staining was used to check the integrity of the isolated RNA and to confirm the presence of equal amounts of RNA in each lane.

Northern blot analysis

Northern blotting was performed as described by Sambrook et al. [15], using Hybond N+ (Amersham). The blotted mRNA was hybridized with a radiolabelled specific mouse *mdr2* cDNA and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The GAPDH cDNA cross-hybridizes with mouse GAPDH RNA and serves as an internal control of the total amount of mRNA per lane. Two probes, kindly provided by Dr. A. H. Schinkel (Dutch Cancer Institute, Amsterdam, The Netherlands) [4] were used: (1) a 0.3 kb *mdr2* cDNA fragment obtained after *PstI*–*EcoRI* digestion of a construct of a *HincII*–*NaeI*-digested fragment of *mdr2* (positions 1875–2168) cloned into *SmaI*–*HincII*-digested pGEM-3Zf(–) as described by Smit et al. [4]; and (2) a 1.3 kb *PstI* fragment of rat GAPDH cDNA. The probes were labelled with ^{32}P using the Random Primers DNA labelling system (Gibco-BRL, Breda, The Netherlands). Hybridization was performed overnight at 65°C in 0.5 M sodium phosphate buffer, pH 7.5, containing 7% (w/v) SDS and 1 mM EDTA. After hybridization, the blots were washed three times with $0.2 \times \text{SSC}/0.1\%$ SDS at 65°C . Radioactivity on the blots was detected by autoradiography on Biomax MR film (Eastman Kodak, Rochester, NY, U.S.A.) followed by detection using a Series 400 Molecular Dynamics phosphorimager. Data collected by the phosphorimager were analysed quantitatively using the Imagequant computer program (Molecular Dynamics).

Bile analysis

Analysis of bile salt species was performed by HPLC using a modification of the method described by Ruben and Berge-Henegouwen [16]. Bile samples were diluted 1:200 (v/v) in eluent (60% methanol/40% 3 mM K_2HPO_4 , pH 3.75), and $20\text{ }\mu\text{l}$ portions of this sample were injected on a C18 Chromosphere column (5 μm pore size) (Chrompack International, Middelburg, The Netherlands) at a flow rate of $800\text{ }\mu\text{l}/\text{min}$. Peaks were detected using an UV detector at 205 nm.

Total phospholipid, cholesterol and bile salt concentrations were determined enzymically as described previously [6].

Data analysis

All data represent means $\pm$ S.D. from at least three animals in each experimental group. Differences between groups were analysed by performing an unpaired two-tailed Student's *t* test.

RESULTS

In order to investigate the effects of the chronic administration of bile salts on biliary lipid secretion and *mdr2* Pgp expression, mice were fed on a control diet to which either 0.1% (w/w) cholate or 0.5% (w/w) UDC was added. In male mice fed either cholate or UDC, bile flow, phospholipid secretion and cholesterol secretion were significantly increased compared with animals on the control diet (Table 1). This was most probably related to the increased bile salt output.

We performed HPLC analyses of bile samples collected during the first 10 min after cannulation to ascertain whether the endogenous bile salt pool had been replaced by the bile salt species supplied in the diet. This was indeed the case (Table 2): cholate feeding led to substantial replacement of endogenous bile salts by taurocholate (> 80% taurocholate), and the same was found for UDC feeding (> 85% TUDC).

Figure 1 shows phospholipid secretion as a function of bile salt secretion. In cholate-fed mice, the amount of phospholipid secreted at each rate of bile salt secretion was 2–3-fold higher than that in control mice (Figure 1A). In mice fed a UDC-containing diet (Figure 1B), the curve deviated slightly from the control curve but the effect was much less marked than for the cholate-supplemented diet.

