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Theaflavin-3,3′-digallate from black tea blocks the nitric oxide synthase by down-regulating the activation of NF-κB in macrophages

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Abstract

Nitric oxide (NO) plays an important role in inflammation and also in multiple stages of carcinogenesis. We investigated the effects of various tea polyphenols, including theaflavin, a mixture of theaflavin-3-gallate and theaflavin-3′-gallate, theaflavin-3,3′-digallate, thearubigin, and (−)-epigallocatechin-3-gallate on the induction of NO synthase in lipopolysaccharide-activated murine macrophages, RAW 264.7 cells. Theaflavin-3,3′-digallate was found to be stronger than (−)-epigallocatechin-3-gallate in inhibiting NO generation and inducible NO synthase protein in activated macrophages, while theaflavin, a mixture of theaflavin-3-gallate and theaflavin-3′-gallate and thearubigin were less effective. Inhibition of NO production was observed when cells were cotreated with theaflavin-3,3′-digallate and lipopolysaccharide. Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses demonstrated that significantly reduced 130-kDa protein and mRNA levels of inducible NO synthase were expressed in lipopolysacchride-activated macrophages with theaflavin-3,3′-digallate, compared to those without theaflavin-3,3′-digallate. Electrophoretic mobility shift assay (EMSA) indicated that theaflavin-3,3′-digallate blocked the activation of nuclear factor κB (NF-κB), a transcription factor necessary for inducible NO synthase induction. Theaflavin-3,3′-digallate also blocked phosphorylation of IκB from cytosolic fraction and reduced lipopolysacchride-induced nuclear accumulation of transcription factor NF-κB p65 and p50 subunits. These results suggest that theaflavin-3,3′-digallate decreases the protein levels of inducible NO synthase by reducing the expression of inducible NO synthase mRNA, and the reduction could be via preventing the activation of NF-κB, thereby inhibiting the induction of inducible NO synthase transcription. It was also demonstrated that the gallic acid moiety of theaflavin-3,3′-digallate is essential for their potent anti-inflammation activity.

Keywords: Theaflavin-3,3′-digallate; (−)-Epigallocatechin-3-gallate; Nitric oxide (NO) synthase, inducible; NF-κB (nuclear factor κB); IκB; Macrophage; RAW 264.7 cell line

1. Introduction

Nitric oxide (NO), a short-lived free radical is an important signalling molecule participating in the physiology and pathophysiology of many systems (Moncada et al., 1992). NO is synthesized in vivo from L-arginine by NO synthase with NADPH and oxygen as cosubstrates (Marletta et al., 1988). Molecular cloning and sequencing analyses revealed the existence of at least three main types of NO synthase isoforms. NO synthase in the endothelium of blood vessels and in brain neurons is constitutive and Ca^{2+}/calmodulin-dependent. This constitutive enzyme synthesizes small amounts of NO triggered by various agonists (e.g., bradykinin, acetylcholine, etc.) that increase intracellular Ca^{2+}. NO synthase in macrophages and hepatocytes is inducible, not detectable in unstimulated cells and requires protein synthesis for expression. Inducible NO synthase produces large amounts of NO several hours after exposure to endotoxin and/or cytokines in macrophages, Kupffer cells, hepatocytes, and fibroblasts. Nitric oxide has a wide biological role in modulating physiological and pathophysiological processes (Moncada et al., 1992; Liu and Hotchkiss, 1995), such as macrophage cytotoxicities, neurotransmissions, neurotoxicities, and regulation of blood pressure. In particular viral infection and

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also in some neuro-inflammatory conditions, NO from inducible NO synthase is protective (Paakkari and Lindsberg, 1995; MacLean et al., 1998). Low concentrations of NO are sufficient, in most cases, to effect these functions. However, during infection and inflammation, in vivo formation of NO is increased suggesting that NO concentrations are well above those found in normal physiological function in infected tissues. High concentrations of NO have been shown to cause deamination of deoxynucleotides and bases within intact DNA in vitro and are mutagenic in vivo (Wink et al., 1991). Exposure of human cells to NO under aerobic conditions results in DNA strand breakage and nitrosative deamination of DNA bases (Nguyen et al., 1992). Therefore, NO can cause DNA damage as well as mutation in human cells.

A portion of the 5′-flanking region of the murine inducible NO synthase gene has been cloned (Weisz et al., 1994). The promoter of the murine gene contains a TATA box and consensus sequences for the binding of transcription factors associated with stimuli that induce inducible NO synthase expression (Xie et al., 1993). These potentially relevant transcription factors, nuclear factor-κB (NF-κB) and interferon regulatory factor have been shown to be functionally important for inducible NO synthase induction. It is well-known that NF-κB is activated by factors such as cytokines, lipopolysaccharide, virus, and agents providing oxidative stress.

