Epigallocatechin-3-gallate, a Green Tea–Derived Polyphenol, Inhibits IL-1β-Dependent Proinflammatory Signal Transduction in Cultured Respiratory Epithelial Cells\(^1,2\)

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ABSTRACT Polyphenolic components of green tea, such as epigallocatechin-3-gallate (EGCG), have potent anti-inflammatory properties. We previously showed that EGCG inhibits tumor necrosis factor-α (TNF-α)–mediated activation of the nuclear factor-κB (NF-κB) pathway, partly through inhibition of IκB kinase (IKK). The NF-κB pathway may also be activated in response to interleukin-1β (IL-1β) stimulation through a distinct signal transduction pathway. We therefore hypothesized that EGCG inhibits IL-1β–mediated activation of the NF-κB pathway. Because the gene expression of interleukin-8 (IL-8), the major human neutrophil chemotactant, is dependent on activation of NF-κB, IL-8 gene expression in human lung epithelial (A549) cells treated with human IL-1β was used as a model of IL-1β signal transduction. The EGCG markedly inhibited IL-1β–mediated IL-1β receptor–associated kinase (IRAK) degradation and the signaling events downstream from IRAK degradation: IKK activation, IκBα degradation, and NF-κB activation. In addition, EGCG inhibited phosphorylation of the p65 subunit of NF-κB. The functional consequence of this inhibition was evident by inhibition of IL-8 gene expression. Therefore, the green tea polyphenol EGCG is a potent inhibitor of IL-1β signal transduction in vitro. The proximal mechanisms of this effect involve inhibition of IRAK-dependent signaling and phosphorylation of p65.

KEY WORDS: transcription factors inflammation signal transduction chemokines polyphenols

During the initial host inflammatory response to an infection or other inciting event, several proinflammatory cytokines are released into the systemic circulation, which, if left unchecked, can ultimately cause a dysregulated inflammatory cascade that results in significant autoinjury to the host (1). The systemic administration of either recombinant interleukin-1β (IL-1β)\(^4\) or tumor necrosis factor-α (TNF-α) rapidly induces a shocklike state in experimental animals (2,3) and causes fever and hypotension in healthy human volunteers (4–6). These two important proinflammatory cytokines appear to orchestrate the inflammatory response through the activation of transcription factors, such as nuclear factor-κB (NF-κB) and activated protein-1, with the subsequent induction of proinflammatory gene expression. Although these two cytokines share many biologic and physiologic properties, the signaling mechanisms that lead to IL-1β–dependent signal transduction are distinct from that of TNF-α–dependent signal transduction.

Nuclear factor-κB belongs to the Rel family of transcription factors, which share common structural motifs for dimerization and DNA binding. Five known subunits belong to the mamalian NF-κB/Rel family: c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), Rel A (p65), and Rel B. Nuclear factor-κB consists of 2 such subunits arranged as either homodimers (e.g., p50/p50) or heterodimers (e.g., p65/p50), although the most common form of activated NF-κB consists of a p65 (Rel A) and p50 heterodimer. Nuclear factor-κB activation appears to be a master switch, or control point, for the expression of a large number of proinflammatory genes, including several cytokines, chemokines, and adhesion molecules (7). Nuclear factor-κB is usually present in the cytoplasm of cells in an inactive state bound to a related inhibitory protein known as IκBα, an association that physically masks the nuclear translocation sequence of NF-κB, thereby retaining it in the cytoplasm.

The regulation of NF-κB activation following stimulation with IL-1β appears to involve at least 2 independent signal transduction pathways. The best-characterized mechanism for

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the activation of NF-κB involves the phosphorylation of the inhibitory protein, IκBα. Interleukin-1β binds to its receptor, the IL-1 receptor type 1 (IL-1R1), which forms a complex with a related accessory protein, IL-1 receptor accessory proteins (IL-1RAcP). This interaction between IL-1R1 and IL-1RAcP is possible due to the presence of a shared region of homology in the cytoplasmic domain of each protein called the Toll/IL-1R domain (8). The cytosolic adaptor protein MyD88 (9) and the Toll-interacting protein (10) interact with this receptor complex, which is a necessary step for association with a serine-threonine kinase, IL-1 receptor–associated kinase (IRAK) (11–13). The IRAK then recruits several additional adaptor proteins, including TNF receptor–associated factor 6 (14), transforming growth factor-β-activated kinase-1 (TAK1), and the TAK1 binding proteins 1 and 2 (14,15). Autophosphorylation of IRAK promotes its dissociation from this complex, which is followed by its polyubiquitination, and subsequent degradation by the 26S proteasome system (16). Interestingly, the kinase activity of IRAK may not be an essential step in the IL-1β signal transduction pathway and may function to terminate signal transduction instead (17–19). Nevertheless, this sequence of events is temporally and functionally associated with the downstream activation of IκB kinase (IKK), which phosphorylates the serine-32 and -36 residues of IκBα (20). Phosphorylated IκBα is targeted for rapid ubiquitination and degradation by the 26S proteasome system, which un.masks the nuclear translocation sequence of NF-κB and allows it to enter the nucleus and bind to the NF-κB consensus sequence to direct the transcription of target proinflammatory genes (20).

