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Inhibitory effects of black tea theaflavin derivatives on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation and arachidonic acid metabolism in mouse ears

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Tea has been shown to possess several health beneficial properties primarily due to its polyphenolic content. The major polyphenolic compounds in black tea leaves are theaflavins (TFs) formed by oxidative coupling of catechins in tea leaves during its processing. In this paper, we report the characterization of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear inflammatory model and the inhibitory effects of major black tea TFs derivatives on this inflammation. In addition, the effect on inflammatory biomarkers, such as proinflammatory cytokines and arachidonic acid metabolites, are reported as well. A single topical application of TPA to ears of CD-1 mice induced a time- and dose-dependent increase in edema as well as formation of proinflammatory cytokine proteins interleukin-1β (IL-1β) and interleukin-6 (IL-6) in mouse ears. A single topical application of equimolar of black tea constituents (TF, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate) strongly inhibited TPA-induced edema of mouse ears. Application of TFs mixture to mouse ears 20 min prior to each TPA application once a day for 4 days inhibited TPA-induced persistent inflammation, as well as TPA-induced increase in IL-1β and IL-6 protein levels. TFs also inhibited arachidonic acid (AA) metabolism *via* both cyclooxygenase (COX) and lipoxygenase pathways. This observation was substantiated by decreased amounts of AA metabolites prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) levels. Combined application of TF and sulindac, a nonsteroidal anti-inflammatory drug resulted a significant synergetic anti-inflammatory effect. Oral administration of TFs or the hot water extract of black tea leaves also significantly inhibited TPA-induced edema in mouse ears. In conclusion, proinflammatory cytokines, IL-1β and IL-6, as well as the intermediated metabolites of AA, PGE_2, and LTB_4 are good biomarkers for inflammation. Black tea constituents, TF and its derivatives, had strongly anti-inflammatory activity *in vivo* which may be due to their ability to inhibit AA metabolism *via* lipoxygenase and COX pathways.

Keywords: Black tea / Sulindac / 12-*O*-tetradecanoylphorbol-13-acetate / Theaflavin / Theaflavin-3,3′-digallate

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1 Introduction

Tea, made from the leaves of *Camellia sinensis*, is the second most widely consumed beverage in the world after water. The most commonly consumed types of tea are green, oolong, and black. While green and oolong tea are consumed in Asian countries, black tea is more popular in western countries and constitutes about 80% of tea consumed worldwide [1, 2].

Tea has been shown to possess numerous health beneficial properties that are attributed to its polyphenolic com-

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Abbreviations: AA, arachidonic acid; **BT**, benzotropolone; **COX**, cyclooxygenase; **EGCG**, (−)-epigallocatechin gallate; **IL-1β, interleukin-1β; IL-6**, interleukin-6; **LTB_4**, leukotriene B_4; **PGE_2**, prostaglandin E_2; **TF**, theaflavin; **TF-3-G**, theaflavin-3-gallate; **TF-3′-G**, theaflavin-3′-gallate; **TF-3,3′-diG**, theaflavin-3,3′-digallate; **TPA**, 12-*O*-tetradecanoylphorbol-13-acetate

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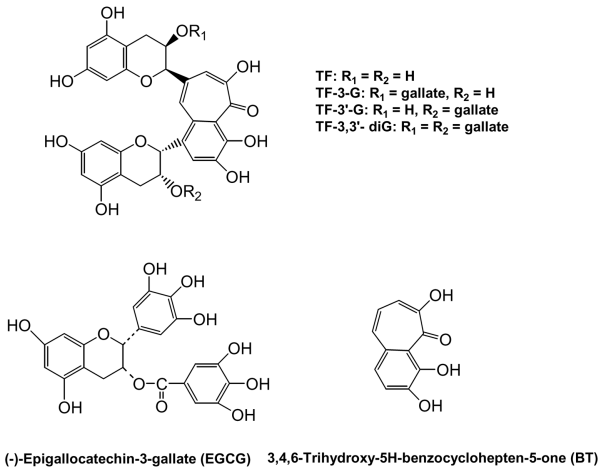

Figure 1. Chemical structures of green tea EGCG, black tea TFs, and 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT).

pounds. The major polyphenolic compounds in green tea, the catechins, are oxidized and polymerized to form theaflavins (TFs) and thearubigins during the processing of black tea by enzymes such as polyphenol oxidase and peroxidase [1 – 5]. TFs contribute to the characteristic flavor and orange-red color of black tea beverage and consist of TF, theaflavin-3-gallate (TF-3-G), theaflavin-3′-gallate (TF-3′-G), and theaflavin-3,3′-digallate (TF-3,3′-diG). The characteristic feature of TFs, which contributes to its flavor and color, is the presence of the seven-membered benzotropolone (BT) ring [1]. The chemical structures of black tea TF derivatives are shown in Fig. 1.

The health-beneficial properties of tea have been extensively studied using several *in vivo* and *in vitro* models. Green and black tea have been shown to possess antioxidant, anticarcinogenic, antimutagenic, antihypertensive, anti-inflammatory, and hypocholesterolemic effects, which have been attributed to its polyphenolic compounds [6 – 13]. Although the biological effects of green tea have been studied in several animal models, the effects of black tea constituents are still being evaluated. This study was undertaken to evaluate the anti-inflammatory potential of the black tea polyphenols.

