Effects of organic anions on biliary lipid secretion in rats

Importance of association with biliary lipid structures

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This study was performed to determine the effects of various organic anions on biliary lipid secretion in rats. We infused bile-salt-pool-depleted rats with sodium taurocholate at a constant rate, with or without various organic anions: Indocyanine Green (ICG), bromosulphophthalein (BSP), BSP-glutathione and Phenol Red (PR). BSP decreased biliary secretion of cholesterol and phospholipids in a dose-dependent manner without affecting bile salt secretion (uncoupling), and this change was fully reversible. In contrast, ICG, BSP-glutathione and PR did not cause such an uncoupling of biliary lipids. In addition, the distribution pattern of each organic anion to various lipid particles was determined by gel-permeation chromatography. BSP was predominantly associated with bile salt micelles, whereas vesicular association was dominant for ICG, and both BSP-glutathione and PR formed only self-aggregations. From these data, we concluded that the uncoupling of biliary lipids from bile salt secretion by BSP resulted from the interaction between BSP and bile salt micelles in the bile canaliculus, and that this interaction inhibited the capacity of bile salts to induce the secretion of phospholipids and cholesterol.

INTRODUCTION

It is known that micelle-forming bile salts can stimulate biliary phospholipid and cholesterol secretion. Although the secretion of cholesterol and phospholipids into bile increases almost linearly with bile salt secretion under physiological conditions [1,2], several organic anions have been shown to cause dissociation of the secretion of biliary lipids from that of bile salts. This uncoupling phenomenon has been described for bromosulphophthalein (BSP) [3], bilirubin [4,5], ampicillin [6,7], iodipamide [5], sodium valproate [8,9], cefoperazone [10] and cyclobutylol [11,12]. However, the mechanism of uncoupling remains poorly understood. Bellringer *et al.* proposed the inhibition of the intracellular movement of vesicles as an uncoupling mechanism in their experiments using ampicillin and valproate [7,8]. Such an intracellular mechanism has also been suggested by Pattison *et al.* for cefoperazone [10]. On the other hand, Monte *et al.* proposed the inhibitory effect of cyclobutylol on lipid transport at the level of the canalicular membrane [11,12]. Alternatively, for ampicillin and sulphated taurothiocholic acid, another mechanism involving physical interaction of the dye with mixed micelles in the bile canaliculus has been suggested [13]. Thus the question of the effects of organic anions on bile secretion and bile formation is complicated.

Organic anions have been found to partition into various biliary lipid particulate species according to their relative hydrophobicity [14,15]. Accordingly, organic anions which are predominantly associated with bile salts in the bile canaliculus may inhibit the capacity of the bile salts to induce secretion of phospholipids and cholesterol into bile. The aims of this study were: (1) to determine whether organic anions that have different hydrophobicities affect biliary lipid secretion differently; and (2) if they do, to explore the mechanism(s) of such effects of organic anions.

MATERIALS AND METHODS

Sodium taurocholate, BSP, Indocyanine Green (ICG) and Phenol Red (PR) were purchased from Sigma Chemical Company

(St. Louis, MO, U.S.A.). BSP-glutathione was prepared *in vitro* as described previously [16]. T.l.c. plates precoated with silica gel (DC-Fertigplatten Kieselgel 60) were purchased from Merck. Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of animals

Male Wistar rats (Hiroshima Jikken Doubutsu, Hiroshima, Japan) weighing 250–300 g were maintained on a standard laboratory diet under a constant light cycle. Animals were anaesthetized with pentobarbital (50 mg/kg body weight) and the bile duct and left femoral vein were cannulated with PE-10 polyethylene tubes (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). Following surgery, the animals were placed in restraining cages and allowed free access to water, and intravenous infusion of 100 mM-NaCl was started at the rate of 0.4 ml/h per 100 g body wt. After the endogenous bile salt pool had been drained for 14 h, animals were infused with sodium taurocholate at a constant rate of 160 nmol/min per 100 g body wt. for 4 h. After 1 h of equilibration, bile was collected hourly into preweighed tubes and bile collection was continued until the end of the experiments. At 4 h after the sodium taurocholate infusion was started, BSP (25, 50, 100 and 150 nmol/min per 100 g body wt.), BSP-glutathione (25 nmol/min per 100 g), ICG (25 nmol/min per 100 g) or PR (50 nmol/min per 100 g) were each infused together with sodium taurocholate (160 nmol/min/100 g). After 3 h the infusion of organic anion was stopped and the sodium taurocholate infusion alone was continued for an additional 4 h. In the present study, ICG (a hydrophobic organic anion), BSP (an intermediately hydrophobic organic anion) and PR (a hydrophilic organic anion) were used to determine the different effects on biliary lipid secretion of anions with different hydrophobicities [14,15].

