Inhibition of Skin Tumor Promoter-caused Induction of Epidermal Ornithine Decarboxylase in SENCAR Mice by Polyphenolic Fraction Isolated from Green Tea and Its Individual Epicatechin Derivatives

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ABSTRACT

Green tea, next to water, is the most popular and commonly consumed beverage in the world, especially in eastern countries. In prior studies we have shown that the polyphenolic fraction isolated from green tea (GTP) exerts antigenotoxic effects in various mutagenicity test systems (Mutat. Res., 223: 273-285, 1989) and that its topical application or oral feeding in drinking water protects against polycyclic aromatic hydrocarbon-induced skin tumor initiation and complete carcinogenesis in SENCAR and BALB/c mice [Cancer Lett., 42: 7-12, 1988; Carcinogenesis (Lond.), 10: 411-415, 1989] and UV B radiation-induced photocarcinogenesis in SKH-1 hairless mice [Carcinogenesis (Lond.), 12: 1527-1530, 1991]. In the present study we assessed the effect of skin application of GTP to SENCAR mice on 12-0-tetradecanoylphorbol-13-acetate (TPA) and other skin tumor promoter-caused induction of epidermal ornithine decarboxylase (ODC) activity. Topical application of GTP to mouse skin inhibited TPA-induced epidermal ODC activity in a dose-dependent manner. The inhibitory effect of GTP was also dependent on the time of its application relative to TPA treatment. Maximum inhibitory effect was observed when GTP was applied 30 min prior to topical application of TPA. GTP application to animals also inhibited the induction of epidermal ODC activity caused by several structurally different mouse skin tumor promoters. In order to identify which of the specific epicatechin derivatives present in GTP is responsible for these inhibitory effects, they were isolated from GTP and evaluated for their inhibitory effects against TPA-caused induction of epidermal ODC activity. Among these, (-)epigallocatechin-3-gallate (EGCG), which was the major constituent present in GTP by weight, exerted the maximum inhibition. EGCG also showed greater inhibitory effects against TPA-caused induction of epidermal ODC activity when compared with several other naturally occurring polyphenols. The results of this study suggest that GTP, specifically its epicatechin derivative EGCG, could provide anti-tumor-promoting effects against a wide spectrum of skin tumor promoters.

INTRODUCTION

Cancer chemoprevention is an important emerging area of research which in addition to providing a practical approach to identifying potentially useful inhibitors of cancer development, also affords opportunities for studying the mechanisms of carcinogenesis (Refs. 1-3 and references therein). For example, the involvement of cytochrome P-450 in the metabolic activation of polycyclic aromatic hydrocarbons leading to tumor initiation was made apparent by studies showing that inhibitors of aryl hydrocarbon hydroxylase, such as synthetic flavone, can diminish 7,12-dimethylbenz[a]anthracene tumorigenesis in mouse skin (4). Inhibition of mouse skin tumor promotion by antiblistering agents, steroids, retinoids, protease inhibitors, antioxidants, inhibitors of arachidonic acid metabolism, polyamine synthesis inhibitors, and protein kinase C inhibitors has clarified and/or established the cascade of events occurring during the promotion stage of multistage carcinogenesis process (5).

It is known that chemical- and UV B radiation-induced carcinogenesis in murine skin and possibly in human skin is a stepwise process of at least three distinct stages: initiation, promotion, and progression (6). Chemoprevention of skin cancer, therefore, refers to the administration of chemical agents to prevent the initiation, and/or promotion, and/or progression events that occur during the multistage process of neoplastic development. Our laboratory has been studying the usefulness and mechanism of action of polyphenols present in fruits, vegetables, and plants as cancer chemopreventive agents (7-13) in the hope of defining an ideal antitumor agent which could be useful in humans.