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 (−)-epigallocatechin-3-gallate, (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epicatechin, (+)-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 as 'fermentation'. Theaflavins (about 1–2% of the total dry weight of black tea), including theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate, possess benzotropolone rings with dihydroxy or trihydroxy substitution systems. About 10–20% of the dry weight of black tea is due to thearubigins, which are more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well-characterized. Pauchong tea or Oolong tea, contains monomeric catechins, theaflavins and thearubigins. Many biological functions of tea polyphenols have been studied (Yang and Wang, 1993), including anti-inflammatory, antioxidative (Ho et al., 1992; Katiyar et al., 1993; Lin et al., 1996), antimutagenic (Shiraki et al., 1994) and anticarcinogenic effects (Huang et al., 1992). Biochemical mechanisms for inhibition of tumorigenesis by tea might be via five mechanisms: (a) antioxidative function, (b) inhibition of nitrosation reactions, (c) modulation of xenobiotic metabolizing enzymes, (d) trapping of activated forms of carcinogens, and (e) inhibition of activities related to tumor promotion and cell proliferation. The anti-inflammatory and cancer preventive characteristics of (−)-epigallocatechin-3-gallate have been well-documented (Lin and Lin, 1997), but the activity of theaflavins and thearubigins has not yet been demonstrated.

In the present study, we first examined effects of theaflavins and thearubigin on NO production in a murine macrophages cell line, RAW 264.7, and further investigated the possible reaction mechanisms of these tea polyphenols.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (*Escherichia coli* 0127:B8), sulfanilamide, naphthylethylenediamine dihydrochloride, dithiothreitol were purchased from Sigma (St. Louis, MO). Acrylamide was purchased from E. Merck (Darmstadt, Germany). 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). The structural formulas of these compounds are shown in Fig. 1. Isotopes were obtained from Amersham Polynucleotide kinase and oligo(dT)18 were obtained from Pharmacia. (−)-Epigallocatechin-3-gallate was purified as described previously (Lin et al., 1996), and its purity was 97%.

2.2. Cell culture

RAW 264.7, which was derived from murine macrophages, was obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free heat-inactivated fetal calf serum (Gibco)/penicillin (100 units/ml)/streptomycin (100 μg/ml). When the cells reached a density of 2×10^6 cells/ml, they were activated by incubation in medium containing lipopolysaccharide (50 ng/ml). Various concentrations of test compounds dissolved in dimethylsulfoxide were added together with lipopolysaccharide.

2.3. Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production by the Griess reaction (Kim et al., 1995). One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined

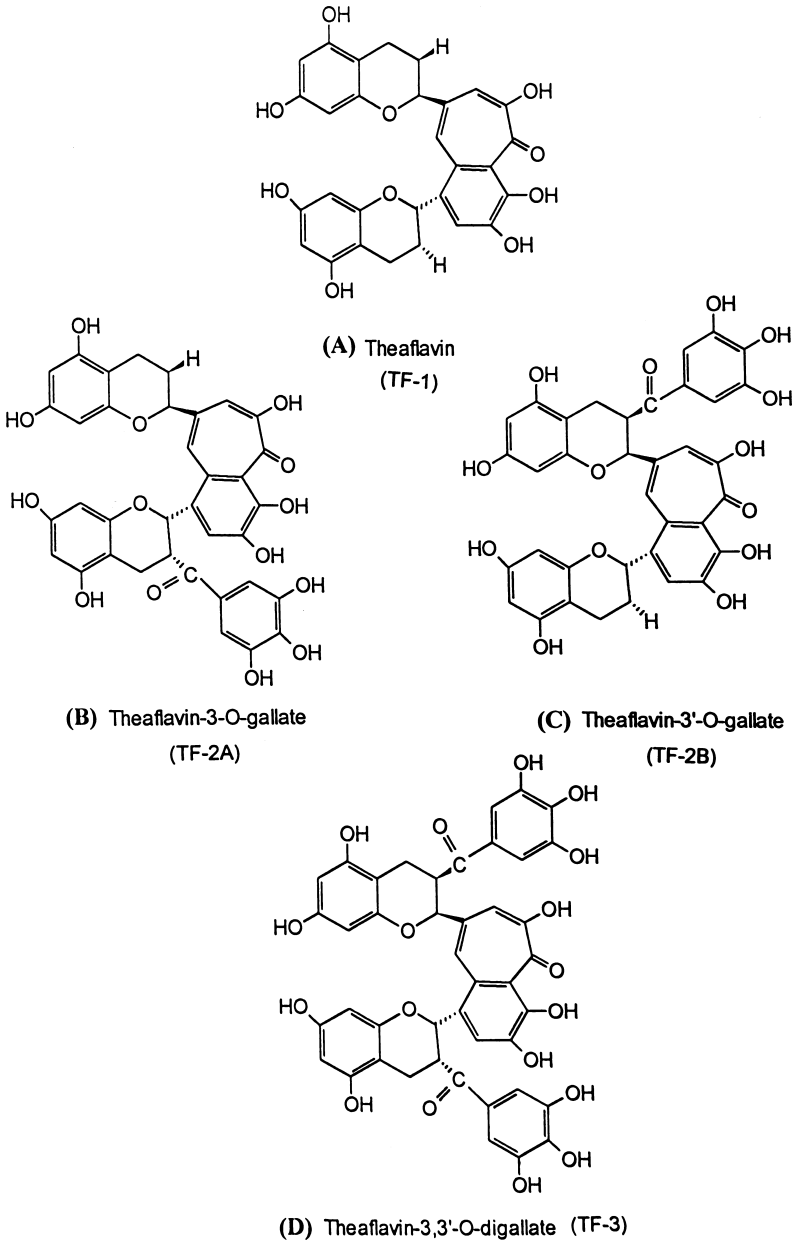

Fig. 1. The structures of theaflavins (TFs). (A) Theaflavin (TF-1), (B) theaflavin-3-*O*-gallate (TF-2A), (C) theaflavin-3′-*O*-gallate (TF-2B), and (D) theaflavin-3,3′-*O*-digallate (TF-3). In the present study, TF-2 is a mixture of TF-2A and TF-2B.

by an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA).

