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THE INHIBITION OF HEPATIC *S*-3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE BY 3,3,5-TRIMETHYLCYCLOHEXANOL AND ITS MANDELIC ACID ESTER, CYCLANDELATE

BRUCE MIDDLETON,* ALIYA MIDDLETON,* ANN MICIAK,* DAVID A. WHITE* and G. DUNCAN BELL†

*Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K.; and †Department of Therapeutics, City Hospital, Nottingham NG5 1PD, U.K.

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Abstract—Rat hepatic HMGCoA reductase was found to be at least 50% inhibited 17 hr after administration of a single oral dose of 3,3,5-trimethylcyclohexanyl mandelate (cyclandelate), a vasoactive substance. This inhibition was also found in rats given the 3,3,5-trimethylcyclohexanol component but only slight inhibition was seen after an equimolar dose of mandelate. The inhibition of HMGCoA was observed both around the high point and near the low point of the diurnal activity cycle. The effect did not persist to 41 hr after treatment. There was no direct inhibition of HMGCoA reductase by trimethylcyclohexanol when added to the assay system *in vitro*. The *in vivo* effect of these inhibitors was specific for HMGCoA reductase. There was no change, neither elevation nor depression, of the amount of microsomal membrane components cytochromes b_5 and P-450, not was the activity of another microsomal enzyme, arylesterase, affected by dosing with cyclandelate or trimethylcyclohexanol.

Cyclandelate is a vasoactive substance [Cyclospasmol (Gist Brocades N.V., Delft, The Netherlands)] consisting of the mandelic acid ester of 3,3,5-trimethylcyclohexanol (TMC).‡ The latter compound is structurally similar to menthol (2-isopropyl,5-methylcyclohexanol) one of a group of cyclic monoterpenes of plant origin that we have shown to decrease hepatic cholesterol synthesis in rats [1] and man [2] by their inhibition of HMGCoA reductase activity *in vivo*. A significant inhibition of hepatic HMGCoA reductase was caused by only those monoterpenes containing an oxygen substituent in the cyclohexane ring [1]. The object of this work was to determine whether a simpler molecule such as TMC retained this activity, and whether its mandelic acid ester (a widely available drug) showed any inhibitory effects on HMGCoA reductase, the rate-determining enzyme of cholesterol biosynthesis [3].

MATERIALS AND METHODS

[3-^{14}C]HMGCoA was purchased from Amersham International Ltd (Amersham, U.K.). [5-^{3}H]Mevalonic acid (DBED salt) was purchased from New England Nuclear Corp. (Boston, MA). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), $NADP^+$, DL-mandelic acid and indoxyl acetate were from Sigma Ltd (Poole, U.K.). Aluminium-backed silica gel precoated sheets for TLC were obtained from Merck (Darmstadt, F.R.G.). Fisofluor scintillation fluid was from Fisons Ltd (Loughborough, U.K.). Cyclandelate and 3,3,5-trimethylcyclohexanol were from Brocades (G.B.) Ltd (Weybridge, U.K.).

Male Wistar rats (220–250 g) were subjected to normal lighting (lit from 08.00 to 20.00) or reversed lighting (lit from 15.00 to 03.00) and were fed a 41B pellet diet *ad lib*. Animals were acclimatized to their lighting schedules for 14 days before use at the wt stated. They were given cyclandelate or TMC in olive-oil by gavage at a dose of 3 or 6 mmoles/kg body wt and controls were given the appropriate vol. of olive-oil. Mandelate was administered by gavage as the sodium salt at 6 mmoles/kg and controls in this case were given the same dose of NaCl. Rats were dosed at 17.00 and killed 17 hr later. Administration of the drugs did not prevent normal feeding and wts of stomachs plus contents *post mortem* were not lower in experimental groups than in controls.

Hepatic microsomal fraction was prepared from freshly excised livers homogenized in four vols of 0.3 M sucrose, 25 mM mercaptoethanol, 10 mM EDTA, pH 7. The supernatant fraction obtained after spinning at 12,000 *g* for 15 min was centrifuged at 100,000 *g* for 1 hr to obtain a microsomal pellet. This was washed by suspension in the above buffer and recentrifugation at 100,000 *g* for 1 hr before being finally suspended in 100 mM phosphate buffer (pH 7.5) containing 10 mM EDTA and 5 mM DTT to a final concn of around 20 mg microsomal protein/ml. Both the yield of microsomal protein per g of liver and the liver wt per 100 g body wt were unchanged by drug treatment.

HMGCoA reductase was assayed in microsomal suspensions at 37° essentially as described in Ref. 1

‡ Abbreviations: HMGCoA, 3-hydroxy-3-methylglutaryl-CoA; TMC, 3,3,5-trimethylcyclohexanol; DBED, dibenzylethylenediamine; EDTA, ethylenediaminetetraacetic acid; DTT, dithothreitol.

