Inhibition of biliary cholesterol and phospholipid secretion during cyclobutyrol-induced hydrocholeresis

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The effects of sodium cyclobutyrate, a synthetic hydrocholeretic drug, on biliary lipid secretion and on the biliary outputs of several plasma-membrane enzymes were investigated in anaesthetized rats. Administration of a single oral dose of cyclobutyrol (0.72 mmol/kg body wt.) reduced biliary concentration and output of cholesterol and phospholipid. However, bile acid secretion was not significantly modified. This uncoupling effect of lipid secretion remained even when the choleretic response to the drug had ceased. It additionally led to a statistically significant decrease in the cholesterol/bile acid and phospholipid/bile acid molar ratios and in the lithogenic index of the bile. The biliary outputs of the plasma-membrane enzymes alkaline phosphatase and γ -glutamyltransferase were markedly reduced by the drug. When cyclobutyrol was administered to rats which had been previously fed with a high-cholesterol diet, the effects of cyclobutyrol persisted, but were less marked. Our results demonstrate that the bile acid-independent choleresis induced by cyclobutyrol (related to its pharmacokinetic effect) is accompanied by a pharmacodynamic action that selectively reduces the secretion of biliary lipids. This is due to an uncoupling of the secretion of cholesterol and phospholipids from that of bile acids. Possible explanations for the biliary response to cyclobutyrol are discussed.

INTRODUCTION

The biliary secretion of cholesterol and phospholipid is related to, and is partially dependent on, bile acid secretion (Carey & Mazer, 1984; Coleman, 1987). However, although bile acids may be needed to stimulate the secretion of biliary lipids, bile acid and biliary lipid secretions are nevertheless separate processes (Barnwell *et al.*, 1984; Coleman, 1987).

Numerous compounds are known to dissociate the secretion of biliary lipids from that of bile acids. Examples of these are ampicillin (Apstein & Russo, 1985), cefoperazone (Pattinson *et al.*, 1987), iodipamide (Apstein & Robins, 1982), ioglycamide (Bell *et al.*, 1978), sulphobromophthalein (Shaffer & Preshaw, 1981), valproic acid (Bellringer *et al.*, 1988b) and other compounds (see the Discussion section). When presented to the liver, all these agents reduce biliary cholesterol and phospholipid secretion, with little or no parallel effect upon bile acid secretion. Some of these organic anions, such as ampicillin, iodipamide, ioglycamide, cefoperazone and valproic acid, are also known to be excreted into bile and to lead to increases in bile flow. Cicloxilic acid has been reported to reduce biliary lipid secretion in the rat (Schiantarelli & Murmann, 1978) and in man (Carulli *et al.*, 1983; Di Padova *et al.*, 1984), but no information exists regarding other drugs of the so-called 'synthetic choleretic' group customarily used in the chemotherapy of several liver diseases. Cyclobutyrol, [α -(hydroxy-1-cyclohexyl)butyric acid] is a known synthetic choleretic that induces a dose-dependent hydrocholeresis (Grandgeorge *et al.*, 1978; Roquet & Jousse, 1960). However, its possible pharmacodynamic action on biliary lipid secretion has not been investigated. Finally, it is known that a high-cholesterol diet enriched with cholic

acid induces hypercholesterolaemia and hypercholesterobilia in the rat (M. J. Monte, A. Estellar & R. Jiménez, unpublished work; Uchida *et al.*, 1977, 1980; Turley & Diestchy, 1982). The purpose of the present study was to examine the possible uncoupling effects of cyclobutyrol on biliary lipid secretion in rats under two different experimental conditions: (1) in animals with normal serum and biliary cholesterol levels, and (2) in animals in which, owing to alterations in their cholesterol metabolism, the serum and biliary cholesterol values were increased by a fat-enriched diet.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats, 34 in number and weighing from 240 to 270 g, were used. The animals were housed in a room maintained at 22 °C with humidity ranging from 45 to 55% and under a constant light cycle (12 h dark/12 h light). The animals were divided into two principal groups which were fed with two different diets: (1) a standard commercial stock diet (Panlab, Barcelona, Spain) with no more than 3% (w/w) of fat; (2) a hypercholesterolaemia-inducing diet; the latter was prepared with the same commercial diet supplemented with 1.5% cholesterol (Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.5% cholic acid (Sigma Chemical Co.) and 3% olive oil. After 20 days on this cholesterol-enriched dietary regimen, the animals were used for the biliary study. All animals had free access to food and water, and were not starved before experiments.