An alternative mechanism for the activation of NF-κB is IκBα-independent and involves direct phosphorylation of the p65 subunit of NF-κB at multiple sites by several candidate kinases (21). In addition, phosphorylation of specific tyrosine residues on IκBα causes activation of NF-κB without the proteolytic degradation of IκBα (22). It is likely that further study in this area will yield additional mechanisms of IκBα-independent NF-κB activation.

Given the important role that NF-κB plays in the regulation of a large number of proinflammatory genes, there is growing interest in targeting NF-κB directly in order to affect the inherent redundancy of the inflammatory cascade. A potential novel, safe, and nontoxic strategy for inhibiting NF-κB activation involves the polyphenolic compounds found in green tea, especially epigallocatechin-3-gallate (EGCG), the major polyphenol present in green tea (23). Apart from their activation of a large number of proinflammatory genes, there is potential novel, safe, and nontoxic strategy for inhibiting NF-κB (26). The probe was labeled with [γ-32P]ATP as substrate, and the resulting proteins were separated electrophoretically using a Novex Mini-Cell System. Gels were dried, exposed overnight, and analyzed using a PhosphorImager screen and Image-Quant software (Molecular Dynamics).

Electromobility gel shift assay. All nuclear extraction procedures were performed on ice with ice-cold reagents as previously described (26). Nuclear proteins were stored at −70°C until used for electromobility gel shift assays (EMSAs).

For this reason, EGCG stock was prepared immediately before each use. The EGCG stock was further diluted to experimental concentrations ranging from 3 to 100 μmol/L in DMEM. Cells were treated with EGCG for 1 h before incubation with IL-1β. Cells not treated with EGCG were preincubated in DMEM alone. The concentration of EGCG used and the duration of treatment did not affect the viability of these cells, as previously reported (26).

Western blot analysis for IRAK and IκBα degradation. Whole cell lysates of treated cells were prepared and electrophoretically separated as previously described (26) on 8 to 16% Tris-glycine gradient gels (Novex) and subsequently transferred to nitrocellulose membranes using the Novex Xcell Mini-Gel system (Novex).

For IRAK immunoblotting, membranes were blocked with nonfat dried milk:PBS (3:97, v:v) for 30 min. Primary antibody against IRAK (Upstate Biotechnology) was applied at a concentration of 2 μg/ml in milk:PBS (3:97) overnight at 4°C. After washing twice with distilled H₂O, the secondary antibody (peroxidase-conjugated anti-rabbit IgG; Stressgen) was applied at a 1:5000 dilution in milk:PBS (3:97) for 1 h.

IκBα immunoblotting was performed as previously described (26), using a primary antibody directed against human IκBα (Santa Cruz Biotechnology). Blots were incubated in commercial enhanced chemiluminescence reagents (ECL; Amersham), and exposed to photographic film (26).

Western blot analysis for phospho-NF-κB (p65). Treated cells were washed twice in ice-cold PBS. Cells were then lysed in ice-cold lysis buffer containing 50 mmol/L Tris (pH 8.0), 110 mmol/L NaCl, 5 mmol/L EDTA, and 1% Triton X-100, to which 100 mmol/L Na₃VO₄, 2 g/L leupeptin, 2 mol/L β-glycerol phosphate, and 100 g/L phenylmethylsulfonyl fluoride were added. Electrophoresis and protein transfer were carried out as described above. For immunoblotting, membranes were blocked in nonfat milk:TBS:Tween (5:9:95; by vol) for 1 h. A primary antibody against phospho-p65 (Cell Signaling Technology) was applied at a 1:1000 dilution in milk:TBS:Tween (5:95; v:v) overnight at 4°C. After washing 3 times with TBS:Tween (99:9:0.1; v:v), secondary antibody (peroxidase-conjugated antirabbit IgG; Stressgen) was applied at a 1:2000 dilution for 1 h. Blots were washed in TBS:Tween twice for 10 min, incubated in commercial enhanced chemiluminescence reagents (ECL; Amersham), and exposed to photographic film.

