Plant-Derived Monoterpenes Suppress Hamster Kidney Cell 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Synthesis at the Post-Transcriptional Level[1]

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ABSTRACT The rate-limiting enzyme for mevalonate and cholesterol synthesis in mammalian cells is 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Control occurs through both transcriptional and post-transcriptional actions signaled by the end product, cholesterol, and by isoprenoid intermediates. End products of plant mevalonate metabolism, i.e., plant-derived isoprenoids, also suppress mammalian HMG-CoA reductase. Previous studies reported that isoprenoids suppress reductase synthesis at a post-transcriptional level. We tested the hypothesis that plant-derived isoprenoids also regulate mammalian HMG-CoA reductase synthesis at a post-transcriptional level by incubating lovastatin-treated C100 cells with mevalonate or a plant-derived isoprenoid (the monoterpenes, limonene, perillyl alcohol or geraniol) either alone or combined with the oxysterol, 25-hydroxycholesterol (25-OH C). Mevalonate decreased HMG-CoA reductase synthesis and mRNA levels by 65 and 66%, respectively ($P < 0.05$). The cyclic monoterpenes, limonene and perillyl alcohol, lowered HMG-CoA reductase synthesis by 70 and 89%, respectively ($P < 0.05$); although neither reduced HMG-CoA reductase mRNA levels ($P = 0.88$). Geraniol, an acyclic monoterpene, suppressed HMG-CoA reductase synthesis by 98% and lowered mRNA levels by 66% ($P < 0.05$). A combination of 25-OH C and either mevalonate or any three monoterpenes reduced HMG-CoA reductase mRNA levels ($P < 0.05$) compared with lovastatin-only treated cells. However, the dual combination of 25-OH C and either mevalonate or a monoterpene resulted in a greater decrease in HMG-CoA reductase synthesis than in mRNA levels. The difference between changes in HMG-CoA reductase synthesis and mRNA levels reflects a specific effect of isoprenoids on HMG-CoA reductase synthesis at the translational level. Mevalonate enhanced HMG-CoA reductase degradation, but no such effect was observed for the monoterpenes. These results indicate that the three plant-derived isoprenoids primarily suppress HMG-CoA reductase synthesis at a post-transcriptional level by attenuating HMG-CoA reductase mRNA translational efficiency. J. Nutr. 133: 38–44, 2003.

KEY WORDS: • *3-hydroxy-3-methylglutaryl-CoA reductase* • *mevalonate* • *isoprenoids* • *monoterpenes*

Plant-derived isoprenoids formed from mevalonate are recognized as potent suppressors of mammalian 3-hydroxy-3-methylglutaryl (HMG)[3]-CoA reductase (1–11). Although some of these studies have established that plant-derived isoprenoids decrease the enzyme mass (5,6,9), the specific levels at which this regulation occurs are not well characterized. The isoprenoid, tocotrienol, has been shown to suppress HMG-CoA reductase levels in HepG2 cells by enhancing degradation of the 97-kDa membrane–associated HMG-CoA reductase protein as well as reducing HMG-CoA reductase synthesis at an undefined post-transcriptional level (9). Although other isoprenoids, the monoterpenes, may regulate HMG-CoA reductase at a post-transcriptional level, no studies have presented data demonstrating at what level these monoterpenes regulate this enzyme. Because the dietary intake of plant-derived isoprenoids has been associated with chemoprevention of cancer (4,12) as well as cholesterol lowering (10), an effect associated with suppression of HMG-CoA reductase, a more thorough understanding of this isoprenoid-mediated regulatory mechanism may enhance our understanding of this chemopreventive effect.

The rate-limiting enzyme of mevalonate biosynthesis in mammalian cells is HMG-CoA reductase (13,14). There are two protein species of HMG-CoA reductase in mammalian cells. One is a 97-kDa form that is associated with the endoplasmic reticulum (ER) (13,15,16). A second 90-kDa form of HMG-CoA reductase is associated with peroxisomes and is thought to play a major role in nonsterol biosynthesis (17,18). The 97-kDa form is translated from a group of heterogenous mRNAs ~5 kb in size (13,16,19,20). Currently, the gene

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[3] Abbreviations used: DFBS, delipidized fetal bovine serum; FBS, fetal bovine serum; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; 25-OH C, 25-hydroxycholesterol;MEM, minimum essential medium; RPA, ribonuclease protection assay; RP S17, ribosomal protein S17; SREBP, sterol regulatory element binding protein.

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coding for the novel 90-kDa HMG-CoA reductase protein is unknown, although this peroxisome-associated form has properties distinct from the ER-associated protein species (21).