Methods

Under sodium pentobarbital anaesthesia (50 mg/kg

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body wt., intraperitoneally) a routine tracheotomy and a median laparotomy were performed. The bile duct and the left carotid artery were cannulated with polyethylene tubing. A cannula was also introduced into the first part of the duodenum to allow periodic administration (each 15 min) of previously collected bile from other non-treated rats in order to maintain the enterohepatic circulation of bile acids (Kuipers *et al.*, 1985; Jiménez *et al.*, 1988). Losses in body temperature were prevented by a thermostatically controlled warming plate, and the rectal temperature was maintained at $37.5 \pm 0.5^\circ\text{C}$. Experiments were started after an equilibration period of 30 min to allow bile flow to stabilize. After collecting two 15 min bile samples under basal conditions, 0.72 mmol of cyclobutylol (sodium cyclobutylate; Laboratorios Ferrer, Barcelona, Spain)/kg body wt. dissolved in 1.5 ml of 0.9% saline was administered orally. Controls received an equal volume of physiological saline only and were used for control purposes. Specimens of bile were taken and immediately stored at -40°C until later assay. After 15 min, 100 μl blood samples were obtained for plasma cholesterol determinations. At the end of the experiments the rats were killed by exsanguination.

Analytical methods. Bile flow was determined gravimetrically without correction for relative density, assuming an absolute bile density of 1.0 g/ml. Bile acid concentrations in bile were assayed by the method of Talalay (1960), with 3α -hydroxysteroid dehydrogenase (EC 1.1.1.50). Cholesterol concentrations in plasma and bile were determined by a modification of the enzymic method of Bolton *et al.* (1980), using a commercial kit

(Cholesterol enzymatique PAP, Biomerieux, Charboniers les Bains, France). Phospholipid concentrations in bile were measured with a commercial enzymic method (Phospholipides enzymatique PAP, Biomerieux) based on that of Takayama *et al.* (1977). The lithogenic index of bile was calculated by the method of Thomas & Hoffman (1973).

Alkaline phosphatase (EC 3.1.3.1) and γ -glutamyl-transferase (EC 2.3.2.2) activities in bile were determined by the methods described by Rej *et al.* (1981) and Persijn & Van der Slik (1976) respectively. Activities were expressed as units/l and their biliary outputs were expressed as $\mu\text{unit}/\text{min}$ per 100 g body wt.

Statistics. Results are expressed as means $\pm$ S.E.M. The data were compared by analysis of variance; when the analysis indicated the presence of a significant difference, the means were compared with the paired *t* test or the pooled *t* test using a significance level of $P = 0.05$ to reject the null hypothesis.

RESULTS

To allow easier comparison between the results obtained from normo- and hyper-cholesterolaemic rats, the values in Figs. 1 and 2 are expressed as percentage variations with respect to the levels before cyclobutylol or saline administration. The amounts corresponding to 100% are shown in Table 1.

In the present study, rats consumed similar amounts of food irrespective of the dietary regimen (approx. 25 g/day; range 21.6–29.3). The rats fed with the

Fig. 1. Effect of cyclobutylol on biliary secretion in normocholesterolaemic rats

Mean percentage changes ($\pm$ S.E.M.) in bile flow (a) and bile acid (b), cholesterol (c) and phospholipid (d) outputs are shown. Cyclobutylol (0.72 mmol/kg) administration (arrow) (■) was performed at 30 min after the beginning of the experiments. Control normocholesterolaemic rats (□) received physiological saline alone. Values represent percentage variations with respect to the preadministration period (0–30 min), the 100% levels for which are shown in Table 1.