Inflammation, defined as the response of tissue to injury, has been implicated in cardiovascular disease, arthritis, and several other chronic disorders [14 – 16]. During inflammation, cells of the immune response migrate to the site of injury and release mediators that help in decreasing the inflammatory response [17]. These mediators of inflammation could be peptide in nature (cytokines) or derived from fatty acids (eicosanoids), primarily AA [17]. The prostaglandins (PGs) and leukotrienes (LTs) are derived from AA by the action of the enzymes cyclooxygenase (COX) and lipoxygenase (LOX), respectively.

Topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to the skin has been shown to induce inflammatory response by attracting inflammatory cells, increasing the levels of proinflammatory mediators and inducing hyperplasia [18]. Based on this, we have developed and characterized the TPA-induced mouse ear inflammatory model. When TPA was applied to mouse ear, in this study it induced edema and an increase in the levels of inflammatory mediators. The anti-inflammatory activities of TF, TF-3-G, TF-3′-G, and TF-3,3′-diG were evaluated in this model. We chose to evaluate the levels of mediators that were formed through different pathways, namely interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and leukotriene B_4 (LTB_4) to understand the mechanisms of action of TF and TF-3,3′-diG.

2 Materials and methods

2.1 Animals and reagents

CD-1 strain mice are the next to Sencar mice with respect to their sensitivity in response to TPA-induced inflammation. Supplies for CD-1 are easy and more economical than Sencar strain mice. Therefore, CD-1 stain mice were use for the mouse ear anti-inflammatory studies. Female CD-1 (4-5-wk-old) mice were purchased from Charles River Breeding Laboratories (Kingston, NY, USA). The mice were kept in our animal facility for at least 1 wk before use. Mice were fed a Purina Laboratory Chow 5001 diet *ad libitum* (Ralston-Purina, St. Louis, MO, USA) and kept on a 12-h light, 12-h dark cycle. Mice were provided drinking water *ad libitum*. TF, TF-3-G, TF-3′-G, TF-3,3′-diG, and 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT) were synthesized as described below. All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2 Preparation of test compounds

2.2.1 Synthesis of TF mixtures

A mixture of TFs was synthesized from green tea polyphenols using enzymatic oxidation methods. Specifically, after filtration, the crude green tea polyphenol (1.8 g, commercial sample containing 80% catechins) was loaded directly onto a Sephadex LH-20 column eluted first with 95% ethanol to remove noncatechin flavonoids, and then the column was eluted with acetone to obtain a mixture of tea catechins (1.34 g). The tea catechins were dissolved in pH 5 buffer (50 mL), which contained 4 mg horseradish peroxidase. While being stirred, 3.0 mL of 3.13% H_2O_2 was added five times for 1 h. The enzymatic reaction solution containing catechins and crude peroxidase turned into a reddish solution during oxidation reaction. The reaction mixture was

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extracted by ethyl acetate (50 mL × 3). After concentration, the residue (0.97 g) was subjected to Sephadex LH 20 column eluted with acetone – water solvent system (from 35 to 50%). A TF mixture (350 mg) was obtained.

The ratios of TFs in the TF mixture were quantified using a Shimadzu LC-2010A HPLC (Tokyo, Japan) equipped with a ternary pump delivery system. The TFs mixture sample (20 μL, 1 mg sample in 1 mL water) were injected onto the column (Luna, 150 mm × 4.6 mm, 3 μm, Phenomenex, Torrance, CA, USA). The mobile phases of HPLC-grade water containing 2% acetic acid and ACN were used at a flow rate of 0.8 mL/min. The initial concentration of ACN was 8%, changed to 20% over 20 min, increased to 35% from 20 to 73 min, and then back to initial concentration for an additional 1 min. The individual TFs were monitored using UV detector at 280 nm. The analysis showed that TF : TF-3-G : TF-3′-G : TF-3, 3′-diG was 1.5 : 3.0 : 1.0 : 3.0.

2.2.2 Synthesis of TFs, TF-3-G, TF-3′-G, and TF-3,3′-diG

TFs, TF-3-G, TF-3′-G, and TF-3,3′-diG were synthesized according to the previously reported method of Sang *et al*. [19].

2.2.3 Synthesis of 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT)

The synthesis of the 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one, the simplest form of BT from a catechol molecule and a pyrogallol molecule, was accomplished by enzymatic coupling method (horseradish peroxide/H_2O_2). A mixture of acetone-pH 5.0 phosphate-citrate buffer (1 : 10 v/v, 50 mL), which contained 4 mg horseradish peroxide was prepared. The buffer solution was divided into two parts of 5 and 45 mL, and pyrogallol (0.1 g), and catechol (0.9 g) were dissolved in them, respectively. Five 1.0 mL aliquots of 3.13% H_2O_2 and pyrogallol were added into catechol buffer solution for a period of 45 min while stirring. The resulting reaction mixture was extracted with ethyl acetate (3 × 50 mL). After concentration, the residue was applied to a Sephadex LH 20 column and eluted with acetone-water (40 : 60 v/v). The components within the confines of orange-red color were collected and concentrated. The residue was applied to a C-18 self-pack column and eluted with methanol-water (35 : 65 v/v) to obtain 60 mg 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT). 1H NMR (DMSO, 500 MHz): 15.0 1H, s (–OH), 9.85 1H, s (–OH), 9.50 1H, s (–OH), 7.48, 1H, d, J = 11.5 (H-5), 7.44 1H, d, J = 8.5 (H-7), 7.39 1H, d, J = 8.5 (H-6), 7.19 1H, d, J = 9.0, 6.76 1H, dd, J = 11.5, 9.0; 13C NMR (DMSO, 125 MHz): 184.4 s (C-1), 154.7 s (C-2), 150.4 s (C-9), 146.1 s (C-8), 135.5 d (C-5), 131.4 s (C-11), 125.0 d (C-6), 122.8 d (C-4), 122.3 d (C-7), 120.2 s (C-10), 118.6 d (C-3) ppm; positive APCI-MS m/z 205 $[M + H]^+$.

