

A Green Tea-Derived Polyphenol, Epigallocatechin-3-Gallate, Inhibits I κ B Kinase Activation and IL-8 Gene Expression in Respiratory Epithelium

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Abstract—Interleukin-8 (IL-8) is a principle neutrophil chemoattractant and activator in humans. There is interest in developing novel pharmacological inhibitors of IL-8 gene expression as a means for modulating inflammation in disease states such as acute lung injury. Herein we determined the effects of epigallocatechin-3-gallate (EGCG), a green tea-derived polyphenol, on tumor necrosis factor- α (TNF- α)-mediated expression of the IL-8 gene in A549 cells. EGCG inhibited TNF- α -mediated IL-8 gene expression in a dose response manner, as measured by ELISA and Northern blot analysis. This effect appears to primarily involve inhibition of IL-8 transcription because EGCG inhibited TNF- α -mediated activation of the IL-8 promoter in cells transiently transfected with an IL-8 promoter-luciferase reporter plasmid. In addition, EGCG inhibited TNF- α -mediated activation of I κ B kinase and subsequent activation of the I κ B α /NF- κ B pathway. We conclude that EGCG is a potent inhibitor of IL-8 gene expression *in vitro*. The proximal mechanism of this effect involves, in part, inhibition of I κ B kinase activation.

KEY WORDS: tumor necrosis factor- α , nuclear factor- κ B, inflammation, signal transduction, I κ B α

INTRODUCTION

The medicinal effects of green tea (*Camellia sinensis*) have a long, rich history. The Buddhist monks of ancient Japan recognized green tea for its medicinal powers and brought it back from China as early as the eighth century. The monk Eisai, considered the "Father of Tea" in Japan, wrote in his book *Maintaining Health by Drinking Tea* in 1211: "Tea is a miraculous medicine for the maintenance of health. Tea has an extraordinary power to prolong life." More recently, there is increasing interest

in the beneficial effects of green tea on health and disease prevention. Epidemiological studies have suggested that the regular consumption of green tea reduces the risk of cancer (1–4). Additional studies have demonstrated the potent anticarcinogenic, antioxidant, and bactericidal properties of green tea or its constituents (4–6). Consumption of green tea may also prove to play an adjunctive role in the treatment of diabetes mellitus, hypertension, and hypercholesterolemia (7).

Although green tea consists of over 2000 components, the flavinol polyphenolic compounds are the most abundant, accounting for nearly 30% of the dry weight of green tea leaves (3). These compounds are commonly known as catechins and include epigallocatechin-3-gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin (EC) (Fig. 1). Apart from their ability to sequester metal ions and scavenge reactive oxygen species (8, 9), the catechins, espe-

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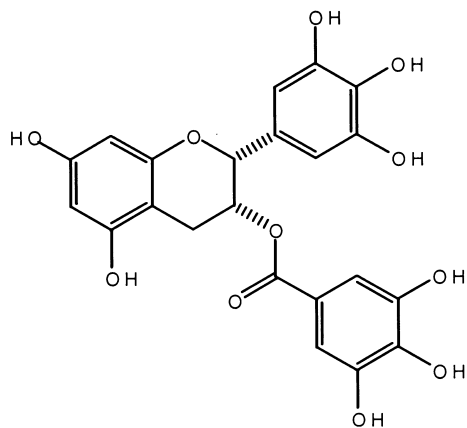


Fig. 1. Chemical structure of the green tea polyphenol, epigallocatechin-3-gallate (EGCG)

cially EGCG, have been shown to inhibit several proteins involved in inflammation, including lipoxygenase (10–12), cyclooxygenase (11, 13, 14), nitric oxide synthase (15, 16), tumor necrosis factor- α (17), xanthine oxidase (18), and nuclear factor κ B (16, 19).

