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TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL INHIBITION OF HMGCR AND PC BIOSYNTHESIS BY GERANIOL IN TWO HEP-G2 CELL-PROLIFERATION–LINKED PATHWAYS

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ABSTRACT

Geraniol, present in the essential oils of many aromatic plants, has *in-vitro* and *in-vivo* antitumor activity against several cell lines. We investigated geraniol's effects on lipid metabolic pathways involved in Hep-G2 cell proliferation and found that geraniol inhibits the mevalonate pathway, phosphatidylcholine biosynthesis, cell growth, and cell-cycle progression (with an arrest occurring at the G0/G1 interphase) and increases apoptosis. The expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)—the rate-limiting step in cholesterol synthesis—was inhibited at the transcriptional and posttranscriptional levels, as assessed by real-time RT-PCR, Western blots, and [^{14}C]HMG-CoA-conversion radioactivity assays. That geraniol decreased cholesterogenesis but increased the incorporation of [^{14}C]acetate into other nonsaponifiable metabolites indicated the existence of a second control point between squalene and cholesterol involved in redirecting the flow of cholesterol-derived carbon toward other metabolites of the mevalonate pathway. That exogenous mevalonate failed to restore growth in geraniol-inhibited cells suggests that, in addition to the inhibition of HMGCR, other dose-dependent actions exist through which geraniol can impact the mevalonate pathway and consequently inhibit cell proliferation. These results suggest that geraniol—a nontoxic compound found in many fruits and herbs—exhibits notable potential as a natural agent for combatting cancer and/or cardiovascular diseases.

Key words: geraniol, HMGCR, phosphatidylcholine, cell proliferation, Hep-G2

INTRODUCTION

Plant isoprenoids are widely known as nontoxic natural compounds that inhibit cell proliferation *in vitro* and *in vivo* with selectivity against tumor cells (Duncan et al. 2004; Mo and Elson 2004). Isoprenoids are also potent suppressors of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR; Nakanishi et al. 1988; Correll et al. 1994; Peffley and Gayen 2003; Chung et al. 2008), the enzyme catalyzing the main rate-limiting step of the mevalonate pathway and cholesterol synthesis in mammalian cells. Transcriptional regulation of this enzyme is modulated by the binding of the sterol-regulatory-element–binding protein (SREBP-2) transcription factor to specific sterol-response elements located in the promoter region of HMGCR gene (Horton et al. 2002). HMGCR catalyzes the conversion of hydroxymethylglutaryl-CoA to mevalonate—it a crucial intermediate in the formation of both cholesterol and nonsterol products that are vitally involved in diverse cellular functions (Goldstein and Brown 1990; Grunler et al. 1994). Although numerous studies have reported the inhibition of HMGCR by isoprenoids, the specific levels of the latter at which the enzyme is regulated have not been well characterized. HMGCR inhibition decreases the elevated cholesterol levels widely required by rapidly growing cancer cells, inhibits the prenylation of certain growth-regulatory proteins that play a key role in controlling cell proliferation, and induces apoptosis (Mo and Elson 1999; Joo and Jetten 2010). The mechanisms by which isoprenoids affect apoptosis are not fully understood; nevertheless, an inhibition of phosphatidylcholine (PC) synthesis has been associated with that cytoregulatory process (Joo and Jetten 2010).

Geraniol, an acyclic isoprenoid monoterpene, occurring in the essential oils of several aromatic plants, is one of the most widely used molecules in the flavor and fragrance industries. Its antitumor activity against many human tumor cells both *in-vitro*

and *in-vivo* have been well documented (Yu et al. 1995; Burke et al. 1997; Carnesecchi et al. 2004; Chen and Viljoen 2010); and because of that property geraniol is thought to represent a new class of cancer chemoprophylactic agents. Although several recent molecular studies on aromatic terpenoids have shown that those compounds modulate the mevalonate pathway by down-regulating HMGCR expression (Chung et al. 2007, 2008), the effects of geraniol in particular have been reported only by Peffley and Gayen (2003). That, however, no reports have appeared in the literature on the action of terpenoids in liver cells, where the mevalonate pathway is the most active, is furthermore highly relevant. In order to understand the mechanisms by which geraniol inhibits cell growth in liver cells, we analyzed the effects of this monoterpene on the mevalonate pathway in general and on HMGCR expression in particular as well as on the biosynthesis of PC in the human-hepatoma cell line Hep-G2 in culture.

MATERIALS AND METHODS

Reagents

[^{14}C]Acetate (54.7 Ci/mol) was purchased from Perkin Elmer Life Science, Inc. (Boston, MA), [^{14}C]3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (57.0 Ci/mol) from DuPont NEN (Boston, MA), and [^{3}H]methylcholine chloride (83 Ci/mmol) from Amersham (UK). Inorganic reagents and solvents were of analytical grade. Dimethylsulfoxide (DMSO) was supplied by Analyticals Carlo Erba (MiIan, Italy) and geraniol (98%), mevalonolactone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and HMG-CoA by Sigma (St. Louis, MO). The sodium salt of simvastatin was prepared by dissolving the drug in ethanol at 60 °C, adding equimolar amounts of NaOH, and incubating at 60 °C for 1 h. The ethanol was then evaporated under nitrogen and the salt dissolved in distilled water at a final concentration of 10 mg/ml.

Cell Culture

Hep-G2 cells were obtained from American Type Culture Collection (ATCC HB-8065) and maintained in 95-cm^2 flasks in nitrocellulose-filtered (0.22 µ pore size; Sartorius, Göttingen) Eagle's minimal essential medium (MEM; Gibco, Invitrogen Corporation) plus 100 µg/ml streptomycin and supplemented with 10% fetal-bovine serum (Natocor, Córdoba, Argentina; filter-sterilized by the manufacturer). For subcultivation of the cells, surface cultures were harvested with trypsin (0.25% [w/v]) in phosphate-buffered saline (PBS: NaCl 137 mM; KCl 2.7 mM, Na_2HPO_4, 10.0 mM, KH_2PO_4 2.0; pH 7.4) and grown at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 in air. For assays, cultures were incubated in serum-containing MEM until they reached the logarithmic growth phase and then washed free of serum and incubated (according to the experiment) in serum-free MEM Zinc option (IMEM-Zo) or in serum-containing MEM supplemented with either 50, 200, 400, or 600 µM geraniol previously dissolved in DMSO such that the final concentration of the vehicle was 0.05% (v/v). That same concentration of DMSO was therefore added to the parallel control cultures. The cells were then incubated for 4, 8, 12, 24, or 48 h according to the assay protocol. Simvastatin (15 µM) and mevalonate (0.5 µM) were added as aqueous solutions.

Viability and cell proliferation

Viability and cell proliferation were determined by trypan-blue–dye-exclusion cell counts in a Neubauer chamber (Phillips 1973) and by the MTT assay (Mosmann 1983) as follows: After treatment, cells were incubated in 24-well microtest plates with 0.5 mg/ml of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St Louis, MO)] in PBS at 37 °C for 3 h. The MTT was then removed from the wells

and acidified with 0.04 M HCl in isopropanol. The absorbance of the reaction product (formazan) was measured at 560 nm with background subtraction at 640 nm with a Beckman Coulter DTX 880 Microplate Reader.

Flow cytometry

Cells (1 x 10^6) were fixed with 70% (v/v) aqueous ethanol at 4 ºC overnight, washed with cold PBS, and incubated with ribonuclease (500 U/ml, RNasa A, Biodynamics, Argentina) at 37 °C for 15 min. Nuclei were stained with propidium iodide (25 µg/ml in PBS containing 0.1% (v/v) Triton X-100). The DNA content was determined with a FACSARia II flow-cytometery cell sorter (BD Biosciences, USA). The distribution of the cells throughout each phase of the cell cycle was calculated through the use of the FlowJo® software version 7.6.2.

In-situ detection of apoptosis

Apoptosis was determined by terminal-deoxynucleotidyl-transferase–dUTP nick-end labelling (the TUNEL assay), as carried out with the *In-Situ* Cell Death Detection Kit, TMR Red (Roche, Mannheim, Germany) according to the manufacturer's protocol. The TUNEL-positive cells were evaluated under an Olympus BX51 fluorescence microscope (Tokyo, Japan), equipped with an Olympus DP70 digital camera and the results analyzed through the use of the ImagePro Plus v5.1 software (Media Cynernetics, Silver Spring, MD). The results were expressed as the percentage of TUNEL-positive cells relative to the total number of cells, the latter being determined by uptake of the fluorescent dye DAPI (4', 6-diamidino-2-phenylindole dihydrochloride; Invitrogen by Life Technologies).