Increased phospholipid secretion at the same rate of bile salt secretion could be caused by the greater phospholipid-extracting capacity of the more hydrophobic taurocholate compared with the more hydrophilic tauromuricholate and TUDC [3, 17], or by increased *mdr2* expression. In order to distinguish between these factors, we studied phospholipid secretion in control and cholate-fed mice after complete replacement of the endogenous bile salt pool by TUDC infusion. We achieved this by infusion of TUDC, at stepwise increased rates, after depletion of the endogenous bile

Table 1 Initial rates of secretion of the major bile constituents in FVB mice

Bile samples were obtained during the first 10 min after cannulation of the gall bladder. Bile samples from both male and female mice fed on control or bile-salt-enriched diets were analysed for bile salt, phospholipid and cholesterol content as described in the Experimental section. Results are means $\pm$ S.D. of the numbers of animals given in parentheses. Data from the male mice fed on the experimental diets were tested for significant differences compared with male mice on the control diet: * $P < 0.05$, ** $P < 0.001$. Data from the female mice were tested for significant differences compared with male mice under the same conditions: † $P < 0.05$, †† $P < 0.001$.

Diet	Bile flow ($\mu\text{l}/\text{min}$ per 100 g)	Rate of secretion (nmol/min per 100 g)		
		Bile salts	Phospholipid	Cholesterol
Male				
Control (7)	6.1 ± 1.7	173 ± 67	10.1 ± 3.4	1.2 ± 0.5
UDC (4)	$9.7 \pm 2.8^*$	$547 \pm 128^{**}$	$28.8 \pm 8.3^{**}$	$6.5 \pm 2.4^{**}$
Cholate (9)	$8.1 \pm 1.4^*$	$321 \pm 91^*$	$43.7 \pm 19.6^{**}$	$8.4 \pm 3.8^{**}$
Female				
Control (7)	5.1 ± 1.7	$393 \pm 81^{\dagger\dagger}$	$37.6 \pm 9.5^{\dagger\dagger}$	$2.3 \pm 1.1^{\dagger}$
UDC (8)	7.7 ± 2.1	701 ± 314	$67.6 \pm 24.7^{\dagger}$	7.3 ± 2.0
Cholate (4)	6.6 ± 0.4	289 ± 167	43.4 ± 17.7	5.9 ± 3.3

Table 2 Bile salt composition in the bile of FVB mice fed the control or experimental diets

The bile salt composition in initial bile samples from mice fed the control or bile-salt-enriched diets, collected during the first 10 min after cannulation, was determined by HPLC. The experiments and animals are the same as in Figure 1. For experimental details, see the Experimental section. Results are means $\pm$ S.D. from the numbers of animals given in parentheses. N.D., not detectable.

Diet	Bile salt species (mol% of total bile salts)				
	Muricholate	Taurocholate	TUDC	Taurochenodeoxycholate	Taurodeoxycholate
Male					
Control (3)	88.2 $\pm$ 0.9	11.8 $\pm$ 0.9	N.D.	N.D.	N.D.
UDC (4)	7.0 $\pm$ 1.7	N.D.	86.8 $\pm$ 5.4	6.2 $\pm$ 4.2	N.D.
Cholate (9)	14.5 $\pm$ 6.6	80.4 $\pm$ 8.3	N.D.	N.D.	6.3 $\pm$ 1.0
Female					
Control (3)	50.8 $\pm$ 0.7	47.4 $\pm$ 2.8	N.D.	1.7 $\pm$ 3.0	N.D.
UDC (3)	6.1 $\pm$ 1.2	N.D.	90.7 $\pm$ 1.1	4.2 $\pm$ 1.5	N.D.
Cholate (3)	14.7 $\pm$ 2.1	85.3 $\pm$ 2.1	N.D.	N.D.	N.D.

Figure 1 Effects of bile salt-supplemented diets on biliary phospholipid secretion capacity

Male FVB mice were fed on a control purified diet (+) or a purified diet supplemented with either (A) 0.1% (w/w) cholate (Δ) or (B) 0.5% (w/w) UDC (∇). Collection and analysis of bile samples was performed as described in the Experimental section. Data represent measurements in individual samples from six bile collection periods during the 90 min after cannulation of the gall bladder. BS, bile salts; PL, phospholipid.

salt pool. TUDC infusion rates exceeded the secretory maximum, and a plateau phase was reached (Figure 2).

