Regulation of biliary lipid secretion
Frijters, C.M.G.

Citation for published version (APA):
Frijters, C. M. G. (2000). Regulation of biliary lipid secretion

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 3

DOWN-REGULATION OF MDR2 P-GLYCOPROTEIN EXPRESSION DURING CHRONIC BILE DIVERSION IN THE RAT

Charles M.G. Frijters, Piter J. Bosma, Raoul J.J.M. Frijters
Regulation of Mdr2 expression during bile diversion

ABSTRACT

Biliary lipid secretion is dependent on both bile salt secretion and the phosphatidylcholine (PC) translocating activity of class III type P-glycoprotein (P-gp), the rate-controlling step in biliary phospholipid secretion. Biliary PC secretion is linearly related to the expression level of Mdr2 P-gp. There is a hyperbolic relation between bile salt and biliary PC secretion and PC secretion increases with hydrophobicity of the bile salt species. Previous studies with bile salt fed mice showed an increase in Mdr2 P-gp expression if the bile salt pool was enlarged by feeding cholate (1). In the present study we investigated the regulatory effect of bile salts on class III type P-gp expression by chronic bile diversion in the rat. Reducing the bile salt pool by bile diversion and subsequent restoration of the enterohepatic cycle of bile salts allowed us to study the regulatory effect on biliary lipid secretion over a broad range of bile salt concentrations. Three experimental groups were formed: 8 days bile diversion, 4 days bile diversion followed by 4 days of restoration of the enterohepatic circulation (EHC) and 8 days bile diversion combined with duodenal infusion of TC during the last 24 or 48 hours. At day 0, 4 and 8 bile was collected during two hours while the rats received a stepwise increased tauroursodeoxycholate infusion. At day 8 rats were sacrificed and total liver mRNA was prepared for quantitative analysis of Mdr2 mRNA by aid of real-time detection of the PCR reaction (LightCycler). Four days of bile diversion resulted in a strong decrease in maximal PL secretion capacity (PLsecMax) to 42% and maximal cholesterol secretion capacity (CHOLsecMAX) was reduced to 32% of control. Prolongation of the bile diversion to 8 days resulted in a further decrease in PLsecMax to 16% whereas CHOLsecMAX remained at the low level of 32% of control. Restoration of the EHC after four days of bile diversion restored PL and cholesterol output capacity at day 8 to 115% and 105% of control value respectively. To confirm that alteration of the bile salt pool was responsible for this regulatory event, taurocholic acid was infused through the duodenal catheter during bile depletion. Twenty-four hours of TC infusion led to a modest restoration (30%) of PLsecMax while 48 h TC infusion further restored phospholipid secretion to 77% and 137% of control levels in two separate animals.

The Mdr2 mRNA levels reflected the maximal secretion levels for biliary lipids; Mdr2 (Pgp3) expression was strongly reduced in animals depleted for eight days and returned to normal values after restoration of the EHC. Duodenal TC infusion partially restored
Chapter III

*Mdr2 (Pgp3)* expression and PLsecMax when given during the last 24 hours, and increased further when TC was infused during the last 48 hours of bile diversion. Our results show that interruption of the enterohepatic circulation of bile components leads to strong down-regulation of *Mdr2* and can be restored by supplying bile salts. This adds evidence to our hypothesis that bile salt levels regulate *Mdr2/Pgp3* expression.
INTRODUCTION

One of the major processes in bile formation is the secretion of bile salts into the canalicular lumen forming primary bile. During passage through the biliary tree primary bile becomes more concentrated and in the gallbladder bile salt concentration reaches maximum. Biliary phospholipid and cholesterol secretion is driven by the presence of bile salts in the canalicular lumen. There is a hyperbolic relation between bile salt and biliary PC secretion and PC secretion increases with hydrophobicity of the bile salt species. Biliary phospholipid secretion is fully controlled by Mdr2 P-glycoprotein in the canalicular membrane. In homozygous \textit{Mdr2}-/- mice no phospholipid secretion was observed. The ATP-dependent PC-translocating activities of murine Mdr2 P-gp and its human homologue MDR3 P-gp is well established and together with their orthologues in other species (for instance rat Mdr2 P-gp (PGP3)) these proteins are classified as class III type P-glycoproteins.

