Biochemical Pharmacology, Vol. 29, pp. 2125–2127.
Pergamon Press Ltd. 1980. Printed in Great Britain.

INHIBITION OF HEPATIC CHOLESTEROL SYNTHESIS AND *S*-3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE BY MONO AND BICYCLIC MONOTERPENES ADMINISTERED *IN VIVO*

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(*Received* 28 *February* 1980; *accepted* 18 *April* 1980)

Abstract—The ability of a variety of mono- and bicyclic monoterpenes to inhibit hepatic HMGCoA reductase measured 17 hr after *in vivo* administration to rats was determined. Of the terpenes tested, menthol and cineole inhibited by 70 per cent, while borneol and methone were slightly less inhibitory (50 per cent) when dosed at the same rate. Limonene, a rapidly metabolized terpene, also showed significant inhibition, while pinene and camphene were without effect. This inhibition of reductase correlated well with inhibition of C_2-flux into non-saponifiable lipid ($r = 0.86$, $P < 0.001$). There were no changes in a variety of other microsomal membrane activities, indicating that the effect was specific rather than due to generalized hepatoxicity. Possible mechanisms for the inhibition of reductase are discussed.

The formation of cholesterol gallstones in both man [1] and hamsters [2] is associated with increased activity of hepatic *S*-3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase. Rowachol, a proprietary choleretic containing the mono- and bicyclic monoterpenes menthol (32% w/v), pinene (17% w/v), menthone (6% w/v), borneol (5% w/v), camphene (5% w/v) and cineole (2% w/v) in olive oil, has been shown recently to cause dissolution of cholesterol gallstones in man [3] and to inhibit hepatic HMGCoA reductase activity in rats [4] when administered *in vivo*. Isler *et al.* [5] screened a wide variety of acyclic semi-, mono-, sesqui- and triterpenes as inhibitors of sterol biosynthesis but found no evidence for any action *in vivo* (with the exception of squalene), although several acyclic terpenes gave very significant inhibitions *in vitro* of acetate incorporation into sterol. This paper reports investigations into the effect of mono- and bicyclic terpenes on rat hepatic HMGCoA reductase activity and sterol biosynthesis after *in vivo* administration.

MATERIALS AND METHODS

[3-^{14}C]HMGCoA, [2-^{14}C]acetate and [1,2-^{3}H]cholesterol were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [5-^{3}H]Mevalonic acid (DBED salt) was purchased from New England Nuclear Corp., Boston, MA. Acetyl Coenzyme A synthetase (E.C. 6.2.1.1), glucose 6-phosphate, glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) and $NADP^+$ were obtained from Boehringer, Mannheim, F.R.G. Fisofluor scintillation fluid was from Fisons U.K. Ltd. Menthol, pinene, menthone, borneol, camphene and cineole were supplied by Rowa Ltd., Bantry, Eire. All terpenes were adjudged to be pure by g.l.c. analysis on 10% Carbowax 20N.

Male Wistar rats (220–250 g) were adapted to a lighting schedule where lights were on from 3:00 p.m. till 3:00 a.m. Rowachol (Rowa Ltd., Bantry, Eire) was administered daily at 2.0 ml/kg body wt and individual terpenes (in olive oil) at 3 mmole/kg body wt (the concentration of menthol in the Rowachol dose). Animals receiving the drug did not become anorexic; examination of stomach contents post mortem revealed no differences from control animals.

Livers were excised and a portion homogenized in the medium of Goldstein and Brown [6]. The supernatant fraction obtained after spinning at 12,000 *g* for 15 min was centrifuged at 48,000 *g* for 1 hr to obtain a microsomal pellet. This fraction was washed by resuspension in the homogenization buffer followed by centrifugation at 48,000 *g* for 1 hr and finally suspended in 100 mM phosphate buffer, pH 7.5, containing 10 mM EDTA and 5 mM DTT to a final concentration of 10–20 mg protein/ml. A second portion of liver was homogenized and a 5000 *g* supernatant fraction prepared by the method of Dugan *et al.* [7].

Assay procedures. The incorporation of [^{14}C] acetate into non-saponifiable lipid was assayed according to Dugan *et al.* [7] except that acetyl CoA synthetase (0.2 units/ml) was included in the incubations. After incubation for 100 min the reaction was terminated by addition of 2.5 ml of 10% ethanolic KOH. Carrier [1,2-^{3}H]cholesterol (1 mg, 20,000 dpm) was added to each tube as recovery standard. Tubes were

‡ Abbreviations used: HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A; DBED, dibenzylethylenediamine; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

saponified for 1 hr and non-saponifiable lipid was extracted into petroleum ether as described by Dugan *et al.* [7]. [^{14}C]Acetate incorporation into non-saponifiable lipid was found to be linear with time and protein under the conditions used. Analysis of this fraction showed that incorporation into sterols accounted for the majority of the radioactivity in all experiments.

HMGCoA reductase was assayed essentially as described by Goldstein and Brown [6] using a preincubation of 10 min and starting the reaction with [3-^{14}C]HMGCoA. The 0.3 ml incubation system contained: DL-[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; dithiothreitol, 5 mM; EDTA, 10 mM; 50–750 μg of microsomal protein and potassium phosphate, pH 7.5, 100 mM. The reaction was terminated after 15 min with 20 μl 10 M HCl and [5-^{3}H] mevalonate (40,000 dpm) was added as recovery standard. After lactonization the mevalonolactone was separated by thin layer chromatography and counted for radioactivity as described by Shapiro *et al.* [8]. Formation of [^{14}C]-mevalonate under these conditions was linear with time and protein and product recovery averaged 90 per cent.

