Inhibition of TPA-Induced Protein Kinase C and Transcription Activator Protein-1 Binding Activities by Theaflavin-3,3′-digallate from Black Tea in NIH3T3 Cells

Yen-Chou Chen,[†] Yu-Chih Liang,[†] Shoei-Yn Lin-Shiau,[‡] Chi-Tang Ho,[§] and Jen-Kun Lin*,[†]

Institutes of Biochemistry and Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, and Department of Food Science, Cook College, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901-8520

Tea is one of the most popular beverages in the world. Several reports have shown that both green tea and black tea were able to inhibit tumor cell proliferation in animal models. In this study, we investigated the inhibitory effects of black tea polyphenols including theaflavin (TF-1), the mixture (TF-2) of theaflavin-3-gallate (TF-2a), and theaflavin-3′-gallate (TF-2b), theaflavin-3,3′-digallate (TF-3), thearubigin (TR), and a major green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced protein kinase C (PKC) and transcription activator protein-1 (AP-1) binding activities in NIH3T3 cells. On analysis of PKC activity with partial purified preparation, TPA (100 ng/mL) treatment was able to elevate membrane-associated PKC activity ∼3-fold, and treatment with TF-3 (20 μM) and EGCG (20 μM) showed 94.5% and 9.4% suppression on TPA-induced PKC activity, respectively. Translocation of PKCα protein from cytosol to membrane was detected in TPA-treated NIH3T3 cells, and TF-3 was able to block its translocation. By in vitro kinase assay using myelin basic protein (MBP) as a PKC-specific substrate, we found that TPA treatment was able to increase PKC kinase activity by detection of phosphorylated MBP protein and TF-3 showed strongest inhibitory effect on its phosphorylation while EGCG was shown to be less effective. We also analyzed the AP-1 binding activity by electrophoretic mobility shift assay and c-Jun gene expression by northern blot and western blot, the results showed that TF-3 is the most potent inhibitor on TPA-induced AP-1 binding activity and c-Jun gene expression among these five tea polyphenols. Our results might provide new molecular basis for understanding the inhibitory effects of tea polyphenols on TPA-mediated tumor promotion.

Keywords: *Theaflavin-3,3′-digallate; PKC; AP-1 binding activity; TPA; tumor promotion; black tea*

INTRODUCTION

Tumor promotion is an important process in carcinogenesis and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was used as a potent tumor promoter in mouse skin models (Slaga, 1983). On the basis of several studies, TPA treatment was able to activate protein kinase C (PKC) (Blumber, 1988; Fujiki et al., 1984), increase ornithine decarboxylase (ODC) activity (Birt et al., 1986) and induce immediate early gene expression through an increase of reactive oxygen species generation (Wu et al., 1992; Grausz et al., 1986). Therefore, an agent that is able to block TPA-mediated cellular responses is thought as a promising antitumor promoter.

Tea is one of the most popular beverage in the world. More than 300 different kinds of tea are produced by different fermentation processes and divided into three general forms: the unfermented greens tea, the partially fermented oolong tea, and the fermented black tea. Both green and black teas have shown anticarcinogenic activity in many animal models in vivo (Katiyar et al., 1993a,b), and the role of tea extracts as cancer chemopreventive agents are of great interest.

The major green tea polyphenols are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). Among these polyphenols, EGCG is the dominant component and has inhibitory effects on epidermal growth factor (EGF) receptor autophosphorylation and LPS-induced iNOS production in our previous studies (Liang et al., 1997; Lin et al., 1997). Black tea leaves are produced through extensive enzymatic oxidation of polyphenols to dark products such as theaflavins and thearubigins (TR). The major theaflavins in black tea are theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3). Oral administration of black tea extract inhibits NMBzA-induced esophageal tumors in rats and NNK-induced lung tumors in mice (Shi et al., 1994; Wang et al., 1994).

