Partial characterization of mechanism(s) by which sulphobromophthalein reduces biliary lipid secretion

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This study was performed to explore the mechanisms by which sulphobromophthalein (BSP) reduces the secretion of biliary lipid using Sprague–Dawley rats (SDR) and mutant rats with congenital conjugated hyperbilirubinaemia bred from SDR (EHBR). We infused the bile-salt-pool-depleted rats with sodium taurocholate at a constant rate of 160 nmol/min per 100 g body wt. with BSP (12.5, 25 and 50 nmol/min per 100 g body wt.) or BSP–GSH (12.5, 25 and 50 nmol/min per 100 g body wt.). The biliary secretion of BSP and BSP–GSH was markedly impaired in EHBR as compared with that in SDR. BSP reduced the biliary secretion of cholesterol and phospho-

lipids in a dose-dependent manner without affecting the secretion of bile salts and composition of fatty acids in phospholipids in SDR, but had no effect on lipid secretion in EHBR. In contrast, BSP–GSH had no such effect on biliary lipids, either in the SDR or EHBR. In addition, the amount of BSP in the liver of EHBR was in the same range as that of SDR. Therefore it is unlikely that an intracellular mechanism is involved in the phenomenon of uncoupling by BSP. We conclude that the uncoupling of biliary lipids from bile-salt secretion by BSP occurs at the level of the bile canaliculus following the secretion of unconjugated BSP.

INTRODUCTION

The biliary secretion of cholesterol and phospholipids appears to be regulated by biliary bile-salt secretion. Under physiological conditions, the secretion of cholesterol and phospholipids into bile increases almost linearly with the secretion of bile salt [1,2]. However, several organic anions have been shown to cause dissociation of the secretion of biliary lipids from that of bile salts (uncoupling). This uncoupling phenomenon has been described for sulphobromophthalein (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 is poorly understood. Bellringer et al. [7,8] proposed the inhibition of the intracellular movement of vesicles as a mechanism in their experiments with ampicillin and valproate. Such an intracellular mechanism has also been suggested by Pattison et al. [10] for cefoperazone. Monte et al. [11,12] proposed an inhibitory effect of cyclobutylol on lipid transport at the level of the canalicular membrane. Alternatively, another mechanism, involving physical interaction of the dye with mixed micelles in the bile canaliculus, has been suggested for ampicillin and sulphated taurothiocholic acid [13]. Thus the effects of organic anions on the secretion of bile and on bile formation are complicated.

We recently reported that BSP produces dissociation of the secretion of biliary lipids from that of bile salts and that the mechanism of the uncoupling phenomenon by BSP was, in part, due 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 [14]. However, the mechanism(s) responsible for the uncoupling phenomenon by BSP have not been elucidated. Therefore, our objective was to expand the observations of our initial study and to confirm the mechanism(s) of the effects of BSP on biliary lipid secretion in Sprague–Dawley rats (SDRs). For this purpose, the effects of BSP and BSP–GSH, the main conjugated form (> 90 %) in rat bile [15], on biliary lipid secretion were compared

in SDR and mutant SDR with hereditary conjugated hyperbilirubinaemia and a deficiency of biliary excretion of organic anions [Eisai hyperbilirubinaemic rat (EHBR)].

MATERIALS AND METHODS

Sodium taurocholate (STC) and BSP were purchased from Sigma. BSP–GSH was prepared *in vitro* as described previously [16]. T.l.c. plates precoated with silica gel (DC-Fertigplatten Kieselgel 60) were purchased from Merck.