RESULTS AND DISCUSSION

The terpene mixture, Rowachol (1.2 g terpene/kg body wt), caused a significant inhibition of hepatic HMGCoA reductase measured at the peak of the diurnal rhythm, 17 hr after dosing (Table 1). The effect was persistent since activity was still 50 per cent inhibited 41 hr after dosing. Preliminary experiments had shown that menthol was active in the above inhibition and the other individual terpenes of the parent drug were also assessed for their inhibitory action. Rats were given terpenes at a molar dose equivalent to that of menthol in the original mixture. Table 1 shows the effect of *in vivo* dosing of the individual terpenes on hepatic reductase measured 17 hr after dosing. Another monocyclic terpene, limonene, not a component of Rowachol, was included because considerable information is available on its metabolism and excretion in the rat [9]. Approximately 70 per cent inhibition was caused by both cineole and menthol (oxy- and hydroxy-substituted, respectively), whereas borneol, a bicyclic hydroxyterpene, and menthone, a keto-monocyclic terpene, inhibited approximately 50 per cent. The other two components of the mixture, pinene and camphene, containing no oxygen substituents had no significant effect. Limonene, like camphene and pinene, has no oxygen substituent but did give significant inhibition of reductase activity. This difference in response may reflect limonene's very rapid metabolism to hydroxy-metabolites in the rat [9].

Table 1. Inhibition of HMGCoA reductase by terpenes*

Terpene administered	HMGCoA reductase activity (nmoles.min^{-1}.mg^{-1})
Control	1.08 ± 0.30 (10)
Rowachol	0.43 ± 0.10 (10)†
Menthol	0.26 ± 0.13 (6)†
Pinene	0.92 ± 0.29 (4)
Menthone	0.45 ± 0.21 (7)†
Borneol	0.44 ± 0.15 (3)‡
Camphene	0.76 ± 0.11 (6)
Cineole	0.27 ± 0.10 (6)†
Limonene	0.61 ± 0.05 (3)§

* Rats were intubated with individual terpenes (3 mmoles/kg) in olive oil, or Rowachol (1.2 g terpene mixture/kg) in olive oil or with olive oil alone (2 ml/kg) and 17 hr later the livers were removed and the microsomal fraction isolated. HMGCoA reductase was assayed as described in Materials and Methods and its activity expressed as nmoles mevalonate produced/min per mg microsomal protein ± S.D. with the number of animals in parentheses. Statistical significance with respect to controls is shown.

† $P < 0.001$.

‡ $P < 0.01$.

§ $P < 0.05$.

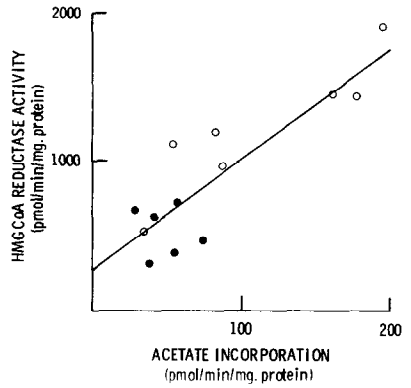

Fig. 1. Correlation of hepatic HMGCoA reductase activity with incorporation of [2-^{14}C]acetate into non-saponifiable lipid after olive oil (○) and cineole (●) treatment *in vivo*. Samples from the same liver were fractionated for microsomal and post mitochondrial supernatant fractions and these were used for the reductase assay and acetate incorporations, respectively. Each point represents the mean of duplicate estimations from individual animals.

Dietschy and Brown [10] have shown that activity of rat hepatic HMGCoA reductase correlates very well with the C_2-flux rate into cholesterol under a variety of dietary and stress conditions; such a correlation holds true after dosing of individual terpenes as illustrated for cineole in Fig. 1. The terpene-induced inhibition of hepatic HMGCoA reductase correlated well ($r = 0.86$, $P < 0.001$) with reduction of incorporation of acetate into non-saponifiable lipid. A major site of action of the terpenes in the inhibition of sterol synthesis was thus at the level of HMGCoA reductase.

Comparison of the structures of the terpenes suggested that only oxygen-containing terpenes or terpenes such as limonene which are rapidly converted to such compounds are the inhibitory agents. The effect is not due to a generalized hepatotoxicity as microsomal yield, cytochromes b_5 and P-450 concentrations, arylesterase and cholesterol 7-hydroxylase activities are unchanged after a single dose of the terpene mixture. Because inhibition of HMGCoA reductase after menthol administration *in vivo* is considerably more potent than the inhi-

bition seen after administering the same amount of menthol plus the other terpene components of Rowachol (Table 1), it is clear that competition can occur between inhibitory and non-inhibitory terpenes.

Isler *et al.* [5] showed that high concentrations (300 mM) of certain acyclic monoterpenes *in vitro* inhibited acetate incorporation into sterol in homogenates but did not investigate the effect on sterol synthesis after pretreating animals with terpenes. A direct effect of the terpenes on HMGCoA reductase is unlikely in the present system because terpene concentrations in the washed microsomal preparations will be much less than that used by Isler *et al.* [5].

Because HMGCoA reductase has a short half-life [11] the observed inhibition might be exerted at the level of enzyme synthesis and/or degradation or conversion into the less active phosphorylated form [12]. Preliminary evidence suggests that, as with inhibition by dietary cholesterol [13], the former is more likely.

Acknowledgements—The authors wish to thank Rowa Ltd. of Bantry, Eire, for the gifts of Rowachol liquid and pure individual terpenes and the Medical Research Council for a research studentship to R.J.C.

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