Dietary β-Ionone Suppresses Hepatic 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity

Suzanne G. Yu,† Naji M. Abuirmeileh,‡ Asaf A. Qureshi,§ and Charles E. Elson*,†

Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706, Jordan University of Science and Technology, Irbid, Jordan, and Advanced Medical Research, 8251 Raymond Road, Madison, Wisconsin 53719

Incorporation of apolar extracts of alfalfa into the diets of chickens effected significant reductions in hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and serum concentrations of cholesterol (total and LDL) and apolipoprotein B. One of several mevalonate-suppressive constituents of the extracts shared characteristics with β-ionone. Dietary β-ionone, a potent inhibitor of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity, was most effective under conditions favoring basal rather than induced reductase activity. This inhibitory action is distinct from that of mevinolin, a competitive inhibitor of reductase activity. The presence of β-ionone in milk was confirmed. This demonstration of the cholesterol-suppressive action of a passive rather than a physiological constituent of milk may explain the conflicting reports of the impact of dairy products on blood cholesterol levels.

Keywords: *β-Ionone, mevalonate synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase, cholesterol, alfalfa*

INTRODUCTION

The widely reported cholesterol-lowering action of alfalfa is generally attributed to the physical impact of alfalfa fibers (Cookson and Fedoroff, 1968; Kelly et al., 1992), β-sitosterol (Cookson and Fedoroff, 1968), and saponins (Malinow et al., 1981) on the absorption of dietary cholesterol and reabsorption of the bile acids. Alfalfa also exerts a cholesterol-lowering action when added to a cholesterol-free diet (Hamilton and Carroll, 1976; Kritchevsky et al., 1977; Malinow, 1982). These studies are generally interpreted as showing that alfalfa fiber and saponins increase the excretion of endogenous cholesterol and the bile acids. An alternative interpretation is suggested by Fitch et al. (1989), who found that hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity is suppressed by petroleum ether soluble constituents of alfalfa.

Studies described in the following sections led to the findings that β-ionone, an end ring analog of β-carotene, and other apolar constituents of alfalfa suppress mevalonate synthesis. The inhibitory action of β-ionone on HMG CoA reductase activity, the rate-limiting step in sterol synthesis, is clearly distinct from the feedback regulation by sterols and the inhibitory action of mevinolin.

MATERIALS AND METHODS

Materials. Pulverized, third cutting, air-dried alfalfa (University of Wisconsin Experimental Station, Madison, WI) was extracted sequentially with petroleum ether (petroleum ether solubles, PES) and methyl alcohol (methyl alcohol solubles, MAS). Chlorophyll and other more polar constituents of the PES were partitioned into methyl alcohol (PES–MA), leaving the more apolar constituents in the PES–PE fraction. Solvents from these extracts and the R_f 0.25 band of the latter (see below) were removed under vacuum. The extracts and two terpenoids, β-ionone (97%) and geraniol (98%), purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), were tested for their impact on avian cholesterol metabolism.

Chromatography. PES–PE extracted materials (30–50 mg) were dissolved in petroleum ether and spotted on silica gel 60 A thin-layer plates (TLC). The plates were developed in a mixture of petroleum ether/diethyl ether/acetic acid (95/5/0.5). Seven bands were identified following exposure to iodine vapor (Kritchevsky and Kirk, 1952). Screening of the chromatographic bands according to the microbiological assay described by Fitch et al. (1989) identified mevalonate-suppressive activity in the R_f 0.25 band.

Larger quantities of the mevalonate-suppressive fraction (R_f 0.25) were isolated from the PES–PE by chromatography on reversed-phase Bond-Elut C_{18} cartridges. Preparation of the 1-mL cartridges consisted of two washes with 1 mL of deionized water followed by two washes with 1 mL of methanol. The PES–PE (0.5 mL, 20 mg of extract/mL) was slowly applied to the column under pressure. Fatty acids and sterols were eluted with two 1-mL washes of petroleum ether, the last trace of which was removed by applying pressure to the column. The mevalonate-suppressive fraction was then eluted with 0.5 mL of methanol. The methanol eluates from several columns were combined, the solvent was removed under vacuum, and the semipurified alfalfa fraction was tested for its impact on cholesterol metabolism.

