Studies on the origin of biliary phospholipid

Effect of dehydrocholic acid and cholic acid infusions on hepatic and biliary phospholipids

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The correlation between the secretion of biliary phospholipid (PL) and bile acid suggests a regulatory effect of bile acid on PL secretion. Bile acids may influence PL synthesis and/or the mobilization of a preformed PL pool. The objective of this study was to determine the contribution of these two sources to biliary PL, by using an experimental protocol in which dehydrocholic acid (DHCA) and cholic acid (CA) were infused to manipulate biliary PL secretion. In control rats, there was a steady state in bile flow, PL secretion and the biliary secretion of newly synthesized phosphatidylcholine (PC). The specific radioactivity of PC in bile was significantly higher than in plasma, microsomes and canalicular membranes. DHCA infusion decreased biliary PC secretion rate by 80 %, and secretion returned to normal values at the transport maximum of CA. The specific radioactivity of biliary PC was decreased by 30 % by DHCA infusion and reached normal values during CA infusion. There were no significant changes in the specific radioactivity of PC in plasma or cellular organelles during infusion of bile acids. These data indicate that: (1) newly synthesized PC contributes a small percentage to biliary PC; thus a preformed pool (microsomal and extrahepatic) is a major source of biliary PL; (2) the contribution of the extrahepatic pool to the biliary PL may be more important than the microsomal pool.

INTRODUCTION

The origin and the secretion mechanism(s) of biliary phospholipids (PL) are not yet fully elucidated. With regard to the origin of biliary PL, Zilversmith & Van Handel (1958) suggested that liver contains an active phosphatidylcholine (PC) pool from which biliary PL may be derived. These studies were soon confirmed elsewhere (Balint *et al.*, 1967; Schersten *et al.*, 1967; Swell *et al.*, 1969; Nilsson & Schersten, 1970). Curstedt & Sjoval (1974) demonstrated that a similar pool of hepatic glycerol-3-phosphate was used for synthesis of PC *de novo* in bile, liver and plasma.

However, since biliary PC is specific in its molecular species and is different from that of total liver and plasma (Balint *et al.*, 1965; Yousef & Fisher, 1976), it may be proposed that a selective process is involved in the secretion of biliary PC. Small (1970) suggested that bile acids may solubilize PC from the bile-canalicular membrane during their secretion into bile through this membrane. Such results can explain, during continuous infusion of bile acids, the depletion of the biliary PL pool and the solubilization of cellular membrane, causing the cholestasis that follows the secretory rate maximum of bile acids (Yousef *et al.*, 1987a,b; Barnwell *et al.*, 1987). The hypothesis of Small (1970), however, cannot explain the difference in the molecular species of biliary PL and canalicular-membrane PC (Yousef & Fisher, 1976; Evans *et al.*, 1976). Yousef & Fisher (1976) showed *in vitro* that bile acids at low concentrations can extract particular species of canalicular-membrane PC, which are similar to those found in bile. Coleman (1987, 1988) suggested that bile acids extract 'microdomains' of PC in the canalicular membranes, which could be replaced by similar species of PC originating

from a preformed PL pool. But this preformed pool was not described by that author, nor by the studies of Yousef *et al.* (1987a) or Barnwell *et al.* (1987), despite their demonstration that the amount of this preformed pool appears to be similar at the secretory-rate maximum of different bile acids.

Balint *et al.* (1971) studied the effect of bile salts, infused intraduodenally in bile-fistula rats, on PC synthesis. They suggested that there was one site within the liver for PC synthesis which provides PC for bile, liver and plasma, and that bile salt stimulated PC synthesis within this common pool. Gregory *et al.* (1975), using isolated perfused rat liver, demonstrated that the infusion of taurocholic acid stimulated the synthesis *de novo* of biliary PC and, in the absence of the CDP-choline:1,2-diacylglycerol cholinephosphotransferase from the canalicular membrane (Gregory *et al.*, 1975; Yousef *et al.*, 1977), they suggested that microsomes are probably the site and the source of biliary PC synthesis. However, Robins & Brunengraber (1982) showed in a similar system that newly synthesized PC only contributes 3–5 % of the biliary secretion and, since the secretion of biliary PL is closely related to the secretion of bile acids, they suggested the presence of a preformed hepatic pool which is directed for biliary secretion. This suggestion is possible, since canalicular membranes possess the enzymic activities necessary for re-arranging fatty acids in the molecular species of PC such as monoacylglycerol acyltransferase (Waite *et al.*, 1977). Thus the hepatic pool of biliary PL may not be necessarily of similar species to biliary PL. However, in a later study Robins *et al.* (1985) showed that exogenous dietary PL can alter the molecular species of biliary PL, suggesting the possibility of an extrahepatic source for them. This suggestion was reinforced by studies in our laboratory in which dietary PC increased the secretion of biliary