It is thought that biliary phospholipid secretion serves to mitigate the detergent action of bile salts and thereby protect the membranes lining the hepatic biliary tract. The detergent capacities of bile salts increase with increasing overall hydrophobicity of the molecule. Female mice of the FVB strain have a more hydrophobic bile salt pool as compared to males and also do have a higher maximal PC secretion rate. Previous experiments in which the bile salt pool of male mice was made more hydrophobic by feeding cholate showed that the maximal PC secretion capacity increased with concomitant elevation of the \textit{Mdr2} mRNA expression (2,3). These results suggested that bile salts counteract their potential destructive effects on membranes by inducing the PC translocation capacities in the canalicular membrane. To substantiate this hypothesis we investigated the influence of reducing the bile salt pool in rats by chronic bile salt diversion. Catheters were placed in common bile duct and duodenum of male Wistar rats. The bile duct catheter was connected to tubing with a swivel joint to start permanent bile diversion. Restoration of the enterohepatic circulation (EHC) was achieved by interconnection of both catheters. Maximal secretion capacity of cholesterol and phospholipid was assessed by infusion of tauroursodeoxycholate (TUDC) at stepwise increased rates (550, 1100, 1650 and 2200 nmol/minx100g body weight). Mdr2 mRNA levels were determined by quantitative PCR using the LightCycler detection method.
These expression levels were normalised to total mRNA expression as determined by 18S ribosomal RNA quantification.

MATERIALS AND METHODS

Animals and surgery
Male Wistar rats weighing 200-250 grams were obtained from Harlan-CPB (Zeist, The Netherlands). Three days before surgery rats were housed individually in the same cages as used for the bile diversion period following the surgery. The animals had free access to water and food. Rat surgery was performed under anaesthesia (hypnorn (fentanyl/fluanisone, 1 ml kg, Janssen Pharmaceuticals, Beerse, Belgium and Midazolam. The rats were equipped with two catheters placed in the common bile duct and in the duodenum respectively. (4). Both catheters were tunneled subcutaneously to the head were they were fixed to the skull. Interconnecting the catheters with a polyethylene loop restored the enterohepatic circulation.

Experimental set-up
At the start of the experiments (day 0) rats were anaesthetised using oxygen mixed with isoflurane 0.6% (Forene®, Abbott Laboratories Ltd. Queensborough, U.K.) at a flow rate of 1 l/min. To deplete the endogenous bile salt pool, polyethylene tubing was connected to the biliary catheter and bile was diverted for a 2.5-hour period. At selected time points samples of ten minutes each were taken. Following this depletion phase an infusion needle was placed into the tail vein and TUDC was infused at stepwise increasing rates. The TUDC infusion rates during this standard bile infusion protocol were 550, 1100, 1650 and 2200 nmol/min per 100 gram body weight, respectively, and the rate was changed every 30 minutes. Bile was collected during this 2 hour period in fractions of 10 minutes each and frozen immediately until further analysis. Regardless of the further experimental set-up all rats received this standard TUDC infusion protocol also at day 4 (96 hours after start experiment) and day 8 (192 hours after start of the experiment). At the end of the TUDC infusion the rats were placed in their cages and the long term bile diversion started (or continued) by interconnecting the bile canula to a long polyethylene tube. Insertion of a swivel joint in this tube allowed the animals to move freely in their
Regulation of Mdr2 expression during bile diversion

cage. The animals were fed ad libitum and had free access to water and to a glucose/saline solution (glucose (5% (\( \text{w/v} \))) and sodium chloride (0.45% (\( \text{w/v} \))) to compensate the loss of salts by diversion. Food and water intake and body weight were determined on a daily base to ensure a good condition of the animal.