In this study, we investigated the effects of five tea polyphenols [TF-1, TF-2 (a mixture of TF-2a and TF-2b), TF-3, TR, and EGCG] on TPA-induced PKC and AP-1 binding activity. Our results provide some molecular basis for understanding inhibitory effects of tea polyphenols on TPA-mediated tumor promotion.

* Author to whom correspondence should be addressed: Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan. Telephone: (886)-2-2356-2213. Fax: (886)-2-2391-8944.

[†] Institute of Biochemistry.

[‡] Institute of Toxicology.

[§] Rutgers, The State University of New Jersey.

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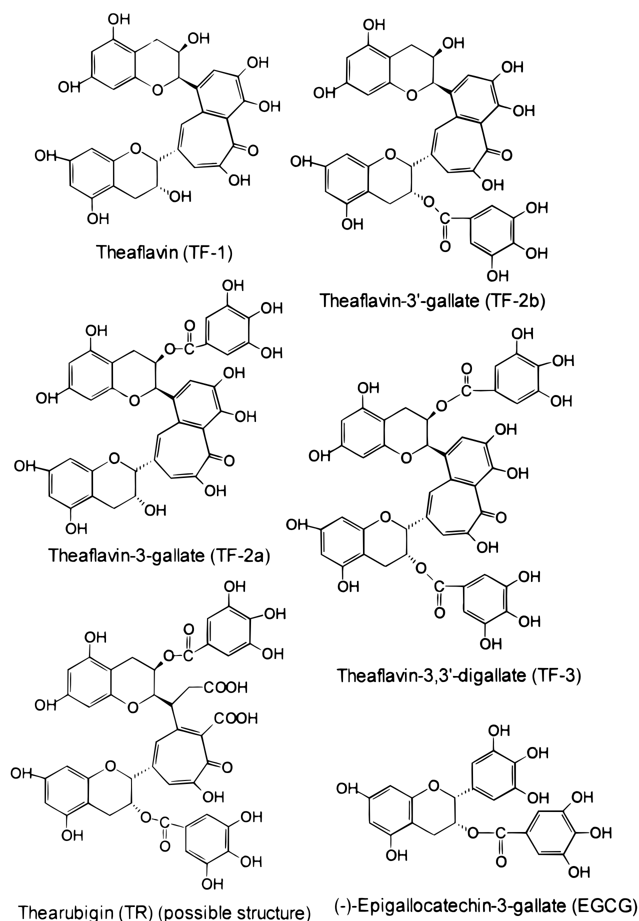


Figure 1. Structures of thearubigin, theaflavins (TFs), and (−)-epigallocatechin-3′-gallate. In the present study, TF-2 is a mixture of TF-2a and TF-2b. The structure of thearubigin is tentative and is suggested by Huang and Ferraro (1992).

MATERIALS AND METHODS

Reagents and Cells. TF-1 (theaflavin), TF-2 (a mixture of theaflavin-3-gallate and theaflavin-3′-gallate), TF-3 (theaflavin-3,3′-digallate), and TR (thearubigin) were isolated from black tea as described previously (Chen and Ho, 1995). EGCG was purified from Chinese tea (Longjing tea, *Camellia sinensis*) as described in our previous report (Lin et al., 1996), and its purity was more than 95%. NIH3T3 cells, the mouse embryonic cells, were obtained from Veterans General Hospital (Taipei, Taiwan, ROC). The cells were maintained in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% glutamine and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Fractionation of Cellular Extract and Analysis of PKC Activity. NIH3T3 cells (6×10^5) were plated in 10-cm Petri dish and maintained at 37 °C in an incubator for 24 h, followed by replacing the medium with serum-free DMEM medium for a further 24 h. After serum-free medium treatment, NIH3T3 cells were treated with 100 ng/mL TPA (Sigma Chemical; 25 μg/mL of stock solution in ethanol) or cotreated with tea polyphenols (TF-1, TF-2, TF-3, TR, or EGCG) and TPA for indicated times. The tea polyphenols were dissolved in 5 μL of dimethyl sulfoxide and added to the inside wall of the Petri dish by micropipet and then mixed gently with medium. After treatment, the cells were harvested with a scraper in 5 mL of PBS, and the cytosolic fraction and membrane fraction were isolated as described in our previous study (Lee and Lin, 1997). The PKC activity was analyzed by a PKC enzyme assay system (RPN77, Amersham, Little Chalfont, Bucks, UK). All the PKC assay procedures were described from the recipe of the kit. The 900 μM PKC substrate−histone IIIS reagent was replaced by 300 μM PKC-specific substrate−myelin basic protein (MBP)