Fig. 2. Effect of cyclobutyrol on biliary secretion in hypercholesterolaemic rats

Mean percentage changes ($\pm$ S.E.M.) in bile flow (a) and bile acid (b), cholesterol (c) and phospholipid (d) outputs are shown. Cyclobutyrol (0.72 mmol/kg) administration (arrow) (■) was performed at 30 min after the beginning of the experiments. Control hypercholesterolaemic rats (□) received physiological saline alone. Values represent percentage variations with respect to the preadministration period (0–30 min), the 100% levels for which are shown in Table 1.

Table 1. Effects of a single oral dose of cyclobutyrol (0.72 mmol/kg body wt.) on biliary secretion in normocholesterolaemic and hypercholesterolaemic anaesthetized rats

Results are mean values $\pm$ S.E.M. for bile flow and biliary concentrations (BC) and secretion rates (SR) of bile acids, cholesterol and phospholipids before (0–30 min) and after (30–90 min) drug administration. There were six to eight animals in each group. * $P < 0.05$, significantly different from pre-test values.

Parameter	Rats... Normocholesterolaemic		Hypercholesterolaemic	
	Before	After	Before	After
Bile flow (μ l/min per 100 g body wt.)	7.11 $\pm$ 0.83	12.00 $\pm$ 0.88*	9.45 $\pm$ 1.01	14.57 $\pm$ 1.91*
Bile acids				
BC (mmol/l)	31.9 $\pm$ 4.4	17.2 $\pm$ 3.8*	29.9 $\pm$ 3.2	17.4 $\pm$ 2.7*
SR (μ mol/min per 100 g)	0.23 $\pm$ 0.04	0.19 $\pm$ 0.04	0.29 $\pm$ 0.03	0.25 $\pm$ 0.04
Cholesterol				
BC (μ mol/l)	0.52 $\pm$ 0.08	0.22 $\pm$ 0.07*	0.42 $\pm$ 0.05	0.23 $\pm$ 0.03*
SR (nmol/min per 100 g)	3.49 $\pm$ 0.63	2.16 $\pm$ 0.26*	4.26 $\pm$ 0.68	2.92 $\pm$ 0.74
Phospholipids				
BC (μ mol/l)	3.35 $\pm$ 0.68	1.44 $\pm$ 0.21*	3.92 $\pm$ 0.47	2.13 $\pm$ 0.58*
SR (nmol/min per 100 g)	23.6 $\pm$ 4.1	14.3 $\pm$ 2.9*	39.7 $\pm$ 4.3	30.6 $\pm$ 4.1

hypercholesterolaemic diet appeared healthy and gained weight at the same rate as those fed with the commercial diet, as described previously by others working with rats fed with diets of compositions similar to those used in the present work (Kawamoto *et al.*, 1981; Garg *et al.*, 1985).

Both in the control animals (physiological-saline-treated rats) and in those subjected to cyclobutyrol administration under basal conditions, mean bile flow, bile acid, cholesterol and phospholipid biliary

concentrations and excretion rates were similar to those reported in the literature for this species (Uchida *et al.*, 1977; Turley & Diestchy, 1981; García-Marín & Esteller, 1984; Apstein & Russo, 1985; Kuipers *et al.*, 1985). Additionally, in both the normo- and hypercholesterolaemic animals, bile flow and biliary bile acid, cholesterol and phospholipid excretion decreased progressively after common-bile-duct cannulation; this reduction over time was very slight in the case of bile flow, but more pronounced for bile acid and cholesterol

excretion. Such a progressive depression in biliary secretion in the rat has been reported previously by several workers (Turley & Dietsch, 1981, 1982; Garcia-Marín & Esteller, 1984; Kuipers *et al.*, 1985, 1987).

Effect of cyclobutyrol on biliary lipid secretion in normocholesterolaemic rats

Cyclobutyrol administration caused a choleretic effect with a peak increase in bile flow after 30–45 min, thereafter decreasing to approaching pre-test values (Fig. 1), similar to what has been described previously (Grandgeorge *et al.*, 1978). The bile acid concentration before cyclobutyrol administration was 31.9 ± 4.4 mmol/l and fell to 17.2 ± 3.8 mmol/l after 60 min ($P < 0.05$). However, compared with the control animals or with the pre-test values, the bile acid secretion rate was not modified significantly during the cyclobutyrol biliary response (Fig. 1; Table 1).

