The Mechanism of Cyclic Monoterpen Inhibition of Hepatic 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in Vivo in the Rat

Richard J. Clegg, Bruce Middleton, G. Duncan Bell, and David A. White

From the Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, United Kingdom and the Department of Therapeutics, City Hospital, Hucknall Road, Nottingham, United Kingdom

Seventeen hours after a single oral dose of the cyclic monoterpenes cineole or menthol, rat liver 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity was inhibited by up to 70%. The transient nature of this effect (no inhibition at 1 h after dosing) was compatible with the rapid metabolism and excretion of these terpenes. Neither menthol, and its major metabolite, menthylglucuronide, nor cineole acted as direct inhibitors of HMG-CoA reductase activity in vitro, although menthol was found to bind to liver microsomes (Kd ~ 0.1 mm). Unlike the short term effects of dietary cholesterol, terpene administration did not affect HMG-CoA reductase activity by modulation of the lipid microenvironment of the enzyme. Thus, following menthol or cineole treatment, we found no deviations from the normal kinetic responses to changes in temperature or in concentration of HMG-CoA. Furthermore, the inhibitory effect was still seen after solubilization of the enzyme from microsomes. The loss of HMG-CoA reductase activity was not associated with increased phosphorylation of the enzyme.

Immunotitration of HMG-CoA reductase from terpene-treated rats showed that activity loss was due to less enzyme molecules (together with some possibly “crippled” enzyme), indicating that rates of enzyme synthesis or degradation had been altered. Since menthol inhibition of reductase was still observed in rats deprived of foods, we conclude that the effect is not mediated by those hormones whose concentration is changed during fasting (insulin, glucagon, and adrenaline).

Previously, we have reported (8) that the individual oxygen-containing cyclic monoterpenes menthone, borneol, and, in particular, menthol and cineole caused inhibition of hepatic HMG-CoA reductase in rats in vivo, and this correlated significantly with the flux of C2 units from acetate into non-saponifiable lipid. The mechanism of this effect is unclear, but it could be similar to the inhibition caused by feeding cholesterol. It has been suggested that cholesterol may modulate HMG-CoA reductase activity by a number of possible mechanisms: 1) altering the activity of the pre-existing enzyme, by changing the local membrane environment (9, 10) or the phosphorylation state (11) of the enzyme; or 2) by decreasing the amount of enzyme protein present (12, 13). In this study, we have investigated the possibility that menthol or cineole administration in vitro inhibited hepatic HMG-CoA reductase activity by one or more of the above, or other, mechanisms.

EXPERIMENTAL PROCEDURES

Materials—[3-14C]HMG-CoA and [3-14H]menthol were prepared by Amersham Corp. [5-3H]Mevalonic acid (DBED salt) was purchased from New England Nuclear. Glucose-6-phosphate dehydrogenase, NADP', glucose 6-phosphate, dithiothreitol, and mercaptoethanol were purchased from Sigma. Fisulaver was from Fisons U.K. Ltd. Menthol and cineole were supplied by Rowa Ltd., Bantry, Eire, and adjudged to be pure by gas-liquid chromatography on 10% Carbopack wax 20N. NCS tissue solubilizer was purchased from Amersham Corp. All other chemicals were obtained from commercial suppliers and were of the highest grade available.

Administration of Terpenes to Rats—Male Wistar rats, 200–250 g, were routinely maintained on a reversed lighting schedule (lights on 1500 to 0300 h). Food and water were available ad libitum. Terpenes were administered in olive oil by gastric tube at a dose of 3 mmol/kg of body weight in a volume of 2 ml/kg. In determining the dose-response relation for menthol, this terpene was given at 0.3, 0.6, 1.0, 2.0, 3.0, and 6.0 mmol/kg of body weight in a volume of 2 ml/kg. Cycloheximide was given in 6.9 NaCl solution (saline) by intraperitoneal injection (1.3 mg/g of body weight) just before terpene treatment. Controls were given olive oil alone. Animals were dosed in this manner at 1630 h on the day preceding the experiment. Animals were killed around 0930 h, to coincide with the midpoint of the dark period. No differences in the weights of livers and stomachs were seen between control and treated rats.