2.3 TPA-induced acute edema and up-expression of cytokine IL-1β and IL-6 protein in ears of CD-1 mice

Both ears of female CD-1 mice were treated with 10 μL of acetone (vehicle control) or test compound in acetone 20 min prior to application of acetone or 1.5 nmol TPA in acetone. The mice were sacrificed 6 h after last of TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Ear samples from each group were pooled, homogenized in phosphate buffered saline, and quantified for the levels of inflammatory mediators using ELISA as described in Section 2.4.2.

2.4 TPA-induced persistent inflammation, up-expression of cytokine IL-1β and IL-6 protein levels, and formation of PGE_2 and LTB_4 levels in ears of CD-1 mice

For persistent inflammation, both ears of female CD-1 mice were treated topically once a day for 4 days with either 10 μL of nacetone (vehicle) or test compound in acetone 20 min prior to each application of acetone or 1.5 nmol TPA in acetone. Ears were in persistent inflammation during the 4 days of TPA treatment. The mice were sacrificed 6 h after the last TPA treatment. Ears punches (6 mm in diameter) were taken and weighed. Ear samples from each group were pooled and homogenized as described in Section 2.4.1.

2.4.1 Preparation of ear homogenate for ELISA assay

Ear tissues were homogenized in a PBS containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM PMSF, 0.1 mM benzethonium, 10 mM EDTA, and 20 U aprotinin *per* mL. The homogenates were centrifuged at 12 000 × g for 60 min at 4°C. The supernatant fraction was used for determination of cytokine protein levels. A two-site sandwich ELISA was used to assay for cytokines.

2.4.2 ELISA assay procedure

The levels of the cytokines, IL-1β and IL-6, protein were quantified using ELISA kit purchased from Biosource International (Camarillo, CA, USA), and the levels of the eicosanoids (PGE_2 and LTB_4) were quantified using ELISA kit purchased from Cayman Chemical (Ann Arbor, MI, USA). The IL-1β and IL-6 ELISA kits follow the same basic procedure. The capture antibody, diluted with PBS, was used to coat a 96-well plate overnight at room tempera-

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ture. The plate was then washed, blocked (1% BSA, 5% sucrose in PBS with 0.05% NaN_3), and washed again. The standards were added to the plate leaving at least one zero-concentration well and one blank well. The diluted samples (1 : 3 – 1 : 8) were then added to the plate. After incubating for 2 h, the plates were washed and the detection antibody was added. After incubating for another 2 h the plates were washed and Streptavidin-HRP was added. After 20-min incubation, the plates were washed, and substrate (H_2O_2) and tetramethylbenzidine were added. After another 20-min incubation, the stop solution (2 N H_2SO_4) was added and the plates were read with a microplate reader at a wavelength of 450 nm.

The PGE_2 and LTB_4 ELISA kits follow the same basic procedure. The well plates are precoated with goat polyclonal antimouse IgG and blocking proteins. The standards and samples are added to the wells and incubated for an hour with tracer and antiserum. After washing, Ellman's Reagent is added for color development. After incubating in the dark, the plate is read by microplate reader at a wavelength of 420 nm.

Statistical analyses were performed using the Student's *t*-test.

3 Results

3.1 Inhibitory effect of TF derivatives TF, TF-3-G, TF-3′-G, and TF-3,3′-diG on TPA-induced edema of mouse ears

The chemical structures of the major green tea constituent, (–)-epigallocatechin gallate (EGCG), and black tea constituents, TF, TF-3-G, TF-3′-G, and TF-3,3′-diG, are shown in Fig. 1. Also shown in Fig. 1 is the structure of 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT), the simplest form of BT from a catechol molecule and a pyrogallol molecule. The anti-inflammatory activity of tea polyphenolic compounds was evaluated in a mouse ear model. A single topical application of TPA-induced ear edema of mice in a time- and dose-dependent manner (data not shown). Topical application of TF derivatives before TPA treatment inhibited TPA-induced edema of mouse ears in a dose-dependent fashion (data not shown). A single topical application of equimolar of BT, (–)-EGCG, TF, TF-3-G, TF-3′-G, and TF-3,3′-diG before TPA treatment strongly inhibited TPA-induced edema in ears of mice (Fig. 2). The degree of anti-inflammatory activity was in the order: TF-3,3′-diG > TF-3-G = TF-3′-G > TF > EGCG > BT. The potential degree of the anti-inflammatory activity of these phenolic compounds was proportional to the number of phenolic groups present in them (Fig. 2). The results suggested that the black tea polyphenolic constituents had higher anti-inflammatory activity than the green tea polyphenolic constituents.

