Retrograde intrabiliary injection of amphipathic materials causes phospholipid secretion into bile

Taurocholate causes phosphatidylcholine secretion, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate (CHAPS) causes mixed phospholipid secretion

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The control of biliary phospholipid and cholesterol secretions by bile acid was studied by using the technique of retrograde intrabiliary injection. Taurocholate (TC), a moderately hydrophobic bile acid, taurodehydrocholate (TDHC), a hydrophilic non-micelle-forming bile acid, and 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate (CHAPS), a detergent, were individually administered by retrograde intrabiliary injection (RII) into the biliary tree, and bile acids, phospholipids and cholesterol subsequently appearing in the bile were measured. TC (1.3 μmol ; 45 μl) injected retrogradely provoked a 3.5-fold increase in biliary phospholipid output for 40 min, as compared with the saline control. Injection of 2.7 μmol of TC (90 μl) caused a 7.5-fold increase in phospholipid output, which reached a peak at 12 min after RII, and phospholipid output continued for 40 min. Cholesterol output was also elicited under these conditions, showing both dose-dependency and extended secretion. Injection of 1.8 μmol of TDHC caused very little increase in either biliary phospholipid or cholesterol. Injection of 0.9 μmol of CHAPS (45 μl) provoked a single substantial peak of phospholipid output in the 3 min bile sample. T.l.c. analysis of the phospholipid extracts of the bile collected after each compound showed, for TC, a single compound which co-migrated with the phosphatidylcholine standard, whereas for CHAPS substantial amounts of other phospholipids were present.

INTRODUCTION

Bile acids and biliary lipids (phospholipids and cholesterol) are important components in bile. Owing not only to their inherent scientific interest, but also to their contribution to gallstone formation, the processes of biliary lipid secretion have recently become a subject of renewed study.

In experiments with orthograde-venous presentation, the infusion of exogenous bile salts into perfused livers or bile-fistula rats results in increased output of endogenous phospholipids and cholesterol into bile. In the physiological range of bile acid infusion, the output of phospholipids keeps pace with the output of bile acids, and a steady phospholipid/bile-salt molar ratio is maintained close to 0.06 (see Rahman *et al.*, 1986).

Although the secretion of bile acids thus appears to be coupled to biliary lipid secretion, the actual point of the interaction(s) remains to be established. Lowe *et al.* (1984) have observed that bile acids are put out into bile before the lipid output and, in other reports, lipid output has been shown to be decreased by several different compounds without influencing the bile acid output (Bellringer *et al.*, 1988*a,b*). These findings indicate that bile acids are secreted into the bile canaliculus independent of lipid secretion, and point to the formation of bile acid-biliary mixed lipid micelles probably taking place in the bile-canalicular lumen at a post-secretory stage of bile acid output (see Coleman, 1987*a,b*).

In model studies with different bile acids, a pre-lytic removal of lipid from the plasma membrane of erythrocytes has been observed (see Coleman, 1987*b*). The immediate origin of the lipid in bile is unknown, but it has been suggested to be derived from specific microdomains of the bile-canalicular membrane, which may be shed into the canalicular lumen as a consequence of a vesiculation initiated by the interaction of bile acids with the membrane (Lowe *et al.*, 1984; Coleman, 1987*b*).

Retrograde intrabiliary injection (RII) (Fujimoto, 1982; Olson & Fujimoto, 1979; Peterson & Fujimoto, 1975) is a technique which allows introduction of materials into the biliary tree directly; the present paper reports its use to study the effects of presenting bile acids to the bile-canalicular membrane and luminal contents directly. The nature of the responses to different bile acids, i.e. to a bile acid, taurocholate (TC), to a hydrophilic non-micelle-forming bile acid, taurodehydrocholate (TDHC), and to the detergent CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate}, are used to gain further information on the processes of biliary lipid secretion and their control.

MATERIALS AND METHODS

Materials

TC and TDHC were obtained from Calbiochem-Behring Corp., Bishops Cleeve, Herts., U.K. The detergent CHAPS and the enzyme assay kits for aspartate

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; TC, taurocholate; TDHC, taurodehydrocholate; RII, retrograde intrabiliary injection.

aminotransferase and γ -glutamyltransferase were obtained from Boehringer Mannheim. Sodium tauro[carbonyl- 14 C]cholate was obtained from Amersham International, Amersham, Bucks., U.K. Plastic cannula tubing (PP10) was obtained from Portex, Hythe, Kent, U.K.

