Inhibition of Tumor Promotion in SENCAR Mouse Skin by Ethanol Extract of Zingiber officinale Rhizome

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ABSTRACT

There is considerable emphasis on identifying potential chemopreventive agents present in food consumed by the human population. Ginger rhizome (Zingiber officinale), known commonly as ginger, is consumed worldwide in cookeries as a spice and a flavoring agent. In this study, we evaluated whether ethanol extract of ginger (GE) possesses anti-tumor-promoting effects in a mouse skin tumorigenesis model. Because skin tumor promoters induced epidermal ornithine decarboxylase (ODC), cyclooxygenase, and lipoxigenase activities, and edema and hyperplasia are conventionally used markers of skin tumor promotion, first, we assessed the effect of GE on these parameters. Preapplication of GE onto the skin of SENCAR mice resulted in significant inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-caused induction of epidermal ODC, cyclooxygenase, and lipoxigenase activities and ODC mRNA expression in a dose-dependent manner. Preapplication of GE to mouse skin also afforded significant inhibition of TPA-caused epidermal edema (56%) and hyperplasia (44%). In long-term tumor studies, topical application of GE 30 min prior to that of each TPA application to 7,12-dimethylbenz(a)anthracene-initiated SENCAR mice resulted in a highly significant protection against skin tumor incidence and its subsequent multiplicity. The animals pretreated with GE showed substantially lower tumor body burdens compared with non-GE-treated controls. The results of our study, for the first time, provide clear evidence that GE possesses anti-skin tumor-promoting effects, and that the mechanism of such effects may involve inhibition of tumor promoter-caused cellular, biochemical, and molecular changes in mouse skin.

INTRODUCTION

To reduce the occurrence of cancer, one promising approach is its prevention, specifically by chemical intervention through minor non-nutrient dietary constituents. Chemoprevention, therefore, is the means of cancer control in which the occurrence of this disease, as a consequence of exposure to carcinogenic agents, can be slowed, blocked, or reversed by the administration of one or more naturally occurring and synthetic compounds (1–4 and references therein). Chemoprevention also deals with the chemotheraphy of precancerous lesions, which are called preinvasive neoplasia, dysplasia, or intraepithelial neoplasia, depending on the organ system (2). Chemopreventive agents can be targeted for intervention at either the initiation, promotion, or progression stage of multistage carcinogenesis (1–4 and references therein). The intervention of cancer at the promotion stage, however, seems to be most appropriate and practical. The major reason for that relates to the fact that tumor promotion is a reversible event at least in early stages and requires repeated and prolonged exposure of a promoting agent (5). For this reason, it is important to identify anti-tumor-promoting agents present in diets consumed by the human population.

Ginger rhizome (Zingiber officinale), commonly known as ginger, is consumed worldwide as a spice and a flavoring agent. Limited in vitro studies have shown that water and organic solvent extracts of ginger as well as some constituents isolated from ginger possess antioxidant properties (6–10). Zingerone, a compound isolated from ginger, has been shown to inhibit nitro blue tetrazolium reduction in a xanthine-xanthine oxidase system, providing the evidence that it scavenges superoxide anions (7). Reddy and Lokesh (8), using liver microsomes, reported that zingerone also inhibits lipid peroxidation, suggesting its antioxidant properties. Similar results were also reported by Jitoe et al. (9) using different ginger extracts. Oral consumption of dried, powdered ginger for 3 months–2.5 years by patients with rheumatoid arthritis, osteoarthritis, or muscular discomfort has been shown to result in relief of pain and swelling (10, 11). The investigators of these studies suggested that the mechanism of ameliorative effects of ginger could be related to the inhibition of PG2 and leukotriene biosynthesis (10, 11). In other studies, ginger oil, obtained by steam distillation of dried ginger, has been shown to be an inhibitor of both cyclooxygenase and lipoxigenase activities (12, 13). Recently, Sharma et al. (14) reported that ginger oil (33 mg/kg), given orally for 26 days to male Sprague-Dawley rats, caused a significant suppression of both paw and joint swelling. This finding suggested that ginger oil possesses antiinflammatory properties.

