Monoterpene regulation of Ras and Ras-related protein expression

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Abstract **Monoterpenes, derived primarily from plants, are products of the isoprenoid biosynthetic pathway and function as chemical messengers with diverse functions. The biochemical bases for these activities are largely undefined. The Ras small GTPase superfamily of proteins consists of isoprenylated proteins that play key roles in signal transduction pathways known to regulate diverse cellular functions. In these studies, we have examined the effects of the monoterpenes on expression of Ras and Ras-related proteins, in the absence and presence of mevalonate depletion. Although prior studies have suggested that monoterpenes inhibit isoprenyl transferases, our studies clearly show that select monoterpenes inhibit up-regulation of Ras and the Ras-related proteins. A structure-activity relationship model for these effects was defined. The ability of monoterpenes to regulate the expression of the Ras-related proteins was found to be independent of effects on cell proliferation or total cellular protein synthesis/degradation. This regulatory function of monoterpenes suggests a role for these plant-derived compounds in altering signal transduction elements.**—Holstein, S. A., and R. J. Hohl. **Monoterpene regulation of Ras and Ras-related protein expression.** ***J. Lipid Res.*** **2003.** 44: **1209–1215.**

Supplementary key words Rap1a • RhoA • RhoB • perillyl alcohol • down-regulation

Monoterpenes are 10-carbon molecules derived from geranyl pyrophosphate, an intermediate in the isoprenoid biosynthetic pathway. These compounds are ubiquitous in the plant and animal kingdoms and function as allomones, pheromones, kairomones, and synomones. Plant extracts containing monoterpenes have been used in the treatment of a wide variety of human diseases dating back to dynastic Egypt (3000 B.C.). In modern times, monoterpenes are utilized as ingredients in cosmetics, food flavorings, and cleaning products. Recently, there has been interest in several monoterpenes because of their chemopreventative and chemotherapeutic properties. The mechanisms underlying these properties are as yet largely unknown.

The Ras superfamily of small GTPase proteins, conserved in all eukaryotes, regulates a diverse array of cellular functions, including cell proliferation, differentiation, cytoskeletal organization, and apoptosis (1–3). The function of these proteins is dependent on posttranslational processing (4, 5). These proteins are modified by isoprenyl transferases through the addition of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl chain. The donors of these lipid chains are the isoprenoids farnesyl pyrophosphate (FPP, C_{15}) and geranylgeranyl pyrophosphate (GGPP, C_{20}), respectively. We have recently demonstrated that, in addition to serving as substrates for isoprenylation reactions, FPP and GGPP regulate the expression of Ras-related proteins through both transcriptional and posttranscriptional mechanisms (6, 7).

Prior studies have suggested an interaction between monoterpenes and isoprenylated proteins. In particular, the metabolites of limonene decrease radiolabeled mevalonate incorporation into small GTPase proteins (8). Although these data were interpreted as resulting from monoterpene inhibition of isoprenyl transferases (8), we clarified that the decrease in farnesylated Ras levels by perillyl alcohol (PA) was a consequence of decreased de novo synthesis of Ras protein (9). We hypothesize that the effect of PA on Ras is generalizable to other naturally occurring monoterpenes and to other small GTPase proteins. Our prior findings with the sesquiterpene FPP and the diterpene GGPP provide an experimental framework to test this hypothesis. The results reported herein reveal structure-function characteristics for monoterpene-mediated regulation of protein expression and suggest an even greater role for these evolutionarily conserved molecules. Thus monoterpenes, principally derived from plants, profoundly influence expression of key regulatory signal transduction elements in animals.

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Abbreviations: FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HRP, horseradish peroxidase; LOV, lovastatin; PA, perillyl alcohol; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Cell culture and reagents

The K562 human erythroleukemia cell line, established from a patient with chronic myelogenous leukemia (10), was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin, amphotericin (2.5 μg/ml), and glutamine (2 mM). Cells were grown at 37°C and 5% CO_2 in T-75 culture flasks. Anti-RhoA, anti-RhoB, anti-Rap1a [specific for unmodified Rap1a (11); catalog number sc-1482], anti-β-tubulin, and anti-goat IgG horseradish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NCC-004 anti-pan RAS antibody (12) was kindly provided by Dr. Setsuo Hirohashi (National Cancer Center, Tokyo). Anti-mouse and anti-rabbit HRP-linked antibodies were obtained from Amersham (Piscataway, NJ). Lovastatin (Lov), FPP, GGPP, and all monoterpenes were purchased from Sigma-Aldrich. [^{35}S]Express Protein Labeling Mix was purchased from PerkinElmer (Boston, MA). Methionine and cystine-deficient RPMI medium was obtained from ICN (Costa Mesa, CA).

