Partial characterization of regulation of biliary lecithin hydrophobicity: association with organic anion-induced solute cholestasis in rats

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We examined the effects of the depletion of bile salts and of the intravenous infusion of sodium taurocholate (STC) with or without bromosulphophthalein (BSP) in rats on the biliary secretion of lipids to clarify the regulatory mechanism(s). Each rat was equipped with a bile-duct cannula to collect bile. After the endogenous bile salt pool was depleted, STC was infused at a constant rate (160 nmol/min per 100 g body wt.) with or without BSP (50, 100, or 150 nmol/min per 100 g body wt.). BSP reduced the biliary secretion of cholesterol and phospholipids dose-dependently without affecting the secretion of bile salts

(uncoupling phenomenon). Compared with the physiological and STC-infused condition, the biliary cholesterol/phospholipid ratio and saturated/unsaturated fatty acid ratio increased under the bile salts depletion and uncoupling phenomenon. Data indicate that the hydrophobicity of biliary lecithin increases with a decrease in the bile salt micelle capacity to induce biliary lipid secretion, resulting in a higher packing density of biliary vesicle. The cholesterol-holding capacity of the biliary vesicle is therefore enhanced during the depletion of bile salts and the uncoupling phenomenon.

INTRODUCTION

The biliary secretion of cholesterol and phospholipids appears to be regulated by the biliary secretion of bile salts. The mechanism of biliary lipid secretion is that the biliary secretion of lipids into the canaliculus follows that of bile salts, and that the micelle-forming bile salts can stimulate the biliary secretion of phospholipids and cholesterol [1]. We recently reported that bromosulphophthalein (BSP) causes the dissociation of the secretion of biliary lipids from that of bile salts, the so called ‘uncoupling phenomenon’, and that the mechanism of the uncoupling phenomenon by BSP may be due in part to an inhibition of the capacity of the bile-salt micelles to induce the secretion of phospholipids and cholesterol by the interaction of BSP with micelles in the bile canaliculus to form hybrid micelles [2]. We also demonstrated that vesicular lecithin containing more saturated fatty acyl chains binds tightly to cholesterol in bile, and therefore, that vesicular cholesterol metastability is affected by the regulation of fatty acyl chain saturation in lecithin [3]. The present study was performed to characterize the regulatory systems for the secretion of biliary lipids, especially cholesterol, and the change in fatty acid composition of biliary lecithin under various conditions, especially the depletion of bile salts and the uncoupling phenomenon.

MATERIALS AND METHODS

Materials

Sodium taurocholate (STC) and BSP were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Animals and experimental protocol

Male Sprague–Dawley rats (Hiroshima Jikken Doubutsu, Hiroshima, Japan) weighing 250–350 g were maintained on a standard laboratory diet under a constant light cycle. They were anaesthetized with pentobarbital (50 mg/kg body wt.), and the bile duct and left femoral vein were cannulated with PE10 polyethylene tubes (Nippon Becton Dickinson Co. Ltd. Tokyo,

Japan). Following surgery, rats were placed in restraining cages and an intravenous infusion of 0.9% NaCl was started at the rate of 0.4 ml/h per 100 g body wt. After 1 h of equilibration, bile was collected hourly for 14 h into preweighed tubes. After the endogenous pool of bile salts had been drained for 14 h, rats were infused with STC at a constant rate of 160 nmol/min per 100 g body wt. for 4 h. At 4 h after STC infusion was started, BSP (50, 100, or 150 nmol/min per 100 g body wt.) was infused together with STC. After 4 h, the infusion of BSP was stopped (Figure 1).

Figure 1 Experimental design

Representative pattern of changes in bile (bars), and of biliary excretion of phospholipid (○), cholesterol (◇) and bile acid (△) in our experiments is shown. At zero time, animals are fitted with a biliary fistula and a femoral vein catheter, and 0.9% NaCl was infused intravenously at the rate of 0.4 ml/h per 100 g body wt. throughout the experiment. After the endogenous bile salt pool had been drained for 14 h, animals received an infusion of STC (160 nmol/min per 100 g body wt.) for 8 h. From 4 h after beginning the STC infusion, BSP (50, 100, or 150 nmol/min per 100 g body wt.) was infused for 4 h ($n = 3$ in each group). Bile was collected every hour and assayed for bile acid, phospholipid, cholesterol and BSP.

Abbreviations used: BSP, bromosulphophthalein; STC, sodium taurocholate; C/P ratio, cholesterol/phospholipid ratio; S/U ratio, saturated/unsaturated fatty acid ratio.

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Analysis of biliary lipids and BSP

The volume of rat bile collected in a 1 h period was determined gravimetrically by assuming a density of 1 g/ml. The concentrations of BSP was measured spectrophotometrically at the absorption maxima (580 nm) after appropriate dilution with 0.05 M NaOH. Total bile acids were measured enzymically using 3 α -hydroxysteroid dehydrogenase. Phospholipids, cholesterol and the fatty acid composition of lecithin were measured by gas-liquid chromatography (GLC) [4]. Lecithin hydrophobicity was estimated by the ratio of saturated fatty acid to unsaturated fatty acid, the S/U ratio.

