Inhibition of biliary cholesterol and phospholipid secretion by cefmetazole

The role of vesicular transport and of canalicular events

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A number of organic anions selectively inhibit the biliary secretion of cholesterol and phospholipids without affecting bile acid secretion. We studied the effect of cefmetazole, a third-generation cephalosporin, on biliary lipid secretion in the rat. Injection of cefmetazole at a dose of 200 $\mu\text{mol/kg}$ body wt. induced a choleretic effect and a significant decrease in the biliary output of cholesterol and phospholipid, without changes in bile acid secretion. The decrease was more marked for cholesterol than for phospholipid secretion, with a significant decrease in their molar ratio in bile. The effects were apparently unrelated to an inhibition of intracellular vesicular transport because, after injection of horseradish peroxidase, both the time course and total amount secreted of the protein did not significantly differ between control animals and those receiving cefmetazole. The secretory rate of the lysosomal marker acid phosphatase was not affected by cefmetazole administration. Biliary outputs of the plasma-membrane enzymes alkaline phosphatase and γ -glutamyltransferase were significantly decreased by the antibiotic. These results point to an effect of cefmetazole at the level of the canalicular membrane.

INTRODUCTION

Cefmetazole sodium (7- β -cyanomethylthioacetamido-7 α -methoxy-3-[[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-3-cephem-4-carboxylate) is a semisynthetic derivative of cephamycin that has a broad antimicrobial spectrum and shows a low toxicity both in experimental animals and in clinical studies (Shindo *et al.*, 1982). It has been reported that this compound induces in the rat a dose-dependent choleretic effect by stimulating the bile-acid-independent flow through the osmotic properties of the compound transported into bile (González *et al.*, 1989). Cefmetazole cholestasis is associated with a decreased biliary secretion of cholesterol and phospholipid, without affecting bile acid secretion (González *et al.*, 1989). Numerous compounds are also known to dissociate the biliary secretion of cholesterol and phospholipid from that of bile acids. Examples of these are ampicillin (Apstein & Russo, 1985; Bellringer *et al.*, 1988*b*), cefoperazone (Pattinson *et al.*, 1987), ceftriaxone (Xia *et al.*, 1990), iodipamide (Apstein & Robbins, 1982), valproic acid (Bellringer *et al.*, 1988*a*), sulphobromophthalein (Shaffer & Preshaw, 1981), bilirubin (Apstein, 1984) or cyclobutylol (Monte *et al.*, 1989, 1990).

The mechanisms and sites responsible for the uncoupling action of these compounds are still unclear, although in some cases an intracellular effect has been suggested. Thus ampicillin and valproic acid might inhibit lipid assembly or delivery, or might affect membrane fusion (Bellringer *et al.*, 1988*a,b*). However, the effect of other compounds, such as cyclobutylol, is most probably exerted at the level of the canalicular membrane, because the secretion of different materials involving vesicles remains unaffected (Monte *et al.*, 1990).

The purpose of our study was to examine the mechanisms of the inhibitory effect of cefmetazole on the biliary secretion of cholesterol and phospholipids in the rat. In order to demonstrate a possible impairment of the vesicular movement of lipids to the

canalicular membrane, the hepatobiliary transport of horseradish peroxidase, a classic tracer of vesicular transport, was investigated.

MATERIALS AND METHODS

Materials

Horseradish peroxidase, 3- α -hydroxysteroid dehydrogenase and all other fine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cefmetazole was purchased from Antibioticos S.A. (Madrid, Spain). Sodium pentobarbital was obtained from Claudio Barcia (Madrid, Spain). All other reagents were of the highest quality available. Distilled deionized water was used throughout.

Methods

Male Wistar rats weighing 240–270 g were used. The animals were housed in a room maintained at 22 °C with humidity in the range 45–55 % and under a constant light cycle (12 h light/12 h dark). They received standard laboratory diet (Panlab, Barcelona, Spain) and water *ad libitum*.

The animals were anaesthetized with sodium pentobarbital (50 mg/kg body wt.) given intraperitoneally and a median laparotomy was performed. The bile duct, right jugular vein and right carotid artery were cannulated with polyethylene tubing. Losses in body temperature were prevented by a thermostatically controlled warming plate, and the rectal temperature was maintained at 37.5 ± 0.5 °C.

