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Suppression of Lipopolysaccharide-Induced Nuclear Factor-κB Activity by Theaflavin-3,3′-Digallate from Black Tea and Other Polyphenols through Down-regulation of IκB Kinase Activity in Macrophages

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ABSTRACT. We investigated the inhibition of IκB kinase (IKK) activity in lipopolysaccharide (LPS)-activated murine macrophages (RAW 264.7 cell line) by various polyphenols including (−)-epigallocatechin-3-gallate, theaflavin, a mixture of theaflavin-3 gallate and theaflavin-3′-gallate, theaflavin-3,3′-digallate (TF-3), pyrocyanidin B-3, casuarinin, geraniin, and penta-*O*-galloyl-β-D-glucose (5GG). TF-3 inhibited IKK activity in activated macrophages more strongly than did the other polyphenols. TF-3 strongly inhibited both IKK1 and IKK2 activity and prevented the degradation of IκBα and IκBβ in activated macrophage cells. The results suggested that the inhibition of IKK activity by TF-3 could occur by a direct effect on IKKs or on upstream events in the signal transduction pathway. Furthermore, geraniin, 5GG, and TF-3 all blocked phosphorylation of IκB from the cytosolic fraction, inhibited nuclear factor-κB (NFκB) activity, and inhibited increases in inducible nitric oxide synthase levels in activated macrophages. These results suggest that TF-3 may exert its anti-inflammatory and cancer chemopreventive actions by suppressing the activation of NFκB through inhibition of IKK activity. BIOCHEM PHARMACOL **59**;4:357–367, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. theaflavin-3,3′-digallate; IκB kinase; nuclear factor-κB; inhibitor κB; inducible nitric oxide synthase; macrophage; RAW 264.7

The transcriptional activator protein NFκB¶ plays a critical role in immune and inflammatory responses [1]. NF-κB is sequestered in the cytoplasm of most cell types by virtue of its association with the IκB family of inhibitor proteins, which includes IκBα, IκBβ, and IκBε. The IκBs bind to the Rel homology domain, which contains the dimerization, nuclear transfer, and DNA binding functions of the NFκB/Rel protein [2–4]. At least two of the IκBs (IκBα and IκBβ) undergo rapid phosphorylation at two conserved N-terminal residues in response to cell stimulation with proinflammatory cytokines (e.g. interleukin-1 or tumor necrosis factor), bacterial LPS, or phorbol ester (12-*O*-tetradecanoyl-phorbol-13-acetate). In the case of IκBα, this occurs at Ser-32 and Ser-36; the corresponding residues in IκBβ are Ser-19 and Ser-23. This phosphorylation targets them for rapid polyubiquitination followed by degradation through the 26S proteasome pathway [5], thereby liberating NFκB, which is then free to translocate to the nucleus and bind to DNA [6, 7].

The mechanism of phosphorylation of the N-terminus of IκB recently has been the subject of intense investigation [8–10]. Immunoprecipitates of both IκB kinase α and IκB kinase β (termed IKK1 and IKK2) can phosphorylate IκBα and IκBβ at the regulatory N-terminal Ser residues, and both IKKs can be activated by NFκB inducing kinase [11–15]. In addition, domain-negative versions of NFκB inducing kinase, IKKα, and IKKβ can inhibit tumor necrosis factor-α- and interleukin 1-induced NFκB activation. Several laboratories have established that signal-induced phosphorylation is accomplished by an intriguingly large IKK complex. Two catalytic subunits of IKK have been identified, cloned [8], and shown to be part of multi-protein complexes in the appropriate size range (M_r 800,000) [16]. IKK1 and IKK2 form homo- and heterodimers with each other, but the active complex appears to be the het-

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¶ *Abbreviations*: NFκB, nuclear factor-κB; LPS, lipopolysaccharide; IκB, inhibitor κB; IKK, IκB kinase; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TF-1, theaflavin; TF-2, the mixture of theaflavin-3-gallate (TF-2a) and theaflavin-3′-gallate (TF-2b); TF-3, theaflavin-3,3′-digallate; TR, thearubigin; EGCG, (-)-epigallocatechin-3-gallate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; and 5GG, penta-*O*-galloyl-β-D-glucose.

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erodimer. IKK appears to be the kinase involved in the signal-inducible degradation of IκB [16].

NO synthase in macrophages and hepatocytes is inducible, not detectable in unstimulated cells, and requires protein synthesis for expression. iNOS produces large amounts of NO several hours after exposure to endotoxin and/or cytokines in macrophages, Kupffer cells, hepatocytes, and fibroblasts. NO has a wide biological role in modulating physiological and pathophysiological processes [17, 18], such as macrophage cytotoxicity, neurotransmission, neurotoxicity, and regulation of blood pressure; low concentrations of NO are sufficient, in most cases, to affect these functions. However, high concentrations of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, play important roles in inflammation and in the multi-stage process of carcinogenesis [19, 20]. Among the most important stimuli for induction of iNOS is bacterial endotoxic LPS [21, 22]. A protein that binds to the 5′-flanking region of the murine iNOS gene has been cloned [23], and several binding sites for transcription factors, including NFκB, ISRE, IRF-1, and Oct, have been identified in the promoter region of the iNOS gene [23–26]. Of these transcription factors, only NFκB has been shown to enhance the expression of the iNOS gene in macrophages exposed to LPS [27].

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 flavanols. The major green tea catechins are EGCG, (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epicatechin, (+)-gallocatechin, and (+)-catechin. Among these polyphenols, EGCG is the dominant component, and it has been shown to inhibit epidermal growth factor receptor autophosphorylation and LPS-induced iNOS production [28, 29]. In the manufacture of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerization leading to the formation of bisflavanols, the TFs, TRs, and other oligomers. TFs account for about 1–2% of the total dry weight of black tea and include TF-1, TF-2a, TF-2b, and TF-3. All TFs possess benzotropolone rings with dihydroxy or trihydroxy substitution systems. About 10–20% of the dry weight of black tea is due to TRs, which are more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well characterized. The phenolic hydroxyl groups in these oxidized products of catechins show antioxidative activity similar to that of catechins. Many biological functions of tea polyphenols have been studied [30], including anti-inflammatory, antioxidative [31–33], antimutagenic [34], and anticarcinogenic effects [35]. The anti-inflammatory and cancer preventive characteristics of EGCG have been well documented [36], but the activities of TFs and TRs have not been demonstrated. In this study, we investigated the effects of TFs, TR, and other polyphenols on IKK activity and the NFκB/IκB system in a murine macrophage cell line, RAW 264.7. Our results provide a molecular basis for understanding the inhibitory effects of tea polyphenols on endotoxin-mediated inflammation.