From all animals bile was diverted during 4 days (96 hours), then the rats were randomly placed in four different experimental groups. In the first group (n=3) bile diversion was prolonged to day 8 (192 hours). In the second group (n=3) the enterohepatic circulation was restored for 4 days (96-192 hours). The animals in the third and fourth group (n=2 each) had a bile diversion of 8 days total (as in the first group) but additionally they received a duodenal TC infusion (30 mM TC in water at 0.33 ml/hour*100 grams body weight) during the last 24 or 48 hours of bile diversion, respectively.

After the last TUDC infusion protocol at day 8 all rats were sacrificed and liver tissue was frozen immediately in liquid nitrogen and stored at -80°C. RNA was isolated from these samples using the guanidinium isothiocyanate method and CsCl2 gradient purification (5). cDNA synthesis was carried out with 1 \( \mu \)g of total RNA using and random hexamer primers and Superscript Reverse Transcriptase II (Life Technologies, Gaithersburg, MD, USA), at 47°C for 45 minutes. Subsequently RNase H incubation was performed for 15 minutes at 37°C. Upon ethanol precipitation cDNA was redissolved in 200 \( \mu \)l of sterile water.

The level of Mdr2 mRNA expression was determined using real-time fluorescence PCR with the LightCycler (Roche, Mannheim, Germany). The PCR was performed with 2\( \mu \)l of cDNA using a ready-to-use reaction mix for LightCycler based PCR (LightCycler-DNA master SYBR Green I (Roche Diagnostics, Mannheim, Germany), according to the manufacturers protocols at an annealing temperature of 56°C. For Mdr2 analysis a primer set was used that resulted in a 385 bp PCR-fragment; forward primer CAGACATCAGGAAGCCACAGG (nt. 1913-1928) and reverse primer CCAGCGAGAACATGTTAC (nt. 2280-2298). A second primer set for 18S ribosomal RNA was used separately to determine the total mRNA expression in each individual sample. The forward 18S ribosomal RNA primer was TGATCCTGCCAGTAGCATATGC (nt. 9-30) and the reverse primer was CTACCATCGAAAGTTGATAGGGC (nt. 358-380) and this set resulted in a PCR-fragment of 371 bp. (6). Both primer sets were used at a final concentration of 0.5 \( \mu \)g. LightCycler PCR using samples from which reverse
transcriptase was omitted only showed primer dimer formation confirming the specificity of PCR-reaction. Performing melting curve analysis that allows discrimination between primer dimers and specific product assessed the specificity of the amplified PCR product. The amount of Mdr2 in the PCR reaction was determined using a standard PCR curve obtained by serial dilution of rat Mdr2 DNA template. For this, cDNA of normal Wistar rats was prepared as described above. A 600 bp fragment of Mdr2 cDNA (nt. 1800-2600) was amplified by PCR using the following primer-set: CCTCCTGCTGGACGAGGCC (nt. 1682-1709) and reverse primer CCTAGGCCCAAGAAGACC (nt.2297-2314). This PCR fragment (632 bp) was cloned into the PCR-II-1 vector (Invitrogen) and checked by sequence analysis. A similar approach was used to obtain 18S ribosomal RNA DNA template, with the same primer set as used for the LightCycler PCR mentioned above. Because several enzymatic steps are involved in this quantitative process some redundancy was built in to assure reliable estimates of mRNA levels. From each liver we performed two separate mRNA isolations and subsequently two separate rounds of cDNA synthesis were applied to each sample. Finally these cDNA factions were all subjected twice to LightCycler PCR.

Bile samples were analysed for PC, bile salt (BS) and cholesterol content using enzymatic assays as described before (7).
Bile flow during the 10-minute sampling period was determined gravimetrically, using pre-weighted sample cups, assuming a density of 1 g/ml.
RESULTS

To test our hypothesis that bile salts regulate Mdr2/Pgp3 mediated hepatobiliary PL secretion we experimentally decreased the bile salt pool in male Wistar rats by prolonged interruption of the enterohepatic circulation. Besides the Mdr2 mediated PL translocation, the presence of biliary bile salts is a prerequisite for lipid secretion (1,8). Therefore, regulatory effects on Mdr2 activity at the functional level were assessed by bile salt (TUDC) infusion into the tail vein which ensured sufficient hepatobiliary secretion of bile salt. Using a standard infusion protocol, in which the bile salt infusion rate was increased in a stepwise manner, the maximal PL output capacity at any moment during manipulation of the enterohepatic circulation could be assessed.

