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The effects of 3,5,5-trimethylcyclohexanol on hepatic cholesterol synthesis, bile flow and biliary lipid secretion in the rat

G.D. Bell, R.J. Clegg*, W.R. Ellis[1], B. Middleton* & D.A. White*

Department of Therapeutics, City Hospital, Nottingham NG5 1PB and Department of Biochemistry*, Queen's Medical Centre, Nottingham NG7 2UH

1 The chemical trimethylcyclohexanol (TMC) is closely related to menthol, the major component of a terpene preparation with known choleretic and cholelitholytic activity. Its effects on hepatic cholesterol synthesis and bile secretion were examined in the rat.

2 In both acute and long-term dosing experiments TMC significantly inhibited hepatic S-3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase. TMC was a potent choleretic, with detectable effects on bile flow at low doses, which also reduced coupling of cholesterol secretion to bile salt secretion. Single large doses tended to lower biliary cholesterol output and caused significant reduction in cholesterol saturation index after biliary diversion for 1h.

3 TMC and its widely prescribed ester cyclandelate, which is rapidly degraded to TMC after ingestion, should be investigated further as potential cholelitholytic treatments in man.

Introduction

The chemical 3,5,5-trimethylcyclohexanol (TMC) is a major component of the widely prescribed vasoactive substance cyclandelate (cyclospasmol, Brocade Ltd, Amsterdam, Holland). Cyclandelate is the mandelic acid ester of TMC; the ester is rapidly hydrolysed in the body into its constituent parts, so that 65% of TMC administered as cyclandelate may be recovered from urine as glucuronide (Brocades Ltd, data on file).

We became interested in TMC because of its structural similarity to a number of cyclic monoterpene compounds which we have investigated. Six such terpenes, including menthol, are combined in Rowachol (Rowa Ltd, Bantry, Ireland), a preparation which we have shown can dissolve gallstones, both alone (Bell & Doran, 1979) and in combination with chenodeoxycholic acid (Ellis, Middleton, White & Bell, 1981). Like the latter, Rowachol favourably alters bile lipid composition (Doran, Keighley & Bell, 1979) and inhibits hepatic S-3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR, EC 1.1.1. 34) in animals (Clegg, Middleton, Bell & White, 1980; Handelsman, Bonorris, Marks & Schoenfield, 1982) and man (Ellis, Bell, Clegg, Middleton & White, 1981). HMGCoAR is rate-limiting for *de novo* cholesterol synthesis from acetyl CoA and is overactive in patients with cholesterol gallstones (Key, Bonorris, Marks, Chung & Schoenfield, 1980).

The chief constituent of Rowachol is menthol, which is closely related to TMC: both are substituted cyclohexanols. We speculated therefore that TMC might have cholelitholytic potential. Accordingly we studied the effects of TMC in the rat on hepatic HMGCoAR activity, bile flow and bile lipid composition.

Methods

Treatment of animals

Male Wistar rats (250–300 g) were acclimatized for 14 days to reversed lighting (lit from 15h 00 min to 03h 00 min) and fed 41B pellet diet with water *ad libitum*, before use at the stated weight. Acute dosing of TMC was by gavage at a dose of 3 mmol kg^{-1} body weight. TMC was dissolved in olive oil, which was given alone in equivalent volume to control animals.

[1]Correspondence to Dr W.R. Ellis, Department of Medicine, St James's Hospital, Leeds LS9 7TF

Dosing was at 17h 00 min on the two days preceding the experiment, or on the day before only, and animals were killed or experimental procedures initiated 17 h after the last dose. Long-term administration of TMC was as a 1 mM or 10 mM solution in the drinking water over 2–6 weeks. TMC did not prevent normal feeding: weights of stomachs plus contents *post mortem* were not lower in experimental animals than in controls and in chronic experiments TMC did not alter weight gain.

Measurement of HMGCoAR

Microsomal fractions were prepared from freshly-removed livers homogenized in 4 volumes of 0.3 M sucrose, 10 mM EDTA and 20 mM mercaptoethanol at pH 7.0. The suspension was centrifuged at 12,000 *g* for 15 min and the resulting supernatant centrifuged at 80,000 *g* for 60 min to yield the microsomal pellet, which was washed by resuspension in homogenization medium followed by further centrifugation at 80,000 *g* for 60 min. The microsomes were resuspended in 100 mM potassium phosphate containing 10 mM EDTA and 5 mM dithiothreitol at pH 7.5 and were assayed for HMGCoAR activity by the method of Balasubramaniam, Goldstein, Faust & Brown, (1976) as previously described (Clegg *et al.*, 1980). Microsomal protein content was determined by the microbiuret method (Burgi, Richterich & Briner, 1967). Arylesterase activity in microsomes was measured according to Shephard & Hubscher (1969). Enzyme activities were expressed as amount of product min^{-1} mg^{-1} microsomal protein.

