Inhibition of 12-*O*-Tetradecanoylphorbol-13-Acetate-Induced Inflammatory Skin Edema and Ornithine Decarboxylase Activity by Theaflavin-3,3′-Digallate in Mouse

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Abstract: Among black tea polyphenols, theaflavins were generally considered to be the most effective in cancer chemoprevention. In this study, we examined the inhibitory effects of black tea polyphenols, including theaflavin (TF-1), a mixture (TF-2) of theaflavin-3-gallate and theaflavin-3′-gallate, theaflavin-3,3′-digallate (TF-3), and the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced edema and ornithine decarboxylase (ODC) activity. Topical application of these polyphenols onto the mouse resulted in inhibition of TPA-induced ear edema and skin epidermal ODC activity. The inhibitory order was as follows: TF-3 > TF-2 ≅ EGCG > TF-1. Western and Northern blots indicated that TF-3 significantly reduced the protein and mRNA levels of ODC in TPA-treated mouse skin and NIH 3T3 cells, whereas EGCG showed less activity. EGCG and TF-3 were able to inhibit the ODC enzyme activity in vitro. Furthermore, TF-3 also significantly reduced the basal promoter activity of the ODC gene in NIH 3T3 cells that were transiently transfected with ODC reporter plasmid. These results suggested that TF-3 was a potential inhibitor of ODC activity and TPA-induced edema and might be effective in cancer chemoprevention.

Introduction

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines and catalyzes the formation of putrescine from ornithine. ODC activity is regulated by gene expression, stability, and transcription rate of ODC mRNA, stability and translation rate of the ODC enzyme, and post-translational modification (1–4). The activity of ODC can be induced by growth-promoting stimuli, including growth factors, steroid hormone, cAMP-elevating agents, and tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (5). Overexpression of ODC in fibroblast cells induced neoplastic transformation (6–8). On the other hand,

ODC is highly expressed in various cancer cells (9). It is possible that agents that inhibit ODC activity may be good candidates for use in cancer chemoprevention and chemotherapy. Indeed, a suicide substrate inhibitor of ODC, α-difluoromethylornithine, is presently being evaluated for the prevention and/or treatment of some cancers in humans (10,11).

Several reports have demonstrated that green tea polyphenols have potent inhibitory effects on tumor promotion, especially (−)-epigallocatechin-3-gallate (EGCG) (12,13). However, increasing numbers of reports have demonstrated that black tea could be as effective as green tea in cancer chemoprevention (14–17). For the manufacture of black tea, the "fermentation" process causes green tea polyphenols to oxidize and form oligomeric flavanols, including theaflavins, thearubigin, and other oligomers. Theaflavins are a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3) (Fig. 1). Thearubigins are the most abundant phenolic fraction of black tea, and their structures are not well characterized. Among black tea components, theaflavins are generally considered to be the effective components for cancer chemoprevention. Several reports have demonstrated that green and black tea could inhibit ODC activity and tumor promotion (18–23), but the action of the individual component of purified black tea polyphenol has not been well investigated. In addition, the mechanisms of action of these tea polyphenols are poorly understood.

In this study, we tested TF-1, TF-2 (a mixture of TF-2a and TF-2b), TF-3, and EGCG to assess their inhibitory effects on TPA-induced ODC activity and ear edema in mouse skin and/or NIH 3T3 cells. To further understand the mechanisms that underlie the inhibitory actions, the effects of individual theaflavins and EGCG on TPA-induced ODC mRNA and protein levels and on the promoter activity of ODC gene in mouse skin and/or NIH 3T3 cells were studied.

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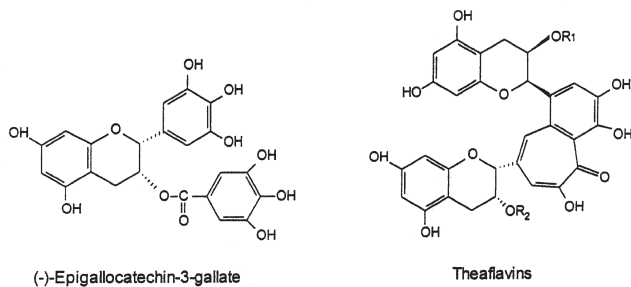
Theaflavins	R_1	R_2
Theaflavin (Theaflavin-1; TF-1)	H	H
Theaflavin-3-gallate (Theaflavin-2a; TF-2a)	Galloyl	H
Theaflavin-3′-gallate (Theaflavin-2b; TF-2b)	H	Galloyl
Theaflavin-3,3′-digallate (Theaflavin-3; TF-3)	Galloyl	Galloyl

Figure 1. Chemical structures of theaflavins and (−)-epigallocatechin-3-gallate (EGCG).