After two 10 min bile samples had been collected under basal conditions, cefmetazole (dissolved in 1 ml of 0.145 M-NaCl) was given intravenously at a dose of 200 $\mu\text{mol/kg}$. Control animals received 0.145 M-NaCl. Bile was further collected for 2 h over 10 min intervals. In a separate series of experiments, horseradish peroxidase (5 mg/kg) was co-injected with cefmetazole or saline

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and bile was collected at 5 min intervals. At the end of the experiments the rats were killed by exsanguination.

Specific determinations

Bile flow was determined gravimetrically without correction for relative density. Bile acid concentrations in bile were determined enzymically by the method of Talalay (1960) as modified by Paumgartner *et al.* (1971). Cholesterol concentrations in bile were determined by a modification of the method of Bolton *et al.* (1980) using a commercial kit (Cholesterol enzymatique PAP; Biomerieux, Charboniers les Bains, France). Phospholipid concentrations in bile were measured by 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 & Hoffmann (1973). Alkaline phosphatase (EC 3.1.3.1) and γ -glutamyltransferase (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. Acid phosphatase (EC 3.1.3.2) was assayed in bile by the method of Hubscher & West (1965). Activities were expressed as units/l. Peroxidase activity in bile was measured spectrophotometrically as described by Putter & Becker (1983). One unit of enzyme activity was taken to be equivalent to a change of 1 absorbance unit/min.

Statistics

Results are expressed as means $\pm$ S.E.M. The data were compared by the Mann-Whitney U test, using a significance level of $P = 0.05$ to reject the null hypothesis. Regression lines were calculated by the least-squares method and compared by a method comparing multivariate normal means (Anderson, 1958).

RESULTS

Fig. 1 shows the changes in bile flow and the secretion of biliary lipids induced by cefmetazole. To allow an easier comparison between the results obtained from control and treated

rats, values are expressed as percentage variations with respect to those before saline or cefmetazole administration. The amounts corresponding to 100 % are shown in Table 1. Administration of the antibiotic led to a choleretic effect with a peak increase in bile flow after 0–10 min, thereafter decreasing to approach pre-administration values. Bile acid secretion slightly and progressively decreased in both control and cefmetazole-treated animals, with no significant difference between both groups. The biliary secretion of cholesterol and phospholipids began to decrease parallel to the increase in bile flow, with a maximum decrease at 20 min after injection (Fig. 1). Values at the end of experiments still remained significantly below those of the controls. When the mean values for the first 30 min after cefmetazole administration

Table 1. Effect of cefmetazole on biliary secretion

Results are means $\pm$ S.E.M. for bile flow and bile acid (BA), cholesterol (Ch) and phospholipid (PI) output in control ($n = 7$) and cefmetazole-treated ($n = 10$) rats before (0–20 min) and after (20–50 min) saline or cefmetazole (200 μ mol/kg) administration: * $P < 0.05$ significantly different from pre-administration values. Values in parentheses are percentage variations with respect to pre-administration values.

Rats	Flow (μ l/min per 100 g)	Output (nmol/min per 100 g)		
		BA	Ch	PI
Control				
Before	6.8 $\pm$ 0.3	215 $\pm$ 24	2.77 $\pm$ 0.16	25.0 $\pm$ 2.1
After	6.6 $\pm$ 0.3 (–3)	194 $\pm$ 26 (–10)	2.65 $\pm$ 0.16 (–4)	24.1 $\pm$ 2.6 (–4)
Cefmetazole-treated				
Before	7.1 $\pm$ 0.7	167 $\pm$ 9	2.90 $\pm$ 0.23	19.7 $\pm$ 1.0
After	12.4 $\pm$ 1.0* (+74)	152 $\pm$ 12 (–9)	1.44 $\pm$ 0.16* (–50)	12.9 $\pm$ 1.1* (–35)

Fig. 1. Effect of cefmetazole on bile flow and on the biliary output of bile acids, cholesterol and phospholipid

Mean percentage changes ($\pm$ S.E.M.) in bile flow and bile acid, cholesterol and phospholipid outputs are shown for control (□) and cefmetazole-treated (■) rats. Cefmetazole (200 μ mol/kg) administration was performed at 20 min after beginning the experiments ($n = 10$). Controls received saline ($n = 7$). Values represent percentage variations with respect to the pre-administration period (0–20 min), the 100 % values of which are shown in Table 1. * $P < 0.05$ significantly different from control values.

