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ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 130–135
Journal of Nutritional Biochemistry

Chemopreventive effects of β-ionone and geraniol during rat hepatocarcinogenesis promotion: distinct actions on cell proliferation, apoptosis, HMGCoA reductase, and RhoA

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Received 17 September 2009; received in revised form 10 December 2009; accepted 11 December 2009

Abstract

Chemopreventive activities of the dietary isoprenoids β-ionone (βI) and geraniol (GOH) were evaluated during the promotion phase of hepatocarcinogenesis. Over 5 consecutive weeks, rats received daily 16 mg/100 g body weight (b.w.) of βI (βI group), 25 mg/100 g b.w. of GOH (GOH group), or only corn oil (CO group, controls). Compared to the CO group, the following was observed: only the βI group showed a decrease in the mean number of visible hepatocyte nodules ($P<.05$); βI and GOH groups had reduced mean number of persistent preneoplastic lesions (pPNLs) ($P<.05$), but no differences regarding number of remodeling PNL (rPNLs) were observed; only the βI group exhibited smaller rPNL size and percentage of liver sections occupied by pPNLs ($P<.05$), whereas the GOH group displayed a smaller percentage of liver sections occupied by rPNLs ($P<.05$); a trend was observed in the βI group, which showed reduced cell proliferation of pPNLs ($P<.10$), and the GOH group had increased apoptosis in pPNLs and rPNLs ($P<.05$); only the βI group displayed reduced total plasma cholesterol concentrations ($P<.05$) and increased hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase mRNA levels ($P<.05$); only the GOH group had lower hepatic membrane RhoA protein levels ($P<.05$); both the βI- and GOH-treated groups had higher hepatic concentrations of βI and GOH, respectively ($P<.05$). Given these data, βI and GOH show promising chemopreventive effects during promotion of hepatocarcinogenesis by acting through distinct mechanism of actions: βI may inhibit cell proliferation and modulate HMGCoA reductase, and GOH can induce apoptosis and inhibit RhoA activation.

Keywords: Hepatocarcinogenesis; Chemoprevention; Isoprenoids; β-ionone; Geraniol

1. Introduction

The consumption of bioactive compounds in food has been previously demonstrated to correlate with reduction in cancer risk [1]. Dietary isoprenic derivatives such as β-ionone (βI), a cyclic isoprenoid present in grapes and wine [2], and geraniol (GOH), an acyclic monoterpene present in lemon and lemongrass [3], represent a promising class of chemopreventive agents [4].

The anticarcinogenic actions of βI and GOH have been described mostly in vitro [5]. Among the few in vivo studies, βI inhibits melanoma [6], breast cancer [7], and colon [8] cancer development, while GOH inhibits growth of pancreatic adenocarcinomas [9]. Both isoprenoids inhibit hepatic preneoplastic lesions (PNLs) when administered continuously during the initiation and selection/promotion phases of hepatocarcinogenesis [10,11].

One proposed chemopreventive mechanism of isoprenoids is the suppression of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase activity, which is frequently increased and deregulated in preneoplastic and neoplastic tissues [5]. This suppression limits farnesyl and geranylgeranyl pyrophosphates [12], which are important for prenylation of proto-oncogenes such as RhoA [12,13], thereby inhibiting cell proliferation and inducing apoptosis [14–16].

In the present study, the chemopreventive activities of βI and GOH were investigated when administered during the promotion phase of the "resistant hepatocyte" (RH) model of hepatocarcinogenesis. Parameters that were evaluated include PNL development, cell proliferation, apoptosis, total plasma cholesterol concentration,

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doi:10.1016/j.jnutbio.2009.12.007

HMGCoA reductase mRNA levels, RhoA activation, and hepatic concentrations of βI and GOH.