It is known from literature that a curvilinear relation exists between biliary PC secretion and biliary bile salt secretion (9). Alterations in factors influencing the PC secretory mechanism, i.e. changes in the level of Mdr2 expression, would lead to differences in the curves describing the relationship between biliary bile salts and PC. The relationship between BS and PC secretion is captured in a mathematical model formulated by Mazer and Carey (10). This model fits their and our experimental data on biliary lipid secretion in the rat nicely.

They deduced the following equation: \( \text{PCsec} = \frac{\text{PCmax} \times \text{BSsec}}{\beta / k + \text{BSsec}} \) (1)

Where \( \text{PCsec} \) is the rate of biliary PC secretion, \( \text{PCmax} \) is the maximal rate of hepatocellular phospholipid synthesis, \( \text{BSsec} \) is the rate of biliary BS secretion, \( \beta \) represents feedback inhibition of phospholipid synthesis by hepatic phospholipid content and \( k \) describes the coupling constant between \( \text{BSsec} \) and \( \text{PCsec} \). At the time this relation was formulated it was not known yet that the PC translocating activity of Mdr2 P-gp (PGP3) plays a crucial role in biliary phospholipid secretion. The maximal phospholipid output in their original formula therefore was determined by the maximal rate of synthesis. Now it is known that Mdr2 P-gp activity is the major regulatory factor in PC secretion (1,11). Therefore the \( \text{PCmax} \) in the formula can be substituted with PLsecMax, the maximal PC secretory rate (12). At high bile salt fluxes the constant \( \beta / k \) in the denominator is of minor importance and PLsec reaches PLsecMax. From studies with mice heterozygous for Mdr2 gene disruption and mice bearing a human MDR3 transgene, it has become clear that PLsecMax is linearly related to P-glycoprotein expression (13) and therefore can be viewed as a measure of Mdr2/Pgp3 expression in our experiments.
Bile diversion.

In accordance with previous studies on bile diverted rats, bile salt output steadily declined during bile diversion to 15% of the initial values. No difference was observed in

![Graph showing bile salt output over time](image)

**Figure 1.** During the first 24 hours of bile diversion bile salt output reduces to approx. 15% of initial values, indicating that the bile salt pool is depleted. During chronic bile diversion BS output remains low. Upon restoration of the enterohepatic circulation (EHC) there is a very strong increase in bile salt output to 2.5-fold higher levels as compared to t=0; this is due to the induction of the enzymes in the biosynthetic pathway of bile salts during bile diversion. Data indicate mean ± stand. Dev. (initial depletion phase n=6; continuous depleted n=3; restored EHC n=3).

the (low) steady state secretion rates for endogenous bile salts between four days and eight days bile diverted rats (Fig. 1). It is known that when the bile salt pool is fully depleted bile salt secretion rate equals its synthetic capacity. Furthermore it has been shown that both specific activity and mRNA levels of cholesterol 7α-hydroxylase, one of the key enzymes involved in bile salt synthesis are upregulated during prolonged bile diversion (14). In our experiment in which rat bile was depleted for four days and subsequently the enterohepatic circulation was restored, the steady state bile salt secretion rates became higher than in controls. This overshoot was most likely due to the increased expression of key enzymes in bile salt synthesis.
Biliary lipid secretion during manipulation of the EHC.
The PC/BS relationship reflecting the "normal" situation in rats receiving the standard TUDC infusion protocol was determined at the start of the experiment. A 2.5 hour bile depletion period prior the bile salt infusion was necessary to prevent the contribution of endogenous bile salts (mainly taurocholate and tauromuricholate) on TUDC evoked PL secretion. HPLC analysis of bile samples taken during TUDC infusion showed that even at moderate infusion rates (1100 nmol/min*100 gram body weight) less than 1.5% of the bile salts originated from the endogenous pool. The maximal TUDC secretion rate that can be reached using the standard infusion protocol is ±1850 nmol/min*100 grams body weight. We observed that during TUDC infusion the bile salt output value increased with each consecutive timepoint and it never reached a plateau (data not shown). Therefore it is unlikely that the maximal TUDC secretion rate we observed is the absolute maximal secretion value and no experimental cholestasis was induced.