Incorporation of [^{14}C]acetate

[^{14}C]acetate (3 µCi/ml in the final culture medium) was added during the final 3 h after either 8 h or 24 h of treatment. The surface cultures were washed 3 times with 5 mL of ice-cold saline solution, detached from the growing surface mechanically with a rubber-tipped spatula, and pelleted at 500 x *g* for 10 min. After resuspension of the sedimented cells in 5 mL of the same solution, an aliquot was used to determine cellular protein content (Lowry et al. 1951), the remaining cell material centrifuged as before, and the pellet processed for lipid extraction (Folch et al. 1957). Cholesterol and other mevalonate-pathway metabolites from the nonsaponifiable fraction were separated by thin-layer chromatography (TLC) on silica gel G after development first in 100% chloroform and then in 3% (v/v) methanol in chloroform, and identified by autoradiography with a Storage Phosphor Screen (GE Healthcare). Quantitative densitometric analyses were performed by the Image J Program. All lipid classes were identified by comparison with a standard mixture containing cholesterol, lanosterol, and squalene added to the same plate.

Analysis of [^{3}H]choline incorporation

Cells were incubated for various lengths o7f time with geraniol-supplemented medium containing 2 µCi/ml [^{3}H]methylcholine chloride during the final 3 h. The cultures were washed twice in PBS, the cells scraped off, and after a thorough mixing of the suspension by pipetting, an aliquot taken for protein determination. After extraction of the cellular lipids according to Folch et al. (1957), the aqueous phase was dried under nitrogen and choline, phosphocholine, and CDP-choline separated on silica-gel TLC plates (Merck) developed in EtOH/2.4% NaCl/H_2O/NH_4OH (50/12.5/37.5/5) (Miquel et al. 1998). The lipids in the chloroform phase were separated in the solvent chloroform/methanol/acetic acid/water (50/37.5/3.5/2). The spots, visualized by exposure to iodine vapors, were

161 scraped off and the radioactivity measured by liquid-scintillation counting (Optiphase
162 Hisafe 3, Perkin Elmer).
163
164 *RNA isolation and real-time PCR analysis*
165 Total RNA was extracted from the Hep G2 cells with the TRI Reagent (Molecular
166 Research Center, Inc., Cincinnati, OH) followed by a DNAase-I treatment (Fermentas Life
167 Sciences). The cDNA was synthesized by means of the kit qScript™ cDNA SuperMix
168 (Quanta Biosciences, Inc). Real-time reverse-transcriptase–polymerase-chain-reaction (RT-
169 PCR) analysis was done through the use of the PerfeCta™SyberGreen FastMix™ and the
170 StratageneMX30005P RT-PCR System. PCR conditions were 94 °C for 4 min, followed
171 first by 40 cycles at 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s and then by a final
172 cycle at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The relative amount of mRNA
173 was calculated by means of the comparative-threshold-cycle method and the qBase v1.3.5
174 program with ß-actin as the reference housekeeping gene. The following oligonucleotide
175 primers were used: SREBP-2 forward primer 5'-CTCTGACCAGCACCCACACT-3' and
176 reverse primer 5'-CACACCATTTACCAGCCATAAG-3', HMGCR forward primer 5′-
177 CTCCAGTACCTACCTTACAGGGATT-3′ and reverse primer 5'-
178 GCTGCTGGCACCTCCA-3′, and ß-actin forward primer 5′-
179 TCAAGATCATTGCTCCTCCTGAG-3′ and reverse pimer 5′-
180 CTCCTGCTTGCTGATCCACA-3′. Detection of the amplified fragments was made by
181 electrophoresis on a 1.8% (w/v) agarose gels with Sybr Green I staining. The assays were
182 performed in triplicate.
183
184 *Western blotting*

Proteins were isolated with the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Proteins were boiled in a sample buffer (with 5% [v/v] ß-mercaptoethanol) for 5 min. Samples were separated on 12.5% (w/v) sodium-dodecylsulfide polyacrylamide gels and adsorbed to polyvinylidene difluoride membranes (Amersham, GE Healthcare) by semidry transfer at 10 V for 1 h in 20% (v/v) methanol in 48 mM Tris pH 8.3, 39 mM glycine. Nonspecific protein-binding sites were blocked by incubation in PBS (pH 7.4) containing 0.05% (v/v) Tween 20 and 5% (v/v) skimmed milk. The membrane was incubated with rabbit anti-HMGCR (Santa Cruz, CA, USA) diluted 1/200 in antibody dilution buffer (2% [v/v] skimmed milk in PBS plus 0.1% [v/v] Tween 20) for 1 h followed by three 5-min washes in the same buffer. Horseradish-peroxidase-conjugated goat anti(rabbit IgG) antibodies (Thermo Scientific) were added to the membrane for 1 h. Immunoreactive bands were detected by enhanced-chemiluminescence Western-blot–detection reagents (Amersham Pharmacia Biotech) and processed manually through the use of common X-ray film developers and fixers. Protein immunoblots were scanned and the density of each band quantified by means of the ImageJ software (Image processing and analysis in Java).

Assay of HMGCR activity

After resuspension of harvested cells in 1.0 ml of a solution containing 50 mM PBS (pH 7.4), 1 mM ethylenediaminetetraacetic acid, and 5 mM D,L-1,4-dithiothreitol (Saucier and Kandutsch 1979; Cavenee et al. 1981) and sonication with the microtip attachment of a Heat-System Ultrasonic W-220F cell disrupter (Plainview, NY) for 10 s at one-half maximum energy and 0–2 °C, the activity of HMGCR was determined through the radioassay described by Brown et al. (1979). Aliquots of the sonicated cells (100 µg in 90 µL of suspension) were preincubated at 37 °C for 30 min, then 100 µL of a solution

containing 0.2 M potassium phosphate (pH 7.4), 40 mM glucose-6-phosphate, 5 mM $NADP^+$, 0.7 unit glucose-6-phosphate dehydrogenase, 20 mM ethylenediaminetetraacetic acid, and 10 mM dithiothreitol were added. The reaction was initiated by the addition of D,L-[3-^{14}C]HMG-CoA (57.1 Ci/mol), to a final concentration of 176 µM in a final assay volume of 200 µL. After 30 min at 37 °C, the incubations were stopped by the addition of 25 µL 6N HCl and the [^{14}C]mevalonate formed converted into the lactone, isolated by TLC, and counted along with an internal standard of [^{3}H]mevalonolactone to correct for the incompleteness of recovery (Shapiro et al. 1974). HMGCR activity was expressed as picomoles of mevalonate synthesized per mg protein per min.

Statistical analyses

Statistical analyses were performed by means of the Student t test. Differences in the data were considered statistically significant at a $p < 0.05$.

RESULTS

When Hep-G2 cells were incubated in serum-containing MEM supplemented with 50, 200, or 400 µM geraniol for 24–48 h, at the higher doses the isoprenoid significantly inhibited cell proliferation (Fig. 1), arrested cells in the G0/G1 interphase of the cell cycle (Table 1), and enhanced apoptosis as evaluated by the Tunel assay (Fig. 2). On the basis of these initial results, the Hep-G2 cells were then incubated with 200, 400, and 600 µM of geraniol (up to a dose intended to be growth-inhibiting) for mevalonate assays. When mevalonate was added to the culture medium along with geraniol, cell growth never became restored the control values (Table 2). As a positive control, we observed that exogenous mevalonate restored cell growth in simvastatin-treated cells.

