Inhibition of Xanthine Oxidase and Suppression of Intracellular Reactive Oxygen Species in HL-60 Cells by Theaflavin-3,3′-digallate, (−)-Epigallocatechin-3-gallate, and Propyl Gallate

Jen-Kun Lin,*,† Ping-Chung Chen,† Chi-Tang Ho,‡ and Shoei-Yn Lin-Shiau§

Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan, Department of Food Science, Rutgers University, Brunswick, New Jersey, and Institutes of Toxicology and Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

The inhibitory effects of five tea polyphenols, namely theaflavin (TF1), theaflavin-3-gallate (TF2), theaflavin-3,3′-digallate (TF3), (−)-epigallocatechin-3-gallate (EGCG), and gallic acid, and propyl gallate (PG) on xanthine oxidase (XO) were investigated. These six antioxidant compounds reduce oxidative stress. Theaflavins and EGCG inhibit XO to produce uric acid and also act as scanvengers of superoxide. TF3 acts as a competitive inhibitor and is the most potent inhibitor of XO among these compounds. Tea polyphenols and PG all have potent inhibitory effects (>50%) on PMA-stimulated superoxide production at 20∼50 μM in HL-60 cells. Gallic acid (GA) showed no inhibition under the same conditions. At 10 μM, only EGCG, TF3, and PG showed significant inhibition with potency of PG > EGCG > TF3. The superoxide scavenging abilities of these six compunds are as follows: EGCG > TF2 > TF1 > GA > TF3 > PG. PG was the most potent inhibitor of PMA-stimulated H_2O_2 production in HL-60 cells. The order of H_2O_2 scavenging ability was TF2 > TF3 > TF1 > EGCG > PG > GA. Therefore, the antioxidative activity of tea polyphenols and PG is due not only to their ability to scavenge superoxides but also to their ability to block XO and related oxidative signal transducers.

Keywords: *Theaflavin-3,3′-digallate; EGCG; xantine oxidase; ROS; superoxide; hydrogen peroxide; allopurinol*

INTRODUCTION

Hydrogen peroxide, hydroxyl radicals, peroxide anions, and superoxide anion are collectively known as reactive oxygen species (ROS). Normal loads of ROS are removed by the enzyme superoxide dismutase (SOD), catalase, and glutathione peroxidase. Excessive amounts of ROS increase the oxidative stress in the body. This accelerates membrane damage, DNA base oxidation, DNA strand breaks, and chromosome aberrations, most of which are involved in the carcinogenesis process (Cerutti, 1985). It has been demonstrated that chemically mediated or phagocytic-release ROS play important roles in mutagenesis and carcinogenesis (Briemer, 1990; Frenkel, 1992). The formation of hydrogen peroxide and oxidized DNA bases by human polymorphonuclear neutrophils stimulated with various tumor promoters correlates well with the in vivo tumor-promoting potencies of the activating chemicals (Frankel et al., 1987).

A tumor promoter such as PMA enhances the generation of ROS accumulation and decreases the ROS detoxification enzymes in both epidermal and inflammatory cells. It triggers ROS accumulation through activation of xanthine oxidase (XO) or the stimulation of PMNs which cause NADPH oxidase activation.

XO is a complex enzyme, containing molybdenum, FAD, and iron/sulfur redox centers, that has been known for at least 95 years. The intestinal mucosa and liver are the richest sources of XO. In the ischemic small intestine, XO is the major source of ROS candidacy, as strengthened by recent analyses of promoter regions of human, mouse, and rat enzymes, which suggest the presence of potential regulatory sites for cytokines known to stimulate generation of ROS.

Tea is one of the most popular beverages in the world. It has been shown that both green tea and black tea have antioxidant activity and are able to inhibit tumor induction (Wang et al., 1994). In recent years, many animal studies and several epidemiological studies have suggested the anticarcinogenic effects of tea (Katiyar, 1996; Bushman, 1998). Extracts of green, black, and other teas inhibited PMA-induced JB6 cell transformation (Jain et al., 1989). Recent studies in our laboratory have demonstrated that both green tea polyphenol EGCG and black tea polyphenol TF3 suppress the EGF-receptor autophosphorylation and proliferative signals in fibroblast cells (Liang et al., 1997, 1999a) and inhibit the activity of inducible nitric oxide synthase in macrophages (Lin and Lin, 1997; Lin et al. 1999a). Furthermore, EGCG can arrest cell division at the G1 phase through inhibiting the cyclin-dependent kinases 2 and 4 and elevating the cdk inhibitors p21 and p27 (Liang et al., 1999b). TF3 can inhibit the activity of protein kinase C and AP-1 binding in NIH 3T3 cells (Chen et

* Please send correspondence to Dr. Jen-Kun Lin, Institute of Biochemistry, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei, Taiwan. Tel: (886)-2-2356-2213. Fax: (886)-2-2391-8944. E-mail: jklin@ha.mc.ntu.edu.tw.

† Institute of Biochemistry, National Taiwan University.

‡ Rutgers University.

§ Institutes of Toxicology and Pharmacology, National Taiwan University.

10.1021/jf000066d CCC: $19.00 © 2000 American Chemical Society
Published on Web 06/27/2000

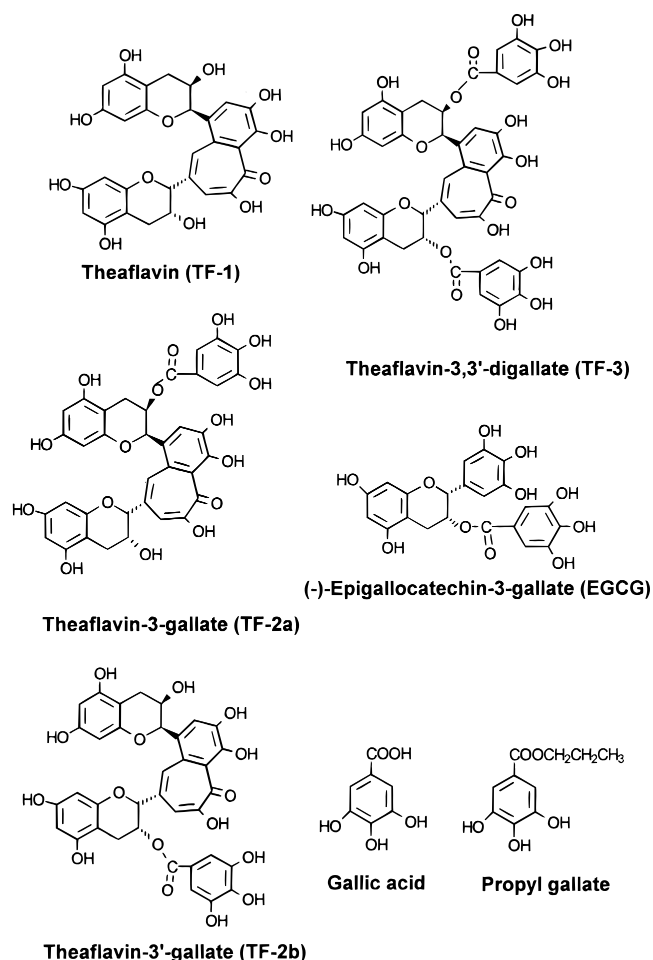

Figure 1. Chemical structures of tea polyphenols.

al., 1999). On the basis of these findings, it has been proposed that the mechanisms of action by tea and its polyphenols on anticarcinogenesis may be through the signal transduction blockade (Lin et al., 1999b). On the other hand, in many investigations of skin carcinogenesis, the phorbol ester PMA was used as a potent tumor promoter in mouse skin. Also, the NADPH oxidase system of neutrophil was found to greatly contribute to O_2^- generation in PMA-treated mouse skin (Roberston et al., 1990). ROS from leukocytes, including O_2^-, plays an important role for continuous and excessive production of chemotactic factors, leading to chronic inflammation and hyperplasia. O_2^- generation inhibitors are agents that effectively inhibit these responses. Since EGCG and theaflavins are antioxidants, it might be of interest to compare the efficacies of these compounds in suppressing the oxidative stress in vitro and in vivo.