Regulation of HMG-CoA reductase occurs by feedback regulation through a complex interplay of transcriptional (13,22,23), post-translational (24–29) and post-transcriptional (1,24–27,30–33) control mechanisms. Feedback regulation is mediated by both mevalonate-derived sterol and isoprenoid or nonsterol intermediates of the cholesterol biosynthetic pathway (13). In addition, HMG-CoA reductase is subject to regulation by mitogenic stimuli, an effect mediated independently of metabolic feedback regulation (33–35).

Transcriptional control of HMG-CoA reductase mRNA levels is mediated primarily by cholesterol or oxygenated derivatives of cholesterol referred to generically as oxysterols (13,22,23). These sterols regulate the post-translational proteolytic processing of the sterol regulatory element binding protein-1 (SREBP-1) (36–38). Low cellular cholesterol levels initiate proteolytic cleavage of the amino terminal fragment from membrane-bound SREBP, which subsequently enters the nucleus and binds to the sterol regulatory element (SRE) located in the 5′-promoter region of the reductase gene (38). This promoter sequence–specific binding increases transcription from the negative sterol regulated promoter elements (14,37,39,40).

Post-translational control of HMG-CoA reductase occurs through a mechanism regulating stability of the 97-kDa membrane associated form of the enzyme (1,13,25,28,29,31,41). Both sterols and isoprenoids or nonsterols have been shown to accelerate degradation of HMG-CoA reductase (2,3,13,25). The membrane-spanning region of reductase, which consists of multiple hydrophobic domains (13,42), is required for sterol or nonsterol-mediated degradation. The isoprenoid that signals accelerated reductase degradation has been identified as farnesol (2,3). It has recently been shown that both mammalian (29) and yeast HMG-CoA reductase (43) undergo regulated degradation through the ubiquitin-proteosome pathway (29,41,43).

Post-transcriptional control of HMG-CoA reductase is mediated through two types of regulatory phenomena. One involves an oxysterol-mediated process that destabilizes HMG-CoA reductase mRNA (23), a process mediated through 1600 bases of 3′ untranslated HMG-CoA reductase mRNA (44). A second post-transcriptional regulatory process involves control of HMG-CoA reductase synthesis at the translational level (1,13,24–26,30,31). This translational control is mediated by isoprenoids or nonsterols, either alone or in combination with oxysterols (1,24–26,30). A nonsterol synthesized between farnesyl diphosphate (FPP) and lanosterol decreases the translational efficiency of HMG-CoA reductase mRNA (30), an effect that appears to be regulated at the level of translation initiation (31).

In this study, we characterized the effects of three plant-derived isoprenoids, the monoterpenes, limonene, perillyl alcohol and geraniol, on HMG-CoA reductase mRNA levels, rates of synthesis and degradation in Syrian hamster kidney cells (C100 cells). These cells express high levels of HMG-CoA reductase because of a stable fourfold amplification of the HMG-CoA reductase gene (45). In addition, regulation of HMG-CoA reductase in C100 cells by oxysterols and mevalonate-derived isoprenoids and nonsterols has been characterized extensively (23,25,29,41). Therefore, this HMG-CoA reductase gene-amplified cell line represents an excellent experimental system with which to define the monoterpene-mediated mechanisms regulating this enzyme.

MATERIALS AND METHODS

Media and cells. The baby hamster kidney cell lines, C100 and SV28, were obtained from Dr. Robert Simoni, Department of Biologic Sciences, Stanford Medical School. C100 cells were grown in minimum essential medium (MEM) supplemented with 5% delipidized (lipid-deficient) fetal bovine serum (FBS) and 5 μmol/L lovastatin as described previously (25,30,31). SV28 cells (45) were grown in MEM supplemented with 5% FBS. During all experiments, cells were grown in either 5% FBS-MEM or 5% delipidized FBS (DFBS-MEM). Lovastatin was provided by Merck Laboratories (Rantan, NJ) and was prepared as described previously (25). Mevalonate was dissolved in deionized water and brought to a pH of 7.0 and final concentration of 1.0 mol/L as described previously (25). The oxysterol, 25-hydroxycholesterol (25-OH C), was dissolved in 100% ethanol to a final concentration of 1.2 mmol/L. The monoterpenes, limonene, perillyl alcohol and geraniol were obtained from Aldrich Chemicals (St Louis, MO). These were dissolved in 100% ethanol to final concentrations of 5, 0.7 and 0.4 mol/L of limonene, perillyl alcohol and geraniol, respectively. Final concentrations for limonene, perillyl alcohol and geraniol in all experiments were 5, 0.7 and 0.4 mmol/L, respectively. The concentration of ethanol in the cell culture medium did not exceed 0.01% (v/v).