Radioiodination

¹²⁵I radioiodination of BSP was performed using the chloramine-T method [17] with a slight modification [18]. BSP (150 µg) was dissolved in 100 µl of 0.25 M sodium phosphate buffer, pH 8.0. Na¹²⁵I (10 µl, 100 mCi/ml; New England Nuclear) was added to BSP solution, followed by 20 µl chloramine-T (2 mg/ml). This solution was vortex-mixed and incubated at room temperature for 10 min. This oxidation step was repeated, and the reaction was stopped by the addition of 50 µl of sodium metabisulphite (2.5 mg/ml). Unlabelled NaI (2 mg/ml) was then added to a final volume of 250 µl so as to competitively displace ¹²⁵I from sites of non-specific absorption. This mixture was immediately applied to an LH-20 column (1 cm × 10 cm, Pharmacia). Free ¹²⁵I was eluted with 0.25 M sodium phosphate buffer, pH 8.0, and the radioiodinated compounds were eluted with methanol. Methanol was removed from the labelled compounds by evaporation under a stream of N₂. No major degradative products were detected by h.p.l.c. The specific radioactivity of the labelled BSP was 1.7 mCi/µmol.

Animals and experimental protocol

Male SDRs and EHBRs weighing 250–300 g were used in all experiments. EHBRs were maintained by inbreeding male homozygotic and female heterozygotic rats at the Eisai

Laboratory (Gifu, Japan). The serum bilirubin level in EHBRs (~ 6 mg/100 ml, about 75% of directly reactive bilirubin) [19] was markedly elevated as compared with SDRs (~ 0.2 mg/100 ml). Rats were maintained on a standard laboratory diet under a constant light cycle and were used for these experiments. Under pentobarbital anaesthesia (50 mg/kg body wt.), the bile duct and femoral vein were cannulated with PE-10 polyethylene tubes (Nippon Becton Dickinson). Following surgery the rats were placed in restraining cages and allowed free access to water. The intravenous infusion of 100 mM NaCl was started at a 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 STC at a constant rate of 160 nmol/min per 100 g body wt. After 1 h of equilibration, bile was collected every 15 min in preweighed tubes. The collection of bile was continued until the end of the experiments. At 2 h after the STC infusion was started, either BSP (12.5, 25, 50 and 100 nmol/min per 100 g body wt.) or BSP-GSH (12.5, 25 and 50 nmol/min per 100 g body wt.) was infused together with STC (160 nmol/min per 100 g body wt.). After 30 min, the infusion of organic anion was stopped and the STC infusion alone was continued for an additional 2 h.

Serum clearance and hepatic content of BSP

Serum clearance and hepatic content of BSP were determined in short-term experiments. For this purpose, 1 h after the bile duct had been cannulated, BSP (300 nmol/100 g body wt.) with a tracer dose of ^{125}I -BSP (20 μCi) was injected as an intravenous bolus via the femoral vein. Blood was collected at 1, 2, 4, 6, 10, 15, 20, 25 and 30 min after injection. To determine the hepatic content of BSP, 10 min after a bolus injection of BSP (300 nmol/100 g body wt.) with a tracer dose of ^{125}I -BSP, the rat was killed and the liver was immediately perfused with cold saline via the portal vein, then excised. The liver was weighed and homogenized for determination of BSP content by liquid-scintillation counting. Radioactivity in serum and liver was measured in a Beckman liquid-scintillation counter.

Analysis of biliary lipid and organic anions

The volume of rat bile collected every 15 min was determined gravimetrically by assuming a density of 1 g/ml. The concentrations of BSP and its GSH conjugate in bile were measured spectrophotometrically at the absorption maxima (580 nm) after appropriate dilution with 0.05 M NaOH. The proportion of BSP and BSP conjugates in bile was determined by t.l.c. [16]. 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 of the supernatant was measured spectrophotometrically. Total bile acids were measured enzymically using 3α -hydroxysteroid dehydrogenase [20]. Phospholipids were determined as described by Bartlett et al. [21]. Total cholesterol and the fatty acid composition of phospholipids were measured by g.l.c. [22].

Statistical methods

All data are expressed as means $\pm$ S.D. Analysis of significant difference was performed using repeated measures analysis of

variance [24] or Student's *t* test. A probability value of < 0.01 was considered statistically significant.