When Hep-G2 cells were incubated in IMEM-Zo, they continued to grow, although at a reduced rate, for up to 48 h. With geraniol present under these conditions quantification of the incorporation of radioactivity into [^{14}C]cholesterol and other nonsaponifiable lipids was used to determine the extent of the conversion of [^{14}C]acetate into that fraction after an 8- and 24-h period of incubation. We found that radioactivity was incorporated into cholesterol, squalene, and lanosterol; while at shorter incubation times radiolabelling was also detected in an unidentified oxysterol with an Rf similar to 25-hydroxycholesterol upon analysis by TLC in two different solvent systems. The results demonstrated that both doses of geraniol diminished [^{14}C]acetate incorporation into cholesterol, but that the labelling in other metabolites was variable. After 8 h of treatment both doses of the isoprenoid increased the incorporation of radioactivity into squalene and the oxysterol. Lanosterol, however, exhibited no significant differences in incorporation at the lower dose of geraniol, and the radioactivity became decreased at the higher dose (Fig. 3A). After 24 h of treatment the lower dose furthermore increased ^{14}C incorporation into squalene, whereas the higher dose decreased the radiolabelling of both squalene and lanosterol (Fig. 3B). All incorporation of radioactivity into the oxysterol became undetectable after 24 h at either dose of the isoprenoid. Radioactivity assays indicated intermediate metabolites generated early in the pathway to lanosterol became redirected away from the normal sequence of mevalonate metabolites by geraniol. Under that condition radiolabel was also found in compounds (probably polar compounds that were not analyzed) generated later in the metabolic pathway. These results together with the increased inhibition of cellular growth by mevalonate at low doses of geraniol (Table 2) clearly indicated that, through an inhibition of the mevalonate pathway downstream from squalene, an increased number of the subsequent intermediates could be shunted towards

the synthesis of other metabolites that, for their part, could diminish HMGCR expression and/or inhibit cellular proliferation.

As determined by real-time RT-PCR, HMGCR-mRNA expression was significantly reduced in cells incubated with 200 μM geraniol for 8 h, whereas a similar decrease in cells incubated with 50 μM for the same period of time was not quite statistically significant (p = 0.0612). SREBP-2-mRNA levels, however, did not vary significantly under either condition (p >0.1; Fig 4). Cells incubated with both doses of geraniol for half that time (4 h), moreover, gave similar results with respect to both gene products (data not shown). These findings indicated that during the first hours of exposure to geraniol (*i. e.*, <12 h) HMGCR-mRNA expression decreased reproducibly without affecting SREBP-2 levels. In contrast, after longer periods of incubation (*i. e.*, 12–48 h) the lower doses of geraniol produced a marginal increase in HMGCR- and SREBP-2-mRNA levels, but with respect to both gene products those elevations were both significantly greater compared to control levels only in cells treated with the higher dose of geraniol (Fig. 5)

Although Western-blot analysis showed that HMGCR-protein levels decreased in cells incubated with both doses of geraniol for 24 h, the reduction in enzymatic activity became significant only with the higher dose of the isoprenoid. Shorter incubation times with geraniol also produced decreases in the HMGCR protein, with incubation times of 4–8 h with 50 μM and 200 μM geraniol producing decreases in protein levels of *ca*. 30% and 50%, respectively (data not shown).

Incubation of Hep-G2 cells with [^{3}H]choline demonstrated that the higher dose of geraniol caused an accumulation of phosphocholine in aqueous medium along with a decrease in [^{3}H]choline incorporation into phosphatidylcholine (Fig. 7). All the other soluble compounds evaluated, however, did not vary with geraniol treatment.

DISCUSSION

We have previously reported that geraniol has multiple effects on lipid metabolism (fatty acids, triglycerides, and cholesterol) and on cell growth in the human-hepatoma cell line Hep- G2 (Polo and de Bravo 2006; Polo et al. 2011). The mevalonate pathway plays a key role in cholesterol synthesis as well as in cell proliferation (Goldstein and Brown 1990). In the present investigation we analyzed the effect of geraniol on the regulation of HGMCR, the main rate-limiting enzyme of the mevalonate pathway; on cell viability; on cell-cycle progression; and on PC biosynthesis—with the production of PC being a pathway associated with apoptosis (Miquel et al. 1998; Lagace et al. 2002). Our data showed that geraniol regulated HMGCR through transcriptional and posttranscriptional mechanisms and that the isoprenoid's regulation is both time- and dose-dependent. HMGCR mRNA and protein levels, along with cholesterol synthesis, decreased during the first 8 h of treatment; while [^{14}C]acetate incorporation into squalene, lanosterol, and an oxysterol became significantly increased. Nevertheless, we observed no significant changes in SREBP-2 expression. In cells incubated with geraniol for over 12 hours, both HMGCR- and SREBP-2-mRNA levels were increased compared to controls; but the protein levels and activity of the enzyme were nevertheless significantly decreased, as was cholesterol synthesis.

In addition, the present results indicated that the depletion of mevalonate (it required for cellular growth) by a decrease in HMGCR expression cannot be the only mechanism of growth inhibition by geraniol because exogenous mevalonate was unable to reverse that growth arrest. Although these results suggest that the relationship between HMGCR inhibition and the decrease in Hep-G2 proliferation are not necessarily causally linked, all the results reported in the present work demonstrate that geraniol (either

directly or indirectly) inhibits HMGCR even at doses that do not inhibit cell proliferation. We proposed that geraniol and/or one or more mevalonate-derived metabolites accumulating during only the first hours of isoprenoid treatment might regulate HMGCR at both a transcriptional and posttranscriptional level, whereas after longer incubation times the effect of geraniol on the mevalonate pathway down-regulates the enzyme by an exclusively posttranscriptional mechanism.

The increase in the incorporation of [^{14}C]acetate into mevalonate-derived metabolites indicated that geraniol probably evoked an inhibition at different metabolic steps between squalene and cholesterol. Although our results indicated that geraniol inhibited the conversion of lanosterol to cholesterol in some manner—in agreement with the findings of Ren and Gould (1994), who demonstrated that perrilyl alcohol blocked the conversion of lathosterol to cholesterol in NIH 3T3 cells—we consider that the main pathway affected by geraniol is likely to be the conversion of squalene to lanosterol. Fig. 8 shows the proposed points of inhibition of the mevalonate pathway by geraniol, as suggested from the results obtained in this study.

Oxysterols, the oxygenated derivatives of cholesterol, play a role in cholesterol homeostasis (Björkhem and Diczfalusy 2004). Although most oxysterols are derived from cholesterol, 24,25-epoxycholesterol (24,25-EC) is produced in a shunt off the mevalonate pathway because the 2,3-oxidosqualene cyclase (OSC)—it catalyzing the highly selective cyclization of 2,3-monoepoxysqualene to lanosterol—also effects the cyclization of 2,3,22,23-diepoxysqualene to 24,25-epoxylanosterol, the immediate precursor of 24,25-EC (Spencer et al. 1985; Brown 2009). That the synthesis of 24,25-EC is favored over cholesterol synthesis under conditions of partial OSC inhibition, whereas complete OSC inhibition results in a decreased synthesis of cholesterol as well as 24,25-EC (Telford et al. 2005) is a fundamental detail in the regulatory action of geraniol (*cf.* the legend to Fig. 8).

In addition, recent findings have demonstrated that 24,25-EC decreased cholesterol synthesis by suppressing SREBP activation and by accelerating HMGCR degradation (Brown 2009).

Accordingly, in the present work, cells incubated with geraniol showed a significantly increased [^{14}C]acetate incorporation into an unidentified oxysterol during the first 8 h of treatment. TLC analyses suggested that this metabolite could be 24,25-EC. In such an instance, the finding of an enhanced ^{14}C incorporation into 24,25-EC and squalene might suggest at least a partial inhibition of the OSC by geraniol (Fig. 8). Moreover, 24,25-EC might prevent SREBP-2 activation with a consequent eventual suppression of HMGCR expression and induction of HMGCR degradation. Although two studies reported that certain isoprenoids reduced SREBP-2 expression (Olivero-Verbel et al. 2010; Cho et al. 2011), our findings suggest that geraniol and/or accumulated mevalonate metabolites could regulate the posttranslational proteolytic processing of SREBP-2 (Wong et al. 2008), or else might inhibit SREBP-2 binding to the HMGCR promoter, as had occurred in cells incubated with linalool (Cho et al. 2011). Another possible explanation for the significant decrease observed in HMGCR-mRNA levels in the absence of a decrease of SREBP-2-mRNA levels—as had been seen in a previous study (Peffley and Gayen 2003)—could be a destabilization or degradation of the HMGCR mRNA. After a 12-h incubation, the decreased [^{14}C]acetate incorporation into cholesterol along with the failure to detect the unidentified oxysterol at that time could be attributed to a complete inhibition of OSC. In such an instance, a feedback regulation of HMGCR would be triggered in cells under low-cholesterol conditions (Goldstein and Brown 1990). Although the mRNAs encoding SREBP-2 and HMGCR were found to be increased, the concentrations of the HMGCR protein and the enzyme's total catalytic activity were not. These data support the notion that geraniol regulates HMGCR at a posttranscriptional

level either by inhibiting HMGCR synthesis or by deregulating enzyme stability as suggested by Peffley and Gayen (2003). Similar mechanisms of control may also regulate the amount of enzyme in cells treated with geraniol during shorter incubation periods (*i. e.*, less than 12 h).