Preparation of Microsomes—Livers were removed and homogenized (except where noted) in 4 volumes of buffer A (0.3 M sucrose, 10 mM EDTA/20 mM mercaptoethanol adjusted to pH 7 with NaOH) in a loose fitting, hand-operated Dounce homogenizer using 6–10 strokes. In some cases, livers were divided and portions homogenized in buffer A containing either 50 mM NaF or 50 mM NaCl. The homogenate was centrifuged at 12,000 × g for 15 min; the supernatant was collected and centrifuged at 80,000 × g for 60 min. The pellet obtained was washed by resuspension in buffer A and centrifuged for a further 60 min at 80,000 × g. The microsomal pellet was resuspended in buffer B (100 mM potassium phosphate containing 10 mM EDTA and 5 mM dithiothreitol, pH 7.5) in a tight fitting, hand-operated homogenizer to give a final protein concentration of approximately 20 mg/ml. Terpene pretreatment did not alter microsomal protein yield per g of liver.

Assay of Hepatic HMG-CoA Reductase Activity—HMG-CoA re-
ductase activity was assayed essentially as described by Balasubramaniam et al. (14) using a preincubation time of 10–15 min, starting the incubation with [3-14C]HMG-CoA and incubating for 15–30 min at 37 °C. The 0.15-ml system contained, unless noted, (RS)-[3-I4C]matography of the antisera on protein A Sepharose affinity columns.

The incubation with [3-I4C]HMG-CoA and incubating for 15–30 min solubilized enzyme with antibody for 30 min at 37 °C in the presence glucose-6-phosphate dehydrogenase, 3 units/ml dithiothreitol, HMG-CoA. Immunotitrations were carried out after preincubating microsomes or try in a heptane/glacial acetic acid/acetone (19:21) system. In this condition was linear with time and protein, product recovery averaging 90%.

Tissue Distribution of Menthol after 17 h—[3-H]Menthol (1 mcg, approximately 250 mcCi) was purified by thin layer chromatography in a heptane/glacial acetic acid/acetone (19:2:1) system. In this system, menthol had an Rf of 0.22 to 0.54. [3-H]Menthol was detected using a Panax tlc scanner and extracted with diethylether. For the binding experiment described below, a quarter of this extract was removed, dried under a stream of nitrogen, and dissolved in ethanol containing 1 µmol of unlabeled menthol. The remaining fraction was dried under a stream of N2, and 1.5 ml of 1.5 m pmol HMG-CoA in olive oil was added for administration to rats. This [3-H]menthol (approximately 80,000 dpm/µmol) was given in a dose of 3.0 mmol/kg of body weight. Urine and feces were collected over the 17-h period before the rats were killed. After killing, samples of a variety of tissues were removed, digested with tissue solubilizer, and counted for radioactivity. Body fluids were counted directly without prior solubilization.

The Binding of [3-H]Menthol to Microsomes—[3-H]Menthol (approximately 24,000 cpm/mg protein), purified as described above, was added at 10 µg-1 nm (final concentration) to rat liver microsomes in potassium phosphate (100 mM), pH 7.5, containing EDTA, 10 mM, to a final volume of 1 ml. The microsomes and [3-H]menthol were thoroughly mixed and applied to a presoaked GF/F filter paper under suction filtration. The filter paper was washed 10 times with 1 ml of ice-cold potassium phosphate buffer and counted for radioactivity.

The Effect of Temperature on HMG-CoA Reductase—HMG-CoA reductase activity was measured, as described above, over a temperature range of 13–37 °C in microsomes from livers of control, menthol-, or cineole-treated rats.

Kinetics of Hepatic HMG-CoA Reductase—HMG-CoA reductase activity was measured in microsomes from control, cineole-, or menthol-treated rats over a range of [3-14C]HMG-CoA concentrations of 10–160 µM. The amount of microsomal protein and the time of incubation were adjusted such that no more than 30% of the active isomer of HMG-CoA-CoA was consumed.

Solubilization of Microsomal HMG-CoA Reductase—This was performed essentially as described by Edwards et al. (17). Microsomes were prepared as above except that livers were homogenized in buffer C (0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, 0.03 M EDTA, pH 7.2) in a tight fitting, motor-driven homogenizer (6 strokes) and centrifuged as previously outlined. The microsomal suspension, approximately 80 mg/mg of protein, was prepared in buffer D (buffer C containing 5 mM dithiothreitol). Aliquots (1 ml) were cooled to −20 °C (at a rate of 6–8 °C/min) and stored overnight. The microsomal suspension was then thawed at 37 °C, an equal volume of 50% glycerol in buffer D added, and rehomogenized in a tight fitting, hand-operated, glass homogenizer. The suspension was incubated at 37 °C for 1 h, diluted 3-fold with buffer D, and rehomogenized in the glass homogenizer. The suspension was centrifuged at 100,000 x g for 1 h by a Beckman rotor. The supernatant was retained.