HPLC analyses of bile demonstrated that TUDC infusion on top of a low baseline bile salt output led to a near-complete

Figure 2 Effect of a cholate-enriched diet on the phospholipid secretion capacity in male mice during intravenous infusion of TUDC

Mice were fed on either a control diet (●) or the same diet to which 0.1% (w/w) cholate was added (Δ). Subsequently the animals were depleted of their endogenous bile salt pool by diverting bile for 90 min through a cannula placed in the gall bladder. Following this depletion phase, TUDC was infused through the tail vein at a start rate of 600 nmol/min per 100 g. Every 30 min this rate was increased by 600 nmol/min per 100 g, until a final infusion rate of 2400 nmol/min per 100 g was reached. Phospholipid (PL) and bile salt (BS) output rates were determined during both the depletion and infusion phases.

(98%) replacement of endogenous bile salts by TUDC in both groups. Figure 2 shows that, during TUDC infusion, the rates of phospholipid output in male mice fed the cholate-enriched diet were still higher than those in mice fed on the control diet, despite the complete replacement of the biliary bile salts by TUDC. This experiment clearly demonstrated that the phospholipid secretion capacity was enhanced by chronic cholate feeding, irrespective of the type of bile salt secreted into the canaliculus. Under these conditions, differences in the ability of the dietary bile salts to extract phospholipid cannot explain the increase in phospholipid secretion; therefore another factor must be involved in regulating phospholipid output.

We hypothesized that the activity of mdr2 Pgp, the rate-controlling step in phospholipid secretion, was induced by chronic feeding of cholate. This hypothesis was supported by Northern blot analysis of the liver mRNA of male mice fed the different bile-salt-enriched diets (Figure 3). It is clear that cholate feeding led to an increase in the mdr2 mRNA level. The quantitative data obtained after scanning the Phosphorimages of

Figure 3 Representative Northern blot of hepatic mRNA from male mice fed purified diets containing cholate or UDC

The blotted mRNA was hybridized with a radiolabelled specific mouse *mdr2* cDNA and a rat GAPDH cDNA as described in the Experimental section. The GAPDH probe cross-hybridizes with mouse RNA and serves as an internal control of the total amount of mRNA per lane. This blot shows two separately isolated RNA samples from each experimental group. Lanes 1 and 2, control mice; lanes 3 and 4, mice fed 0.5% (w/w) UDC; lanes 5 and 6, mice fed 0.1% (w/w) cholate.

Table 3 Specific hybridization of *mdr2* mRNA

Quantitative data are shown of *mdr2* RNA on Northern blots of liver RNA derived from male or female mice fed either purified control diet or a purified diet enriched with 0.1% (w/w) cholate or 0.5% (w/w) UDC. Individual ratios of *mdr2*/GAPDH signals were determined, and the mean value of this ratio in each group was normalized to the ratio found in the group fed the control diet. The data are given as a percentage of the control, and are means $\pm$ S.D. from four animals in each group. Significant difference compared with control: * $P < 0.05$.

Mice	Diet	<i>mdr2</i> mRNA/GAPDH mRNA (% of control)
Male	Control	100 $\pm$ 20
	UDC	97 $\pm$ 14
	Cholate	142 $\pm$ 25*
Female	Control	100 $\pm$ 17
	UDC	79 $\pm$ 16
	Cholate	111 $\pm$ 20

the blots are presented in Table 3. Dietary cholate increased the amount of mRNA hybridizing with the *mdr2*-specific probe by 42% compared with the control diet. In contrast, mice fed a UDC-enriched diet showed no change in *mdr2* mRNA level. Northern blot analysis of the liver mRNA of mice fed a cholate-rich diet for different time periods revealed that this up-regulation of *mdr2* Pgp mRNA had reached a maximum after 1 day of cholate feeding (results not shown).