Cellular treatment and RNA isolation. C100 cells were incubated in MEM-FBS for 24 h. The medium was replaced with DFBS-MEM containing 25 μmol/L lovastatin alone, or lovastatin plus mevalonate (10 mmol/L), limonene, perillyl alcohol or geraniol. In addition, a parallel series of treatments included the addition of 1.2 μmol/L 25-OH C to cells treated with mevalonate and the three monoterpenes. Cells were incubated for an additional 16 h; the medium was removed and RNA isolated by the single-step acid guanidinium thiocyanate/phenol/chloroform extraction procedure (46) as modified by Choi et al. (23).

Measurement of RNA levels. A ribonuclease protection assay (RPA) as described by Peffley and Gayen (31) was used to measure levels of HMG-CoA reductase and ribosomal protein S17 (RP S17) mRNA. EcoRI linearized pRedD2 and SP6 were used to transcribe antisense RNA transcripts complementary to reductase mRNA. Antisense transcripts complementary to RP S17 were synthesized from AvaII-restricted pRP S17 using T7 RNA polymerase. RNA was synthesized and labeled with [^{32}P] CTP as described previously (31). Each hybridization assay contained ~2.5 kBq of [^{32}P]-labeled probe; hybridizations were done at 50°C for 16 h. An RPA II kit (Ambion, San Antonio, TX) was used for subsequent protocols. RNase A and T1 were added to the hybridization mixes to digest unhybridized probe. Hybridized or protected RNA species representing either HMG-CoA reductase or RP S17 mRNAs were resolved by electrophoresis on 6–8% polyacrylamide/7 mol/L urea gels. The gels were exposed to X-ray film and relative changes in mRNA estimated by densitometric scanning of autoradiographs.

Radioimmunoprecipitation of HMG-CoA reductase. C100 cells (1×10^6) were inoculated into 60-mm cell culture plates and incubated for 24 h in FBS-MEM. To determine the rate of synthesis, the medium was changed to DFBS-MEM supplemented with 25 μmol/L lovastatin and either mevalonate, limonene, perillyl alcohol or geraniol in the presence or absence of 25-OH C. Cells were then labeled with 5.55 MBq [^{35}S] methionine [ICN Radiochemicals, Irvine, CA; 2.70×10^{-8} mol/L] in methionine-free medium for 1 h as described previously (25). Labeling was done in the presence of lovastatin, mevalonate, 25-OH C and the three monoterpenes as described above. HMG-CoA reductase degradation rates were determined by labeling cells with [^{35}S] methionine as described above in methionine-free DFBS-MEM supplemented with 25 μmol/L lovastatin. The medium was then changed to DFBS-MEM plus methionine supplemented with mevalonate, limonene, perillyl alcohol or geraniol.

HMG-CoA reductase immunoprecipitation was done as described previously by Peffley and Gayen (30). Modifications to the lysis protocol were made according to Correl and Edwards (2). Medium was removed from the culture plates and cells lysed directly in 1 mL of lysis buffer (10 mmol/L sodium phosphate, pH 7.5, 5 mmol/L EGTA, 5 mmol/L EDTA, 100 mmol/L NaCl, 1% (v/v) Triton X-100, 1 g/L SDS, 5 g/L sodium deoxycholate, 50 μmol/L leupeptin and 1.0

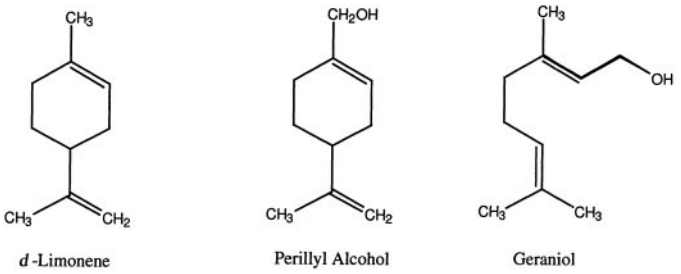

FIGURE 1 Structures of the monoterpenes, *d*-limonene, perillyl alcohol and geraniol

mmol/L phenyl methyl sulfonate). Lysates were cleared of insoluble material by centrifugation at 10,000 × *g* at 4°C for 15 min. A HMG-CoA reductase antibody generated against rat HMG-CoA reductase and known to cross-react with hamster HMG-CoA reductase was added to the lysates and HMG-CoA reductase immunoprecipitated, electrophoresed and autoradiographed as described previously (25). The 97-kDa protein species corresponding to immunoprecipitated HMG-CoA reductase was excised from the gel, and the amount of [^{35}S] methionine estimated by liquid scintillation spectrometry.

Statistical methods. Assessment of treatment-mediated effects on HMG-CoA reductase mRNA levels and rates of synthesis utilized unpaired *t* tests. HMG-CoA reductase degradation rates were estimated by linear regression analysis (24). Statistical analyses were done with StatView software (Abacus Concepts, Berkeley, CA).