In this study, we investigated the effects of tea polyphenols (TF1, TF2, TF3, and EGCG) and gallates (gallic acid and propyl gallate) (Figure 1) on the activity of a ROS-producing enzyme, xanthine oxidase. We compared the inhibitory effects of these six compounds on PMA-stimulated superoxide and hydrogen peroxide production in HL-60 cells. Their scavenging activities to exogenous superoxide and hydrogen peroxide were also studied. The structure−activity relationship among these compounds was discussed.

MATERIALS AND METHODS

Reagents. Lucigenin (M8010), luminol (A8264), phorbol-12-myristate-13-acetate (PMA) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), scopoletin, horseradish peroxidase (HRP), gallic acid, propyl gallate, xanthine oxidase, xanthine, and uric acid were purchased from Sigma (St. Louis, MO). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Aldrich (Milwaukee, WI), and DMSO was purchased from Merck (D-6100 Darmstadt, Germany). Theaflavin (TF1), a mixture of theaflavin-3-gallate and theaflavin-3′-gallate (TF2), and theaflavin-3,3′-digallate were isolated from black tea as described previously (Chen and Ho, 1995). Black tea leaves was extracted with hot water (90 °C). The aqueous extract was first extracted with chloroform to remove caffeine and chlorophyll and then extracted with ethyl acetate to recover total black tea polyphenols. TF1, TF2, and TF3 were isolated from the total polyphenols by column chromatography on Sephadex LH-20 eluting with 30% acetone. (−)-Epigallocatechin-3-gallate (EGCG) was purified from Chinese tea (Longjing tea, *Camellia sinensis*) as described in our previuos report (Lin et al., 1996). Briefly, the total green tea polyphenols were obtained by stepwise extraction of Longjing tea with hot water, choloroform, and then ethyl acetate. The pure EGCG was isolated from the total green tea polyphenols by column chromatography on Sephadex LH-20 eluting with 95% ethanol. The purities of theaflavins and EGCG were assessed by HPLC (Lin et al., 1996). The chemical structures of these tea polyphenols and gallates are illustrated in Figure 1.

Cell Culture and Differentiation Induction. Human promyelocytic lukemia cell line (HL-60) was cultured in RPMI 1640 (a Gibco BRL product, Cat. No. 31800-022; this culture medium contains L-glutamine, but no sodium bicarbonate) supplemented with 15% heat-inactivated fetal bovine serum and 50 U/mL penicillin-streptomycin. Cells were cultured in a humidified atmospheric incubator at 37 °C in 5% CO_2. To induce myeloid differentiation, cells were seeded at a density of 5×10^5 cells/mL and were cultivated for 5 days in RPMI 1640 containing 1.24% DMSO. The characteristics of mature cells were determined by smaller cell size, decreased nucleoli-to-cytoplasm ratio, and pyknotic changes in nuclear chromatin. Cell numbers were counted using a hemocytometer, and cell viability was >98% as evidenced by trypan blue staining.

Chemiluminescence Assay. Superoxide production of PMA-stimulated HL-60 was determined by lucigenin-amplified chemiluminescence (CL) and luminol-dependent CL as described (Gyllenhammar, 1987). CL was assessed by luminometers (models 1251, LKB, Bromma, Sweden) and recorded as millivolts (Palmblad et al., 1984). A total of 2.5×10^5 cells in Hanks' balanced salt solution (HBSS) with 200 μM lucigenin or 20 μM luminol were preincubated with various compounds for 5 min, readings were started by 100 ng/mL PMA, and CL was measured and recorded for 120 min. The total amount of superoxide produced during the assay period was obtained by integrating the area under the curve (in mV min^{-1}).

H_2O_2 Assay. H_2O_2 production by HL-60 cells was determined by measuring HRP-mediated scopoletin oxidation, as previously described (Harpe et al., 1985). A total of 2.5×10^5 cells per well were placed in the assay mixture which was prepared immediately before use from stock solution and consisted of 30 μM scopoletin, 1 mM NaN_3, and 1 purogallin unit/mL, in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Different concentrations of tested compounds (theaflavins, EGCG, gallic acid, propyl gallate) were preincubated for 5 min, then 100 ng/mL PMA was added to the mixture, the plate was placed in a filter fluorometer (CytoFluor 2300, Millipore), and the fluorescence, in arbitrary unit, was recorded for each well. The plate was then transferred to an incubation chamber maintained at 37 °C. After 90 min, the fluorescence in each well was again recorded using the CytoFluor.

Determination of Hydrogen Peroxide by DCF-DA in the Flow Cytometer. DCF-DA is a nonfluorescent probe which becomes fluorescent in the presence of hydrogen peroxide. This dye is a stable nonpolar compound that readily diffuses into the cells and is hydrolyzed by intracellular esterase to yield dichlorodihydrofluorescein (DCFH), which is trapped within the cell. Hydrogen peroxide or low molecular weight peroxides, produced during oxidative respiratory burst, oxidize DCFH to highly fluorescent 2′, 7′-dichlorofluorescein (DCF). HL-60 (1×10^6 cells /mL) were suspended in HBSS

with 50 μM DCF-DA and the desired concentration of tested compounds at 37 °C for 30 min. The cells were stimulated with the addition of 100 ng/mL PMA for a further 30 min and were analyzed in the FACSCAN laser flow cytometry (Becton Dickenson, San Jose, CA).

Estimation of Superoxide Scavenging Activity. The superoxide-producing system was set up by mixing phenazine methosulfate (PMS), NADH, and oxygen (air), and the production of superoxide was estimated by nitroblue tetrazonium method. The reaction mixture (3 mL) containing 10 μM phenazine methosulfate, 78 μM NADH, and 25 μM nitroblue tetrazolium in 0.1 M phosphate buffer pH 7.4 was incubated for 2 min at room temperature and read at 560 nm against a blank containing PMS. Different concentrations of compounds were preincubated for 2 min before adding NADH.