MATERIALS AND METHODS

Reagents

LPS (*Escherichia coli* 0127: E8), sulfanilamide, naphthylethylenediamine dihydrochloride, and DTT were purchased from the Sigma Chemical Co. Acrylamide was purchased from the E. Merck Co. TF-1, TF-2, TF-3, and TR were isolated from black tea as described previously [35]. EGCG was purified from Chinese tea (Longjing tea, *Camellia sinensis*) as described in our previous report [30], and its purity was greater than 97%. 5GG (Fig. 1) and geraniin were isolated from the leaves of *Macaranga tanarins (L.)* as described previously [37]. Other polyphenols such as pyrocyanidin B-3 and casuarinin (Fig. 1) were isolated from the roots of *Rosa taiwanensis* Nakai as described previously [38].

Cell Culture

RAW 264.7 cells, which were derived from murine macrophages, were obtained from the American Type Culture Collection. RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. When the cells reached a density of 2–3 $\times$ 10^6/mL, they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Various concentrations of test compounds dissolved in DMSO were added together with LPS.

Cytotoxicity Assay

The RAW 264.7 cells were cultured at a density of 2 $\times$ 10^5 in a 6-well plate. The polyphenols studied were added to the medium 18 hr after the inoculation. The cells were harvested after 18 hr. The viability was determined by trypan blue exclusion and microscopic examination.

IKK

IKK was assayed as performed by Yamaoka *et al.* [39], with some modifications. Whole cell extracts were lysed with Gold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/mL of aprotinin, and 10 μg/mL of leupeptin] for 30 min at 4°. The cell lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4°. The cell extracts were subjected to immunoprecipitation with specific anti-IKK1 and anti-IKK2 antibodies (Santa Cruz Biotechnology) in TNT buffer [200 mM NaCl, 20 mM Tris–HCl (pH 7.5), and 1% Triton X-100, supplemented with 300 μM sodium orthovanadate, 2 μM PMSF, 10

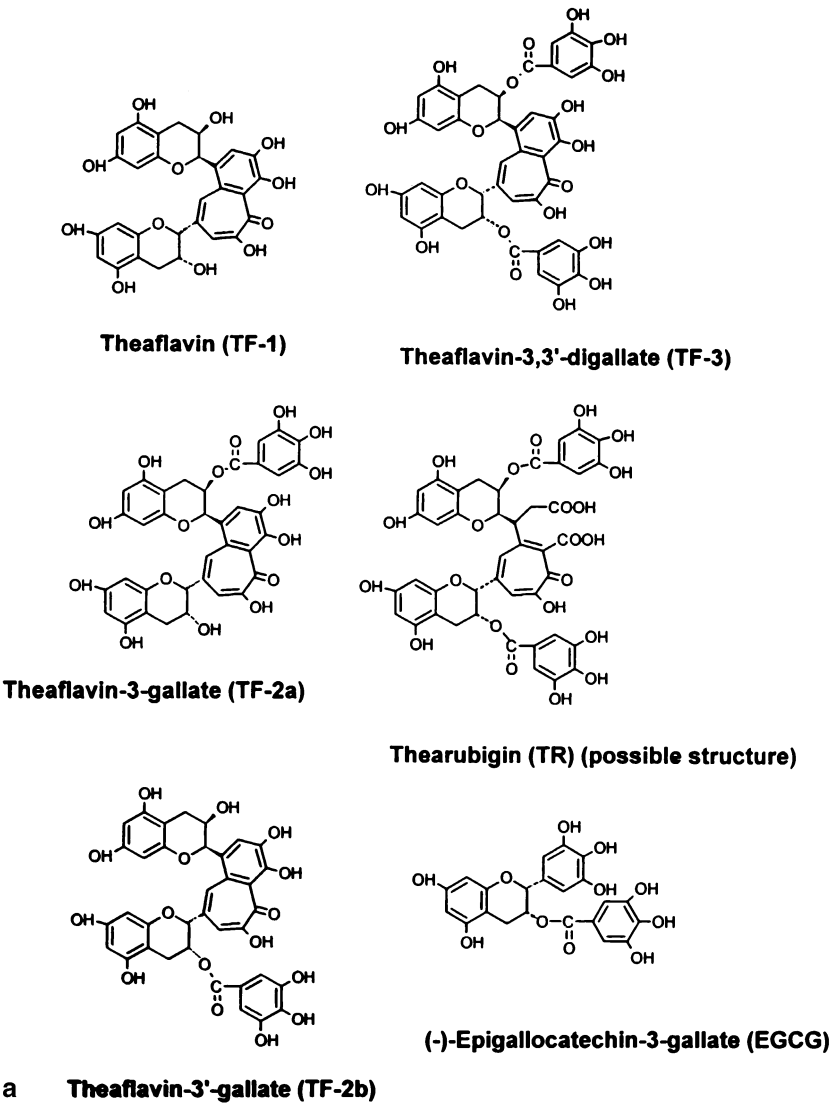

FIG. 1. Structures of selected polyphenols.

μg/mL of aprotinin, and 1 μg/mL of leupeptin]. The IKK–antibody complex was precipitated with protein-A Sepharose beads for 18 hr at 4°, washed three times with TNT buffer, and finally washed three times with kinase buffer [20 mM HEPES, 10 mM $MgCl_2$, 300 μM sodium orthovanadate, 20 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT, and 50 mM NaCl (pH 7.5) supplemented with 2 μM PMSF, 10 μg/mL of aprotinin, and 1 μg/mL of leupeptin]. The purified enzyme was assayed in kinase buffer incubated with a GST-IκBα (1–317) fusion protein (Santa Cruz Biotechnology) as the substrate. Kinase reactions were run for 30 min at 30° using 10 μCi of [γ-^{32}P]ATP, and terminated by the addition of 5x SDS–PAGE sample buffer and boiling for 10 min. The reaction products were resolved by SDS–PAGE in 10% gels, visualized by autoradiography with Kodak X-Omat film for 3 hr at room temperature, and quantitated by densitometry (IS-1000 Digital Imaging System).