Although additional studies are needed to demonstrate an inhibition of OSC by geraniol, isoprenoids have thus far never been implicated in the regulation of that enzyme *per se*.

A number of investigations have demonstrated that isoprenoids inhibit cell proliferation (Duncan et al. 2004; Mo and Elson 2004), and certain studies have linked this observation to an inhibition of HMGCR by isoprenoids (Goldstein and Brown 1990; Edwards and Ericsson 1999). The relevance of this work, as well as that of previous studies we have recently published, resides in the finding that geraniol both decreases HMGCR levels and inhibits cell proliferation. Since depleted mevalonate levels should consequently diminish the pools of farnesyl and other phosphorylated products that, in turn, isoprenylate various proteins vital for cell proliferation (such as members of the Ras family), our results suggest that this effect is very likely not be the main mechanism by which geraniol inhibits the prenylation of certain proteins. Further studies are will no doubt provide new insights about this question in Hep-G2 cells. The growth of tumor cells has been shown to reflect a “positive” balance between two processes, cell division and cell death. Crowell and Elson (2001), have pointed out that the isoprenoid-mediated impact on cell division and apoptosis might be explained by mevalonate starvation, but what remains to be determined is the initiating process triggered by isoprenoids.

In the present experiments the incubation of geraniol-treated Hep-G2 cells with [^{3}H]choline resulted in an accumulation of labelled phosphocholine in the cells along with a decrease in phosphatidylcholine labelling. We attributed this observation to a possible

inhibition of CTP-phosphocholine cytidylyltransferase (Fig. 8). These results, along with the above findings, would suggest that the dual inhibitions of the mevalonate pathway and phosphatidylcholine synthesis by geraniol could produce both a decrease in cell proliferation and an increase in apoptosis. The data here pointing to the central role of a common component of essential oils in regulating two complex metabolic pathways could contribute to a more effective design of drugs for combatting cancer and/or cardiovascular diseases.

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LEGENDS TO THE FIGURES

Fig. 1. *Effect of geraniol on cell proliferation of Hep-G2 cells.* Hep-G2 (25 x 10^3 cells/well) were seeded on 24-well microtest plates and treated with geraniol (50 and 200 µM) for 48 h (in serum-free IMEM-Zo medium for the last 24 h). Viability and cell proliferation were determined (A) by trypan-blue–dye-exclusion cell counts in a hemocytometer and (B) by the MTT assay. Data are the means ± SD of 12 replicate wells per dose performed in 3 separate experiments. (**) $p < 0.01$ *vs*. control.

Fig. 2. *Effect of geraniol on apoptosis of Hep G2 cells.* Cells were plated on coverlips in 6-well plates and treated with geraniol (50 and 200 µM) for 48 h (in serum-free IMEM-Zo for the last 24 h). (A) The percentage of apoptotic cells were determined by Tunel assay. The Tunel-positive cells were counted in 15 random fields with 1,000 cells from three experimental situations. (**) $p < 0.01$ from control (the unpaired Student t-test). Fluorescence micrographs of (B) control cells and (C) 200 µM geraniol-treated cells showing apoptotic cells (Tunel-positive = arrow) and nonapoptotic nuclei staining with DAPI.

Fig. 3. *[^{14}C]acetate incorporation into cholesterol and other nonsaponifiable lipids of the mevalonate pathway in Hep G2 cells*. Cells (3 x 10^6 per flask) were seeded in 95-cm^2 flasks and treated with geraniol (50 and 200 µM) in IMEM-Zo for (A) 8 h and (B) 24 h. [^{14}C]acetate was added during the final 3 h. Data are the means ± SD of 4 individual samples. (*) $p < 0.05$ vs control, (**) $p < 0.01$ *vs*. control.

Fig. 4. *Expression of (A) SREBP-2- and (B) HMGCR-mRNA in Hep G2 cells.* Cells were seeded in 25-cm^2 flasks and treated with geraniol (50 and 200 µM) in IMEM-Zo for 8 h. Expression of mRNA was determined by comparative real-time RT-PCR. Data are expressed as the mean ± SD of triplicate measurements from three independent experiments. (*) $p < 0.05$ *vs*. control, (**) $p < 0.01$ *vs*. control.

Fig. 5. *Expression of SREBP-2 and HMGCR-mRNA in Hep G2 cells over time.* Cells were seeded in 25-cm^2 flasks and treated with geraniol (50 and 200 µM) in IMEM-Zo for (A,B) 12 h, (C,D) 24 h, and (E,F) 48 h. mRNA expression was determined by comparative real-time RT-PCR. Data are expressed as the mean ± SD of triplicate measurements from three independent experiments. (*) $p < 0.05$ *vs*. control, (**) $p < 0.01$ *vs*. control.

Fig. 6. *Effect of geraniol on HMGCR protein levels in Hep G2 cells.* Cells were seeded in 95-cm^2 flasks and treated with geraniol (50 and 200 µM) in IMEM-Zo for 24 h. Cell lysates (A) were fractionated by 12.5% (w/v) sodium-dodecylsulfide polyacrylamide-gel electrophoresis followed by immunoblotting, and (B) the activity of HMGCR was determined by radioassay. Data are expressed as the mean ± SD of triplicate measurements from three independent experiments. (*) $p < 0.05$ *vs*. control, (**) $p < 0.01$ *vs*. control.

Fig. 7. *[^{3}H]choline incorporation into phosphatidylcholine and its precursors in geraniol-treated Hep G2 cells.* Cells (3 x 10^6) were seeded in 95-cm^2 flasks, treated with (50 and 200 µM) geraniol in IMEM-Zo for 24 h, and then exposed to [^{3}H]choline for 3 h. Phosphatidylcholine and its precursors were analyzed for radioactivity incorporation. Data are the means ± SD of 4 individual samples. (*) $p < 0.05$ *vs*. control (**), $p < 0.01$ *vs*. control.

Fig. 8. *Proposed model for the regulation of the mevalonate pathway and phophatydyl-choline synthesis by geraniol.* A low dose of geraniol (50 µM) elevates oxysterols and squalene within the mevalonate pathway through a partial inhibition of oxidosqualene cyclase (*lower arrow*). The resulting elevation in squalene levels and deviation of carbon through the 24,25-epoxycholesterol shunt, in turn, inhibit mevalonate production through a negative-feedback control of HMGCoA reductase at the transcriptional level (*upper arrow pointing right*). A higher dose of geraniol (200 µM) regulates HMGCoA-reductase levels directly through a reduction in messenger translation and an inhibiton of enzymic catalytic activity (*upper arrow pointing left*). Geraniol at this level also effects a complete inhibition of oxidosqualene cyclase to block the synthesis of both 2,3-monoepoxysqualene and 24,25-epoxycholesterol (*lower arrow*). Finally, geraniol at the higher dose also elevates phosphocholine production and decreases phosphatidylcholine synthesis, probably through an inhibiton of CDP-phosphocholine cytidylyltransferase (*middle arrow*).