Perfusion of isolated liver

Male Wistar rats (370–400 g) were used throughout. The bile duct was cannulated with PP10 polyethylene tubing under pentobarbital anaesthesia. The liver was isolated *in situ* as described by Hems *et al.* (1966), and was perfused at a flow rate of 15–17 ml/min with 150 ml of Krebs–Ringer bicarbonate solution at pH 7.4, containing 2 mM- CaCl_2 , 5 mM-glucose, 1% bovine serum albumin, a physiological amino acid mixture and 20% (v/v) packed human erythrocytes. This solution was recycled, gassed continuously with O_2/CO_2 (19:1) and maintained at $37 \pm 0.5^\circ\text{C}$ within a thermostatically controlled cabinet (Rahman *et al.*, 1986).

For the initial 60 min of perfusion, the isolated liver received bile-acid-free perfusion medium; during this period the output of both bile acids and lipids in 3 min bile collections declined to the values indicated at 0 min (Figs. 1–3).

Retrograde intrabiliary injection (RII)

Saline (45 μl) containing 30 mM-TC, 40 mM-TDHC or 20 mM-CHAPS was injected manually from a syringe into the biliary tract via the bile-duct cannula. In another group of rats, 90 μl of saline containing 30 mM-TC was injected into the biliary system. Control rats received 90 μl of saline. The injection rate in each experiment was approx. 45 $\mu\text{l/s}$. Immediately after the injection, the syringe was detached from the bile cannula, and bile samples were collected at 3 min intervals into pre-weighed tubes (based on the method of Olson & Fujimoto, 1979).

Chemical and enzyme assays

Phospholipid present in bile was determined by the method of Bartlett (1959) after lipid extraction by the method of Bligh & Dyer (1959). Cholesterol in bile was analysed by g.l.c. as trimethylsilyl ether derivatives, by a slight modification of the method of Vanlerenberghe & Cassaigne (1968). Bile acids in bile were assayed by using hydroxysteroid dehydrogenase (EC 1.1.1.50) as described by Coleman *et al.* (1979). γ -Glutamyltransferase was assayed with a Boehringer test kit.

For phospholipid chromatography, lipid extracts of bile were prepared by the methods of Bligh & Dyer (1959) or of Folch *et al.* (1957). The extracts were dried down, taken up in a minimum volume of chloroform and chromatographed as described by Skipski *et al.* (1964), with authentic phosphatidylcholine and total liver phospholipids as standards.

Radioactivity recovery experiments

Sodium tauro[carbonyl- 14 C]cholate (0.5 μCi ; sp. radioactivity 55.7 $\mu\text{Ci}/\mu\text{mol}$) was mixed with non-radioactive taurocholate to a final concentration of 30 mM. A 45 μl portion of this was administered by retrograde intrabiliary injection as described above. Bile samples were collected at 3 min intervals for radioactivity determination. Perfusion fluid was collected after a single pass through the liver at 15 s intervals for 6 min

immediately after intrabiliary injection: it was centrifuged at 1400 *g* for 1 min, and the erythrocyte-free supernatant was taken for radioactivity determination. At 12 min after the injection, the liver was cut out, weighed, homogenized in a Potter–Elvehjem homogenizer, and a small sample was taken for radioactivity counting in a toluene-based scintillant containing Triton X-100/toluene (1:10, v/v).

RESULTS

Taking into consideration the dead space of the biliary cannula (about 5 μl), a total volume of either 40 μl or 85 μl of material was injected manually into the bile-canalicular lumen. The volume of 40 μl is approximately the volume of the biliary tree (see the Discussion section).

Effects of taurocholate

The bile-flow rate after the intrabiliary injection of TC or saline at 0 min is shown in Fig. 1(a). A peak of bile-flow rate after saline presentation was observed, and is probably related to the distensible property of the biliary tree, such that part of the fluid injected is rapidly returned to the collection tube. A smaller peak was observed when 45 μl of TC was injected; injection of 90 μl of TC caused an even smaller increase in bile flow as compared with the 90 μl of saline. After this peak, the bile-flow rates for both saline and 1.3 μmol of TC returned to basal values, but a further decrease in flow was seen after 2.7 μmol of TC.