Activation of nuclear factor- κ B (NF- κ B) acts as a master switch or control point for the expression of a large number of proinflammatory genes (20–22). NF- κ B, a member of the Rel family of transcription factors, is usually present in the cytoplasm of the cell in an inactive state bound to a related inhibitory protein known as I κ B. I κ B physically masks the nuclear translocation sequence of NF- κ B, thereby retaining it in the cytoplasm in an inactive state. A common pathway for the activation of NF- κ B occurs when I κ B is phosphorylated by the recently described I κ B kinase, specifically at the serine-32 and -36 residues of I κ B (23–26). Phosphorylated I κ B is targeted for rapid ubiquitination, which results in degradation by the 26S proteasome. Degradation of I κ B unmasks the nuclear translocation sequence of NF- κ B, allowing NF- κ B to enter the nucleus and direct transcription of target genes (24). The current data strongly support a central role for this pluripotent transcription factor and support the concept of therapeutic strategies targeting the NF- κ B pathway.

Interleukin-8 (IL-8) is a primary chemoattractant peptide for neutrophils, thereby playing a central role in many inflammatory conditions such as acute lung injury and sepsis. IL-8 gene expression is regulated in part by NF- κ B (26). The green tea polyphenol EGCG has been shown to inhibit 26S proteasome activity *in vitro* and *in vivo*, thereby leading to the accumulation of I κ B and inhibiting NF- κ B activation (27). Accordingly, in the

current experiments we determined the effect of EGCG on *in vitro* IL-8 gene expression and on activation of the NF- κ B pathway in cultured human respiratory epithelial cells.

METHODS

Cell Culture

All experiments involved A549 cells (American Type Culture Collection, Bethesda, MD), a human lung adenocarcinoma cell line representative of distal respiratory epithelium. These cells have previously been shown to be a useful model of IL-8 gene regulation (28, 29). Cells were maintained in a room air/5% CO₂ incubator at 37°C using Dulbecco's modified eagle's medium (DMEM) containing 8% FBS and 1% penicillin/streptomycin (Gibco BRL, Rockville, MD).

Experimental Conditions

IL-8 gene expression was induced by treating cells with 2 ng/ml of human TNF (Boehringer Mannheim, Indianapolis, IN). Epigallocatechin gallate (EGCG, Sigma Chemical Co., St. Louis, MO) was diluted in filtered phosphate-buffered saline to a stock concentration of 10 mM and further diluted to experimental concentrations ranging from 3–100 μ M in DMEM. Cells were treated with EGCG for 1 hr before incubation with TNF- α . Cells not treated with EGCG were preincubated in DMEM alone.

Enzyme-Linked Immunosorbent Assay (ELISA)

Immunoreactive IL-8 concentrations were measured in the medium of treated cells using a commercially available sandwich ELISA (Biosource, Camarillo, CA). All procedures were performed as recommended by the manufacturer.

Northern Blot Analysis

Total cellular RNA was recovered using the Trizol reagent (GIBCO BRL). RNA was quantified by spectrophotometry (260 nm) and 15 μ g of total RNA per condition underwent electrophoresis on 1% agarose gels containing 3% formaldehyde. Ethidium bromide staining and brief ultraviolet (UV) illumination confirmed integrity of the RNA after electrophoresis.

RNA was subsequently transferred to nylon membranes (Micro Separations Inc., Westboro, MA) and UV auto-crosslinked (UV Stratalinker 1800; Stratagene, La Jolla, CA). After a 4 hr prehybridization at 42°C, membranes were hybridized overnight with a radiolabeled human IL-8 cDNA probe (Allen, 2000). The cDNA was labeled with a α -[³²P]deoxycytidine triphosphate (specific activity, 3,000 Ci/mM; New England Nuclear Research Products, Boston, MA) by random priming (Pharmacia, Piscataway, NJ). The hybridized filters were washed at 53°C using 2 × sodium chloride/0.1% sodium dodecyl sulfate (SDS) and 25 mM NaHPO₄/1mM ethylenediaminetetraacetic acid (EDTA)/0.1% SDS solutions. After washing, exposure was carried out overnight and analyzed using a PhosphorImager screen and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Transient Transfections and Luciferase Assays

IL-8 promoter activity was measured using a plasmid containing the -97 bp 5' flanking region of the IL-8 gene cloned into a luciferase reporter plasmid (pGL2; Promega, Madison, WI). Cells were transiently transfected with the IL-8 promoter-luciferase reporter plasmid in duplicate, in six-well plates, at a density of 200,000 cells per well by incubation with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) and serum free DMEM overnight. After transfection, cells were washed once with PBS and pre-treated with EGCG for 1 hr and subsequently treated with human TNF- α for 6 h. Cellular proteins were extracted and analyzed for luciferase activity according to the manufacturer's instructions (Promega) using a Berthold AutoLumat LB953 luminometer. Luciferase activity was corrected for total cellular protein and reported as fold induction over control cells (cells that were transfected and treated with medium alone).