2.4. Western blots

Total cellular extract was prepared using radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS/1% aprotinin). Total protein (for inducible NO synthase and α-tubulin), cytosolic fractions (for IκB), or nuclear fractions (for p65 and p50) containing 50 μg protein were separated on sodiumdodecylsulfate-polyacrylamide minigels (8% for inducible NO synthase and 10% for IκB, p65, and p50), and transferred to Immobilon PVDF membrane (Millipore). The membrane was incubated overnight at 37°C with 10% bovine serum albumin in phosphate-buffered saline to block nonspecific immunoglobulins, and then incubated with anti-macNOS monoclonal antibody (Transduction Laboratories), anti-IκB-P polyclonal antibody (Bio. Lab.), anti-p65, anti-p50 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-α-tubulin monoclonal antibody (Oncogene Science). Inducible NO synthase, IκB-P, p65, p50, and α-tubulin protein were detected by chemiluminescence (ECL, Amersham).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Total RNA (1 μg) was converted to cDNA with 1 μM oligo(dT)18, 0.5 mM of each dNTP, 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, RNase inhibitor (1 U/μl) and Moloney-murine leukemia virus reverse transcriptase (10 U/μl) at 42°C for 1 h. The amplification of inducible NO synthase cDNA was performed by incubating 20 ng equivalents of DNA in 100 mM Tris–HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 200 μM dNTPs and 50 U/ml of Super *Taq* DNA polymerase with the following oligonucleotide primers: 5′-CCCTTCCGAAGTTTCTGG-CAGCAGC-3′ and 5′-GGCTGTCAGAGAGCCTCGTG-GCTTTGG-3′. The cDNA sequence of GAPDH was also amplified as control in a similar way using the following primers: 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′. A thermal cycle of 45 s at 95°C, 45 s at 65°C and 2 min at 72°C was used for 30 cycles. PCR products were analyzed on 1.8% agarose gels (Brouet and Ohshima, 1995).

2.6. Preparation of extracts and electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic extracts were prepared by a modified method of Chen et al. (1995). At the end of culture, the cells were suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice, vortexed for 10 s. Nuclei were pelleted by centrifugation at 12,000 × *g* for 20 s. The supernatants containing cytosolic proteins were collected. Pellet containing nuclei was resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000 × *g* for 2 min and stored at −70°C. For EMSA, each 10 μg nuclear extract was mixed with the labelled double-stranded NF-κB oligonucleotide, 5′-AGTTGA**GGGGACTTTCCC**AGGC-3′, and incubated at room temperature for 20 min. The incubation mixture included 1 μg of poly (dI–dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl). The DNA–protein complex was electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5 × TBE buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA). A double-stranded mutated oligonucleotide, 5′-AGTTGA**GGCGACTTTCCC**AGGC-3′, was used to examine the specificity of binding of NF-κB to DNA. The specificity of binding was also examined by competition with the unlabelled oligonucleotide.

3. Results

3.1. Inhibition of NO generation by theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate

The inhibition of NO generation in the lipopolysaccharide-activated macrophages with theaflavins, thearubigin, and(−)-epigallocatechin-3-gallate were investigated (Fig. 2). Theaflavin-3,3′-digallate was found to significantly reduce the NO generation. Comparison of the inhibition of NO generation by these compounds gave the following order: theaflavin-3,3′-digallate > (−)-epigallocatechin-3-gallate > a mixture of theaflavin-3-gallate and theaflavin-3′-gallate > thearubigin > theaflavin. No significant cytotoxicity of these compounds were observed under the experimental conditions as demonstrated by the Western blotting data (Figs. 3 and 4).

3.2. Inhibition of inducible NO synthase protein by theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate

Theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate were examined to determine whether they affect inducible NO synthase protein in macrophages activated with lipopolysaccharide for 18 h (Fig. 3). Theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate were found to significantly reduce the amount of inducible NO synthase protein, while a mixture of theaflavin-3-gallate and theaflavin-3′-gallate, theaflavins, and thearubigin showed