582 Table 1

583 Effect of Geraniol on cell-cycle distribution in Hep-G2 cells

Geraniol (μM)	Cell-cycle distribution (%)			
	GO/G1	S	G2/M	Sub G1 (apoptosis)
Control	54.3 ± 2.6	36.1 ± 7.5	7.6 ± 2.1	8.4 ± 2.3
50	50.9 ± 2.8	36.5 ± 8.2	7.8 ± 2.7	12.6 ± 4.8
200	59.3 ± 3.7	31.1 ± 9.8	7.9 ± 1.1	11.9 ± 2.9
400	72.4 ± 2.9*	19.9 ± 6.0	9.3 ± 1.8	10.7±1.3

584

585 Table 1. Hep-G2 cells were seeded in 25-cm^2 flasks and grown in serum-containing MEM
586 in the absence (control) or presence of geraniol at the indicated concentrations for 48 h.
587 Cells were stained with propidium iodide and analyzed for DNA content by flow
588 cytometry. Values are means ± SEM (n = 3). *p <0.05 *vs*. control for same cell-cycle
589 phase

590

591 Table 2.

592 Effect of exogenous mevalonate on the viability of geraniol-treated Hep-G2 cells.

	HEP-G2 viability (%)	
	- MVL	+ MVL
Control	100 ± 18.0	105.0 ± 15.1
Geraniol 200 µM	91.2 ± 6.1	73.1 ± 2.6**
Geraniol 400 µM	77.9 ± 14.8	76.5 ± 10.1
Geraniol 600 µM	52.8 ± 6.1	47.9 ± 0.6
Simv 15 µM	60.6 ± 3.0	98.6 ± 5.2**

593

594 Hep-G2 cells were seeded in 24-well microtest plates (25 x 10^3 cells/well) with serum-
595 containing MEM and treated with geraniol (200, 400, and 600 µM) or simvastatin (15
596 µM) and mevalonate (0.5 µM) concurrently for 48 h. Viability was determined by the
597 MTT assay. Data are the means ± SD of 12 replicate wells per dose performed in 3
598 separate experiments. (**) $p < 0.01$ *vs*. cells without mevalonate. MVL, mevalonate; +/-
599 MVL, with or without MVL; Simv, simvastatin

600

601

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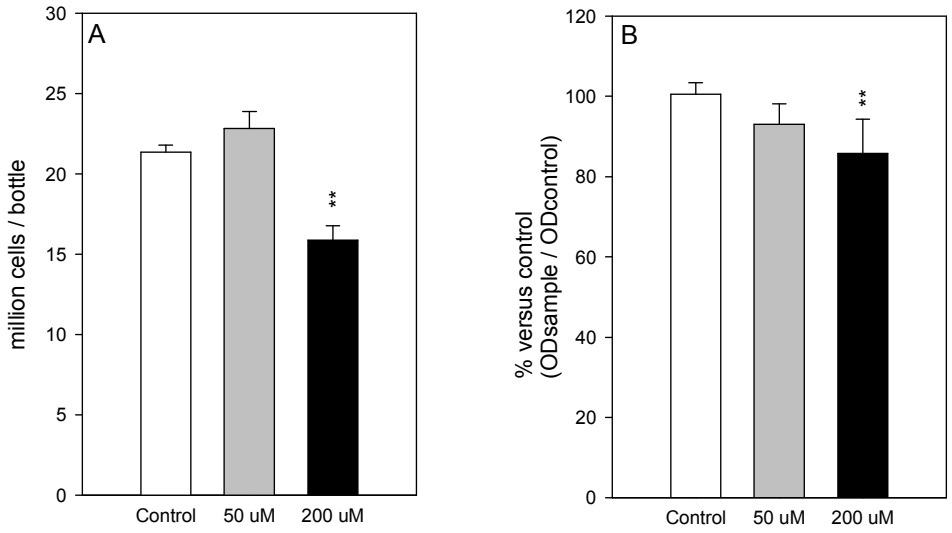
Figure 1.



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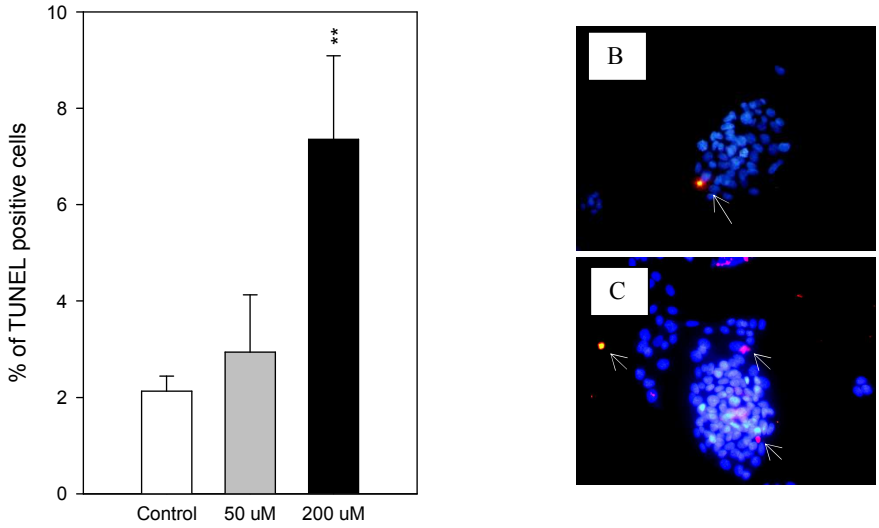

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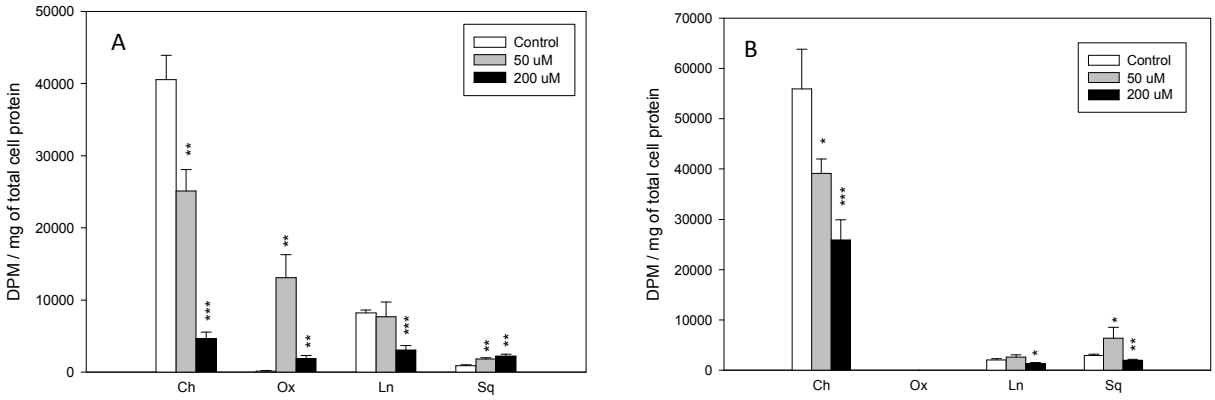

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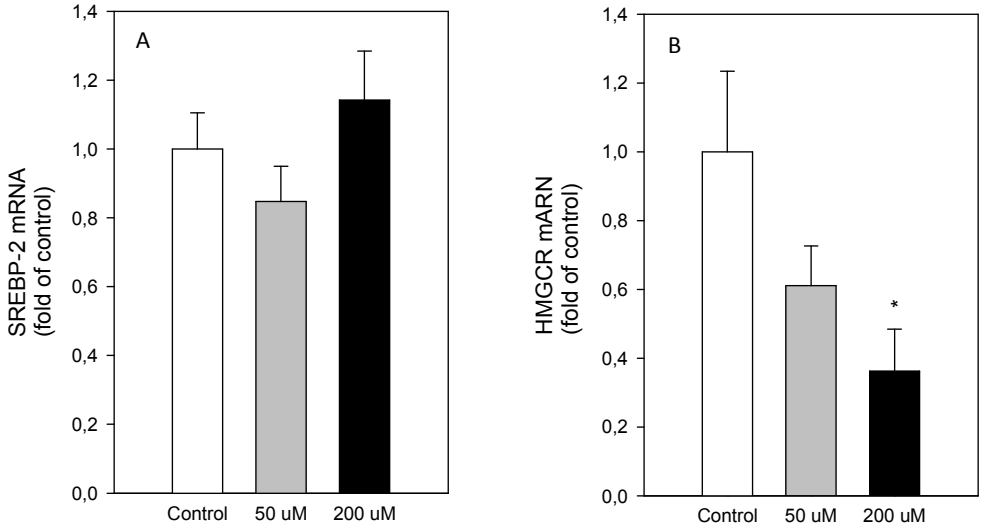