Western Blots

Total cellular extracts were prepared in Gold lysis buffer. Aliquots containing 30–50 μg of total protein (for IKK1, IKK2, IκBα, IκBβ, iNOS, and α-tubulin) were separated on SDS–polyacrylamide minigels (8% for IKK1, IKK2, and iNOS, and 10% for IκBα, IκBβ, and α-tubulin) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was blocked overnight at room temperature with blocking solution [20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 1% BSA, and 0.1% sodium azide] and then incubated with anti-IκBα, IκBβ, IKK1, or IKK2 polyclonal antibodies (Santa Cruz Biotechnology), anti-macNOS monoclonal antibody (Transduction Laboratories), anti-IκB-P polyclonal antibody (Bio. Lab.), or anti-α-tubulin monoclonal antibodies (Oncogene Science Inc.) at room temperature for 1 hr. iNOS, IKK1, IKK2, IκBα, IκBβ, and α-tubulin proteins were detected by chemiluminescence (ECL, Amersham).

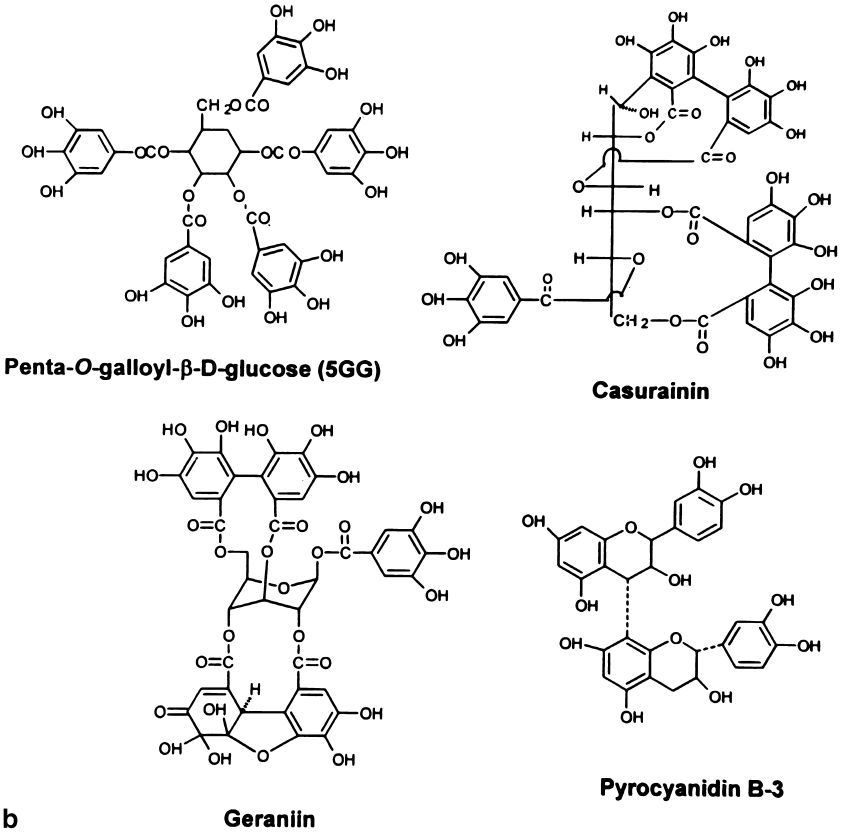

FIG. 1. Continued.

Electrophoretic Mobility Shift Assays for NFκB

Nuclear and cytoplasmic extracts were prepared according to a modification of the method described by Lin and Lin [29]. Briefly, 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 DTT, 0.5 mM PMSF] for 10 min on ice, and vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 *g* for 20 sec, and the supernatants, containing cytosolic proteins, were collected. Then the pellets were suspended in buffer C [20 mM HEPES (pH 7.6), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF] for 30 min on ice. The supernatants, containing nuclear proteins, were collected by centrifugation at 12,000 *g* for 20 min and stored at −70°.

For electrophoretic mobility shift assays, 6 μg of each nuclear extract was mixed with the ^{32}P-labeled double-stranded NFκB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-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 DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl]. The DNA/protein complex was electrophoresed on 5% nondenaturing polyacrylamide gels in 0.5x Tris/borate/EDTA buffer (0.0445 M Tris, 0.0445 M borate, 0.001 M EDTA). The specificity of binding also was examined by competition with the unlabeled oligonucleotide. Radioactive bands were detected by autoradiography.

Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by George *et al.* [40] with some modifications. RAW 264.7 cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco). Then the cells were transfected with the pNFκB-Luc plasmid reporter gene (Stratagene) using LipofectAMINE^{TM} reagent (Gibco, NRL, Life Technologies, Inc.) pGFPemd-cmv control plasmid (Packard) was co-transfected as an internal control for transfection efficiency. After another 24 hr of incubation, the medium was replaced with complete medium. After 24 hr, the cells were trypsinized, and equal numbers of cells were plated in 12-well tissue culture dishes for 18 hr. Then the cells were incubated with 100 ng/mL of LPS and TF-3 for 3 hr. Each well was washed twice with cold PBS and harvested in 150 μL of lysis buffer [0.5 M HEPES (pH 7.8), 1% Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$]. Luciferase activity was assayed by means of the LucLite^{TM} luciferase reporter gene kit (Packard BioScience Co.) with 100 μL of cell lysate used in each assay. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.02 min/well, following a 5-min adaptation in the dark. Luciferase activities were determined and normalized on the basis of pGFPemd-cmv expression.

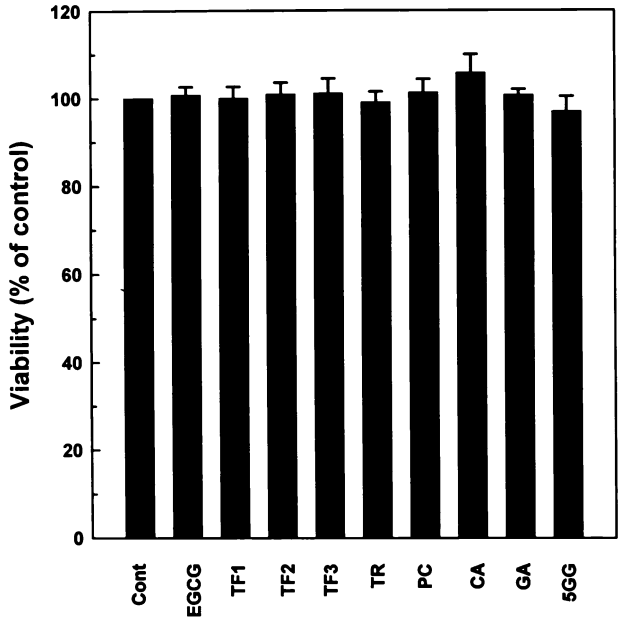

FIG. 2. Cytotoxic effects of the polyphenols in RAW 264.7 cells. Cytotoxicity was estimated by trypan blue exclusion using a hemocytometer chamber. The percent survival of RAW 264.7 cells is shown following 18 hr of exposure to various polyphenols (30 μM). Cont: control; EGCG: (−)-epigallocatechin-3-gallate; TF-1: theaflavin; TF-2: theaflavin-3-gallate and theaflavin-3′-gallate; TF-3: theaflavin-3,3′-digallate; TR: thearubigin; PC: pyrocyanidin B-3; CA: casuarinin; GA: geraniin; 5GG: penta-O-galloyl-β-D-glucose. The number of cells in the control was 2×10^5. Data are expressed as the means ± SEM of triplicate wells of experiments performed with two independent cell cultures.