In view of the antiinflammatory and antioxidative activities of ginger extract, as well as its inhibitory potential against cyclooxygenase and lipoxigenase activities (6–13), we considered that ginger extract may possess significant anti-tumor-promoting potential. In this study, we assessed the anti-tumor-promoting effect of ginger extract on SENCAR mouse skin and delineated the mechanism of such an effect.

MATERIALS AND METHODS

Chemicals

TPA, mezerein, (–)-indolactam V, n-dodecane, anthralin, formamide, SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate (pH 7.0)), and D,L-ornithine were purchased from Sigma Chemical Co. (St. Louis, MO). DMBA and benzoyl peroxide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Arachidonic acid, 5-HETh, 8-HETh, 12-HETE, 15-HETh, PGE2, PGF20, and PGG2 were purchased from Biomol Research Laboratories, Inc. (Plymouth, PA). [1-14C]Arachidonic acid (52 mCi/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). D,L-[1-14C]Ornithine (58 Ci/mmol) was purchased from Amersham Searle (Chicago, IL). [α-32P]dCTP (6000 Ci/ mmol) and a GeneScreen nitrocellulose membrane were purchased from DuPont-New England Nuclear. cDNA of G3PDH was obtained from Stratagene (La Jolla, CA). Guanidine thiocyanate was a product of Fluka (Ronkonkoma, NY), and agarose was obtained from Fisher Scientific (Springfield, NJ). The chemicals used for RNA isolation and Northern blot analysis were of molecular biology grade and RNase free. The mouse ODC cDNA probe (15) was a generous gift from Ajit K. Verma (University of Wisconsin

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Comprehensive Cancer Center, Madison, WI). All other chemicals were obtained in the purest forms commercially available. Fresh ginger was purchased from a local vegetable market.

Animals

Six-to-seven-week-old female SENCAR mice were obtained from Harlan-Sprague-Dawley (Indianapolis, IN). These mice were housed four or five per cage and were acclimatized for 1 week before use, subjected to a 12-h light/12-h dark cycle, and housed at 24 ± 2°C and 50 ± 10% relative humidity. Animals were fed a Purina chow diet and water ad libitum.

Isolation of GE

Fresh ginger was cut into small pieces, crushed, macerated with ethanol, and filtered through muslin cloth. The filtrate thus obtained was centrifuged at 2500 rpm for 15 min at 4°C, and the supernatant was collected. This extraction procedure was repeated three times, and all the supernatants were pooled and evaporated to dryness in a rotary vacuum evaporator. A viscous, oily mass thus obtained was designated GE. This extract was dissolved in methanol:acetone (1:1, v/v) and used in all the experiments. The total extractable ethanol soluble fraction was 3.6% of fresh ginger (w/w). It is useful to mention here that other investigators (12, 13) used ginger oil that was extracted by steam distillation, and this oil was a mixture of camphene, limonen, linalool, citral, and bornel compounds.

Treatment of Animals for Short-Term, in Vivo Studies

The animals were shaved on the dorsal side of the skin, divided into three groups of eight each, and treated topically on the shaved area with either 0.2 ml vehicle (methanol:acetone, 1:1, v/v), TPA (5 μg/animal), or GE (2 or 4 mg/animal), followed 30 min later with TPA (5 μg/animal). All the test compounds were applied in 0.2 ml vehicle. Animals were sacrificed either 6 h after the TPA treatment (for ODC activity and ODC mRNA expression) or after 24 h (for cyclooxygenase and lipoxygenase activities), and the treated area of the skin was used for the determination of enzyme activities. The epidermis was separated from the whole skin and homogenized in 0.1 M Tris-HCl buffer (pH 7.2) using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) and 100,000 x g supernatant, and microsomal fractions were prepared as described earlier (16).

Assays of Enzyme Activities

ODC Activity. ODC activity was determined using 0.4 ml epidermal 100,000 x g supernatant fraction/assay tube by measuring the release of 14CO2 from the d,L-[14C]ornithine by the method of O'Brien et al. (17) as described by Verma et al. (18). The details of the assay procedure were described earlier (19). Enzyme activity is expressed as pmol CO2 released/h/mg protein. Protein concentration was determined by the method of Bradford (20).