An unexpected observation of our study was that phospholipid output in female mice on a control diet was significantly greater than that in males (Table 1). This greater phospholipid output was accompanied by significantly greater bile salt output. If the greater phospholipid output in female mice was completely dependent on the observed increased bile salt output, the hyperbolic relationship between phospholipid output and bile salt output should be the same for both male and female mice. Figure 4 (top panel) shows, however, that in mice fed the control

Figure 4 Effects of bile salt-supplemented diets on phospholipid secretion capacity

Male (●) or female (○) FVB mice were fed on a purified control diet alone (top) or supplemented with either 0.5% (w/w) UDC (middle) or 0.1% (w/w) cholate (bottom). Collection and analysis of bile samples was performed as described in the Experimental section. Data represent measurements in individual samples from six bile collection periods during the 90 min after cannulation of the gall bladder.

diet phospholipid output rates in males were 30–50% of those in females at all rates of bile salt output. This clearly demonstrates that, when fed on a control diet, female mice have an intrinsically greater phospholipid secretion capacity than males, and that the higher phospholipid output levels in females are not caused by the elevated bile salt output alone. Feeding a UDC-enriched diet resulted in only a slightly increased phospholipid output, and again phospholipid output was greater in females (Figure 4,

Figure 5 Northern blot of hepatic mRNA from female mice fed purified diets containing cholate or UDC

The conditions were identical to those described for Figure 3. This blot shows RNA samples from each experimental group. Lane 1, controls; lane 2, mice fed 0.5% (w/w) UDC; lane 3, mice fed 0.1% (w/w) cholate.

middle panel). Figure 4 (bottom panel) shows that feeding mice on a diet containing the more hydrophobic bile salt, cholate, induced an increase in phospholipid secretion in male but not female mice. Thus, with cholate feeding, the phospholipid output levels for males and females at a given bile salt output became similar. These results imply a gender difference in phospholipid output rates in FVB mice.

To ascertain whether the dissimilar rates of phospholipid output between the sexes were due to differences in bile salt composition, we performed HPLC analyses of bile samples collected during the first 10 min after cannulation from both males and females fed the different diets. On a control diet, the bile of female mice contained a 4-fold higher proportion of taurocholate than that of male mice (47% and 12% respectively; Table 2). Cholate feeding led to a marked replacement of endogenous bile salts by taurocholate (> 80% taurocholate) in both male and female mice, and the same effect was found for UDC feeding (> 85% TUDC). Thus females have an intrinsically higher bile salt output, and this bile contains more hydrophobic bile salts under control conditions than that from corresponding males, which could explain the higher phospholipid output.

Northern blot analysis of liver material from female mice showed that, on the control diet, the basal level of *mdr2*-specific mRNA in female mice was 2.2 times higher than in males. In females, *mdr2* expression was not further induced by taurocholate feeding (Figure 5). Our results reveal a close correlation between *mdr2* mRNA levels and phospholipid secretion capacity, suggesting that *mdr2* Pgp levels are up-regulated by hydrophobic bile salts.

The data in Table 1 show that induction of phospholipid secretion was accompanied by an increase in cholesterol secretion, as expected [1,13]. Figure 6 shows that, during TUDC infusion (as described in Figure 2), cholesterol and phospholipid output remained coupled in mice on both the control and cholate-enriched diets.

DISCUSSION

Regulation of *mdr2* Pgp by bile salts

The results presented here show that feeding male mice on a cholate-rich diet increases the expression of *mdr2* Pgp, either

Figure 6 Relationship between phospholipid and cholesterol secretion in male mice fed a control diet or a diet supplemented with 0.1% (w/w) cholate

After a depletion phase, mice received TUDC infusions at a stepwise increased rate, as described for Figure 2. ∇ , Mice fed a control diet; $\square$, cholate-fed mice.

directly or indirectly. We have shown previously that *mdr2* Pgp activity is a rate-controlling step in the secretion of phospholipids into bile [5]. Therefore phospholipid secretion reflects the functional level of canalicular-localized *mdr2* Pgp. Indeed, in the present study, increased *mdr2* Pgp expression was accompanied by an increase in the maximal capacity of phospholipid secretion into bile.