Materials and Methods

Materials

TPA was purchased from Sigma (St. Louis, MO) and DL-[^{14}C]ornithine and [γ-^{32}P]ATP from Amersham (Arlington Heights, IL). Individual theaflavins and EGCG were purified as previously described (24,25). The mouse fibroblast cell line NIH 3T3 was cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (GIBCO BRL, Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Animals

Female Balb/c mice (3–4 wk old) were purchased from the animal center of the National Taiwan University Hospital (Taipei, Taiwan) and kept in the animal facility for 1–2 wk before use. Mice were provided drinking water ad libitum. The dorsal region of each mouse was shaved with electric clippers ≥3 days before treatment with TPA. Only mice that did not show signs of hair regrowth were used.

Measurement of Mouse Ear Edema

Female Balb/c mice (3–4 animals/group) were pretreated with vehicle (15 μl acetone), individual theaflavins, or EGCG on both ears for 30 min and then treated with TPA (1 nmol) for 7.5 h. After TPA treatment, the mice were sacrificed by cervical dislocation, and ear punch biopsies were taken and weighed.

Preparation of Epidermal Homogenates

Mice (5–6 wk old) were pretreated with vehicle (200 μl acetone) or individual theaflavins or EGCG for 30 min and

then treated with TPA (5 nmol/animal/200 μl acetone) for 5.5 h. After TPA treatment, the mice were sacrificed by cervical dislocation, and the dorsal area of the skin was removed. To remove the epidermis from the dermis, the skin was plunged into a 58°C water bath for 30 s and then immediately submerged in an ice water bath as described by Slaga et al. (26). The epidermis was removed from the dermis by gentle scraping on an ice block, bisected, frozen in liquid N$_2$, and kept at −75°C until analyzed. For the determination of ODC activity, one-half of each skin sample was homogenized for 45 s in 1 ml of ice-cold homogenizing buffer containing 50 mM Tris buffer (pH 7.5), 0.1 mM pyridoxal phosphate, and 0.1 mM EDTA and centrifuged at 15,000 g for 30 min. The supernatant fractions were removed and stored overnight at −20°C before determination of ODC activity as described earlier (27,28). Protein concentration was determined by the protein-dye binding procedure described by Bradford (29) using a protein assay kit (Bio-Rad, Richmond, CA).

Western Blot

Equal amounts of total cellular protein (50 or 100 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described previously (30). The membrane was then incubated with an anti-ODC antiserum (Sigma), probed with anti-mouse IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and visualized using enhanced chemiluminescence kits (Amersham). The densities of the bands were quantitated by a computer densitometer (IS-1000 Digital Imaging System).

Reverse Transcription-Polymerase Chain Reaction and Probe

Total RNA was isolated from cultured cells, and cDNA was prepared as previously described (31,32). The amplification of ODC cDNA was performed by incubating 500-ng equivalents of cDNA in 100 mM Tris·HCl buffer (pH 8.3) containing 500 mM KCl, 15 mM MgCl$_2$, 0.1% gelatin, dNTP at 200 μM each, and 50 U/ml Super *Taq* DNA polymerase with the following oligonucleotide primers: 5′-AGCAGCTTTACTAAGGACGAGTTT-3′ and 5′-CTCACACCAATGACGTCAATATTT-3′. The cDNA sequence of glyceraldehyde-3-phosphate dehydrogenase was also amplified as a control by the same method using the following primers: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Thermal cycle conditions were as follows: 1 cycle at 95°C for 5 min, 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and 1 cycle at 72°C for 10 min. Polymerase chain reaction products were analyzed on 1% agarose gels. The ODC cDNA probe was prepared according to the products of ODC cDNA and fully described in our previous report (31).