2. Methods and materials

2.1. Chemicals

βI [4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3(E)-buten-2-one, 95%], GOH (*trans*-3,7-dimethyl-2, 6-octadien-1-ol, 98%), 2-acetylaminofluorene (2-AAF), 5-bromo-2′-deoxyuridine (BrdU), diethylnitrosamine (DEN), and 3,3′-diaminobenzidine were purchased from Sigma (St. Louis, MO, USA); commercial diet was purchased from Purina (Campinas, Brazil); corn oil (CO), Mazola (São Paulo, Brazil); polyclonal anti-placental glutathione *S*-transferase (GST-P) rabbit antibody was purchased from Medical and Biological Laboratories (Tokyo, Japan); monoclonal anti-RhoA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-BrdU rat antibody, secondary biotinylated antibody, and streptavidin-biotin-peroxidase complex (StrepABComplex/HRP Duet, mouse/rabbit) were purchased from Dako (Glostrup, Denmark); the Illustra RNAspin Mini RNA Isolation kit and Hybond-ECL chemiluminescence kits were purchased from GE Healthcare (Chicago, IL, USA); SuperScript II reverse transcriptase, 10× Invitrogen reverse transcriptase-polymerase chain reaction (RT-PCR) buffer and Platinum Taq DNA Polymerase were purchased from Invitrogen (Carlsbad, CA, USA); MEM-PER reagent and the BCA protein assay kit were purchased from Pierce (Rockford, IL, USA). Other chemicals were of the highest available quality.

2.2. Animals and experimental protocol

Male Wistar rats (45–50 g) from the colony of the Faculty of Pharmaceutical Sciences of the University of São Paulo (São Paulo, Brazil) were maintained under standard environmental conditions. After 1 week of adaptation, with the exception of eight rats not submitted to any experimental procedure (Normal [N] group), 48 animals were submitted to the RH model of hepatocarcinogenesis, as described previously [17]. Briefly, for initiation, animals received an intraperitoneal (i.p.) dose of DEN (20 mg/100 g body weight [b.w.]) dissolved in 0.9% NaCl solution. After a recovery period of 2 weeks, the initiated hepatocytes were selected/promoted by 6 intragastric doses of 2-AAF, the first 4 (2 mg/100 g b.w.) on 4 consecutive days before partial (2/3) hepatectomy and the remaining 2 (0.75 and 2 mg/100 g b.w.) on day 2 and day 4 thereafter, respectively. One week after hepatectomy, animals were treated with βI (βI group: 16 mg/100 g b.w.) or GOH (GOH group: 25 mg/100 g b.w.) dissolved in CO (0.25 ml/100 g b.w.). Animals receiving only CO (CO group: 0.25 ml/100 g b.w.) were used as controls. All treatments were conducted by gavage, daily for 5 consecutive weeks during the promotion phase of hepatocarcinogenesis. All animals were euthanized 8 weeks after DEN administration. Two hours before euthanasia, rats received a single i.p. injection of BrdU (10 mg/100 g b.w.) dissolved in dimethyl sulfoxide and saline (1:3 v/v). The study was approved by the Faculty of Pharmaceutical Sciences of the University of São Paulo Ethics Committee for Animal Research (Protocol number 63).

2.3. Visible hepatocyte nodules counting

After euthanasia, the liver was removed from each animal, weighed and examined on the surface for the presence of visible hepatocyte nodules of varied sizes and a generally whitish or yellowish color, which is different from the hepatic parenchyma.

2.4. Double staining immunohistochemistry for GST-P and BrdU

Representative fragments of each liver lobe were fixed in methacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for 24 h and subsequently embedded in paraffin. Serial histological sections of liver samples were processed for double staining immunohistochemical reactions in order to detect PNLs (foci/nodules) positive for GST-P (anti GST-P antibody 1:500) and hepatocytes positive for BrdU (1:400), as previously described [18].

2.5. Apoptosis evaluation

Hepatic apoptotic bodies (ABs) were quantified by fluorescence microscopy [11] using a Nikon microscope (Tokyo, Japan) equipped with an epifluorescence unit. Identification of ABs was confirmed using morphological criteria established by Goldsworthy et al. [19]. The number of ABs in each group was counted in all PNLs and surrounding normal tissue transections within the liver sections. Reading of the ABs was performed by scanning the whole slide, and the results were expressed as the number of ABs/mm^2 of pPNLs, rPNLs or the surrounding area.

2.6. Total plasma cholesterol concentration

Blood was collected by puncture of the abdominal aorta at the time of euthanasia. Immediately after collection, it was placed in centrifuge tubes containing EDTA and centrifuged at 3500×*g* at 4°C for 10 min. Total plasma cholesterol concentration was determined using an enzymatic-spectrophotometric technique. Analysis was performed at 500 nm with a Hitachi U 110 spectrophotometer (Tokyo, Japan).