Table 2. Effect of cefmetazole on biliary bile acid/(cholesterol + phospholipid) (BA/Ch + PI) molar ratio, cholesterol/phospholipid (Ch/PI) molar ratio and lithogenic index (LI) of bile

Results are means $\pm$ S.E.M. for control ($n = 7$) and cefmetazole-treated ($n = 10$) rats before (0–20 min) and after (20–50 min) saline or cefmetazole (200 μ mol/kg) administration: * $P < 0.05$ significantly different from pre-administration values. Values in parentheses are percentage variations with respect to pre-administration values.

Rats	BA/(Ch + PI)	Ch/PI	LI
Control			
Before	7.65 $\pm$ 0.81	0.110 $\pm$ 0.016	0.33 $\pm$ 0.02
After	7.12 $\pm$ 0.69 (–7)	0.107 $\pm$ 0.018 (–3)	0.36 $\pm$ 0.02 (+9)
Cefmetazole-treated			
Before	7.38 $\pm$ 0.81	0.128 $\pm$ 0.009	0.38 $\pm$ 0.03
After	10.54 $\pm$ 0.85* (+43)	0.099 $\pm$ 0.009* (–23)	0.24 $\pm$ 0.02* (–37)

Fig. 2. Effect of cefmetazole on the relationship between bile flow and the biliary output of bile acids

Values correspond to 20–60 min samples for controls ($y = 4.734 + 0.017x$; $n = 28$; $r = 0.729$; $P < 0.01$) ($\square$) and cefmetazole-treated ($y = 7.305 + 0.018x$; $n = 36$; $r = 0.862$; $P < 0.01$) ($\blacksquare$) animals.

Fig. 3. Effect of cefmetazole on the relationship between the biliary outputs of cholesterol and bile acids

Values correspond to 20–50 min samples for controls ($y = 1.342 + 0.006x$; $n = 21$; $r = 0.731$; $P < 0.01$) ($\square$) and cefmetazole-treated ($y = 0.625 + 0.004x$; $n = 27$; $r = 0.582$; $P < 0.01$) ($\blacksquare$) animals.

were compared with those corresponding to the pre-administration period (0–20 min) (Table 1), it was found that, although there was a significant increase in bile flow, bile acid output did not significantly change and there was a significant decrease in both cholesterol and phospholipid outputs. The degree of inhibition was higher for the cholesterol than for the

Table 3. Effect of cefmetazole on the biliary outputs of alkaline phosphatase (ALP), γ -glutamyltransferase (γ -GT) and acid phosphatase (ACP)

Results are means $\pm$ S.E.M. in control ($n = 7$) and cefmetazole-treated ($n = 10$) rats before (0–20 min) and after (20–50 min) saline or cefmetazole (200 μ mol/kg) administration: * $P < 0.05$ significantly different from pre-administration values. Values in parentheses are percentage variations with respect to pre-administration values.

Rats	Enzyme output (μ -units/min per 100 g)		
	ALP	γ -GT	ACP
Control			
Before	190 $\pm$ 26	1207 $\pm$ 171	243 $\pm$ 75
After	182 $\pm$ 20 (–4)	1052 $\pm$ 214 (–13)	271 $\pm$ 50 (+12)
Cefmetazole-treated			
Before	220 $\pm$ 25	1086 $\pm$ 192	402 $\pm$ 112
After	116 $\pm$ 21* (–42)	359 $\pm$ 159* (–67)	371 $\pm$ 150 (–8)

Fig. 4. Effect of cefmetazole on the secretion of horseradish peroxidase

Mean values $\pm$ S.E.M. for the biliary output (a) and cumulative biliary secretion (b) are shown for control ($\square$) and cefmetazole-treated ($\blacksquare$) rats. Cefmetazole (200 μ mol/kg) plus horseradish peroxidase (5 mg/kg) were administered (arrow) at 20 min after the start of experiments ($n = 6$). Controls received saline plus horseradish peroxidase ($n = 6$).

phospholipid output. No significant changes were found for any of the above parameters in the control group.