Northern Blot

Total RNA was isolated as described by Chomczynski and Sacchi (32). For Northern blot, 20 μg of total RNA were separated by electrophoresis on a 1.2% agarose gel containing 6.7% formaldehyde and transferred to a Hybond-N nylon membrane (Amersham). After they were baked at 80°C for 2 h, the membranes were prehybrized for 12–16 h at 42°C in hybridization buffer (50% formamide, 6× sodium chloride-sodium citrate, 10× Denhart's solution, 10 mM EDTA, 0.1% sodium dodecyl sulfate, and 100 μg/ml single-stranded salmon sperm DNA). Hybridization was carried out for 16 h at 42°C with ODC cDNA probe (3×10^6 cpm/ml). Then the membranes were washed and autoradiographied with X-ray film (Kodak XAR-5) using an intensifying screen at −80°C (33).

Plasmids

The mouse 1.6- and 0.6-kb ODC-chloramphenicol acetyltransferase (CAT) promoter plasmids were generously provided by Dr. Olli A. Janne (University of Helsinki, Helsinki, Finland) and Dr. Chaim Kahana (Weizmann Institute of Science, Rehovot, Israel), respectively. The 1.6- and 0.6-kb ODC-CAT promoter constructs contain the region from −1658 to +16 nt and from −270 to +343 nt, respectively, relative to the transcription start site of the mouse ODC gene promoter (34,35).

Transient Transfection and CAT Assay

NIH 3T3 cells were cultured in a 100-mm dish; then the medium was replaced with serum-free Opti-MEM (GIBCO). Cells were transfected with the ODC-CAT reporter plasmids and internal control plasmid (β-galactosidase) using LipofectAMINE (GIBCO). After 24 h of incubation, the medium was replaced with complete medium and continually incubated for another 24 h. Transfected cells were pretreated with theaflavins or EGCG (15 μM) for 30 min and then treated with 200 nM TPA for 4 h. Cells lysates were collected by a rubber policeman and extracted by three cycles of freezing and thawing; then the lysates were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected, and CAT activity was assayed by the two-phase fluor diffusion technique (36). Briefly, 30 μl of supernatant were incubated at 65°C for 15 min to inactivate endogenous transacetylases. The assay was performed in a reaction mixture of 1.25 mM chloramphenicol (Sigma), 125 mM Tris (pH 7.8), and 0.2 μCi of [^{3}H]acetyl coenzyme A (Amersham) in a reaction volume of 0.2 ml. The reaction mixture was overlaid with 5 ml of water-immiscible scintillation fluid (Ecoscint O, National Diagnostics) and incubated at 37°C for 2 h. The activity of the ^{3}H-acetylated chloramphenicol was measured on a scintillation counter (Beckman). β-Galactosidase activity was determined by chemiluminescent assay according to the manufacturer's instructions (Roche) and used to normalize the transfection efficiency.

Results

Inhibition of Theaflavins and EGCG on TPA-Induced Edema and ODC Activity in Mouse Skin

Various concentrations of individual theaflavins and EGCG were tested for their abilities to inhibit TPA-induced ear edema. As shown in Table 1, TF-3 had the best effect on the inhibition of ear edema induced by TPA. Application of 0.1, 0.5, or 1 μmol of TF-3 inhibited TPA-induced edema by 23.6%, 73.8%, or 93.2%, respectively. TF-2 and EGCG also exhibited strong inhibition on TPA-induced ear edema (Table 1) but were less effective than TF-3.

Next, we studied the effects of TPA on ODC activity in mouse dorsal skin, and the time-dependent study showed the maximum induction of ODC activity at 5.5 h after treatment with 5 nmol of TPA (data not shown). The inhibitory effects of individual theaflavins and EGCG on TPA-induced ODC activity were examined, and the results are shown in Table 1. Topical application of 1 μmol of TF-1, TF-2, TF-3, or EGCG inhibited TPA-induced ODC activity by 18.2%, 36.3%, 48.6%, or 27.8%, respectively. The inhibition of individual theaflavins and EGCG on TPA-induced ODC activity was dose dependent. These results indicated that TF-2 and EGCG inhibited ODC activity but were less effective than TF-3.

Inhibition of Theaflavins and EGCG on TPA-Induced ODC Protein and mRNA in Mouse Skin

To understand the mechanisms, we examined the effects of individual theaflavins and EGCG on TPA-induced protein and mRNA expression of ODC in mouse skin. As shown in Fig. 2, topical application of TF-3 (2.5 μmol) significantly inhibited TPA-induced protein and mRNA levels of ODC concomitantly with inhibition of ODC activity, whereas TF-2 and EGCG were less effective.