The R_f 0.25 band, dissolved in acetonitrile, was fractionated by chromatography on a C_{18} column (0.5 μ, 24 cm × 4.2 mm) with methanol as the eluting solvent at a flow rate of 0.5 mL/min. The HPLC system had a stop-flow capacity which permitted the scanning of each peak. The second fraction, UV absorption maximum of 305 nm, had mevalonate-suppressive activity. A preliminary GLC/MS analysis of this fraction revealed peaks in the mass range 180–267 with a major peak at 236 (UV absorption maximum of 305 nm). This peak and a 192 mass constituent suppressed the growth of *Halobacterium halobium* when tested according to the procedure of Fitch et al. (1989). The 192 mass peak matched the library profile of β-ionone. β-Ionone proved to be a potent, mevalonate-reversible suppressor of *H. halobium* growth.

† University of Wisconsin.
‡ Jordan University of Science and Technology.
§ Advanced Medical Research.

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Table 1. Impact of Alfalfa Extracts on Serum Cholesterol and Apolipoprotein A-1 and B Concentrations and Hepatic HMG CoA Reductase Activity[a]

	cholesterol		apolipoproteins		
diet	total[b] (mg/dL)	HDL[b] (mg/dL)	A-1[c] (mg/dL)	B[c] (mg/dL)	HMG CoA reductase[c,d]
control	189 ± 9[a,e]	105 ± 5[a]	151 ± 4[a]	35 ± 3.0[a]	1232 ■ 179[a]
PES	148 ± 5[c]	97 ± 6[c]	147 ± 7[bc]	29 ■ 2.9[bc]	1050 ± 123[ab]
PES–PE	141 ± 6[c]	96 ± 8[c]	149 ± 3[ab]	27 ± 3.0[d]	1086 ± 277[ab]
R_f 0.25	121 ± 6[e]	89 ± 7[d]	151 ± 3[a]	23 ± 2.2[ef]	870 ± 109[b]
PES–MA	131 ± 5[d]	95 ± 7[c]	148 ± 3[bc]	25 ± 2.7[de]	858 ± 93[b]
MAS	176 ± 7[b]	100 ± 5[b]	149 ± 4[ab]	32 ± 1.2[ab]	1053 ± 81[ab]

[a] Six groups of 2-week-old White Leghorn chicks, predominantly cockerels, were fed the basal diet supplemented with 200 ppm of the alfalfa fractions for 24 days. The control diet consisted of ground corn (61.1%), 44% soybean meal (32.5%), corn oil (1.0%), dicalcium phosphate (1.8%), calcium carbonate (1.8%), *dl*-methionine (0.3%), iodized salt (0.5%), and vitamin–mineral mixture (1.0%). the vitamin and mineral mixture supplies, per kilogram, of complete diet, 4500 IU of vitamin A, 596 ICU of vitamin D_3, 30 IU of vitamin E, 1.5 mg of vitamin K, 71 mg of choline, 1.8 mg of thiamin, 32.5 mg of niacin, 3.75 mg of riboflavin, 3.0 mg of pyridoxine, 8 mg of calcium panthothenate, 0.014 mg of vitamin B_{12}, 0.13 mg of Na_2SeO_3, 92 mg of $ZnSO_4$, and 68 mg of MnO_2. Grit (5.0%) was incorporated into the finished diet. The birds were fasted for 36 h and refered for 54 h prior to sacrifice to induce HMG CoA reductase activity (treatment $P < 0.01$). Values in columns lacking a common superscript are significantly different ($P < 0.05$). [b] $n = 12$. [c] $n = 6$. [d] Picomoles of mevalonate per milligram of microsomal protein per minute. [e] SD.