Western blot analysis

Cells (1×10^6 cells/ml) were incubated with Lov and various monoterpenes (added as DMSO solutions) for 24 h. Cells were lysed as previously described (6) and protein content was determined using the Lowry method (13). Equivalent amounts of cell lysate were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the appropriate antibodies. HRP-linked secondary antibodies and ECL Western blotting reagents (Amersham Biosciences, Inc.) were employed according to manufacturer's protocols.

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Cells were seeded (5×10^4 cells/150 μl per well) in 96-well flat-bottom plates. Cells were incubated with Lov and monoterpenes at 37°C for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously described (14). The absorbance for control cells was defined as an MTT activity of 100%.

[^{35}S]methionine experiments

For pulse experiments, cells were incubated in methionine- and cystine-free RPMI medium with or without monoterpenes and/or Lov and pulsed with [^{35}S]methionine (120 μCi/10×10^6 cells) for 4 h. For pulse-chase experiments, cells were incubated in methionine- and cystine-free RPMI medium and pulsed with [^{35}S]methionine (120 μCi/10×10^6 cells) for 4 h. Cells were then washed with complete RPMI medium plus 10 mM methionine, 3 mM cysteine, and 10% FCS and incubated for 4 h in the presence or absence of monoterpenes and/or Lov. Cells were lysed and trichloroacetic acid (TCA) precipitation was performed. Tissue solubilizer was added to the TCA precipitates, and samples were counted via liquid scintillation counting. Counts were normalized per microgram total protein.

Statistics

The relationship between monoterpene activity in terms of effects on Ras-related protein levels (**Table 1**) and activity in the MTT assay (**Table 2**) was analyzed via the Wilcoxon rank sum test ($\alpha = 0.05$) (15). The effect of Lov on monoterpene-induced changes in MTT activity was analyzed via the Wilcoxon signed-rank test ($\alpha = 0.05$) (15). Two sample Student's *t*-tests were performed to determine whether there was a significant difference between control cells and monoterpene-treated cells (with or without Lov) in the [^{35}S]methionine experiments (**Table 3**). To account for multiple comparisons (n = 16), the Bonferroni correction (15) was performed and significance defined at $\alpha = 0.003$.

TABLE 1. Effects of monoterpenes on Ras, Rap1a, RhoA, and RhoB protein levels in the presence or absence of mevalonate depletion

	− Lov[a]				+ Lov[b]			
	Ras	Rap1a	RhoA	RhoB	Ras	Rap1a	RhoA	RhoB
(*R*)-PA	↓↓	–	↓	–	↓↓↓	↓↓	↓↓↓	↓↓↓
(*S*)-PA	↓↓↓	–	↓	–	↓↓↓	↓↓	↓	↓↓↓
(*S*)-perillaldehyde	–	–	–	–	↓↓	↓↓	↓↓	↓↓
(*S*)-perillic acid	–	–	–	–	–	–	↓	↓
(*R*)-limonene	–	–	–	–	–	–	–	–
(*S*)-limonene	–	–	–	–	–	–	–	–
Limonene oxide	–	–	–	–	–	–	–	–
Myrtenol	–	–	–	–	↓	↓	↓	↓
Myrtanol	–	–	–	–	↓↓	↓	↓↓	↓
(+)-Menthol	–	–	–	–	–	–	–	–
(−)-Menthol	–	–	–	–	↓↓	↓	–	↓
Isomenthol	–	–	–	–	↓	↓	↓	↓↓
Neomenthol	–	–	–	–	↓	–	↓	↓↓↓
(−) Menthone	–	–	–	–	–	–	–	–
Carveol	–	–	–	–	↓↓	–	–	↓
Dihydrocarveol	–	–	–	–	↓	–	↓	↓
Carvone	–	–	–	–	↓	–	↓	↓↓↓
Sobrerol	–	–	–	–	–	–	–	–
Geraniol	–	–	–	–	–	–	–	–
Linalool	–	–	–	–	–	–	–	–

Lov, lovastatin. PA, perillyl alcohol; K562 cells were incubated for 24 h with monoterpenes (0.5 mM) in the absence or presence of mevalonate depletion (10 μM Lov). Western blots with subsequent densitometric analyses were performed.