RESULTS

The monoterpenes reduce synthesis of HMG-CoA reductase at a post-transcriptional level. Cellular lovastatin treatment inhibits endogenous mevalonate and cholesterol biosynthesis (13,24,47). Consequently, HMG-CoA reductase mRNA and protein levels are elevated because feedback suppression of HMG-CoA reductase transcription as well as its synthetic rate from reductase mRNA are decreased (13). Therefore, the level at which various compounds control HMG-CoA reductase can be evaluated by supplementing cell culture medium with a specific compound, and after a suitable period of incubation, measuring HMG-CoA reductase mRNA levels and rates of synthesis. If drug-mediated regulation of HMG-CoA reductase synthesis occurs only at the transcriptional level, a direct correlation between changes in HMG-CoA reductase mRNA levels and synthetic rate is anticipated. On the other hand, a greater degree of change in the rate of synthesis compared with that for HMG-CoA reductase mRNA levels would imply some type of post-transcriptional regulation of HMG-CoA reductase synthesis as described previously (1,13,24–26,30). Because HMG-CoA reductase is also regulated at the level of degradation, we also determined its half-life under various treatment conditions, and these values are also reported in this study.

The isoprenoids selected for this study were the monoterpenes, limonene, perillyl alcohol and geraniol (**Fig. 1**) because they have been shown in previous studies to suppress HMG-CoA reductase activity at levels readily achievable in cellular and animal studies (4–7,9–11,48). However, the specific nutrient-gene interactions mediating monoterpene regulation of HMG-CoA activity are not well characterized. Because these plant-derived isoprenoids are structurally similar to those from the mammalian mevalonate biosynthetic pathway, we wanted to test the hypothesis that their effects on reductase synthesis would be similar to those mediated by mevalonate in lovastatin-treated hamster cells

All three of these monoterpenes alone suppressed synthesis of the 97-kDa protein species corresponding to ER-associated HMG-CoA reductase (**Fig. 2**). However, the magnitude of the reductions was not the same for the three monoterpenes (**Table 1**). When added alone to lovastatin-treated cells, limonene decreased reductase synthesis by ~70% ($P < 0.05$), whereas a 65% reduction ($P < 0.05$) was mediated by 10 mmol/L mevalonate. Perillyl alcohol, an oxidation product of limonene, decreased reductase synthesis by 89% ($P < 0.05$). Geraniol was the most potent regulator of reductase and decreased synthesis by ~98% ($P < 0.05$).

The levels of HMG-CoA reductase mRNA were also determined under identical experimental conditions by RPA. The autoradiographs corresponding to these experiments are shown in **Figure 3**. HMG-CoA reductase mRNA appears as a 400-bp protected RNA species. As a control, antisense probe corresponding to RP S17 mRNA was added to each hybridization. This control mRNA appears as a 180-bp protected RNA species and does not respond to changes in cellular mevalonate and cholesterol biosynthesis or any of the treatment conditions known to affect HMG-CoA reductase mRNA (30).

With both limonene and perillyl alcohol treatments, HMG-CoA reductase mRNA levels did not differ ($P = 0.88$) from those in cells treated only with lovastatin (Fig. 3; Table 1). Therefore, because HMG-CoA reductase mRNA levels were unchanged in the presence of limonene and perillyl alcohol, whereas under the same conditions, HMG-CoA reductase synthesis was significantly decreased, these two monoterpenes appear to act only at a post-transcriptional level. This regulation appears to be analogous to the post-transcriptional control of the reductase mediated by endogenous mevalonate-derived nonsterols described in earlier studies (1,24–26,30, 31,41).

In contrast, the monoterpene, geraniol, decreased HMG-CoA reductase mRNA levels by ~66% ($P < 0.05$) when added to lovastatin-treated cells (Fig. 3; Table 1). In this respect, geraniol was unique because its behavior was similar to that of sterols such as cholesterol or 25-OH C (13,22,25). However, this decrease in mRNA levels was less than that for the rate of synthesis (98%), indicating that geraniol also