Nitrite Assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [41]. 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 with an enzyme-linked immunosorbent assay plate reader (Dynatech MR-7000; Dynatech Laboratories).

RESULTS

Inhibition of IKK1 Activity by Selected Polyphenols in RAW 264.7 Cells

The chemical structures of the selected polyphenols used in this study are illustrated in Fig. 1. In an attempt to explore the effects of the selected polyphenols on the inhibition of IKK activity in RAW 264.7 cells, the cytotoxicities of the polyphenols were evaluated by trypan blue assay and microscopic examination. It was apparent that there were no cytotoxic effects of the polyphenols at 30 μM for 18 hr (Fig. 2). Because LPS is an activator of IKK, we studied the inhibitory effects of these nine polyphenols on LPS-induced IKK activity in macrophage cells. RAW 264.7 cells were exposed to each of the selected polyphenols (30 μM) and LPS (100 ng/mL) for 10 min. To directly measure IKK activity in cells, IKK1 was immunoprecipitated and assayed using recombinant GST-IκBα (1–317) as a substrate. Figure 3 illustrates the relative effect of selected polyphenols on IKK1 activity at a concentration of 30 μM. After stimulation with LPS, GST-IκBα fusion protein was phosphorylated strongly, indicating stimulation of IKK activity. Basal IKK1 activity also was found in unstimulated RAW 264.7 cells. Several of the selected polyphenols inhibited IKK1 activity. The most effective of the tested compounds was TF-3, which inhibited LPS-induced IKK activity by about 80%, followed by geraniin, 5GG, EGCG, TF-1, and TF-2, whereas the inhibitory effects of TR, pyrocyanidin, and casuarinin on LPS-induced IKK1 activity were rather low. Western blot analysis showed that the level of IKK1 protein was not changed by incubation with polyphenols (Fig. 3A), suggesting that the inhibition of LPS-induced IKK1 activity by the polyphenols was not due to decreased expression of IKK1.

Inhibition of Kinase Activity of IKK1 and IKK2 by TF-3 in Macrophages

Because TF-3 was the most potent inhibitor of IKK in RAW 264.7 cells, we studied the effect of different concentrations of this polyphenol on IKK activity. As it has been reported that IKK1 and IKK2 need to form a heterodimer for maximal enzyme activity [15], we also tested the effect of TF-3 on IKK2 activity in RAW 264.7 cells by means of an immunocomplex kinase assay. Stimulation of RAW 264.7 cells with LPS caused a marked increase in IKK1 and IKK2 activity as measured after 10 min. TF-3, at 10–30 μM, inhibited IKK1 and IKK2 activity in a concentration-dependent manner (Fig. 4). Western blot analysis showed that the protein levels of IKK1 and IKK2 were not changed. These results further confirmed that TF-3 inhibited the activity of the IKK complex (IKK1 and IKK2) in LPS-stimulated RAW 264.7 cells, and that these effects were not due to decreased protein levels.

Effect of Geraniin, 5GG, and TF-3 on Phosphorylation of IκB

To determine whether the inhibitory action of TF-3 was due to its effect on IκB phosphorylation, the cytoplasmic levels of IκB-P were examined by western blot analysis. Incubation of macrophages with LPS for 15 min caused marked phosphorylation of cytosolic IκB. From the blot it appeared that geraniin and 5GG lowered phosphorylation to near basal levels, whereas TF-3 also blocked basal-level phosphorylation (Fig. 5).

Inhibition of LPS-Induced Degradation of IκBs

It has been reported that IKK can phosphorylate IκBα and IκBβ, thereby targeting them for degradation through the