Determination of Xanthine Oxidase Activity. The enzyme activity was measured spectrophotometrically by determining uric acid formation at 295 nm (U-3210 Hitachi) with xanthine as substrate (Kalckar, 1947). The assay system consisted of a 1 mL reaction mixture containing 0.1 M potassium phosphate buffer pH 7.4, 0.004U XO, and xanthine as substrate. All inhibitors were preincubated with enzyme for 5 min, and the reaction was started by addition of xanthine. The reference cuvette was identical, and only the enzyme was absent. The IC_{50} and equations of dose−response curves were analyzed with Sigma plot in Windows 95 based on the experimental data.

RESULTS

Inhibition of Xanthine Oxidase by Tea Polyphenols. Xanthine oxidase activity was determined by the formation of uric acid, which has a maximum absorption in OD 295 nm, providing the basis for a spectrophtometric assay. The linear standard curve of uric acid is constructed and expressed as $y = 0.01296x$, $r^2 = 0.999$ (data not shown).

The effects of six polyphenols on XO activity were tested, and the dose−response curves and IC_{50} values for these six compounds are shown in Figures 2 and 3. The IC_{50} values of both gallic acid and propyl gallate on the inhibition of XO are more than 200 μM. The inhibition ability of theaflavins and EGCG are as follows: TF3 > TF2 > EGCG > TF1. Theaflavin-3,3′-digallate (TF3) (IC_{50}: 4.5 μM) was found to be the most effective inhibitor of XO among the polyphenols tested. In this system, the IC_{50} value for the well-known XO inhibitor allopurinol is 0.68 μM (Figure 3).

The type of inhibition of XO activity by TF3 is analyzed; the results indicate that TF3 acts as a competitive inhibitior (Figure 4), and this inhibition will be discussed later.

PMA-Induced ROS Production of Differentiated HL-60. Incubation of HL-60 with 1.24% DMSO for 0−5 days and then PMA-stimulated ROS production of HL-60 were assayed by DCF-DA (data not shown), and the ability of PMA-induced ROS production is dependent on the time of DMSO treatment. DMSO has been known to induce HL-60 differentiation, which exhibits morphological alteration as well as functional maturity, e.g., the ability to generate superoxide, H_2O_2, and increased myeloperoxidase and phagocytic activities (Nagy et al., 1993).

Total ROS production was detected by the luminol-derived CL assay. PMA-induced ROS production was constitutively expressed at least 50 min (Figure 5A). Both superoxide and hydroxyl radical could be detected by the luminol-derived CL, and only the CL of the hydroxy radical was inhibited by DMSO (Figure 5B). The CL of superoxide was specifically detected by the

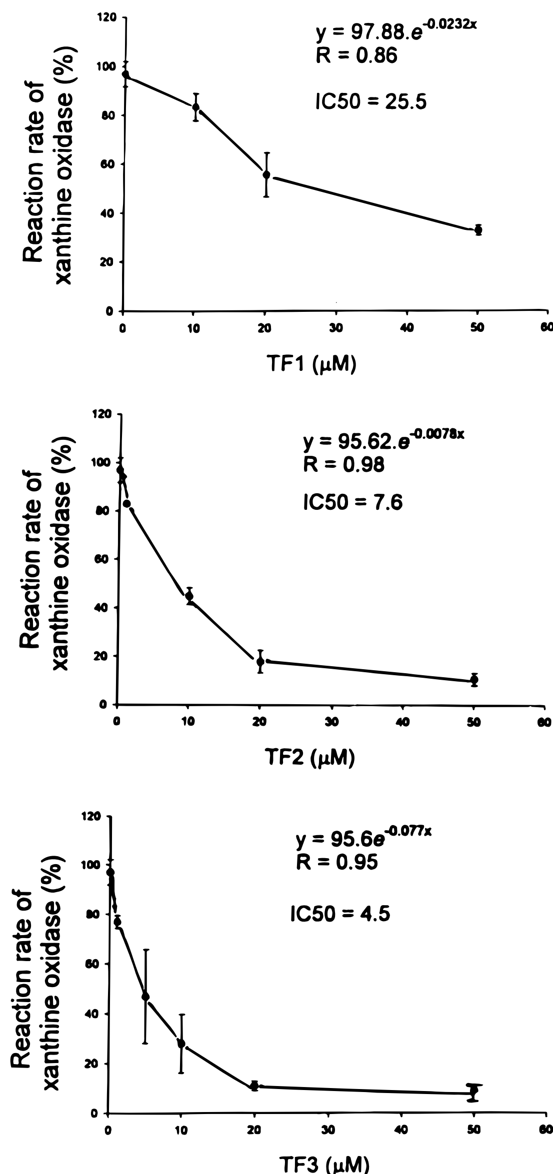

Figure 2. Inhibition of xanthine oxidase by theaflavins in vitro. The formation of uric acid was determined spectrophotometrically at 295 nm as described in the Materials and Methods section. IC_{50} values were calculated from the regression line, $y = ae^{-bx}$ (for TF1, TF2, and TF3).

lucigenin-derived CL method and could not be inhibited by DMSO (Figure 5C).

In the following experiments of the CL assay, CL was recorded for 120 min after PMA treatment, and the effects of compounds were compared with PMA alone. The inhibitory effects of tea polyphenols on PMA-stimulated ROS production were analyzed. The IC_{50} value of tested compounds on PMA-stimulated ROS production was less than 1 μM. DMSO (0.2%) has inhibitory effects in this assay system. (33%), but 10 μM of tested compounds have almost 90% inhibition. It seemed that ROS was effectively scavenged by these compounds.

Effects of Phytopolyphenols on $O_2^{\bullet-}$ Production in PMA-Stimulated HL-60 Cells. Three independent experiments were performed to determine the effect of the selected tea polyphenols on $O_2^{\bullet-}$ production by PMA-stimulated HL-60 cells over a wide concentration range (Figure 6).

Gallic acid has no inhibition effects on $O_2^{\bullet-}$ production by PMA-stimulated HL-60 cells, but propylgallate was