Immunotitration—Anti-HMG-CoA reductase IgG fraction was the kind gift of Dr. P. A. Edwards (University of California, Los Angeles). It was raised in rabbits against purified rat liver HMG-CoA reductase (18), and the purified IgG fraction was obtained after affinity chromatography of the antisera on protein A Sepharose affinity columns. Immunotitration were carried out after preincubating microsomes or solubilized enzyme with antibody for 30 min at 37 °C in the presence of 0.5% Triton X-100. Residual HMG-CoA reductase activity was determined as described above. Immunotitration were expressed as equivalence points defined as nanomoles of mevalonate formed per min per µl of antibody.

Aryl Esterase Assay—Aryl esterase activity was measured as described by Shephard and Hübischer (19). Indoxyl acetate (0.84 mM final) was added to microsomal suspensions in potassium phosphate, 100 mM, pH 6.8, and the activity was measured by the increase in absorbance at 386 nm at 30 °C. Cytochrome b5 and Cytochrome P-450 Activity—These cytochromes were assayed as described by Omura and Sato (20). Cytochrome P-450 content is expressed as the difference in absorbance between 440 and 490 nm, and cytochrome b5 content as the difference in absorbance between 423 and 490 nm/mg of microsomal protein.

Protein—After the initial precipitation of protein with 10% trichloroacetic acid, and resuspension of the pellet in 0.5 ml of 1.0 M NaOH, protein was assayed by the microbiuret method of Burg et al. (21) using bovine serum albumin as a standard.

Statistical Analysis—Results are expressed as means ± S.D., and significance of the difference in means is calculated by Student's t test.

Fig. 1 shows a dose-response curve for menthol inhibition, expressed as the percentage of inhibition of HMG-CoA reductase activity (measured 17 h after administration) against increasing menthol dose. At the usual dose of 3.0 mmol/kg of body weight the inhibition had reached maximum. However, after a further 24 h (41 h after administration) no inhibition was seen in either menthol- or cineole-dosed animals. This transitory inhibition is consistent with the rapid excretion of terpenes (16) and is in accord with the rapid turnover of hepatic HMG-CoA reductase (1). To show that terpenes do not cause a hepatotoxic effect resulting in a general decrease in microsomal protein, the activities of HMG-CoA reductase and aryl esterase and the amounts of cytochrome b5 and cytochrome P-450 were measured after 4 consecutive daily doses of menthol or cineole at 3 mmol/kg of body weight. The half-life of cytochrome P-450 is between 7 and 48 h (22, 23), that of b5 is 2–3 days (24), and the average half-life of microsomal protein is 1–2 days (25), so that any general, nonspecific changes in the synthesis or degradation of these proteins should, therefore, be observed. Prolonged terpene treatment caused no reduction in aryl esterase activity or cytochrome b5.

RESULTS

Fig. 1. Dose-response curve for inhibition by menthol. Data expressed as a percentage of HMG-CoA reductase activity against increasing menthol dose. Control HMG-CoA reductase activities were 1.53 ± 0.42 pmol/mg/min. Each point represents the mean percentage ± S.D. for 3 or 4 rats. A single dose of menthol was given in olive of by gastric tube at between 0.3 and 6.0 mmol/kg of body weight in a volume of 2 ml/kg at 1630 h on the day preceding the experiment. Rats were killed at 0930 h. HMG-CoA reductase activity was measured as described under "Experimental Procedures."
and cytochrome P-450 amounts, although HMG-CoA reductase activity was decreased by 40-45%. Treatment with cineole or menthol gave arylesterase activities of 4.4 ± 0.8 (n = 4) μmol/min/mg in both cases, compared to controls of 3.9 ± 0.8 (n = 4); cytochrome b₅ amounts of 43.5 ± 3.5 (n = 4) and 42.5 ± 3.1 (n = 4), ΔE₆₅₀/mg, respectively, compared to controls of 32.3 ± 5.6 (n = 4); cytochrome P-450 amounts of 58.9 ± 12.3 (n = 4) and 65.1 ± 5.8 (n = 4), ΔE₆₅₀/mg, respectively, compared to controls of 48.8 ± 9.7 (n = 4).