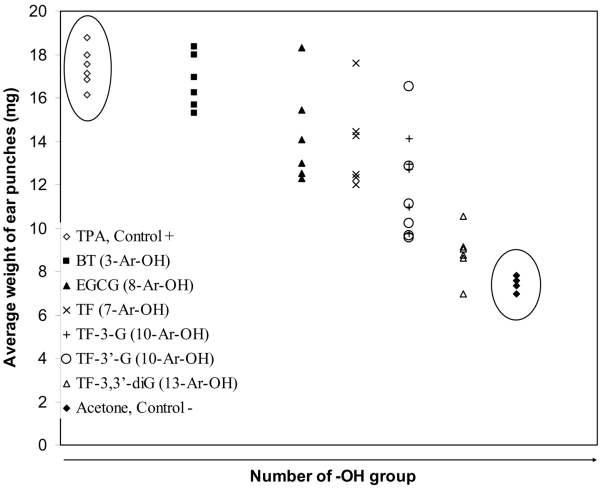

Figure 2. Inhibitory effect of topical application 3,4,6-trihydroxy-5*H*-benzocyclohepten-5-one (BT), EGCG, and black tea TFs on TPA-induced edema of mouse ears.

3.2 Inhibitory effect of TF mixture on TPA-induced persistent inflammation and up-expression of proinflammatory cytokine IL-1β and IL-6 protein levels in mouse ears

TF mixture contains TF, TF-3-G, TF-3′-G and TF-3,3′-diG. The ratio of TF : TF-3-G : TF-3′-G : TF-3,3′-diG was approximately equal to 1.5 : 3.0 : 1.0 : 3.0 (see Section 2). Topical application of TPA (0.8 nmol) to ears of the mice once a day for 4 days induced persistent inflammation and up-expression of IL-1β and IL-6 protein levels in ears during 4-day TPA treatment period. Inflammation (ear thickness) and up-expression of proinflammatory cytokines were linear up to 4 days in a time-dependent fashion (data not shown). Both ears of CD-1 mice were treated topically with 0.25, 0.50, and 1.00 μmol of TFs 20 min prior to each TPA (0.8 nmol) treatment once a day for 4 days. The mice were sacrificed 6 h after the last TPA treatment. Under these conditions, ears showed persistent inflammation during the 4-day TPA treatment period. Persistent inflammation in ears was evaluated by measuring average weight of ear punches (6 mm in diameter). Topical application of 0.25, 0.50, and 1.0 μmol of TFs inhibited TPA-induced persistent inflammation in ears by 53, 72, and 97%, respectively (Table 1). In addition, 0.25, 0.50, and 1.00 μmol of TFs also blocked TPA-induced up-expression of IL-1β by 87, 93, and 99%, respectively (Table 1). Topical application of 0.25, 0.50, and 1.00 μmol of TFs to ears before TPA treatment also greatly reduced TPA-induced up-expression of IL-6 protein levels by 43, 55, and 98%, respectively.

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Table 1. Inhibitory effect of topical application of TFs on TPA-induced persistent inflammation and up-expression of proinflammatory cytokine proteins IL-1β and IL-6 in mouse ears

Treatment	Inflammation average weight ear punches		Proinflammatory IL-1β protein levels, pg/mg		Proinflammatory IL-6 protein levels, pg/mg	
	Mean ± SE, mg	% Inhibition	Mean ± SE, pg/mg	% Inhibition	Mean ± SE, pg/mg	% Inhibition
Acetone + acetone	8.10 ± 0.30[a)]	–	0.95 ± 0.15[a)]	–	0.34 ± 0.21	–
Acetone + TPA (0.8 nmol)	14.10 ± 0.48	–	88.50 ± 4.00	–	3.95 ± 0.02	–
TFs (0.25 μmol) ± TPA	10.90 ± 0.65[a)]	↓53	12.80 ± 1.05[a)]	↓87	2.40 ± 0.03[a)]	↓43
TFs (0.50 μmol) + TPA	9.78 ± 0.50[a)]	↓72	7.10 ± 2.05[a)]	↓93	1.95 ± 0.04[a)]	↓55
TFs (1.00 μmol) + TPA	8.31 ± 0.35[a)]	↓97	1.92 ± 0.15[a)]	↓99	0.40 ± 0.03	↓98

Both ears of female CD-1 mice (7–8-wk-old; six mice *per* group) were treated topically with 10 μL of acetone or TF mixture in acetone 20 min prior to topical application of acetone or TPA (0.8 nmol) in acetone once a day for 4 days. Mice were sacrificed 5 h after the last TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Ear samples were pooled and homogenized in a phosphate buffer for cytokine protein ELISA triplicate assays.

[a)] a) Statistically different from the group 2 (acetone + TPA) ($p < 0.05$) as determined by the Student's *t*-test.