Avian Studies. White Leghorn chicks were fed a commercial cholesterol- and alfalfa-free starter mash (Table 1). The birds were housed at the University of Wisconsin Poultry Research Laboratory. All sacrifices were made between 8:00 and 10:00 a.m. Birds were anesthetized with CO_2. Blood samples were held at room temperature for 1 h and then centrifuged for 15 min at 3000 rpm. Serum cholesterol and apolipoprotein A1 and B concentrations were estimated with Sigma cholesterol reagent and Apo A-1 and Apo B RIA kits. Livers were processed and assayed for HMG CoA reductase activity (Philipp and Shapiro, 1979) as previously described by Fitch et al. (1989).

Milk. Milk samples examined for the presence of mevalonate-suppressive alfalfa metabolites were obtained from a cow in the University of Wisconsin—Madison Department of Dairy Science herd. The cow (no. 3286) was fed a 60% alfalfa test ration. After collection, the milk was held at 4 °C for 24 h prior to saponification. The saponified milk was extracted three times with 100 mL of petroleum ether. The extracts were combined and the solvent was evaporated under vacuum. Chromatographic procedures outlined above yielded a fraction (UV absorption maximum of 288 nm) which had mevalonate-suppressive activity. HPLC was also performed on a normal phase silica column (30 cm × 3.4 mm) with an eluant mixture of 99.5% hexanes/0.5% 2-propanol at flow rate of 0.7 mL/min. The stop-flow scan identified a peak, UV absorption maximum of 288, which had mevalonate-suppressive activity. The chromatographic mobility and UV absorption spectrum of this constituent were same as those of a β-ionone standard subjected to these procedures. A modest change in the proportion of 2-propanol in the eluting solvent resulted in similar changes in the elution time and absorption maxima of both the metabolite and the standard. For example, doubling the proportion of 2-propanol caused a shift in the absorption maxima of each from 288 to 293 nm, the UV maximum for β-ionone. The mass spectral analysis of the purified metabolite gave a molecular ion peak at 192 with a fragmentation pattern nearly identical to that of the β-ionone standard.

Statistical Evaluation. Treatment effects were evaluated by analysis of variance. When the F test indicated a significant treatment effect, the differences between means were analyzed by a protected LSD test.

Table 2. Impact of Diets Supplemented with 250 ppm of β-Ionone and Geraniol on Cholesterol Metabolism[a]

	cholesterol			
treatment	total (mg/dL)	HDL (mg/dL)	HMG CoA reductase[c]	wt gain (g)
control	120 ± 20[b]	70 ± 8	468 ± 116	404 ± 42
β-ionone	80 ± 6	56 ± 6	110 ± 56	447 ± 48
geraniol	70 ± 11	54 ± 8	252 ± 141	407 ± 59

[a] Groups of 4-week-old White Leghorn pullets were fed the diets (Table 1) for 4 weeks. [b] $n = 8$, SD. [c] Picomoles of mevalonate per milligram of microsomal protein per minute. Treatment ($P < 0.01$) for all parameters.

RESULTS

The impacts of diets containing 200 ppm of the crude apolar extracts of alfalfa on avian cholesterol metabolism are shown in Table 1. Hepatic HMG CoA reductase activity was induced by a short-term fasting/refeeding regimen. Under these conditions constituents of the PES and MAS suppressed hepatic HMG CoA reductase activity by 15%. The residue remaining after removal of the MAS was devoid of mevalonate-suppressive activity (not shown). PES constituents were more potent than the MAS constituents in lowering serum cholesterol and apolipoprotein B concentrations. The PES constituents were partitioned between petroleum ether (PES–PE) and methyl alcohol (PES–MA). The PES–MA proved to be more effective in suppressing hepatic HMG CoA reductase activity and concomitantly in lowering serum cholesterol and apolipoprotein B concentrations (Table 1). On finding that weight gain was impaired by this chlorophyll-rich extract, we directed our efforts toward the isolation of the cholesterol-suppressive agent from the PES–PE extract. The R_f 0.25 obtained by chromatography of this fraction contained mevalonate-suppressive constituent(s). This action is confirmed by results of the feeding trial (200 ppm R_f 0.25 band) which show a 30% decrease in hepatic HMG CoA reductase activity with concomitant reductions in serum cholesterol and apolipoprotein B concentrations (Table 1).