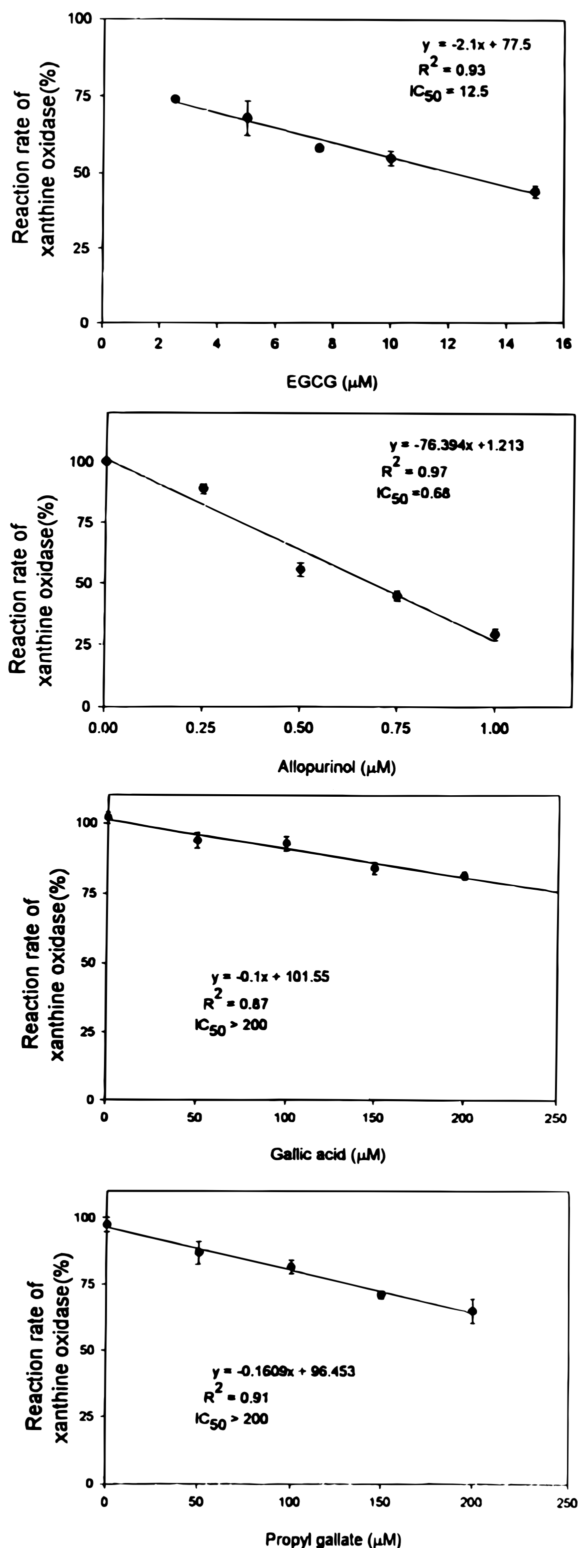

Figure 3. Inhibition of xanthine oxidase by EGCG, gallates, and allopurinol. The reaction mixture is as described in Figure 2 except that EGCG, gallic acid, propyl gallate, and allopurinol were used instead of theaflavins. The linear equation is obtained by Sigma plot based on the experimental data. $y = ax + b$ (for EGCG, gallic acid, and propyl gallate); here, x is the log of the concentration of tested compound, and y is the relative activity of enzyme (%).

the most potent inhibitor at 10 μM. The inhibition ability of these compounds was significantly high, and the degree of inhibition was highly dependent on their concentrations. At 10 μM, the order of inhibition was

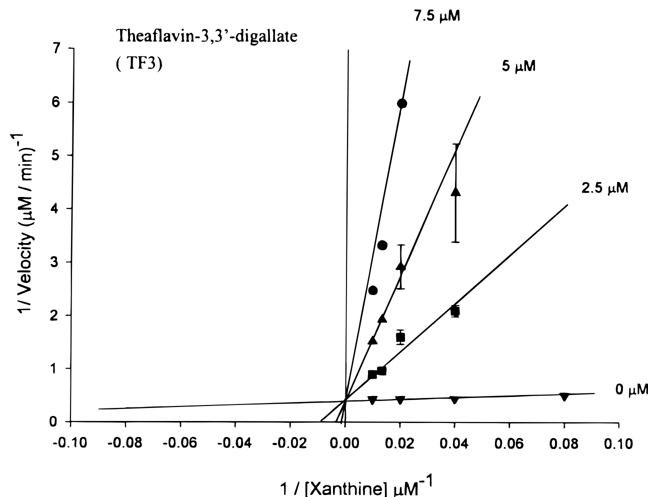

Figure 4. Inhibition of xanthine oxidase by theaflavin-3,3′-digallate. Lineweaver–Burk plots for the inhibition of xanthine oxidase by theaflavin-3,3′-digallate with xanthine as substrate are shown. It appears that a competitive inhibition pattern is demonstrated.

PG > EGCG > TF3 > TF1 > TF2. When the concentration was 20 or 50 μM, the order of inhibition was TF3 > TF1 > EGCG > TF2 > PG. The degree of superoxide scavenging activity of these compounds was estimated as described in Materials and Methods, and the results are presented in Figure 7: EGCG ($IC_{50} = 7.4$ μM) > TF2 ($IC_{50} = 15.6$ μM) > TF1 ($IC_{50} = 13.6$ μM) > GA ($IC_{50} = 42.5$ μM) > TF3 ($IC_{50} = 40.5$ μM) > PG ($IC_{50} = 50.4$ μM).

The inhibition of PMA-stimulated $O_2^{\bullet-}$ production of HL-60 was comparable to the direct scavenging of $O_2^{\bullet-}$ by the tested compounds. The results imply that EGCG, TF1, and TF2 play a major role in extracellular scavenging activity. The effects of TF3 and propyl gallate on $O_2^{\bullet-}$ production in PMA-stimulated HL-60 cells are possibly involved in the signal transduction of the PKC activation pathway that is stimulated by PMA.

Effects of Tea Polyphenols on H_2O_2 Production of PMA-Stimulated HL-60 Cells. Studies of the effects of the selected tea polyphenols on H_2O_2 production by PMA-stimulated HL-60 cells were performed in three independent experiments. The results show that propyl gallate was the most potent inhibitor of PMA-induced H_2O_2 production in HL-60 at the concentration 5–20 μM (Figure 8A). The inhibitory abilities of TF1, TF2, TF3, EGCG, and gallic acid were significant between 5 and 50 μM, but less significant different between 1 and 5 μM. Figure 8B indicates the scavenging effects of tested compounds on exogenous H_2O_2, the potency of H_2O_2 scavenging being TF2 > TF3 > TF1 > EGCG > propylgallate > gallic acid. TF2 was the most potent scavenger of exogenous H_2O_2.

DISCUSSION

Chemoprevention has had a potential impact on cancer incidence rates through the modulation of initiation and promotion stages (Szarka, 1994). Increasing epidemiological studies have suggested that the contents of phytochemicals such as tea polyphenols are ubiquitously present in tea and may reduce the risk of cancer through their role in the metabolism of carcinogens, in hormonal binding, in regulation of gene expression, in antioxidant enzymatic activities, and in scavenging of