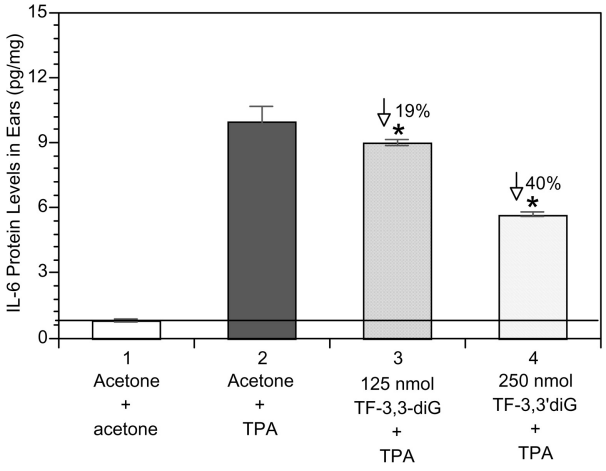

Figure 3. Inhibitory effect of theaflavin-3,3′-di-G (TF-3,3′-diG) on TPA-induced persistent inflammation and up-expression of IL-6 protein in ears of the CD-1 mouse. Both ears of female CD-1 mice (7-wk-old; six mice *per* group) were treated topically with 125 and 250 nmol of TF-3,3′-diG 20 min prior TPA (0.8 nmol) treatment once a day. These treatments were continued for 4 days (total four treatments). Mice were sacrificed 6 h after the last TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Ear samples from each group were pooled together and homogenized in a phosphate buffer. Supernatant fraction was used for cytokine ELISA assay. *Statistically different from the group 2 (acetone + TPA) ($p < 0.05$) as determined by the Student's *t*-test.

These results indicated that TFs markedly inhibited TPA-induced persistent inflammation and up-expression of proinflammatory cytokines IL-1 and IL6 in mouse ears. Topical application of 125 and 250 nmol of pure TF-3,3′-diG 20 min prior to each TPA (0.8 nmol) treatment once a day for 4 days inhibited TPA-induced up-expression of proinflammatory cytokine IL-6 protein levels in ears by 19 and 40% (Fig. 3), respectively.

3.3 Inhibitory effects of TFs and pure TF-3,3′-diG on TPA-induced increases in AA in ears of the mice

Levels of AA metabolites PGE_2 and LTB_4 were measured in TPA-induced mouse ear model. Topical application of TPA (0.8 nmol) to ears of the mice once a day for 4 days induced linear incraese in PGE_2 and LTB_4 levels (data not shown). Topical application of 0.25, 0.50, and 1.00 μmol of TFs 20 min prior to each TPA treatment once a day for 4 days inhibited TPA-induced increase in PGE_2 levels in ears by 21, 41, and 80%, respectively (Table 2), and inhibited increase in LTB_4 levels in ears by 93, 98, and 100%, respectively (Table 2). The results showed that TFs had a stronger inhibitory effect on LTB_4 than PGE_2 levels in ears. Although black tea TF mixture inhibited formation of both PGE_2 and LTB_4, TFs were shown to have a stronger inhibitory effect on the lipoxygenase pathway than on the COX pathway. We also examined the effect of highly pure TF-3,3′-diG on TPA-induced AA metabolism in mouse ears. Topical application of 125 and 250 nmol of TF-3,3′-diG to ears of the mice prior to each 0.8 nmol of TPA treatment once a day for 4 days markedly inhibited TPA-induced AA metabolism in ears by decreasing TPA-induced increase in PGE_2 and LTB_4 levels in ears (Fig. 4). Topical application of 125 and 250 nmol of TF-3,3′-diG 20 min prior to each TPA (0.8 nmol) treatment once a day for 4 days inhibited TPA-induced increase in PGE_2 levels in ears by 19 and 47%, respectively (Fig. 4A). Topical application of 125 and 250 nmol of TF-3,3′-diG 20 min prior to each TPA (0.8 nmol) treatment once a day for 4 days inhibited TPA-induced increase in LTB_4 levels in ears by 43 and 82%, respectively (Fig. 4B).The results indicated that TF-3,3′-diG also had a stronger inhibitory effect on the lipoxygenase pathway than on the COX pathway.

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Table 2. Inhibitory effect of topical application of TF mixture on TPA-induced formation of PGE_2 and LTB_4 in mouse ears

Treatment	Formation of PGE_2 levels in ears		Formation of LTB_4 levels in ears	
	Mean ± SE, pg/mg	% Inhibition	Mean ± SE, pg/mg	% Inhibition
Acetone + acetone	1000 ± 75[a)]	–	1.9 ± 0.17[a)]	–
Acetone + TPA (0.8 nmol)	2400 ± 616	–	9.8 ± 0.21	–
TFs (0.25 μmol) + TPA	2100 ± 260[a)]	21.4	2.53 ± 0.21[a)]	92.8
TFs (0.50 μmol) + TPA	833 ± 144[a)]	40.5	2.12 ± 0.18[a)]	97.5
TFs (1.00 μmol) + TPA	1277 ± 81[a)]	80.2	1.18 ± 0.04[a)]	> 100.0

Both ears of female CD-1 mice (7 – -8-wk-old; six mice *per* group) were treated topically with 10 μL of acetone or TF mixture in acetone 20 min prior each topical application or 10 μL acetone or TPA (0.8 nmol) in acetone. The mice were sacrificed 5 h after the TPA treatment. Ear samples were pooled and homogenized in a phosphate buffer. Supernatant fraction was used for PGE_2 and LTB_4 ELISA triplication assays.