Table 2. The effect of cyclandelate or TMC on the activity of hepatic HMGCoA reductase at 2 times in the diurnal cycle

Animals killed at	HMGCoA reductase activity (nmoles/min/mg)		
	Controls	Cyclandelate	TMC
D7	1.58 ± 0.15 (3)	0.86 ± 0.13* (3)	
D7	0.99 ± 0.09 (4)		0.44 ± 0.13† (4)
L2	0.41 ± 0.05 (4)	0.12 ± 0.01† (4)	0.22 ± 0.05* (4)

Rats were maintained on a 12-hr light and dark schedule and killed at the seventh hour of dark (D7) or the second hour of light (L2) 17 hr after being intubated with olive-oil (controls) or cyclandelate or TMC (both at 3 mmoles/kg) in olive-oil. HMGCoA reductase activity is expressed as nmoles/min/mg. Results are means ± S.E.M. with the number of animals in parentheses.

* $P < 0.05$ with respect to controls.

† $P < 0.01$ with respect to controls.

using a preincubation of 10 min and starting the reaction with [3-^{14}C]HMGCoA. The 0.15-ml system contained *RS*-[3-^{14}C]HMGCoA (3000 dpm/nmole), 100 μM; $NADP^+$, 2.5 mM; glucose 6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 3 U/ml; DTT, 5 mM; EDTA, 10 mM; 25–300 μg of microsomal protein and potassium phosphate, pH 7.5, 100 mM. The reaction was stopped after 15–30 min with 10 μl of 10 M HCl and [5-^{3}H]mevalonate (40,000 dpm) added as recovery standard. After lactonization the mevalonolactone was separated by TLC as described by Shapiro *et al.* [4] and counted for ^{14}C and ^{3}H radioactivity in Fisofluor. Formation of [^{14}C]mevalonate under these conditions was linear with time and protein and recovery averaged 90%. The enzyme activity was expressed as nmoles of mevalonate formed/min per mg of microsomal protein.

Arylesterase activity was determined at 30° by following the formation of indoxolol at 386 nm from the enzymic hydrolysis of indoxyl acetate as described by Shephard and Hubscher [5]. The activity was expressed as μmoles of indoxolol formed/min/mg microsomal protein.

Cytochromes P-450 and b_5 were assayed as described by Omura and Sato [6] and their content was expressed as nmoles/mg of microsomal protein.

Table 1. The effect of cyclandelate and its individual constituents TMC and mandelate on the activity of hepatic HMGCoA reductase

Treatment	HMGCoA reductase activity (nmoles/min/mg)
Controls (olive-oil)	1.47 ± 0.14 (6)
Cyclandelate	0.56 ± 0.08 (6)*
TMC	0.57 ± 0.13 (5)*
Controls (NaCl)	1.30 ± 0.07 (6)
Mandelate	0.92 ± 0.13 (6)†

Rats were given a single dose (6 mmoles/kg) of cyclandelate, TMC or mandelate 17 hr prior to removal of liver near the time of peak enzyme activity. HMGCoA reductase activity was measured in the microsomal fraction and expressed as nmoles/min/mg ± S.E.M. with the number of animals in parentheses.

* $P < 0.01$ with respect to appropriate controls.

† $P < 0.02$ with respect to appropriate controls.

Microsomal protein content was determined by the micro-biuret method [7] using bovine serum albumin as standard after initial precipitation by 10% (w/v) trichloroacetic acid and subsequent dissolution in 1 M NaOH.

RESULTS AND DISCUSSION

In the rat, hepatic HMGCoA reductase shows considerable diurnal variation in activity, being maximal around the mid-point of the dark period and minimal at the middle of the light period [8]. In our initial experiments rats were dosed at around the second hour of the light period (L2) and killed 17 hr later at the seventh hour of the dark period (D7) when the enzyme activity was near maximal. Under these conditions (Table 1) a single dose of cyclandelate of 6 mmoles/kg body wt caused 62% inhibition of HMGCoA reductase activity ($P < 0.01$). The same inhibition (61%, $P < 0.01$) was caused by an equimolar dose of TMC (the alcohol component of the cyclandelate ester). Mandelate, the acid component of cyclandelate, gave only 30% inhibition at the same high dose. At a lower dose of mandelate (3 mmoles/kg) a 26% inhibition was observed but did not reach statistical significance. In contrast the same lower doses of cyclandelate and TMC retained their significant inhibitor effects (Table 2). It has been reported that orally administered cyclandelate is rapidly hydrolysed [9] so that we can tentatively conclude that the major part of the inhibition of hepatic HMGCoA reductase caused by oral cyclandelate is due to the release of TMC which could thus be the main active agent.