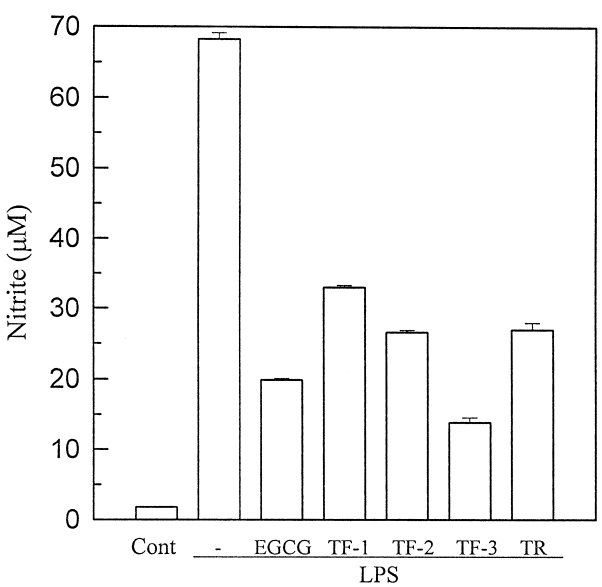

Fig. 2. Effects of theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate on nitrite release in culture medium of activated macrophages with lipopolysaccharide. RAW 264.7 cells were cotreated with lipopolysaccharide (50 ng/ml) and theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate (10 μM) for 18 h. At the end of the incubation time, the culture medium was collected for nitrite assay. Each data represented mean ± SE for three determinations.

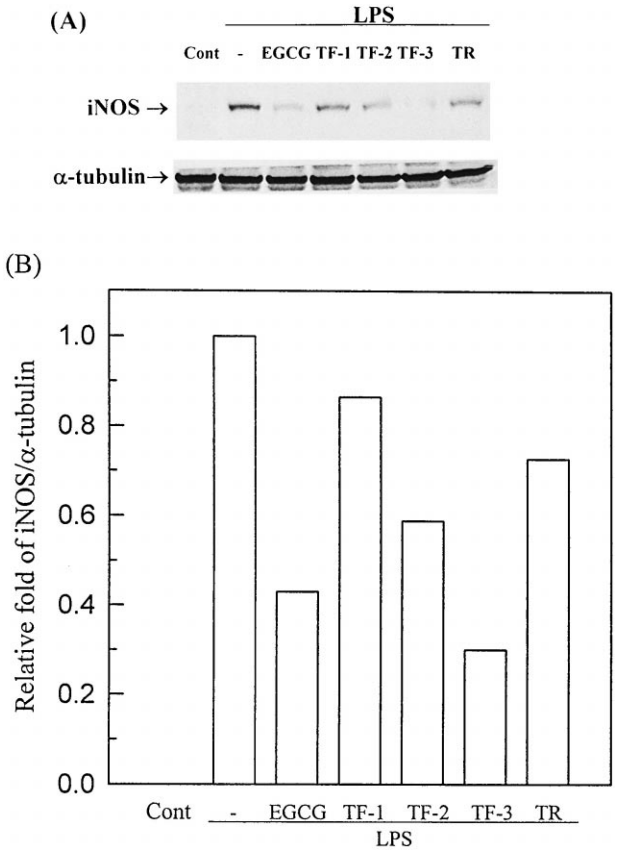

Fig. 3. Western blotting analysis of inducible NO synthase in activated macrophages. RAW 264.7 cells were cotreated with lipopolysaccharide (50 ng/ml) and theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate (10 μM) for 18 h. At the end of the incubation time, the total protein was extracted for inducible NO synthase protein and α-tubulin analysis (A). (B) Band intensities were quantified by densitometry (IS-1000 Digital Imaging System). This experiment was repeated three times with similar results.

less inhibitory effect at the same dose (10 μM); the pattern of inhibitory activity was parallel to the generation of NO (Fig. 2).

3.3. Effects of theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate on inducible NO synthase protein and NO generation

The dose-response of theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate on the inhibition of 130 kDa inducible NO synthase protein and NO generation are shown in Fig. 4A, B and C. Theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate were found to significantly