[a] A less than 20% decrease in protein levels compared with untreated control (−), 20–39% decrease in protein level compared with untreated control (↓), 40–59% decrease in protein levels compared with untreated control (↓↓), and a 60–70% decrease in protein levels compared with untreated control, respectively (↓↓↓).

[b] A less than 20% decrease in protein levels compared with Lov-treated control (−), 20–39% decrease in protein level compared with Lov-treated control (↓), 40–59% decrease in protein levels compared with Lov-treated control (↓↓), and 60–79% decrease in protein levels compared with Lov-treated control (↓↓↓).

RESULTS

To investigate the effects of PA on Ras-related protein expression, K562 cells were incubated with either (*R*)- or (*S*)-PA (**Fig. 1**) in the absence or presence of Lov, an HMG-CoA reductase inhibitor. As shown in **Fig. 2**, unlike Lov, neither terpene enantiomer induced an accumulation of unmodified Ras (as indicated by the presence of the more slowly migrating band) or unmodified Rap1a, or induced an up-regulation of RhoA or RhoB. Instead, treatment with the PA enantiomers resulted in concentration-dependent decreases in Ras and RhoA. PA concentrations of 0.5 mM yielded 51–67% decreases in Ras and 33–39% decreases in RhoA protein levels. In addition, PA (0.5 mM) was found to decrease the up-regulation of Ras, Rap1a, RhoA, and RhoB induced by mevalonate depletion by 75%, 50–59%, 37–63%, and 70%, respectively (Table 1). An immunoblot of β-tubulin is shown as a control; incubation with PA does not alter β-tubulin levels.

TABLE 2. Effects of monoterpenes on cell proliferation

	MTT Activity	
	− Lov	+ Lov
Control	100.0 (3.6)	97.1 (1.4)
(*R*)-PA	59.6 (7.6)	61.3 (4.3)
(*S*)-PA	71.4 (9.2)	74.3 (6.0)
(*S*)-perillaldehyde	80.8 (3.7)	69.5 (3.5)
(*S*)-perillic acid	86.3 (3.5)	84.8 (3.0)
(*R*)-limonene	21.5 (4.1)	24.4 (2.6)
(*S*)-limonene	23.1 (3.0)	19.2 (0.5)
Limonene oxide	86.9 (6.3)	84.5 (5.6)
Myrtenol	79.4 (3.9)	76.7 (3.6)
Myrtanol	66.9 (5.9)	59.6 (4.8)
Carveol	79.9 (3.6)	75.3 (4.3)
Dihydrocarveol	49.3 (2.6)	52.8 (12.8)
(−)-Carvone	69.5 (5.1)	70.1 (2.2)
(+)-Menthol	51.8 (7.5)	54.1 (7.2)
(−)-Menthol	53.7 (5.6)	54.6 (4.9)
Isomenthol	55.1 (5.8)	61.9 (3.5)
Neomenthol	60.5 (7.0)	56.3 (8.7)
(−)-Menthone	60.2 (8.7)	59.0 (3.3)
Sobrerol	89.9 (3.3)	83.5 (4.4)
Linalool	65.4 (4.5)	62.3 (6.1)
Geraniol	62.6 (7.5)	67.6 (4.6)

K562 cells were incubated with monoterpenes (0.5 mM) in the absence or presence of Lov (10 μM) for 24 h. MTT assays were performed as described in Materials and Methods. The MTT activity of the treated cells is expressed as a percentage of the MTT activity of the untreated cells for each time point. Data are presented as the mean (± SD) (n = 4).