One consituent of the R_f 0.25 band had properties in common with β-ionone. Our second feeding trial compared the impact of this end-ring analog of β-carotene with that of geraniol (Elson et al., 1989) on cholesterol metabolism. Experimental diets containing 250 ppm of geraniol and β-ionone were fed to 4-week-old pullets for 4 weeks. The treatments significantly lowered serum cholesterol and HDL cholesterol (Table 2). Serum values reported on Tables 1 and 2 reflect group differences in age and sex. Differences in the protocols, the fasting/refeeding regimen (Table 1), and the fed regimen (Table 2) account for the 3-fold difference in the hepatic HMG CoA reductase activities of the control groups. Under fed conditions the treatments suppressed HMG CoA reductase activity by more than 50% with β-ionone being the more potent suppressor. The negative finding was a treatment-mediated 20% decrease in HDL cholesterol.

Reducing the concentration of dietary β-ionone and geraniol to 100 ppm gave more promising results (Table 3). β-Ionone elicited a 15% decrease in serum total cholesterol and a 9% increase in HDL cholesterol. Hepatic HMG CoA reductase activity in the 5-week-old cockerels following fasting and refeeding was decreased by 45%. Geraniol decreased serum cholesterol and hepatic HMG CoA reductase activity by 10% and 20%, respectively. Parallel groups of birds were fed diets formulated with pentobarbital to induce the hepatic microsomal P450

Table 3. Impact of β-Ionone and Geraniol on Cholesterol Metabolism and Interactions with Pentobarbital and Mevinolin[a]

treatment	cholesterol[b] total (mg/dL)	cholesterol[b] HDL (mg/dL)	HMG CoA reductase[c]	wt gain	cockerels (n)
control	134 ± 4[a g]	74 ± 11[bc]	1065 ± 132[b]	287 ± 34	7
+ pentobarbital[d]	133 ± 6[a]	73 ± 9[c]	1029 ± 117[b]	266 ± 58	6
+ mevinolin[e]			1519 ± 126[a]		6
β-ionone[f]	113 ± 4[c]	81 ± 10[a]	566 ± 163[d]	351 ± 127	8
+ pentobarbital[d]	130 ± 8[a]	77 ± 5[b]	912 ± 238[c]	266 ± 58	5
+ mevinolin[e]			1099 ± 55[b]		5
geraniol[f]	121 ± 14[b]	75 ± 6[b]	838 ± 88[c]	259 ± 25	8
+ pentobarbital[d]	137 ± 21[a]	71 ± 6[c]	1016 ± 255[b]	249 ± 50	6

[a] White Leghorn chicks (2 weeks of age) were distributed into two groups of 24 (control and β-ionone) and four groups of 16 (control + pentobarbital, β-ionone + pentobarbital, geraniol, and geraniol + pentobarbital). The birds were fed the diet (Table 1) for 17 days, fasted for 2 days, and refed for 3 days to induce HMG CoA reductase activity. The data represent values for cockerels (treatment $P < 0.01$). Values in columns lacking common superscript are significantly different ($P < 0.05$). [b] Blood was taken before the fasting/refeeding regimen. [c] Picomoles of mevalonate per milligram of microsomal protein per minute. [d] 20 ppm. [e] 500 ppm fed only during refeeding period. [f] 100 ppm. [g] SD.

monooxygenase activity that degrades the isoprenoids (Licht and Coscia, 1978; Chada and Madyastha, 1984; Austin et al., 1988). When coupled with this treatment, neither isoprenoid had an impact on cholesterol concentration or HMG CoA reductase activity (Table 3).

The third phase of this trial offers insight into the β-ionone-mediated supression of HMG CoA reductase activity. Mevinolin, a competitive inhibitor of HMG CoA reductase activity, induces the synthesis of the reductase protein. This increase in protein can be detected by enzyme assay in vitro after the inhibitor has been removed or diluted (Goldstein and Brown, 1990). The impact of mevinolin is recorded in Table 3; adding 500 ppm of mevinolin to the control diet during the refeeding phase increased HMG CoA reductase activity by 50% (Table 3). Under conditions which maximally (fasting/refeeding and mevinolin) or moderately (fasting/refeeding) induce hepatic HMG CoA reductase activity, dietary β-ionone (100 ppm) suppressed the activity by 500 pmol of mevalonate (mg of microsomal protein)$^{-1}$ min^{-1}. As previously noted, the protocol for the second study (Table 2) did not include a regimen to induce HMG CoA activity. Under this protocol dietary β-ionone suppressed HMG CoA reductase activity by 350 pmol of mevalonate (mg of microsomal protein)$^{-1}$ min^{-1}.