Abbreviations used: CA, cholic acid; DHCA, dehydrocholic acid; PL, phospholipid; PC, phosphatidylcholine.

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PC without affecting its molecular species of fatty acids (Rioux *et al.*, 1988). The objective of the present study was to attempt to estimate the contribution of the different sources suggested for biliary PC by using an experimental protocol in which the secretion of biliary PL was altered by dehydrocholic acid (DHCA) (Yousef *et al.*, 1990) or cholic acid (CA) (Yousef *et al.*, 1987a,b; Barnwell *et al.*, 1987) infusions, as compared with control rats in steady-state conditions.

MATERIALS AND METHODS

In these experiments, we used infusions of DHCA and CA because it has been established that DHCA drastically decreases, and CA infusions increase, the secretion of biliary PL (Yousef *et al.*, 1987a, 1990; Barnwell *et al.*, 1987). This provided us with a means to manipulate the biliary PL secretion in the rat.

Male Sprague-Dawley rats (Charles River, Saint-Constant, Quebec, Canada) weighing an average of 308 ± 4 g were used in these studies. The animals were maintained in controlled temperature (22°C) and light cycle (19:00–07:00 h darkness) as previously described (Yousef *et al.*, 1990). Under pentobarbital anaesthesia (48 mg/kg body wt., intraperitoneally), the bile duct was cannulated with a PE-10 catheter and bile was collected for 90 min in 30 min batches. The animals were then infused in the right jugular vein through a PE-50 catheter with 3% (w/v) BSA solution in 0.9% NaCl for 30 min, and bile was collected in 10 min batches. This was followed by infusion of 2 and 3 μmol of DHCA/min per 100 g body wt. (these doses were selected since they were shown previously to decrease the secretion of biliary PL; Yousef *et al.*, 1990). Each dose was infused for 30 min, and bile was collected in 10 min samples. At the end of DHCA infusion, 1, 2 and 3 μmol of CA/min per 100 g body wt. were infused in stepwise-increasing doses (these doses cause a maximum increase in the secretion of biliary PL; Yousef *et al.*, 1987a; Barnwell *et al.*, 1987); each dose was infused for 30 min, and bile was collected in 10 min batches. A group of 6–8 rats was killed at the end of DHCA infusion, at the maximum rate of PL secretion, which corresponded to the period at the end of infusion of the 2 μmol of CA, and at the end of the experiments. Controls rats were treated similarly, without infusion of DHCA or CA. All animals were injected intravenously with 1.5 μCi of [^{14}C]choline chloride (Amersham; sp. radioactivity 56 $\mu\text{Ci}/\mu\text{mol}$) per 100 g body wt. 3 h before the end of the experiment, and the body temperature was maintained at 37°C by using a rectal probe and a thermostatically controlled i.r. lamp. The plasma and liver were removed, and the livers were fractionated into two liver-cell plasma-membrane fractions, one enriched in canalicular complexes and the other mainly containing sinusoidal and lateral membranes. Microsomes were also obtained as previously described by this laboratory (Yousef *et al.*, 1984; Yousef & Tuchweber, 1984). The purity of the cellular fractions was estimated by using glucose-6-phosphatase (EC 3.1.3.9) as microsomal marker, leucine aminopeptidase (EC 3.4.11.1) as canalicular marker and 5'-nucleotidase (EC 3.1.3.5) as sinusoidal marker (Yousef *et al.*, 1984; Yousef & Tuchweber, 1984). These markers indicated that the fractions obtained were satisfactorily pure and similar to those used previously in other metabolic studies in the laboratory.