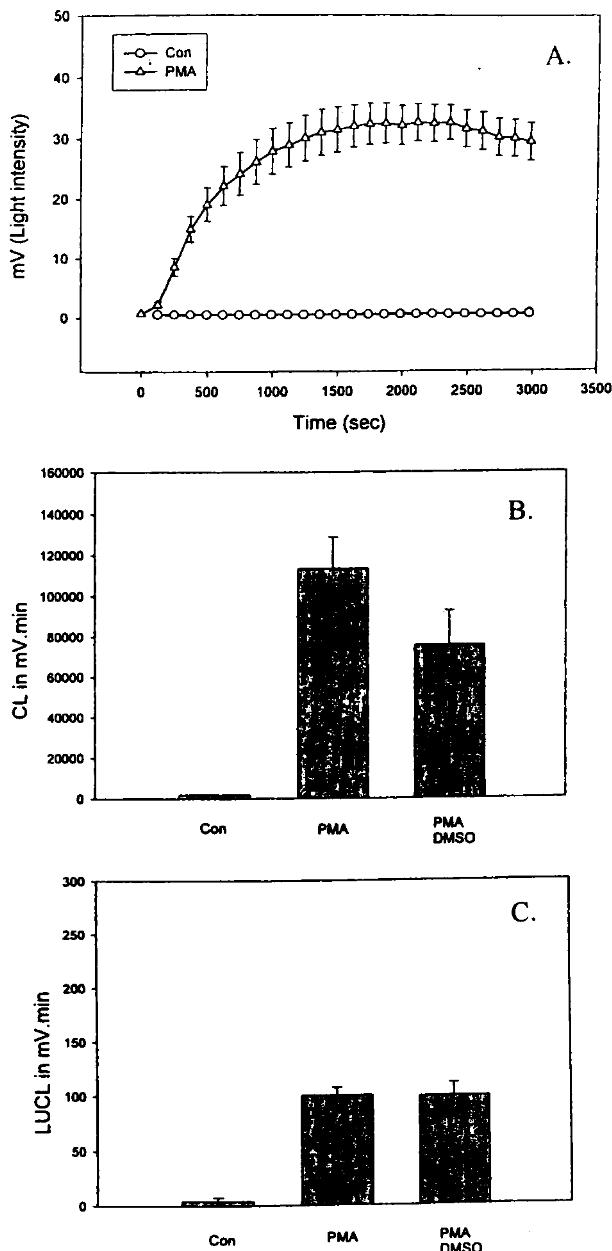

Figure 5. Differential determination of total ROS and superoxide production in PMA-stimulated HL-60 cells by luminol- and lucigenin-derived chemiluminescence. HL-60 cells were stimulated with 100 ng/mL PMA, and chemiluminescence (CL) was measured and recorded for 120 min as described in the Materials and Methods section. The CL response was represented by the average integration (mV min^{-1}). (A) Representative profile of the luminol-derived CL responses of HL-60 cell culture containing 2.5×10^5 cells with or without 100 ng/mL PMA. (B) Representation of the luminol-derived CL for total ROS production. Please note that the hydroxyl radical portion of ROS was inhibited by DMSO. (C) Representation of the lucigenin-derived CL (LUCL) for superoxide production, which was insensitive to DMSO.

free radicals (Lin et al., 1999; Kelloff et al., 1996). Several studies have shown that the differentiated HL-60 cells possess the phagocytic properties and the capability of generating ROS upon stimulation (Wei et al., 1993; Nagy, 1993). These phagocyte-generated oxidants are known to act as complete carcinogens and to cause harmful biological effects via several pathways, i.e., damaging DNA, regulating gene expression, and modulating signal transduction pathways (Weitzman, 1990; Frenkel, 1992). It is well known that generation

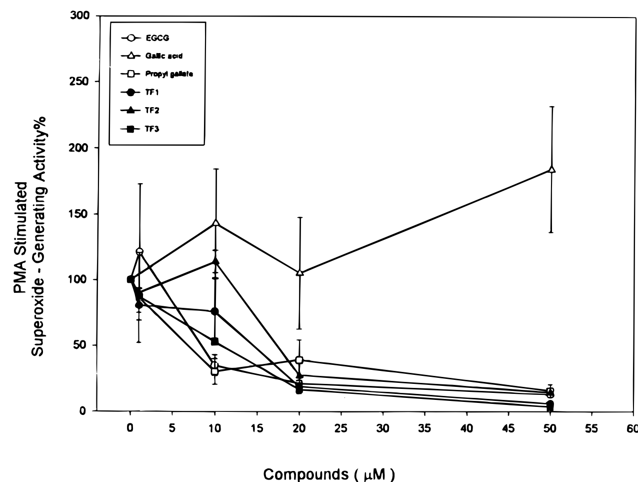

Figure 6. Inhibition of PMA-stimulated superoxide production in HL-60 cells by tea polyphenols. HL-60 cells were induced to differentiation for 5 days by 1.24% DMSO. These cells were treated with various concentrations of the following compounds: EGCG, gallic acid, TF1, TF2, TF3, and PG for 5 min. They were then stimulated with 100 ng/mL PMA for 2 h. The superoxide production was determined by the lucigenin CL method.

of ROS is associated with the initiation and promotion of carcinogenesis.

Green tea is widely used as a beverage in China, Japan, and other Asian countries, whereas black tea is more popular in western countries. In recent years, many animal studies and several epidemiological studies have suggested the anticarcinogenic effects of tea (Katiyar, 1996). Extracts of green, black, and other teas inhibited PMA-induced JB6 cell transformation (Jain, 1989). However, the mechanisms of action by which these chemicals block carcinogenesis are under intensive investigation (Lin and Lin, 1997; Lin et al., 1999a, 1999b; Liang et al., 1999b).

The purpose of this study was to investigate the possible role of tea polyphenols and gallates in the prevention of carcinogenesis. Because oxidative DNA damage is considered to be relevant to carcinogenic processes (Weitzman, 1990; Frenkel, 1992), we have evaluated the possible anticarcinogenic effects by determining the effect of tea polyphenols on PMA-induced ROS generation and xanthine oxidase activity.

Human carcinogenesis is known to proceed through multiple stages, such as initiation, promotion, and progression (Lin et al., 1999). Tumor promotion, which is a complex and important process in carcinogenesis, is generally studied using PMA as a tumor promoter in mouse skin models (Slaga, 1983; Lee and Lin, 1997). Both green tea and black tea have shown the ability to suppress PMA-mediated tumor-promoting effects on mouse skin (Katiyar et al., 1993; Wang et al., 1994); however, the active components that are responsible for the effects are still not well characterized. PMA is known to induce H_2O_2 production by phagocytic cells and epidermal cells (Robertson, 1990) through increasing XO activity (Reiners, 1987) and by diminishing antioxidant enzyme activities (Solanki et al., 1981). Recent reports show that, in double PMA treated mouse skin, ROS from leukocytes including superoxide play an important role leading to chronic inflammation and hyperplasia. Superoxide generation inhibitors are effective in inhibiting this tumor promotion response.

Tea polyphenols and propylgallate all have potent inhibitory effects on PMA-stimulated superoxide pro-