**Tissue Distribution of Menthol**—Table I shows the distribution of menthol-derived [³H] at 17 h after dosing, expressed as the percentage of the dose given. The total accounted for 63% of the administered radioactivity. 52% appeared in the urine, and this is consistent with the known rapid metabolism and excretion of oral menthol as its urinary glucuronide (16). A very low percentage (0.8%) of the dosed radioactivity remained in the liver after 17 h, although HMG-CoA reductase was approximately 70% inhibited at this time. This would imply a very high affinity of the enzyme for menthol if menthol were acting as a direct inhibitor of the enzyme.

**[³H]Menthol Binding to Microsomes and Lack of Direct Effects on HMG-CoA Reductase Activity**—Fig. 2 shows the binding of [³H]menthol (both specific and nonspecific) to untreated liver microsomes over a concentration range of 0.01-0.5 mM. Menthol appeared to bind to liver microsomes with a Kᵢ value of 81 mM. The solubility product of menthol was exceeded at concentrations greater than 0.5 mM, and terpene crystals precipitated. However, under these same conditions neither menthol (Fig. 2) nor cineole (data not shown) exerted any direct inhibitory effect on HMG-CoA reductase activity. Menthol glucuronide, the major hepatic metabolite of this terpene, was equally ineffective at altering HMG-CoA reductase activity over the concentration range 0.1-10 mM. Therefore, neither terpene alone nor its metabolite exert any direct inhibition on the enzyme.

**The Effect of Menthol or Cineole Dosing on the Thermal Kinetics of Microsomal HMG-CoA Reductase—Mitropoulos and Venkatesan (10) and Sabine and James (9) have suggested that short term (12 h) cholesterol feeding can change the microsomal membrane microenvironment of HMG-CoA reductase and thus decrease its activity. These changes were seen as the abolition of the usual breaks (at 19 °C and 28 °C) in the Arrhenius plots of log HMG-CoA reductase activity against 1/T.** Fig. 3 shows an Arrhenius plot for control and menthol-treated rats. Both plots exhibited breaks at 19 °C.

**TABLE I**

Distribution of menthol-derived [³H] at 17 h after dosing

<table>
<thead>
<tr>
<th>Tissue or fluid</th>
<th>Given dose</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Feces</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum*</td>
<td></td>
<td>0.31</td>
</tr>
</tbody>
</table>

*Fat assumed to be 10% of total body weight.

*Volume of serum assumed to be 7.5 ml/rat.

**Fig. 2.** The binding of [³H]menthol to liver microsomes and the lack of direct inhibition of HMG-CoA reductase by menthol. Liver microsomes from an untreated rat were prepared as described under "Experimental Procedures." The binding of [³H] menthol (approximately 24,000 dpm/nmol) at between 0.01 and 0.5 mM (final concentration) in 100 mm potassium phosphate, pH 7.5, containing EDTA, 10 mM, in a final volume of 1 ml. After mixing, microsomes were applied to a presoaked Whatman GF/F filter paper under suction filtration, washed 10 times with ice-cold potassium phosphate buffer, and counted for radioactivity. ○, the specific activity of HMG-CoA reductase measured after binding menthol to microsomes. Aliquots of menthol were added directly to microsomes in the standard HMG-CoA reductase assay system (prior to preincubation) to give a final menthol concentration between 0.01 and 0.5 mM. HMG-CoA reductase activity was then assayed as described under "Experimental Procedures."

**Fig. 3.** Arrhenius plot of loga HMG-CoA reductase activity against 10⁴/T for control (●) and menthol-treated (Δ) rats. Menthol in olive oil was given by gastric tube at 3.0 mmol/kg of body weight in a volume of 2 ml/kg at 1630 h on the day preceding the experiment. Rats were killed at 0930 h. Tissues were solubilized for counting as described under Experimental Procedures. Body fluids were counted directly without solubilization.

and 28 °C despite a constant menthol-induced inhibition of HMG-CoA reductase activity of about 70% over the entire temperature range. A similar result (not shown) was observed for cineole-treated rats. Thus terpene pretreatment did not significantly alter this measure of enzyme membrane interaction.
The Effect of Menthol Dosing on the $K_m$ for HMG-CoA—Mitropoulos and Venkatesan (10) have shown that short term cholesterol feeding alters some kinetic parameters of microsomal HMG-CoA reductase. They observed a decrease in $V_{max}$ and an approximately 67% decrease in the $K_m$ value for HMG-CoA and have interpreted this effect in terms of altered enzyme microenvironment. After menthol treatment, a large decrease (73%) in $V_{max}$ was observed, but no significant change in the $K_m$ for HMG-CoA was seen for microsomal enzyme from animals in normal or reversed lighting conditions (Table II). Cineole treatment gave very similar results (not shown); thus, although it decreased $V_{max}$ by 82%, the $K_m$ value for HMG-CoA was decreased by only 10%. By this measure, neither menthol nor cineole pretreatment changed the enzyme activity by altering its microenvironment.