RESULTS

As shown in Figure 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, newly synthesized bile salts were secreted at a constant rate [8], so that the rate of biliary lipid secretion was virtually constant for at least the next 6 h. When rats that had been diverted for 14 h were infused with STC (160 nmol/min per 100 g body wt.) in the next procedure, the output of bile salt, phospholipid and cholesterol rapidly became constant and remained stable throughout the 6 h infusion. Under these conditions, the output of bile salt (175 ± 16 nmol/min per 100 g body wt.), phospholipid (35 ± 13 nmol/min per 100 g body wt.) and cholesterol (3.4 ± 2.0 nmol/min per 100 g body wt.) in EHBRs was similar to that in SDRs (186 ± 15 , 37 ± 12 , 3.7 ± 1.9 nmol/min per 100 g body wt. respectively). In contrast, bile flow in EHBRs (4.4 ± 1.1 μl /min per 100 g body wt.) was lower than that in SDRs (5.2 ± 1.2 μl /min per 100 g body wt.) ($n = 5$). Based on the results of this preliminary study, further experiments were performed during constant STC infusion at 160 nmol/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 STC, either BSP (25 nmol/min per 100 g body wt.) or BSP-GSH (25 nmol/min per 100 g body wt.) was infused. Figure 2 shows the secretion of bile salt, BSP (Figure 2a) and BSP-GSH (Figure 2b) into bile of SDRs and in EHBRs. Biliary secretion of BSP and BSP-GSH was markedly impaired in EHBRs. In contrast, BSP and BSP-GSH were secreted into bile in SDRs at a recovery rate of $\sim 95\%$ without affecting bile-salt secretion. Figure 3 shows the effect of organic anions on the secretion of phospholipids and cholesterol. Organic anions did not affect the biliary secretion of phospholipids or cholesterol in EHBRs (Figure 3a). In contrast,

Figure 1 Experimental design

A representative pattern of changes in the biliary excretion of phospholipid (■), cholesterol (□) and bile acids (●) is shown. At zero time 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 received an infusion of STC (160 nmol/min per 100 g body wt.) for 4 h. From 1 h after the beginning of the STC infusion, each organic anion was infused for 30 min. Bile was collected every 15 min and assayed for bile acid, phospholipid, cholesterol and organic anions.

Figure 2 Biliary excretion of BSP, BSP-GSH and bile salts in SDRs and in EHBRs

Animals were infused with BSP (a; 25 nmol/min per 100 g body wt.) or BSP-GSH (b; 25 nmol/min per 100 g body wt.) for 30 min. Organic anions did not affect the biliary excretion of bile salts in SDR (▲) or in EHBR (△). Biliary secretion of BSP (a; ●) and BSP-GSH (b; ○) was markedly impaired in EHBR. In contrast, BSP (a; ●) and BSP-GSH (b; ●) were secreted into bile of SDR at a recovery rate of ~95%. S.D.s have been omitted for clarity.

only BSP reduced the biliary secretion of biliary lipids in SDRs (Figure 3b). At the BSP infusion rate of 25 nmol/min per 100 g body wt., the output of phospholipids and cholesterol was significantly reduced ($33 \pm 3\%$ and $23 \pm 8\%$ respectively; Figure 3b). After the cessation of BSP infusion, the secretion of phospholipids and cholesterol returned to control levels (Figure 3b).