Tea from the Camellia sinensis species of the Theaceae family is one of the most ancient beverages and, next to water, the most widely consumed beverage in the world. In the mid-1980s, it was shown that the water extract of green tea exhibits antigenotoxic effects in mutagenicity test systems (14, 15). Studies from our laboratory have shown that (a) when added in vitro, GTP* and its constituent epicatechin derivatives interact with hepatic cytochrome P-450 and inhibit the cytochrome P-450-dependent mixed function oxidase enzymes in skin and liver (16); and that (b) GTP and its epicatechin derivatives possess antimutagenic effects in several test systems (17). In addition, we showed that topical application of GTP or its administration in drinking water affords substantial protection against polycyclic aromatic hydrocarbon-induced tumor-initiating activity in murine skin tumorigenesis bioassay systems (18, 19). Similarly, GTP in drinking water was found to afford significant protection against UV B radiation-induced photocarcinogenesis in SKH-1 hairless mice (20). Furthermore, EGCG, the major polyphenolic constituent in GTP, has been shown to protect against teleocin-1-caused tumor promotion in 7,12-dimethylbenz[a]anthracene-initiated female CD-1 mouse skin (21). In the present study we assessed the effect of the preapplication of GTP on TPA-induced epidermal ODC activity in SENCAR mice and identified the specific epicatechin derivative responsible for such effects associated with GTP.

MATERIALS AND METHODS

Chemicals. TPA, mezerein, (-)-indolactam V, H2O2, anthralin, n-dodecanes, 1,2-naphthylamine, phenylurethane, quercetin, a-GA, b-GA, and EC were purchased from Sigma Chemical Co. (St. Louis, MO). Benzoyl peroxide, tert-butylperoxide, tannic acid, and ellagic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,2-Dimethylhydrazine dihydrochloride was purchased from research chemicals, Inc. (Berkeley, CA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 1/9/92; accepted 4/24/92.

The abbreviations used are: GTP, polyphenolic fraction isolated from green tea; TPA, 12-O-tetradecanoylphorbol-13-acetate; HPLC, high-pressure liquid chromatography; EGCG, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; ODC, ornithine decarboxylase; a-GA and b-GA, 18α- and 18β-glycyrrhetinic acid.

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were purchased from Aldrich Chemical Co. (Milwaukee, WI). d,L-[14C]Ornithine (58 Ci/mmol) was from Amersham Searle (Chicago, IL). All other chemicals were obtained in the purest form commercially available.

Preparation of GTP. GTP from green tea leaves (a product of Korea; distributed by Hanni, Inc., Los Angeles, CA) was prepared by the method described earlier (19, 20) with some modifications. In brief, dried green tea leaves (100 g) were suspended and extracted twice with hot water (80°C) and thrice with 80% ethanol (700 ml each time) under nitrogen. The total combined extract (3.5 liters) was concentrated under vacuum to 1 liter and extracted with an equal volume of chloroform to remove most of the caffeine and pigments. The aqueous layer, thus obtained, was extracted thrice with ethyl acetate (800 ml each time) under nitrogen, and the total organic soluble fraction (2.4 liters) was concentrated under vacuum. The residue obtained was dissolved in water (50 ml) and freeze-dried. The light-brown solid matter obtained was called GTP.

HPLC Analysis of GTP. The HPLC system comprised two Waters model 510 pumps and a model 680 automatic gradient controller. A Waters 4-μm Nova Pak C-18 column (3.9 mm x 15 cm) was used along with a sample loop of 2 ml capacity. The absorbance of the eluate was monitored at 280 nm using a Shimadzu SPD-6A UV-visible spectrophotometer detector and recorded on a Shimadzu C-R3A Chromatopak integrating recorder. The mobile phase was a mixture of water (solvent A) and methanol (solvent B). For HPLC analysis, GTP was dissolved in 10% aqueous methanol (2 mg/ml, w/v) and small aliquots (50–100 μl) were subjected to reverse-phase HPLC using a Waters model WISP 710B autoinjector. The gradient system used in terms of percentage of solvent B was: 0 min, 10%; 0–35 min, a linear increase to 30%; 35–45 min, 30% isocratic; and beyond 45 min, 100%. The individual polyphenolic constituents were eluted under these conditions at a uniform flow rate of 1 ml/min throughout the HPLC run. Identification of the individual epicatechin derivatives was based on the comparison of the retention times of unknown peaks to those of reference standards either obtained commercially (EC) or provided by Dr. R. Saljö (Vegetable and Ornamental Corp., Research Station, Japan) and Dr. Matsuizaki (Food Research Laboratories, Mitsui Nurin Co., Japan) (EGC, EGCG, ECG).