The Effect of Menthol or Cineole Dosing on the Apparent HMG-CoA Reductase Activity after Solubilization from Microsomes—It might be expected that HMG-CoA reductase, when solubilized, would lose most of its surrounding lipid, and, therefore, not be under such microenvironmental control as suggested by Mitropoulos and Venkatesan (10). HMG-CoA reductase, solubilized by freeze-thawing in the absence of detergent, from microsomes of menthol- or cineole-treated rats, showed an equivalent inhibition as was seen with the microsomal enzyme from the same animals (Table III).

Effect of Menthol or Cineole Dosing on the Phosphorylation State of HMG-CoA Reductase, Measured in the Presence or Absence of Fluoride—Acute regulation of HMG-CoA reductase by a phosphorylation-dephosphorylation cycle has been demonstrated in vitro (reviewed by Gibson and Ingebritsen (11)). Inactivation is catalyzed by an Mg$^{2+}$-ATP-dependent kinase and activation by a fluoride-sensitive phosphoprotein phosphatase. Both these processes appear to operate in vivo (25). The activity in fluoride-containing medium divided by the activity in chloride medium (where both media contain EDTA) can be thought of as expressing the in vivo HMG-CoA reductase activity as a fraction of the total. This ratio was shown by Brown et al. (27) to be about 0.2 under a variety of physiological conditions. As shown in Table IV, we find this ratio to be around 0.12 both for control and cineole-treated rats. Significantly, inhibition by cineole was evident in enzyme preparations isolated both in the presence and absence of fluoride. This inhibition by cineole seen 17 h after dosing was not accompanied by changes in the degree of phosphorylation. Inhibition of hepatic HMG-CoA reductase by increased phosphorylation within 2 h of feeding with mevalonolactone or cholesterol has been demonstrated by Arebalo et al. (28, 29). No changes in HMG-CoA reductase activity (isolated in the presence or absence of fluoride) are observed 2 h after dosing rats with cineole. Thus, in this respect, inhibition by cineole may differ from that caused by cholesterol or mevalonolactone.

### Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (reverse lighting)</td>
<td>0.24</td>
<td>1.0</td>
</tr>
<tr>
<td>Menthol (reverse lighting)</td>
<td>0.28</td>
<td>1.0</td>
</tr>
<tr>
<td>Control (normal lighting)</td>
<td>0.24</td>
<td>1.0</td>
</tr>
<tr>
<td>Menthol (normal lighting)</td>
<td>0.24</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis indicate number of animals.

### Table III

The effect of solubilization of microsomal HMG-CoA reductase on observed terpene inhibition

Terpenes in olive oil were given by gastric tube at 3 mmol/kg of body weight in a volume of 2 ml/kg at 1630 h on the day preceding the experiment. Rats were killed at 0930 h. Microsomal HMG-CoA reductase was solubilized by freeze-thawing and incubation in 25% glycerol, as described under "Experimental Procedures." HMG-CoA reductase activity was measured as described under "Experimental Procedure."

### Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HMG-CoA reductase activity after isolation</th>
<th>Activity in F medium (ratio of means)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (reverse)</td>
<td>0.19 ± 0.01</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>Cineole (reverse)</td>
<td>0.20 ± 0.01</td>
<td>0.015 ± 0.001</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis indicate number of animals.

### Table V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HMG-CoA reductase activity after isolation</th>
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</tr>
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<tbody>
<tr>
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<td>0.20 ± 0.01</td>
<td>0.015 ± 0.001</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis indicate number of animals.
FIG. 4. The immunotitration of HMG-CoA reductase activity from control and menthol-treated rats. Menthol in olive oil was given by gastric tube at 3.0 mmol/kg of body weight in a volume of 2 ml/kg at 1630 h on the day preceding the experiment. Rats were killed at 0930 h. Immunotitrations were carried out after the preincubation of microsomes with antibody for 30 min in the presence of 0.5% Triton. Residual HMG-CoA reductase activity was determined as described under “Experimental Procedures.” a, an immunotitration in the presence of increasing aliquots of antibody with a constant amount of microsomal protein for control (0.23 mg of microsomal protein, □) and menthol-treated (0.65 mg of microsomal protein, □) animals. b, an immunotitration in the presence of increasing aliquots of microsomal protein with a constant amount of antibody (1 µl) for control (○) and menthol-treated (●) animals.