Cyclooxygenase Activity. Epidermal microsomal cyclooxygenase activity was determined by the method described earlier (21). In brief, 150 μl reaction mixture contained 12 μM [14C]arachidonic acid (~400,000 dpm), 1 mM epinephrine, 1 mM glutathione and ~10 μg epidermal microsomal protein in 50 mM potassium phosphate buffer (pH 7.4). After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 μL 0.2 M HCl. The radioactive prostaglandin metabolites (PGF2α, PGF2α, and PGD2) of [14C]arachidonic acid were detected by TLC as described earlier (21). The radioactivity profile of each metabolite was determined by counting the samples in a Beckman LS-6K SC liquid scintillation counter (Beckman Instruments, Fullerton, CA) equipped with automatic external standardization.

Lipoxygenase Activity. Epidermal cytosolic lipoxygenase activity was determined by the method described earlier (21). In brief, 150 μl reaction mixture contained [14C]arachidonic acid (5 nmol, ~460,000 dpm), 2 mM calcium chloride, and ~400 μg epidermal 100,000 x g cytosolic protein in 100 mM Tris-HCl (pH 7.2). After incubation for 30 min at 37°C, the reaction was terminated by the addition of 10 μL 0.2 M HCl. The HETE metabolites of arachidonic acid were extracted as described elsewhere (21) and subjected to normal-phase, high-pressure liquid chromatography using a 3.9 x 300-mm μ-PORASIL column (Waters Associates, Milford, MA) at a flow rate of 0.7 ml/min. The column eluate was monitored at 236 nm using a Shimadzu (Columbia, MD) variable length detector; and 0.1-ml fractions were collected, radioactivity was determined in each fraction, and the radioactive profile was compared with the 236-nm UV absorption profile of authentic HETEs subjected to high-pressure liquid chromatography under the identical conditions.

Edema and Hyperplasia

To assess the inhibitory effect of preapplication of GE on TPA-induced edema, 1-cm-diameter punches of skin from vehicle-, TPA- or GE- and TPA-treated animals were removed, made free of fat pads, and weighed quickly. After drying for 24 h at 50°C, the skin punches were reweighed, and the loss of water content was determined. The difference in the amount of water gain between the control vehicle and TPA represented the extent of edema induced by TPA, whereas that between the control vehicle and GE plus TPA represented the inhibitory effect of GE. For the hyperplasia study, skin was removed, fixed in 10% formalin, and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, and stained with hematoxylin and eosin. For each section of the skin, the thickness of the epidermis from the basal layer to the stratum comeum was measured at five equidistant interfollicular sites using an Olympus (Palo Alto, CA) light microscope equipped with an ocular micrometer.

Isolation of Total RNA and Northern Blot Analysis

The treated area of the dorsal skin was removed. The epidermis was separated and homogenized using a tissue homogenizer (Tissuezurer; Tekmar, Cincinnati, OH) in 4 M guanidine thiocyanate lysis buffer, and total RNA was extracted by the method of Chomczynski and Sacchi (22) with some modification as described earlier (23). The concentration of total RNA was determined by measuring the absorbance at 260 nm in a Beckman DU 640 spectrophotometer. Total RNA (20 μg) was electrophoresed through a 1.2% agarose gel containing 6% formaldehyde and transferred onto a nitrocellulose membrane. The membrane was UV cross-linked (1200 millijoules/cm2), vacuum-dried at 80°C for 2 h, prehybridized at 42°C for 2–3 h, and hybridized to the 32P-labeled ODC cDNA probe pOD48 (specific activity, 1.5 x 106 cpm/ml) overnight at 42°C. The ODC cDNA, a fragment of about 2.1 kb, was labeled with [α-32P]dCTP using a random primer DNA-labeling kit (GIBCO-BRL, Gaithersburg, MD). After hybridization, the nitrocellulose membrane was washed twice for 15 min each in low-stringency buffer (2X SSC and 1.0% SDS) at room temperature and once in high-stringency buffer (1X SSC and 0.5% SDS) at 45–50°C. The membrane was autoradiographed using Kodar XAR-5 film with intensifying screens at ~70°C. After stripping, the same membrane was rehybridized to 32P-labeled G3PDH cDNA to verify equal loading of RNA onto the gel. The autoradiograms were scanned with a densitometer (Micromet Internation Inc., Taiwan, Republic of China), and the results were integrated and normalized to the value for G3PDH.