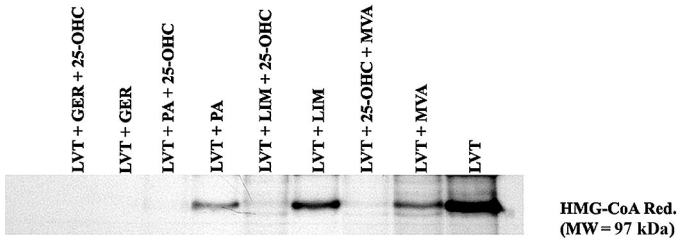

FIGURE 2 Fluorograph representing the effects of mevalonate-derived isoprenoids on 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase synthesis in C100 cells. Cells (1×10^6) were incubated for 16 h in delipidized fetal bovine serum-minimum essential medium (DFBS-MEM) plus 25 μmol/L lovastatin (LVT) alone or supplemented with mevalonate (MVA at 10 mmol/L) or mevalonate (10 mmol/L) plus 25-hydroxycholesterol (25-OH C at 1.2 μmol/L), limonene (LIM at 0.5 mmol/L) or limonene (0.5 mmol/L) plus 25-OH C (1.2 μmol/L), perillyl alcohol (PA at 0.6 mmol/L) or perillyl alcohol (0.7 mmol/L) plus 25-OH C (1.2 μmol/L) and geraniol (GER at 0.4 mmol/L) or geraniol plus 25-OH C (1.2 μmol/L). Cells were labeled for 1 h with [^{35}S]-methionine and reductase solubilized with TritonX-100 and immunoprecipitated with an HMG-CoA reductase specific antibody. HMG-CoA reductase appears as a 97-kDa protein species. The fluorograph shown is representative of three independent determinations.

TABLE 1

Effects of isoprenoids on HMG-CoA reductase synthesis, mRNA levels, and degradation in lovastatin-treated Syrian hamster cells[1]

Treatment	Rate of synthesis[2]	mRNA levels[3]	Half-life[4]
	%		*h*
Lovastatin	100 ± 15	100 ± 5	10.0 ± 1.2
Lovastatin + Mevalonate (10 mmol/L)	35 ± 5	34 ± 3	4.0 ± 0.4*
Lovastatin + Mevalonate (10 mmol/L) + 25-OH C (1.2 μmol/L)	5.6 ± 0.6	21 ± 3*	ND
Lovastatin + Limonene (5 mmol/L)	30 ± 6*	115 ± 7	10.5 ± 1.3
Lovastatin + Limonene (5 mmol/L) + 25-OH C (1.2 μmol/L)	5.2 ± 0.4*	21 ± 5*	ND
Lovastatin + Perillyl alcohol (0.7 mmol/L)	11 ± 3	112 ± 5	16.0 ± 1.9*
Lovastatin + Perillyl alcohol (0.7 mmol/L) + 25-OH C (1.2 μmol/L)	6.2 ± 0.5*	27 ± 6*	ND
Lovastatin + Geraniol (0.4 mmol/L)	1.4 ± 0.3*	34 ± 6*	9.6 ± 1.0
Lovastatin + Geraniol (0.4 mmol/L) + 25-OH C (1.2 μmol/L)	<0.5	14 ± 2*	ND

[1] Abbreviations: HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; 25-OH C, 25-hydroxycholesterol; ND, not determined.

[2] Rates of reductase synthesis were based on the dpm of ^{35}S-labeled immunoprecipitable reductase from 1×10^6 cells after a 1-h labeling period. Values were corrected for incorporation of ^{35}S-methionine on the basis of the amount of trichloroacetic acid precipitable ^{35}S-labeled protein for each treatment condition. Rates of reductase synthesis in lovastatin-treated cells were set at 100% and all other values are reported as a percentage of this value. Values are means ± SD, $n = 3$.

[3] Reductase RNA values were determined by ribonuclease protection assay and corrected for RNA loading effects on the basis of the amount of ribosomal protein (RP) S17 mRNA. Corrected reductase RNA values in lovastatin-treated cells were set at 100% and all other values are reported as a percentage of this value. Values are means ± SD, $n = 3$.

[4] Cells were labeled with [^{35}S] methionine in the presence of lovastatin and the ^{35}S-labeled reductase immunoprecipitated at various time points after addition of mevalonate, limonene, perillyl alcohol or geraniol as indicated in the table. ^{35}S-labeled reductase was immunoprecipitated and quantitated as described in the Materials and Methods. The half-life and error (± SD) were determined as described previously (24). * Different from the lovastatin control. $P < 0.05$.

regulated reductase levels at a post-transcriptional level similar to limonene and perillyl alcohol. Whether geraniol has a direct effect on transcription factors mediating reductase transcription as described for cholesterol and other oxysterols remains to be determined.

Adding 10 mmol/L mevalonate to lovastatin-treated cells also decreased ($P < 0.05$) HMG-CoA reductase mRNA levels by ~65% after an incubation period of 16–20 h (Fig. 3; Table 1). This magnitude of decrease in mRNA levels was comparable to the decrease in the rate of synthesis under similar treatment conditions (Table 1). In previous studies, adding high concentrations of mevalonate to hepatocytes generated sterols, which are regulators of HMG-CoA reductase transcription (49). Therefore, this decrease in reductase mRNA levels could most likely be attributed to increased sterol production from mevalonate through the cholesterol biosynthetic pathway.