[a)] Statistically different from the group TPA alone ($p < 0.05$) as determined by the Student's *t*-test.

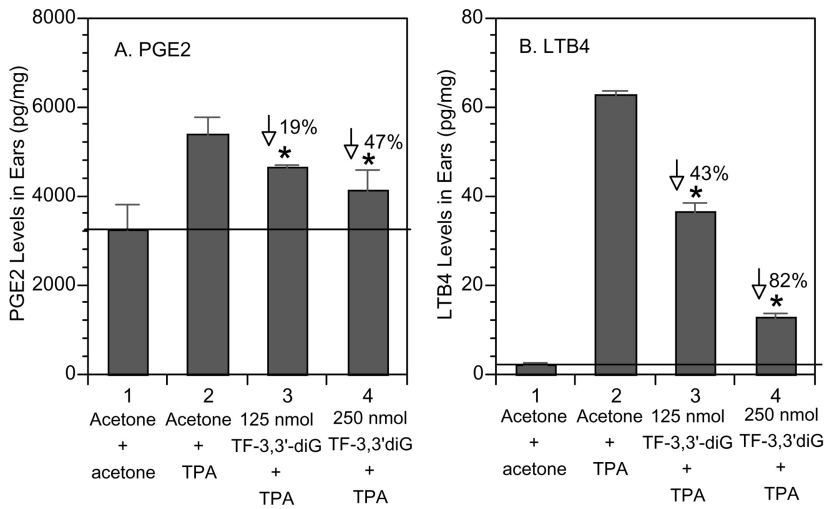

Figure 4. Inhibitory effect of TF-3,3′-diG on TPA-induced arachidonic acid in ears of the CD-1 mouse. Both ears of female CD-1 mice (7-wk-old; six mice *per* group) were treated topically with 125 and 250 nmol of TF-3,3′-diG 20 min prior TPA (0.8 nmol) treatment once a day. These treatments were continued for 4 days (total four treatments). Mice were sacrificed 6 h after the last TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Ear samples from each group were pooled together and homogenized in a phosphate buffer. Supernatant fraction was used for cytokine ELISA assay. *Statistically different from the group 2 (acetone + TPA) ($p < 0.05$) as determined by the Student's *t*-test.

3.4 Synergetic inhibitory effect of TF with sulindac on TPA-induced persistent inflammation and up-expression of proinflammatory cytokine IL-6 in ears

TF and TF-3,3′-diG had a stronger inhibitory effect on the LTB_4 than PGE_2 levels in mouse ears. The results indicated that TF mixture and pure TF-3,3′-diG resulted in stronger inhibition effect on the lipoxygenase pathway than on the COX pathway. Combination of TF with sulindac, a COX inhibitor, would be more effective to inhibit both the lipoxygenase and the COX pathways. Figure 6 showed synergetic inhibitory effect by combination of TF with sulindac on TPA-induced persistent inflammation (Fig. 5A), and on TPA-induced up-expression of proinflammatory cytokine IL-6 in ears of the mice (Fig. 5B). Topical application of 125 nmol TF, 300 nmol sulindac, and 125 + 300 nmol sulindac 20 min prior to each TPA (0.8 nmol) treatment once a day for 4 days inhibited TPA-induced persistent inflammation in ears by 18, 22, and 50%, respectively (Fig. 5A). Topical application of 125 nmol TF, 300 nmol sulindac, and 125 + 300 nmol sulindac 20 min each TPA (0.8 nmol) treatment once a day for 4 days inhibited TPA-induced up-expression of IL-6 protein levels in ears by 11, 34, and 80%, respectively (Fig. 5B).

3.5 Inhibitory effect of oral administration of TF mixture on TPA-induced edema of mouse ears

We also evaluated whether oral administration of TF mixture could inhibit TPA-induced ear edema of the mouse. Figure 6 showed that the mice were given oral gavages with 5 mg of TFs 60 min and 5 mg TFs 20 min prior to topical application of TPA. Oral administration of TFs to mice inhibited TPA-induced local ear edema by 69%,which indicated that TFs could be absorbed and transported to the ear tissue.

4 Discussion

A growing understanding of the pivotal role of AA metabolic pathways in a number of inflammatory and neoplastic

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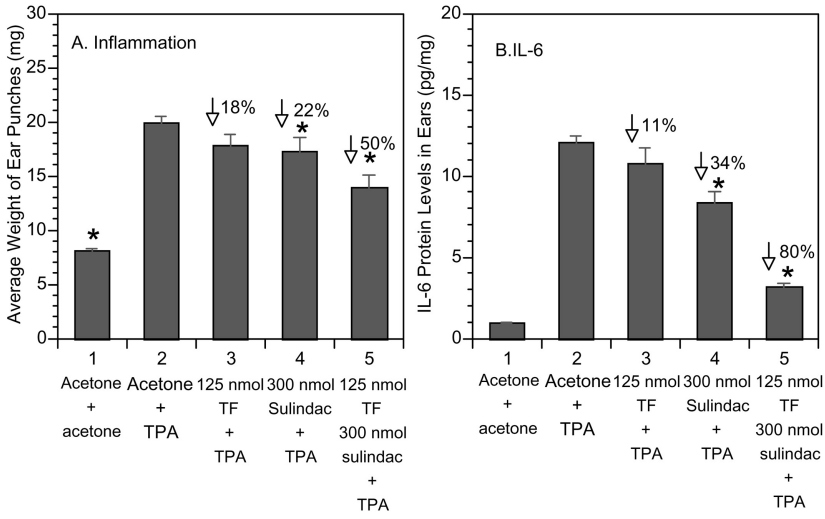