Inhibition of Theaflavins and EGCG on TPA-Induced ODC Activity and mRNA Expression in NIH 3T3 Cells

To further understand the mechanisms of action of these tea polyphenols, we examined the effects of individual theaflavins and EGCG on TPA-induced ODC activity in NIH 3T3 cells. In NIH 3T3 cells, individual theaflavins and EGCG showed an inhibitory effect on TPA-induced ODC activity (Fig. 3A) similar to that shown by mouse skin. Treatment with TF-1, TF-2, TF-3, or EGCG at 15 μM each inhibited the TPA-induced increase in ODC activity by 21.1%, 36.5%, 52.3%, or 32.1%, respectively. The reverse transcription-polymerase chain reaction showed that TF-3

Table 1. Inhibitory Effects of Theaflavins and EGCG on TPA-Induced Ear Edema and Epidermal ODC Activity in Mouse[a,b]

Treatment[c]	Weight per Punch, mg	Treatment[d]	ODC Activity, pmol CO_2/h/mg protein
		Experiment 1	
Acetone	6.4 ± 0.4*	Acetone	256 ± 58*
TPA only	15.3 ± 0.5	TPA only	2,708 ± 413
TPA + TF-1 (0.1 μmol)	14.5 ± 0.3 (9.0)	TPA + TF-1 (1 μmol)	2,261 ± 285 (18.2)
TPA + TF-2 (0.1 μmol)	14.8 ± 0.5 (5.6)	TPA + TF-2 (1 μmol)	1,819 ± 389 (36.3)
TPA + TF-3 (0.1 μmol)	13.2 ± 0.4* (23.6)	TPA + TF-3 (1 μmol)	1,516 ± 190* (48.6)
TPA + EGCG (0.1 μmol)	14.5 ± 0.5 (9.0)	TPA + EGCG (1 μmol)	2,036 ± 332 (27.8)
		Experiment 2	
Acetone	6.8 ± 0.2*	Acetone	353 ± 83*
TPA only	15.2 ± 0.3	TPA only	4,238 ± 556
TPA + TF-1 (0.5 μmol)	11.0 ± 0.3* (50.0)	TPA + TF-1 (2.5 μmol)	2,855 ± 352* (35.6)
TPA + TF-2 (0.5 μmol)	9.4 ± 0.5* (69.0)	TPA + TF-2 (2.5 μmol)	1,856 ± 288* (61.3)
TPA + TF-3 (0.5 μmol)	9.0 ± 0.3* (73.8)	TPA + TF-3 (2.5 μmol)	1,487 ± 391* (70.8)
TPA + EGCG (0.5 μmol)	10.1 ± 0.4* (60.7)	TPA + EGCG (2.5 μmol)	2,128 ± 414* (54.3)
		Experiment 3	
Acetone	7.4 ± 0.4*	Acetone	128 ± 49*
TPA only	16.2 ± 0.3	TPA only	2,109 ± 432
TPA + TF-1 (1 μmol)	9.5 ± 0.2* (76.1)	TPA + TF-1 (5 μmol)	1,033 ± 317* (54.3)
TPA + TF-2 (1 μmol)	8.4 ± 0.3* (88.6)	TPA + TF-2 (5 μmol)	501 ± 237* (81.2)
TPA + TF-3 (1 μmol)	8.0 ± 0.4* (93.2)	TPA + TF-3 (5 μmol)	358 ± 101* (88.4)
TPA + EGCG (1 μmol)	8.7 ± 0.4* (85.2)	TPA + EGCG (5 μmol)	637 ± 201* (74.3)

a: Values are means ± SE; values in parentheses represent percent inhibition.

b: Statistical significance is as follows: *, significantly different from 12-*O*-tetradecanoylphorbol-13-acetate (TPA) alone (*P* < 0.05, Student's *t*-test).

c: Both ears of mice (5–6 wk old) were pretreated topically with vehicle (15 μl acetone), individual theaflavins, or (−)-epigallocatechin-3-gallate (EGCG) for 30 min and then treated with TPA (1 nmol) for 7.5 h. After TPA treatment, mice (*n* = 2–3/group) were sacrificed, and 6.5-mm-diameter ear punches were weighed. TF-1, theaflavin; TF-2, mixture of theaflavin-3-gallate and theaflavin-3′-gallate; TF-3, theaflavin-3-3′-digallate.

d: Mice (5–6 wk old) were pretreated topically with vehicle (200 μl acetone), individual theaflavins, or EGCG for 30 min and then treated with TPA (5 nmol) for 5.5 h. After TPA treatment, mice (*n* = 3/group) were sacrificed, and epidermal ornithine decarboxylase (ODC) activity was determined.

significantly inhibited the mRNA level of ODC induced by TPA (Fig. 3B).