Oxysterols enhance the post-transcriptional effects of monoterpenes on reductase synthesis. The oxysterol, 25-OH C, has been shown to synergistically enhance the post-transcriptional suppression of HMG-CoA reductase synthesis by mevalonate-derived isoprenoids (24,25). Therefore, in this study, we added 25-OH C along with the three monoterpenes to lovastatin-treated cells to determine whether they could similarly enhance the post-transcriptional regulation of HMG-CoA reductase synthesis. As expected, when 25-OH C was added to lovastatin-treated cells containing any of the three monoterpenes, there was an ~70% decrease ($P < 0.05$) in reductase mRNA levels (see Fig. 3; Table 1). These results agree with those reported previously for the effects of 25-OH C on HMG-CoA reductase mRNA levels where this oxysterol was shown to mediate a decrease in HMG-CoA reductase transcription (23). However, as shown in Figure 2 and summarized in Table 1, the rate of synthesis in the presence of oxysterols and these three isoprenoids was decreased more than could be accounted for by changes in HMG-CoA reductase mRNA levels. With limonene and 25-OH C, there was a 95% decrease in HMG-CoA reductase synthesis, but only a 70% decrease in the corresponding mRNA level. Similarly, an 80% decrease in perillyl alcohol plus 25-OH C–treated cells was accompanied by a 94% reduction in synthesis. Geraniol plus 25-OH C treatment resulted in a 66% decrease in HMG-CoA reductase mRNA levels but a >99% reduction in HMG-CoA reductase synthesis. This occurred also in cells treated with mevalonate and 25-OH C where HMG-CoA reductase mRNA levels and rates of synthesis were decreased by 70 and 95%, respectively.

Monoterpenes do not enhance degradation of HMG-CoA reductase. Mevalonate-derived isoprenoids enhance degradation of HMG-CoA reductase in mammalian cells either alone (16,18) or in the presence of sterols (24–26). Therefore, we also assessed the effects of these three plant-derived isoprenoids on HMG-CoA reductase degradation in the presence of lovastatin. Degradation rates were compared with those in

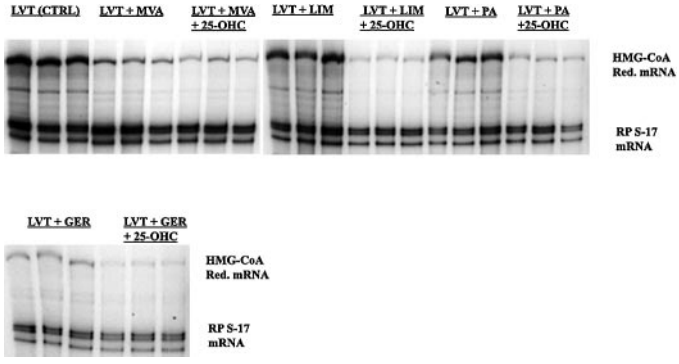

FIGURE 3 Autoradiograph showing the effects of lovastatin (LVT), mevalonate (MVA), limonene (LIM), perillyl alcohol (PA) and geraniol, (GER) either alone or in combination with 25-hydroxycholesterol (25-OH C) on the regulation of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase mRNA levels in C100 cells. Cellular treatments were as described in the legend to Figure 2. RNA was isolated from cells and levels of reductase and ribosomal protein (RP) S17 mRNA were measured by an RNase protection assay. The three lanes under each treatment condition represent three independent mRNA measurements. All three determinations for each treatment condition were conducted within one experiment using the same ^{32}P-labeled antisense RNA probes for both HMG-CoA reductase and RPS17 mRNA.