Skin Tumorigenesis

Female SENCAR mice were used in DMBA- and TPA-induced, two-stage skin tumorigenesis protocol (21). The dorsal side of the skin was shaved using electric clippers, and the mice with hair cycles in the resting phase were used for tumor studies. In each group, 20 animals were used. Tumorigenesis was initiated in the animals by a single topical application of 12.5 nmol DMBA in 0.2 ml vehicle on the dorsal shaved skin, and 1 week later, the tumor growth was promoted with twice-weekly applications of 4 nmol TPA in 0.2 ml vehicle. To assess the anti-skin tumor-promoting effect of GE, various doses of GE (1, 2, or 4 mg/animal), which produced significant inhibition against TPA-caused induction of ODC, cyclooxygenase, and lipoxygenase activities, were applied topically 30 min prior to each TPA application in different groups. Treatment with TPA alone or various doses of GE plus TPA was repeated twice weekly up to the termination of the experiments at 20 weeks. One group of animals was treated with 0.2 ml vehicle alone and served as a negative control to assess spontaneous tumor induction. To test whether GE itself possesses tumor-promoting effects, a group of animals was initiated with the 12.5-nmol dose of DMBA and 1 week later was treated with GE (4 mg/animal) twice weekly up to the end of the experiment. Animals in all the groups were watched for any apparent signs of toxicity, such as weight loss or mortality, during the entire period of study. Skin tumor formation was recorded weekly, and tumors larger than 1 mm in diameter were included in the cumulative number if they persisted for 2 weeks or more. The tumors were diagnosed histologically at the termination of the experiment.
INHIBITION OF SKIN TUMORIGENESIS BY GINGER

Statistical Analysis

Student's t test was used in enzymatic studies. In tumorigenesis experiments, the statistical significance of difference in terms of tumor incidence and multiplicity between the TPA and GE-plus-TPA groups was evaluated by the Wilcoxon rank sum and \( \chi^2 \) tests. An advantage of the Wilcoxon rank sum test is that its validity does not depend on any assumption about the shape of the distribution of tumor multiplicities, whereas the Cochran-Mantel-Haenzel statistical test was used to evaluate the statistical significance of the decreasing number of tumors with increasing doses of GE.

RESULTS

Inhibitory Effect of GE on TPA-caused Induction of Epidermal ODC Activity. To determine the optimal effective dose of GE against the TPA-caused induction of epidermal ODC activity in SENCAR mice, groups of animals were treated topically with varying doses of GE (1, 2, or 4 mg GE/animal) 30 min prior to topical application of TPA (2.5 \( \mu \)g/animal). As shown in Fig. 1A, pretreatment of animals with GE resulted in a dose-dependent inhibition of the TPA-caused induction of epidermal ODC activity. At the maximum dose of GE (4 mg) used in this study, 67% inhibition (\( P < 0.0005 \)) was observed (Fig. 1A). Similarly, at lower doses, GE also resulted in a significant inhibition (46–55%; \( P < 0.005 \)). Topical application of GE alone of up to 4 mg/animal was without any effect on basal enzyme activity and did not cause any induction of epidermal ODC activity.

The effect of preapplication of GE on TPA-caused induction of epidermal ODC activity was also studied as a function of time after the TPA application. Consistent with published studies (23–25), the maximum induction of epidermal ODC activity after the single topical application of TPA was observed at 6 h, which started declining after that period and reached an almost basal level by 16 h. When GE (4 mg/animal) was applied 30 min prior to each topical application of TPA, significant inhibition was observed at all the time points studied, with a similar pattern as observed with TPA alone (Fig. 1B).