Fig. 1(b) shows that bile-acid output after saline injection remained low throughout the collection, whereas after the injections of TC bile-acid output peaks were observed approx. 3 min after injection; the peak then fell abruptly back close to basal values, continuing only slightly above basal values until the end of the experiment. The injection of 0.5 μCi of [14 C]TC (1.3 μmol) resulted in a recovery of $59.5\% \pm 3.9\%$ in bile, $10.7\% \pm 1\%$ in the liver and $12.2\% \pm 3\%$ in the perfusion fluid, with 17.5% unaccounted for ($n = 3$), within the time scale of the initial peak (12 min).

The phospholipid output into bile is shown in Fig. 1(c). After the two different amounts of TC, phospholipid outputs were found to increase, but the output profiles were different. A 1.3 μmol amount of TC caused approx. 3.5-fold increase in phospholipid output as compared with both the saline control and its basal value; the peak also occurred 6 min after RII, declined slightly and was then maintained at an output of about 2.3 nmol/min. A 2.7 μmol amount of TC provoked approx. 7.5-fold increase at the peak, or 3-fold increase as compared with the control or its basal value, respectively. The peak occurred 12 min after RII and then declined slowly to about 3 $\mu\text{mol/min}$.

The cholesterol output is shown in Fig. 1(d); the lower TC administration produced a small and continuous increase in output as compared with the control, with an apparent small late peak at 24 min, whereas the higher TC administration produced a substantial and prolonged increase; the peak and decline are different from those of phospholipid. Detailed interpretations of the relative output of phospholipids and cholesterol are complicated by the differences in the points at which analyses were made; samples for cholesterol determination were pooled over a 6 min period, whereas those for phospholipid were 3 min samples.

Fig. 1. Effects of taurocholate or saline on (a) bile flow, (b) bile acid output, (c) phospholipid output and (d) cholesterol output

Livers were perfused recyclically for 60 min to cause partial depletion of the endogenous bile acid pool before the retrograde injection of materials: 45 μl (○) or 90 μl (□) of 30 mM-TC or 90 μl of saline (●) was then injected manually at 0 min into the biliary tree via a biliary cannula at a rate of approx. 45 $\mu\text{l}/\text{s}$. Values before 0 min represent values of the appropriate parameters. All values represent means of three experiments; the error bars represent S.E.M.

Fig. 2. Effects of taurodehydrocholate on (a) bile flow, (b) bile acid output, (c) phospholipid output and (d) cholesterol output

Procedures of liver perfusion and retrograde injection were the same as described in Fig. 1, but 45 μl of 40 mM-TDHC (○) was used. The same control group as in Fig. 1 was used (●). All values represent means of three experiments and the bars represent S.E.M.

Effects of TDHC and CHAPS

TDHC (1.8 μmol) and CHAPS (0.9 μmol) were similarly injected, as described in the Materials and methods section. Fig. 2(a) shows the bile-flow rate after the injection of TDHC (45 μl) as compared with the saline

control. A larger peak was observed, indicating that TDHC may have induced a choleresis, especially since the volume of TDHC injected is less than that used in the control. The bile acid output after TDHC showed a much lower and broader peak than that after TC,

Fig. 3. Effects of CHAPS on (a) bile flow, (b) bile acid output and (c) phospholipid output

Operational procedures were the same as described in Fig. 1, but 45 μL of 20 mM-CHAPS (○) was used. Values represent averages of two experiments; the bars represent ranges. The same control group as in Fig. 1 was used (●).

representing partly endogenous bile acid secretion and partly hydroxy derivatives of TDHC. In contrast with the effect of TC, TDHC only caused a small increase in phospholipid (Fig. 2b). Although the cholesterol amount shown in Fig. 2(c) was higher than the control, its basal value was also higher than the control, and thus TDHC did not appear to elicit appreciable cholesterol secretion when presented to the liver via the biliary tract.