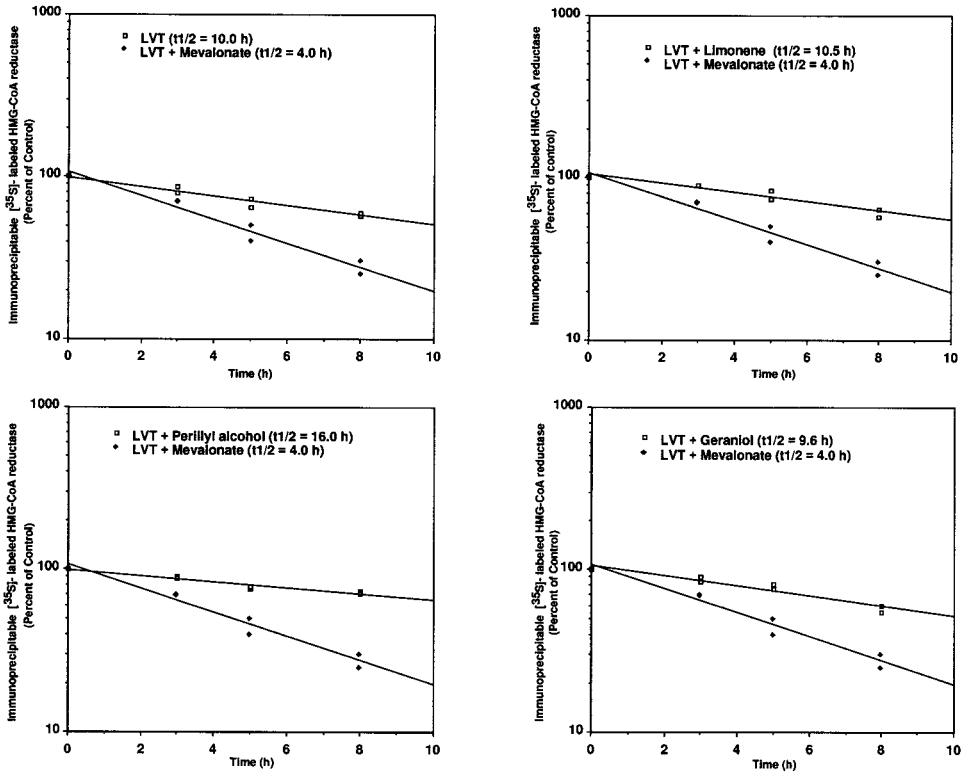

FIGURE 4 Effects of mevalonate-derived isoprenoids on degradation of [^{35}S]-labeled 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. SV28 cells incubated with 25 μmol/L lovastatin (LVT) were labeled with [^{35}S] methionine. The medium was then changed to delipidized fetal bovine serum-minimum essential medium (DFBS-MEM) plus methionine supplemented with LVT alone or LVT plus mevalonate (10 mmol/L), limonene (0.5 mmol/L), perillyl alcohol (0.7 mmol/L) or geraniol (0.4 mmol/L). Labeled reductase was measured at various times by immunoprecipitation. [^{35}S]-labeled reductase (~333 Bq) was immunoprecipitated from LVT-treated cells at time 0 in each experiment. Each time point is represented by two independent determinations. The half-life for HMG-CoA reductase under each treatment condition was estimated from the slope of the line fit to the data by least-squares analysis as described by Peffley and Sinensky (24).

mevalonate-treated Syrian hamster SV-28 cells. These cells represent the parental cell line from which the lovastatin resistant cell line, C100, was selected (45). The lower HMG-CoA reductase levels in SV28 cells allows for greater resolution of isoprenoid-mediated degradation effects on endoplasmic-associated HMG-CoA reductase (25). The half-life of reductase in lovastatin-treated cells was 10.0 ± 1.2 h; the addition of mevalonate to lovastatin-treated cells accelerated degradation of the 97-kDa species; under these conditions, the half-life was 4.0 ± 0.4 h ($P < 0.05$) (**Fig. 4**, Table 1). In contrast, limonene, perillyl alcohol and geraniol did not enhance reductase degradation (Table 1). Curiously, the monoterpene, perillyl alcohol, appeared to stabilize ER membrane–associated HMG-CoA reductase and increase the half-life from 10.0 ± 1.2 to 16.0 ± 1.9 h ($P < 0.05$) when added to lovastatin-treated cells (Fig. 4; Table 1).

DISCUSSION

This is the first study to clearly define the multiple regulatory steps at the molecular level through which plant-derived monoterpenes mediate their effects on HMG-CoA reductase in mammalian cells. Previous studies have investigated the role of these monoterpenes in HMG-CoA reductase regulation, but these have focused primarily on enzyme activity (4–6). A more complete understanding of these effects is essential given the consequences of these monoterpenes in tumor cell proliferation, an effect potentially mediated through the post-transcriptional inhibition of HMG-CoA reductase (4–6,12). Because consumption of these monoterpenes has been linked to chemoprevention of certain cancers (12), this information will be useful in defining more clearly the relationship between HMG-CoA reductase suppression and this unique nutrient-gene interaction to this phenomenon in tumor cells.

The cyclic monoterpenes, limonene and perillyl alcohol clearly affected HMG-CoA reductase synthesis primarily at a post-transcriptional level through a mechanism that appears to affect primarily the translation of HMG-CoA reductase mRNA. These findings resemble the effect of endogenous mevalonate-derived isoprenoids or nonsterols, which decrease reductase synthesis without any corresponding decrease in HMG-CoA reductase mRNA (1,24–26,30,31). This effect of mevalonate-derived isoprenoids or nonsterols on HMG-CoA reductase synthesis is mediated through a mechanism regulating the efficiency of translation initiation (31). It remains to be determined whether limonene and perillyl alcohol regulate HMG-CoA reductase synthesis at the same step of protein translation.

Geraniol was unique because it not only affected HMG-CoA reductase synthesis at the translational level but also lowered HMG-CoA reductase mRNA levels. However, the effect on translation was far greater than that on HMG-CoA reductase mRNA levels. In general, monoterpenes and other terpenoid derivatives do not regulate transcription of sterol-responsive genes such as HMG-CoA reductase. In this regard, geraniol appears to have properties analogous to side-chain sterols that regulate mRNA levels. At the present time, it is unknown whether this regulation is at the level of transcription through a mechanism involving sterol regulatory element binding protein (50). Alternatively, the geraniol-mediated effect could involve reductase transcript stability as has been reported in an earlier study from this laboratory that demonstrated oxysterol-enhanced degradation of HMG-CoA reductase mRNA (23,44).