Nuclear Protein Extraction

All nuclear extraction procedures were performed on ice with ice-cold reagents. Treated cells were washed twice with PBS and harvested by scraping. Cells were pelleted in 1 ml of PBS at 6,000 rpm for 5 min. The pellet was washed twice with PBS and resuspended in lysis buffer (10 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid [HEPES], pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% v/v Nonidet P-40, 1 mM dithiothreitol [DTT], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was incubated

on ice for 5 min and centrifuged at 4°C at 6,000 rpm. The supernatant was discarded and one cell pellet volume of extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 M EDTA, 1.5 mM MgCl₂, 25% v/v glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min. Nuclear proteins were isolated by centrifugation at 14,000 rpm at 4°C for 15 min. Protein concentrations of the resultant supernatants were determined using the Bradford assay. Nuclear proteins were stored at -70°C until used for electromobility gel shift assays (EMSA).

EMSA

The NF- κ B oligonucleotide probe used for EMSA (5'-GTGGAATTCCTCTGA-3') corresponds to the NF- κ B site in the IL-8 promoter and was synthesized at the University of Cincinnati DNA Core Facility (Cincinnati, OH) (28). The probe was labeled with γ -[³²P]adenosine triphosphate using T4 polynucleotide kinase (GIBCO BRL) and purified in Bio-Spin chromatography columns (BioRad).

For EMSA 10 μ g of nuclear proteins were preincubated with EMSA buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCL, pH 7.9, 25 mM KCL, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly [d(I-C)], 12% glycerol vol/vol, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide probe for an additional 10 min. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:biacrylamide) and run in 0.5X TBE (45 mM Tris-HCL, 45 mM boric acid, 1 mM EDTA) for 1 hr at constant current (30mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 hr, and exposed to photographic film at -70°C with an intensifying screen.

Western Blot Analysis

Treated cells were washed once in PBS and lysed in ice-cold lysis buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, and PMSF (100 μ g/ml). Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). Whole cell lysates containing 50 μ g of protein were boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2- β -mercaptoethanol). Proteins were separated electrophoretically on 8–16% Tris-glycine gradient gels

(Novex, San Diego, CA) and subsequently transferred to nitrocellulose membranes (Novex) using the Novex Xcell Mini-Gel system. For immunoblotting, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) for 1 hr. Primary antibody against the inducible isoform of I κ B (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:250 dilutions for 1 hr. After washing twice with TBS containing 0.05% Tween 20 (TTBS), secondary antibody (peroxidase-conjugated goat antirabbit immunoglobulin G, Stressgen, Victoria, British Columbia) was applied at 1:10,000 dilution for 1 hr. Blots were washed in TTBS twice for 10 min, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham, Buckinghamshire, England), and exposed to photographic film.

IKK Assay

Treated cells were washed with PBS containing 1 mM PMSF, 100 mM Na₃VO₄, 2 mM PNPP, and 210 mU/ml aprotinin (Sigma, St. Louis, MO). Cells were scraped and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 100 μ M Na₃VO₄, 2 mM PNPP, and 210 mU/ml aprotinin. Immunoprecipitation of the cell extract was performed using anti-IKK γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the cell-extract-antibody to pull down the immunoprecipitated IKK. The pellet was washed several times, resuspended in kinase assay buffer containing 100 μ M ATP, 6 μ g GST-I κ B α as substrate, and 0.5 μ l γ -[³²P]ATP, and incubated for 30 min at 30°C. The reaction was stopped in an ice bath and electrophoresis was performed in a Novex Mini-Cell System for 90 min at 140 volts. Following several washes, the gel was dried on a BioRad gel drying apparatus. Dried gels were exposed overnight and analyzed using a PhosphorImager screen and Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Cell Viability

Cell viability after 24 hr exposure to increasing concentrations of EGCG was determined by measuring the remaining mass of attached cells as previously described (30). Briefly, cells were washed once in PBS to remove dead or detached cells. The remaining attached

cells were lysed in 0.5 M NaOH and the DNA concentration was measured by spectrophotometry. Percent viability was calculated as the absorbance of treated cells/absorbance of control cells \times 100.