Animals and Treatment. Six-week-old female SENCAR mice were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). SENCAR mice were used in the present study because they are the most studied mouse strain for the causation, mechanism, and chemoprevention of cutaneous carcinogenesis (Refs. 6 and 22 and references therein). Additionally, this mouse strain respond well to TPA and other structurally different skin tumor promoter-caused induction of epidermal ODC (Ref. 22 and references therein). The animals were shaved with electric clippers, and Nair depilatory was applied 1 day prior to the experiments. Animals were divided into several groups of eight animals each. All treatments were done topically on the shaved areas, which later were used for cytosol preparation and ODC activity determination. In the control group, the animals received a single topical application of 0.2 ml acetone only. In experimental groups, the animals were treated either with a single topical application of known skin tumor promoters in 0.2 ml acetone only or with GTP or individual epicatechin derivatives isolated from GTP in 0.2 ml acetone 30 min prior to the skin application of tumor promoters in 0.2 ml acetone. The details of treatment protocols of various skin tumor promoters, GTP, and/or individual epicatechin derivatives in relation to their doses, time of application, and killing of animals after treatments are provided at appropriate places in "Results." The animals were killed by cervical dislocation, and the dorsal treated skin was removed and made free of connective tissue and fat. The epidermis was separated from the whole skin by brief heat treatment at 52°C for 30 s, and the epidermal 100,000 g supernatant fraction was prepared as described earlier (23).

Assay of ODC Activity. ODC activity was determined utilizing 0.4 ml of epidermal 100,000 g supernatant fraction per assay tube by measuring the release of [14C]CO2 from the d,L-[14C]Ornithine by the method of O'Brien et al. (24) as described by Verma et al. (25). The details of the assay procedure are described earlier (26). Enzyme activity is expressed as pmol CO2 released/h/mg protein. Protein concentration was determined by the method of Bradford (27).

Inhibitory Effect of GTP on TPA-induced Epidermal ODC Activity. In order to determine the optimal effective dose and the 50% inhibitory dose of GTP against TPA-induced epidermal ODC activity in SENCAR mice, various groups of animals were treated topically with varying doses of GTP (0.5–24 mg GTP in 0.2 ml acetone/animal) 30 min prior to the topical application of TPA (5 μg in 0.2 ml acetone/animal). As shown in Fig. 2, pretreatment of animals with GTP resulted in a dose-dependent inhibition of TPA-induced epidermal ODC activity. At the highest dose of GTP (24 mg), as much as 90% inhibition was observed (Fig. 2). Similarly, at small doses GTP resulted in significant inhibition. The effective dose of GTP resulting in 50% inhibition of the levels of epidermal ODC activity induced by the topical application of 5 μg TPA was found to be 1.9 mg/animal (Fig. 2). GTP alone up to 24 mg in 0.2 ml acetone does not cause any induction of epidermal ODC activity (data not shown).

Based on the data shown in Fig. 2, a dose of 2 mg GTP, which produced a 55–60% inhibition of TPA-induced epidermal ODC activity, was selected for further experiments.
The effect of the preapplication of GTP on TPA-induced epidermal ODC activity was also studied as a function of time after the TPA application. As shown in Fig. 4, consistent with published studies (28, 29), 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 was almost at the basal level by 14 h. When GTP (2 mg in 0.2 ml acetone) was applied 30 min prior to topical application of TPA, significant inhibition was observed at all the time points studied, in a pattern similar to that observed with TPA alone (Fig. 4).

Table 1. Qualitative and quantitative analysis of GTP in relation of epicatechin derivatives by reverse-phase HPLC

<table>
<thead>
<tr>
<th>Percentage of total GTP (w/w)</th>
<th>Percentage of dry green tea leaves* (w/w)</th>
<th>Percentage of total epicatechin derivatives* (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total epicatechin derivatives</td>
<td>68.5</td>
<td>5.14</td>
</tr>
<tr>
<td>EGc</td>
<td>3.7</td>
<td>0.28</td>
</tr>
<tr>
<td>EGCG</td>
<td>42.0</td>
<td>3.15</td>
</tr>
<tr>
<td>EC</td>
<td>4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>ECG</td>
<td>18.8</td>
<td>1.41</td>
</tr>
</tbody>
</table>

*GTP (100–200 μg) was subjected to reverse-phase HPLC on a Waters 4-μm Nova Pak C-18 column under the conditions detailed in “Materials and Methods.” The quantitative data shown here in this table in relation to total epicatechin derivatives as well as individual epicatechin derivatives are based on the printout obtained by using a Shimadzu C-R3A chromatopak integrating recorder programmed for the area normalization method.