2.7. HMGCoA reductase RT-PCR analysis

Total hepatic RNA (six animals per group) was isolated with the Illustra RNAspin Mini RNA Isolation kit. The RNA quality was checked by gel electrophoresis. One microgram of total RNA was reverse transcribed with 1 U of SuperScript II reverse transcriptase. Two microliters of cDNA template was submitted to PCR with 5 μl of 10× RT-PCR buffer (2.5 mM $MgCl_2$ final concentration), 1 μl of dNTP mix (10 mM of each dNTP), 1 μl of forward and reverse gene-specific primers (10 mM), 1.25 U Platinum Taq DNA polymerase and PCR-grade water to make a 50 μl final volume. The PCR conditions were as follows: the RT reaction was performed at 50°C for 50 min, and initial PCR activation was performed at 95°C for 15 min, followed by 35 cycles [94°C (denaturation) for 45 s, 58.8°C (annealing) for 75 s, and 72°C (extension) for 60 s], with a 10-min final extension at 72°C. HMGCoA reductase primers (323 bp PCR product) were as follows: forward 5′-CATGATTTC-CAAGGGTACGG-3′ and reverse 5′-GGGCACATGCAATGTAGATG-3′. β-actin primers (290 bp PCR product) were as follows: forward 5′-CGTTGACATCCGTAAAGACCTCTA-3′ and reverse 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ [20]. PCR products were separated on 2% agarose gels, and DNA was visualized by ethidium bromide staining. Densitometric analysis of the bands was performed with the Bio-Rad Molecular Analyst software (Los Angeles, CA, USA).

2.8. RhoA Western blot analysis

Membrane protein extracts were prepared from liver samples using the MEM-PER reagent. Protein concentration was determined with the BCA protein assay kit. Samples of membrane extracts (50 μg) were separated by electrophoresis on 15% denaturing polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), in 1× Tris-glycine buffer. Proteins were transferred from the gel to a nitrocellulose membrane and then blocked overnight at 4°C with phosphate buffered saline (PBS) containing 5% powdered milk. After washing with PBS buffer containing 0.1% Tween 20, the membrane was incubated for 2 h at room temperature with the primary anti-RhoA antibody (1:1,000), followed by incubation with the secondary antibody conjugated to horseradish peroxidase. The membrane was developed using the Hybond-ECL chemiluminescence kit. An X-ray film was then exposed to the membrane, resulting in approximately 24-kDa bands, corresponding to the expected molecular weight of RhoA. Densitometric analysis of the bands was performed with Molecular Analyst software. As an internal control, membranes probed for RhoA were stained with Coomassie blue [10,11].

2.9. βI and GOH hepatic concentrations

βI and GOH were quantified according to Su et al. [21], with modifications. Liver samples from 6 animals of each group were powdered in mortars with liquid N_2 and homogenized in a Potter with 400 μL absolute ethanol plus 10 ng methyl-β-ionone or 10 ng thymol [internal standards (IS)] for βI and GOH quantification, respectively. Homogenates were centrifuged for 15 min at 10,000×*g* (4°C). Supernatants were collected and transferred to clean injection vials, and 2 μl were injected in a gas chromatography/mass spectrometry system. βI and GOH standard solutions, plus the same amount of IS added to the samples, were used for calibration curves that were calculated using the analyte/IS peak-area ratios versus the nominal concentrations of each standard. Samples and standards were injected in an Agilent GC HP6890 (Palo Alto, CA, USA) equipped with a CP Wax 58-FFAP

Table 1
Body and liver weights and quantification of visible hepatocyte nodules of rats treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis

Group	No. of rats	Final weight (g) *	Liver weight (g) *	Relative liver weight (g/100 g b.w.) *	Incidence of nodules (%)	Mean no. of nodules per rat *
CO	8	308±9	11.2±0.8	3.6±0.2	100	188±49
βI	12	297±6	10.5±0.6	3.5±0.1	100	77±26[a]
GOH	11	298±6	9.9±0.4	3.3±0.1	100	87±30

[a] Statistically significant difference (P<.05) when compared to the CO group (ANOVA followed by Tukey test); ANOVA, analysis of variance.
* Values are means±S.E.M.