The effect of the diurnal variation in HMGCoA reductase activity upon the occurrence of inhibition 17 hr after oral cyclandelate or TMC was investigated and the results are presented in Table 2. The rats were killed at D7 around the diurnal activity peak or at L2 near the activity trough. In controls, the ratio of reductase activity at D7 to that at L2 was 2.4 to 3.9 but at both times a single pretreatment with cyclandelate or TMC resulted in 46–72% inhibition ($P < 0.05$) of the enzyme activity. Thus, the inhibition was not influenced by the normal diurnal variation of HMGCoA reductase activity and neither did cyclandelate nor TMC pretreatment affect the diurnal rhythm itself since the ratio of activity at D7

Table 3. Arylesterase activity and content of cytochromes P-450 and b_5 after cyclandelate, TMC or mandelate treatment

Treatment	Arylesterase activity (μmoles/min/mg)	Cytochrome content	
		P-450 (nmoles/mg)	b_5 (nmoles/mg)
Controls (olive-oil)	4.20 ± 0.46 (6)	0.69 ± 0.06 (4)	0.18 ± 0.01 (4)
Cyclandelate	4.37 ± 0.37 (6)	0.65 ± 0.13 (4)	0.19 ± 0.03 (4)
TMC	4.11 ± 0.58 (5)	0.70 ± 0.10 (3)	0.19 ± 0.03 (3)
Controls (NaCl)	5.15 ± 0.58 (3)	0.56 ± 0.06 (4)	0.17 ± 0.02 (3)
Mandelate	5.85 ± 0.46 (3)	0.74 ± 0.02 (3)	0.16 ± 0.01 (3)

Rats were given a single dose (6 mmoles/kg) of cyclandelate, TMC or mandelate 17 hr prior to removal of liver and preparation of microsomal fractions. Arylesterase activity is expressed as μmoles/min/mg. Cytochrome P-450 and b_5 amounts are given as nmoles/mg. Results are means ± S.E.M. with the number of animals in parentheses.

to that at L2 after treatment was an average of 2.4, not significantly altered from controls. The diurnal variation in HMGCoA reductase activity is linked to the cyclic pattern of food intake in the rat via hormonal changes [10] so that the inhibition caused by cyclandelate and TMC is unlikely to be mediated by the same hormones since the diurnal cycle was not apparently affected. We can rule out any direct effect of the compounds on HMGCoA reductase since the inclusion of TMC at 0.2 mM in the assay system did not affect the enzyme activity. It is unlikely that the concn of TMC in washed microsomes 17 hr after administration would even approach this value. The effect of TMC, like that of its analogue menthol [11], was not seen 41 hr after administration of a single dose, the activity of HMGCoA reductase in TMC-treated rats (3 mmoles/kg) being 0.54 ± 0.01 (3) nmoles/min/mg compared to a control value of 0.44 ± 0.05 (3) nmoles/min/mg. We have shown that the same dose of menthol is rapidly excreted in the urine [11], presumably as the glucuronide [12] and it is probable that TMC is treated similarly. This would then account for the lack of effect 41 hr after dosing.

The inhibitory effect of cyclandelate or TMC on HMGCoA reductase activity was specific and not due to a rapid general hepatotoxicity. Table 3 shows the results of a single large dose of cyclandelate and its components on the activities or amounts of microsomal membrane proteins. The activity of arylesterase, an enzyme unconnected with cholesterol metabolism, was not affected. Furthermore cytochromes P-450 and b_5 were unchanged in amount after cyclandelate or TMC treatment. Mandelate administration increased cytochrome P-450 content by 31%.

The results of the study of the effects of TMC conform with the suggestion [1] that oxygen substitution in cyclohexane ring of cyclic monoterpenes is necessary to yield inhibition of HMGCoA reductase. In particular, the inhibition by TMC resembles that caused by menthol [11] in four ways: (1) the effect is independent of the point in the diurnal cycle of HMGCoA reductase activity at which the animal was killed, (2) the effect is not directly exerted on the catalytic activity of the enzyme, (3) the effect does not persist to 41 hr after dosing, and (4) other microsomal enzymes are not affected. It remains to be seen whether TMC, like menthol [11], decreases the amount of HMGCoA reductase protein after treatment.

In summary, cyclandelate, a widely available vasoactive substance, has been shown to cause the specific inhibition of hepatic HMGCoA reductase after oral administration. This effect is probably due to the trimethylcyclohexanol component of the drug. Whether cyclandelate or TMC exert any inhibitory effects on the process of sterol synthesis *in vivo* is the subject of a further investigation.

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