Calculations are based on considering the total epicatechin derivatives as 100% GTP.

Fig. 2. Dose-dependent inhibition of TPA-induced epidermal ODC activity in SENCAR mice by GTP. Eight animals in each group were treated topically either with 0.2 ml acetone or with the indicated doses of GTP in 0.2 ml acetone at various time intervals before or after the skin application of 5 μg TPA in 0.2 ml acetone. As shown in Fig. 3, the inhibitory effect of GTP was dependent on the time of its application in relation to TPA application. The maximum inhibition was observed when GTP was applied 30 min prior to TPA application. Comparable results were obtained when GTP was applied 15–25 min prior to TPA application (data not shown). Although not that profound, the topical application of GTP between 3 h before and 2 h after TPA application was also significantly effective in inhibiting the TPA-caused induction of epidermal ODC activity (Fig. 3).

Fig. 3. Effect of time of single GTP application before or after TPA application on the inhibition of TPA-induced epidermal ODC activity in SENCAR mice. Eight animals in each group were treated topically either with 0.2 ml acetone or with 2.0 mg GTP in 0.2 ml acetone before or after the indicated time of TPA application (5 μg in 0.2 ml acetone). Animals were killed 6 h after the TPA treatment, and ODC activity was determined as described in “Materials and Methods.” Each data point represents mean ± SE of four individual values; the epidermis from two animals were pooled for each determination. The basal ODC activity in control animals receiving acetone alone was 69 ± 7 pmol CO₂ released/h/mg protein. The application of 5 μg TPA alone caused the induction of epidermal ODC activity to the level of 2046 ± 142 pmol CO₂ released/h/mg protein. For statistical analysis of the data, Student’s t test was applied between TPA alone and GTP plus TPA groups. *P < 0.005 in case of TPA alone versus 1 to 24 mg GTP dosage plus TPA.
Inhibitory Effect of GTP on Epidermal ODC Induction Caused by Structurally Different Mouse Skin Tumor Promoters. In addition to its inhibitory effect against TPA-induced epidermal ODC, GTP was also evaluated for its inhibitory effects against epidermal ODC induced by several structurally different mouse skin tumor promoters. The doses of various tumor promoters used in the present study were based on the published studies by us as well as by others (Refs. 26, 29, and 30 and references therein). As shown by the data in Table 2, the topical application of GTP (2 mg in 0.2 ml acetone/animal) 30 min prior to the topical application of these tumor-promoting agents resulted in 45 to 61% inhibition of epidermal ODC induction caused by these agents; however, a maximal inhibitory effect was observed in the case of TPA, although it was comparable to that with \( \text{H}_2\text{O}_2 \) and benzoyl peroxide (Table 2).

Inhibitory Effects of Individual Epicatechin Derivatives Isolated from GTP on TPA-Induced Epidermal ODC. To identify which of the specific epicatechin derivatives present in GTP is involved in exerting the inhibitory effect against TPA-induced epidermal ODC activity, we evaluated the inhibitory effects of individual epicatechin derivatives isolated from GTP. For the isolation of individual epicatechin derivatives, GTP was subjected to HPLC under the conditions described in “Materials and Methods.” Each peak for EGC, EGCG, EC, and ECG was collected manually, lyophilized, and dissolved in acetone.

In the experiments utilizing GTP, the quantity of each of the epicatechin derivatives present in 2 mg GTP was applied topically 30 min prior to the application of TPA, and ODC activity was determined under similar experimental conditions. As shown by the data in Table 3, maximum inhibition (63%) was found to be associated with EGCG, the major constituent present in GTP (Table 1), followed by EGC (43%), ECG (38%), and EC (17%), as compared to 49% inhibition observed with GTP. Furthermore, in order to determine (a) if there is any loss of inhibitory effect of GTP during its fractionation on HPLC, and (b) that the inhibitory effect of GTP is only due to the epicatechin derivatives which constitute 68.5% of total GTP, all four epicatechin derivative fractions isolated from GTP (EGC, EGCG, EC, and ECG) were combined together in the same ratios in which they occur in unfractionated GTP, and this reconstituted GTP preparation was evaluated for its inhibitory effect against TPA-induced epidermal ODC activity. As shown by the data in Table 3, the reconstituted GTP preparation showed inhibition almost comparable to that observed with the unfractionated GTP preparation (51 and 49%, respectively).