To determine whether the effects of PA were specific, additional monoterpenes were examined. The results of these experiments are summarized in Table 1. Perillaldehyde, an oxidized form of PA, decreased the up-regulation of Ras-related proteins induced by mevalonate depletion. Perillic acid, a more highly oxygenated monoterpene, displayed reduced activity, with effects observed only for RhoA and RhoB. Neither enantiomer of limonene nor limonene oxide had an effect. The structurally related alcohols myrtenol and myrtanol were found to decrease the up-regulation of Ras, RhoA, and RhoB induced by Lov.

TABLE 3. Effects of monoterpenes on total cellular protein synthesis and degradation

	Pulse		Pulse-chase	
	− Lov	+ Lov	− Lov	+ Lov
Control	100.0 (8.4)	105.4 (8.5)	100.0 (5.7)	99.9 (14.9)
(*R*)-PA	84.8 (22.2)	86.9 (9.8)	89.5 (12.4)	87.5 (7.0)
(*S*)-PA	96.3 (2.9)	91.7 (1.0)	89.0 (23.0)	92.9 (11.7)
Myrtenol	94.6 (8.0)	94.6 (21.3)	105.5 (14.5)	101.6 (10.2)
Carveol	89.0 (12.6)	95.1 (11.7)	95.6 (8.6)	105.9 (29.1)
(+)-Menthol	76.4 (2.8)	70.5 (4.7)	98.4 (7.5)	83.2 (14.6)
(−)-Menthol	83.6 (7.7)	82.9 (9.2)	99.9 (7.8)	101.8 (12.4)
Isomenthol	92.5 (9.8)	90.3 (12.7)	90.9 (5.2)	94.4 (8.7)
Sobrerol	97.8 (5.3)	105.4 (8.2)	99.9 (11.5)	94.5 (9.0)

For pulse experiments, K562 cells were incubated in the presence of monoterpenes (0.5 mM), Lov (10 μM), and [^{35}S]methionine for 4 h. For pulse-chase experiments, cells were pulsed with [^{35}S]methionine for 4 h and then chased in the presence of monoterpenes (0.5 mM) and/or Lov (10 μM) for 4 h. Trichloroacetic acid precipitation of whole-cell lysate was performed, and samples were counted via liquid scintillation counting. The radioactivity (counts per minute) was normalized per μg of protein and then expressed as a percentage of control. Data are presented as the mean (± SD) (n = 3).

Other alcohols, including menthol, carveol, and dihydrocarveol, also decreased Lov-induced up-regulation of Ras-related proteins, albeit to varying degrees. The diol sobrerol and the acyclic linalool and geraniol were inactive. Interestingly, the activity of menthol was dependent on the specific stereochemistry. As shown in **Fig. 3**, while (+)-menthol had only a minimal effect on Lov-induced up-regulation of Ras-related proteins, (−)-menthol significantly decreased Ras and RhoB levels and also diminished Rap1a levels. Isomenthol had similar effects as (−)-menthol and also decreased RhoA levels. Neomenthol displayed slightly different effects by decreasing the mevalonate depletion-induced up-regulation of Ras, RhoA, and RhoB (Table 1). The effects of the ketones carvone and menthone were also examined, and while menthone was inactive, carvone did decrease the induced up-regulation of Ras, RhoA, and RhoB.

In order to exclude the possibility that the effects of the monoterpenes are a consequence of diminished cell proliferation, MTT assays were performed. As shown in Table 2, the monoterpenes (at 0.5 mM) impaired proliferation to varying degrees, ranging from MTT activities of 21.5% [(R)-limonene] to 86.9% (limonene oxide), expressed as percent of control. Although some of the compounds significantly depressed MTT activity, there was no correlation between the ability of the monoterpenes to influence Ras-related protein expression and the effects on MTT activity as determined by the Wilcoxon rank sum test ($P > 0.05$). Despite the differing activities of the menthol isomers observed in Fig. 3, all of the menthol isomers displayed equivalent MTT activities. The addition of Lov did not significantly alter the cytotoxic characteristics of the monoterpenes as determined by the Wilcoxon signed rank test ($P > 0.05$).