Cell viability after 24 hr exposure to increasing concentrations of EGCG was also determined with the use of a colorimetric assay based on the ability of mitochondria in viable cells to reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as previously described (30). Percent viability was calculated as the absorbance of treated cells/absorbance of untreated cells \times 100.

Statistical Analysis

Differences in immunoreactive IL-8 levels, luciferase activity, and cell viability between the experimental groups were evaluated by one-way analysis of variance and Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

EGCG Inhibits TNF- α -Mediated Production of Immunoreactive IL-8

In these experiments, we determined the effect of EGCG on TNF- α -mediated production of immunoreactive IL-8. Treatment with TNF- α alone markedly increased the production of immunoreactive IL-8 compared with control cells treated with media alone (Fig. 2). Pretreatment with EGCG decreased the production of immunoreactive IL-8 in a dose-dependent manner. Cell viability was greater than 90% after exposure to all experimental conditions (data not shown).

EGCG Inhibits TNF- α -Mediated IL-8 mRNA Expression

After demonstrating that EGCG inhibited TNF- α -mediated immunoreactive IL-8 production, we next determined the effect of EGCG on TNF- α -mediated expression of IL-8 mRNA using Northern blot analysis. As shown in Fig. 3, treatment with TNF- α alone significantly increased IL-8 mRNA expression compared to control cells treated with media alone. Pretreatment with EGCG inhibited the expression of IL-8 mRNA in a dose-dependent manner (Fig. 3, lanes 3–6). Collectively, these data and the previous data involving immunoreactive IL-

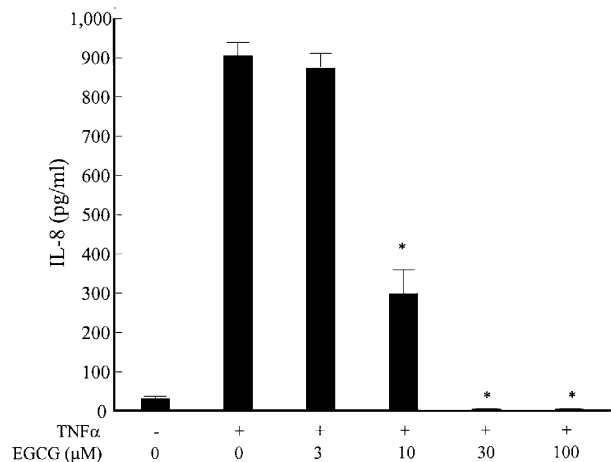


Fig. 2. ELISA results demonstrating the effects of EGCG on TNF- α -mediated production of immunoreactive IL-8. Control cells were maintained in a basal growth medium. TNF- α -treated cells were treated with TNF- α (2 ng/ml) for 24 hr. EGCG-treated cells were treated with EGCG at increasing concentrations (3–100 μ M) for 1 hr before the addition of TNF- α for 24 hr. Data represent the mean \pm SEM of four separate experiments with each condition performed in triplicate. * P < 0.05 versus TNF- α alone.

8 demonstrate that EGCG is a potent inhibitor of TNF- α -mediated IL-8 gene expression in A549 cells.

EGCG Inhibits TNF- α -Mediated Activation of the IL-8 Promoter

Previous work with A549 cells demonstrated that IL-8 gene expression is regulated at the transcriptional level (1, 12). Given the observed effect of EGCG on TNF- α -mediated IL-8 gene expression, we next sought

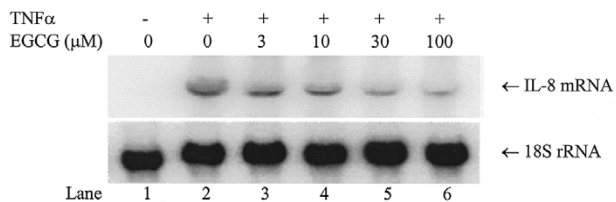


Fig. 3. Representative Northern blot demonstrating the effect of EGCG on TNF- α -mediated expression of IL-8 mRNA. Control cells (lane 1) were maintained in basal growth medium. TNF- α -treated cells (lane 2) were treated with TNF- α (2 ng/ml) for 2 hr. EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 μ M) for 1 hr before the addition of TNF- α (2ng/ml) for 2 hr. The blot is representative of three separate experiments with similar results. All blots were reprobbed with a radiolabeled oligonucleotide corresponding to 18S rRNA to normalize for differences in total RNA loading (1).