One of the aims of the present study was to identify which of the epicatechin derivatives present in GTP is the most effective inhibitor of TPA-induced epidermal ODC activity, and since the data summarized in Table 3 are based on the quantity of each epicatechin derivative present in 2 mg GTP, we also evaluated the inhibitory effects of the topical application of equal quantities of individual epicatechin derivatives, on a molar basis, on TPA-induced epidermal ODC activity. As shown by the data in Table 4, the topical application of 4 \( \mu \)mol of EGC, EGCG, EC, or ECG in 0.2 ml acetone/animal 30 min prior to the topical application of 5 \( \mu \)g TPA in 0.2 ml acetone resulted in the significant inhibition of TPA-induced epidermal

Table 2 Inhibitory effect of GTP on epidermal ODC induction caused by structurally different skin tumor promoters in SENCAR mice*  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose of tumor promoter (µg)</th>
<th>ODC activity (pmol CO(_2) released/h/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>77±9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>5 µg</td>
<td>2059±160</td>
<td>61</td>
</tr>
<tr>
<td>GTP + TPA</td>
<td>5 µg</td>
<td>795±53</td>
<td></td>
</tr>
<tr>
<td>Mezerein</td>
<td>5 µg</td>
<td>2872±255</td>
<td>52</td>
</tr>
<tr>
<td>GTP + Mezerein</td>
<td>1378±101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)Indolactam V</td>
<td>25 µg</td>
<td>1140±107</td>
<td>47</td>
</tr>
<tr>
<td>GTP + (−)Indolactam V</td>
<td>604±38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthralin</td>
<td>50 µg</td>
<td>498±45</td>
<td>40</td>
</tr>
<tr>
<td>GTP + Anthralin</td>
<td>299±25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>5 µg</td>
<td>387±31</td>
<td>58</td>
</tr>
<tr>
<td>GTP + ( \text{H}_2\text{O}_2 )</td>
<td>163±21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPO/</td>
<td>20 mg</td>
<td>776±49</td>
<td>59</td>
</tr>
<tr>
<td>BPO + GTP</td>
<td>318±29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Dodecane</td>
<td>25 mg</td>
<td>118±12</td>
<td>52</td>
</tr>
<tr>
<td>GTP + n-Dodecane</td>
<td>57±10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Eight animals in each group were treated topically either with 0.2 ml acetone or with 2 mg GTP in 0.2 ml acetone/animal 30 min prior to the skin application of indicated doses of tumor promoters in 0.2 ml acetone. In case of benzoyl peroxide and tert-butyleroxide, animals were killed 24 h after the treatment, whereas in rest of the cases animals were killed 6 h after the treatment by cervical dislocation. The ODC activity was determined in epidermal 100,000 × g supernatant fraction.

Each value represents the mean ± SE of four individual values; the epidermis from two animals were pooled for each determination. Student's \( t \) test was applied between tumor promoter and GTP plus tumor promoter groups.

Highly significant versus TPA promoter alone, \( P < 0.0005 \).
Significant versus TPA promoter alone, \( P < 0.005 \).
Significant versus tumor promoter alone, \( P < 0.01 \).
BPO, benzoyl peroxide; t-BuPO, tert-butyleroxide.
ODC activity. However, even on a molar basis, the maximum inhibitory effect was associated with EGCG (Table 4).

Since in a number of studies from this laboratory (13, 26, 31) as well as by other investigators (Refs. 5, 29, and 32 and references therein), various naturally occurring polyphenols have been shown to be inhibitors of TPA-induced epidermal ODC activity and mouse skin tumor promotion, and since in the present study we found EGCG to be a potent inhibitor of TPA-induced epidermal ODC activity, we also evaluated the comparative inhibitory effects of various known naturally occurring polyphenols with EGCG. As shown by the data in Table 4, of all the polyphenols evaluated, EGCG showed the maximum inhibitory effect against TPA-induced epidermal ODC activity. At a dose of 4 μmol EGCG, an inhibition as high as 90% was observed, whereas EGC, EC, ECG, nordihydroguaiaretic acid, quercetin, tannic acid, ellagic acid, α-GA, and β-GA produced only 55–70% inhibition at the same molar dose (Table 4). However, application of any of the naturally occurring polyphenols alone does not induce epidermal ODC activity (data not shown).