Inhibition of Theaflavins and EGCG on the Enzyme Activity of ODC In Vitro

To understand whether the inhibitory effects of these tea polyphenols on ODC activity were due to a direct inhibition of ODC intrinsic enzyme activity, we used the cell lysate of TPA-treated NIH 3T3 cells. TF-3 and EGCG were able to directly inhibit ODC activity ~50% at 15 μM. These results indicated that TF-3 and EGCG could directly inhibit ODC activity in vitro and possibly in vivo (Fig. 4).

Inhibition of Theaflavins and EGCG on Promoter Activity of the ODC Gene in NIH 3T3 Cells

To further investigate the importance of theaflavins and EGCG and TPA in modulating expression of the ODC gene, transient transfection was performed using two mouse ODC-CAT promoter constructs. When the cells were treated with TPA, endogenous ODC activity significantly increased (Fig. 3A). However, promoter activity of the 1.6-kb ODC-

CAT construct slightly increased, and the 0.6-kb construct was virtually unresponsive to treatment with TPA (Fig. 5). Furthermore, TF-3 strongly inhibited the basal promoter activity about eightfold (1.6-kb ODC-CAT), and the inhibitory order was as follows: TF-3 > TF-2 > EGCG > TF-1. These results indicated that theaflavins and EGCG could affect basal promoter activity of the ODC gene (−1658 to +343 nt), but no components were detectable in the cloned 5′-flanking region of the ODC gene tested here in response to TPA.

Discussion

Our previous reports and reports by others demonstrated that black tea polyphenols could be as effective as green tea polyphenols and even stronger than green tea polyphenols in cancer chemoprevention (37–40). The present study demonstrated that topical application of TF-3 strongly inhibited TPA-induced ear edema, epidermal ODC activity, and protein and mRNA levels of ODC in mouse. The related compounds TF-2 and EGCG were also active as inhibitors but were less effective than TF-3. Moreover, TF-3 strongly inhibited TPA-induced ODC activity, ODC enzyme activity, and basal promoter activity of the ODC gene (−1658 to +343
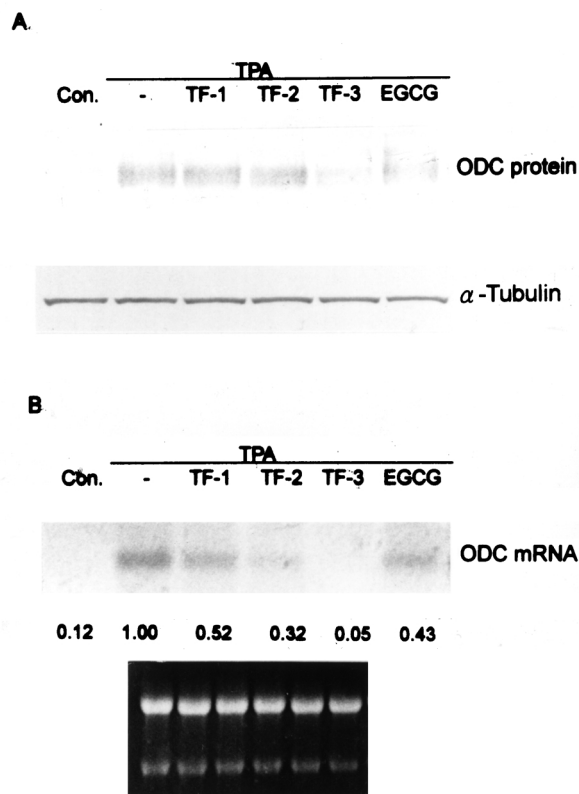
A.