It is known that the extent of biliary phospholipid secretion depends on the hydrophobicity of the secreted bile salts; hydrophobic bile salts such as taurodeoxycholate and taurochenodeoxycholate induce greater phospholipid secretion per mol of bile salt than hydrophilic bile salts such as TUDC. Taurocholate has an intermediate hydrophobicity. The increased phospholipid secretion capacity induced by cholate feeding was not only caused by this phenomenon; when both cholate-fed and control mice were infused with TUDC, leading to a near-complete replacement of endogenous bile salts by TUDC, cholate-fed mice still had a significantly greater phospholipid secretion capacity (Figure 2).

Elegant studies by Haussinger et al. [18] have shown that TUDC can induce recruitment of pericanalicular-localized vesicles containing canalicular transport proteins. Recruitment of these vesicles leads to an acute induction of transport capacity in the canalicular membrane, and probably also occurs in our experimental setting. Both control and cholate-fed mice received the same TUDC challenge and, within the experimental time frame, this will lead to maximal induction of transport capacity. Therefore the observed difference in phospholipid output between cholate-fed and control mice is solely dependent on the dietary regime. As a plateau is reached in TUDC excretion, the observed phospholipid secretion rates at the saturating level can be related to the maximal functional expression of *mdr2* Pgp.

Thus we have shown an induction of *mdr2* Pgp at the functional as well as the mRNA level. The selective induction of *mdr2* Pgp makes sense. One of the important functions of biliary lipid secretion is to counteract the detergent action of bile salts in the biliary tree. This is clearly demonstrated by the liver pathology of *mdr2* (−/−) knockout mice. In the absence of biliary lipid secretion, these animals suffer from hepatocyte necrosis, ductular

proliferation, portal inflammation and finally primary hepatocellular carcinoma [4,19].

In the light of the greater cytotoxicity of hydrophobic bile salts, it is quite understandable that the expression of *mdr2* Pgp is regulated by bile salts and that this regulation depends on hydrophobicity. When bile salts of greater hydrophobicity are secreted into the canaliculus, more lipid is required to inactivate the lytic action of these bile salts.

A regulatory function for bile salts has been described previously for key enzymes involved in bile salt synthesis. Bile salts differentially regulate the activity of 7α -hydroxylase and sterol 27-hydroxylase by altering the transcriptional activity of these genes [20–24]. The potency of suppression of these enzymes by bile salts was shown to be dependent on hydrophobicity and linked to the bile acid structure [25].

Besides this direct effect on *mdr2* Pgp gene expression, bile salts might also regulate phospholipid secretion into bile by affecting post-translational processing of the *mdr2* Pgp. Bile salts are known to induce the protein kinase C-mediated phosphorylation of a range of different endogenous substrates [26–30], and it was reported recently that repression of cholesterol 7α -hydroxylase transcription by bile salts is mediated through protein kinase C [31]. Furthermore, protein kinase C-mediated phosphorylation of the human multidrug-resistance Pgp has been shown to modulate drug transport and cell volume-activated chloride channels [32–36]. Because of the close relationship between the multidrug-resistance Pgp and *mdr2* Pgp, it seems likely that the activity of *mdr2* Pgp may be regulated by its protein kinase C-mediated phosphorylation. Our results show, however, that regulation also occurs, at least partially, at the mRNA level.

Another hypothetical mechanism for the activation of *mdr2* expression is by a signal derived from a perturbation of the lipid pool rather than a direct effect of bile salts. Such a signal could be derived, for example, from the extent of saturation of phosphatidylcholine-transfer protein with phosphatidylcholine. This transfer protein is thought to be involved in the intracellular supply of phosphatidylcholine to the canalicular membrane [37].

The proposed regulation of phospholipid secretion by increasing *mdr2* Pgp mRNA levels is in agreement with the recently published findings of Chianale et al. [38]. They described the induction of *mdr2* Pgp gene expression and phospholipid secretion in mice fed on fibrate-rich diets [38].