Table 1. Effects of Tea Polyphenols on the TPA-Induced PKC Activity in HIH3T3 Cells

treatment[a]	PKC activity (pmol of P/mg of protein) cytosol	membrane	ratio of Mem/Mem+Cyt
control	2011 ± 98	708 ± 21	0.26
TPA (100 ng/mL)	1042 ± 25	1930 ± 55	0.65
TPA+			
TF-1 (20 μM)	1069 ± 52	1911 ± 77	0.64
TF-1 (40 μM)	1242 ± 47	1638 ± 34	0.56
TF-2 (20 μM)	1070 ± 31	1596 ± 43	0.60
TF-2 (40 μM)	1276 ± 29	1672 ± 88	0.57
TF-3 (20 μM)	1931 ± 62	871 ± 19	0.31
TF-3 (40 μM)	2149 ± 89	775 ± 10	0.27
TR (20 μM)	1035 ± 16	1886 ± 58	0.64
TR (40 μM)	1138 ± 34	1932 ± 81	0.63
EGCG (20 μM)	1104 ± 11	1814 ± 43	0.62
EGCG (40 μM)	1560 ± 21	1338 ± 23	0.46

[a] Cells were treated with TPA (100 ng/mL) only or TPA plus various concentration of tea polyphenols (TF-1, TF-2, TF-3, TR, and EGCG) as indicated above for 1 h. Cells were then harvested, and subcellular fractions prepared by centrifugation as described under Materials and Methods. PKC activity was determined for cytosolic and membrane fractions. Values represent means ± SEM of three separate experiments.

synthetic oligopeptide, because MBP is a more specific substrate for PKC than histone IIIS. This oligopeptide consists of 11 amino acids for 4th to the 14th amino acid (QKRPSQRSKY) (UBI, Lake Placid, NY). The reaction was carried out by incubation at 25 °C for 10 min. The reaction mixture was spotted onto P-81 binding paper, the spotted papers were washed three times with 5% trichloroacetic acid and dried in air in the hood, and the radioactivity was detected by a β counter.

Western Blotting. Equal amounts of proteins (30 μg) (estimated by bicinchoninic acid assay, Promega Corp., Madison, WI), which were prepared as described previously, were mixed with 2× sample buffer and resolved by 10% SDS−polyacrylamide gel electrophoresis for PKCα detection. Proteins were electrotransferred to an immobilon membrane (PVDF; Millipore Corp., Bedford, MA), and equivalent protein loading was verified by staining the membrane with reversible dye amido black (Sigma Chemical Co.). This was followed by overnight blocking with a solution composed of 20 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 4% nonfat dry milk, and 0.1% sodium azide. The membrane was incubated with anti-PKCα polyclonal antibody or anti-c-Jun monoclonal antibody (Oncogene Science, Inc., Manhasset, NY) for 3 h, and with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G antibody for another 1 h. Protein was visualized by incubation with colorigenic substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as suggested by the manufacturer (Sigma Chemical Co.).