Table 2
Morphometric analysis of GST-P positive PNLs of rats treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis

Group	No. of rats	Number of GST-P positive PNLs per cm^2 *		Size of GST-P positive PNLs (mm^2) *		Area of liver section occupied by GST-P positive PNLs (%) *	
		pPNL	rPNL	pPNL	rPNL	pPNL	rPNL
CO	8	12±4	62±6	0.49±0.10	0.39±0.07	7±3	25±4
βI	12	2±1[a]	92±12	0.33±0.20	0.22±0.04[a]	1±1[a]	16±3
GOH	11	4±1[a]	52±5	0.40±0.10	0.25±0.03	2±1	12±2[a]

p, persistent; r, remodeling.
[a] Statistically significant differences (P<.05) when compared to the CO group (ANOVA followed by Tukey test or Kruskal–Wallis test).
* Values are means±S.E.M.

(Varian 25 m×0.32 m×0.2 μm) column. Injection was done in the splitless mode, and the injection port was maintained at 200°C. The carrier gas was helium, at a flow rate of 1.0 ml/min. The oven temperature was maintained at 50°C for 3 min and raised to 250°C at 20°C/min. The column effluent was monitored by an Agilent mass selective detector HP5973 using electron impact ionization with an ion source at 70 eV and 200°C.

2.10. Statistical analysis

Results are expressed as mean±S.E.M., and all analyses were conducted with STATISTICA 8.0 (Statsoft), adopting 5% as the significance level (P<.05).

3. Results

3.1. Body and liver weights, incidence and mean number of visible hepatocyte nodules

Table 1 presents the data on body and liver weights and incidence and mean number per rat of visible hepatocyte nodules. No differences (P>.05) were observed between the different experimental groups regarding the final body weights and absolute and relative liver weights, which indicates that βI and GOH did not cause toxicity at the dosages that were used. Additionally, no differences (P>.05) were observed between the different experimental groups regarding incidence of nodules. When compared to the CO group (controls), the βI and GOH groups displayed a lower mean number of hepatocyte nodules, although statistical significance (P<.05) was reached only in the βI group.

3.2. Morphometric quantification of GST-P positive PNLs

Table 2 shows results from the morphometric quantification of GST-P positive PNLs. Compared to the CO group, the βI and GOH groups had reduced (P<.05) numbers of pPNLs, while no statistical differences (P>.05) were observed regarding the number of rPNLs. Additionally, no differences (P>.05) were observed between the isoprenoid-treated groups regarding these parameters. Compared to the CO group, there was no difference in pPNL and rPNL size in the GOH group, while the βI group exhibited rPNLs (but not pPNLs) with smaller size (P<.05). Compared to the CO group, βI group had pPNLs that occupied a smaller (P<.05) area of the liver section while GOH group had rPNLs that occupied a smaller (P<.05) area of the liver section.

3.3. Evaluation of cell proliferation and apoptosis

Fig. 1 shows the quantification of BrdU-stained hepatocytes/mm^2 and hepatic ABs/mm^2 of PNLs and the surrounding tissue. In all groups, pPNLs, but not rPNLs, had increased cell proliferation compared to their respective surrounding normal tissue (P<.05). Compared to the CO group, the βI group showed a trend towards reduced cell proliferation in pPNLs (P<.10), while no differences (P>.05) were observed in the GOH group. In addition, no differences were observed between all groups regarding rPNL cell proliferation. In all groups, pPNLs and rPNLs exhibited increased apoptosis compared to their respective surrounding normal tissue (P<.05). However, compared to the CO group, the GOH group had increased apoptosis in pPNLs and rPNLs (P<.05), while no differences (P<.05) were observed in the βI group.