The biliary concentration and secretion of cholesterol and phospholipid started to fall in parallel with the increase in bile flow. By 45 min, they were 50 and 53% respectively of the values before cyclobutyrol administration (Fig. 1). Subsequently, these values remained low and, at the end of the experiments, phospholipid secretion was still 50–60% below the control values ($P < 0.05$).

Table 2 ('NCh') shows the effects of cyclobutyrol on the cholesterol/bile acid, cholesterol/phospholipid and phospholipid/bile acid molar ratios in normocholesterolaemic rats. Cyclobutyrol significantly reduced the cholesterol/bile acid and phospholipid/bile acid ratios, without changing the cholesterol/phospholipid ratio. The lithogenic index of bile before drug administration, 0.39 ± 0.01 , fell to 0.29 ± 0.02 after cyclobutyrol ($P < 0.05$).

Effect of cyclobutyrol on biliary lipid secretion in hypercholesterolaemic rats

After 20 days feeding of a high-cholesterol diet *ad libitum*, serum cholesterol levels were 3-fold higher than those found in the standard diet-fed rats (5.34 ± 0.43 mmol/l, $n = 6$, compared with 1.49 ± 0.36 mmol/l, $n = 6$ respectively). Basal biliary secretion in these hypercholesterolaemic rats was stimulated (A. Esteller & R. Jimenez, unpublished work; Uchida *et al.*, 1977, 1980; Turley & Dietsch, 1982; see also the Discussion section). Bile flow and bile acid, cholesterol and phospholipid secretion rates were 33, 26, 22 and 68% higher respectively than in the normocholesterolaemic animals (Table 1).

The effect of the same oral dose of cyclobutyrol on biliary secretion in the hypercholesterolaemic rats is shown in Table 1 and Fig. 2. The choleretic response had a similar duration and intensity in both the normocholesterolaemic and hypercholesterolaemic cyclobutyrol-treated animals (Figs. 1 and 2). Again, whereas the bile acid output was not significantly modified by the drug with respect to pre-test values or with respect to the untreated group (Fig. 2), cholesterol and phospholipid secretion rates were decreased (Table 1, Fig. 2). Despite this, the inhibitory effect of cyclobutyrol was lower than in the normocholesterolaemic treated rats. In the case of cholesterol secretion, this had totally recovered after 60–90 min of cyclobutyrol administration (Fig. 2). The effect of biliary cholesterol saturation was also less

Table 2. Biliary cholesterol/bile acid (Ch/BA), cholesterol/phospholipids (Ch/PL) and phospholipids/bile acid (PL/BA) molar ratios in normocholesterolaemic (NCh) and hypercholesterolaemic (HCh) rats before and after the administration of an oral dose of 0.72 mmol/kg body wt. of cyclobutyrol

Results are mean values $\pm$ S.E.M. for six to eight animals. * $P < 0.05$, significantly different from pre-test values; values in parentheses are percentage variations with respect to pre-administration values.

Rats	Ratio...	Ch/BA	Ch/PL	PL/BA
NCh				
Before		16.1 ± 1.8	148 ± 13	114 ± 8
After		$10.3 \pm 1.2^*$	142 ± 15	$88 \pm 9^*$
		(-36)	(-4)	(-23)
HCh				
Before		12.1 ± 2.1	107 ± 9	117 ± 7
After		10.0 ± 1.9	109 ± 12	$101 \pm 5^*$
		(-17)	(-2)	(-13)

Fig. 3. Biliary outputs (b) alkaline phosphatase and (a) γ -glutamyltransferase in normocholesterolaemic rats before and after physiological saline ($\square$) or cyclobutyrol ($\blacksquare$) administration

Oral administration (arrow) of cyclobutyrol (0.72 mmol/kg body wt.) or physiological saline was performed at 30 min after the beginning of the experiments. Activities are expressed as μ units/min per 100 g body wt.; values are means $\pm$ S.E.M. for five animals in each group; * $P < 0.05$, significantly different from pre-test values.

important in the hypercholesterolaemic animals (Table 2, 'HCh').