Total RNA Isolation and Northern Blot Analysis. NIH3T3 cells were grown in 10-cm dishes to approximately 80% confluence and were then serum-starved for 24−36 h. TPA, with or without tea polyphenols, were added to the medium for 60 min, and the cells were harvested for RNA extraction. Total RNA was isolated as described by Chomczynski and Sacchi (Chomcynsk and Sacchi, 1987). For northern blots, 25 μg of total RNA per lane was fractionated by electrophoresis on 1.2% agarose gel containing 6.7% formaldehyde. RNA was transferred to Nylon filters (Bio-Rad, Richmond, CA), incubated, prehybridized, and hybridized for 12−16 h at 42 °C in hybridization buffer (6× SSC (sodium chloride and sodium citrate), 50% deionized formamide, 10× Denhart's solution, 10 mM EDTA, 0.1% SDS) containing approximately 1×10^6 cpm/mL of probes (Maniantis et al., 1989). The filters was then washed successively in 2× SSC, 0.1× SDS, 0.5× SSC, 0.1% SDS, 0.1% SSC, and 0.1% SDS for 15 min in each solution at room temperature. Filters were then exposed to X-ray film (Kodak XAR-5) between two intensifying screens at −70 °C.

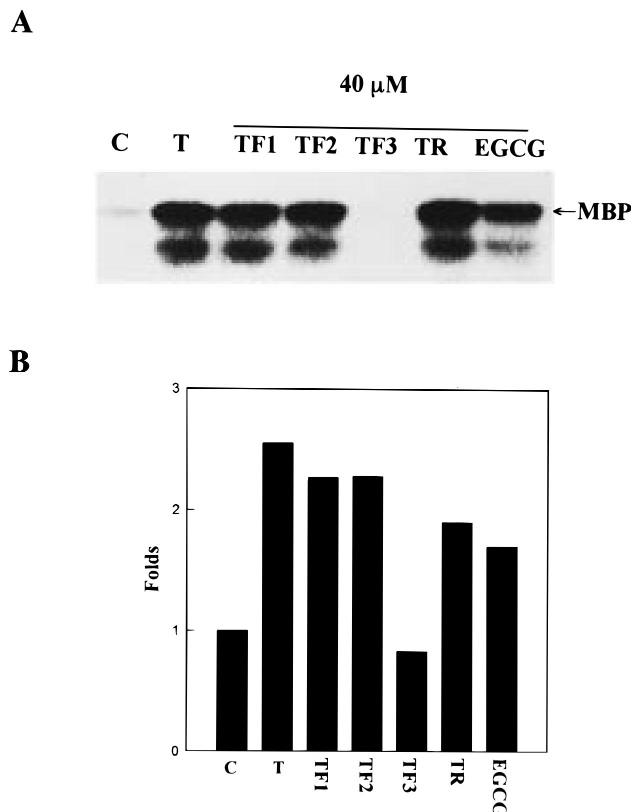


Figure 2. Effects of theaflavins, thearubigin, and EGCG on TPA-induced PKC activity. (A) Serum-starved NIH3T3 cells were treated with 100 ng/mL of TPA only (T) or cotreated TPA and various tea polyphenols (TF-1, TF-2, TF-3, TR, or EGCG) for 1 h. In vitro kinase assay was performed as described under Materials and Methods. (B) Band intensities were quantified by densitometry (IS-1000 Digital Imaging System). This experiment was repeated three times with similar results.