Inhibition of TPA-caused Induction of Epidermal ODC mRNA Expression by GE. In the next series of experiments, we assessed the effect of skin application of GE on TPA-caused, enhanced expression of ODC mRNA in the epidermis. Northern blot analysis revealed that topical application of TPA (5 \( \mu \)g) resulted in a marked increase in the concentration of epidermal ODC mRNA. Skin application of GE prior to that of TPA resulted in a significant inhibition against TPA-caused induction of epidermal ODC mRNA expression in a dose-dependent manner (Fig. 2, top panel). Densitometric scanning of these blots indicated that, under the experimental conditions used, the inhibition varied from 40–70% in GE-pretreated animals (Fig. 2, bottom panel). Fig. 2, middle panel, shows the equal loading of RNA samples in each lane.

**Fig. 1.** A, inhibitory effect of GE on TPA-caused induction of epidermal ODC activity in SENCAR mice. Eight animals in each group were treated topically with either 0.2 ml vehicle alone or indicated doses of GE (1, 2, or 4 mg/animal) in 0.2 ml vehicle 30 min prior to that of 2.5 \( \mu \)g TPA in 0.2 ml vehicle. Animals were killed by cervical dislocation 6 h after the TPA treatment, and ODC activity was determined as described in "Materials and Methods." Data represent the mean ± SEM of four values; epidermis from two animals was pooled for each determination. P < 0.005–0.0005 in the case of TPA alone versus 1, 2, or 4 mg GE plus TPA. B, time-dependent inhibitory effect of GE on TPA-induced epidermal ODC activity in SENCAR mice. Eight animals in each group were treated topically with vehicle or 4 mg GE 30 min prior to that of 5.0 \( \mu \)g TPA, as described in Fig. 1A, and animals were killed 6 h after the TPA treatment. In each case, three epidermis samples were pooled, and the total RNA was isolated. Fractionation of RNA by agarose gel electrophoresis, Northern blotting, and hybridization to the \( ^{32}P \)-labeled ODC cDNA probe are described in "Materials and Methods."
Inhibitory Effects of GE on TPA-induced Epidermal Cyclooxygenase and Lipoxygenase Activities. The effect of preapplication of GE on TPA-caused induction of epidermal cyclooxygenase activity is shown in Table 1. As quantitated by the formation of PGE$_2$, PGF$_2x$, and PGD$_2$, the application of TPA to the SENCAR mouse skin resulted in a significant induction in epidermal cyclooxygenase activity. The application of GE prior to that of TPA resulted in a significant inhibition of TPA-induced epidermal cyclooxygenase activity, as evident by the quantitative analysis of PG metabolite formation. Compared with TPA application alone, preapplication of 2 mg GE to mouse skin resulted in 47, 42, and 51% inhibition (P < 0.0005) of PGE$_2$, PGF$_2x$, and PGD$_2$ formation, respectively. Preapplication of 4 mg GE to that of TPA was found to result in 80, 72, and 66% (P < 0.0005) inhibition of PGE$_2$, PGF$_2x$, and PGD$_2$ formation, respectively (Table 1).

The effect of preapplication of GE on TPA-caused induction of epidermal lipoxygenase activity is shown in Table 1. As quantitated by the formation of 8-HETh and 5-HETh, the application of TPA to the SENCAR mouse skin resulted in significant inductions in 8- and 5-lipoxygenase activity in the epidermis. The application of 2 or 4 mg GE prior to that of TPA was found to result in significant inhibition of TPA-induced epidermal lipoxygenase activity, as evident by the quantitative analysis of different HETE metabolite formations from arachidonic acid. Compared with TPA alone, prior application of 2 or 4 mg GE showed 38–72% (P < 0.0005) or 69–92% (P < 0.0005) inhibition in 8-HETE and 5-HETE formation, respectively, as shown in Table 1. The application of GE alone at the dose of 4 mg did not produce any change in epidermal cyclooxygenase and lipoxygenase activities when compared with only vehicle-treated control animals (Table 1).