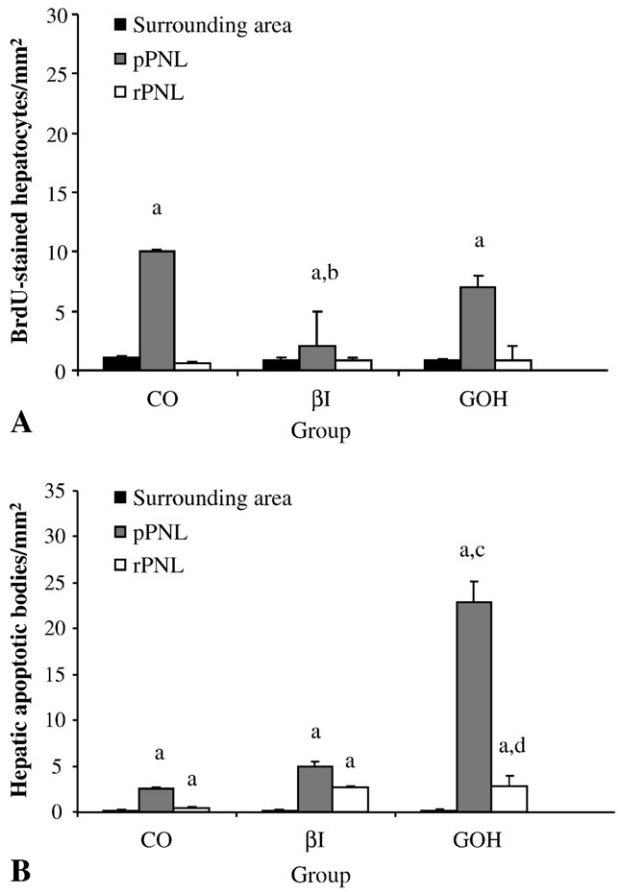

Fig. 1. (A) Quantification of BrdU-stained hepatocytes and (B) hepatic ABs of PNLs and their respective surrounding tissue of rats treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis. p, persistent; r, remodeling. Values are means±S.E.M., n=8 (CO group), n=12 (BI group), and n=11 (GOH groups). [a]Statistically significant differences (P<.05) when compared to respective surrounding tissue area (Wilcoxon's test). [b]Marginally significant difference (P<.10) when compared to pPNLs of the CO group (ANOVA followed by Tukey test). Statistically significant differences (P<.05) when compared to [c]pPNLs and [d]rPNLs of the CO group (ANOVA followed by Tukey test).

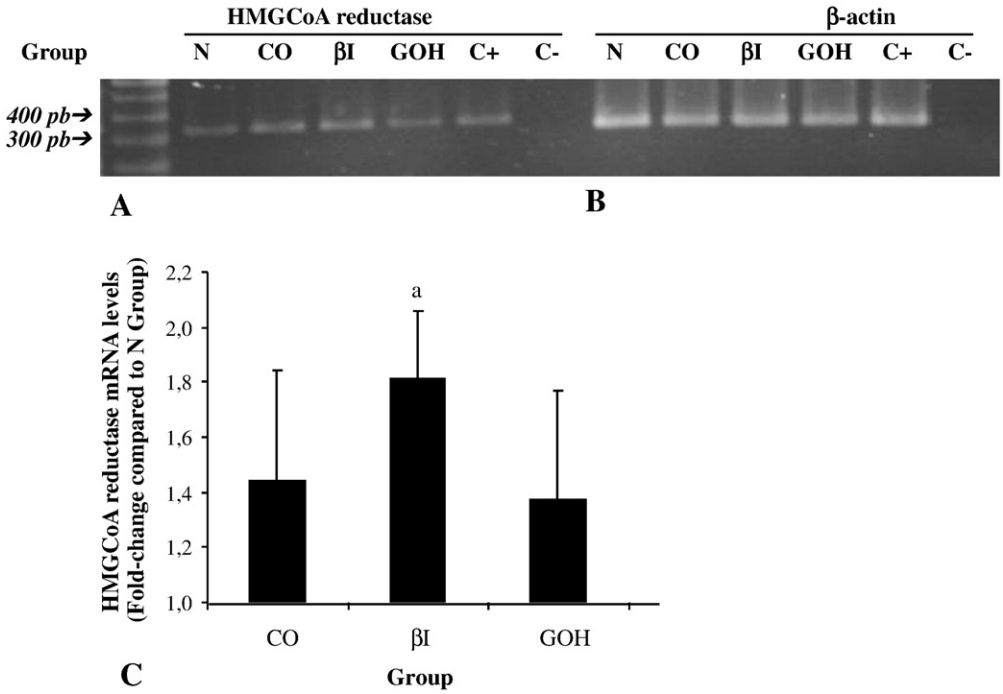

Fig. 2. (A) HMGCoA reductase and (B) β-actin RT-PCR analysis performed with hepatic RNA extracted from normal Wistar rats and animals treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis. Representative sample from 1 rat of each group. (C) Quantification of HMGCoA reductase mRNA levels in liver samples of normal Wistar rats and animals treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis. Results are expressed in relation to the HMGCoA reductase mRNA levels of normal animals, which were considered to be 1. C+, positive control; C-, negative control. Values are means±S.E.M., $n=4$. [a] Statistically significant difference ($P<.05$) when compared to the CO group (Student's t test for unpaired data).