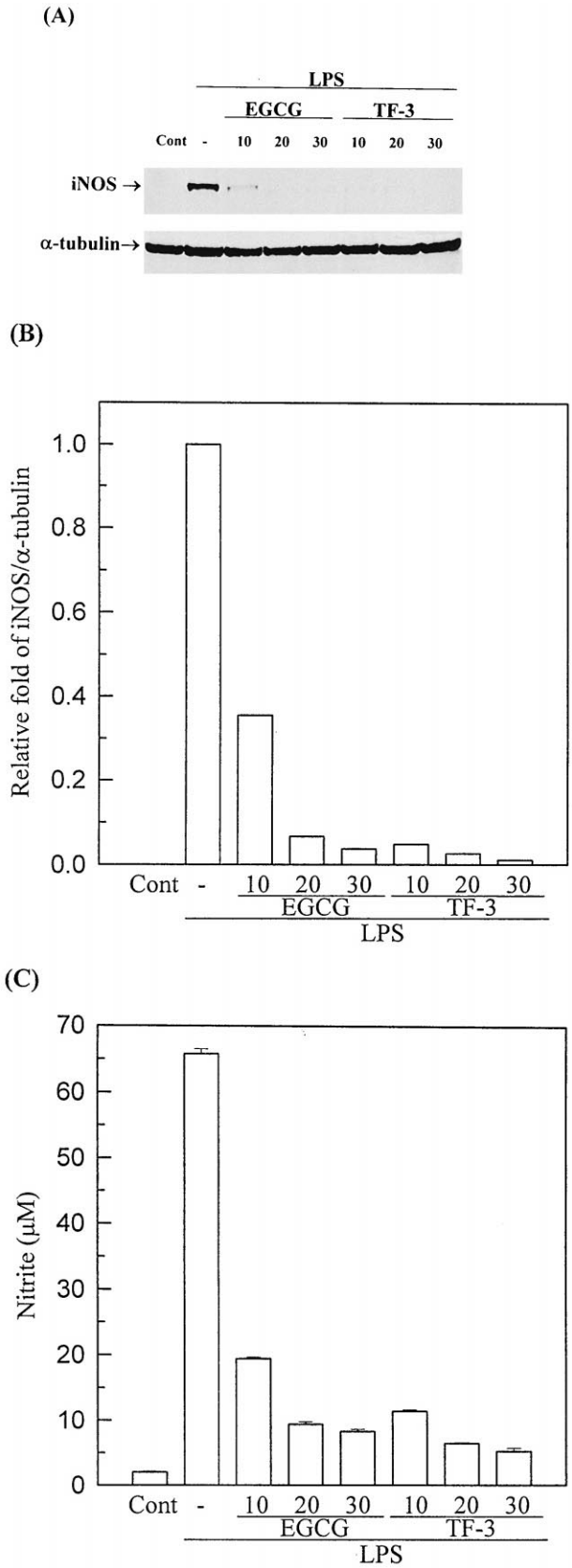

Fig. 4. Dose response of inhibition of lipopolysaccharide-dependent inducible NO synthase activation by theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate. Western blotting analysis of inducible NO synthase (A) and nitrite release (C) in culture medium of activated macrophages with or without theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate. Murine macrophages were incubated with lipopolysaccharide (50 ng/ml) and various concentrations of theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate for 18 h. (B) Band intensities were quantified by densitometry (IS-1000 Digital Imaging System). This experiment was repeated three times with similar results.

reduce the amount of inducible NO synthase protein and NO generation with dose-dependent manner. At 10, 20, and 30 μM, theaflavin-3,3′-digallate inhibited the levels of inducible NO synthase protein by 95, 97, and 99%, respectively; and inhibited NO generation by 85, 93, and 95%, respectively. At 10, 20, and 30 μM, (−)-epigallocatechin-3-gallate inhibited the levels of inducible NO synthase protein by 65, 93, and 96%, respectively; and inhibited NO generation by 73, 88, and 90%, respectively. It is obvious that the black tea polyphenol theaflavin-3,3′-digallate is more effective than the green tea polyphenol (−)-epigallocatechin-3-gallate on this inhibition.

3.4. Inhibition of inducible NO synthase expression by theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate

In order to investigate whether the suppression of inducible NO synthase protein in tea polyphenols treated macrophages was due to reduce inducible NO synthase mRNA or not, an RT-PCR analysis for total mRNA samples extracted from RAW 264.7 cells was carried out. The amplification of cDNA with primers specific for mouse inducible NO synthase and GAPDH (as control gene) was shown in Fig. 5. Macrophages were activated with lipopolysaccharide with and without theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate (10 μM) for 6 h, harvested, and assayed for inducible NO synthase mRNA expression by RT-PCR. We found that significantly lower levels of inducible NO synthase mRNA were expressed in macrophages activated by lipopolysaccharide in the presence of theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate, than in its absence. These experiments were repeated three times with similar results. In unstimulated cells, there was no detectable inducible NO synthase mRNA. Coincubation of macrophages with lipopolysaccharide plus theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate caused almost complete suppression of inducible NO synthase mRNA after 6 h incubation, and a weak suppression was detectable in the presence of lipopolysaccharide plus a mixture of theaflavin-3-gallate and theaflavin-3′-gallate, theaflavin, or thearubigin.

3.5. Effect of theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate on lipopolysaccharide-induced nuclear proteins with NF-κB binding activity

To determine whether the decreased inducible NO synthase mRNA was mediated through inhibition of inducible NO synthase transcription by suppression of NF-κB activation, an electrophoretic mobility shift assay on nuclear extracts of macrophages by using a consensus oligonucleotide for NF-κB binding was performed. Incubation of