The effects of the monoterpenes described in Table 1 might be because of global effects on protein synthesis and degradation. To establish specificity for the Ras-related proteins, [^{35}S]methionine pulse and pulse-chase experiments were performed. As shown in Table 3, both enantiomers of PA, myrtenol, carveol, three menthol isomers, and the inactive sobrerol did not significantly alter [^{35}S]methionine incorporation into total protein pools, as measured by TCA precipitation. Statistical significance was established through the two-sample Student's *t*-tests in which the Bonferroni correction was applied for α to correct for multiple comparisons (15). In addition, the presence of Lov, either alone or in combination with the monoterpenes, did not alter de novo protein synthesis. As a control, cells were incubated with cycloheximide, a known inhibitor of protein synthesis, which decreased [^{35}S]methionine incorporation to 20% of control (unpublished observations). The effect of the monoterpenes on protein degradation was examined in pulse-chase experiments. Treatment with the monoterpenes, either alone or in combination with Lov, did not significantly alter the loss of [^{35}S]methionine from total protein pools (Table 3).

We previously demonstrated that both naturally occurring and synthetic 15- and 20-carbon isoprenoids influence the expression of Ras-related proteins. In this context, we examined the effects of combining monoterpenes

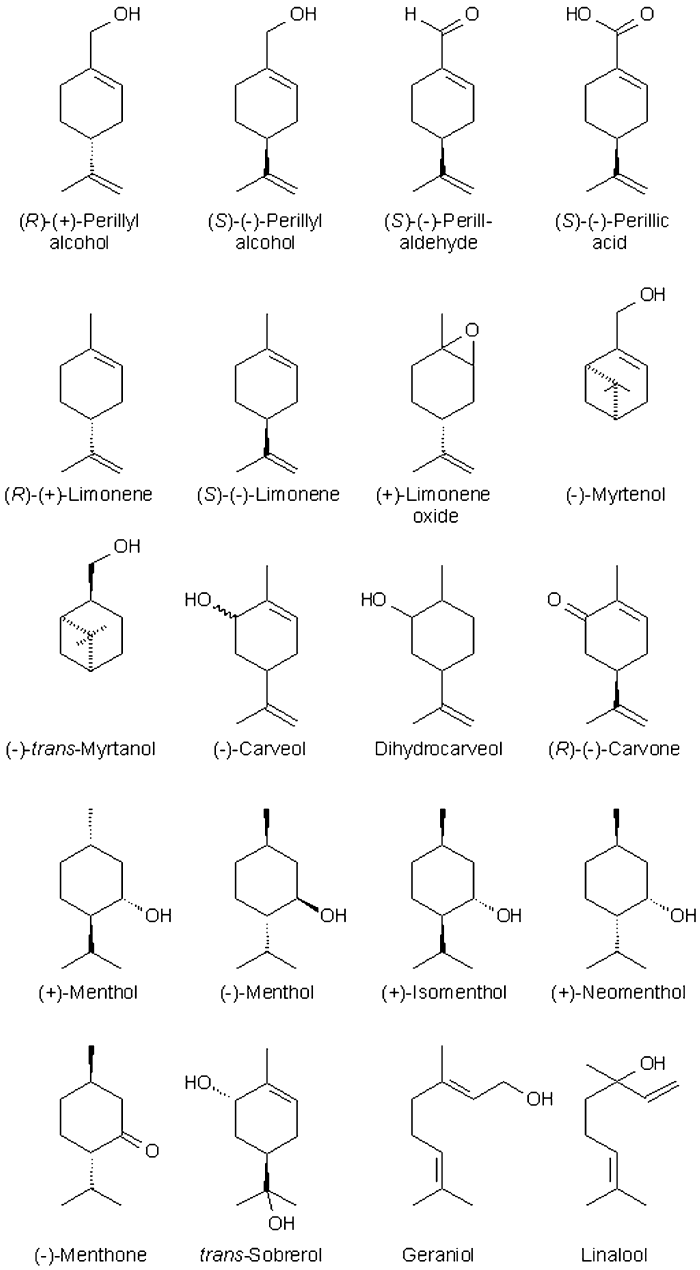

Fig. 1. Structures of monoterpenes.