B

0.12   1.00   0.52   0.32   0.05   0.43

Figure 2. Effects of theaflavins and EGCG on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) protein and mRNA levels in mouse skin. Groups of mice were pretreated with 1 μmol of individual theaflavins or EGCG for 30 min and then treated with TPA (5 nmol) for 5.5 h. After TPA treatment, mice were sacrificed, and total cell lysates and RNA were isolated. A: total cellular protein (100 μg/lane) was separated on 10% sodium dodecyl sulfate-polyacrylamide gels and blotted with antibody specific for ODC and α-tubulin. B: total RNA (25 μg) was successively hybridized to ^{32}P-labeled ODC probes. TF-1, theaflavin; TF-2, theaflavin-3-gallate + theaflavin-3′-gallate; TF-3, theaflavin-3,3′-digallate. 28S and 18S RNA were used as control for gel loading. Band intensities were quantified by densitometry, and relative amounts of radioactivity are shown presented at bottom.

A

B

Figure 3. Effects of theaflavins and EGCG on TPA-induced ODC activity and mRNA levels in NIH 3T3 cells. Serum-starved cells were pretreated with individual theaflavins or EGCG (15 μM) for 30 min and then treated with 200 nM TPA for 5.5 or 5 h. A: total cell lysate (100 μg) was analyzed for ODC activity. Values are means ± SE of triplicate tests. B: total RNA was isolated, and ODC mRNA was analyzed by reverse transcription-polymerase chain reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

nt). Clearly, the fermentation process of black tea did not diminish the beneficial chemopreventive property, contrary to general belief. The cancer chemopreventive effect of black tea polyphenols could be attributed to the theaflavins, especially TF-3. Thearubigins are the most abundant phenolic fraction of black tea, but they did not significantly inhibit edema and ODC activity (data not shown). It is likely that other unidentified components of black tea also have beneficial roles in cancer chemoprevention.

The 1.6- or 0.6-kb ODC-CAT promoter construct was transfected into NIH 3T3 cells, and no change of the promoter activity in response to TPA stimuli was observed. It is possible that TPA regulatory sequences lie outside the region of −1658 to +343 nt that we used for constructs. The 1.6-kb mouse ODC promoter possesses strong basal activity in cultured mouse cells (35), and the first 200 nt of the promoter exhibited ∼50% of the activity of a 1.6-kb construct. This result indicated that the proximal promoter contains elements important for transcriptional activation (34). There
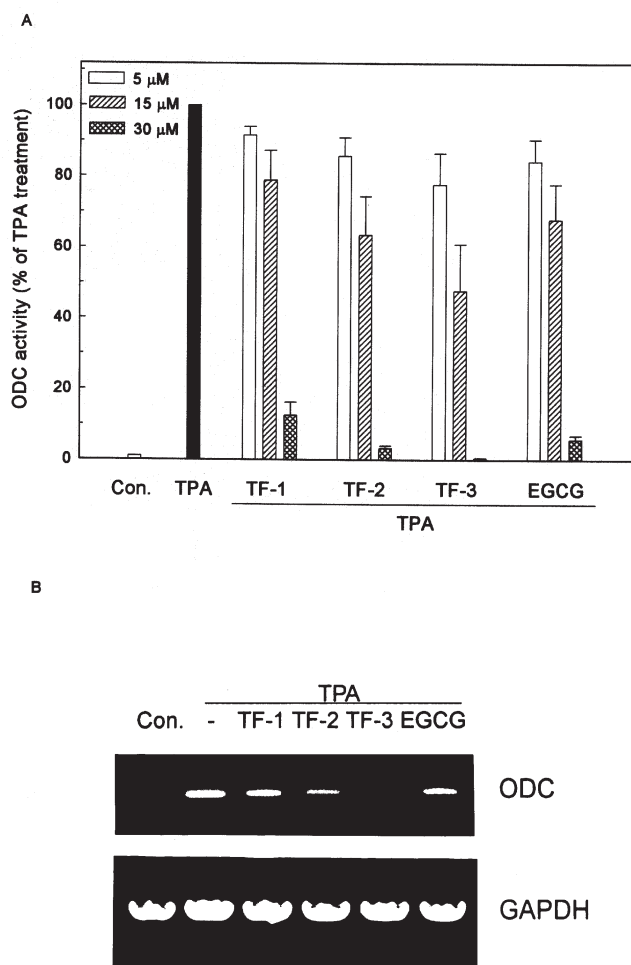
are several potential regulatory elements for *trans*-acting factor sites within the first 380 nt of the mouse promoter, such as a TATA box at −31 nt, a cAMP response element (CRE)-like element at −48 nt, a putative CAAT box at −81 nt, and four or five Sp1-binding sites (45). In the present study, we indicated that TF-3 strongly inhibited 1.6- and 0.6-kb ODC-CAT promoter activity, and further experimental investigation is required to identify the elements that are involved in this inhibition.