Figure 5. Combination of TF with sulindac on TPA-induced persistent inflammation and up-expression of IL-6 protein levels in mouse ears. Both ears of female CD-1 mice (6–7-wk-old; six mice *per* group) were treated topically with 10 μL of acetone or test compound in acetone 20 min prior to each topical application of 10 μL acetone or TPA (0.8 nmol) in acetone once a day. These treatments were continued for 4 days (total four treatments). Mice were sacrificed 6 h after the last TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Twelve ear samples were pooled and homogenized in a phosphate buffer. Supernatant fraction was used for IL-6 protein ELISA triplicate assays. Data are mean ± SE. *Statistically different from the group 2 (acetone + TPA) ($p < 0.05$) as determined by the Student's *t*-test.

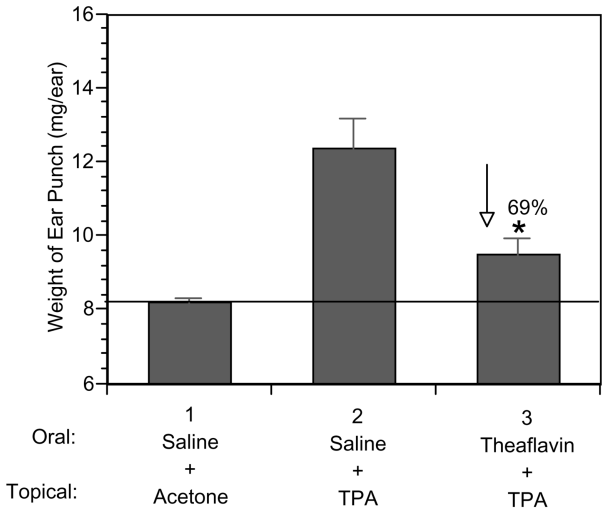

Figure 6. Inhibitory effect of oral gavages of TF mixture on TPA-induced ear edema of the CD-1 mouse. Female CD-1 mice (five mice *per* group) were given gavages of 1 mL of normal saline or TF mixture in normal saline 60 and 20 min prior to topical application of acetone or TPA (1 nmol) in acetone. Mice were sacrificed 6 h after TPA treatment. Ear punches (6 mm in diameter) were taken and weighed. Data are mean ± SE from ten ear samples. *Statistically different from the group 2 (saline + TPA) as determined by the Student's *t*-test.

disorders [20, 21] resulted in an increased interest in natural anti-inflammatory ingredients affecting AA metabolism [22]. In this paper, the anti-inflammatory activity of TF and its derivatives were evaluated in the mouse ear model. The results of the present study demonstrate that topical application of the black tea polyphenolic constituents TF and its derivatives onto the ears of the mice strongly inhibited TPA-induced inflammation, and up-expression of proinflammatory cytokines IL-1β and IL-6, protein levels and decreased TPA-induced formation of PGE_2 and LTB_4 in ears of the female CD-1 mice. The degree of their anti-inflammatory activity is proportional to the number of phenolic groups (Fig. 2). TF and its derivatives have more number of the phenolic groups than that of green tea constituent EGCG (Fig. 1). Our results confirmed that TF and its derivatives had a stronger anti-inflammatory activity than EGCG *in vivo* (Fig. 2). We have recently developed a mouse ear model for persistent inflammation. Topical application of TPA once a day for 4 days resulted in persistent inflammation, up-expression of proinflammatory cytokines, and a linear increase in PGE_2 and LTB_4 level in mouse ears. Application of TF and its derivatives to ears of the mouse prior to each TPA treatment once a day for 4 days inhibited persistent inflammation, up-expression of IL-1β and IL-6 protein levels, as well as inhibited TPA-induced increases in PGE_2 and LTB_4 levels in ears. Inhibition of AA metabolism by TF and its derivatives plays a very important role in their anti-inflammatory activity *in vivo*. Our data demonstrated that TF and its derivatives had a stronger inhibitory effect on the lipoxygenase pathway than on the COX pathway (Table 2 and Fig. 4). Addition of sulindac in combination with TF or TF-3,3′-diG produced a synergetic inhibitory effect on TPA-induced ear inflammation. Oral administration of TFs also inhibited TPA-induced ear inflammation (Fig. 6). Further studies are needed to demonstrate whether the *in vivo* anti-inflammatory activity of TF and its derivatives is due to their parent compounds or their metabolites. Prolonged persistent inflammation (chronic inflammation) causes and accelerates to develop certain degenerative diseases such as atherosclerosis, arthritis, aging, and cancer [16, 23–25]. Many nutraceuticals including fruits, vegetables, green tea, and black tea polyphenolic compounds are reported to have anti-inflammatory activity to protect against certain chronic related degenerative diseases [16, 23–26].