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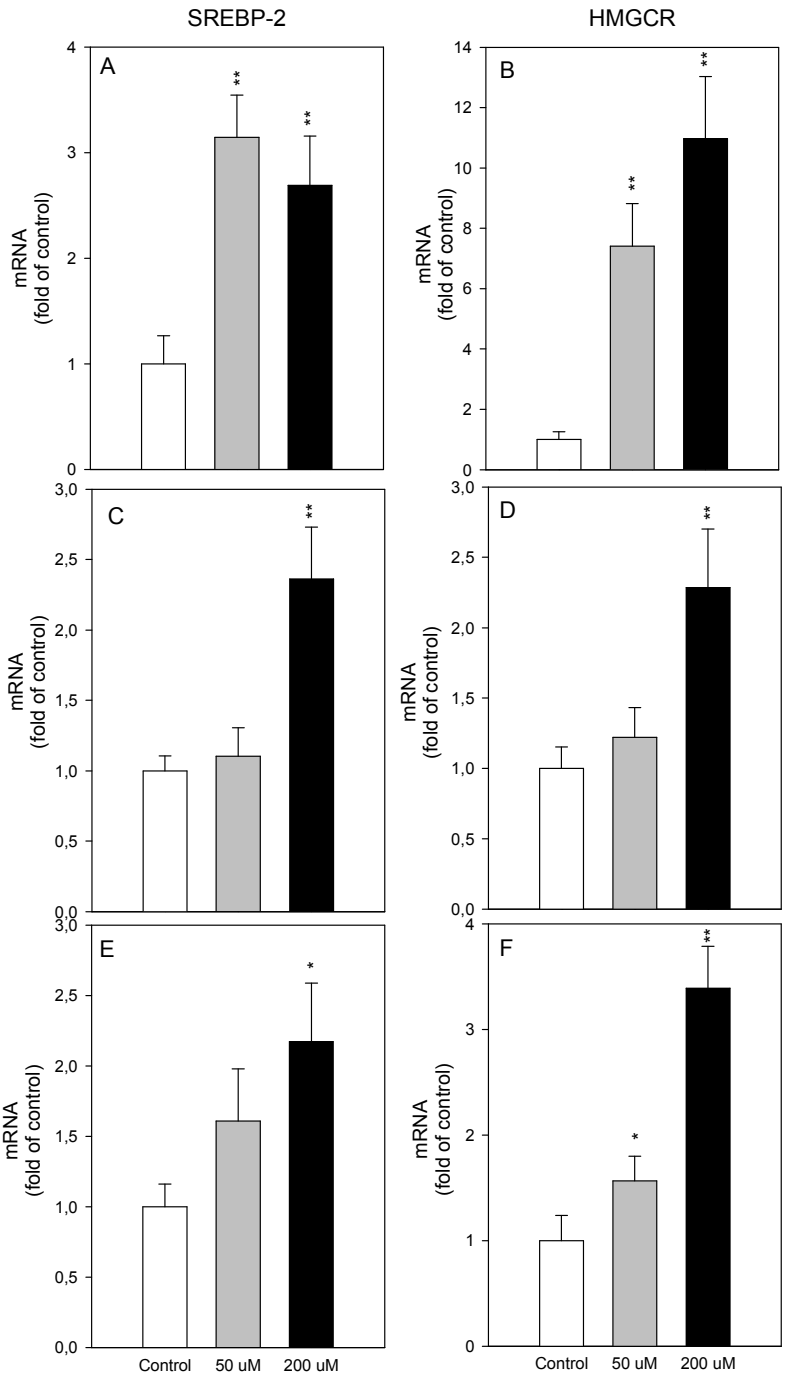
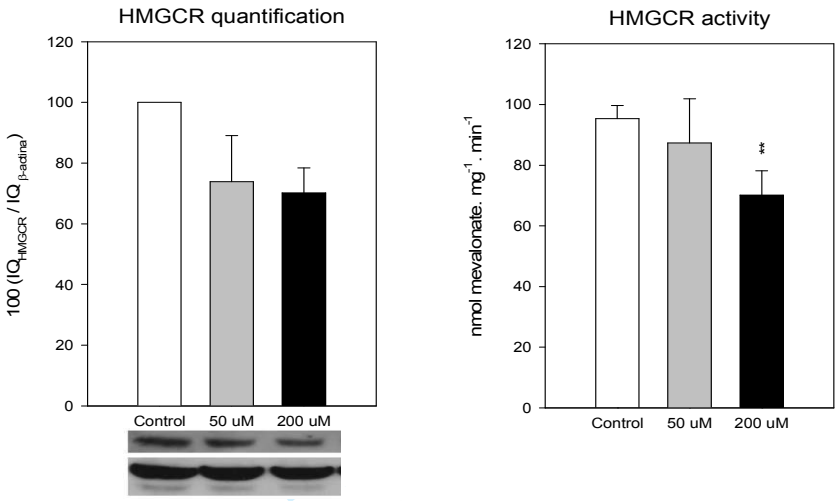

SREBP-2
HMGCR
A
B
C
D
E
F
mRNA (fold of control)
Control
50 uM
200 uM

607

Figure 6.



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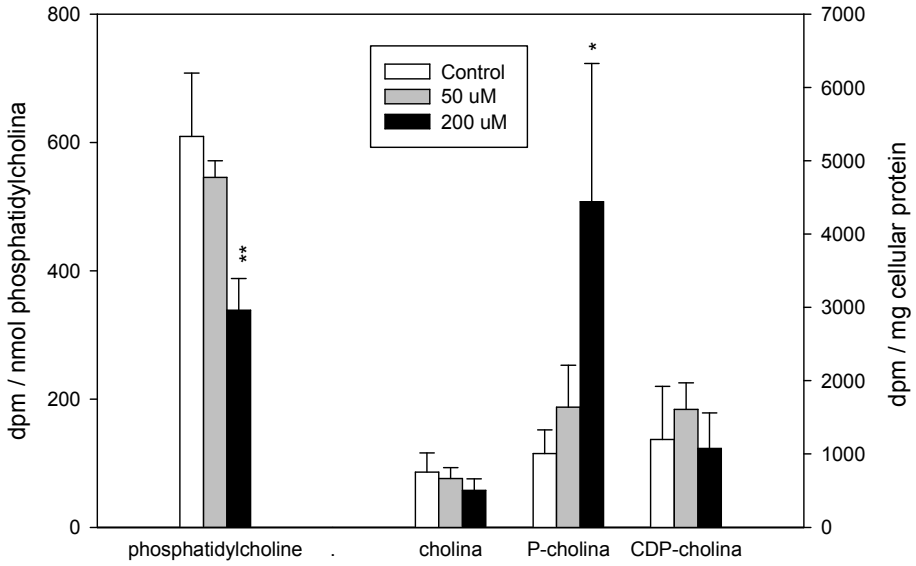

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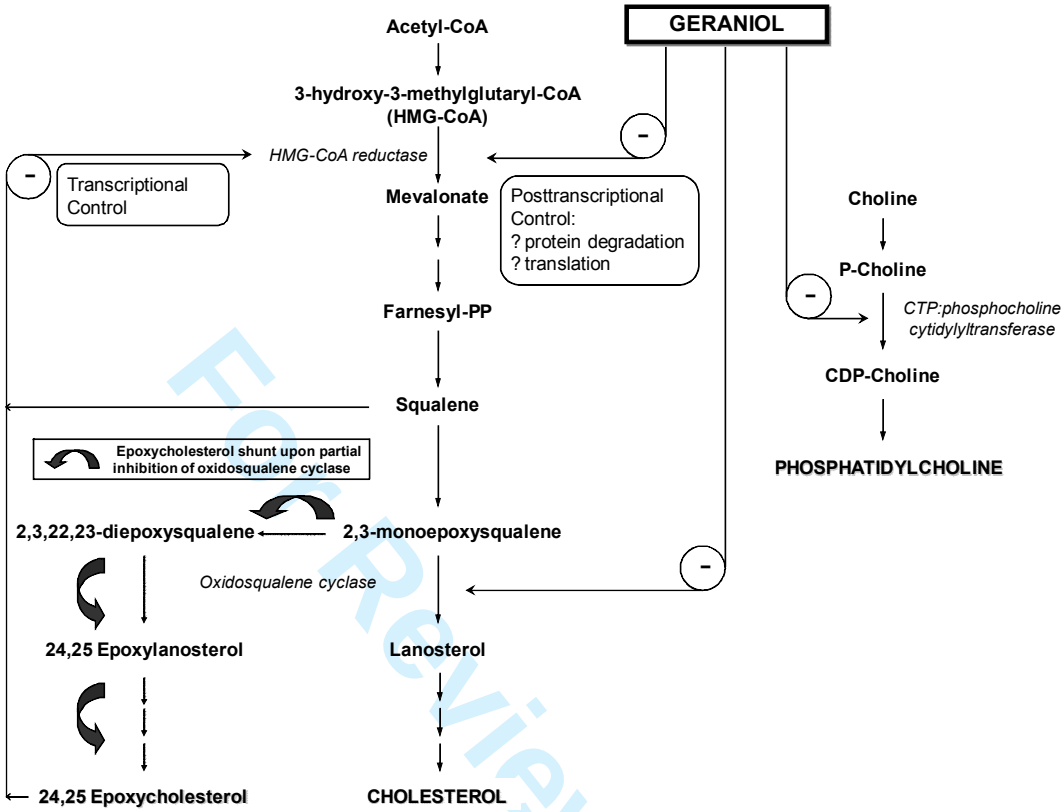