Analysis of biliary lipids and organic anions

The volume of rat bile collected in 1 h periods was determined gravimetrically by assuming a density of 1 g/ml. The concentrations of organic anions in bile were measured

Abbreviations used: BSP, bromosulphophthalein; ICG, Indocyanine Green; PR, Phenol Red.

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spectrophotometrically at their respective absorption maxima (BSP and BSP-glutathione, 580 nm; ICG, 805 nm; PR, 560 nm), after appropriate dilution with 0.05 M-NaOH. The proportions of BSP and BSP conjugates in bile were determined by t.l.c. [17]. The solvent system was acetone/water/0.1 M-NaOH (81:15:4, by vol.), and the bands of BSP and BSP conjugates were detected by spraying with 0.05 M-NaOH. The bands separated by t.l.c. were scraped off and dissolved separately in 2 ml of 0.05 M-NaOH. After centrifugation (1500 g, 10 min), the BSP concentration in the supernatant was measured spectrophotometrically. Total bile acids were measured enzymically using 3 α -hydroxysteroid dehydrogenase [18]. Phospholipids were determined as described by Bartlett [19] and cholesterol was measured by g.l.c. [20].

Gel-permeation chromatography of rat bile

A 1 h fraction of bile collected during the period of maximal secretion of each organic anion was applied to a Sepharose CL-4B column (1.5 cm $\times$ 40 cm) that had been pre-equilibrated with Hepes/NaCl (10 mM-Hepes, 150 mM-NaCl, pH 7.5) containing 4 mM-EDTA and 0.02% (w/v) Na₂S₂O₃, and eluted with the same buffer at the rate of 1 ml/min, with the eluate collected in 1.5 ml fractions. The excluded volume (V_0) and included volume (V_i) of the column were determined using Blue Dextran 2000 (Sigma) and [¹⁴C]sucrose respectively. Column fractions were assayed for bile acids, phospholipids, cholesterol and organic anions. For the fractions containing BSP, t.l.c. was performed in order to determine the proportions of conjugated and unconjugated BSP.

Statistics

All data are expressed as means $\pm$ S.D., and the statistical analysis for data significance was performed using repeated measures analysis of variance [21]. A probability of < 0.01 was considered significant.

RESULTS

As shown in Fig. 1, the endogenous pool of bile salts and biliary lipid secretion remained stable for 3 h, but then declined rapidly. After 12 h of diversion, bile flow was $4.3 \pm 0.6 \mu\text{l/min}$ per 100 g body wt., bile salt output was $20 \pm 8 \text{ nmol/min}$ per

Fig. 1. Experimental design

A representative pattern of changes in bile flow (bars), and of biliary excretion of phospholipid (○), cholesterol (□) and bile acid (●) in our experiments is shown. At time zero, animals were fitted with a biliary fistula and a femoral vein catheter, and 100 mM-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 were given an infusion with sodium taurocholate (STC) at a rate of 160 nmol/min per 100 g body wt. for 11 h. From 4 h after the STC infusion began, each organic anion was infused for 3 h. Bile was collected hourly and assayed for bile acid, phospholipid, cholesterol and organic anions.

Fig. 2. Effects of organic anions on bile flow and on the output of bile salts, phospholipids and cholesterol in bile

Animals were infused with (a; 25 nmol/min per 100 g body wt.), ICG (b; 25 nmol/min per 100 g), BSP-glutathione (c; 25 nmol/min per 100 g) or PR (d; 50 nmol/min per 100 g). The effects on bile flow, total bile acid, phospholipid, and cholesterol are expressed as the mean percentage ($\pm$ S.D.) of the value before the infusion of organic anions. Values are from measurements taken before (□), during (■), and after (▨) infusion ($n = 4$ in each group). Abbreviations: BF, bile flow; TBA, total bile acids; PL, phospholipid; CHOL, cholesterol. * Significantly different from control ($P < 0.01$).