Inhibitory Effects of GE on TPA-caused Epidermal Edema and Hyperplasia. Because the dose of 4 mg GE/animal applied topically afforded optimum and significant inhibition against TPA-caused induction of ODC, cyclooxygenase, and lipoxygenase activities, this dose of GE was selected to determine whether its application on the skin affords protection against TPA-caused edema and hyperplasia. As determined by the weight of the 1-cm-diameter punch of dorsal skin, the application of TPA to SENCAR mouse skin showed a significant edema (Table 2). The skin application of GE, 30 min prior to that of TPA, showed 56% inhibition (P < 0.0005) against TPA-caused edema.

The effect of preapplication of GE on TPA-caused induction of epidermal hyperplasia is shown in Fig. 3. To determine the induction in mean epidermal thickness by TPA and inhibition by GE, epidermal thickness was measured at five equidistant points between interfollicular spaces along the length of the epidermis from the dermoeipidermal junction to the top of the stratum corneum, and all five values were averaged and reported as the mean epidermal thickness in μm. Similarly, cell layers were also counted from the dermoeipidermal junction to the bottom of the stratum corneum to determine the mean vertical thickness in terms of cell layers in the epidermis. As shown in Fig. 3, application of TPA (5 μg), followed 24 h later by the killing of the animals (Fig. 3, middle panel), resulted in a significant increase in mean epidermal thickness (72.1 ± 5.1 μm) and mean vertical thickness in terms of epidermal cell layers (5.9 ± 0.2) when compared with the skin of only vehicle-treated animals (15.5 ± 0.8 and 1.8 ± 0.2 μm, respectively; Fig. 3, top panel). However, the preapplication of GE to that of TPA resulted in 41% (P < 0.005) inhibition in the induction of epidermal thickness (48.8 ± 3.2 μm) and 44% (P < 0.005) inhibition in terms of vertical cell layers (4.1 ± 0.2 μm), as shown in Fig. 3, bottom panel. The application of TPA also resulted in mixed cell infiltration in the dermis, which comprised mostly neutrophils with some mononuclear cells admixed (Fig. 3, middle panel); this effect of TPA in the dermis was also inhibited by the preapplication of GE (Fig. 3, bottom panel). The application of GE alone, however, did not induce epidermal edema or hyperplasia in these experiments, because the mean epidermal thickness (16 μm) and mean vertical thickness in terms of epidermal cell layers (2.1 μm) were comparable with those observed in vehicle-treated animals (data not shown).

Additionally, microscopic examination of the sections of skin biopsies also revealed that interstitial cell spaces were larger, and cells were spangiotic in nature in the TPA-treated group of animals compared with the vehicle- and GE-plus-TPA-treated groups of animals. This shows that preapplication of GE to that of TPA inhibited TPA-caused infusion of edema in animal skin. It is also noteworthy to mention that, in the microscopic observations of the skin sections, the number of cells in the mitotic phase was 10 when counted in each of three skin samples of 1-cm length, whereas under similar conditions, only 2 and 5 cells in this phase were observed in the vehicle- and GE-plus-TPA-treated groups, respectively. This observation demonstrates further that skin application of ginger 30 min prior to TPA...
application inhibits TPA-induced stimulation of cell proliferation through mitosis.

**Anti-Skin Tumor-promoting Effects of GE.** As shown by data in Fig. 4, topical application of GE prior to that of TPA in DMBA-initiated SENCAR mouse skin resulted in a dose-dependent inhibition of skin tumorigenesis. This inhibition was evident when tumor data were considered as the percentage of mice with tumors (Fig. 4, top panel) and the number of tumors per mouse (Fig. 4, bottom panel). At the termination of the experiment at 20 weeks, compared with 100% animals with skin tumors in non-GE-treated group, 90, 65, and 50% of the animals exhibited the appearance of skin tumors in the 1, 2, and 4 mg GE-treated groups of animals, respectively. The tumor incidence data at the dose of 1 mg GE were not significantly different from those of the non-GE-treated control group ($\chi^2$ test). However, at the 2- and 4-mg doses of GE individually, they were significantly different compared with those of the non-GE-treated group ($P < 0.05$ or 0.005; $\chi^2$ test). At the termination of the experiment, compared with a total of 330 tumors in the non-GE-treated group of animals, 195, 92, and 74 tumors per group in the 1, 2, and 4 mg GE-treated groups, respectively, were recorded. Compared with the non-GE-treated group, such decreases in the total numbers of tumors in the GE-treated groups corresponded to 40, 72, and 77% inhibition, respectively. In addition, when tumor multiplicity data were evaluated using Cochran-Mantel-Haenzel statistical test, a linear trend of decrease in tumor incidence was evident with increasing doses of GE ($P < 0.01$). When these tumor data were considered in terms of tumor volume per mouse, at the termination of the experiment at 20 weeks, preapplication of 1, 2, and 4 mg GE resulted in 54, 85, and 91% inhibition, respectively ($P < 0.03–0.0001$) in the sizes of developing papillomas (Table 3).