Effect of cyclobutyrol on biliary outputs of alkaline phosphatase and γ -glutamyltransferase

The results describing the biliary outputs of the plasma-membrane enzymes alkaline phosphatase and γ -

glutamyltransferase are shown in Fig. 3. After cyclobutyrol administration the biliary secretion rates for both enzymes were markedly reduced. A tendency towards a recovery of pre-test values was observed from 60–90 min post-administration.

DISCUSSION

In the present paper we have shown that cyclobutyrol, a classic synthetic hydrocholeretic drug, has a selective effect in reducing cholesterol and phospholipid biliary secretion. However, it does not affect bile acid secretion, either in rats under basal conditions or when the secretion of cholesterol, phospholipids and bile acid was increased by feeding a high-cholesterol diet. Similar findings have been reported for other hydrocholeretic drugs such as cicloxicil acid (Carulli *et al.*, 1983; Di Padova *et al.*, 1984) and other compounds such as ampicillin (Apstein & Russo, 1985; Bellringer *et al.*, 1988a), bilirubin (Apstein & Robins, 1982), cefoperazone (Pattinson *et al.*, 1987), iodipamide (Apstein & Robins, 1982), ioglycamide (Bell *et al.*, 1978), lysine acetylsalicylate (Erlinger *et al.*, 1975), sulphobromophthalein (Shaffer & Preshaw, 1981), valproic and octanoic acids (Bellringer *et al.*, 1988b) and other substances (Kuipers *et al.*, 1987) in different normocholesterolaemic experimental models. However, despite numerous studies, the hepatocellular mechanisms involved in the uncoupling effect are still unclear (see Coleman *et al.*, 1988, for a discussion). A number of explanations could account for this dissociation of lipid secretion in the cyclobutyrol experiments.

Firstly, cyclobutyrol might reduce cholesterol and phospholipid secretion into bile by interfering with their intracellular synthesis. However, Turley & Dietschy (1981) have demonstrated that cholesterol destined for biliary secretion in the rat is largely independent of newly synthesized cholesterol. Since cyclobutyrol reduces phospholipid biliary output (and cholesterol secretion partially depends on phospholipid secretion; Carey & Mazer, 1984), if cyclobutyrol does affect phospholipid synthesis this might account for the inhibition of the biliary output of both compounds, as proposed by other authors. We think that, in view of the speed of the response, this explanation is also unlikely.

Another mechanism that has been postulated to explain the effects of these agents is related to their hydrocholeretic action. The increase in bile flow seen after cyclobutyrol administration is the result of an osmotic choleresis caused by the biliary elimination of the drug (Grandgeorge *et al.*, 1978), as has been proposed for the anions mentioned above. Erlinger *et al.* (1975) have suggested that lysine acetylsalicylate depresses biliary lipid secretion simply as a result of its diluting canalicular bile and reducing micellar bile acid concentrations. Again, we propose that this cannot be the only explanation for our results, since cholesterol and phospholipid biliary outputs remain low when cyclobutyrol-induced choleresis has disappeared. In addition, bilirubin and octanoic acid, which cause a similar degree of inhibition in biliary lipid secretion, have no choleretic effect in the rat (Apstein & Robins, 1982; Bellringer *et al.*, 1988b).

Cholesterol and phospholipids in bile are secreted at the biliary pole of the hepatocyte, probably via vesicle-mediated transfer, whereas bile salts, under non-stimulated conditions, are probably transported across