Statistical methods

Data are expressed as means $\pm$ S.D. Differences between groups were performed using repeated-measures analysis of variance or Student's *t*-test. A value of *P* < 0.05 was considered statistically significant.

RESULTS

The endogenous pool of bile salts and of biliary lipid secretion remained stable for 3 h, then declined rapidly as shown in Figure 1. After 12 h of diversion, bile salts were secreted at a constant rate as described previously [2,5]. Bile salt output was 22.9 ± 9.9 nmol/min per 100 g body wt. The outputs of phospholipids and cholesterol were 3.5 ± 2.8 and 0.9 ± 0.6 nmol/min per

Figure 2 Change in C/P ratio, S/U ratio and output of biliary lipids during change in biliary output of bile acid

Relative changes in C/P ratio (■), S/U ratio (▲), cholesterol (●) and phospholipids output (◆) in bile, compared with continuous STC infusion (160 nmol/min per 100 g body wt.), are shown. The output of cholesterol and phospholipids was significantly reduced by bile acid depletion (*P* < 0.05), whereas C/P ratio and S/U ratio were significantly increased (*P* < 0.05).

Table 1 Relative composition of biliary lipids under various conditions

Values were expressed as mean $\pm$ S.D. (*n* = 3, each). Values given in parentheses indicate concentration of BSP infusion (in nmol/min per 100 g body wt.)

	C/P	S/U
Control	0.14 $\pm$ 0.04	1.09 $\pm$ 0.06
Bile acid depletion	0.31 $\pm$ 0.07 ^a	1.29 $\pm$ 0.14 ^a
STC	0.12 $\pm$ 0.03 ^b	0.92 $\pm$ 0.04 ^b
BSP (50) + STC	0.18 $\pm$ 0.06 ^c	1.19 $\pm$ 0.12 ^c
BSP (100) + STC	0.20 $\pm$ 0.10 ^c	1.24 $\pm$ 0.15 ^c
BSP (150) + STC	0.23 $\pm$ 0.00 ^c	1.30 $\pm$ 0.23 ^c

The C/P ratio in bile and the S/U ratio in biliary phospholipids were significantly increased by bile-salt depletion when compared with control (^a*P* < 0.05), whereas these values were significantly decreased by continuous STC infusion (^b*P* < 0.05). In addition, these values were significantly increased by BSP infusion when compared with those during continuous STC infusion without BSP in a dose-dependent manner (^c*P* < 0.05).

100 g body wt. respectively. The cholesterol/phospholipid ratio (C/P ratio) was 0.31 ± 0.07 , and the S/U ratio was 1.29 ± 0.14 (Table 1). When rats that had been diverted for 14 h were infused with STC in the next procedure, the output of bile salts, phospholipids and cholesterol rapidly became constant and remained stable throughout the 8 h infusion. Under these conditions, the output of bile salts was 167.3 ± 10.7 nmol/min per 100 g body wt., and the output of phospholipids and cholesterol was 22.1 ± 8.5 and 2.4 ± 0.5 nmol/min per 100 g body wt. respectively (C/P ratio 0.12 ± 0.03 and S/U ratio 0.91 ± 0.04 ; Table 1). After a steady state of bile-salt secretion had been achieved and maintained by the continuous infusion of STC, BSP was infused at a dose of 50 nmol/min per 100 g body wt. BSP did not effect the output of bile salt, but it decreased the output of phospholipid and cholesterol. The C/P ratio and the S/U ratio were increased (0.18 ± 0.06 and 1.19 ± 0.12 respectively; Table 1). To examine the dose-dependency of the inhibitory effect of BSP, we infused several doses of BSP (50, 100, and 150 nmol/min per 100 g body wt.) into rats (*n* = 3 in each group). We also investigated the details of the composition of biliary lecithin fatty acid during the depletion of bile salts and the

Table 2 Fatty acid composition of biliary phospholipids under various conditions

The proportion of palmitic acid significantly increased (^a*P* < 0.05), and that of linoleic acid significantly decreased (^a*P* < 0.05) during bile-salt depletion and BSP infusion relative to the continuous STC infusion. These changes during BSP infusion were dose-dependent. The fatty acid compositions of phospholipids are expressed as means $\pm$ S.D. Values given in parentheses indicate concentration of BSP infusion (in nmol/min per 100 g body wt.)