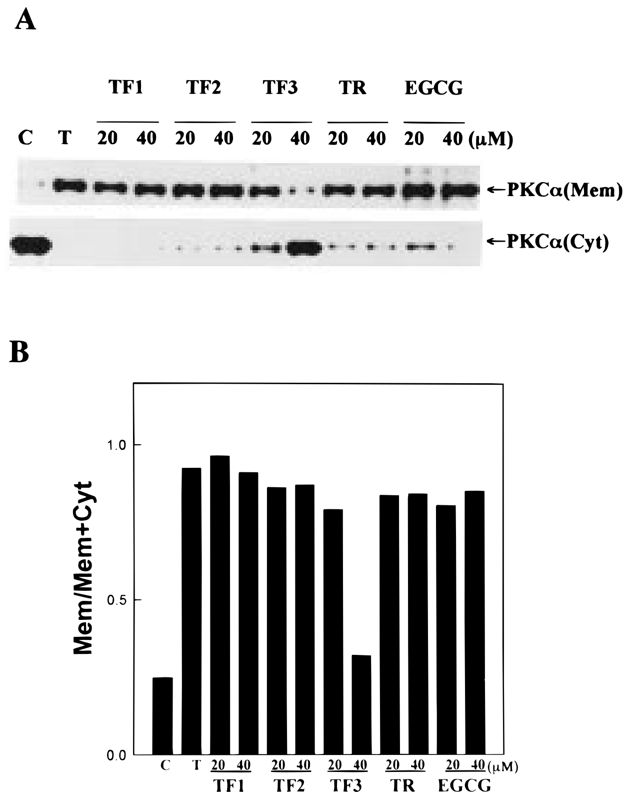


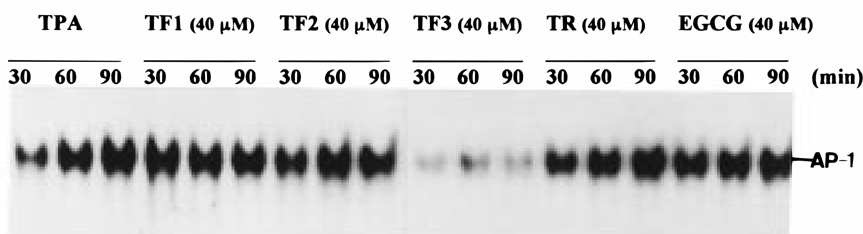
Figure 3. Inhibition of TPA-induced PKCα translocation by tea polyphenols. (A) Serum-straved NIH3T3 cells were treated with 100 ng/mL TPA only or cotreated with TPA and various doses (20 or 40 μM) of tea polyphenols for 1 h, and the cytosolic and membrane fractions were extracted as described previously. Detection of PKCα protein was performed by western blotting using anti-PKCα monoclonal antibody. (B) Band intensities were quantified by densitometry (IS-1000 Digital Imaging System) and the ratio of Mem/Mem+Cyt was indicated. This experiment was repeated three times with similar results.

In Vitro PKC Activity Assay. The membrane proteins (100 μg) were mixed with anti-PKCα antibody (1 μg) and incubated at 4 °C for 1 h, followed by adding A plus G agarose [an agarose conjugated with protein A from *Staphylococcus aureus* and protein G from *Streptococcus* sp. Group C (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] for further 16 h. The antigen/antibody conjugated to protein A was washed 3 times with lysis buffer and once with kinase buffer (20 mM β-glycerophosphate, pH 7.3, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether-*N,N,N,N*-tetraacetic acid (EGTA), 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). The complex was then incubated for 15 min at 30 °C in 10 μL of kinase buffer with 50 μM ATP, 10 μCi of ^{32}P-γ-ATP, and 1 μg of myelin basic protein (MBP, UBI) as a PKC-specific substrate. The reaction was terminated by adding 2× SDS−sample buffer, and the sample was analyzed by 10 SDS−polyacrylamide gel electrophoresis. Gels were then dried and exposed to X-ray film (Kodak XAR-5).

Electrophoresis Mobility Shift Assay (EMSA). The cells extracts and the gel mobility−shift assay were performed as described elsewhere (Chen et al., 1998). An equal amount of nuclear protein (5 μg) (estimated by bicinchoninic acid assay, Promega Corp., Madison, WI) from each sample was incubated with ^{32}P-labeled double oligonucleotides containing the binding sequence of AP-1 element (Oncogene Science, Inc., Manhasset, NY), and electrophoresis on 5% TBE−acrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film (Kodak XAR-5) at −70 °C for 24 h.