100 g body wt., and outputs of phospholipids and cholesterol were 7 ± 3 and $0.8 \pm 0.3 \text{ nmol/min}$ per 100 g body weight respectively. Beyond this time, newly synthesized bile salts were secreted at a constant rate [1], so that the biliary lipid secretion rate was virtually constant for at least the next 12 h. When rats that had had their bile flow diverted for 14 h were infused with sodium taurocholate (160 nmol/min per 100 g body wt.) in the

Fig. 3. Inhibition of cholesterol and phospholipid secretion in relation to BSP secretion in bile

Biliary cholesterol (●) and phospholipid (■) and unconjugated BSP (○) secretion are shown ($n = 4$, in each group). * Significantly different from control ($P < 0.01$).

next procedure, the outputs of bile salt, phospholipid and cholesterol rapidly became constant and remained stable throughout the 11 h infusion period. Under these conditions, bile flow was $6.1 \pm 0.9 \mu\text{l}/\text{min}$ per 100 g body wt., bile salt output was $176 \pm 7 \text{ nmol}/\text{min}$ per 100 g, and outputs of phospholipids and cholesterol were 37 ± 5 and $2.1 \pm 0.4 \text{ nmol}/\text{min}$ per 100 g respectively ($n = 5$). Based on the results of this preliminary study, further experiments were performed during constant sodium taurocholate infusion at $160 \text{ nmol}/\text{min}$ per 100 g body wt.

Effects of organic anions on biliary lipid secretion

After a steady state of bile salt secretion had been achieved and maintained by the continuous infusion of sodium taurocholate, each organic anion was infused at a dose of 25 (ICG, BSP and BSP-glutathione) or 50 (PR) nmol/min per 100 g body wt. The output of organic anions into bile was as follows (in nmol/min per 100 g body wt.): ICG, 15 ± 2 ; BSP, 23 ± 2 ; BSP-glutathione, 23 ± 2 ; and PR, 16 ± 2 . Fig. 2 shows the effects of organic anions on bile flow and on the secretion of bile salts, phospholipids and cholesterol into bile. All organic anions except for ICG significantly increased bile flow (ICG, $98 \pm 8\%$; BSP, $107 \pm 2\%$; BSP-glutathione, $118 \pm 4\%$; PR, $116 \pm 5\%$ of control). None of the four organic anions affected bile salt output, and only BSP affected biliary lipid output. At the BSP infusion rate of $25 \text{ nmol}/\text{min}$ per 100 g body wt., the output of phospholipids and cholesterol was significantly decreased ($33 \pm 3\%$ and $23 \pm 8\%$ respectively; Fig. 2a). After the cessation of BSP infusion, bile flow and the secretion of phospholipids and cholesterol returned to control levels (Fig. 2a). In contrast, ICG, BSP-glutathione and PR did not affect the output of phospholipids and cholesterol (Figs. 2b, 2c and 2d). In addition, when BSP-glutathione was infused, very little deconjugation occurred and the percentage of unconjugated BSP was below 1%.

To examine the dose-dependence of the inhibitory effect of BSP on biliary lipid secretion, rats were infused with BSP at 25, 50, 100 and 150 nmol/min per 100 g body wt. ($n = 4$ in each group). Further, we also examined the changes in the quantity of non-conjugated BSP in relation to BSP secretion into the bile. As shown in Fig. 3, there was a correlation between the biliary output of BSP and unconjugated BSP, and there was an inverse non-linear correlation between the biliary output of BSP and of lipids. With increasing BSP output the inhibition of phospholipid and cholesterol secretion became more pronounced, with no corresponding change in bile salt secretion. In contrast, no decrease in phospholipid and cholesterol was found in the experiments with other organic anions (ICG, BSP-glutathione and PR)

Fig. 4. Gel-permeation elution profiles

Rat bile produced during maximal secretion of each organic anion was applied to a Sepharose CL-4B column and eluted with Hepes/saline buffer. Elution profiles of cholesterol were similar to those of phospholipids (●) and are not shown in the Figures for clarity. ICG (a, ■) was incorporated into the vesicular fractions, while unconjugated BSP (b, ■) was predominantly associated with bile salt (○) micelles. In contrast, self-aggregation was predominant for conjugated BSP (b, □) and PR (c, ■).

at the doses producing comparable unconjugated BSP output (G. Yamashita, S. Tazuma & G. Kajiyama, unpublished work).

Gel-permeation chromatography

Fig. 4 shows the preferential distribution patterns of various organic anions to biliary lipid particles. Almost all of the ICG was incorporated into the vesicular fractions (Fig. 4a), while BSP was predominantly associated with bile salt micelles (Fig. 4b). In contrast, self-aggregation was predominant for conjugated BSP and PR (Figs. 4b and 4c). When the organic anions were chromatographed alone in the same system, all of them were eluted at V_t .