To examine the dose-dependency of the inhibitory effect of BSP and BSP-GSH on biliary lipid secretion, SDRs were infused with BSP (12.5, 25, 50 and 100 nmol/min per 100 g body wt.) or BSP-GSH (12.5, 25 and 50 nmol/min per 100 g body wt.) respectively. At BSP infusion rates of 12.5, 25, 50, 100 nmol/min per 100 g body wt., biliary outputs of unconjugated BSP were 7.0 ± 1.2 , 13.8 ± 2.0 , 22.2 ± 5.1 , 38.5 ± 4.6 nmol/min per 100 g body wt respectively. The rest of the BSP infusion was secreted into bile as conjugated forms, primarily as BSP-GSH. In addition, when BSP-GSH was infused, very little deconjugation occurred and the percentage of unconjugated BSP was less than 1%. Figure 4 shows the effect of biliary output of unconjugated BSP and BSP-glutathione on the output of bile salts, cholesterol and phospholipids in SDRs. There was an inverse non-linear correlation between the biliary output of BSP and lipids without an effect on output of bile salts. With increasing

Figure 3 Effect of BSP and BSP-GSH on the secretion of phospholipid and cholesterol in SDR and in EHBR

Animals were infused with BSP-GSH (a; 25 nmol/min per 100 g body wt.) or BSP (b; 25 nmol/min per 100 g body wt.). The effect of each organic anion on the secretion of phospholipid [SDR (●); EHBR (○)] and cholesterol [SDR (■); EHBR (□)] is shown. Organic anions did not affect the secretion of phospholipids or cholesterol in EHBR (a). In contrast, only BSP reduced the biliary secretion of biliary phospholipid and cholesterol in SDR. S.D.s have been omitted for clarity. * Indicates a significant reduction ($P < 0.01$).

Figure 4 Relationship between biliary output of lipids and organic anions

Biliary phospholipids (●) and cholesterol (■) are expressed as mean percentages $\pm$ S.D. There was an inverse non-linear correlation between the biliary output of unconjugated BSP and that of lipids, phospholipids and cholesterol (a). In contrast, no reduction of phospholipid and cholesterol was found in BSP-GSH (b). * Indicates a significant reduction ($P < 0.01$).

Table 1 Effect of BSP (100 nmol/min per 100 g body wt.) on fatty acid composition of phospholipids

The fatty acid compositions of phospholipids are expressed as means $\pm$ S.D. No alteration in fatty acid composition of phospholipids by BSP infusion was evident.

	Composition (%)					
	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Others
Before infusion	52.1 $\pm$ 4.4	1.6 $\pm$ 1.1	8.5 $\pm$ 3.0	6.9 $\pm$ 2.3	29.1 $\pm$ 3.9	1.8 $\pm$ 1.4
After infusion	53.5 $\pm$ 5.4	1.4 $\pm$ 1.0	9.5 $\pm$ 3.2	8.1 $\pm$ 1.9	25.8 $\pm$ 4.2	1.7 $\pm$ 1.1

Figure 5 Plasma elimination of BSP in SDR (○) and EHBR (●)

Plasma elimination of BSP at 10 min after BSP (300 nmol/100 g body wt. + trace dose of 125 I-BSP) is shown. There was no difference between the SDR (○) and EHBR (●). S.D.s have been omitted for clarity.

BSP output, the inhibition of phospholipid and cholesterol secretion became more pronounced, with no corresponding change in bile salt secretion. In contrast, no reduction of phospholipid or cholesterol was found in BSP-GSH.

Table 1 shows the effect of BSP (100 nmol/min per 100 g body wt. infusion) on the fatty acid composition of phospholipids. There was no significant change in the fatty acid composition in phospholipids despite the drastic reduction of phospholipid in bile.

Serum clearance and hepatic content of BSP

To exclude the possibility that the impaired bile secretion of BSP in EHBRs was due to an impaired hepatic uptake of BSP, the serum clearance and hepatic content of BSP were measured. Figure 5 shows the serum clearance of BSP. The clearance of BSP in EHBR was slightly delayed as compared with that of SDR, but did not differ significantly from that of SDR 20 min after the bolus injection of BSP. The hepatic content of BSP in EHBR (74.1 $\pm$ 6.9 %) was similar to that of SDR (69.1 $\pm$ 7.1 %) 10 min after the bolus injection of BSP.