As expected a curvilinear relation between PC and BS secretion was observed in agreement with what has been reported for rats and other species before (Fig 2A). Fitting the data with equation 1, PLsecMax was determined to be 75 nmol/min*100 gram body weight. After four days of bile diversion the PL secretion was determined again by the same TUDC infusion protocol. The PC output was drastically decreased (Fig 2B) at any given bile salt output. The PLsecMax only reached 42% of the control value at day 0 suggesting a decreased expression of Mdr2. At this point a mild reduction in TUDC (to 84% of control) secretion capacity was observed in some of the rats.

Prolonging the bile depletion period to 192 hours showed (Fig 2C) that the PC secretion capacity at any given bile salt output was even further decreased. The PLsecMax had decreased to 16% of the value obtained at the start of the diversion period. The maximal level of TUDC secretion that was reached at this point was about 84% of the control situation, like day 4. To ascertain that the effects on PC secretion could be contributed to the diversion of bile alone, the enterohepatic circulation of 4 rats was restored by shunting the biliary and duodenal canula after 96 hours of bile diversion until the end of the experiment at 192 hours. In these animals the PC output capacity was considerably increased compared to 4 days or 8 days of bile diversion (Fig 2D). The maximal output capacity reached 115% of the control values showing that the inhibiting effect of bile salt diversion on PC secretion capacity could be reversed totally by restoration of the enterohepatic cycle. Although the data in figure 2 indicate that the PC output in some
Figure 2. Phospolipid output capacity under different experimental conditions. A standard infusion of TUDC (550, 1100, 1650, 2250 nmol/min*100 gram bw.) was used. (A) PLsec in controls; (B) and (C) PLsec during 4 or 8 days of bile diversion respectively; (D) PLsec in animals of which the bile was diverted for 4 days followed restoration of the enterohepatic cycle of biliary components. The data were fit using the formula describing PLsec as discussed in the text.

animals is much higher than in others, analysis of the data from individual rats showed that the PLsecMax values of every animal normalised, upon restoration of the EHC, to the values determined for each individual at the start of the experiment. Restoration of the normal enterohepatic cycling led also to increased maximal bile salt secretion capacity compared to four or eight days of bile diversion; these values now reached 105% of control values.

The biliary secretion of cholesterol is not exclusively dependent on mdr2 P-gp activity. It has been suggested that cholesterol is partly secreted in association with PC, and that this mechanism depends on Mdr2 P-gp mediated PC translocation and the formation of
vesicles on the canalicular outer membrane leaflet. These vesicles then are shed from the membrane in the presence of bile salts. On the other hand in the absence of mdr2 P-gp activity still a fraction of cholesterol is secreted into bile upon infusion of bile salts. This secretion most probably is caused by direct extraction of cholesterol in bile salt micelles present in the canalicular lumen, a mechanism which has been suggested already for many years (15-19).