3.4. Total plasma cholesterol

Regarding the total plasma cholesterol concentration, the following results were obtained: 55±2 mg/dl (N group), 76±4 mg/dl (CO group), 68±2 mg/dl (βI group) and 70±3 mg/dl (GOH group). Compared to the N group, the CO group had higher total plasma cholesterol concentrations ($P<.05$; Student's t test for unpaired data). In contrast, compared to the CO group, the βI group had reduced total plasma cholesterol concentrations ($P<.05$; Student's t test for unpaired data). No differences ($P>.05$) in total plasma cholesterol concentrations were observed between the CO and GOH groups.

3.5. HMGCoA reductase RT-PCR analysis

Fig. 2 shows the HMGCoA reductase RT-PCR analysis performed with total RNA extracted from normal rat livers and entire livers (nodules+non-nodular surrounding tissues) of animals from the CO, βI and GOH groups. Compared to the N group, the CO group had a 1.4-fold increase in HMGCoA reductase mRNA levels, and compared to the CO group, the βI group had a 1.3-fold increase in HMGCoA reductase mRNA level ($P<.05$), while no difference was observed in the GOH group ($P>.05$).

3.6. RhoA western blot analysis

Fig. 3 shows the RhoA immunoblot analysis performed with membrane protein extracts of normal rat livers and entire livers (nodules+non-nodular surrounding tissues) of animals from the CO, βI and GOH groups. Compared to the N group, the CO group had a 1.4-fold increase in RhoA membrane levels, and compared to the CO group, the GOH group had reduced hepatic membrane RhoA protein levels ($P<.05$), while no differences were observed in the βI group

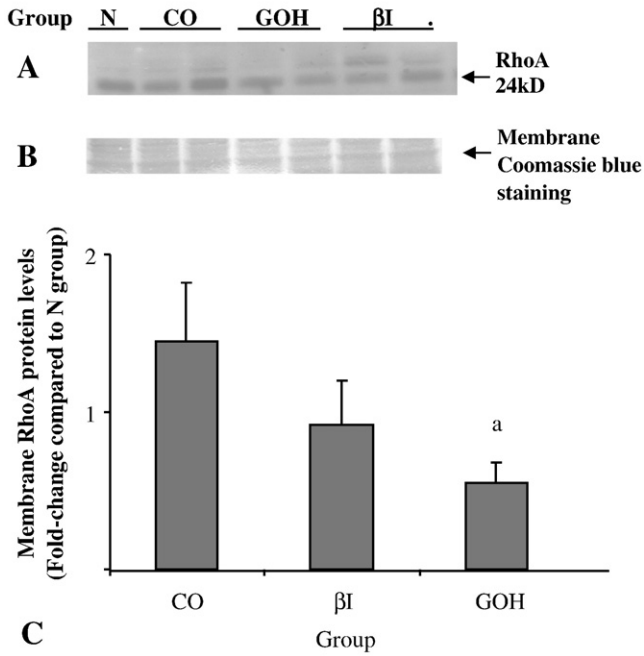

Fig. 3. (A) RhoA western blot analysis performed with membrane proteins extracted from normal Wistar rats and animals treated with CO, βI, or GOH during the promotion phase of hepatocarcinogenesis. Representative samples from 2 animals of the CO, βI, and GOH groups. (B) Coomassie blue staining of the membrane as an equal protein loading control. (C) Quantification of membrane RhoA levels in liver samples of normal Wistar rats and animals treated with CO, βI, or GOH during the promotion phase of hepatocarcinogenesis. Results are expressed in relation to the hepatic RhoA protein levels of normal animals, which were considered to be 1. Values are means±S.E.M., $n=4$. [a] Statistically significant difference ($P<.05$) when compared to the CO group (Student's t test for unpaired data).