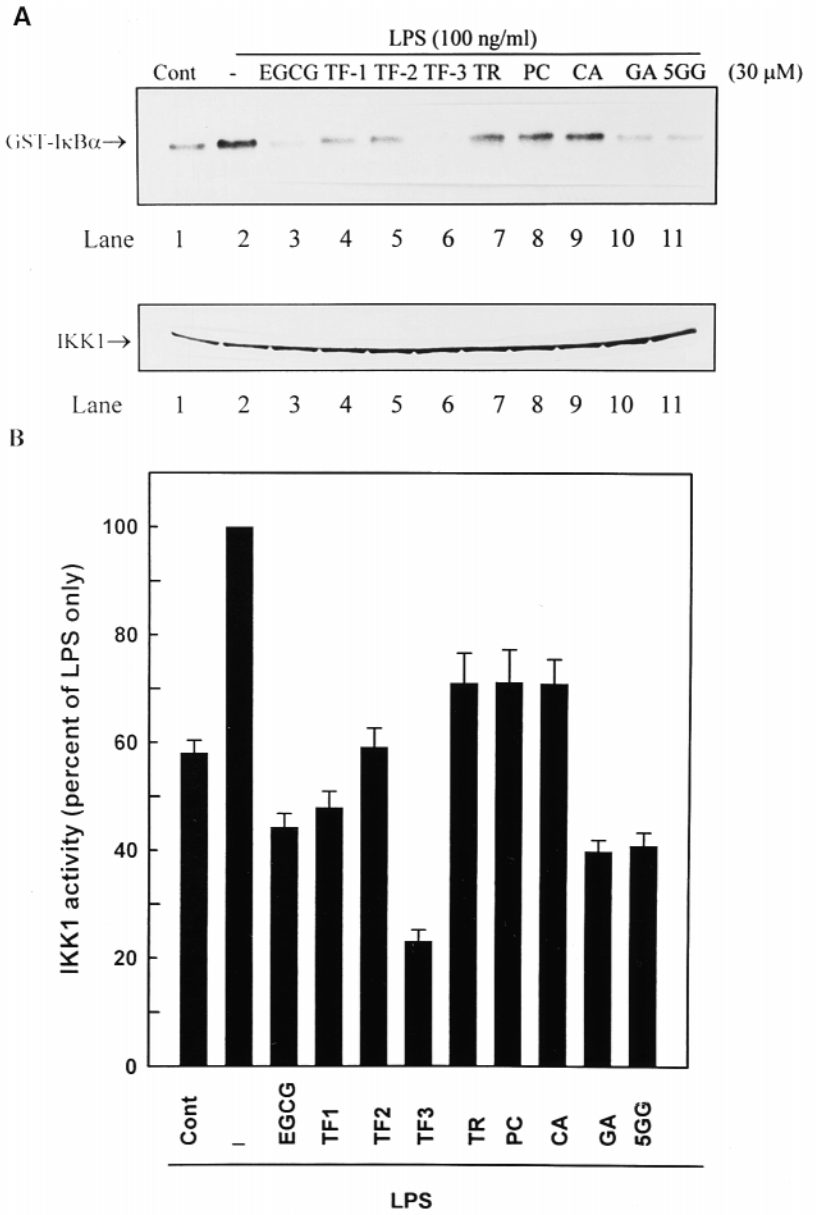

FIG. 3. Analysis of IKK1 expression and activation in murine macrophages (RAW 264.7) treated with LPS and various polyphenols. (A) Immunoprecipitation kinase assays of whole-cell extracts. RAW 264.7 cells were cotreated with 100 ng/mL of LPS with or without various polyphenols (30 μM) or DMSO (0.03%) solvent for 10 min. Cells were harvested, and IKK1 activity in the soluble fractions was analyzed using immune complex kinase assays as described in Materials and Methods. IKK1 was immunoprecipitated with the anti-IKK1 antibody, and the activity in the immune complexes was assayed by using GST-IκBα (1–317) as a substrate. PC: pyrocyanidin B-3; CA: casuarinin; GA: geraniin. (B) Quantitation of the phosphorylated GST-IκBα was performed by densitometric analysis (IS-1000 Digital System) of the kinase assay. Data are expressed as the means ± SEM of the percent of maximal phosphorylated GST-IκBα observed with LPS, as determined in three independent experiments.

ubiquitin–proteasome pathway [5]. To determine whether TF-3 affected degradation of IκBs, we determined the levels of IκBα and IκBβ in RAW 264.7 cells after incubation for 30 min with 100 ng/mL of LPS and 0–30 μM TF-3. Western blot analysis of cell extracts with antibodies specific for IκBα and IκBβ showed that LPS alone caused remarkable reductions in the levels of both IκBs, and TF-3 blocked these reductions in a concentration-dependent manner (Fig. 6).

Inhibition of LPS-Induced NFκB Activity by Geraniin, 5GG, and TF-3

Because the degradation of IκBs results in NFκB activation, we investigated the effects of geraniin, 5GG, and TF-3 on NFκB activation by means of electrophoretic mobility shift assays and transient transfection. Incubation of macrophages with LPS (100 ng/mL) for 1 hr markedly increased binding of the NFκB target DNA sequence probe by nuclear NFκB, and this induction of NFκB binding activity

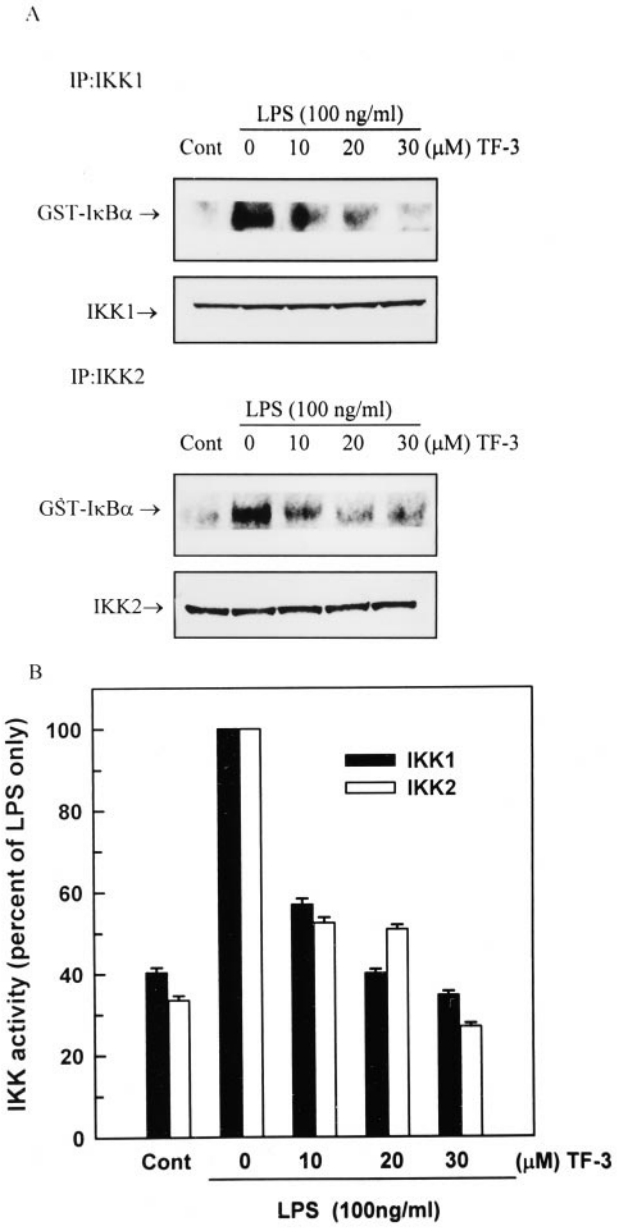

FIG. 4. Concentration-dependent inhibition by TF-3 of LPS-induced IKK activity in murine macrophages (RAW 264.7). Whole cell extracts were prepared from RAW 264.7 cells treated with the indicated concentration of TF-3 and 100 ng/mL of LPS or solvent only for 10 min. IKK1 and IKK2 activity assays were carried out as described in Materials and Methods. (A) Effects of TF-3 on IKK1/2 activity. A part of each extract was used to measure IKK1 activity (upper panel), and another part was used to measure IKK2 activity by immune complex kinase assay (bottom panel). (B) Phosphorylated GST-IκBα was quantitated by densitometric analysis (IS-1000 Digital System) of the kinase assay. Data are expressed as the means ± SEM of the percent of maximal phosphorylated GST-IκBα observed with LPS, as determined in three independent experiments.

by LPS was inhibited markedly by coincubation with TF-3, geraniin, or 5GG (Fig. 7A). Moreover, similar results were obtained with transient transfection. When the pNFκB-Leu reporter plasmid was cotransfected with pGFPemd-cmv control plasmids into RAW 264.7 cells, TF-3 inhibited LPS-induced NFκB transcriptional activity in a concentration-dependent manner (Fig. 7B). At a concentration of 30 μM, TF-3 had the greatest inhibitory potency of the polyphenols tested, followed by 5GG and geraniin (Fig. 7C). Taken together, these results suggested that TF-3 might block LPS-induced NFκB activation by inhibiting IKK activity, which could perturb the degradation of IκBα and IκBβ.