### TABLE V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity HMG-CoA reductase</th>
<th>Equivalence point (1) nmol/mg/min</th>
<th>(2) %</th>
<th>Equivalence point (1) nmol/min/µl antibody</th>
<th>(2) %</th>
</tr>
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<tbody>
<tr>
<td>Control (5)*</td>
<td>1.020 ± 0.47</td>
<td>100</td>
<td>0.164</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Cineole (5)</td>
<td>0.254 ± 0.140</td>
<td>25</td>
<td>0.112</td>
<td>68</td>
<td>0.37</td>
</tr>
<tr>
<td>Menthol (5)</td>
<td>0.310 ± 0.147</td>
<td>30</td>
<td>0.086</td>
<td>52</td>
<td>0.58</td>
</tr>
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*Numbers in parenthesis indicate number of animals.

The effect of fasting on the inhibition of HMG-CoA reductase by menthol

Animals were deprived of food for 24 h prior to being given menthol in olive oil by gastric tube at 3.0 mmol/kg of body weight in a volume of 2 ml/kg at 1630 h on the day preceding the experiment. The rats were killed at 0930 h. HMG-CoA reductase activity was measured as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic HMG-CoA reductase activity pmol/min/mg</th>
<th>(1)</th>
<th>(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed control (3)*</td>
<td>1090 ± 188</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Fasted control (3)</td>
<td>133 ± 88</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Fasted menthol (3)</td>
<td>17.6 ± 8.5</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

*Numbers in parenthesis indicate number of animals.

The transient nature of the specific inhibition of HMG-CoA reductase by menthol and cineole is compatible with the rapid metabolism and excretion of these terpenes. This effect is not related to a direct terpene inhibition of HMG-CoA reductase, even though menthol can bind to microsomal particles. Terpene-inhibited particulate HMG-CoA reductase does not show deviations from its normal responses to changes in temperature or variations in the concentrations of HMG-CoA, indicating that no modulation of the lipid microenvironment occurs following terpene treatment. Furthermore, the inhibitory effect is still seen after the enzyme is solubilized. The loss of HMG-CoA reductase activity is not associated with increased phosphorylation of the enzyme. Immunotitration of HMG-CoA reductase from terpene-treated rats shows that the activity loss is associated with less enzyme molecules as well as with “crippled” enzyme, indicating that terpenes alter enzyme mass by changing the rates of synthesis and/or deg-
radation. It is possible that terpene ingestion could alter the concentration of hormones that control the mass of the enzyme. To test this possibility, rats were deprived of food for 24 h before they were dosed with menthol. After a further 17 h the menthol inhibition of HMG-CoA reductase was still observed, even though the control activity was itself considerably depressed by fasting. This indicates that terpene inhibition of HMG-CoA reductase is unlikely to be mediated by those hormones (insulin, glucagon, and adrenaline) whose concentration is changed by food intake or fasting.

It is relevant here to compare inhibition of HMG-CoA reductase by monoterpenes with that exerted by cholesterol derivatives. Cyclic monoterpenes are products of the isoprenoid biosynthetic pathway in plants. Certain monoterpenes (such as menthol and cineole) can, like cholesterol, regulate the rate-limiting step of this pathway in mammalian liver. Previously we have shown (8) that the capacity to inhibit hepatic HMG-CoA reductase is seen only in those cyclic monoterpenes in which oxygen is a substituent. This is strikingly similar to the requirements for the inhibition of hepatic and extra-hepatic HMG-CoA reductase activity by sterols, where oxy-substituents of cholesterol (such as 25-hydroxy- or 7-ketocholesterol) are far more potent inhibitors than cholesterol itself (30-32). Furthermore, the oxy- and hydroxy-substituted cyclic monoterpenes cineole and menthol exert their inhibitory effect in the same manner as the oxy-substituted cholesterol derivatives, by decreasing the mass of HMG-CoA reductase enzyme. We, therefore, propose that these plant cyclic monoterpenes could be acting as simple analogues of the inhibitory substituted cholesterol derivatives and could exert their effects by the same (but as yet unknown) mechanism.

Acknowledgments—We wish to thank J. Paxton and J. Hatton for their help in the preparation of this paper.

REFERENCES