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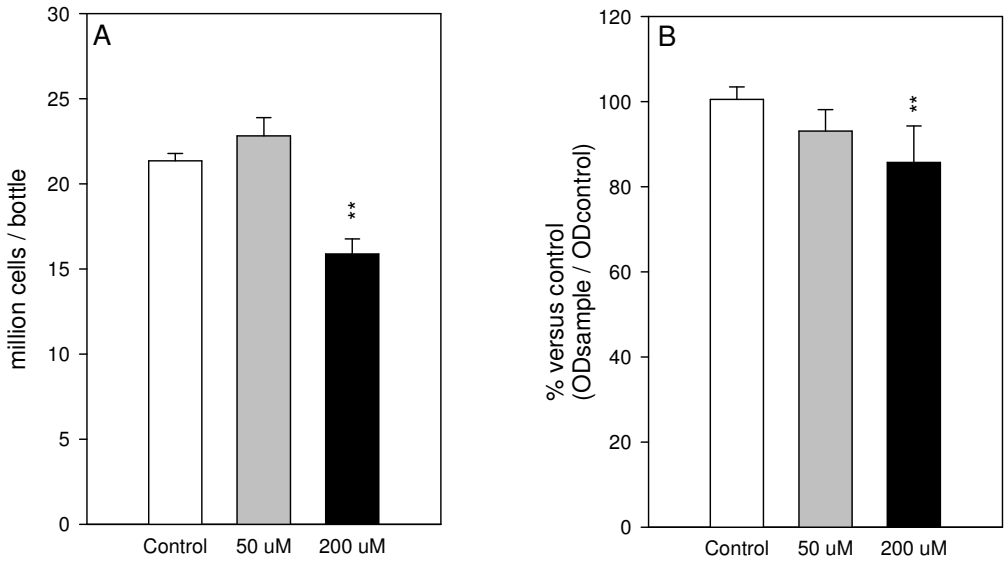

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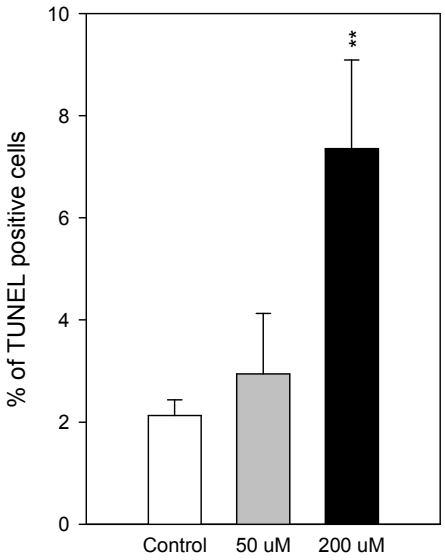

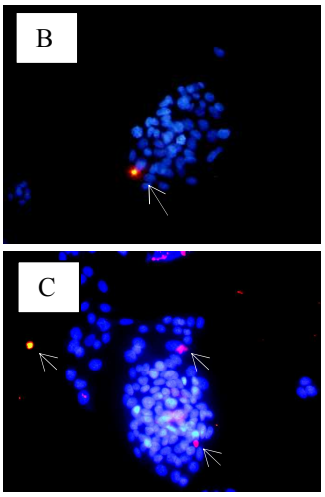

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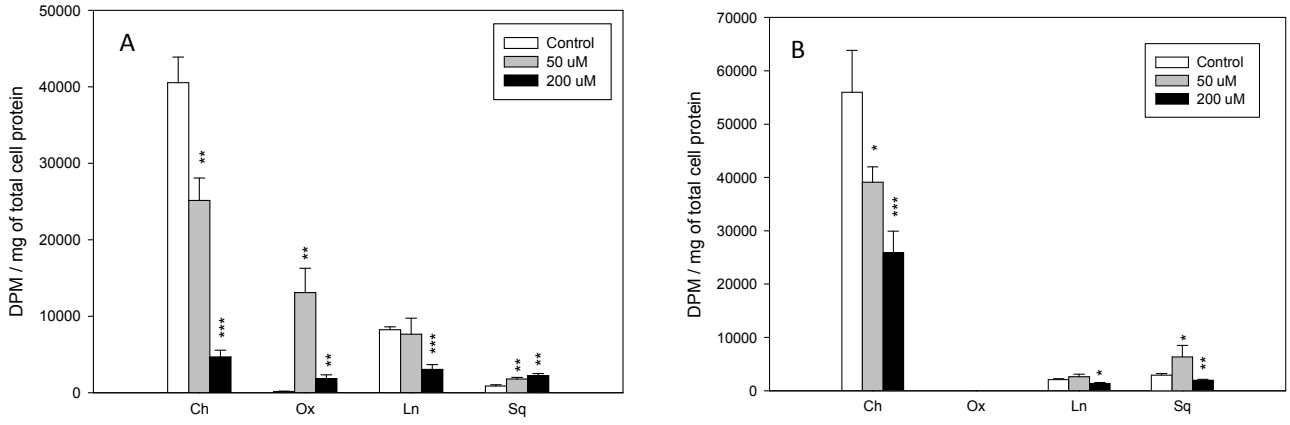