to determine the effect of EGCG on TNF- α -mediated activation of the IL-8 promoter. Cells were transiently transfected with an IL-8 promoter-luciferase reporter plasmid and exposed to the experimental conditions. Treatment with TNF- α alone induced luciferase activity nearly thirty-fold above control cells that were transfected and treated with media alone (Fig. 4). Pretreatment with 30 μ M and 100 μ M EGCG significantly inhibited TNF- α -mediated induction of luciferase activity, whereas pretreatment with 3 μ M and 10 μ M did not significantly inhibit TNF- α -mediated induction of luciferase activity. These data indicate that EGCG inhibits activation of the IL-8 promoter.

EGCG Inhibits TNF- α -Mediated Activation of NF- κ B

As IL-8 gene expression is regulated, in part, by NF- κ B, we next determined the effect of EGCG on TNF- α -mediated activation of NF- κ B using EMSA. Treatment with TNF- α alone increased activation of NF- κ B compared with control cells (Fig. 5, lanes 1 and 2). The specificity of this band in A549 cells treated with TNF- α

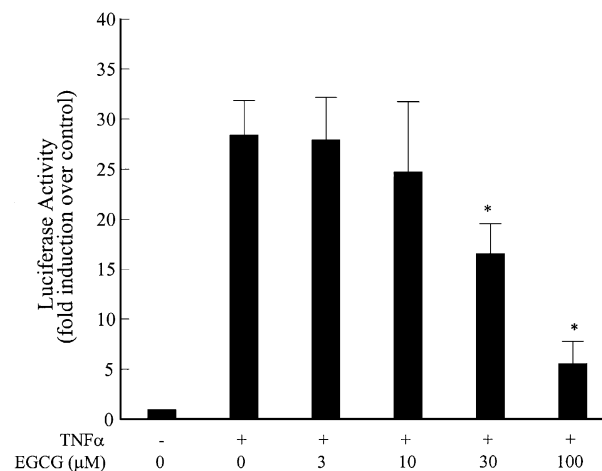


Fig. 4. Luciferase assay demonstrating the effect of EGCG on TNF- α -mediated activation of the IL-8 promoter. Cells were transiently transfected with an IL-8 promoter-luciferase promoter plasmid containing the -97 bp 5' flanking region of the IL-8 gene promoter, which contains the NF- κ B binding site. Control cells were maintained in basal growth medium. TNF- α -treated cells were treated with TNF- α (2 ng/ml) for 6 h. EGCG-treated cells were treated with EGCG at increasing concentrations (3–100 μ M) for 1 hr before the addition of TNF- α (2 ng/ml) for 6 hr. Data are expressed as fold induction over control cells and are corrected for total cellular protein. Data represent the mean \pm SEM of five separate experiments with each condition carried out in duplicate. * P < 0.05 versus TNF- α alone.

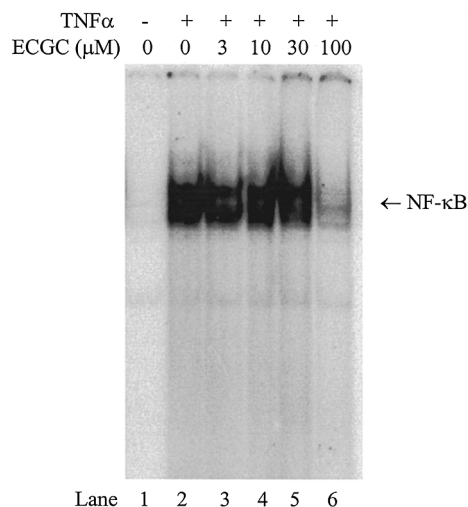


Fig. 5. EMSA demonstrating the effect of EGCG on TNF- α -mediated activation of NF- κ B. Control cells (lane 1) were maintained in basal growth medium. TNF- α -treated cells (lane 2) were treated with TNF- α (2 ng/ml) for 1 hr. EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 M) for 1 hr before the addition of TNF- α (2 ng/ml) for 1 hr. The gel is a representative of four experiments with similar results.