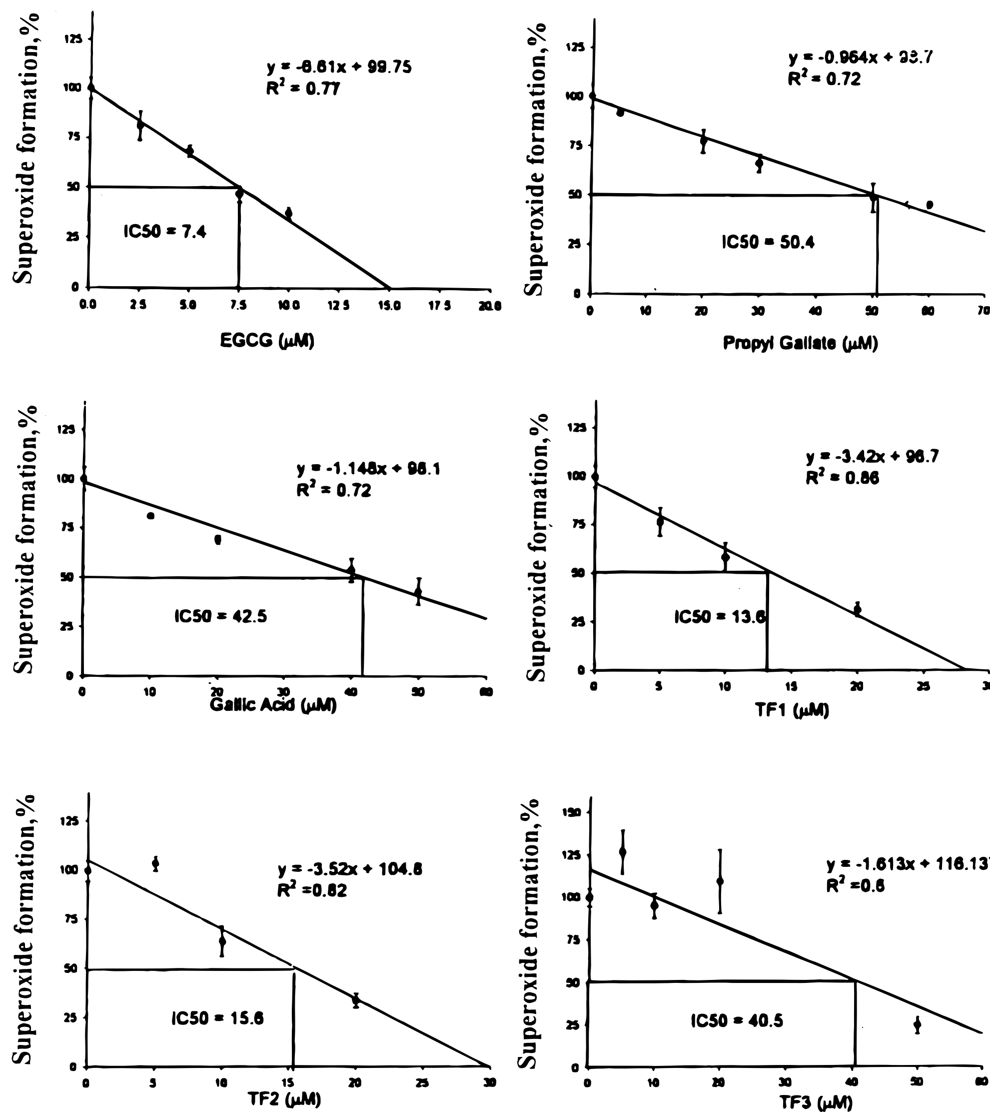

Figure 7. Superoxide scavenging effects of tea polyphenols in vitro. The testing reaction mixtures containing 10 μM phenazine methosulfate, 78 μM NADH, 25 μM nitroblue tetrazolium, and different concentrations of tea polyphenols in 0.1 M phosphate buffer pH 7.4 were incubated at room temperature (26 °C) for 2 min. The formation of superoxide was determined spectrophotometrically at 560 nm against a blank containing phenazine methosulfate alone.

duction between 20 and 50 μM (Figure 6), whereas gallic acid (GA) has no effect on the inhibition of superoxide production by PMA. From these data we suggest that PG and TF3 inhibition of ROS production is not only by its scavenging ability but mostly through modulating the signal transduction of NADPH oxidase and XO activation. Several oxidative signal transducers such as receptor tyrosine kinase, protein kinase C, PI-3-kinase, and I κ B kinase might be involved in this oxidative activation. Further studies on the association of these oxidative signal transducers with cellular ROS production is highly recommended.

Several flavonoids are good scavengers of ROS by either directly inhibiting the mechanisms of generation or scavenging already formed ROS in the target tissues (Kandaswami and Middletown, 1997). The natural antioxidants from tea were reviewed by Ho et al. (1997). It seemed that theaflavins were more effective in DPPH radical and lipoxygenase inhibition while catechins were more effective in superoxide inhibition (Ho et al., 1997). In the present study, we have extended this line of studies to XO inhibition (Figures 2 and 3) and scavenging ROS in HL-60 cells (Figures 6 and 8) and in the phenazine methosulfate system (Figure 7). It appeared that theaflavins were more effective than EGCG in scavenging ROS in HL-60 cells.

Meanwhile, ROS are increasingly being cited as agents of normal signal transduction (Burdon, 1995), and while their source is usually uncertain, xanthine oxidase (XO) is often a likely candidate. Although TF3 (IC_{50}: 4.5 μM) is about 6−7 times less potent than allopurinol (IC_{50}: 0.68 μM), it may still be a good inhibitor of XO in vivo. Several mechanisms have been proposed to explain the in vitro antimutagenic and the in vivo anticancer properties of tea. For instance, antioxidant properties reduce oxidative stress by scavenging the ROS and inhibiting hydroxyl radical formation. Tea polyphenols are particularly good in vivo antioxidants, due to their amphibious polar properties. Inhibition of XO in vivo can be studied by measuring plasma concentrations of the substrate (xanthine and hypoxanthine) and the product (uric acid). The lowering of uric acid in the urine can also be measured (Kojiam, 1984). Inhibition of XO can lower the ROS load of the body, thereby making endogenous reducing equivalents available for other detoxification reactions. The present work indicates that tea polyphenols may have a novel mechanism, namely the limited inhibition of XO, and

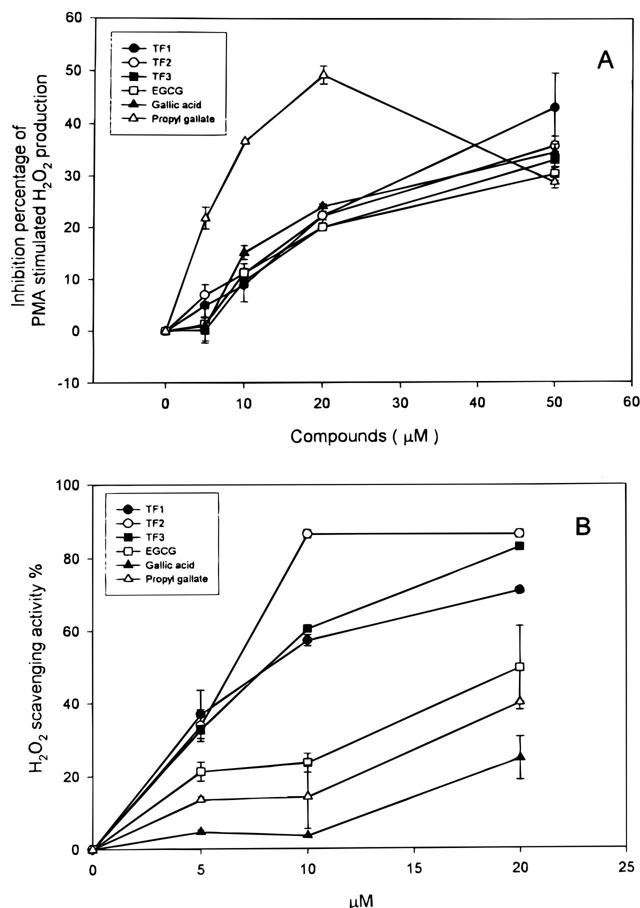

Figure 8. Inhibition of PMA-stimulated H_2O_2 production in HL-60 cells and the scavenging effect on exogenous H_2O_2 by tea polyphenols. (A) Studies of the effects of tea polyphenols on H_2O_2 production by PMA-stimulated HL-60 cells were performed as described in the Materials and Methods section. The data were presented as follows: percentage of inhibition = (H_2O_2 production in control − H_2O_2 production in test)/(H_2O_2 production in control) × 100%. (B) H_2O_2 (5 μM) was added to the reaction mixture containing different concentrations of tested tea polyphenols, and after 90 min, the residual H_2O_2 was measured by the DCFH-DA method as described in the Materials and Methods section.