DISCUSSION

BSP has been shown to decrease the biliary secretion of phospholipids and cholesterol without affecting the secretion of bile salts in the dog [3]. We recently reported that BSP produces uncoupling in Wistar rats and that its mechanism 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 micelles in the bile canaliculus [14]. In the present study, in order to further elucidate the mechanism(s) of the uncoupling phenomenon by BSP, we compared the effects of BSP and BSP-GSH on biliary lipid secretion in SDRs and EHBRs. EHBR shows a defective hepatobiliary transport of various organic anions, including bilirubin, BSP and BSP-GSH, but an almost normal secretion of bile acids [19,25]. In addition, bilirubin and BSP elimination from the serum after bolus injection of bilirubin or BSP in EHBR did not differ from that of SD rats [19,25]. Although the exact mechanism of secretory defect of organic anions in EHBR has not been elucidated, a recent study indicated that it is related to canalicular membrane transport [19].

We reproducibly observed the apparent uncoupling of biliary phospholipids and cholesterol from bile salt secretion by BSP in a dose-dependent manner in SDRs, which was similar to our previous study in Wistar rats [14]. In contrast, this uncoupling phenomenon was not observed in EHBRs with a defect in biliary secretion of BSP. Further, the hepatic content of BSP in EHBRs was in the same range in SDRs. These data strongly suggest that the intracellular mechanism does not involve the uncoupling phenomenon by BSP. In addition, Takikawa et al. [19] demonstrated that the profile of cytosolic binding protein, which binds to both bile acids and organic anions, was similar in EHBRs to that of SDRs.

The inhibition of the organic-anion-transport process of the canalicular membrane may be responsible for the uncoupling phenomenon. Despite the possibility that BSP-GSH may share, in part, the pathways for biliary secretion with unconjugated BSP at the canalicular membrane level, BSP-GSH did not produce the uncoupling phenomenon in SDRs, whereas unconjugated BSP did. Therefore, an inhibition of the canalicular membrane level is unlikely to be a crucial factor in this phenomenon produced by BSP infusion.

The mechanism of bile secretion includes the primary secretion of bile salts into the canaliculus, followed by that of lipids. The subsequent association of bile salts and lipids results in the formation of mixed micelles [22]. On this basis the interaction between organic anions and bile salts in the bile canaliculus could inhibit the capacity of bile salts to induce the secretion of phospholipids and cholesterol into bile.

Thus the uncoupling phenomenon by BSP seemingly resulted from an interaction between BSP and bile-salt micelles in the bile canaliculus. However, it is known that BSP is excreted into bile as conjugated and unconjugated forms, with the conjugated form being primarily (> 90 %) BSP-glutathione in rats [16]. We therefore also examined the effect of BSP-GSH on biliary lipid secretion. The uncoupling phenomenon was not found when BSP-GSH was infused at the same biliary output as BSP, which caused a drastic reduction in biliary lipid secretion. It was recently found that organic anions partition into various biliary

lipid particulate species according to their relative hydrophobicities [14,18,23]. BSP was predominantly associated with bile-salt micelles, whereas Indocyanine Green, a more hydrophobic organic anion, was co-eluted with the vesicle fractions, and relatively hydrophilic organic anions such as BSP-GSH were found as small self-aggregates [14,18]. Among these organic anions, only BSP decreased biliary lipid secretion without affecting the secretion of bile salts. [14].

We also examined the effects of BSP on the fatty acid composition of phospholipids, and found no significant change in the fatty acid composition of phospholipids, despite the drastic reduction of phospholipid in bile. These data suggest that the inhibitory effect of BSP affects a general, rather than a specific, vesicular pathway.

We conclude that the uncoupling of biliary lipids from bile salts during BSP infusion is, in part, due to the inhibition of the capacity of the bile-salt micelles to induce the secretion of phospholipids and cholesterol. This inhibition appears to be caused by the interaction of BSP with micelles in the bile canaliculus.

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