Previous studies describing the effects of mevalonate-derived isoprenoids or nonsterols on reductase have established that a sterol such as 25-OH C acts as a cofactor that enhances the translational effect in a synergistic fashion (24–26). Similarly, in this study, the oxysterol 25-OH C also increased the isoprenoid-mediated translational effect compared with treat-

ments with these three monoterpenes alone. The exact mechanism for this synergism is unknown, although a previously published study from this laboratory has provided evidence that oxysterols in combination with mevalonate-derived isoprenoids may modify proteins binding to the mRNA (31). In turn, this altered protein binding may decrease translation of HMG-CoA reductase mRNA.

One of the most unique findings in this study was that none of these three monoterpenes enhanced degradation of the 97-kDa form of ER-associated HMG-CoA reductase. This is in contrast to other isoprenoids such as farnesol and tocotrienol that decrease the half-life of ER-associated HMG-CoA reductase as well as decrease synthesis of the reductase at a post-transcriptional level (3,9,30,51). Clearly, there must be a structural-functional relationship that determines whether the isoprenoid inhibits synthesis or both synthesis and degradation of HMG-CoA reductase. It is unknown whether these isoprenoids act directly on some aspect of the translational control mechanism to regulate HMG-CoA reductase synthesis or act indirectly by enhancing dephosphorylation of FPP to form farnesol. Case et al. (52) proposed that cyclic and acyclic monoterpenes increase allyl pyrophosphate pyrophosphatase activity and thereby decrease the levels of FPP available for farnesylation of various proteins. The subsequent increase in farnesol levels then triggers a decrease in reductase synthesis and in certain cases, increases degradation of HMG-CoA reductase in the ER. Recently, Gardner and Hampton (43) and Wihovsky et al. (53) presented evidence that in yeast, FPP provides a signal that specifically increases degradation of ER-associated HMG-CoA reductase without activating ER-localized degradation processes in general. An FPP-derived signal in mammalian cells also increases the degradation of ER-associated HMG-CoA reductase (2,3), suggesting that the molecular processes for HMG-CoA reductase degradation may be conserved in both yeast and mammalian cells. Therefore, it appears that the monoterpenes used in this study may not provide the isoprenoid-derived signal required for direct activation of this ER-associated degradation process. Alternatively, each of the three monoterpenes used in this study may not be able to increase farnesol levels sufficiently to activate this degradation process indirectly (52). However, it is clear from this study that a currently unidentified structural/functional relationship must exist among the isoprenoids in terms of providing distinct signals for either suppression of HMG-CoA reductase synthesis, enhancement of HMG-CoA reductase degradation, or as in the case of farnesol (43,52,53) or tocotrienols (9), dual regulatory signals.

These studies have established that mevalonate-derived monoterpenes from plants control HMG-CoA reductase synthesis primarily through a mechanism that appears to modulate the translational efficiency of reductase mRNA. This observation is important because recent studies have established that plant-derived monoterpenes as well as the related isoprenoids, tocotrienol and farnesol, suppress tumor cell growth and induce apoptosis (4,12,54,55). This isoprenoid-mediated effect is very specific for tumor cells, and the growth and viability of normal cells are largely unaffected by these lipids (12,54,55). The hypothesis has been proposed that isoprenoid-mediated suppression of HMG-CoA reductase in tumor cells has a major role in suppressing tumor cell growth as well as inducing apoptotic cell death (12). HMG-CoA reductase in tumor cells is elevated because it is resistant to the suppressive effects of sterols, an effect referred to as sterol-resistant regulation (56–59). This attenuation of sterol-mediated gene expression may be a consequence of constitutive activation of signal transduction pathways mediating tumor cell growth, cell survival and suppression of apoptosis (60,61). However, HMG-CoA reductase expression remains very sensitive to isoprenoid-mediated suppression at the post-transcriptional level. Isoprenoid-mediated suppression of HMG-CoA reductase has been associated with the arrest of tumor cells in the G_1-phase as well as the initiation of apoptotic cell death in a manner analogous to that of the reductase inhibitor lovastatin (62–64). Several studies have established the efficacy of lovastatin as a cancer chemopreventive agent (64–66). Therefore, isoprenoids represent a similar chemopreventive agent that is readily available in dietary constituents. Current studies are directed toward understanding the mechanism of isoprenoid-mediated HMG-CoA reductase suppression at the translational level as well as the effects of isoprenoids on other pathways mediating tumor cell growth and apoptosis that are dependent on mevalonate biosynthetic activities.

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