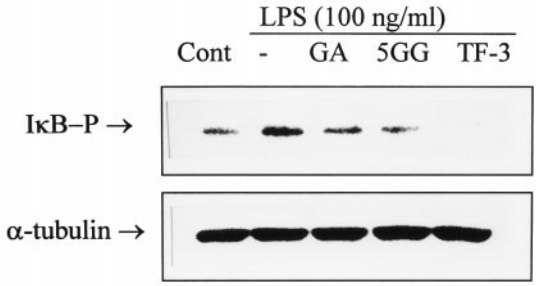

FIG. 5. Inhibition of LPS-induced IκB phosphorylation by geraniin (GA), 5GG, and TF-3. Murine macrophages (RAW 264.7) were incubated with LPS (100 ng/mL) and a 30 μM concentration of GA, 5GG, or TF-3 for 15 min. Cont: control, DMSO (0.03%). Cytosolic fractions were prepared and analyzed for the content of IκB-P protein by western blot. This experiment was repeated three times with similar results.

Inhibition of iNOS Expression by Geraniin, 5GG, and TF-3

We next investigated whether geraniin, 5GG, and TF-3 could affect iNOS protein levels in macrophages activated with LPS. All three polyphenols markedly reduced the amount of iNOS protein in LPS-stimulated cells (Fig. 8). TF-3 markedly reduced the iNOS protein level in a concentration-dependent manner (Fig. 8A and B). At a

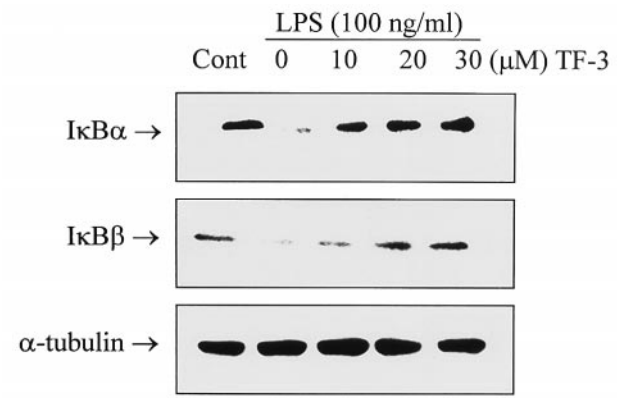

FIG. 6. Concentration-dependent stabilization of IκBα and IκBβ by TF-3 in murine macrophages (RAW 264.7) exposed to LPS. RAW 264.7 cells were treated with the indicated concentrations of TF-3 and 100 ng/mL of LPS for 30 min. Total protein extracts were separated by SDS–PAGE through 10% gels and analyzed by western blot analysis as indicated in Materials and Methods. Similar results were obtained in three independent experiments.