CHAPS caused an increase in bile flow, but this was delayed as compared with the effect of saline (Fig. 3a). No overall change in bile acid output (Fig. 3b) was observed after CHAPS, but a substantial effect on phospholipid output (Fig. 3c) was observed at 3 min; this 3 min phospholipid peak then returned sharply back to the basal value. No cholesterol determinations were performed in this series of experiments.

Analysis of lipids secreted after TC or CHAPS injections

Analyses of the phospholipids present in bile samples were performed on lipid extracts prepared by extraction as described by Bligh & Dyer (1959) or Folch *et al.* (1957). Only a single compound, co-migrating with the phosphatidylcholine standard, appeared in the lipid extracts (by either extraction method) after TC injection (see Fig. 4). However, the injection of CHAPS produced not only phosphatidylcholine but also substantial amounts of other phospholipids, i.e. phosphatidylethanolamine, phosphatidylserine, sphingomyelin etc. (Fig. 4).

Assessment of cell damage during retrograde injections

The damage to the liver by the RII technique was monitored by measuring aspartate aminotransferase and γ -glutamyltransferase in both the bile samples and the perfusate. No activity of aspartate aminotransferase or increase in γ -glutamyltransferase activity was detected in the perfusate, indicating that little or no damage was inflicted at the sinusoidal pole of the cell. However, a small increase in activities of both enzymes was detected in bile after injection of 90 μL of TC; a larger increase was detected in bile after CHAPS injection. However, the damage in both cases is very small, since the aspartate aminotransferase activity in bile relative to the total activity in the liver is minimal, i.e. < 0.01 % (Table 1).

DISCUSSION

In the present report a standard injection solution of 40 μL (excluding the 5 μL of the biliary cannula) was used; this has been estimated by Olson & Fujimoto (1979) to be the distended biliary-tree volume. Thus 40 μL of injection solution may represent more closely the tidal volume of the biliary tree, and will be expected to be pushed up high into the bile canaliculi. Little damage to the liver is caused by this retrograde injection volume of 45 μL , or of 90 μL , as shown by the very small release of aspartate aminotransferase and γ -glutamyltransferase, which are indicators of hepatocellular damage.

The biliary peak of TC after injection of 45 μL results in ~ 60 % recovery of TC in the bile in the first peak. The material entering the perfusion fluid (~ 12 %) entered within the first 1 min and probably represents material passing the tight junctions, which may have become transiently more permeable, owing to back pressure during the injection of TC. This TC, together with that recovered in the liver, may be responsible for the slight elevation in bile acid concentrations in the period 6–36 min, and is greater than the recoveries of other materials after retrograde administration (see Peterson & Fujimoto, 1975; Olson & Fujimoto, 1979; Fujimoto, 1982).

Retrograde injection of bile acid brought about substantial secretion of phospholipid into the bile. Phospholipid secretion is not coincident with the tidal volume of the biliary tree, and reaches a peak some 3–6 min later (Fig. 1c). This is reminiscent of the lag time for phospholipid secretion seen in kinetic orthograde venous perfusion experiments (Lowe *et al.*, 1984). In contrast, the phospholipid secretion peak elicited by retrograde intrabiliary injection of CHAPS (Fig. 3b) is virtually coincident with the tidal-volume peak.

Fig. 4. Species profile of biliary phospholipids before, during and after RII of TC or CHAPS

The conditions for RII of TC (2.7 μ mol) or CHAPS (0.8 μ mol) are given in the legends to Figs. 1 and 3 respectively. Samples at each time point were pooled as indicated, and 50 μ l portions (100 μ l of zero-time sample, TC) were extracted and chromatographed as described in the Materials and methods section, with a total liver lipid extract as standard (Std.). When the plates were scraped and the appropriate spots quantified by digestion and phosphate determination, the CHAPS-elicited material in lane 2 contained 45% phosphatidylcholine (PC), 23% phosphatidylethanolamine (PE), 12% phosphatidylserine/phosphatidylinositol (PS/PI), 6% sphingomyelin (SP) and 14% lysophosphatidylcholine (LPC).

