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Effects of *Ginkgo biloba* extract (EGb 761) and quercetin on lipopolysaccharide-induced signaling pathways involved in the release of tumor necrosis factor-α

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Abstract

Administration of bacterial lipopolysaccharide (LPS) to laboratory animals and cultured macrophages induces tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine. Pretreatment with *Ginkgo biloba* extract (EGb 761) inhibited the *in vivo* production of TNF-α (measured by ELISA) after challenge with LPS. To begin to understand the mechanism of this inhibition, we evaluated the *in vitro* effects of EGb 761 and its flavonoid component, quercetin, on LPS-treated RAW 264.7 macrophages. Pretreatment with EGb 761 or quercetin concentration-dependently inhibited TNF-α release, as measured by the L929 fibroblast assay. Northern blotting demonstrated that quercetin inhibited LPS-induced TNF-α mRNA, but did not alter its half-life. Activation of mitogen-activated protein kinases (MAPKs) and the redox-sensitive transcription factors, nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), are key events in the signal transduction pathways mediating TNF-α induction. Phosphorylation of extracellular signal-related kinases 1 and 2 (ERK 1/2), p38 MAPK, and Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), members of the MAPK family, was analyzed by western blotting. Our results suggest that quercetin is unique in its ability to inhibit TNF-α transcription by inhibiting the phosphorylation and activation of JNK/SAPK and, therefore, suppressing AP-1–DNA binding [assessed by electrophoretic mobility shift analysis (EMSA)]. Results from western analysis, EMSA, and transient transfections suggest that EGb 761 diminishes LPS-induced NF-κB but has no effect on LPS-induced TNF-α transcription. Both EGb 761 and quercetin inhibited ERK1/2 phosphorylation and p38 MAPK activity, which are important in the post-transcriptional regulation of TNF-α mRNA.

Keywords: TNF-α; MAPK; Quercetin; EGb 761; Macrophage

1. Introduction

The standardized extract of *Ginkgo biloba*, EGb 761, is the most commonly prescribed herbal remedy in France and Germany. It has been used in the treatment of peripheral vascular, cardiovascular, and cerebrovascular disease and acute mountain sickness, all of which have components associated with oxidative stress [1,2]. The antioxidant properties of EGb 761 have been proposed to underlie its beneficial effects. *In vitro*, it is a potent free radical scavenger and inhibitor of NADPH-oxidase, which significantly decreases superoxide radical, hydrogen peroxide, and hydroxyl radical production in human neutrophils stimulated with phorbol ester [3,4]. EGb 761 is composed of 24% flavonoid glycosides; rutin, the glycoside of quercetin, accounts for 11.1%. In addition to flavonoids, EGb 761 consists of unique terpenes (3% bilobalide and 3% ginkgolides A, B, and C) [5]. It has yet to be determined whether the effects of EGb 761 are caused by a single active ingredient or the combined action of its many components.

Exposure of laboratory animals or cells in culture to bacterial LPS triggers gene induction and the generation of

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Abbreviations: EGb 761, *Ginkgo biloba* extract; LPS, lipopolysaccharide; ROIs, reactive oxygen intermediates; TNF, tumor necrosis factor; IL, interleukin; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; TLR, Toll-like receptor; LBP, lipid binding protein; NF-κB, nuclear factor-κB; IKK, IκB kinase; AP-1, activator protein 1; ATF, activating transcription factor; CRE, cyclic AMP response element; CREB, cyclic AMP binding protein; C/EBP, CCAAT/enhancer binding protein; ARE, AU-rich element; -UTR, untranslated region; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; MEKK, MAPK/ERK kinase kinase; MEK, MAPK/ERK kinase; JNK/SAPK, Jun N terminal kinase/stress-activated protein kinase; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; and ECL, enhanced chemiluminescence.

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ROIs by monocytes and macrophages. The inducible genes encode pro-inflammatory cytokines and enzymes such as TNF-α, IL-6, COX-2, and iNOS, which up-regulate the host defense systems but unfortunately also contribute to pathological conditions such as bacterial sepsis, ischemia/reperfusion injury, chronic inflammatory disease, and the down-regulation of hepatic drug-metabolizing enzymes. Recent observations suggest that the murine TLR4, in concert with CD14 and LBP, transduces the cellular responses to LPS in a molecular framework analogous to the IL-1 signaling cascade [6]. In turn, many signal transduction pathways are activated, including MAPK pathways, and transcription factors such as NF-κB/Rel and the AP-1 complex [7].

The induction of TNF-α production upon stimulation by LPS results from both an enhancement of gene transcription and translational de-repression [8]. The TNF-α promoter contains potential binding sites for several transcription factors, including NF-κB, AP-1, CRE, and CCAAT/enhancer binding protein β (C/EBPβ, also called NF-IL6) [9]. Transcriptional control appears to be mediated primarily by NF-κB, whereas the TNF-α CRE is critical for autoregulation [10,11]. Post-transcriptional regulation (mRNA instability and translational repression) is governed by an ARE in the 3′-UTR of TNF-α mRNA. Three well-defined MAPK cascades, ERK, p38 MAPK, and JNK/SAPK, are activated upon stimulation with LPS. Several reports have described the importance of these pathways in LPS-induced TNF-α secretion in murine macrophages [12,13].