IκB kinase assay. The IκBα kinase assay was performed as previously described (26). Briefly, cell extracts were immunoprecipitated using anti-IKKy antibody (Santa Cruz Biotechnology). The kinase reaction was performed using ATP, GST-IκBα, and γ-[32P]ATP as substrate, and the resulting proteins were separated electrophoretically using a Novex Mini-Cell System. Gels were dried, exposed overnight, and analyzed using a PhosphoImager screen and Image-Quant software (Molecular Dynamics).

Materials and methods

Cell culture. Epithelial A549 cells (American Type Culture Collection), a human lung adenocarcinoma cell line representative of the distal respiratory epithelium, were maintained in an incubator with room air:CO₂ (95:5, v:v) at 37°C, using DMEM containing 8% FBS and 1% penicillin/streptomycin (Gibco BRL).

Experimental conditions. Cells were treated with either 1 μg/L of human IL-1β (Boehringer Mannheim) or vehicle. Epigallocatechin gallate (EGCG; Sigma Chemical) was added in filtered PBS to a stock concentration of 10 mmol/L. We noticed an oxidative color change and deterioration in the anti-inflammatory effects noted below when the EGCG stock was used after 24 h (data not shown), and
EGCG (μmol/L) 0 0 3 10 30 100
IL-1β - + + + + + 

Lane 1 2 3 4 5 6

FIGURE 1 Representative Western blot analysis demonstrating the effects of EGCG on IL-1β-mediated IRAK degradation in A549 cells. The control cells (lane 1) were maintained in basal growth medium. The IL-1β-treated cells (lane 2) were treated with IL-1β (1 μg/L) for 30 min. The EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 μmol/L) for 1 h before the addition of IL-1β (1 μg/L) for 5 min. The gel is representative of 3 experiments with similar results.

Northern blot analysis. Total cellular RNA was electrophoretically separated and subsequently transferred to nylon membranes (MicroSeperations) and UV autocrosslinked (UV Stratalinker 1800; Stratagene) as previously described (26). After 4 h of prehybridization at 42°C, membranes were hybridized overnight with a radiolabeled human IL-8 cDNA probe. The cDNA was labeled with a γ[32P]deoxyctydine triphosphate (specific activity = 3000 Ci/mmol; New England Nuclear Research Products) by random priming (Pharmacia). After washing, the hybridized filters were exposed overnight and analyzed using a PhosphorImager screen and ImageQuant software (Molecular Dynamics).

Enzyme-linked immunosorbent assay. Immunoreactive IL-8 concentrations in the media of treated cells were measured using a commercially available sandwich ELISA (Biosource). All procedures were performed as recommended by the manufacturer.

Statistical analysis. Differences in immunoreactive IL-8 level, luciferase activity, and cell viability among the experimental groups were evaluated by one-way ANOVA and Student-Newman-Keuls test. Values of P < 0.05 were considered significant.

RESULTS

Interleukin-1β-mediated degradation of IRAK. Treatment with IL-1β caused nearly complete degradation of IRAK compared to control cells, whereas preincubation with 30 and 100 μmol/L EGCG inhibited this degradation (Fig. 1).

Interleukin-1β-induced IKK activation. Treatment with IL-1β increased IKK activity compared to untreated control cells. Consistent with the IRAK degradation data above, 30 and 100 μmol/L EGCG almost completely suppressed IL-1β-induced activation of IKK (Fig. 2).

Interleukin-1β-mediated degradation of IκBα. Treatment with IL-1β caused marked degradation of IκBα compared to control cells. Consistent with the previous data involving IRAK degradation and IKK activation, 30 and 100 μmol/L EGCG inhibited IL-1β-mediated IκBα degradation (Fig. 3).

Interleukin-1β-mediated activation of NF-κB. Treatment with IL-1β increased the activation of NF-κB compared to control cells, as determined by EMSA. Consistent with the effects of EGCG on IL-1β-induced degradation of IRAK and activation of IKK, pretreatment with 30 and 100 μmol/L EGCG inhibited activation of NF-κB (Fig. 4). However, lower concentrations of EGCG (3 and 10 μmol/L) also moderately inhibited NF-κB activation, suggesting that additional, IκBα-independent mechanisms of NF-κB inhibition may play a role.