with isoprenoid pyrophosphates in mevalonate-depleted cells. As shown in **Fig. 4**, at a concentration of 0.1 mM, (*R*)-PA had no effect on Ras or RhoA levels either alone or in combination with FPP or GGPP. At this concentration, (*R*)-PA minimally decreased the Lov-induced up-regulation of the Ras-related proteins (10% decreases for Ras, Rap1a, and RhoA, and a 40% decrease for RhoB). Submaximal concentrations of FPP and GGPP (2.5 μM) partially decreased the Lov-induced up-regulation of Ras (35% by FPP), Rap1a (35% by GGPP), RhoA (17% by GGPP), and RhoB (40% by FPP and 60% by GGPP). The combination of PA and FPP or GGPP was more effective in decreasing the up-regulation of the Ras-related proteins than either one alone. For Ras, the combination of PA and FPP resulted in a 50% decrease in Ras levels compared with Lov-treated cells. For Rap1a, the combination of PA and GGPP further reduced unmodified Rap1a to 40% of Lov-only levels. Interestingly, addition of FPP to PA also resulted in a decrease in Rap1a levels compared with the effects induced by PA alone in Lov-treated cells. Addition of either FPP or GGPP to PA decreased RhoA levels by an additional 20%. Finally, the combination of PA with either FPP or GGPP resulted in the loss of detectable RhoB protein in Lov-treated cells.

DISCUSSION

These results establish and define a role for monoterpenes in alteration of levels of Ras and Ras-related proteins. In addition to the previously studied limonene and PA, we now demonstrate that many structurally related monoter-

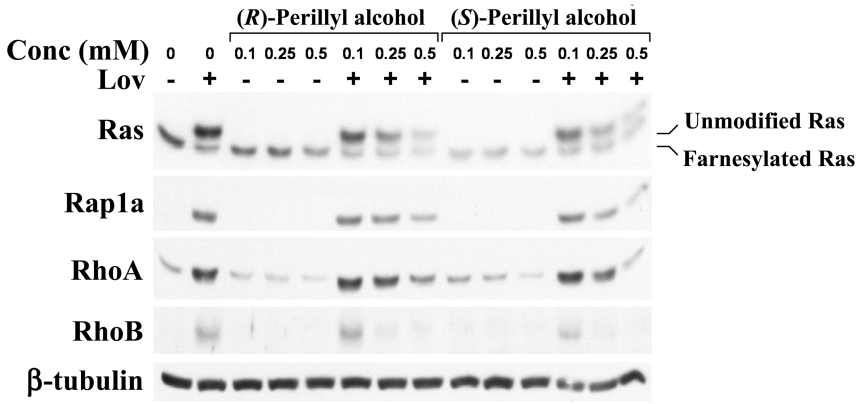

Fig. 2. Effects of perillyl alcohols (PAs) on Ras-related protein levels. K562 cells were incubated with lovastatin (Lov) and/or (*R*)- or (*S*)-PA for 24 h. These immunoblots were developed as described in Materials and Methods. Each lane contains an equivalent amount of protein from cell lysate. The blots reflect one study that is representative of four independent experiments.

penes display such activity. While PA alone decreases Ras and RhoA levels (9) (Fig. 2), it and other monoterpenes significantly decrease the up-regulation of Ras-related proteins that results from mevalonate depletion. The use of Lov enables monoterpene structure-activity determinations and improves clarification of the initial studies involving monoterpenes. Early experiments demonstrated a reduction of radiolabeled mevalonate into small GTPase protein pools in the presence of select monoterpenes (8). Although these results were interpreted as being a result of monoterpene-mediated inhibition of isoprenyltransferases, in vitro enzyme experiments have revealed that very high concentrations of monoterpenes are required for minimal inhibition (16). Furthermore, Western blots (e.g., Fig. 2) have shown that treatment with monoterpenes fails to result in the accumulation of unmodified Ras (a farnesylated protein) or unmodified Rap1a (a geranylgeranylated protein). Thus, the decrease in radiolabeled mevalonate incorporation into Ras-related proteins cannot be a consequence of monoterpene inhibition of isoprenyltransferases. Our findings reconcile these otherwise incongruous results. It is important to note that the labeling experiments were performed in Lov-treated cells (8). Lov induced an up-regulation of small GTPases, and subsequent treatment with monoterpenes decreased this up-regulation. This has the overall result of a decreased pool of proteins to be labeled and explains the earlier data.