Bile flow was determined gravimetrically by assuming a density of 1 g/ml. The total PL in bile was determined by the Bartlett (1959) method. The PL in bile and cellular fractions was then extracted, and PC was determined by an enzymic method using a kit (Gurantz *et al.*, 1981) provided by Wako Chemicals, Dallas, TX, U.S.A. Total PL was also determined in the lipid extract of the cellular fractions (Bartlett, 1959). Total radioactivity was measured in bile and lipid extracts of the cellular fractions with

a Beckman liquid-scintillation counter equipped with an external standard as previously described (Yousef & Tuchweber, 1984). The specific radioactivity of PC in bile and cellular organelles was calculated as the percentage of ^{14}C injected per μmol or nmol of PC.

The data were statistically analysed by ANOVA and Student's *t* test for non-equal groups; $P < 0.05$ was considered to be statistically significant.

RESULTS

Bile flow and phospholipid secretion (Figs. 1 and 2; Table 1)

Fig. 1 shows that DHCA infusion significantly increased bile flow from 1.95 ± 0.45 $\mu\text{l}/\text{min}$ per g of liver to a maximum of 4.00 ± 0.32 $\mu\text{l}/\text{min}$ per g of liver. During CA infusion, bile flow remained at the same level until the end of the 1 μmol infusion dose, at which time bile flow declined significantly from the maximum secretion rate.

Fig. 1. Bile flow in control rats (●) and in rats infused with DHCA (2 and 3 $\mu\text{mol}/\text{min}/100$ g body wt.), followed by infusion of 1, 2 and 3 μmol of CA (▲).

Each dose was infused for 30 min. Bile was collected for 120 min before the start of the infusion, and [^{14}C]choline was injected 90 min after cannulation. Values are means $\pm$ S.D.; * significantly different from control ($P < 0.05$).

Fig. 2. Phospholipid secretion rate (nmol/min per g of liver) in control rats (●) or rats infused with DHCA and CA (▲) as in Fig. 1.

* Significantly different from control ($P < 0.05$).

Fig. 2 shows that, during DHCA infusion, PL secretion declined significantly, from 6.5 nmol/min per g of liver (before DHCA infusion) to 1.4 nmol/min per g of liver at the end of the infusion. PL secretion started to increase with infusion of CA to a maximum value of 7.8 nmol/min per g of liver, which was achieved at the end of the 2 μ mol infusion. PL secretion declined thereafter to reach a value of 3.4 nmol/min per g of liver at the end of the experiment. In control experiments, bile flow and PL secretion remained constant (no significant changes) during the entire period of the experiments. Since bile flow and PL secretion declined significantly at the end of the 2 μ mol and during the 3 μ mol of CA infusion, it was considered that this is a cholestatic period induced by the infusion of CA, as previously shown (Yousef *et al.*, 1987a; Barnwell *et al.*, 1987), and therefore it represents a pathological state. Thus the results of the incorporation of [¹⁴C]choline during the cholestasis phase will not be considered in this study. Only data on controls, DHCA infusion and the maximum PL secretion during CA infusion will be reported here.

Total PL and PC contents of microsomes, bile-canalicular and sinusoidal membranes (Table 1)

Table 1 shows that PC as a percentage of total PL was significantly higher in microsomes as compared with liver cell plasma-membrane fractions. Infusion of DHCA resulted in increase in total microsomal PL and PC (+26 and +24 % of control values). Therefore, PC as a percentage of total PL remained unchanged (63 and 61 % for control and DHCA-treated respectively). In the canalicular membrane obtained after DHCA infusion, the total PL was not affected, but PC was decreased (–24 % of control value). Therefore the PC as a percentage of total PL was decreased from 48 to 39 %. There were no changes in the sinusoidal-membrane total PL or PC.