DISCUSSION

Chemical carcinogenesis in murine skin is a stepwise process of at least three distinct stages: initiation; promotion; and progression (6, 22). Studies in murine skin have shown that even a lapse of up to 1 year between the application of initiator and the beginning of promoter treatment produces a tumor response similar to that observed when promotion is begun only 1 week after initiation, with the carcinogen administered as in a classical two-stage skin carcinogenesis protocol (6, 22). In addition, initiated cell populations in animals may persist throughout their lifetime but may not result in visible tumors in the absence of further treatment, indicating that initiation alone is not sufficient to elicit a tumor response. These studies have aroused an interest in defining the mechanism of tumor promotion and in finding new agents capable of affording protection against tumor promotion.

The morphological, biochemical, and physiological changes occurring in mouse skin as a result of the topical application of phorbol esters like TPA are reviewed extensively (Ref. 22 and references therein). The induction of ODC activity followed by an increase in the levels of polyamines, the induction of dark basal keratinocytes, the induction of sustained epidermal hyperplasia following multiple application, the ability to induce reactive oxygen species formation, and the interaction with and activation of specific receptor protein kinase C are the effects best studied in either mouse skin or keratinocytes following TPA treatment (Ref. 22 and references therein). Of all these effects, the induction of ODC activity is considered to be closely associated with, although not sufficient to produce the tumor-promoting activity of a variety of tumor promoters (6, 22). ODC is involved in polyamine biosynthesis, which plays an essential role in cell proliferation and differentiation (33). The importance of the induction of epidermal ODC in skin tumor promotion is evident from the fact that several inhibitors of ODC are capable of inhibiting tumor promotion in murine skin (5, 29, 22). For example, a variety of compounds such as n-propyl gallate, flavonoids, retinoids, and antioxidants which inhibit ODC induction have also been found to be effective inhibitors of TPA-mediated tumor promotion (5, 29, 32). As in to these reports, in the present study topical application of GTP prior to that of TPA resulted in the significant dose-

### Table 4 Inhibition of TPA-caused induction of epidermal ODC in SENCAR mice by various naturally occurring polyphenols

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC activity (pmol CO₂ released/h/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>20.5 ± 5</td>
<td></td>
</tr>
<tr>
<td>Methanol + acetone</td>
<td>188 ± 17</td>
<td>90</td>
</tr>
<tr>
<td>TPA</td>
<td>192 ± 12</td>
<td></td>
</tr>
<tr>
<td>GTP + TPA</td>
<td>110 ± 10</td>
<td></td>
</tr>
<tr>
<td>EGC + TPA</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
<tr>
<td>ECGG + TPA</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
<tr>
<td>EC + TPA</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
<tr>
<td>EC + TPA</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
<tr>
<td>GTP + TPA + GTP (R)</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
<tr>
<td>GTP (R) + TPA</td>
<td>100 ± 10</td>
<td>65</td>
</tr>
</tbody>
</table>

* Eight animals in each group were treated topically either with 0.2 ml acetone or methanol + acetone (50:50, v/v), or with 4 μmol naturally occurring polyphenol in 0.2 ml acetone [in case of EA in 0.2 ml methanol + acetone (50:50 v/v)] 30 min prior to the skin application of 5 μg TPA in 0.2 ml acetone. Animals were killed 6 h after the TPA application by cervical dislocation, and the ODC activity was determined in the epidermal 100,000 x g supernatant fraction.

### Table 3 Inhibition of TPA-caused induction of epidermal ODC activity in SENCAR mice by GTP and its epicatechin derivatives

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Quantity of individual epicatechin derivative in 2 mg GTP (μg)</th>
<th>μmol of individual epicatechin derivative applied topically (molecular weight)</th>
<th>ODC activity (pmol CO₂ released/h/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>20.5 ± 5</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>192 ± 12</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP + TPA</td>
<td>110 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGC + TPA</td>
<td>100 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECGG + TPA</td>
<td>100 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC + TPA</td>
<td>100 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP + TPA + GTP (R)</td>
<td>100 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP (R) + TPA</td>
<td>100 ± 10</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Eight animals in each group were treated topically either with 0.2 ml acetone, or with 2 mg GTP, or the quantity of individual epicatechin derivative shown in the table, or with reconstituted GTP (1370 μg epicatechin derivatives) in 0.2 ml acetone 30 min prior to the skin application of 5 μg TPA in 0.2 ml acetone. Animals were killed 6 h after the TPA application by cervical dislocation, and the ODC activity was determined in the epidermal 100,000 x g supernatant fraction.