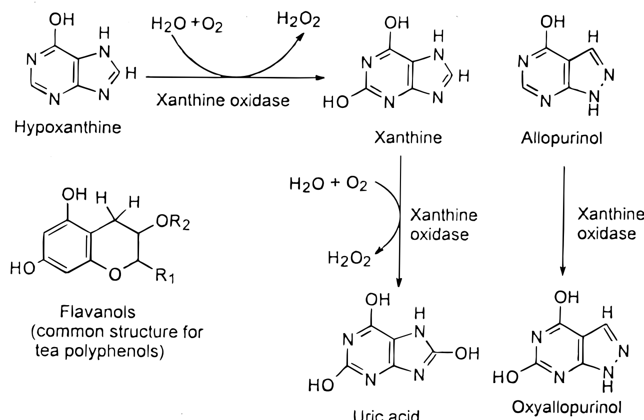

Figure 9. Structural similarities among hypoxanthine, xanthine, uric acid, allopurinol, oxyallopurinol, and flavanols. Both xanthine and hypoxanthine are substrates for XO, while allopurinol is considered as a "suicide substrate" for XO. The structural features of flavanols are similar to that of allopurinol and xanthine.

thereby reduce ROS production in the body. Therefore, it is possible that tea polyphenols counteract the promotional effects of PMA through inhibiting oxidant formation by leukocyte and epidermal XO.

XO catalyzes oxidation of both hypoxanthine and xanthine to uric acid while reducing O_2 to O_2^- and H_2O_2 (Figure 9). Allopurinol, a powerful inhibitor of XO allopurinol, is structurally related to xanthine and is oxidized by an enzyme to give oxyallopurinol, which binds tightly to the active site of XO and causes inhibition. Hence allopurinol, or oxyallopurinol, has been called a "suicide sustrate" or " suicide product" of XO. It is interesting to note that the chemical structure of the A-ring of flavanols has many analogies to the structure of xanthine and uric acid (Figure 9). Therefore, like allopurinol, EGCG may act as "suicide substrate" for XO. The similarities of the dose−response curves of allopurinol and EGCG for XO inhibition (Figure 3) strongly supported this possibility. On the other hand, the chemical structures of theaflavins including TF1, TF2a, TF2b, and TF3 are more bulky and have two A-rings of flavanols linked by a fused seven-member ring (Figure 1). These structural features may provide more interaction sites for XO, as indicated by the hypobolic characteristics of dose−response curves (Figure 2).

The kinetics of inhibition of XO by several flavanols (catechins) and flavonols have been investigated. Most flavanols including chrysin, luteolin, kaempferol, quercetin, myricetin, and isorhamnetin inhibited XO activity in a mixed type mode (Nagao et al., 1997), while the major green tea polyphenol EGCG inhibited XO activity competitively (Aucamp et al., 1997). In the same report, a mixed type inhibition was described for other catechins including (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin gallate and several flavonols such as quercetin and kaempferol. In the present study, theaflavin-3,3′-digallate (TF3), like EGCG, inhibited XO activity competitively (Figure 4).

The scavenging effects of green tea polyphenols (catechins) on ROS in vitro have been reported by Nanjo et al. (1999) and by Hu et al. (1995). In the present study, we have demonstrated the scavenging effect on ROS in HL-60 cells. Furthermore, we have demonstrated that the black tea polyphenols TF1, TF2, and TF3 show an even stronger inhibitory effect than EGCG on ROS production (Figures 6 and 8).

Tea polyphenols may act at an earlier stage than has previously been suspected, by suppressing ROS production through inhibiting XO, rather than only scavenging the already formed ROS. This could partly explain some of the beneficial properties attributed to tea, e.g., antimutagenic and anticarcinogenic effects which are all mediated by ROS (Ho et al., 1997; Kandaswami and Middletown, 1997; Lin et al., 1999).

LITERATURE CITED

Aucamp, A.; Gaspar, A.; Hara, Y.; Apostolides, Z. Inhibition of xanthine Oxidase by catechin from tea (Camellia sinensis). *Anticancer Res.* **1997**, *17*, 4381−4386.

Breimer, L. H. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Mol. Carcinog.* **1990**, *3*, 188−197.

Burdon, R. H. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Rad. Biol. Med.* **1995**, *18*, 775−794.

Bushman, J. L. Green tea and cancer in humans: A review of the literature. *Nutr. Cancer* **1998**, *31*, 151−159.

Cerutti, P. A. Prooxidant states and tumor promotion. *Science* **1985**, *227*, 375−380.

Chen, C. W.; Ho, C. T. Antioxidant properties of polyphenols extracted from green and black tea. *J. Food Lipids* **1995**, *2*, 35−46.

Chen, Y. C.; Liang, Y. C.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Inhibition of TPA-induced protein kinase C and transcription activator protein-1 binding activity by theaflavin-3,3′-digallate from black tea in NIH 3T3 cells. *J. Agric. Food Chem.* **1999**, *47*, 1416−1421.

Frenkel, K. Carcinogen-mediated oxidant formation and oxidative DNA damage. *Phamacol. Ther.* **1992**, *53*, 127−166.

Frenkel, K.; Chrzan, K. Hydrogen peroxide formation and DNA base modification by tumor promoter-activated polymophonuclear leukocytes. *Carcinogenesis* **1987**, *8*, 455−460.

Gyllenhamer, H. Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J. Immunol. Methods* **1987**, *97*, 209−213.

Ho, C. T.; Chen, C. W.; Wanasundara, U. N.; Shahidi, F. Natural antioxidants from tea. In: *Natural Antioxidants: Chemistry, Health Effects, and Application*; Shahidi, F., Ed.; AOCS Press: Champaign, IL, 1997; pp 213−223.

Hu, J. P.; Colomme, M.; Lasure, A.; DeBruyne, T.; Pierers, L.; Vlietinck, A.; Vanden Berghe, D. A. Structure−activity relationship of flavonoids with superoxide scavenging activity. *Biol. Trace Elem. Res.* **1995**, *47*, 327−331.

Harpe, J. D. L.; Nath, C. F. An semi-automated microassay for H_2O_2 release by human blood monocyte and mouse peritoneal macrophage. *J. Immunol. Methods* **1985**, *78*, 323−336.