Table 1. Total phospholipids (TPL) and PC in microsomes and bile-canalicular and sinusoidal membranes after DHCA (initial) and CA (subsequent) infusions

Control: without bile salt infusion; Experiment: with bile salt infusion. Results (nmol/g of liver) are expressed as means $\pm$ S.E.M. (n = number of experiments); * significantly different from control (P < 0.05).

	DHCA		CA	
	Control	Experiment	Control	Experiment
Bile (PC)	354 $\pm$ 55 (n = 8)	134 $\pm$ 28* (n = 8)	269 $\pm$ 24 (n = 7)	381 $\pm$ 63 (n = 7)
Microsomes				
PC	933 $\pm$ 16 (n = 5)	1156 $\pm$ 54 (n = 5)	892 $\pm$ 21 (n = 5)	947 $\pm$ 70 (n = 5)
TPL	1485 $\pm$ 28 (n = 5)	1868 $\pm$ 34 (n = 5)	1249 $\pm$ 51 (n = 4)	1557 $\pm$ 23 (n = 5)
Bile-canalicular membranes				
PC	37.7 $\pm$ 2.7 (n = 4)	28.5 $\pm$ 2.5* (n = 5)	43.4 $\pm$ 4.53 (n = 4)	28.1 $\pm$ 2.7* (n = 4)
TPL	79.1 $\pm$ 15.4 (n = 5)	73.8 $\pm$ 11.6 (n = 5)	96.9 $\pm$ 7.6 (n = 5)	64.6 $\pm$ 10.2* (n = 5)
Sinusoidal membrane				
PC	48.6 $\pm$ 10.0 (n = 5)	46.4 $\pm$ 10.6 (n = 4)	70.4 $\pm$ 20.6 (n = 5)	61.1 $\pm$ 10.8 (n = 5)
TPL	167.5 $\pm$ 28.9 (n = 5)	157.5 $\pm$ 34.2 (n = 4)	135.7 $\pm$ 12.5 (n = 5)	149.2 $\pm$ 37.2 (n = 5)

Fig. 3. Biliary secretion of [¹⁴C]PC in control rats (▲) or rats infused with DHCA and CA (●)

* Significantly different from control (P < 0.05).

CA infusion had a similar effect on microsomes to that of DHCA; total PL was increased after CA infusion by 24 %, but the PC did not change significantly, therefore the PC as a percentage of total PL was decreased from 71 % to 61 %. Compared with the controls, both total PL and PC in the canalicular membranes were decreased by a similar percentage (36 % and 34 % respectively). Therefore the PC as a percentage of total PL remained constant in CA infusion (45 %). As in DHCA, sinusoidal-membrane total PL and PC were not affected by CA infusion.

Biliary secretion of [¹⁴C]PC

Fig. 3 shows that, in control rats 90 min after the injection of [¹⁴C]choline, the biliary secretion of [¹⁴C]PC reached a maximum and remained in a steady state for 120 min (the maximum period for the experiments). On the other hand, the secretion of [¹⁴C]PC was significantly decreased during the infusion of 3 μ mol of DHCA/min per 100 g body wt. and then rose when CA was infused, to reach a maximum at the end of the infusion of 2 μ mol of CA/min per 100 g body wt.

PC specific radioactivity in bile, plasma, microsomes and bile-canalicular membranes (Table 2)

Table 2 gives the specific radioactivities of PC in bile, plasma, microsomes and the liver cell plasma-membrane fractions obtained during DHCA and CA infusions, as compared with their controls. In control studies, the specific radioactivity of biliary PC was significantly higher than that in plasma, microsomes and liver-cell plasma-membrane fractions, but all these other specific radioactivities were similar. DHCA infusion significantly decreased the biliary PC specific radioactivity (–43 % of control), and the specific radioactivity of microsomal PC was also decreased, by 32 % of the control value. However, plasma and liver-cell plasma-membrane fraction specific radioactivities remained unchanged. In CA infusion, biliary PL specific radioactivity increased significantly compared with DHCA infusion (+29 %), but was not significantly different from control.