Using the method of best fit on all available data a curvilinear relation was found that included both mechanistic aspects of cholesterol secretion: \[ \text{CHOLsec} = A_0 \cdot B \text{sec} + A_1 \cdot P \text{sec} \] (2). In this equation \( A_0 \) en \( A_1 \) are constants and the first term could be regarded as "extraction-term" and the second as "vesiculation-term". This empirical equation was further used to fit the data of cholesterol secretion. The secretion of biliary cholesterol during the different stages of interruption and restoration of the enterohepatic circulation showed roughly a similar pattern as phospholipid secretion. During bile depletion the maximal cholesterol output decreased to 32% of control at 4 days. Prolonging the depletion with another 96 hours did not lead to a further decrease in CHOLsecMAX (32%). Restoration of the EHC during the last 96 hours of the experiment resulted in near normal values of cholesterol secretion capacities (105%), as was the case in phospholipid secretion. Also in this situation cholesterol output values from individual rats were not within narrow ranges, but cholesterol output capacities for each individual reached the values found in these rats at the start of the experiment (day 0).

**Effects of duodenal bile salt infusion.**

So far the data can not exclude a role for other biliary constituents than bile salts in regulating the expression of *Mdr2* and concomitantly biliary lipid secretion. To address this point into more detail bile salt (TC) was infused through the duodenal catheter during the last 24 or 48 hours of a total of 192 hrs of bile depletion. Taurocholic acid was chosen because it is the main bile salt found in normal rat bile. The TC infusion rate was 0.33 ml/hour*100 grams and with an concentration of 30 mM that results in 720 \( \mu \text{mol/day} \). This is roughly 1.5 times the amount of bile salt secreted in the normal situation in the rat (4,20) and is in the same order used in previous similar experiments by Pandak et al. (14,21). Functional assessment of biliary lipid secretion with the standard TUDC infusion protocol was not influenced by the duodenal TC infusion because it was preceded by a standard 90 minutes depletion phase. HPLC analysis of the
bile samples showed that the maximal molar contamination of TUDC with TC was less than 0.5%. In both rats receiving a TC infusion for the last 24 hours a slight increase in maximal PC secretion capacity could be detected; 30% of control as opposed to 16% in 8 days diverted rats (figure 3A). In rats that received duodenal TC during a period of 48 hours the maximal PC secretion capacity was more strongly increased although these increases were different in both rats. One of the rats (rat B) reached 77% of control value and in the second one (rat A) secretion rates above normal were reached (137%) as shown in figure 3B. Due to the small group it is not known exactly what levels can be reached but this is a strong indication that normalising the bile salt pool leads to reestablishment of biliary phospholipid secretion.

The cholesterol secretion curves for rat receiving duodenal TC infusion are shown in

![Figure 3: Phospholipid secretion curves for bile diverted rats receiving duodenal TC infusion. Fig. 3A shows the phospholipid secretion in 8 days bile diverted rats that received a duodenal TC infusion during the last 24 hours (circles). The curves obtained from fitting the data with the formula describing PLsec, as described in the text, are shown for these rats (solid line). The dotted line represents the curve describing PLsec in control rats (day 0). Fig 3B shows similar curves for 8 days bile diverted rats that received the duodenal TC infusion during the last 48 hours. Here the curves are calculated for both animals in this group; rat A and rat B (see text). The dotted line represents the curve describing PLsec in control rats (day 0).](image-url)
Regulation of Mdr2 expression during bile diversion

Figure 4. In contrast to PL secretion, the capacity of cholesterol secretion was not increased in rats receiving the 24 hours TC infusion; in both animals it remained low at 32% of control (fig. 4A). Duodenal infusion of TC during a 48 hours period showed that in rat A CHOLsecMAX increased to 65% of controls while rat B remained at 32%, the same level as found for eight days bile diverted rats (Fig 4B).

**Figure 4**: Cholesterol secretion curves for bile diverted rats receiving duodenal TC infusion. Fig. 4A shows the cholesterol secretion in 8 days bile diverted rats that received a duodenal TC infusion during the last 24 hours (circles). The curves obtained from fitting the data with the formula describing CHOLsec, as described in the text, are shown for these rats (solid line). The dotted line represents the curve describing CHOLsec in control rats (day 0). Fig 4B shows similar curves for 8 days bile diverted rats that received the duodenal TC infusion during the last 48 hours. Here the curves are calculated for both animals in this group; rat A and rat B (see text). The dotted line represents the curve for PLsec in control rats (day 0).