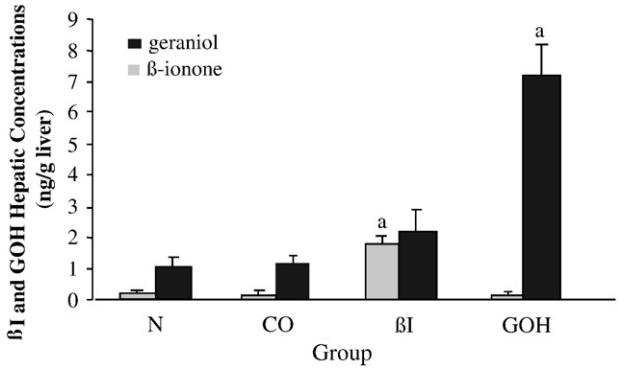

Fig. 4. Hepatic concentrations of βI and GOH of normal Wistar rats and animals treated with CO, βI, or GOH during the promotion phase of the RH model of hepatocarcinogenesis. Values are means±S.E.M., n=6. [a]Statistically significant differences ($P<.05$) when compared to the CO group (ANOVA followed by Tukey test).

($P>.05$). No differences were observed among all groups regarding hepatic cytoplasmic RhoA protein levels ($P>.05$; data not shown).

3.7. Hepatic concentrations of βI and GOH

Fig. 4 shows the hepatic concentrations of βI and GOH in normal Wistar rats and animals submitted to the RH model and treated with CO, βI or GOH. Compared to the N group, there was no difference in βI and GOH hepatic concentrations in the CO group ($P>.05$). Compared to the CO group, the βI hepatic concentration was higher in the βI group ($P<.05$). Compared to the CO group, there was no difference in the hepatic concentration of βI in the GOH group ($P>.05$). Compared to the CO group, the GOH hepatic concentration was higher in the GOH group ($P<.05$). Additionally, compared to the CO group, there was no difference in the GOH hepatic concentration in the βI group ($P>.05$).

4. Discussion

Previous studies have demonstrated the chemopreventive activity of βI [10] and GOH [11] when administered continuously during the initiation and selection/promotion phases of the RH model of hepatocarcinogenesis. Due to the experimental design of these studies, the observed protective effects were attributed to the isoprenoid-mediated modulation of carcinogen metabolism. Since reducing the initiation phase to a zero level is impossible, the most effective intervention would be at the promotion phase, which can be interrupted and in some cases reversed [22]. Therefore, the present study was conducted in order to evaluate if both substances would display anticarcinogenic effects when given specifically during promotion.

In the present study, βI and GOH showed inhibitory action on both macroscopic and microscopic hepatic PNLs. To our knowledge, this is the first study to report the chemopreventive activities of βI and GOH specifically during promotion of hepatocarcinogenesis. It is noteworthy that, in our study, these isoprenoids presented inhibitory effects when dissolved in corn oil, a dietary fat shown to enhance carcinogenesis in several organs, including rat liver [23,24]. Considering the scarcity of isoprenoid chemopreventive studies with an experimental design similar to the present one, emphasis should be given on further investigations that would address the protective potential of these compounds during the promotion phase of hepatocarcinogenesis.

An important feature of hepatocarcinogenesis models is that they allow the identification of two PNL phenotypes: the few persistent ones (2–5%), considered precursors of hepatocarcinoma, and the great majority (95–98%) that remodel to a normal-appearing liver over time [25]. PNL persistence has been associated with a block in remodeling by redifferentiation [26] and apoptosis [27]. In the present study, inhibition of pPNL development represents an important chemopreventive mechanism of βI and GOH.

Inhibition of cell proliferation and induction of apoptosis have been suggested to be the main anticarcinogenic mechanisms of isoprenoids [4,6]. Specifically during hepatocarcinogenesis, βI and GOH exert these actions in total PNLs (persistent and remodeling ones) when administered continuously during the initiation and promotion phases of the RH model [10,11]. In the present study, βI may have inhibited cell proliferation in pPNLs but did not induce apoptosis in either pPNLs or rPNLs. In contrast, GOH induced apoptosis but did not inhibit cell proliferation in either pPNLs or rPNLs. Inhibition of cell proliferation and induction of apoptosis are therefore potential mechanisms related to the chemopreventive activities of βI and GOH, respectively.