The specific radioactivities of microsomal and liver-cell plasma-membrane-fraction PC were not significantly different between DHCA and CA treatments and controls. However, plasma PC after CA infusion was significantly lower than after DHCA infusion (–49 %) and the control (–30 %). Therefore it appears that the specific radioactivity of bile is changed by DHCA and CA infusions. The specific radioactivity of bile-canalicular and sinusoidal membranes was not altered by any treatment.

Table 2. Specific radioactivity of PC in bile, microsomes, bile-canalicular and sinusoidal membranes and plasma after DHCA (initial) and CA (subsequent) infusions

Control: without bile-salt infusion; Experiment: with bile-salt infusion. Results are expressed as means $\pm$ S.E.M. All values are $\times 10^{-3}$; n = number of experiments; * significantly different from control ($P < 0.05$).

	DHCA		CA	
	Control	Experiment	Control	Experiment
Bile (% of ^{14}C / nmol of PC)	86 $\pm$ 8 ($n = 4$)	49 $\pm$ 6* ($n = 5$)	73 $\pm$ 4 ($n = 6$)	63 $\pm$ 13 ($n = 4$)
Microsomes (% of ^{14}C / nmol of PC)	50 $\pm$ 7 ($n = 5$)	34 $\pm$ 2 ($n = 5$)	33 $\pm$ 4 ($n = 5$)	25 $\pm$ 4 ($n = 5$)
Bile-canalicular membranes (% of ^{14}C / nmol of PC)	56 $\pm$ 21 ($n = 4$)	51 $\pm$ 8 ($n = 5$)	41 $\pm$ 3 ($n = 5$)	54 $\pm$ 6 ($n = 4$)
Sinusoidal membranes (% of ^{14}C / nmol of PC)	53 $\pm$ 5 ($n = 4$)	41 $\pm$ 7 ($n = 4$)	38 $\pm$ 6 ($n = 4$)	37 $\pm$ 4 ($n = 5$)
Plasma (% of ^{14}C / μmol of PC)	51 $\pm$ 10 ($n = 4$)	55 $\pm$ 8 ($n = 3$)	40 $\pm$ 8 ($n = 3$)	28 $\pm$ 4 ($n = 3$)

DISCUSSION

In this study we wished to test the hypothesis of the specificity of the biliary PL pool, to determine the role of microsomal and canalicular membranes and newly synthesized PC in the secretion of biliary PL, in an attempt to identify the contribution of these sources to the secretion of biliary PC. These studies utilized [^{14}C]choline to label the newly synthesized PC (which is mainly synthesized via the CDP-choline pathway) and compared control animals in a steady state with respect to PL secretion and synthesis with animals infused with two bile acids (DHCA and CA), with different effect on biliary PL secretion (Yousef *et al.*, 1987a, 1990; Barnwell *et al.*, 1987).

Table 2 clearly shows that under physiological conditions of bile formation (control animals) the specific radioactivity of biliary PC was significantly higher than that of hepatic or plasma PC, suggesting that newly synthesized biliary PL must be derived from a specific pool utilizing the CDP-choline pathway. This is in agreement with previous results (Zilversmith & Van Handel, 1958; Balint *et al.*, 1967; Schersten *et al.*, 1967; Swell *et al.*, 1969; Nilsson & Schersten, 1970), which hypothesized the existence of a specific pool for bile PL secretion, but does not appear to accord with the results of Curstedt & Sjoval (1974), demonstrating a similar pool of hepatic glycerol-3-phosphate for synthesis *de novo* of PC in bile, liver and plasma. This difference may be due to the fact that biliary PC is mainly composed of *sn*-1- $\text{C}_{16:0}$ and *sn*-2- $\text{C}_{18:1}$ or - $\text{C}_{18:2}$ PCs, which are preferentially synthesized via the CDP-choline pathway, as compared with PCs in other cellular organelles and plasma, such as *sn*-1- $\text{C}_{18:0}$ and *sn*-2- $\text{C}_{20:4}$ or - $\text{C}_{22:6}$ PCs, which are preferentially synthesized by methylation of phosphatidylethanolamine. In our results, when DHCA was infused, total biliary PC secreted was decreased by 62% compared with control (134 versus 354 nmol/h per g of