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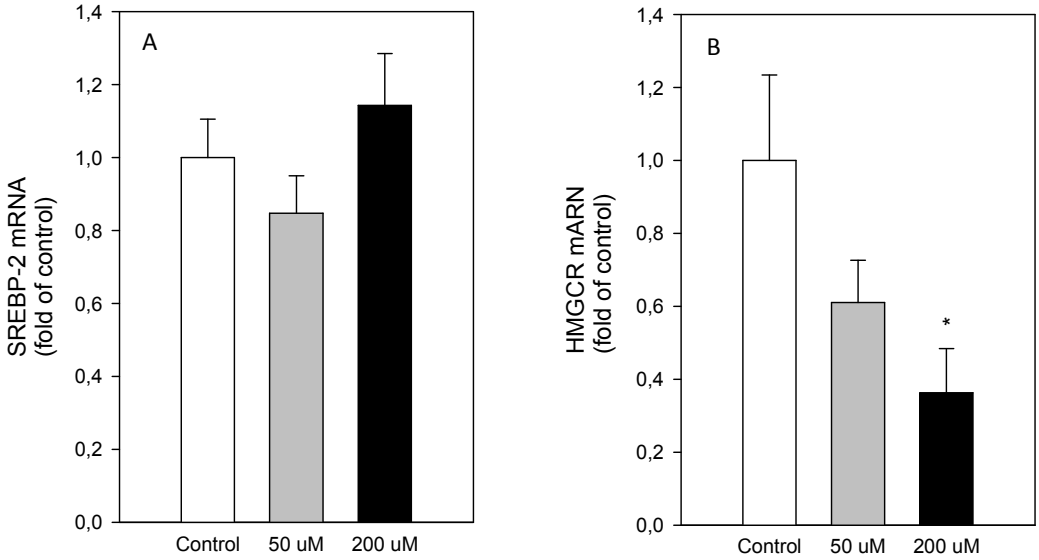
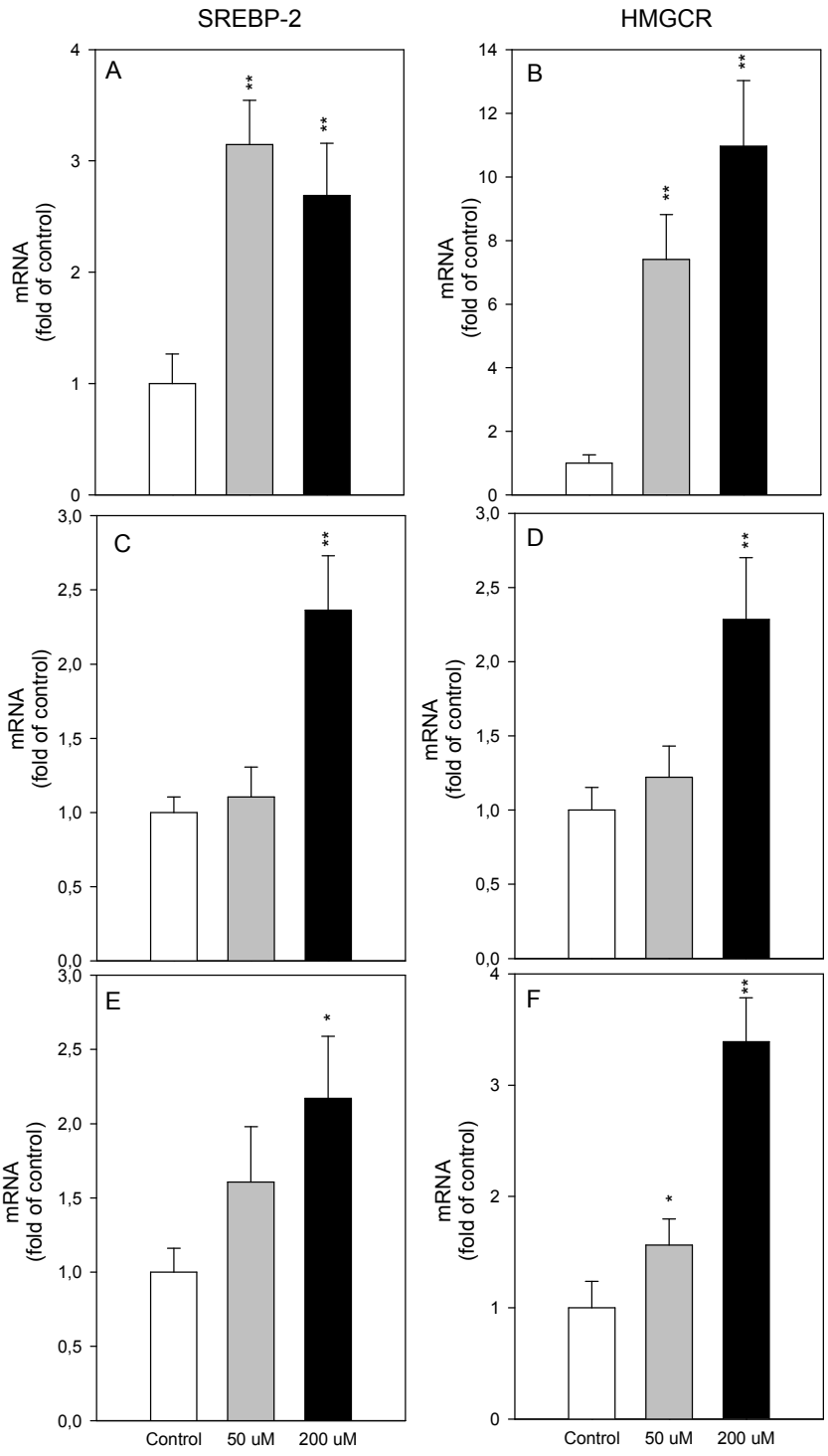


Figure 5.


SREBP-2
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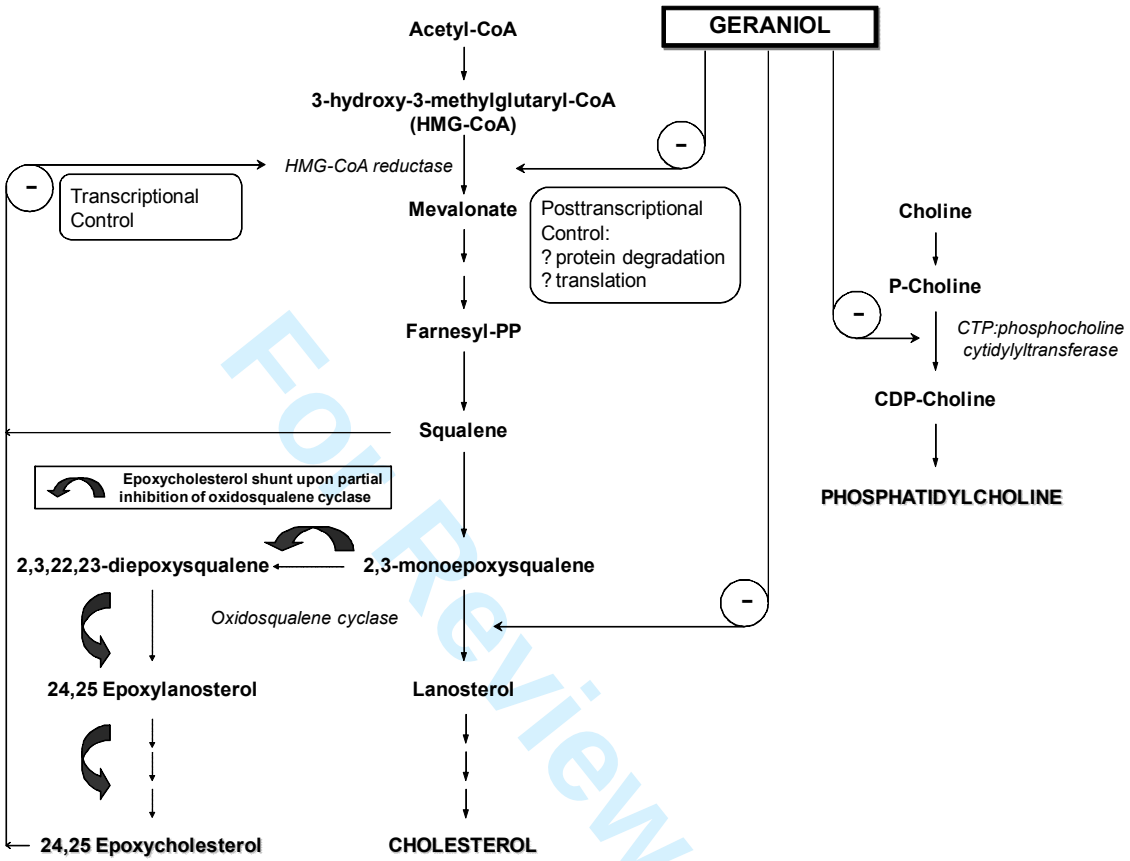

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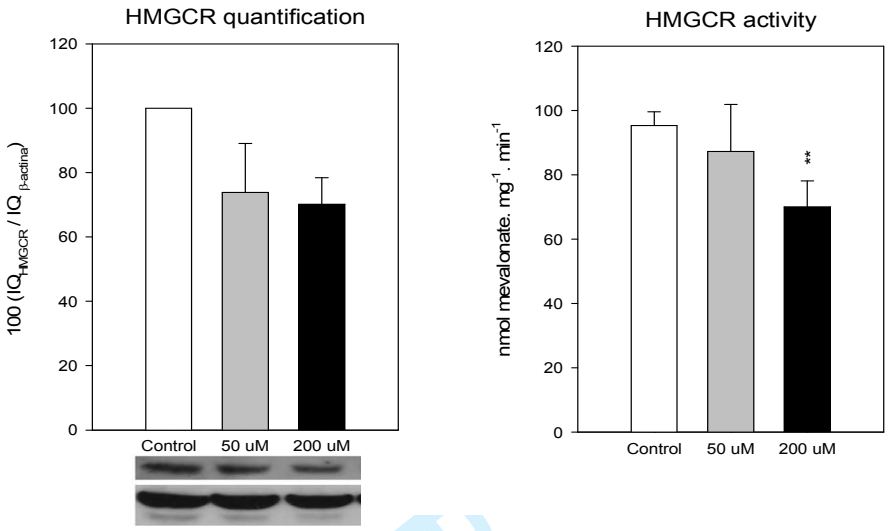

Figure 7.