The effects of monoterpenes on Ras-related protein levels in mevalonate-depleted cells allow for the clarification of the structural basis for this activity. PA is the most active monoterpene in Lov-treated and untreated cells. Interestingly, the stereochemistry at carbon 4 of the menthane skeleton (**Fig. 5**) is not important, as both the (*R*)- and (*S*)-enantiomers displayed similar results. Further evaluation of the functional group requirement at the tail position was accomplished with myrtenol. This compound, albeit to a lesser extent than PA, depressed Ras-related protein levels in mevalonate-depleted cells. This finding indicates a minimal need for flexibility at the C4 position. An oxygenated substituent at the C7 position is, however,

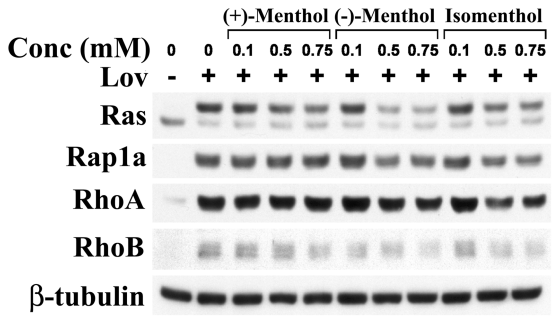

Fig. 3. Menthol isomers display differential effects on Lov-induced up-regulation of Ras-related proteins. K562 cells were incubated for 24 h with Lov and varying concentrations of menthols. These immunoblots were developed as described in Materials and Methods. Each lane contains an equivalent amount of protein from cell lysate. The blots reflect one study that is representative of three independent experiments.

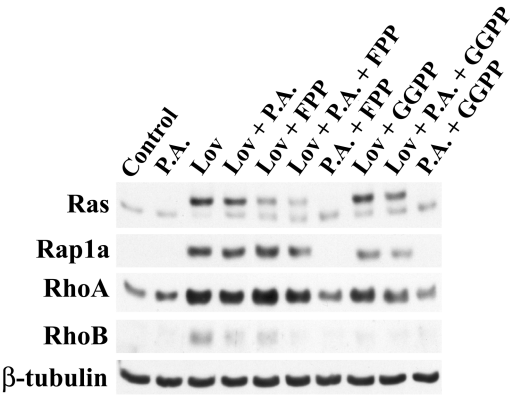

Fig. 4. Effects of PA and isoprenoid pyrophosphates on Ras-related protein levels. K562 cells were incubated for 24 h with 0.1 mM (*R*)-PA, 10 μM Lov (Lov), 2.5 μM farnesyl pyrophosphate (FPP), or 2.5 μM geranylgeranyl pyrophosphate (GGPP). These immunoblots were developed as described in Materials and Methods. Each lane contains an equivalent amount of protein from cell lysate. The blots reflect one study that is representative of two independent experiments.

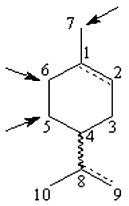

Fig. 5. Structure-activity relationship model for monoterpene regulation of Ras-related protein levels. The essential features of an active menthane derivative include an oxidized substituent (arrow) at either C3/C5, C6, or C7, single or double bonds at C1-2 and C8-9, and *R* or *S* configuration at C4.

required. Specifically, the order of activity at that position was found to be alcohol>aldehyde>acid. Limonene, the deoxygenated form, was inactive. The exact position of the alcohol is less important, because monoterpenes with alcohols at position C3/5 (menthols) or C6 (carveol, dihydrocarveol) retain their effects on Ras-related proteins; however, introduction of a second alcohol at position 8 (sobrerol) abrogates the down-regulation. While a ketone at position 5 (menthone) yields an inactive compound, carvone, an oxidized derivative of carveol, is active. The double bond at C1-2 appears dispensable, in that carveol and myrtenol are similar in activity to dihydrocarveol and myrtanol, respectively. A six-membered ring is a required structural component as the open-chain alcohols geraniol and linalool are inactive. Finally, it is interesting to note that differences exist between the menthol isomers: (−)-menthol, isomenthol, and neomenthol are active, although to differing extents, while (+)-menthol is inactive. All of the effects described appear to be independent of more generalizable biological functions, in that there is no correlation with MTT activity and none of the agents altered total cellular protein synthesis or degradation.