RESULTS

The chemical structures of tea polyphenols that were used in this study are illustrated in Figure 1. EGCG is prepared from Chinese tea (Longjing tea, *C. sinensis*) as described in our previous report (Lin et al., 1996), and theaflavins (TF-1, TF-2, and TF-3) are purified from black tea leaves as described previously (Chen and Ho, 1995). TF-2 is a mixture of TF-2a and TF-2b. As TPA is an activator of PKC, we studied the inhibitory effects of these five tea polyphenols on TPA-induced PKC activity using partially purified preparation of PKC from NIH3T3 cells. As shown in Table 1, TF3 is the most potent inhibitor of PKC activity among these five polyphenols, and the inhibitory order is TF-3 > EGCG > TF-2 > TF-1 > TR. In TPA-treated NIH3T3 cells, the PKC activity in membrane fraction was elevated about 3-fold, and TF3, EGCG, and TF2 (20 μM) resulted in 86.6, 9.4, and 27.3% suppression of TPA-induced PKC activity, respectively. When the concentrations of these compounds were elevated to 40 μM, the inhibition of PKC activity was 94.5, 64.7, and 21.1%, respectively. To further confirm the effects of tea polyphenols on PKC activity, an in vitro kinase assay was performed using PKC-specific substrate myelin basic protein (MBP). The results showed that TPA treatment was able to increase of membrane-associated PKC activity about 3-fold and TF3 treatment reduced TPA-induced PKC activity to basal level. EGCG, TF1, and TF2 also showed slightly inhibitory effects on TPA-induced PKC activity (Figure 2). Translocation of PKC protein from cytosol to membrane is a well-known biomarker of TPA-induced PKC



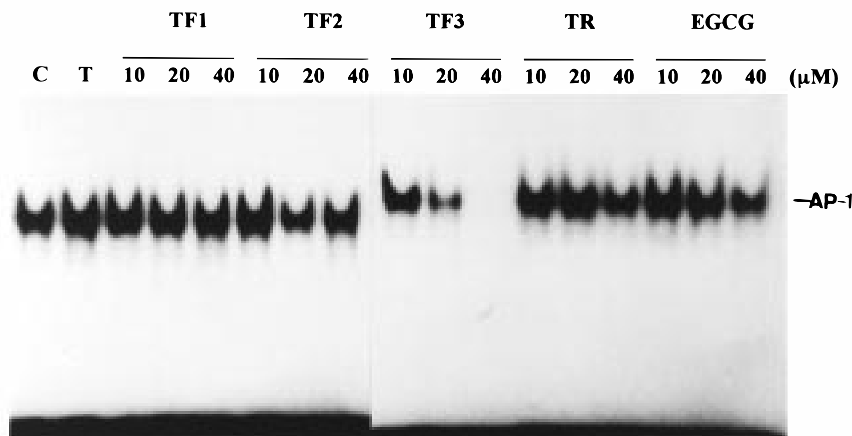


Figure 4. Inhibition of TPA-induced AP-1 binding activity by tea polyphenols. (A) Serum-starved NIH3T3 cells were treated with 100 ng/mL TPA (T) or cotreated with TPA and various tea polyphenols (40 μM) for indicated times (30, 60, or 90 min), and analysis of AP-1 binding activity was performed by EMSA. (B) Cells were treated with 100 ng/mL TPA or cotreated with 100 ng/mL TPA and various doses of tea polyphenols (10, 20, or 40 μM) for 90 min and AP-1 binding activity was performed.

activation. Upon TPA treatment, almost all of PKCα protein was translocated from cytosolic fraction to membrane fraction, and TF3 treatment showed the remarkable blocking effects on its translocation (Figure 3). On the basis of these data, it seems that TF3 is the most potent inhibitor on TPA-induced PKC activation among these five tea polyphenols.