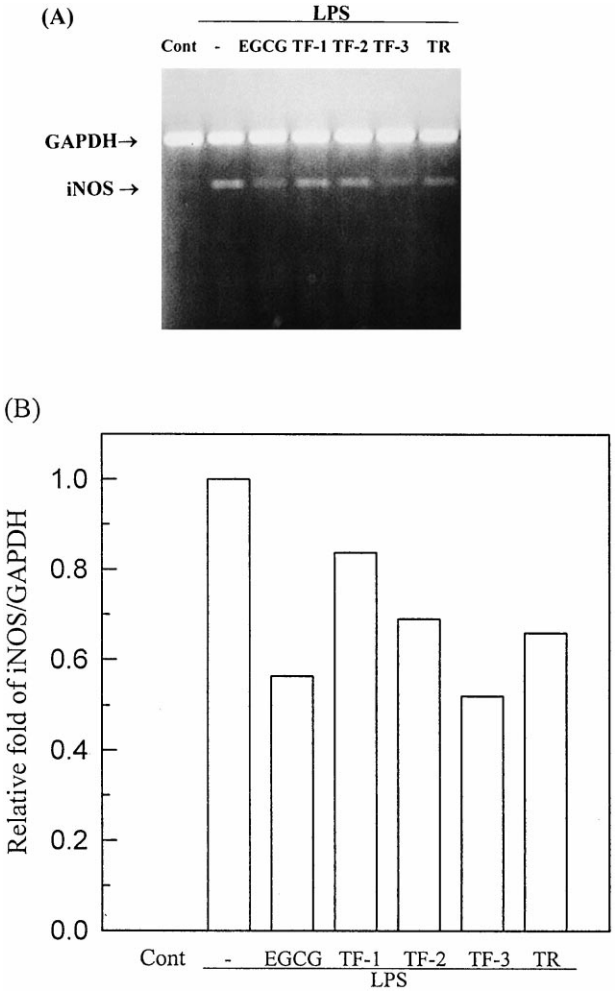

Fig. 5. RT-PCR analysis of mRNA expression of inducible NO synthase. (A) RAW 264.7 cells were incubated in the presence of lipopolysaccharide (50 ng/ml) with or without theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate (10 μM) for 6 h. (B) Band intensities were quantified by densitometry. This experiment was repeated three times with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

macrophages with lipopolysaccharide (50 ng/ml) for 1 h markedly increased this activity. The induction of specific NF-κB binding with NF-κB site by lipopolysaccharide was markedly inhibited by coincubation with theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate (10 μM) (Fig. 6). On the other hand, theaflavin, a mixture of theaflavin-3-gallate and theaflavin-3′-gallate, or thearubigin (10 μM) showed less effective.

3.6. Effects of theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate on phosphorylation of IκB

To determine whether the inhibitory action of theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate was due to its effect on IκB phosphorylation, the cytoplasmic levels of IκB-P protein were examined by Western blot analysis. After 45 min activation of macrophages with

lipopolysaccharide, the cytosolic IκB protein was significantly phosphorylated. The lipopolysaccharide-induced IκB phosphorylation was dramatically inhibited by coincubation lipopolysaccharide plus theaflavin-3,3′-digallate, whereas (−)-epigallocatechin-3-gallate, theaflavin, a mixture of theaflavin-3-gallate and theaflavin-3′-gallate, or thearubigin showed less effective (Fig. 7A). These results suggested that inhibition of NO production by theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate occurred via the prevention of NF-κB activation.

3.7. Reduction of nuclear NF-κB level by theaflavins, thearubigin, and (−)-epigallocatechin-3-gallate

The above results suggested that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate could significantly reduce inducible NO synthase expression by blocking inducible NO synthase promoter activation. Since activation of transcription factor NF-κB is necessary for inducible NO synthase induction, we tested if theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate perturbed the distribution of NF-κB subunits (p65 and p50) as assessed by nuclear accumulation. As shown in Fig. 7B, coincubation with lipopolysaccharide plus theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate decreased the nuclear NF-κB proteins (p65 and p50). These results suggested that inhibition of NO production by theaflavin-3,3′-digallate or (−)-epigallocatechin-3-gallate occurred via blocking the phosphorylation of IκB protein and then preventing the translocation of NF-κB protein, and finally suppressing the NF-κB activation.

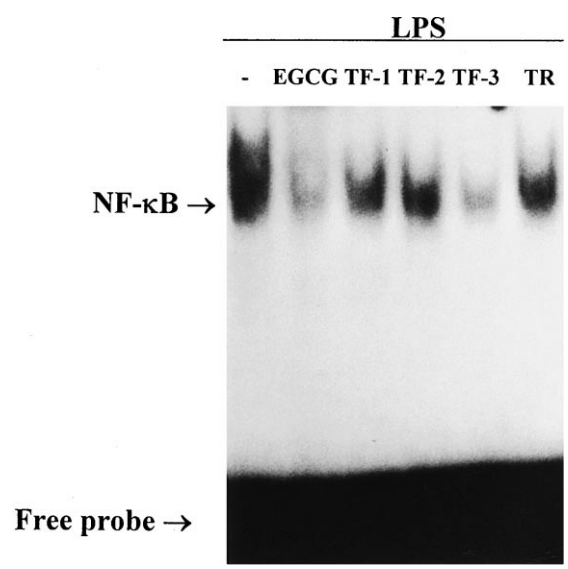

Fig. 6. Electrophoretic mobility shift assay using a 5′-end-labeled consensus oligonucleotide for NF-κB binding and nuclear extracts from murine macrophages. Murine macrophages were cotreated with lipopolysaccharide (50 ng/ml) with or without 10 μM of theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate, then incubation for 1 h. This experiment was repeated three times with similar results.