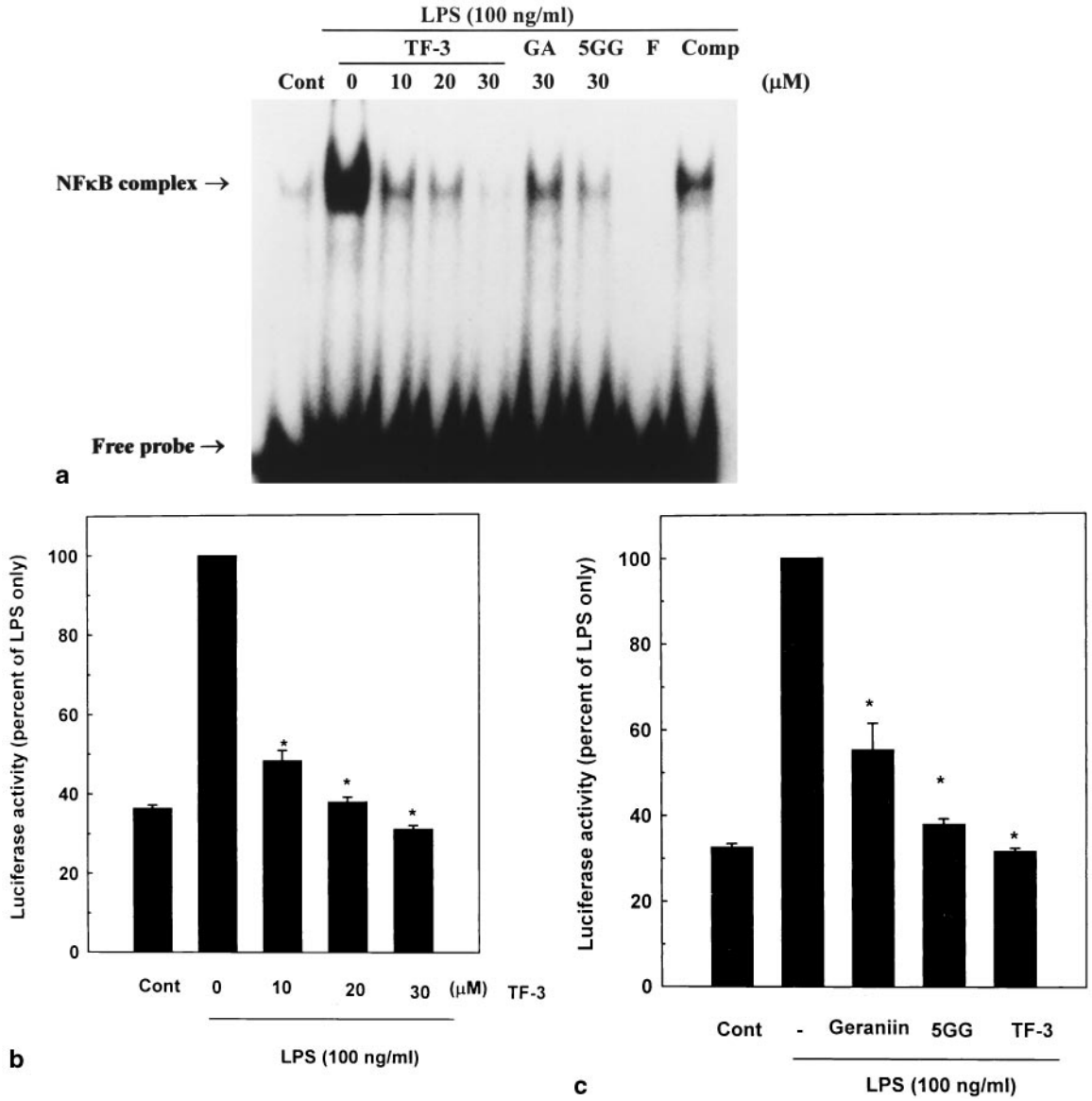

FIG. 7. Inhibition of LPS-induced NFκB activity by geraniin (GA), 5GG, and TF-3. Nuclear extracts were prepared from murine macrophages (RAW 264.7) treated with 100 ng/mL of LPS and the indicated concentration of TF-3, GA, or 5GG for 1 hr. Electrophoretic mobility shift assays were carried out as described in Materials and Methods, and the binding of NFκB from nuclear extracts to [^{32}P]NFκB oligonucleotide is shown. The position of the NFκB–DNA complex is indicated with an arrow. The specificity of NFκB binding activities was tested by competition with a 20-fold excess of unlabeled consensus oligonucleotide. F: free probe; Comp: competition. (B) Cells were cotransfected with 2.5 μg of pNFκB-Luc and pGFPemd-cmv plasmids. After transfection, cells were subcultured in 12-well plates, and then were cotreated with 100 ng/mL of LPS and different concentrations of TF-3 for 3 hr. (C) Cells were treated with 100 ng/mL of LPS and 30 μM GA, 5GG, or TF-3 for 3 hr. Luciferase activities were determined and normalized on the basis of pGFPemd-cmv expression, as described in Materials and Methods. Data are expressed as the means ± SEM of the percent of maximal luciferase activity observed with LPS only (about 3.2 × 10^4 cps) as determined in three independent experiments. Key: (*) $P < 0.001$ vs LPS treatment (Student's *t*-test).

concentration of 10 μM, TF-3 decreased iNOS protein levels by 91%, and no iNOS could be detected after incubation with 20 μM TF-3. 5GG and geraniin at 30 μM inhibited iNOS protein levels by 100 and 78%, respectively (Fig. 8C and D).

Inhibition of NO Generation by Geraniin, 5GG, and TF-3

Of the polyphenols tested, TF-3 inhibited LPS-stimulated NO generation the most strongly; however, geraniin, 5GG, and TF-3 all markedly reduced NO generation in a concentration-dependent manner (Fig. 9). At concentrations of 10, 20, and 30 μM, geraniin inhibited NO generation by 25, 37, and 54%, respectively. At concentrations of 10, 20, and 30 μM, 5GG inhibited NO generation by 53, 63, and 77%, respectively. At concentrations of 10, 20, and 30 μM, TF-3 inhibited NO generation by 77, 89, and 92%, respectively. Inhibition of NO production was not due to cytotoxicity, as determined with the trypan blue exclusion assay (Fig. 2).