was demonstrated in a previous report. Pretreatment with 100 μ M EGCG decreased formation of the NF- κ B/DNA complex (Fig. 5, lane 6), whereas lower concentrations of EGCG did not detectably affect NF- κ B binding. These data indicate that EGCG inhibits activation of the IL-8 promoter, in part, by inhibiting activation of NF- κ B.

EGCG Inhibits TNF- α -Mediated Degradation of I κ B

After demonstrating that EGCG inhibits TNF- α -mediated activation of NF- κ B, we next examined the effects of EGCG on I κ B degradation. Treatment with TNF- α resulted in significant degradation of I κ B compared to control cells treated with media alone (Fig. 6, lanes 1 and 2). Consistent with the EMSA data, EGCG inhibited TNF- α mediated I κ B degradation only at the highest dose studied, 100 μ M (Fig. 6, lanes 3–6).

EGCG Inhibits IKK Activity

We next determined the effect of EGCG on IKK activity. As shown in Fig. 7, TNF- α increased IKK activity nearly eleven-fold above the activity in untreated control cells. Consistent with the I κ B α data above, EGCG inhibited the TNF- α -mediated increase in IKK activity only at a concentration of 100 μ M (Fig. 7, lane 6).

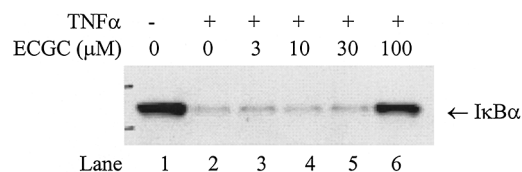


Fig. 6. Representative Western blot analysis demonstrating the effect of EGCG on TNF- α -mediated degradation of I κ B α . Control cells (lane 1) were maintained in basal growth medium. TNF- α -treated cells were treated with TNF- α (2 ng/ml) for 30 min. EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 μ M) for 1 hr before the addition of TNF- α (2 ng/ml) for 30 min. The gel is representative of three experiments with similar results.

DISCUSSION

Despite major advances in critical care medicine, acute lung injury remains a significant cause of morbidity and mortality in critically ill patients. *Chemotactic cytokines* (chemokines) represent a large family of peptides that play primary roles in the recruitment and activation of leukocytes during inflammation. IL-8, a member of the CXC family of chemokines is the principle neutrophil chemoattractant and activator in humans (31). Neutrophil infiltration appears to be an early and important event in the pathophysiology of acute lung injury, and it appears that IL-8 has an important role in medi-

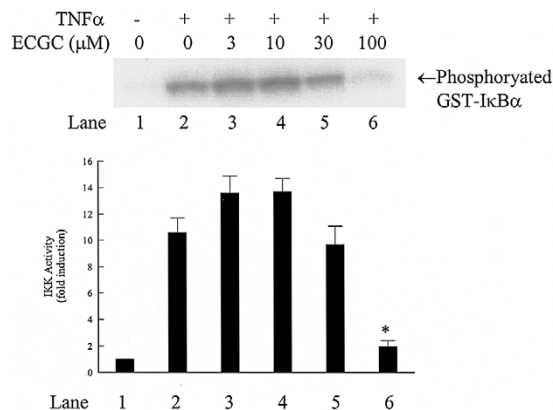


Fig. 7. Representative IKK assay (top panel) demonstrating the effect of EGCG on TNF- α -mediated activation of the IKK complex. Control cells (lane 1) were maintained in basal growth medium. TNF- α -treated cells (lane 2) were treated with TNF- α for 5 min. EGCG-treated cells (lanes 3–6) were treated with increasing concentrations of EGCG (3–100 μ M) for 1 hr before the addition of TNF- α (2 ng/ml) for 5 min. The gel is representative of three experiments with similar results. The bottom panel shows the effect of EGCG on TNF- α -mediated activation of IKK activity as measured by densitometry. Data are expressed as fold induction over control cells and represent the mean \pm SEM of three separate experiments. * P < 0.05 versus TNF- α alone.

ating this process (32–34). The importance of IL-8 in the pathophysiology of acute lung injury is further implicated by several studies demonstrating increased levels of IL-8 in the serum and bronchoalveolar lavage (BAL) fluid of patients with acute lung injury (35–37).