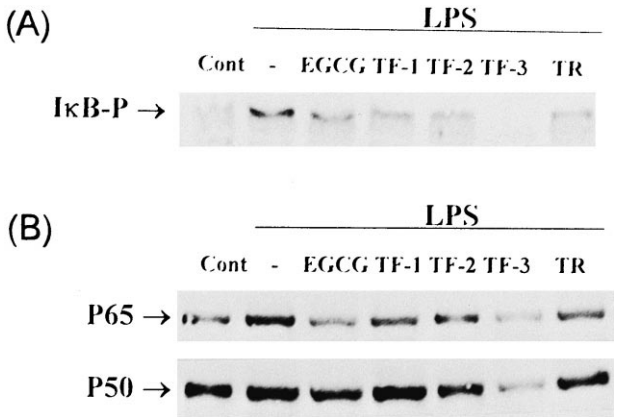

Fig. 7. (A) Lipopolysaccharide-mediated IκB phosphorylation is blocked by the theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate. Murine macrophages were cotreated with lipopolysaccharide (50 ng/ml) with or without 10 μM of theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate, then incubation for 45 min. Cytosolic fractions were prepared and analyzed for the content of IκB-P protein by Western blotting. This experiment was repeated three times with similar results. (B) TF-3 and EGCG reduced nuclear NF-κB levels. RAW 264.7 cells were cotreated with lipopolysaccharide (50 ng/ml) with or without theaflavins, thearubigin, or (−)-epigallocatechin-3-gallate (10 μM) and then incubation for 45 min. The nuclear fractions were prepared and analyzed for the content of p65 and p50 protein by Western blotting. This experiment was repeated three times with similar results.

4. Discussion

Theaflavins and thearubigins, are known to be contained in the infusions of oolong tea and black tea (Whitehead and Temple, 1992), though the content in the infusion of oolong tea is lower than that of black tea. As shown in Fig. 1, which shows the structures of several theaflavins, these components in fermented tea leaves still have many phenolic hydroxy groups. Thearubigin is also known to be a mixture of heterogeneous polyphenols. The phenolic hydroxy groups in these oxidized products of catechins are expected to show antioxidative activity in the same way as catechins. Recently, in our laboratory, we have demonstrated that (−)-epigallocatechin-3-gallate, (−)-epigallocatechin, and gallic acid inhibit induction of inducible NO synthase in murine peritoneal macrophages activated with lipopolysaccharide (Lin and Lin, 1997). The results indicated that the galloyl group and the hydroxy group at the 3′ position on (−)-epigallocatechin-3-gallate were responsible for its strongly anti-inflammatory property. These tea polyphenols have phenol rings that act as electron traps to scavenge peroxy radicals, superoxide-anions and hydroxyl radicals (Ho et al., 1992; Katiyar et al., 1993; Yang and Wang, 1993; Lin et al., 1996). In this study, we also demonstrated that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate strongly inhibited the

inducible NO synthase in a murine macrophage cell line, RAW 264.7. The inhibition of inducible NO synthase protein level was in the following order: theaflavin-3,3′-digallate > (−)-epigallocatechin-3-gallate > a mixture of theaflavin-3-gallate and theaflavin-3′-gallate > thearubigin > theaflavin. Among theaflavins, theaflavin-3,3′-digallate, which has two gallic acid moieties, exhibited the strongest anti-inflammation activity as judged by its suppression on inducible NO synthase induction. Theaflavin, which has no gallic acid moiety, exhibited the least inhibitory effects. From these results, we conclude that gallic acid moiety is important for theaflavins to express anti-inflammation activity. Therefore, we suggest that in addition to the reduction of inducible NO synthase expression, these compounds may block peroxynitrite and nitrite production through inhibiting oxidative reactions. Based on these results, it may be expected that theaflavins in black tea, oolong tea and (−)-epigallocatechin-3-gallate in green tea could play a favorable role in our daily lives in the prevention of a number of diseases including cardiovascular diseases, neurodegenerative diseases, cancers, and aging, for which lipid peroxides or active oxygen are relevant.

Mammals are in permanent contact with gram-negative bacteria and their lipopolysaccharide (Schletter et al., 1995). Low doses of lipopolysaccharide are thought to be beneficial for the host, e.g., in causing immunostimulation and enhanced resistance to infections and malignancies. On the other hand, the presence of a large amount of lipopolysaccharide, leads to dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, and multi-organ failure. Lipopolysaccharide stimulates host cells (mainly monocytes/macrophages, but also endothelial cells, smooth muscle cells, and neutrophils) to produce and release endogenous mediators (e.g., NO, $O_2 \cdot^-$, H_2O_2, etc.). There are several mechanisms by which intracellular elevated NO can exert genotoxic effects after reacting with oxygen. These include formation of carcinogenic *N*-nitroso compounds, direct deamination of DNA bases (Beckman et al., 1990; Wink et al., 1991), DNA single strand breakage and oxidation of DNA after formation of peroxynitrite ($ONOO^-$) and/or hydroxyl radicals ($HO\cdot$) (Emanuela, 1998). The inducible NO synthase isoform can produce high, persistent concentrations of NO upon induction with endotoxin alone or in combination with cytokines in many cell types and is expressed in the resting state in other cells, potentially resulting in cytotoxicity, tissue damage, or DNA damage. NO reacts with oxygen to form NO_2, which dimerizes to N_2O_4. N_2O_4 dismutates spontaneously in water to form nitrates and nitrites (Marletta et al., 1988). Nitrate can be microbiologically reduced to nitrite (Lin and Lai, 1982), which can then interact with dietary substrates such as amines or amides to produce *N*-nitroso compounds. The formation of carcinogenic *N*-nitrosamines resulting from elevated NO formation has been demonstrated in cell cultures and in vivo (Iyengar et al., 1987). It was also found that with L-arginine depletion, inducible NO synthase produced both $O_2 \cdot^-$ and NO, leading to $ONOO^-$ formation. This inducible NO synthase-derived $ONOO^-$ resulted in nitrotyrosine formation, and this could be inhibited by inducible NO synthase blockade (Xia and Zweier, 1997). Our results demonstrated that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate were the potent inhibitors of inducible NO synthase protein; therefore, they may block the formation of *N*-nitroso compounds and peroxynitrite or hydroxyl radicals, and could thus inhibit carcinogenesis.

Theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate decreased the protein levels of inducible NO synthase by reducing the expression of inducible NO synthase mRNA. At the gene level, the expression of murine macrophage NO synthase is largely regulated by transcriptional activation. The promoter of the inducible NO synthase gene contains two major discrete regions synergistically functioning for binding of transcription factors (Muller et al., 1993; Xie et al., 1994; Thanos and Maniatis, 1995; Baldwin, 1996): one for NF-κB that is mainly activated by lipopolysaccharide, and the other one for interferon-related transcription factors such as interferon regulatory factor 1. NF-κB is a mammalian transcription factor that controls a number of genes important for immunity and inflammation. NF-κB is composed mainly of two proteins, p50 and p65. In its unstimulated form, NF-κB is present in the cytosol bound to the inhibitory protein IκB. After induction of cells by a variety of agents, IκB becomes phosphorylated and triggers a proteolytic degradation of IκB, then NF-κB is released from IκB and translocated to the nucleus. But in 1996, Imbert et al. reported an alternative mechanism of NF-κB activation (Imbert et al., 1996). Stimulation of Jurkat T cells with the protein tyrosine phosphatase inhibitor and T cell activator pervanadate led to NF-κB activation through tyrosine phosphorylation, but not degradation of IκB. Agents that have been described as NF-κB activators include mitogens, cytokines, lipopolysaccharide, TPA, and cAMP. Reactive oxygen intermediates (ROIs) have also been proposed to be involved in the activation of NF-κB (Baldwin, 1996). This is based on the observations that treatment of some cells with H_2O_2 can activate NF-κB. Theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate have been demonstrated as antioxidants (Ho et al., 1992; Shiraki et al., 1994; Lin et al., 1996); we found that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate can block activation of NF-κB. This is consistent with the observation that certain antioxidants such as *N*-acetyl cysteine or pyrrolidine dithiocarbamate can block activation of NF-κB by blocking the signal-induced phosphorylation of IκB.

We demonstrated that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate inhibit the activation of transcription factor NF-κB in murine macrophages, thereby inhibiting the induction of inducible NO synthase tran-

scription. Similar finding was made by Kleinert et al.; they observed that dexamethasone decreased the activity of the inducible NO synthase promoter and reduced the formation of cytokine-induced NF-κB complexes that bind to the NF-κB site in the human inducible NO synthase promoter (Kleinert et al., 1996). They suggested that the dexamethasone-activated glucocorticoid receptor is likely to interact with the cytokine-activated NF-κB complex, thereby repressing the binding of this complex to the NF-κB response element in the 5′-flanking sequence of the inducible NO synthase gene.

Phosphorylation of proteins appears to play a significant role in lipopolysaccharide signalling pathways, including protein tyrosine kinases, mitogen-activated protein kinase, protein kinase C, G protein, protein kinase A, and ceramide-activated protein kinase (Sweet and Hume, 1996). Lipopolysaccharides activate protein kinase C, which in turn induces NADPH oxidase activity (Bastian and Hibbs, 1994). NADPH oxidase is located in the plasma membrane and uses intracellular NADPH to produce extracellular $O_2 \cdot^-$. $O_2 \cdot^-$ is unstable and rapidly dismutates (within milliseconds) to form H_2O_2. H_2O_2 can cross cell membranes rapidly, while other ROIs such as $O_2 \cdot^-$ and HO· cannot. Once inside the cell, H_2O_2 can react with iron or copper according to the Fenton reaction to form HO·. In our recent reports, we found that HO· radical is rapidly generated after H_2O_2 enters into the cell, then HO· can activate protein tyrosine kinase (Lee et al., 1996). The role of a protein tyrosine kinase has also been implicated in NF-κB activation by lipopolysaccharide, ultraviolet, and hypoxia (Bastian and Hibbs, 1994). Therefore, we postulated that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate were likely through inhibition of lipopolysaccharide-induced phosphorylation and degradation of IκB. The step in the signal transduction pathway of NF-κB activation inhibited by theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate might be at or before the phosphorylation step of NF-κB. Thus, we concluded that theaflavin-3,3′-digallate and (−)-epigallocatechin-3-gallate prevents the activation of transcription factor NF-κB, which in turn leads to decreased transcription of the inducible NO synthase gene.

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