**DISCUSSION**

Cancer chemoprevention has become an important area of cancer research, which, in addition to providing a practical approach to identifying potentially useful inhibitors of cancer development, also affords opportunities to study the mechanisms of carcinogenesis (1–4). Limited preliminary studies (6–13) provide evidence that water or organic solvent extracts of ginger possess antioxidant and antiinflammatory properties. This study was designed to provide a substantial amount of mechanistic data to show the chemopreventive potential of GE by using carcinogenesis-associated biochemical end points in a mouse skin tumorigenesis model.

ODC is the rate-limiting enzyme in the biosynthesis of polyamines, which appears to be a prerequisite for cell proliferation, differentiation, and neoplastic transformation. The induction of ODC has been suggested to play a significant role in tumor promotion. Initial studies with the mouse skin model (25, 26) and subsequently with other organ systems (27, 28) showed an excellent correlation between the induction of ODC activity and the tumor-promoting ability of a variety of substances. Because tumor formation can be prevented by the agents that block induction of ODC or by inhibitors of arachidonic acid metabolism, including indomethacin (29, 18), ODC inhibition was shown to be a promising tool for screening inhibitors of tumorigenesis (29, 18). In the present study, topical application of GE prior to that of TPA resulted in a significant inhibition of TPA-induced epidermal ODC activity (Fig. 1). It is reasonable to believe that GE application inhibited the action of the tumor promoter and/or the enzymatic pathway(s) that regulates the ODC induction rather than interacting directly with the enzyme. In addition, our data obtained from Northern blot analysis demonstrate that prior application of GE to
Further research is needed to evaluate a possible effect of GE on the translation of ODC mRNA. Furthermore, the inhibition of TPA-induced epidermal ODC activity by GE at each time point studied suggests that GE does not delay the peak of TPA-induced ODC activity.

The topical application of the phorbol ester TPA to mouse skin or its treatment in certain epidermal cells is known to result in a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion (30–32). The induction of inflammation in skin mediated by TPA is believed to be governed by cyclooxygenase- and lipoxygenase-catalyzed metabolites of arachidonic acid, specifically, PGs and HETEs, respectively (30–32). Such an assumption is supported strongly by the studies showing that inhibitors of various parts of these metabolic pathways inhibit skin tumor promotion (32–35). These inhibitory studies support the involvement of arachidonic acid metabolism pathways in skin tumor promotion (32–35).

Cyclooxygenases, the microsomal enzymes, play an important role in cutaneous inflammation, cell proliferation, and skin tumor promotion (32, 35, 36, and references therein). Arachidonic acid, released predominantly from skin phosphatidylcholine by the activation of skin phospholipase A₂ due to a variety of mechanical, chemical, or hormonal stimuli, undergoes oxidative metabolism via the cyclooxygenase pathway, resulting in the formation of PGs such as PGE₂, PGF₂α, and PGD₂ (37 and references therein). In addition to dominant inflammatory effects, TPA application also produces epidermal hyperproliferation, including ODC induction (25), associated with epidermal hyperplasia. Although the results of the present study show the inhibitory effects of GE against TPA-caused induction of epidermal cyclooxygenase activity in the SENCAR mouse (Table 1), it also correlates with the inhibitory effect of GE against TPA-caused induction of skin edema (Table 2) and hyperplasia (Fig. 3).