In all experimental groups no significant differences in total liver phospholipid or cholesterol content could be detected (table 1).
Analysis of Mdr2 (Pgp3) mRNA levels in rat liver.

Following the hypothesis that PLsecMax is a measure for mdr2/Pgp3 expression we investigated whether the functional differences observed during manipulation of the enterohepatic circulation coincided with changes in Mdr2 mRNA expression. Rat livers were harvested at the end of the experiment and mRNA was isolated. Quantitative data on Mdr2 and 18S ribosomal RNA levels were obtained using real-time fluorescence PCR with the LightCycler. The levels of 18S RNA were used to normalise Mdr2 mRNA levels. These data are presented in table 2.

Table 2: Quantification of rat liver Mdr2 mRNA levels. The expression levels of each individual animal were normalised to the expression levels of ribosomal 18S RNA. The normalised Mdr2 RNA levels are expressed as percentage of control. Because of the limited number of animals (n=2 or 3) the range of normalised expression values for each group is given between brackets.
The relative expression levels of *Mdr2* in rat liver and the observed maximal PL secretion capacities of the different experimental groups are depicted in figure 3. Because of the fairly large differences in maximal PL secretion capacities between the two rats receiving 48 hours of duodenal TC infusion, these rats are depicted separately (rats A and B). From this figure it can be seen that *Mdr2* expression levels and maximal PL capacity closely correlate.

![Figure 3.](image)

**Figure 3.** This figure describes the close correlation between maximal phospholipid secretion capacity and *Mdr2* expression. The values were determined from the curves in figs. 2 and 3. It is clear that bile diversion leads to dramatic downregulation of *Mdr2* which can be (slowly) restored by duodenal TC infusion. Levels of mRNA expression were determined from quantitative PCR (LightCycler) as displayed in table 2.
DISCUSSION

The secretion of BS into the canicular lumen is a prerequisite for the secretion of phospholipids into bile. However when Mdr2 P-glycoprotein is not functioning properly in the canicular membrane phospholipid secretion will not take place despite the presence of bile salts in the canicular lumen (1). All data available suggest that these Class III P-gps function in biliary lipid secretion by the translocation of PC from the canicular membrane leaflet facing the cytosol of the hepatocyte to the membrane leaflet facing the canicular lumen (8). It is thought that bile salts in the canicular space play a role in extracting phospholipid from the membrane leaflet facing the canicular space although the exact mechanism by which they act is not known (8,22). When pathophysiological conditions block the hepatobiliary phospholipid secretion mediated by class III P-gps, while bile salt secretion is normal, liver pathology will develop. Mice that are homozygous for a disruption of the Mdr2 gene developed nonsuppurative inflammatory cholangitis, which is believed to be secondary to the defect (1,23). Furthermore it has been shown that a group of patients suffering from progressive familial intrahepatic cholestasis, with high serum levels of γ-glutamyl transferase have mutations in the MDR3 gene (24,25). It is thought that these pathologic effects occur because the detergent action of bile salts is not counteracted by the presence of biliary lipids.

Previously we have shown that female mice (FVB) have higher maximal PC secretion rates as compared to males (3,26). These female mice also had a bile salt composition that was more hydrophobic because of proportional increased levels of TC species. We hypothesised that increased lipid extraction capacity of bile would lead to increased P-glycoprotein mediated PC secretion capacity in a regulated fashion. This was substantiated by the increased PC secretion capacity and Mdr2 P-gp levels that were found in male mice of which the hydrophobic bile salt pool was enlarged by feeding TC (3). The current study shows that bile salts are indeed able to regulate biliary lipid secretion through regulation of Mdr2 mRNA expression. Reducing the bile salt pool by chronic bile diversion results in substantially decreased Mdr2 mRNA levels and a reduction in maximal PC secretion capacity. When the EHC is restored both phospholipid secretion capacity and Mdr2 mRNA expression increase. Duodenal infusion of TC showed that increasing the bile salt load alone is sufficient to increase these parameters and that no other biliary constituents are required to achieve this. It is known that choline deficiency leads to reduced PC secretion in rats (27) This can be contributed to reduced
PC synthesis via the CDP-choline pathway. In a separate experiment (not shown) we showed that suppletion of choline to the their drinking water had no effect on the reduced maximal PC secretion rates during bile diversion. This is in agreement with previous studies of choline-depleted rats and prolonged bile diversion (28,29). Furthermore total liver PL content was identical in the different experimental groups used throughout this study.