Bile collection procedure

Anaesthesia was induced by intraperitoneal pentobarbitone, 50 mg kg^{-1}, and a jugular venous catheter was inserted for maintenance of anaesthesia and fluid loss replacement with 0.9% w/v NaCl solution (saline). At laparotomy the common bile duct was occluded and cannulated for total bile diversion into collecting vessels, which were changed every 30 min in the acute dosing experiment or every 60 min in the long-term low-dose experiment; the animals remained anaesthetized on heated tables throughout. Bile samples were stored at −20 °C before analysis.

Estimation of biliary lipids

Total bile salts were measured by the 3α-hydroxysteroid dehydrogenase method (Carr & Drekter, 1956), phospholipids by the method of Bartlett (1959) and cholesterol by gas-liquid chromatography (Mitropoulos, Balasubramaniam & Myant, 1973). Lipids were extracted from the bile before cholesterol and bile salt estimations (Folch, Lees & Sloane-Stanley, 1957). Cholesterol saturation indices were calculated according to Thomas & Hofmann (1973) using the solubility criteria of Hegardt & Dam (1971). Bile flow and biliary lipid secretion rates were expressed in units 24 h^{-1} 100 g^{-1} body weight; these values were calculated from biliary output in the first 2 h after cannulation, i.e. before bile salt pool depletion begins to affect secretion rates (Mok, Perry & Dowling, 1974). In the chronic low-dose experiment, where bile collection continued for up to 12 h, bile salt pool size was computed by the 'washout' method of Eriksson (1957) as modified by Myant & Eder (1961). Thus the lowest bile salt secretion was assumed to represent basal hepatic synthesis and was subtracted from each of the preceding hourly outputs, whose remainders were then added together to give an estimate of the pool size. For these animals, 4 in each group, which survived for 9 h or more, secretion data were available over a wide range of values and valid linear regression analysis of the relationship between salt secretion and both bile volume and cholesterol output was possible. These calculations could not be made in the cases of the 2 animals of each group in which death (from respiratory difficulty) occurred before the 'low point' in bile salt secretion and before secretion and flow had fallen far enough to demonstrate significant within-animal linear correlations.

Statistical comparisons were made using Student's *t* test.

Table 1 Hepatic microsomal enzyme activities

	*Acute dosing**		*Chronic dosing†*	
	HMGR	*AE*	*HMGR*	*AE*
Controls	0.74 ± 0.33 (14)	5.1 ± 1.1 (11)	0.90 ± 0.35 (6)	4.7 ± 1.4 (6)
TMC-treated	0.42 ± 0.12 (11)	5.9 ± 1.7 (5)	0.41 ± 0.13 (6)	3.4 ± 1.2 (6)
	($P < 0.01$)	(NS)	($P < 0.01$)	(N.S.)

HMGR: HMGCoA reductase activity (nmol min^{-1} mg^{-1} microsomal protein); AE: arylesterase activity (μmol min^{-1} mg^{-1} microsomal protein). Values are mean ± s.d., number of observations in parentheses.

* 2 doses of 3 mmol kg^{-1} given, 41 and 17 h before sacrifice. Controls had equivalent volumes of olive oil vehicle.

† dose given was a 10 mM aqueous solution substituted for drinking water for 6 weeks.

Table 2 Bile secretion—acute-dose experiment

a *Outputs, 100 g^{-1} body weight 24 h^{-1}*

	Volume (ml)	*Cholesterol* (μmol)	*Bile salts* (μmol)	*Phospholipid* (μmol)
Control (24)	10.89 ± 2.31	4.20 ± 1.82	351 ± 119	50.8 ± 20.0
TMC-treated (8)	20.76 ± 4.07 ($P<0.001$)	3.02 – 1.47 (NS)	341 ± 103 (NS)	43.5 ± 17.4 (NS)

b *Saturation index in successive samples*

	0–30 min	*30–60 min*	*60–90 min*	*90–120 min*
Control (24)	0.228 ± 0.096	0.238 ± 0.103	0.235 ± 0.095	0.248 ± 0.097
TMC-treated (9)	0.186 ± 0.047 (NS)	0.174 ± 0.061 (NS)	0.160 ± 0.047 ($P<0.05$)	0.171 ± 0.024 ($P<0.05$)

Animals were given 3,5,5-trimethylcyclohexanol (TMC), 3 mmol kg^{-1} or olive oil vehicle alone, 17 h prior to cannulation. Values are mean ± s.d. with numbers of observations in parentheses.

Results

Hepatic enzyme activities

TMC administration significantly reduced HMGCoAR activity both in two acute doses (57% inhibition) and in low dose over 6 weeks (68% inhibition) (Table 1). The effect was specific in that activity of arylesterase, a microsomal marker enzyme unconnected with cholesterol synthesis, was not significantly inhibited.

Bile collection experiments

Bile flow was doubled by a single large dose of TMC, but this was not accompanied by an increase in bile salt secretion or a significant change in secretion of the other biliary lipids (Table 2). Mean cholesterol secretion was somewhat lower in the treated group and the saturation index was significantly reduced during the second hour of collection.