DISCUSSION

Although the treatment groups in these studies differed in age, sex distribution, and level of dietary β-ionone, the results suggest that the potency of β-ionone as a suppressor of HMG CoA reductase activity was greatest under fed conditions. Fasting/refeeding with adjunctive mevinolin and, less effectively, fasting/refeeding induce HMG CoA reductase activity at the level of transcription (Goldstein and Brown, 1990). Under these respective conditions β-ionone effected 25% and 50% decreases in reductase activity. Our finding of the greater impact of β-ionone on HMG CoA reductase activity under fed conditions, a 75% decrease in reductase activity, is consistent with that reported for other isoprenoid products plant metabolism. Menthol (Clegg et al., 1982) and the tocotrienols (Parker et al., 1993) modulate cholesterol synthesis through the post-transcriptional suppression of HMG CoA reductase activity. This suppression, characterized by parallel decreases in enzyme mass and activity, is similar to that attributed to the non-sterol component of the multivalent feedback regulation of HMG CoA reductase as described by Goldstein and Brown (1990).

Mevalonate synthesis is suppressed by a variety of secondary products of plant isoprenoid metabolism (Elson and Yu, 1994). These minor constituents account in part for the cholesterol-lowering action of essential oils (Elson et al., 1989; Qureshi et al., 1988; Fitch et al., 1989), and fiber-rich products including barley (Qureshi et al., 1986), oats (Peterson and Qureshi, 1993), and alfalfa. β-Ionone and its oxygenated analogs are widely distributed in fruits and other plant products in conjugated and free forms (Wilson et al., 1981; Winterhalter et al., 1990; Takeoka et al., 1990; Sefton and Williams, 1991; Krammer et al., 1991 Charles et al., 1991; Pabst et al., 1991; Humpf et al., 1991; Voilley et al., 1991).

The initial report by Mann and Sperry (1974) that whole milk products lower serum cholesterol levels has been confirmed and extended by human and animal studies (Bogualawski, and Wrobel, 1974; Malinow and McLaughlin, 1975; Howard and Marks, 1977; Nair and Mann, 1977; Ahmed et al., 1979; Papa et al., 1982; Stoll et al., 1991). Contrary to these findings, other tests fail to show a cholesterol-lowering action of milk and milk products (Hussi et al., 1981; McNamara et al., 1989). The rationale for the current work is that passive constituents of milk, collectively a class of mevalonate-derived, apolar plant metabolites, suppress HMG CoA reductase activity. β-Ionone has an odor variously described as woody, violet, or mildly haylike (Suyama et al., 1983). These odors are characteristic of the "off-flavor" of milk, which has been attributed to products of the thermal or photochemical mediated oxidation of β-carotene (Dufour and Haertle, 1990) and, in fortified milk, of retinyl palmitate (Suyama et al., 1983). Our detection of β-ionone in alfalfa and in milk that was neither processed nor fortified points to the passive transfer of this isoprenoid as well as the carotenoid constituents of forages and silages into milk. The findings of cholesterol-suppressive factors in skimmed milk (Malinow and McLaughlin, 1975; Richardson, 1978; Kritchevsky et al., 1979; Dull et al., 1983) suggest that more polar constituents such as those present in the PES–MA and MAS extracts of alfalfa might be passive constituents of milk. Alternatively, the finding that β-ionone binds to β-lactoglobulin, the major globular protein in the whey fraction, may explain the presence of a cholesterol-suppressive agent in skimmed milk (Dufour and Haertle, 1990, 1991). Milk provides a route through which cholesterol-suppressive constituents of forages and silages enter the human diet. These results may reconcile the contradictory reports of the impact of milk and other dairy products on cholesterol metabolism.

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