TPA is a potent tumor promoter and is known to affect a variety of biochemical processes, including the induction of ODC activity. The induction of ODC, which is the first enzyme in mammalian polyamine biosynthesis, is an essential event of tumor promotion. In the present study, we found that TF-3 significantly inhibited ODC activity in mouse skin and NIH 3T3 cells. To further elucidate the mechanism of TF-3-mediated activity, we determined the influence of TF-
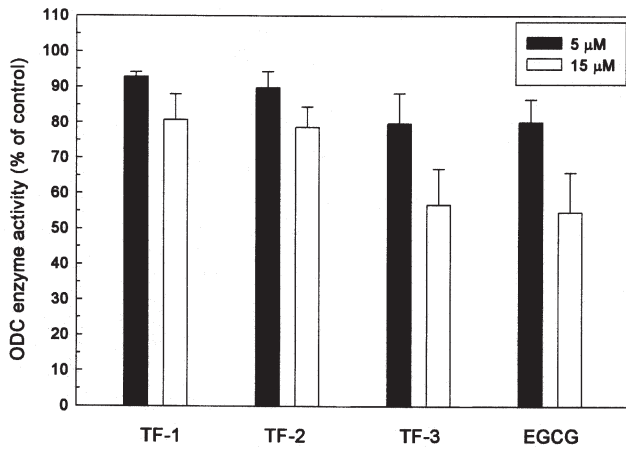
Figure 4. Effects of theaflavins and EGCG on enzyme activity of ODC in vitro. ODC activity was induced in serum-starved NIH 3T3 cells by 5.5 h of treatment with TPA (200 nM). Total cell lysates (200 µg) were incubated with individual theaflavins or EGCG, and ODC activity was determined. Values are means ± SE of triplicate tests. ODC activity of cell lysate alone was 1,012 pmol CO_2/h/mg protein.
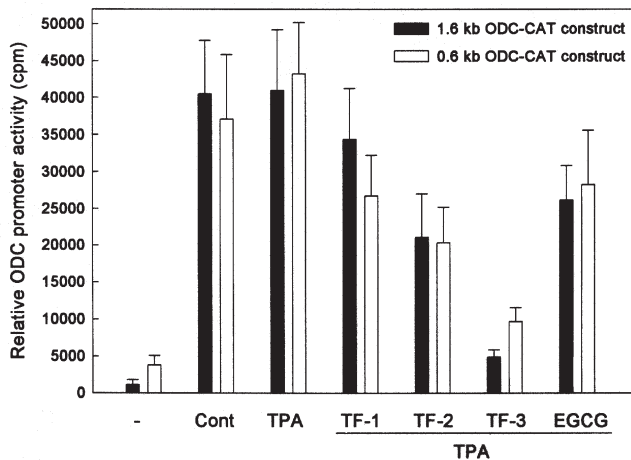

Figure 5. Effects of theaflavins and EGCG on ODC promoter activity. NIH 3T3 cells were cotransfected with 1.6- or 0.6-kb mouse ODC promoter constructs and β-galactosidase expression plasmid as internal control. Transfected cells were pretreated with theaflavins or EGCG (15 µM) for 30 min and then treated with 200 nm TPA for 4 h. Chloramphenicol acetyltransferase (CAT) activity was assayed in cell extracts. Values are means ± SE of triplicate tests.

3 treatment on TPA-induced mRNA and protein expression of ODC in mouse skin and NIH 3T3 cells. The results indicate that TF-3 significantly inhibited the mRNA and protein levels of ODC. In addition, TF-3 also inhibited the enzyme activity of ODC in an in vitro assay. It was possible that suppression of ODC activity by TF-3 might directly inhibit activity of the ODC enzyme and not through inhibition of the message and protein levels. Moreover, TF-3 inhibited the TPA-induced ODC expression and also inhibited basal transcription of the ODC gene.

In conclusion, these results suggested that TF-3 was a potential inhibitor of ODC activity and TPA-induced edema and might be useful in cancer chemoprevention.

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