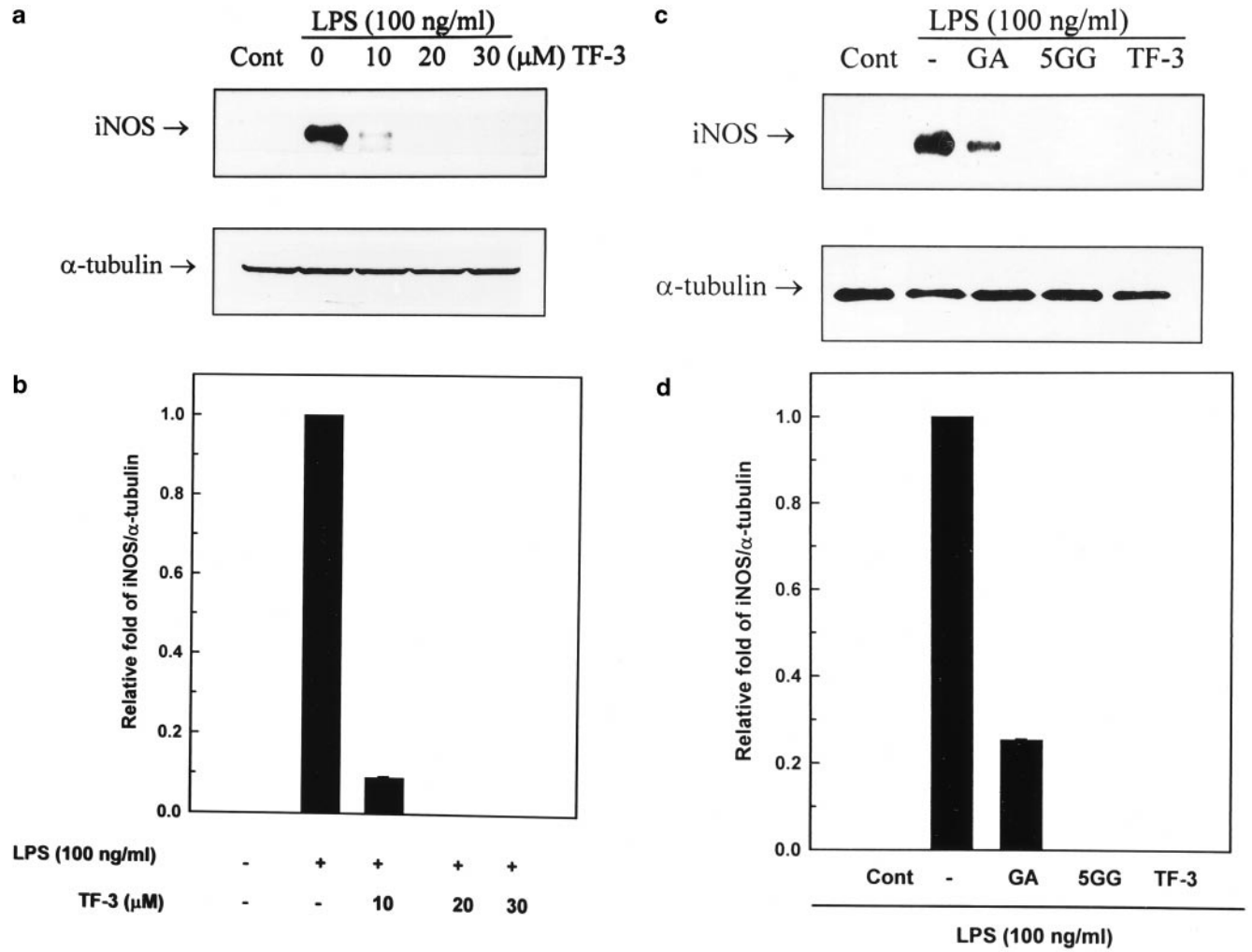

FIG. 8. Inhibition of LPS-dependent iNOS activation by geraniin (GA), 5GG, and TF-3. (A) Murine macrophages (RAW 264.7) were cotreated with 100 ng/mL of LPS and various concentrations of TF-3 (0–30 μM) for 18 hr. At the end of incubation, total protein was extracted for analysis of iNOS protein and α-tubulin by western blot. (B) Band intensities were quantitated by densitometry (IS-1000 Digital Imaging System). (C) RAW 264.7 cells were cotreated with 100 ng/mL of LPS and 30 μM GA, 5GG, or TF-3 for 18 hr. Total protein was isolated for western blot analysis of iNOS and α-tubulin. (D) Band intensities were quantitated by densitometry (IS-1000 Digital Imaging System). Data in panels B and D are expressed as the means ± SEM of the ratio of maximal protein expression observed with LPS, as determined in three independent experiments. The ratio of iNOS to α-tubulin protein expression observed with LPS alone was set at 1. The relative level was calculated as the ratio of iNOS to α-tubulin protein expression, which was determined by densitometric analysis of the immunoblots.

DISCUSSION

A recent study of ours demonstrated that EGCG, (-)-epigallocatechin, and gallic acid inhibit induction of iNOS in murine peritoneal macrophages activated with LPS [29], and that the galloyl group and the hydroxyl group at the 3′ position on EGCG are responsible for its strong anti-inflammatory property. These tea polyphenols have phenol rings that act as electron traps to scavenge peroxyl radicals, superoxide anions, and hydroxyl radicals and prevent oxidation of iron [30–32, 36].

Mammals are in constant contact with Gram-negative bacteria and their LPS [42]. Low doses of LPS are thought to be beneficial for the host, e.g. in causing immunostimulation and enhancing resistance to infections and malignancies. On the other hand, the presence of large amounts of LPS can lead to dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, and multi-organ failure. LPS stimulates host cells (mainly monocytes/macrophages, but also endothelial cells, smooth muscle cells, and neutrophils) to produce and release endogenous mediators such as NO. There are several mechanisms by which elevated levels of intracellular NO can exert genotoxic effects after reacting with oxygen; these include formation of carcinogenic *N*-nitroso compounds, direct deamination of DNA bases [43, 44], DNA strand breakage, and oxidation of DNA after formation of peroxynitrite and/or hydroxyl radicals. The iNOS isoform can produce high, persistent concentrations of NO upon induction with endotoxin alone or in combination with cytokines in many

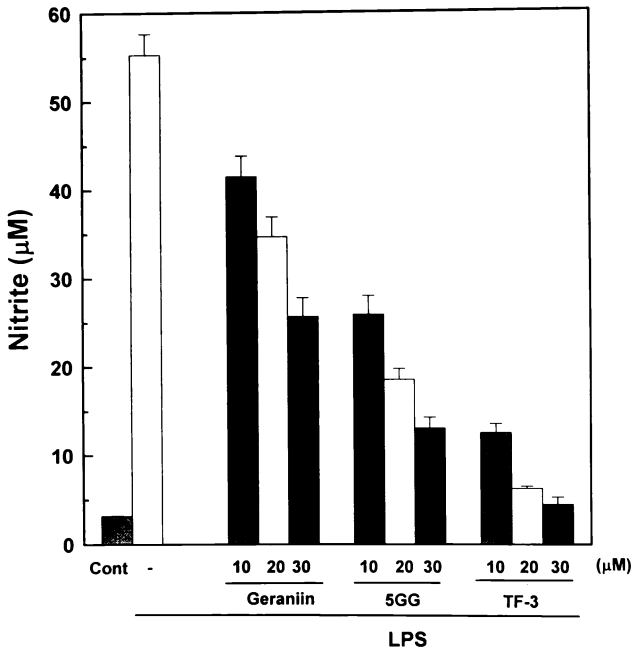

FIG. 9. Effects of geraniin, 5GG, and TF-3 on nitrite release in culture medium of LPS-activated macrophages. RAW 264.7 cells were cotreated with 50 ng/mL of LPS and different concentrations of geraniin, 5GG, or TF-3 for 18 hr. At the end of incubation, the culture medium was collected for nitrite assay. Data are the means ± SEM of three determinations.

cell types. iNOS is expressed in the resting state in other cells, potentially resulting in cytotoxicity, tissue damage, or DNA damage. Our results demonstrated that geraniin, 5GG, and TF-3 are potent inhibitors of iNOS; therefore, they may block the formation of *N*-nitroso compounds and peroxynitrite or hydroxyl radicals, and thus could inhibit carcinogenesis.

At the gene level, the expression of murine macrophage iNOS is regulated largely by transcriptional activation. The promoter of the iNOS gene contains two major discrete regions that function synergistically to bind transcription factors [4, 27, 45, 46]: one for NFκB that is activated mainly by LPS, and the other one for interferon-related transcription factors such as interferon regulatory factor 1. NFκB is a mammalian transcription factor that controls a number of genes important for immunity and inflammation. Central to the activation of NFκB are two IKKs. A critical step in activating NFκB is the rapid depletion of cytoplasmic IκBα protein by proteolytic degradation. This is triggered by phosphorylation of IκBα at two amino-terminal serine residues; binding of ubiquitin then targets the phosphorylated IκBα for degradation by the ubiquitin–proteasome pathway.

In this study, we demonstrated that TF-3 strongly inhibits IKK activity in a murine macrophage cell line, RAW 264.7. Other polyphenols, including geraniin, 5GG, EGCG, TF-1, and TF-2, had moderate inhibitory activities, whereas TR, pyrocyanidin B-3, and casuarinin were less inhibitory. The results suggested that the reduction of IKK activity by TF-3 could be mediated by a direct effect on the IKKs or on events upstream from IKKs in the signal transduction pathway. Our findings also point to a possible pathway for the inhibition of NFκB activation by TF-3: TF-3 first inhibits LPS-induced IKK activity (either directly or indirectly), which prevents the degradation of IκBα and IκBβ and thereby blocks NFκB activation. Furthermore, TF-3 strongly reduced the levels of iNOS protein, and this might be a consequence of reduced activation of NFκB. Indirectly, TF-3 exerts these effects by reducing NO levels. Therefore, we suggest that TF-3 could exert its anti-inflammatory and cancer chemopreventive actions by suppressing the activation of NFκB through inhibition of IKK activity.

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