Table 2 shows the calculated bile acid/(cholesterol + phospholipid) molar ratio, the cholesterol/phospholipid molar ratio and the lithogenic index of bile in the control and cefmetazole-treated rats. The former was significantly increased with respect to the pre-administration period in animals injected with the drug. The cholesterol/phospholipid ratio and the lithogenic index of bile were significantly decreased.

When the relationship between bile flow and bile acid output was studied both in the controls and in the animals receiving cefmetazole (Fig. 2), the drug was found to induce a 55%

increase in the ordinate intercept of the regression line ($P < 0.01$), with no modification in the slope. Fig. 3 shows the quantitative relationship between biliary outputs of cholesterol and bile acid. In this case, both the ordinate intercept and the slope of the regression line were smaller ($P < 0.05$) for the cefmetazole-treated rats than for the controls.

The biliary outputs of the two canalicular-membrane enzymes alkaline phosphatase and γ -glutamyltransferase were significantly decreased after cefmetazole administration in the group of treated animals, but there was no change with respect to the pre-administration value in the controls. The biliary output of the lysosomal enzyme acid phosphatase was not significantly affected by the antibiotic (Table 3).

Fig. 4 shows the biliary output and cumulative biliary secretion of horseradish peroxidase when the protein was injected together with saline or cefmetazole. Neither the peak time of appearance in bile nor the total amount secreted differ significantly between the controls and the animals receiving cefmetazole.

DISCUSSION

Our data show that cefmetazole administration to rats causes a marked increase in bile flow, accompanied by a simultaneous impairment in the biliary secretion of cholesterol and phospholipids. However, bile acid secretion, transcytosis and biliary lysosomal discharge remain unaffected. Different explanations can be put forward to explain these effects and the uncoupling of biliary lipid secretion.

Interference in the intracellular synthesis of cholesterol and phospholipid can be ruled out. As previously indicated for cyclobutylol (Monte *et al.*, 1990) or cefoperazone (Pattinson *et al.*, 1987), the maximal effect on biliary lipid secretion occurs very quickly. Moreover, biliary cholesterol secretion in the rat is largely independent of newly synthesized cholesterol (Turley & Dietschy, 1981).

Cholesterol and phospholipids are packaged and secreted in vesicles that are brought to the canalicular membrane of the hepatocytes via a microtubular system (Crawford *et al.*, 1988), whereas bile acids are transported across the hepatocytes as discrete molecules (Barnwell *et al.*, 1984). For valproic acid, it has been demonstrated that not only phospholipid secretion but also that of endogenous serum albumin, BSA or triacylglycerol are impaired in the rat (Bellringer *et al.*, 1988a). All these molecules are transported to the canalicular membrane via vesicle-mediated transport; thus it has been proposed that valproic acid may inhibit the movement of secretory vesicles within the hepatocytes through a mechanism that still needs to be fully elucidated. Similar effects have been reported for ampicillin (Bellringer *et al.*, 1988b).

Horseradish peroxidase is a glycoprotein of 40 000 Da that has been found to be transferred into bile both by microtubular-dependent and -independent vesicular pathways and by sieving through the tight junctions (Hayakawa *et al.*, 1990; Kan *et al.*, 1989; Lowe *et al.*, 1985). In rats under basal conditions, the microtubule-dependent vesicular pathway accounts for 90% of the biliary output (Hayakawa *et al.*, 1990), and hence horseradish peroxidase is a useful tracer of vesicular transport in hepatocytes (Okanoue *et al.*, 1984; Román *et al.*, 1990). In our study, neither the time course nor the total amount of the protein secreted was modified in animals receiving cefmetazole with respect to the controls. The uncoupling effect of the antibiotic is therefore unlikely to involve vesicles, interaction with microtubules or the microtubules themselves. Additionally, the fact that the time course of horseradish peroxidase secretion within the first 10 min after injection was unaffected also rules out the possibility of changes in junctional permeability induced by cefmetazole.