Arachidonic acid is released from membrane phospholipids by phospholipase activity in the skin by a number of stimuli, and phorbol ester tumor promoters are some of them (38). Depending on their nature, different lipoxygenases catalyze the metabolism of arachidonic acid, resulting in the formation of HETEs (32, 36, 39, and references therein). Lipoxygenase-catalyzed metabolites of arachidonic acid have been considered to play an important role in many physiological and pathophysiological events, including inflammation and growth regulation; the presence of these metabolites in the mammalian epidermis has been reported by several investigators (35, 36, 38, 40, 41). Although 12-lipoxygenase is characterized predominantly in the normal murine epidermis (42, 43) along with small amounts of 5-, 8-, 12-, and 15-lipoxygenases (21, 44), treatment of a mammalian skin or cell-free extract with TPA results in the induction of 8-lipoxygenase, identified by the generation of 8-HETE (21, 45).

### Table 3

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total tumor volume/group (mm³)</th>
<th>Tumor volume/mouse (mm³)</th>
<th>Average tumor volume/mouse (mm³)</th>
<th>% inhibition (volume/mouse)</th>
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<tr>
<td>DMBA + TPA</td>
<td>11,506</td>
<td>575 ± 42</td>
<td>35 ± 11</td>
<td>102</td>
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<tr>
<td>DMBA + 1 mg GE + TPA</td>
<td>5,250</td>
<td>263 ± 36</td>
<td>17 ± 4</td>
<td>83</td>
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<tr>
<td>DMBA + 2 mg GE + TPA</td>
<td>1,670</td>
<td>84 ± 19*</td>
<td>18 ± 2</td>
<td>85</td>
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<tr>
<td>DMBA + 4 mg GE + TPA</td>
<td>983</td>
<td>49 ± 9#</td>
<td>13 ± 3</td>
<td>91</td>
</tr>
</tbody>
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*Significant versus TPA alone; *P < 0.03.

**Highly significant versus TPA alone; *P < 0.001.

**Highly significant versus TPA alone; *P < 0.0001.
As observed in the present study, applications of TPA on SENCAR mouse skin resulted in the highly significant induction of 8-lipoxygenase activity (Table 1). The results of several studies (45 and references therein) suggest that 8-lipoxygenase-catalyzed arachidonic acid metabolites, specifically 8-HETE, play an important role in skin tumor promotion, at least in cases of phorbol ester-type skin tumor promoters (45). In the present study, skin application of GE also showed significant inhibition against TPA-caused induction of epidermal lipoxygenase activity. Such an inhibitory effect of GE may be responsible for the TPA-caused induction of epidermal ODC and epidermal edema and hyperplasia, as observed in the present study. Moreover, the application of GE to mouse skin results in the rapid accumulation of inflammation, as observed in the present study. The mechanisms of GE action indicate that GE is a potent inhibitor of arachidonic acid metabolism via the lipoxygenase and cyclooxygenase pathway and that GE is an antioxidant and scavenger of free radicals.

The results in Fig. 4 and Table 3 show the protective effects of skin application of GE on TPA-caused tumor promotion in DMBA-initiated SENCAR mouse skin. The preapplication of GE to that of TPA showed protective effects when the tumor data were considered as the total number of tumors or tumors per mouse (Fig. 4) and the tumor volume per mouse or average volume per tumor (Table 3). These chemopreventive and anti-tumor promotion observations in murine skin by GE can be explained by the biochemical mechanisms studied in the present study. Additionally, the present study showed that the inhibitory effects of GE on TPA-induced increases in the levels of ODC mRNA in the mouse epidermis provide an explanation for the inhibitory effect of GE on TPA-induced ODC enzyme activity and suggest additional evidence that GE inhibits the expression of genes that play a role in tumor promotion. These data provide highly useful information for further study to elucidate the exact nature of the compounds present in ginger responsible for chemopreventive effects.

REFERENCES


Inhibition of Tumor Promotion in SENCAR Mouse Skin by Ethanol Extract of *Zingiber officinale* Rhizome

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