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The metabolism of AA *via* the lipoxygenase and COX pathways results in formation of reactive oxygen species and other free radicals, and studies in mouse epidermis cells have indicated that inhibitors of AA metabolism can diminish the chemiluminescence's response that is associated with the generation of free radicals [27]. Topical application of benzoyl peroxide onto ears of mice induced inflammation in ears, which suggested that oxygen free radical induces inflammation (unpublished observation). Black tea constituents TF and its derivatives inhibit AA metabolism *via* the lipoxygenase and COX pathways and thus inhibit formation of reactive oxygen species and inflammation. In addition, some proinflammatory cytokines can trigger the up-regulation of other proinflammatory cytokines and chemokines, as well as increase the expression of many cellular adhesion molecules (CAMS), selectins, integrins, and immunoglobulins [28]. TF and its derivatives also inhibit TPA-induced up-regulation of proinflammatory cytokines and thus block IL-1β to induce up-expression of other proinflammatory cytokines and chemokines. Consequently, TF and its derivatives had strong anti-inflammatory activity due to their ability to inhibit AA metabolism and ability to inhibit overexpression of proinflammatory cytokines, IL-1β and IL-6.

5 References

[1] Robertson, A., in: K. Willson, M. Clifford (Eds.), *Tea: Cultivation to Consumption*, Chapman and Hall, London 1992, pp. 555 – 601.

[2] Bokuchava, M. A., Skobeleva, N. I., *Crit. Rev. Food Sci. Nutr.* 1980, *12*, 303 – 370.

[3] Pan, M. H., Lin-Shiau, S. Y., Ho, C.-T., Lin, J. H., Lin, J. K., *Biochem. Pharm.* 2000, *59*, 357 – 367.

[4] Mahanta, P., Baruah, H., *J. Agric. Food Chem.* 1992, *40*, 860 – 863.

[5] Sang, S., Yang, C. S., Ho, C.-T., *Phytochem. Rev.* 2004, *3*, 229 – 241.

[6] Apostolides, Z., Balentine, D. A., Harbowy, M. E., Weisburger, J. H., *Mutat. Res.* 1996, *359*, 159 – 163.

[7] Chen, H. Y., Yen, G. C., *Mutat. Res.* 1997, *393*, 115 – 122.

[8] Kuroda, Y., Hara, Y., *Mutat. Res.* 1999, *436*, 69 – 97.

[9] Lin, J. K., Liang, Y. C., *Proc. Natl. Sci. Counc. Rep. China B* 2000, *24*, 1 – 13.

[10] Crouvezier, S., Powell, B., Keir, D., Yaqoob, P., *Cytokine* 2001, *13*, 280 – 286.

[11] Duffy, S. J., Keaney, J. F., Jr., Holbrook, M., Gokce, N. *et al.*, *Circulation* 2001, *104*, 151 – 156.

[12] Wolfram, R. M., Oguogho, A., Efthimiou, Y., Budinsky, A. C., Sinzinger, H., *Prostaglandins Leukot. Essent. Fatty Acids* 2002, *66*, 529 – 533.

[13] Davies, M. J., Judd, J. T., Baer, D. J., Clevidence, B. A. *et al.*, *J. Nutr.* 2003, *133*, 3298S – 3302S.

[14] Coussens, L. M., Werb, Z., *Nature* 2002, *420*, 860 – 867.

[15] Splettstoesser, W., Schuff-Werner, P., *Microscopy Res. Technique* 2002, *57*, 441 – 455.

[16] Huang, M. T., Ghai, G., Ho, C.-T., *Comp. Rev. Food Sci. Food Safety* 2004, *3*, 127 – 139.

[17] Ferreira, S. H., in: J. C. Houck (Ed.), *Chemical Messengers of the Inflammatory Process*, Elsevier/North-Holland Biomedical Press: Washington, D.C. 1979, pp. 113 – 152.

[18] Lewis, J., Adams, D., *Carcinogenesis* 1987, *8*, 889 – 898.

[19] Sang, S., Lambert, J. D., Tian, S., Hong, J. *et al.*, *Bioorg. Med. Chem.* 2004, *12*, 459 – 467.

[20] Claria, J., Romano, M., *Curr. Pharmaceut. Design* 2005, *11*, 3431 – 3447.

[21] Lötzer, K., Funk, C. D., Habenicht, A. J. R., *BBA – Mol. Cell Biology Lipids* 2005, *1736*, 30 – 37.

[22] Grzanna, R., Lindmark, L., Frondoza, C. G., *J. Med. Food* 2005, *8*, 125 – 132.

[23] Saade, N. E., Nasr, I. W., Massaad, C. A., Safieh-Garabedian, B. *et al.*, *Brit. J. Pharm.* 2000, *131*, 1317 – 1324.

[24] O'Byrne, K. J., Dalglei, A. G., *Brit. J. Cancer* 2001, *85*, 473 – 483.

[25] O'Byrne, K. J., Dalgleish, A. G., Browning, M. J., Steward, W. P., Harris, A. L., *Eur. J. Cancer* 2000, *36*, 151 – 169.

[26] Weisburger, J. H., *Eur. J. Cancer Prevention* 2001, *11 Suppl 2*, 51 – 57.

[27] Fischer, S. M., Adams, L. M., *Cancer Res.* 1985, *45*, 3130 – 3136.

[28] Saklatvala, J., Dean, J., Clark, A., *Biochem. Soc. Symp.* 2003, *70*, 95 – 106.

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