It is clear that in bile diverted rats choline is also depleted. The discrepancy between our results and the reduced PC secretion in choline deficient rats as described by Robins et al. (27) can probably be explained by the longer depletion period others (28,29) and we used. It was shown that in choline deficient rats PC synthesis switches to transmethylation instead of synthesis from choline (30) and this process takes longer then the 10 hour depletion used by Robins et al. (27). Interesting was the finding that, in rats receiving duodenal TC infusion for 48 hours, maximal cholesterol secretion did not exceed 65% of normal secretion while in these rats PL secretion capacity was much more increased. The cholesterol content in these livers was not different from controls. However growing insight in the origin of biliary cholesterol does not designate newly hepatic synthesised cholesterol as the most important pool but cholesterol originating from high density lipoproteins (31-36). Recent studies suggest that this cholesterol does not enter the cell but laterally diffuses along the plasmamembrane and is secreted very rapidly (34). In the present study no information on HDL levels were obtained but it is known that mdr2-/- mice that also lack biliary lipids in their intestine have reduced levels of HDL cholesterol (37). In bile diverted rats (8 days) cholesterol synthetic capacity is increased (4) and recently it has been shown that the bile fraction of newly synthesised cholesterol was increased 3-5 times as compared to normal rats (38), while total liver cholesterol remained the same. Therefore it is possible that in contrast to normal rats, in bile diverted rats most of the cholesterol is originating from newly synthesised cholesterol. In bile diverted rats, where cholesterol secretion is low because of decreased mdr2 expression, the upregulated synthesis of cholesterol is probably sufficient to keep up with biliary cholesterol secretion. However in bile diverted rats receiving duodenal TC infusion, bile salts can resume their negative control on cholesterol synthesis. In addition, the up-regulation of bile salt synthesis in bile salt diverted rats may further drain the cholesterol pool. Thirdly, because bile diversion is continued during TC infusion, the HDL level in plasma will remain low. As a consequence, in that situation cholesterol supply
could become limiting and could explain why the CHOLsecMAX does not increase in a similar way PLsecMax does.

The current experiments confirm our previous finding that bile salts are able to regulate biliary lipid secretion by regulating the expression of the Mdr2 mRNA. Recently it has been demonstrated that bile salts are natural ligands for a nuclear receptor called Farnesoid X receptor (FXR) (39,40). Formation of a bile salt-receptor complex increases the affinity for coactivator proteins such as SRC-1 (40), that is the key step in the formation of active transcription complexes (41). It has been shown that such a nuclear bile salt signalling pathway may have both negative and positive regulatory effects on different genes involved in cholesterol homeostasis (40). It is not known whether this FXR is involved in regulation of class III P-glycoprotein genes. The relative slow effect bile salt infusion has on the restoration of Mdr2 P-gp levels, only 15% increase within 24 hours, makes it unlikely that BS resort their effect through direct binding to such a receptor. However, to exclude this, more experimental data is needed, for instance on the response of Mdr2 P-gp to different concentrations of infused bile salt. No sequences similar to the bile salt responsive elements identified in cholesterol 7α-hydroxylase or intestinal bile acid binding-protein could be identified in the promoter regions of human MDR3 or rat Mdr2 promoter regions (not shown). Taken together the present data strongly support our previous conclusion that the type and amount of bile salt present in the circulating bile salt pool are capable of regulating biliary lipid secretion (2,3). Such a regulatory feature would be in agreement with the proposed bile salt toxicity neutralising function of biliary phospholipids.
REFERENCES


Regulation of Mdr2 expression during bile diversion