Bile flow was also significantly increased by low-dose TMC given for 2 weeks. Although there were no significant changes, mean bile salt secretion was greater in the treated group and mean cholesterol and phospholipid secretion lower (Table 3). Saturation indices were not significantly altered by treatment. The mean slopes of the regression lines relating bile salt secretion (x axis) to bile flow and to cholesterol secretion (y axis) were respectively 0.016 ± 0.002 (s.d) (controls), 0.023 ± 0.004 (TMC-treated), $P<0.05$ and 0.014 ± 0.002 (controls), 0.009 ± 0.002 (TMC-treated), $P<0.02$ ($n=4$ in each group). This points to the bile salt dependency

Table 3 Bile secretion – chronic-dose experiment

a *Outputs 100 g^{-1} body weight 24 h^{-1}*

	Volume (ml)	*Cholesterol* (μmol)	*Bile salts* (μmol)	*Phospholipid* (μmol)
Control (6)	13.30 ± 1.18	4.70 ± 1.30	284 ± 81	61.1 ± 8.3
TMC-treated (6)	16.45 ± 1.85 ($P<0.01$)	3.07 ± 1.67 (NS)	350 ± 95 (NS)	54.4 ± 17.5 (NS)

b *Saturation index in successive hourly collections*

	0–1 h	*1–2 h*	*2–3 h*	*3–4 h*
Control	0.246 ± 0.059 (6)	0.245 ± 0.061 (6)	0.278 ± 0.082 (6)	0.270 ± 0.073 (4)
TMC-treated	0.205 ± 0.067 (6) (NS)	0.212 ± 0.072 (6) (NS)	0.278 ± 0.099 (6) (NS)	0.292 ± 0.086 (5) (NS)

Animals were given 3,5,5-trimethylcyclohexanol (TMC), 1 mM in drinking water, for 2 weeks. Values are mean ± s.d. with number of observations in parentheses.

of the choleresis at this dose and suggests reduced coupling of cholesterol secretion to that of bile salts. Bile salt pool size was unaffected by treatment (43.62 ± 3.16 μmol $100\,g^{-1}$ body weight in controls, 41.46 ± 2.88 TMC-treated, $n = 4$ in each group).

Discussion

Besides Rowachol, presently available orally-administered cholelitholytic drugs include chenodeoxycholic acid and ursodeoxycholic acid. Both of these improve biliary cholesterol saturation in man by reducing biliary cholesterol secretion relative to that of bile salts (Dowling, 1979; Bouchier, 1980). The exact mechanism of this effect remains a matter for debate but both bile acids inhibit HMGCoAR in man or experimental animals (Key *et al.*, 1980; Handelsman *et al.*, 1982). Although the synthetic pathway may contribute only one-third or less of biliary cholesterol output (Schwartz, Berman, Vlahcevic, Halloran, Gregory & Swell, 1978); Turley & Dietschy, 1981) under most circumstances, there is evidence to suggest that unsuppressed HMGCoAR may account for resistance to treatment with bile acids, particularly in obese subjects (Maton, Murphy & Dowling, 1980). Thus it is of interest that TMC also inhibits HMGCoAR both at high and low doses.

The mechanism of inhibition by TMC is not yet known but is likely to resemble that of menthol and cineole which cause a specific decrease in the amount of HMGCoAR protein in rat liver (Clegg, Middleton, Bell & White, 1982). An effect on synthesis of the enzyme (whose turnover is rapid) is postulated. There is no direct effect of TMC (0.2 mM) when added to the reaction mixture *in vitro*.

Our biliary secretion data show that TMC acts as a choleretic in two ways: a single large dose produced a massive hydrocholeresis without alterations in bile salt secretion. In a lower dose (equivalent to less than one 400 mg cyclandelate tablet per day in man) the increased flow was accompanied by a trend towards increased bile salt secretion. Although the latter was not statistically significant, the relationship between the two was changed, suggesting some bile salt dependency of the choleresis in this group. Although choleretic potency is not specifically associated with cholelitholytic activity, at least two non-bile salt choleretics have been shown to affect biliary cholesterol saturation favourably in man – florantyrone (Zimmon, Kerner, Aaron, Raicht, Mosbach & Kessler, 1976) and cicloxilic acid (Carulli, Fenenderes & Ponz de Leon, 1978). Thus it is encouraging that TMC in the single-dose experiment showed a tendency to improve the saturation index of rat bile. Lower dose treatment did not have this effect, but a favourable alteration in the relationship between bile salt and cholesterol secretion, analogous to that found in man with chenodeoxycholic acid treatment (Northfield, La Russo, Hofmann & Thistle, 1975), was detected.

There are clearly dangers in extrapolating data obtained from experimental models to man, particularly in the case of the rat, which has neither gallbladder nor saturated bile and in which, to our knowledge, chenodeoxycholic acid itself has not been shown to improve biliary cholesterol saturation. Nonetheless we conclude from these studies that TMC and, by implication, its mandelic acid ester cyclandelate, deserve further investigation of their potential as cholelitholytic agents in man.

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