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EGCG (μmol/L) 0 0 3 10 30 100
IL-1β - + + + + + 

Lane 1 2 3 4 5 6

FIGURE 2 Representative IκB kinase assay demonstrating the effect of EGCG on IL-1β-mediated activation of IκB kinase in A549 cells. The control cells (lane 1) were maintained in basal growth medium. The IL-1β-treated cells (lane 2) were treated with IL-1β (1 μg/L) for 3 min. The EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 μmol/L) for 1 h before the addition of IL-1β (1 μg/L) for 5 min. The gel is representative of 3 experiments with similar results.

FIGURE 3 Representative Western blot analysis demonstrating the effects of EGCG on IL-1β-mediated IκBα degradation in A549 cells. The control cells (lane 1) were maintained in basal growth medium. The IL-1β-treated cells (lane 2) were treated with IL-1β (1 μg/L) for 30 min. The EGCG-treated cells (lanes 3–6) were treated with EGCG at increasing concentrations (3–100 μmol/L) for 1 h before the addition of IL-1β (1 μg/L) for 30 min. The gel is representative of 3 experiments with similar results.

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Phosphorylation of p65. Phosphorylated p65 was detected within 30 min after stimulation with IL-1β, whereas preincubation with EGCG caused a dose-dependent decrease in phosphorylated p65 concentration (Fig. 5).

Interleukin-1β-mediated expression of the IL-8 gene. Treatment with IL-1β induced nearly 5-fold the luciferase activity in cells transfected with an IL-8 promoter–luciferase reporter plasmid, compared to control cells that were transfected and treated with media alone. Pretreatment with EGCG inhibited luciferase activity in a dose-dependent manner, with significant inhibition at 30 and 100 μmol/L EGCG (Fig. 6). Furthermore, IL-1β treatment alone increased IL-8 mRNA expression (measured by Northern blot analysis) compared to control cells treated with media alone, whereas pre-
treatment with EGCG inhibited the expression of IL-8 mRNA in a dose-dependent manner (Fig. 7). The effects noted for IL-8 mRNA were corroborated by measurement of IL-8 peptide levels by ELISA. Treatment with IL-1β alone markedly increased the production of immunoreactive IL-8 compared to control cells treated with media alone, whereas pretreatment with EGCG decreased the production of immunoreactive IL-8 in a dose-dependent manner (Fig. 8). Collectively, these data demonstrate that the inhibitory effects of EGCG on IL-1β-mediated NF-κB activation are associated with the inhibition of IL-8 gene expression.

DISCUSSION

A large body of indirect and direct evidence links the NF-κB pathway to the dysregulated inflammation that is characteristic of diseases such as sepsis and acute respiratory distress syndrome. Several of the genes that comprise the complex
network contributing to this dysregulated inflammation are regulated at the transcriptional level by NF-κB, including the cytokines IL-1β and TNF-α; chemokines such as IL-6, IL-8, and macrophage chemotactic protein-1; cell adhesion molecules such as vascular cell adhesion molecule 1 and intercellular adhesion molecule 1; growth factors such as granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor; as well as additional proinflammatory genes such as inducible nitric oxide synthase. There appears to be a correlation between increased NF-κB activity and the severity of illness and mortality in critically ill patients (27–29). In addition, studies with in vivo animal models of lethal septic shock demonstrate that inhibition of NF-κB activation reduces mortality (30,31). These data support the general hypothesis that increased NF-κB–dependent inflammation directly contributes to the outcome of inflammation-mediated organ injury and strongly support the concept of therapeutic strategies targeting the NF-κB pathway. An attractive feature of this strategy is the fact that NF-κB activation appears to be a master switch, or control point, for the expression of a large number of proinflammatory genes. Thus, targeting NF-κB may potentially affect the inherent redundancy of the inflammatory cascade.

Recent epidemiological studies link the regular consumption of tea with a decreased risk of cancer (32). In addition, a recent study indicates that consumption of as little as 2 cups (473 mL) of tea per day is associated with a reduction in mortality during the 12-mo period following an acute myocardial infarction (33). Considerably less is known regarding the effective EGCG concentration required to modulate the inflammatory pathway. Alternatively, EGCG and related compounds could potentially be used as a nutritional supplement in patients with inflammatory disease processes. The next steps to further substantiate these assertions are to test the efficacy of green tea–derived polyphenols such as EGCG in animal models of inflammation-associated organ injury and to further elucidate the mechanisms by which these compounds modulate proinflammatory signal transduction pathways.

**LITERATURE CITED**

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