Loss in the down-regulation mechanism of hepatic HMGCoA reductase and of cholesterologenesis, as well as increases in liver nodules cholesterol have been previously described in rats submitted to the RH model [28]. These alterations could potentially explain the higher plasma cholesterol levels observed in this study in control animals compared to normal animals. Previous studies have demonstrated that administration of βI and GOH to rats, mice and birds inhibits HMGCoA reductase and decreases total plasma cholesterol [29,30]. Additionally, we previously observed that βI, but not GOH, decreases total plasma cholesterol concentrations when administered to rats during the initiation and promotion phases of the RH model [10,11]. In this study, similar results have now been observed in animals treated with these isoprenoids during promotion. Reduction in total plasma cholesterol could be due to inhibition of HMGCoA reductase activity [10,11,16].

Increased HMGCoA reductase expression was observed in control rats submitted to the RH model. Isoprenoids post-transcriptionally inhibit HMGCoA reductase [5,31]. Interestingly and unexpectedly, βI treatment increased hepatic HMGCoA reductase mRNA levels when compared to control animals. These data are similar to another study that used farnesol in the RH model [11]. This apparently paradoxical result could be attributed to a compensatory induction of HMGCoA reductase gene transcription following an eventual degradation of the enzyme by βI. Such up-regulation has been described in the livers of rats treated with lovastatin, a competitive inhibitor of HMGCoA reductase [32] and a potent inhibitor of cell proliferation [33]. Although HMGCoA reductase has been pointed as the main target of dietary isoprenoids chemopreventive actions [5,31], it is possible that in our study cell proliferation inhibition by βI is independent of any effects on the mevalonate pathway, as suggested by Duncan et al. [4]. GOH did not have any effects on total plasma cholesterol or HMGCoA reductase mRNA levels, suggesting that the enzyme does not constitute a target for the chemopreventive activity of GOH. Growth inhibition of MCF-7 cells by both βI and GOH has been shown to be mediated entirely through a mechanism that is independent of HMGCoA reductase regulation [4].

Rho GTPases are major regulators of signal transduction pathways and play key roles in processes including actin dynamics, cell cycle progression, cell survival and gene expression, whose deregulation may lead to carcinogenesis [34]. RhoA overexpression at both the mRNA and protein level has been previously observed in hepatocarcinoma and correlates with poor prognosis [35]. In the present study, increased membrane localization of RhoA was observed in control animals compared to normal ones. RhoA hyperactivation during preneoplastic stages suggests that it is a relevant molecular alteration early in hepatocarcinogenesis. Treatment with GOH reduced membrane localization of RhoA without cytoplasmic

alterations. Similar effects on Ras and RhoA have been described for the monoterpene perillyl alcohol in leukemia culture cells [13]. In contrast, βI did not reduce membrane localization of RhoA. A previous report demonstrated that inhibition of the RhoA pathway in endothelial cells with a specific inhibitor results in apoptosis [36]. This suggests that the effects of GOH on the induction of apoptosis could potentially involve this pathway. Interestingly, the monoterpene actions on RhoA occur without inhibition of HMGCoA reductase. GOH could have blocked RhoA isoprenylation through geranilgeranil transferase inhibition [4,5].

Because perturbations in the mevalonate pathway have been linked to diseases such as cancer, it is of the utmost importance to quantify isoprenoids in diseased tissue or in tissue after drug administration [37]. In our study treatment with βI or GOH increased their respective hepatic concentrations. This indicates that it is feasible to achieve hepatic levels sufficient for chemoprevention after daily ingestion of these compounds.

The observation that the two isoprenoids βI and GOH inhibit liver carcinogenesis by inhibiting promotion phase is fundamental to our understating of the chemopreventive effects of these isoprenoids. Although these compounds act through distinct mechanisms (i.e., inhibition of cell proliferation by βI and induction of apoptosis and inhibition of RhoA activation by GOH), both dietary isoprenoids represent promising suppressive chemopreventive agents.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are indebted to Miss Silvania M.P. Neves for providing the care and maintenance of the animals.

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