	Composition (%)					
	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Others
Control	36.2 $\pm$ 0.5	1.1 $\pm$ 0.4	12.3 $\pm$ 1.3*	7.9 $\pm$ 0.6	37.1 $\pm$ 0.9*	5.5 $\pm$ 2.0
Bile acid depletion	41.8 $\pm$ 2.9*	1.1 $\pm$ 0.3*	11.5 $\pm$ 0.7	9.4 $\pm$ 0.5*	31.3 $\pm$ 2.8*	4.9 $\pm$ 2.5
STC	34.4 $\pm$ 0.2	0.7 $\pm$ 0.1	10.4 $\pm$ 0.9	7.6 $\pm$ 0.6	41.4 $\pm$ 0.7	5.4 $\pm$ 2.4
STC + BSP (50)	42.0 $\pm$ 2.1	1.0 $\pm$ 1.3	9.1 $\pm$ 0.3	7.8 $\pm$ 2.1	36.2 $\pm$ 1.1	3.9 $\pm$ 1.8
STC + BSP (100)	44.7 $\pm$ 3.0*	1.3 $\pm$ 0.5	8.8 $\pm$ 2.0	10.1 $\pm$ 0.7*	31.5 $\pm$ 1.4*	3.6 $\pm$ 1.2
STC + BSP (150)	46.7 $\pm$ 3.2*	1.0 $\pm$ 0.5	8.2 $\pm$ 1.3*	8.9 $\pm$ 0.7	33.4 $\pm$ 2.4	1.1 $\pm$ 1.1

uncoupling phenomenon (Table 2). The level of palmitic acid increased, while that of linoleic acid decreased under those conditions. These effects depended on the output of BSP. As shown in Figure 2, there was an inverse proportion between the biliary output of BSP and lipids, but the C/P ratio and the S/U ratio rose in proportion to biliary output of BSP.

DISCUSSION

Several organic anions produce a dissociation of the secretion of biliary lipids from that of bile salts, known as uncoupling. This phenomenon has been described for BSP [2,6], bilirubin [7,8], ampicillin [9,10], iodipamide [8], sodium valproate [11,12], cefoperazone [13] and cyclobutylol [14,15]. The mechanism of such uncoupling is unknown, and the effects of these anions on bile secretion and bile formation are complex. We conducted the present study to evaluate the secretion of biliary lipids under various conditions, especially the depletion of bile salts and the uncoupling phenomenon produced by BSP. BSP decreases the biliary secretion of phospholipids and cholesterol without affecting the secretion of bile salts. The present study provides additional insights into changes in lipid composition. First, we observed an increase in the biliary C/P ratio as compared with that seen in the physiological and the STC-infused conditions. This change became more pronounced with an increase in BSP output. Secondly, the S/U ratio in biliary lecithin also increased during the depletion of bile salts and the uncoupling phenomenon, and was proportional to the biliary BSP output. It seems unlikely that the uncoupling phenomenon with BSP was due to the inhibition of synthesis of cholesterol and phospholipids. The cholesterol that was destined for biliary secretion remained unaffected, even when its rate of synthesis was varied over a wide range [16]. Newly synthesized phosphatidylcholine accounts for only 3% of the biliary phospholipid secretion in the rat [17]. We recently reported that the mechanism of uncoupling in the case of BSP is due, in part, to an inhibition of the capacity of the bile-salt micelles to induce the secretion of phospholipids and cholesterol by the interaction of BSP with the micelles in the bile canaliculus [2]. The change in S/U ratio found in the present study indicates that an interaction between BSP and the bile-salt micelles in the canaliculus leads to a change in selectivity for biliary lecithin species (Figure 3). Such selectivity is also caused by depletion of bile salts (Figure 2). This phenomenon can be explained as follows: the affinity of hydrophilic lecithin for bile salt is stronger than that of hydrophobic lecithin. Therefore, with a reduction in ability of bile-salt micelles that induce lipid secretion, i.e. the bile salt-depleted or uncoupling condition, the secretion of hydrophilic lecithin is decreased, and the proportion of hydrophobic lecithin secretion is relatively increased. This phenomenon may suggest the existence of a bile-salt-independent, or at least less dependent, mechanism of biliary lipid secretion. Similarly, previous investigations provided a high C/P ratio in bile-salt-depleted bile as evidence for a less bile-salt-dependent mechanism of biliary lipid secretion [1,18–23]. Although the physicochemical significance is yet to be established, bile lipid metastability is, in part, modulated by such a less bile-salt-dependent mechanism of biliary lipid secretion [24]. As a result of this, the proportion of saturated fatty acids of lecithin exceeds that of unsaturated fatty acids during the bile-salt-depleted and the uncoupled condition. Consequently, the S/U ratio increased, which means that the biliary lecithin hydrophobicity increased. This phenomenon suggests that biliary vesicles have high packing arrangements for saturated fatty acids during depletion of bile

Figure 3 Relationship between C/P ratio, S/U ratio, biliary lipid output and biliary BSP output

Effects of BSP infusion on biliary lipid secretion, C/P ratio (■), S/U ratio (▲), cholesterol (●) and phospholipids output (◆) were shown. The output of cholesterol and phospholipids was significantly decreased by BSP infusion in a dose-dependent manner ($P < 0.05$), whereas the C/P ratio in bile and the S/U ratio in biliary phospholipids were significantly increased ($P < 0.05$).

salts and in the uncoupling condition. We, therefore, believe that the holding capacity of cholesterol in biliary vesicle is increased, and, consequently, cholesterol secretion is increased. This phenomenon seems understandable in regard to the increase in biliary cholesterol secretion. The increase in S/U and C/P ratios in the biliary lipids during bile-salt depletion and the uncoupling condition may improve the decrease in membrane fluidity resulting from the accumulation of cholesterol in the canalicular membrane during cholestasis.

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