DISCUSSION

Several organic anions are known to decrease the biliary secretion of cholesterol and phospholipids, without affecting bile salt secretion (so-called uncoupling) [13]. Despite numerous studies, the mechanism(s) of this phenomenon, which may not be the same for all organic anions, is still unclear. In the present

study, we compared four organic anions that have different hydrophobicities [14,15]. Although none of these organic anions affected bile salt secretion, only BSP decreased the biliary secretion of phospholipids and cholesterol. It is known that BSP is excreted into bile in both conjugated and unconjugated forms, with the conjugated form being primarily (> 90%) BSP-glutathione in rats [17]. Therefore we also examined the effect of BSP-glutathione on biliary lipid secretion. The uncoupling phenomenon was not found when BSP-glutathione was infused at the same dose as BSP, which caused a drastic reduction in biliary lipid secretion. Thus it seemed that the uncoupling phenomenon was based entirely upon the effect of unconjugated BSP.

Several mechanisms could account for the uncoupling phenomenon caused by BSP administration. First, choleresis due to BSP might reduce the bile salt concentration in the canalicular cavity and consequently decrease lipid solubilization by bile salts. BSP has a slightly hydrocholeretic action, as shown in Fig. 2(a). The increase in bile flow caused by BSP infusion appeared to result from osmotic choleresis, since gel-permeation chromatography revealed that a considerable amount of conjugated BSP was present in the bile as self-aggregations. However, despite the fact that PR and BSP-glutathione have an even more hydrocholeretic action, they did not cause uncoupling in this experiment. Thus hydrocholeresis is unlikely to be a crucial factor in this phenomenon.

Second, BSP might reduce cholesterol and phospholipid synthesis. Turley & Dietschy [22] demonstrated that cholesterol destined for biliary secretion was unaffected even when cholesterol synthesis was varied over a wide range in the rate. Further, Robins & Brunengraber [23] demonstrated that newly synthesized phosphatidylcholine accounts for only 3% of biliary phospholipid secretion in the rat. In contrast, BSP affected biliary lipid secretion rapidly in the present study. Accordingly, it is quite unlikely that the uncoupling phenomenon with BSP was due to the inhibition of synthesis of cholesterol and phospholipids.

The third possibility is that BSP may interfere with intracellular transport of vesicles, which is a dominant vehicle for cholesterol and phospholipids in the liver [24,25]. Monte & Coleman [12] demonstrated that cyclobutylol, which causes an uncoupling phenomenon, does not inhibit general vesicular transport. Further, Verkade *et al.* [13] have recently reported that ampicillin and sulphated tauroolithocholic acid decrease biliary cholesterol and phospholipids in normal rats. However, they did not find the same response in Groningen Yellow rats, which have a hereditary defect in the hepatobiliary transport of various organic anions, despite the fact that intrahepatic amounts of the anions were in the same range or higher than those in normal rats. These workers suggested that the uncoupling mechanism is localized inside the bile canaliculus.

The recent understanding of the mechanisms of bile secretion includes the following points: (1) micelle-forming bile salts can stimulate biliary phospholipid and cholesterol secretion, (2) bile salt output precedes biliary lipid output by several minutes [24], reflecting the contribution of a rapid cytosolic diffusion pathway for bile salts and a slower membrane-based pathway involving biliary lipid [26,27], (3) the primary secretion of bile salts into the canaliculus is followed by that of lipids, and (4) the subsequent association of bile salts and lipids results in the formation of mixed micelles [24]. On this basis, the interaction between organic anions and bile salts in the bile canaliculus might inhibit the capacity of bile salts to induce the secretion of phospholipids and cholesterol into bile. Organic anions partition into various biliary

lipid particulate species according to their relative hydrophobicities [14,15], and the intermediately hydrophobic organic anions bind preferentially to bile salt micelles. In the present study, gel-permeation chromatography of bile-containing organic anions revealed that BSP was predominantly associated with bile salt micelles, but that ICG, a more hydrophobic organic anion, was co-eluted with vesicle fractions, and the relatively hydrophilic organic anions such as conjugated BSP and PR were found as small self-aggregates. Although some phase shifts could occur in our gel-permeation chromatographic study because of lack of bile acids in an eluent, this effect would be minor and unlikely to drastically alter the partitioning of organic anions to lipid particulate species. In fact, very little change was found in the relative affinities of organic anions to vesicles and micelles in the presence of bile acids at an intermicellar concentration in an eluent (G. Yamashita, S. Tazuma & G. Kajiyama, unpublished work). Therefore we suggest that the uncoupling of biliary lipids from bile salts during BSP infusion is due in part to inhibition of the capacity of the bile salt micelles to induce the secretion of phospholipids and cholesterol, and that this inhibition is seemingly caused by the interaction of BSP with micelles in the bile canaliculus.

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