Lysosomal content is secreted into bile by movement of the lysosomes via the microtubular system. Since cholesterol and phospholipids apparently originate from a preformed pool of lipids (Cronholm *et al.*, 1983), the above secretory route could be involved in the transport of some of the endogenous lipids to the bile canaliculus (Rahman & Coleman, 1987a,b). Although this hypothesis still needs further confirmation, the fact remains that in our experiments the appearance in bile of the lysosomal marker enzyme acid phosphatase was not significantly modified by cefmetazole. If therefore appears that the antibiotic does not affect biliary lysosomal discharge.

The incorporation of cefmetazole into mixed micelles, which might interfere with the solubilization of biliary lipids, also seems unlikely. Although different molecules that uncouple biliary lipid secretion are associated with mixed micelles, at least for the cephalosporin, ultracentrifugation studies with cefoperazone show no significant interaction with bile acid micelles (Pattinson *et al.*, 1987). Moreover, if cefmetazole were incorporated into mixed micelles, changes in the choleretic capacity of bile acids would be expected (Monte *et al.*, 1990). However, in our experiments no significant differences in the slopes of the regression lines relating bile flow and bile acid secretion were found between the control and cefmetazole-treated animals.

In our opinion, the most probable effect of cefmetazole would be exerted at the level of the canalicular membrane, because the secretion not only of biliary lipids but also that of enzymes, such as alkaline phosphatase and γ -glutamyltransferase, was significantly decreased. The presence of these enzymes in bile involves a microvesiculation of regions of the canalicular membrane (Coleman, 1987), which would therefore seem to be affected by cefmetazole. For other uncoupling organic anions, the effects on plasma-membrane enzymes such as 5'-nucleotidase or alkaline phosphodiesterase I have been reported (Monte *et al.*, 1990). Uncoupling effects at the level of the membrane or canalicular lumen have also been proposed, based on studies on the Groningen Yellow mutant rat, which expresses a genetic defect for the secretion of different organic anions, but not of bile acids. It has been shown that these mutant animals, although they reach a high intracellular concentration of sulphated lithocholic acid or ceftriaxone, do not show the uncoupling effects present in normal rats (Oude Elferink *et al.*, 1989; Verkade *et al.*, 1989).

The impairment of biliary lipid secretion induced by cefmetazole was proportionally greater for cholesterol than for phospholipid, causing a decrease in the cholesterol/phospholipid molar ratio in bile. A similar effect has been previously reported for other organic anions such as bromosulphophthalein (Shaffer & Preshaw, 1981), ampicillin (Apstein & Russo, 1985) or cyclobutylol (Monte *et al.*, 1990). Different studies have suggested the existence of a source of biliary cholesterol that could be secreted by mechanisms relatively independent from bile acids (Barnwell *et al.*, 1984; Rahman & Coleman, 1986). In our experiments, not only the slope but also the ordinate intercept of the regression line relating cholesterol and bile acid secretion were decreased, which could be interpreted as indicating that cefmetazole decreases the secretion of both the bile acid-dependent and -independent pathways of cholesterol secretion. This inhibition of the second pathway of cholesterol secretion would account for the differential effect with respect to phospholipid secretion. An inhibition of the bile acid-independent fraction of cholesterol secretion has also been suggested for cyclobutylol on the basis of the decrease in cholesterol/phospholipid molar ratio found at low bile acid secretory rates (Monte *et al.*, 1990).

In any case, it is clear that cefmetazole gives rise to a bile less saturated in cholesterol, as shown by the decreased lithogenic index. As speculated by other authors (Apstein & Russo, 1985; Monte *et al.*, 1989), it would be worthwhile to investigate the

relevance of the uncoupling effect caused by this and other drugs to the mechanisms of gallstone formation and dissolution.

In summary, for cefmetazole it appears that the inhibitory action on cholesterol and phospholipid biliary secretion is exerted at the level of the canalicular membrane.

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