the cell as discrete molecules rather than in vesicles (Coleman, 1987; Crawford *et al.*, 1988). It is therefore possible that cyclobutyrol and other organic anions might exert their effect by impairing the vesicular movement of lipid and other compounds to the canalicular membrane, possibly by dysfunctions or alterations affecting the cytoskeletal system. Recently, Bellringer *et al.* (1988a,b) have reported that ampicillin and valproic acid not only decrease biliary cholesterol and phospholipid secretion, but also the sinusoidal secretion of a number of proteins that are secreted or transcytosed via vesicular movement. However, cefmetazole, another antibiotic that, like ampicillin, induces choleresis and reduces cholesterol and phospholipid biliary outputs in the rat, does not affect the hepatobiliary transport of horseradish peroxidase, a classic tracer of vesicular transport (R. Jiménez, F. Cava & J. González, unpublished work). As Fig. 3 shows, the biliary outputs of the plasma-membrane enzymes assayed in normocholesterolaemic rats were also significantly reduced during the initial period, in which the biliary secretion of cholesterol and phospholipid was depressed by cyclobutyrol. A number of studies have demonstrated that the liver secretes bile acid in a pattern containing a high proportion of potentially membrane-damaging bile acids, especially those that form micelles. These bile acids stimulate the extraction of several hepatocyte plasma-membrane enzymes from the canalicular membrane (see Barnwell *et al.*, 1984; Coleman, 1987). The fact that the biliary outputs of alkaline phosphatase and γ -glutamyltransferase after cyclobutyrol administration are reduced suggests that cyclobutyrol affects their transcellular movement and/or that the compound induces changes in the structure of the bile canalicular membrane; these would inhibit the removal of enzymes (and cholesterol?). This would thus limit, by direct interaction with the membrane and/or the bile acids, their removal capacity.

It is known that, under physiological conditions, biliary lipids exist as mixed micelles with bile acids. Current evidence indicates that biliary mixed-micelle formation occurs in the canaliculus. Bile acids are transported into the canalicular lumen and the lipids added subsequently (Barnwell *et al.*, 1984; Coleman, 1987). A number of anions that inhibit biliary lipid secretion have been reported to be physically associated with mixed micelles (Scharschmidt & Schmid, 1978). The incorporation of cyclobutyrol into bile acid micelles could interfere with the subsequent solubilization of biliary lipid. We have no direct evidence to confirm or disprove this hypothesis, although such an association has not been found for cefoperazone (Pattinson *et al.*, 1987). Moreover, Indocyanine Green, which has been found to be associated with bile acids micelles, does not uncouple biliary lipid secretion from bile acid secretion (Shaffer & Preshaw, 1979).

However, in the hypercholesterolaemic rats the cyclobutyrol-induced inhibition of biliary lipid secretion was less intense and returned to control values within 1 h. In these animals, the basal biliary secretion of bile acids was enhanced as compared with normocholesterolaemic non-treated rats; in this sense it is known that excess cholesterol in the diet markedly increases the conversion of cholesterol into bile acids. Additionally these response patterns of bile acid metabolism are consistent with increases in blood cholesterol levels in the rat and other species (Uchida *et al.*, 1977, 1980; Turley

& Dietschy, 1982). Accordingly, the enhanced bile acid synthesis due to the dietary cholesterol increases the biliary bile acid outputs, and hence those of cholesterol and phospholipids, as well as bile flow. It is possible that the presence of more bile acids prevents the effects of cyclobutylol on lipids, as has been reported for cefoperazone (Pattinson *et al.*, 1987) and bilirubin (García-Marín & Esteller, 1984). This would therefore favour a physical interaction between cyclobutylol and bile acid micelles as a possible explanation for the phenomenon.

Whatever is found to be the mechanism or mechanisms implied in the biliary lipid response to the administration of cyclobutylol, it does give rise to a bile that is less saturated in cholesterol (Table 2). Similar phenomena have been reported for ampicillin (Apstein & Russo, 1985) and cicloxilic acid (Schiantarelli & Murmann, 1978; Di Padova *et al.*, 1984) and we therefore agree with those authors in the potential importance of these compounds in influencing cholesterol gallstone formation and dissolution.

In summary, our data indicate that cyclobutylol induces a transient choleresis and that this effect is accompanied by a marked reduction in cholesterol and phospholipid biliary secretion and in the biliary output of plasma-membrane enzymes. However, there are no significant modifications in the bile acid secretion rates. Although further work is still required to elucidate the exact mechanism of the uncoupling action of the so-called 'synthetic choleretics' in biliary lipid secretion, a study of the pharmacodynamic effects of this and of the other habitually used hydrocholeretic drugs is of interest not only because it could help in understanding the biochemistry and the physiology of biliary lipid secretion, but also because it might have implications in the formation and dissolution of cholesterol gallstones and in the future development of new dissolving drugs.

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