Jain, A. K.; Shimoi, K.; Nakamura, Y.; Kada, T.; Hara, Y.; Tomita, I. Crude tea extracts decrease the mutagenic activity of MNNG in vitro and in intragastric tract. *Mutat. Res.* **1989**, *210*, 1−8.

Kalckar, H. M. Differential spectrophotometry of purine compounds by means of specific enzyme. *J. Biol. Chem.* **1947**, *167*, 429−443.

Kandasmami, C.; Middletown, E., Jr. Flavanoids as antioxidants. In: *Natural Antioxidants: Chemistry, Health Effects, and Application*; Shahidi, F., Ed.; AOCS Press: Champaign, IL, 1997; pp 174−203.

Katiyar, S. K.; Mukhtar, H. Tea in chemoprevention of cancer: Epidemiologic and experimental studies (Review). *Int. J. Oncol.* **1996**, *8*, 221−238.

Kelloff, G. J.; Boone, C. W.; Crowell, J. A.; Steele, V. E.; Lubert, R. A.; Doody, L. A.; Malone, W. F.; Hawk, E. T.; Sigman, C. C. New agents for cancer chemoprevention. *J. Cell. Biochem.* **1996**, *26s*, 1−28.

Kojima, T.; Nishina, T.; Kitamura, M.; Hosova, Y.; Nishioka, K. Biochemical studies on the purine metabolism of four easer with hereditary xanthinuria. *Clin. Chim. Acta* **1984**, *137*, 189−198.

Lee, S. F.; Lin, J. K. Inhibitory effects of phytopolyphenols on the TPA-induced transformation, PKC activation and c-jun expression in mouse fibroblast cells. *Nutr. Cancer* **1997**, *28*, 177−183.

Liang, Y. C.; Lin-Shiau, S. Y.; Chen, C. F.; Lin, J. K. Suppression of extracellular signals and cell proliferation through EGF receptor binding by (−)-epigallocatechin-3-gallate. *J. Cell. Biochem.* **1997**, *67*, 55−65.

Liang, Y. C.; Chen, Y. C.; Lin, Y. L.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Suppression of extracellular signals and cell proliferation by the black tea polyphenol theaflavin-3,3′-digallate. *Carcinogenesis* **1999a**, *20*, 733−736.

Liang, Y. C.; Lin-Shiau, S. Y.; Chen, C. F.; Lin, J. K. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (−)-epigallocatechin-3-gallate. *J. Cell. Biochem.* **1999b**, *75*, 1−12.

Lin, J. K.; Liang, Y. C.; Lin-Shiau, S. Y. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.* **1999b**, *58*, 911−915.

Lin, Y. L.; Juan, I. M.; Liang, Y. C.; Lin, J. K. Composition of polyphenols in fresh tea leaves and association of their oxygen-radical absorbing capacity with anti-proliferative actions in fibroblast cells. *J. Agric. Food Chem.* **1996**, *44*, 1387−1394.

Lin, Y. L.; Lin, J. K. (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-κ B. *Mol. Pharmacol.* **1997**, *52*, 465−472.

Lin, Y. L.; Tsai, S. H.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Teaflavin-3,3′-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF κ B in macrophages. *Eur. J. Pharmacol.* **1999a**, *367*, 379−388.

Lundqvist, H.; Follin, P.; Khalfan, L.; Dahlgreh, C. Phorbol myristate acetate induced NADPH oxidase activity in human neutrophils: only half the story has been told. *J. Leukocyte Biol.* **1996**, *59*, 270−279.

Nagao, A.; Seki, M.; Kobayashi, H. Inhibition of xanthine oxidase by Flavonoids. *Biosci. Biotechnol. Biochem.* **1999**, *63* (10), 1767−1790.

Nagy, K.; Pasti, G.; Bene, L.; Nagy, I. Induction of granulocytic maturation in HL-60 human leukemia cells by free radicals a hypothesis of cell differentiation involving hydroxyradicals. *Free Radical Res. Commun.* **1993**, *19*, 1−15.

Nanjo, F.; Mori, M.; Goto, K.; Hara, Y. Radical scavenging activity of tea catechins and their related compounds. *Biosci. Biotechnol. Biochem.* **1999**, *63* (9), 1621−1623.

Palmblad, J.; Gyllenhammar, H.; Lindgren, J. A.; Malmsten, C. L. Effects of leukotrienes and f-Met-Leu-Phe on oxidative metabolism of neutrophils and eosinophils. *J. Immunol.* **1984**, *132*, 3041−3045.

Robertson, F. M.; Beavis, A. J.; Oberysryn, T. M.; O'Connell, S. M.; Dokidos, A.; Laskin, D. L.; Laskin, J. D.; Reiners, J. J., Jr. Production of hydrogen peroxide by murine epidermal keratinocytes following treatment with the tumor promoter 12-*O*-tetradecanoylphorbol- 13-acetate. *Cancer Res.* **1990**, *50*, 6062−6067.

Reiners, J. J., Jr.; Pence, B. C.; Barcus, M. C. S.; Cantu, A. R. 12-*O*-tetradecanoylphorbol-13-acetate-dependent induction of xanthine dehydrogenase and conversion to xanthine oxidase in murine epidermis. *Cancer Res.* **1987**, *47*, 1775−1779.

Slaga, T. J. Overview of tumor promotion in animals. *Environ. Health Prospect.* **1983**, *50*, 3−14.

Szarka, C. E.; Grana, G.; Engstrom, P. F. Chemoprevention of cancer. *Curr. Probl. Cancer* **1994**, *18*, 16−79.

Solanki, V.; Rana, R. S.; Slaga, T. J. Diminution of mouse epidermal superoxide dismutase and catalase activities by tumor promoters. *Carcinogenesis* **1981**, *2*, 1141−1146.

Takeuchi, T.; Nakajima, M.; Morimoto, K. Relationship between the intracellular reactive oxygen species and the induction of oxidative DNA damage in human neutrophil-like cells. *Carcinogenesis* (Lend.) **1995**, *17*, 1543−1548.

Wang, Z. Y.; Huang, M. T.; Lou, Y. R.; Yie, J. G.; Reuhl, K.; Newmark, H. L.; Yang, H. C. T.; C. S.; Conney, A. H. Inhibitory effects of black tea, green tea, decaffeinated black tea and decaffeinated green tea on UVB light-induced skin carcinogenesis in 7,12-dimethylbenz[*a*]anthracene-initiated SKH-1 mice. *Cancer Res.* **1994**, *54*, 3428−3435.

Weitzman, S. A.; Gordon, C. I. Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. *Blood* **1990**, *76*, 655−663.

Wei, H.; Frenkel, K. Relationship of oxidative events and DNA damage to in vive tumor promoting activity. *Carcinogenesis* **1993**, *14*, 1195−1201.

Received for review January 11, 2000. Revised manuscript received April 24, 2000. Accepted April 24, 2000. This study was supported by the National Science Council Grants NSC89-2316-B002-016 and NSC88-EPA-2-002-021 and by the National Health Research Institute Grant DOH 88-HR-403.

JF000066D