liver). This decrease cannot be explained by decreased secretion of newly synthesized PC, since the contribution of synthesis *de novo* to total PC secreted was decreased by only 12.5 nmol/h per g of liver (the synthesis *de novo* of biliary PC was 16.0 nmol/h per g of liver in control and 3.5 nmol/h per g in DHCA treatment). The decrease in biliary PL secretion with DHCA cannot also be explained by a decrease in microsomal total PL or PC content, since both showed an increase during DHCA infusion. However, DHCA caused a 24% decrease in the canalicular-membrane PC, without affecting the total membrane PL. These data may suggest that part of the PL secreted during DHCA infusion could have originated from the canalicular membrane, possibly by direct solubilization of microdomains of PC in this membrane by the endogenous bile acids secreted during DHCA infusion. The decrease in PC in the canalicular membrane was 9.2 nmol/g of liver, which can account for 6.9% of the total PL during DHCA infusion (134 nmol/g of liver). However, this hypothesis needs to be confirmed by analysis of the molecular species of PC in the bile-canalicular membrane and bile. Therefore, it may be suggested that the main cause of the decrease in biliary PL secretion during DHCA infusion could be failure to mobilize the pre-formed pool of PL, which is used for biliary secretion and the repletion mechanism involved in the canalicular membrane. This failure may explain the increase in microsomal PC during DHCA infusion.

CA infusion (after DHCA treatment), on the other hand, increased biliary PL secretion rate by 287%, but the specific radioactivity was only increased by 29% compared with that of PL secreted during DHCA treatment. The newly synthesized PC contributed very little (3.4%) to the total biliary PC secreted. Similarly, the canalicular-membrane contribution was only 3.5% of biliary PC secreted. The total PL in microsomes was increased, but not the PC, which was decreased from 71% to 61%. It can be assumed that the decrease in PC represents what was used in biliary secretion and canalicular repletion, and contributed 125 nmol/g of liver to the increased biliary PC, or 32.9%. From these data it can be calculated that the contribution of newly synthesized PC and canalicular PC (re-supplied possibly from a special hepatic microsomal pool), as well as that of microsomes, is about 40% of the biliary PC. Thus an extrahepatic pool directed to biliary secretion may contribute a major part (approx. 60%) to biliary lipid secretion. The circulating lipoprotein may constitute the extrahepatic pool of biliary PL previously suggested by Kawamoto *et al.* (1987). Bile acids are normally transported in blood bound to albumin (Roda *et al.*, 1982) and, to a lesser extent, to lipoprotein (Darmer *et al.*, 1979; Salvoli *et al.*, 1985). The hepatic uptake of lipoproteins can be related to the uptake of circulating bile salts by the liver (Malavolti *et al.*, 1987). It has been shown that 16–48% of serum bile acids could be bound to lipoproteins in patients with extrahepatic cholestasis (Hedenborg & Norman, 1985), and that the binding of bile acids to serum albumin decreases with increasing bile-acid concentration (Chitranukroh & Billing, 1983). It is possible that, during infusion of CA, lipoprotein uptake increases and could contribute to the enhanced secretion of biliary lipids. However, this suggestion must await direct testing. Therefore the circulating lipoprotein may constitute the extrahepatic pool of biliary PL previously suggested by Kawamoto *et al.* (1987).

Since the percentage of PC in the canalicular membrane remained unchanged during CA infusion, it may be assumed that a repletion mechanism is in place to preserve the integrity of the membrane (Coleman, 1987, 1988). The higher specific radioactivity of the canalicular-membrane PC compared with that in microsomes during CA infusion suggests that the same biliary PC pool present in the microsomes may be involved in this repletion process.

In conclusion, these data provide evidence that preformed pools of PC are a main source of biliary PC secreted in bile. It further confirms previous data showing that newly synthesized PC contributes a small percentage to the biliary secretion of PC. It can be suggested that the preformed pools could be extrahepatic and microsomal, and that the contribution of the extrahepatic pool may be more important than that of the microsomal pool. However, interaction may exist between all the sources of biliary PC, and this may be related to the repletion mechanism in the canalicular membrane after depletion of specific microdomains of canalicular PC.

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