* GTP (R), reconstituted GTP.

* Significant versus TPA alone, P < 0.005.

* Highly significant versus TPA alone, P < 0.0005.

* Not significant versus TPA alone, P < 0.1.
INHIBITION OF ODC BY EPICATECHINS IN GREEN TEA

dependent inhibition of TPA-induced epidermal ODC (Fig. 2). Since the effect of GTP application was more profound when it was applied prior to TPA as compared to the effect observed after TPA application (Fig. 3), it is reasonable to assume that GTP application inhibited the action of tumor promoters and/or the enzymatic pathway(s) which regulates the ODC induction rather than interacting directly with the enzyme. Furthermore, the inhibition of TPA-induced epidermal ODC by GTP at each time point studied (Fig. 4) suggests that GTP does not delay the peak of TPA-induced ODC.

The results of the present study showing the inhibitory effect of GTP on epidermal ODC induction (Table 2) suggest that GTP may be of significant use in protecting against several tumor promoters with diversified mechanisms of action (Refs. 5, 6, 22, 29, 30, 35–42 and references therein). In fact, in our ongoing experiments, when assessed in an anti-tumor-promotion protocol in 7,12-dimethylbenz[a]anthracene-initiated SENCAR mouse skin, the pretreatment of animals topically with GTP before each skin application of TPA resulted in significant protection in terms of total number of tumors per mouse as well as percentage of mice with tumors when compared to non-GTP-pretreated animals.3 A preliminary report by Huang et al. (43), however, has also shown the inhibitory effects of GTP against TPA-caused epidermal ODC induction and skin tumor promotion in CD-1 mice.

In recent years, we and others have shown that green tea leaves are rich in epicatechin derivatives (17, 18), specifically EGCG (21, 44), and suggested that the anti-mutagenic (15, 16, 18, 45–47), hypolipaemic (48), anticarcinogenic (19, 20, 44), antioxidant (49–51), and antibacterial (52, 53) activities of water extracts of green tea and/or GTP may primarily be due to these epicatechin derivatives. In the present study when analyzed on HPLC, GTP separated into six sharp peaks, of which four were identified as EGC, EGCG, EC, and ECG, the epicatechin derivatives (Fig. 1), accounting for 68.5% of total GTP (Table 1). When these epicatechin derivatives were assessed individually against the TPA-caused induction of epidermal ODC activity, both on the basis of the quantity of each epicatechin derivative present in GTP (Table 3) and on a molar basis (Table 4), EGCG was found to be the most effective inhibitor and constitutes the major part of the GTP (42%, w/w; Table 1). Similarly, when compared with other naturally occurring polyphenols with known inhibitory effects against TPA-induced epidermal ODC and skin tumor promotion (Refs. 5, 13, 26, 29, 31, 32 and references therein), EGCG was most effective when tested at an equimolar dosage (Table 4). Based on the chemical structure of EGCG, it seems reasonable that the gallate and galloyl moieties at positions 2 and 3, respectively, in EGCG make it highly active against various inhibitory effects. Finally, since the inhibitory effect observed with reconstituted GTP (Table 3), obtained by mixing only the four epicatechin derivative fractions and not the unidentified peak and the caffeine fractions (Fig. 1), were comparable with that of unfraccionated GTP, it seems reasonable to conclude that the inhibitory effects of GTP observed in the present study may be due to the epicatechin derivatives present therein.

Chemoprevention is a means of cancer control in which disease occurrence is prevented by the administration of one of a combination of several chemical agents (2, 3, 54–58). One of the exciting findings in this field is the presence of these compounds in the human diet, which makes it possible that simple changes in dietary habits could significantly lower cancer risk (2, 54–58). The results of present study, in conjunction with prior publications from our laboratory and others, suggest that GTP may prove to be a useful chemopreventive agent against some forms of human cancers induced by environmental agents and that more detailed studies on EGCG could lead to new strategies for recommending changes in dietary habits and for developing other foods with desired cancer chemopreventive agents.

REFERENCES


Inhibition of Skin Tumor Promoter-caused Induction of Epidermal Ornithine Decarboxylase in SENCAR Mice by Polyphenolic Fraction Isolated from Green Tea and Its Individual Epicatechin Derivatives


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