Effects of gender on *mdr2* Pgp expression

It was observed that the basal phospholipid secretion capacity in male and female FVB mice differed significantly. Female mice fed on the control diet had a higher phospholipid secretion capacity than comparable males, and this was accompanied by a higher *mdr2* mRNA level. The increased basal *mdr2* expression in female mice may be related to the difference in bile salt composition compared with that in males. The predominant bile salt species in both sexes were taurocholate and muricholate, but on the control diet the bile of female mice contained a 4-fold greater proportion of the more hydrophobic taurocholate than the bile of males. Feeding mice on the cholate-rich diet caused the endogenous bile salt pools of both male and female mice to be replaced to a similar extent, so that in both sexes the bile consisted of more than 80% taurocholate. Under this condition the relationships between phospholipid output and bile salt output in male and female FVB mice became very similar. The conclusion from these data must be that *mdr2* Pgp is already induced in female mice, due to the higher taurocholate content in

their bile. Taurocholate feeding induces *mdr2* expression in males to the same maximum level that is reached in females. These data suggest that differences in bile salt composition could be the sole factor responsible for the observed difference in biliary phospholipid output rates between the sexes. If this was the only cause of the gender differences in *mdr2* expression, one would expect that, under all conditions resulting in comparable biliary bile salt compositions, including UDC feeding, no differences in *mdr2* Pgp mRNA levels between male and female mice would occur. Although UDC feeding led to a small decrease in the *mdr2* Pgp mRNA level in female mice, this was not significant, and the mRNA level in female mice remained higher than in male mice fed on the same diet. At this time we have no explanation for this difference, but it may involve a gender-specific factor. An obvious possibility is the marked structural relationship between bile salts and steroid hormones. It is plausible that oestrogens regulate *mdr2* Pgp expression by a mechanism that is similar to that of bile salts. Regulation of expression of the *mdr2* Pgp gene could be similar to that of the 7α -hydroxylase gene, which involves binding of bile salts to a bile-acid-responsive-element binding protein. Our data are in agreement with the greater gender-dependent expression of the *mdr2* homologue, Pgp3, in female rats described by Furuya et al. [39]. In that study female rats consistently expressed higher amounts of Pgp3 in liver than male rats. Further study of the mechanism of bile-salt-induced up-regulation of *mdr2* Pgp should eventually lead to an explanation for the observed gender-dependent differences in biliary phospholipid secretion.

Effects on cholesterol secretion

Bile salts also drive cholesterol secretion, and many groups have observed a strict coupling between phospholipid and cholesterol secretion. This was interpreted to suggest that cholesterol and phospholipid are secreted as intact units [1]. In line with this, we found that cholesterol secretion is deficient in the *mdr2* (–/–) knockout mouse. It became also clear, however, that this relationship can be, at least partly, uncoupled: in heterozygous *mdr2* (+/–) knockout mice, phospholipid secretion is decreased, but cholesterol secretion is normal. Furthermore, in *mdr2* (–/–) mice, cholesterol secretion can be increased by infusion of more hydrophobic bile salts such as taurocholate [4] or taurodeoxycholate. However, with hydrophilic bile salts such as TUDC, coupling between cholesterol and phospholipid secretion seems to remain nearly complete. Infusion of this bile salt into *mdr2* (–/–) mice results in very low cholesterol secretion, whereas infusion into *mdr2* (+/–) mice elicits cholesterol secretion that is intermediate between that of *mdr2* (–/–) mice and controls, as is observed for phospholipids [40]. In line with this is the observation (Figure 6) that the relationship between cholesterol and phospholipid secretion in male mice with induced *mdr2* Pgp expression increases in the same proportion when biliary lipid secretion is driven by TUDC infusion.

In summary, our data suggest that the level of *mdr2* gene expression is regulated by the type of bile salt that is present in the circulation. This could be demonstrated both at the mRNA level and at the functional level of biliary lipid secretion. In addition, *mdr2* gene expression may also be regulated by a gender-specific factor, the nature of which is not known at present.

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