Table 1. Assessment of liver damage

Bile samples were collected after RII of designated materials into the biliary tree and assayed as described in the Materials and methods section. Aspartate aminotransferase values were expressed as the percentage of the total enzyme activity in bile as a total activity in the liver. γ -Glutamyltransferase was expressed as total enzyme activity (munits) per μ l of bile. Under each material is indicated the amount (μ mol) used and the volume injected. All values are averages of two readings.

Enzyme	Sample	Saline (90 μ l)	TC (1.3 μ mol; 45 μ l)	TC (2.7 μ mol; 90 μ l)	CHAPS (0.8 μ mol; 45 μ l)
Aspartate aminotransferase (%, $\times 10^{-3}$)					
	-10-0 min	0.06	0.07	0.07	0.07
	0-10 min	0.08	0.15	1.28	9.9
	10-20 min	0.06	0.12	0.19	2.1
	20-30 min	0.06	0.12	0.04	0.67
γ -Glutamyltransferase (munits/ μ l)					
	-10-0 min	0.2	0.2	0.2	0.2
	0-10 min	0.2	0.3	0.8	1.1
	10-20 min	0.2	0.2	0.6	0.6
	20-30 min	0.2	0.2	0.4	0.3

The amount of phospholipid secretion elicited by retrograde TC injection is increased with the amount of TC originally presented; the amounts secreted are not linearly related, and thus some form of threshold may be operating. The increased secretion is sustained for a substantial period, i.e. over 40 min, after the original presentation. During this time only a small amount of bile acids is being secreted. The phospholipid:bile acid ratio after the secretion varies from about 0.05, before injection, to approx. 0.01 at the tidal volume peak; it subsequently increases to about 0.12 and 0.28, respectively, at peak phospholipid secretion for the two bile acid injection doses. The higher value subsequently declined towards the lower value in the later time periods. These values are considerably in excess of the 0.06 seen during extensive orthograde TC infusion (see Rahman *et al.*, 1986) and must represent the secretion of the phospholipid in the form of material with a relatively high phospholipid:bile acid ratio. The concentration of bile acid in the bile in the later stages of the experiment is of the order of 2 mM or less, considerably lower than the critical micellar concentration of TC.

One possibility for the secretion of lipid into the bile is the effect of the amphipathic TC acting as a detergent-like molecule and causing the membrane of the bile canaliculus to vesiculate outwards and release vesicular material into the bile. The need for an amphipathic molecule is shown by the relative ineffectiveness of TDHC in these experiments (Fig. 2). The detergent molecule CHAPS is capable of releasing lipid into the bile, but in this case the release is rapid and, unlike that provoked by TC, is not prolonged (Fig. 3).

A further difference between the effects of TC and CHAPS is seen in the nature of the lipids released. TC appears to release phosphatidylcholine [after lipid extractions by the methods of Bligh & Dyer (1959) or of Folch *et al.* (1957)], whereas CHAPS provokes the release of a wider population of lipids. Such lipids, phosphatidylethanolamine, sphingomyelin etc., more resemble those of the canalicular membrane, and it may therefore be that CHAPS causes a non-specific vesiculation of membrane in a fairly general sense. On the other hand, TC elicits the release of a unique phospholipid profile which is almost exclusively phosphatidylcholine, and this resembles the pattern of native biliary lipid. The effect of TC in the biliary tree, or on the canalicular membrane, is thus more specific than that of CHAPS, and may thus involve specific micro-domains of the canalicular membrane. There is a delay in the secretion of the lipid, and this delay could be due to the time taken for intercalation

into, and subsequent processing of, the membrane by the bile acid.

The secretion of lipid after initial exposure to TC (but not to CHAPS) is continuous, and suggests that somehow the bile acid has signalled the liver cell of its presence and elicited a continuous response. This effect is well documented for orthograde perfusion (see Rahman & Coleman, 1987). The signalling, when elicited by retrograde injection, may be operating in part extracellularly, i.e. the intercalation into the membrane and the loss of lipid micro-domain, and in part intracellularly, e.g. by intracellular interaction with the cytoskeleton, 'biliary lipid supply vesicles' or with lipid synthesis.

Bile acids are also capable of bringing about the secretion of cholesterol. The effects on cholesterol secretion are, however, somewhat less marked than those on phospholipid secretion.

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