Previous studies have shown that IL-8 gene expression is induced in a cell type-specific and stimulus-specific manner (38). Mutational analysis of the IL-8 promoter region suggest that the transcription factor NF- κ B is central to the regulation of IL-8 gene expression following exposure to TNF- α in A549 cells (26, 39). Therefore, inhibition of NF- κ B would be expected to modulate the effects of TNF- α -mediated induction of IL-8. Herein we show that the green tea-derived polyphenol, EGCG, inhibits TNF- α -mediated expression of the IL-8 gene in A549 cells, at least partially through a mechanism involving the inhibition of IKK, and subsequent activation of the I κ B/NF- κ B pathway. While this work was in progress, a recent report demonstrated that EGCG inhibits NF- κ B activation through the inhibition of IKK activity in an intestinal epithelial cell line (19). In addition, Pan et al (40) previously showed that theaflavin-3,3'-digallate and EGCG inhibited the phosphorylation of I κ B through the inhibition of IKK in cultured macrophages.

Our studies confirm and expand the results of these earlier studies. We demonstrate that EGCG inhibited TNF- α -mediated luciferase activity in A549 cells transiently transfected with an IL-8 promoter-luciferase reporter plasmid, suggesting that inhibition occurs at the promoter level. Inhibition of the luciferase activity, however, occurred only at the highest doses of EGCG studied. In contrast, EGCG inhibited TNF- α -mediated production of immunoreactive IL-8, as well as TNF- α -mediated expression of IL-8 mRNA, at lower concentrations that did not detectably affect the IL-8 promoter.

While NF- κ B appears to be central to the regulation of IL-8 gene expression following exposure to TNF- α in A549 cells (26, 38), additional transcription factors such as AP-1 and NF-IL-6 may also play a role. The 5' flanking region of the IL-8 gene contains putative binding sites for several transcription factors, including AP-1, NF- κ B, and NF-IL6. Several studies have suggested that, unlike the NF- κ B site, the AP-1 and NF-IL6 sites are not essential for induction of the IL-8 gene (41). Studies specifically addressing TNF- α -mediated induction of IL-8 in A549 cells have further supported the notion that IL-8 gene expression only requires an intact NF- κ B site (42). In our experiments, we used a luciferase reporter

plasmid containing the -97 bp 5' flanking region of the IL-8 gene, which contains the NF- κ B binding site. The binding site for the transactivating protein AP-1 lies further upstream from the region cloned into our luciferase reporter plasmid. Our data would suggest that EGCG inhibits TNF- α -mediated IL-8 gene expression through an additional promoter site such as AP-1 or NF-IL-6, or alternatively by affecting the stability of the mRNA transcript. Further studies will be required to address the effect of EGCG on these additional transcription factors, specifically pertaining to the effects on IL-8 gene expression in response to TNF- α in A549 cells.

In summary, the green tea derived polyphenol, EGCG, is a potent inhibitor of IL-8 gene expression. The mechanism of this effect involves, in part, inhibition of IKK activation and subsequent activation of the I κ B/NF- κ B pathway. Thus, EGCG and related compounds may present a novel strategy for modulating inflammatory processes that occur during acute lung injury and other forms of critical illness. The next step to further substantiate this assertion will be to test the efficacy of green tea-derived polyphenols in animal models of inflammation-associated organ injury and to further understand the mechanisms by which these polyphenols modulate inflammation-associated signal transduction pathways.

With regard to green tea consumption, we are not aware of any direct data from which we can determine the amount green tea consumption that would be required to achieve the inhibitory effects that we documented *in vitro*. Indirect data, however, may suggest that this may be feasible. For example, one cup of green tea (240 ml) can contain up to 200 mg of EGCG (43). In addition, a very recent study indicates that consumption of at least 2 cups of tea per day may reduce the mortality rate following myocardial infarction (44).

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