AP-1 binding activity was located downstream of PKC activation in the TPA-induced signaling pathway, so we investigated whether these five tea polyphenols could suppress TPA-induced AP-1 binding activity by EMSA (Figure 4). When quiescent NIH3T3 cells were treated with 100 ng/mL TPA, AP-1 binding activity was induced and increased with a treating time of 90 min (Figure 4A). With exposure of quiescent NIH3T3 cells to 100 ng/mL TPA with 10, 20, and 40 μM tea polyphenols for 90 min, the results showed that TF3 was able to inhibit TPA-induced AP-1 binding activity in a dose-dependent manner, and the concentration that caused 50% inhibition (IC_{50}) is ~15 μM (Figure 4B). Upon 40 μM treatment for 90 min, TF3, EGCG, TF2, TF1, and TR resulted in 90, 56, 50, 40, and 20% suppression of TPA-induced AP-1 binding activity, respectively. Competition studies were performed to confirm the specificity of binding complex. The AP-1 complex disappeared on addition of 25-fold molar excess of unlabeled AP-1 consensus oligonucleotides, but in contrast, the addition of 25-fold molar excess of unlabeled oligonucleotides containing AP-1 mutant sequence did not affect its binding activity (data not shown).

TPA can stimulate the transient expression of c-Jun, which is a protooncogene, so we investigated whether these five tea polyphenols could suppress TPA-induced c-Jun transcription and translation. When quiescent NIH3T3 cells were treated with 100 ng/mL TPA, c-Jun mRNA was transiently induced and reached a maximal level in 60−90 min (Huang et al., 1991). Exposure of quiescent cells to 100 ng/mL TPA with different concentrations of tea polyphenols was performed, and c-Jun mRNA and protein were analyzed by northern and western blots (Figure 5A,B). The results indicated that TF-2, TF-3, and EGCG were able to inhibit TPA-induced c-Jun gene expression at mRNA and protein levels, and TF-3 appeared to be the strongest one.

DISCUSSION

Tea is one of the most popular beverages in the world because of its attractive flavor and aroma. Polyphenols are the most significant group of tea components, especially the catechin group of the flavanols. The major green tea catechins are EGCG, EGC, ECG, EC, (+)-gallocatechin, and (+)-catechin. In the manufacture of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase dependent oxidative polymerization leading to the formation of bisflavanols, theaflavins, thearubigins, and other oligomers in a process commonly known

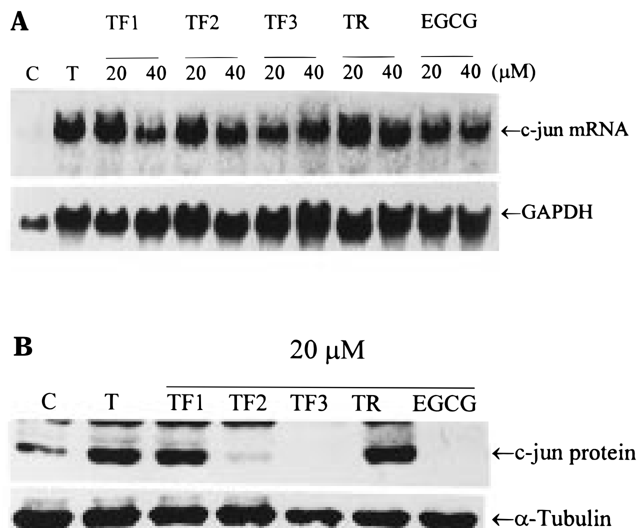


Figure 5. Effects of tea polyphenols on TPA-induced c-Jun gene expression. (A) Serum-starved NIH3T3 cells were treated with 100 ng/mL TPA (T) or cotreated with 100 ng/mL TPA and various doses of tea polyphenols (20 or 40 μM) for 1 h, and analysis of c-Jun mRNA was performed by northern blot as described under Materials and Methods. (B) Serum-starved cells were treated with 100 ng/mL TPA (T) or cotreated with TPA and 20 μM indicated tea polyphenols for 1 h, and detection of c-Jun protein was performed by western blot using anti-c-Jun antibody.

as "fermentation". Theaflavins (TFs, about 1−2% of the total dry weight of black tea), including theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b), and theaflavin-3,3′-digallate (TF-3), possess benzotroprolone rings with dihydroxy or trihydroxy substitution systems. About 10−20% of the dry weight of black tea is due to thearubigin (TR), which are more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well characterized.