These observed results lend themselves to the development of a structure-activity relationship model for regulation of Ras-related protein levels. Figure 5 depicts the essential features of an active monoterpene. Not readily appreciated by this simplified model are the striking differences in activity that occur in monoterpenes with multiple stereocenters and differing 3-D structures. For example, menthol contains three stereogenic centers and the orientation of each imparts different activities with regard to Ras-related protein expression (Fig. 3, Table 1). It is interesting to note that different menthol isomers have been shown to induce different pharmacological effects on nasal sensory nerve endings (17, 18). Also, differences in the 3-D structures are important, as evidenced by the comparison of myrtenol with PA (Table 1).

Although the precise mechanism for monoterpene activity on Ras-related protein expression is not yet defined, our results narrow the possibilities. That these effects are more prominent under mevalonate depletion conditions could be because of interference of monoterpene action by intermediates in the isoprenoid pathway; however, this is unlikely because low concentrations of either FPP or GGPP enhance the activity of PA (Fig. 4). This suggests that the reason mevalonate depletion is required is because this condition increases the expression of Ras-related proteins (6). Without this increase, the relatively long half-life of Ras and its related proteins (6) limits detection of monoterpene-mediated effects on the synthesis of these proteins. We have previously demonstrated that FPP and GGPP differentially decrease expression of select Ras-related proteins. FPP decreases Ras and RhoB, whereas GGPP affects Rap1a, RhoA, and RhoB in a manner independent of protein isoprenylation (7). The monoterpene effects are clearly different. PA decreases the levels of all of these proteins, whereas the related active monoterpenes always affect Ras and RhoB, and to a lesser extent RhoA and Rap1a (Table 1). Even within closely related monoterpenes, there are different patterns of activity. For example, while (+)-menthol does not alter levels of the Ras-related proteins, (−)-menthol affects Ras, Rap1a, and RhoB, isomenthol affects all four proteins, and neomenthol alters Ras, RhoA, and RhoB levels in the presence of Lov. These findings further support the hypothesis that there are distinct mechanisms underlying the effects of the monoterpenes as compared with the isoprenoid pyrophosphates, and that differences exist even amongst the related monoterpenes.

Our current studies focus on common monoterpenes selected from the estimated 1,000 naturally occurring examples (19). More specifically, in addition to being used as flavorings, monoterpenes such as PA and its derivatives, the menthols, and carveol and its derivatives are used for medicinal purposes. Although the disease processes against which these compounds may display activity are numerous, the underlying pharmacological effects also are numerous and include antiirritant and antiinflammatory activities. The biochemical bases for these pharmacological effects are not known. Monoterpenes have been extensively studied as potent allelochemicals in plant-insect interactions. The plant-generated monoterpenes broadly influence insect behaviors via largely unknown molecular mechanisms. Some of these compounds may inhibit isoprenyltransferases (8), and while it is tempting to attribute biological effects to such inhibition, the concentrations of monoterpenes required for these effects are in the range of 1.5 mM to 10 mM (16). Our findings on monoterpene-induced suppression of Ras-related protein levels occur in lower concentrations (0.25–0.75 mM). We propose that these latter effects may be responsible for some of these activities and that our structure-activity relationship analysis will provide the tools to further evaluate this hypothesis. It is conceivable that the pattern of the specific Ras-related proteins down-regulated by a single monoterpene may predict the resultant biological effects. Ras and Ras-related proteins are evolutionarily highly conserved and regulate fundamental cellular processes (20, 21). Not only are the biochemical pathways for monoterpene synthesis also evolutionarily conserved, but the mevalonate-dependent pathway is also highly regulated by isoprenoids themselves (22, 23). It has been well established that isoprenoids are necessary for proper intracellular localization and function of the Ras-related proteins (4, 5). Our current studies advance the understanding of

the relationship between these proteins and isoprenoids to reveal that plant-derived isoprenoids influence expression of mammalian Ras superfamily proteins. Further studies will clarify the consequences of these induced changes in Ras proteins as applied to the biological effects that have been ascribed to the monoterpenes.

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