Human carcinogenesis is known to proceed through multiple stages, such as initiation, promotion, and progression (Weinstein, 1988). Tumor promotion, which is a complex and important process in carcinogenesis, is generally studied using TPA as tumor promoter in mouse skin models (Slaga, 1983; Lee and Lin, 1997). Both green tea and black tea have shown the ability to suppress TPA-mediated tumor-promoting effects on mouse skin (Katiyar et al., 1993a); however the active components which performed the effects were still not well characterized. In this study, we investigated the effects of five tea purified polyphenols (TF-1, TF-2, TF-3, TR, and EGCG) on TPA-induced PKC and AP-1 binding activities. The results indicated that TF-3 strongly inhibited TPA-induced PKC and AP-1 binding activity, and the inhibitory order is TF-3 > EGCG > TF-2 > TF-1 > TR. Among theaflavins, TF-3, which has two gallic acid moieties, exhibited the strongest inhibition on TPA-induced effects. We concluded that the gallic acid moiety is important for theaflavins to perform antitumor promotion activity.

The gene expression which can be transiently induced by TPA is one of the events that triggers the onset of tumor promotion. The number of known inducible genes has grown to at least 30 (Carter, 1987; Lerman and Colburn, 1987), of which c-Jun is one of the immediate early protooncogenes closely related to cell growth and transformation (Ryder and Nathans, 1988; Ryseck et al., 1988). It has been reported that induction of c-Jun gene expression is greatly increased in early stages and gradually declines in the late stages of two-step carcinogenesis in C3H10 T1/2 cell models (Wu et al., 1992). According to this point, overinduction of c-Jun by TPA in the early stages of tumor progression may be one of the events leading to carcinogenesis (Nishizuka, 1989), and agents that are able to inhibit TPA-induced c-Jun gene expression might exert their cancer chemopreventive action. In this study, we demonstrated that TF-2, TF-3, and EGCG were able to inhibit TPA-induced c-Jun gene expression at both mRNA and protein levels, and TF-3 showed the most potent inhibitory effect on the c-Jun induction.

AP-1 (activator protein 1) complex comprises members of fos and jun protein families (Nishina et al., 1990). Jun family members can form low-affinity dimers among themselves and high-affinity heterodimers with the fos family components (Ransone and Verma, 1990). Induction of AP-1 binding activity was detected by a plethora of extracellular stimuli including serum, growth factors, and TPA (Nishizuka, 1989), and involvement in the pathway of tumor cell proliferation. In this study, treatment of quiescent NIH3T3 cells with 100 ng/mL TPA was able to elevate AP-1 binding activity, and TF-3 showed potent inhibition on TPA-induced AP-1 binding activity. Others (TF-1, TF2, TR, and EGCG) also showed slight, but less effective, inhibitory effects on AP-1 binding.

In conclusion, we present herein basic data describing various tea polyphenols which may inhibit TPA-induced PKC and AP-1 binding activities. On the basis of this study, TF-3 is an active component in black tea that effectively blocks various TPA-induced gene activations, and it also appears to be a better inhibitor on TPA-induced PKC and AP-1 binding activities than green tea polyphenol EGCG. Our results provide the molecular basis for understanding the active inhibition components of black tea on the TPA-mediated tumor promotion.

ABBREVIATIONS USED

TF-1, theaflavin; TF-2a, theaflavin-3-gallate; TF-2b, theaflavin-3′-gallate; TF-3, theaflavin-3,3′-digallate; TR, thearubigin; EGCG, (−)-epigallocatechin-3-gallate; PKC, protein kinase C; AP-1, transcription activator protein-1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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