Our recent studies addressed the hypothesis that the flavonoid quercetin inhibits LPS-induced production of TNF-α and nitrite in the macrophage cell line RAW 264.7 [14]. In addition to EGb 761, quercetin is present in wine and in a wide variety of fruits, vegetables, and herbs. *In vitro*, it is a powerful antioxidant and a non-specific inhibitor of various tyrosine and serine/threonine kinases, including MAPKs [15]. Quercetin inhibits the purified recombinant IκB kinases, IKK1 and IKK2, by serving as a mixed-type inhibitor versus ATP [16]. Unexpectedly, in our previous studies, quercetin inhibited LPS-induced TNF-α release at both transcriptional and post-transcriptional levels, but did not inhibit NF-κB–DNA binding [14]. In the present study, we examined the mechanism by which EGb 761 and its aglycone component, quercetin, inhibit TNF-α by investigating the effects of these natural product antioxidants on the LPS-induced stimulation of MAPK, NF-κB, AP-1, and CRE. Our results show that EGb 761 and quercetin have selective effects on TNF-α and the MAPK cascade.

2. Materials and methods

2.1. Reagents

Escherichia coli LPS, serotype 0127:B8, quercetin, penicillin/streptomycin, DMSO, Denhardt's reagent, sodium orthovanadate, and E-toxate® reagent were from the Sigma Chemical Co. Antibodies to p50, p65, c-Rel, c-Jun/AP-1, ATF-2, and IκBα were from Santa Cruz Biotechnology. Antibodies to phospho-MAPK (Erk1/2), phospho-p38 MAPK, and phospho-JNK/SAPK, and the p38 MAP kinase assay kit were from New England Biolabs. The antiphosphotyrosine antibody 4G10 was a contribution from Dr. Brian Druker (Oregon Health Sciences University). The protease inhibitors aprotinin, leupeptin, Pefabloc, and E-64 and the selective MAPK inhibitors SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole] and UO126 [1,4-diamino-2,3-dicyano-1,4-bis(phenylthio)butadiene] were from Calbiochem. PMSF was from the Boehringer Mannheim Corp. NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), AP-1 (c-jun) (5′-CGC TTG ATG AGT CAG CCG GAA-3′), and CRE (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) consensus oligonucleotides, Passive Lysis Buffer, and luciferin were from Promega. Recombinant mouse TNF-α was from R&D Systems. Dulbecco's minimal essential medium (DMEM), RPMI, TRIzol® reagent, and salmon sperm DNA were from Gibco BRL. The pAP-1-luc, pNF-κB-luc, pCRE-luc, and negative control pCIS-CK cis-reporter plasmids were from Stratagene. SuperFect Transfection Reagent and the Endo-Free Plasmid Maxi Kit were from Qiagen. DNA Etox columns were from Sterogene. Fetal bovine serum (FBS) was from Research Sera. Tissue culture plates were from Fisher Scientific. [^{32}P]ATP and GeneScreen Plus® membranes were from DuPont NEN. Bradford and D_c protein assay reagents were from Bio-Rad. TNF-α ELISA kit and recombinant mouse interferon-γ were from Endogen. PVDF membranes were from Millipore. Nitrocellulose membranes were from Schleicher & Schuell. ECL™ western blotting detection reagents were from Amersham Life Science. SuperSignal® Ultra chemiluminescent substrate was from Pierce. The mouse macrophage RAW 264.7 and L929 fibroblast cell lines were obtained from the American Type Culture Collection. C57BL/6 mice were from Jackson Laboratories. EGb 761 was a gift from Dr. Willmar Schwabe GmbH.

2.2. Animals

Male C57BL/6 mice, 8 weeks old, were allowed free access to food and water at all times and were allowed to acclimatize to the facilities for 7 days before use. Immediately prior to treatment, EGb 761 was dissolved in sterile saline, adjusted to pH 7.4, and administered intraperitoneally to the mice at a dose of 0, 20, 50, or 100 mg/kg for 7 days. On day 7, LPS (dissolved in sterile saline) was injected intraperitoneally at a dose of 1.3 mg/kg body weight. One hour after LPS injection, the mice were killed by CO_2 asphyxiation. All procedures were approved by the Institutional Animal Care and Use Committee of Oregon Health Sciences University.

2.3. Serum

Blood samples were collected by cardiac puncture. Blood was allowed to clot for 2 hr at room temperature or overnight at 4° before being centrifuged at 2000 *g* for 20 min at 4°. Serum was removed and stored at −70°.

2.4. Cell culture

RAW 264.7 cells were cultured in phenol red-free DMEM and L929 fibroblasts in RPMI containing 50 units/mL of penicillin, 50 μg/mL of streptomycin, 44 mM sodium bicarbonate, and 10% FBS at 37° in humidified air containing 5% CO_2. For preparation of RNA, nuclear extracts, or cell lysates for western blot analysis, RAW cells were plated in 2.5 mL of medium in 5.5-cm dishes, were cultured for 2 days until cells reached 80% confluency (approximately 2×10^7 cells/dish), and then were treated as described in the text. For western blot analysis of p44/42 MAPK, cells were cultured for 2 days in 0.5% FBS to reduce the basal levels of MAPK phosphorylation. For determination of TNF-α, RAW cells were plated in 1 mL of medium in 24-well plates, were cultured for 2 days to 80% confluency (approximately 1×10^6 cells/well), and then were treated. For the p38 MAPK immunoprecipitation-kinase assay, cells were plated in 10-cm tissue culture dishes at a density of 6×10^6 cells/dish and were treated 48 hr later. In all cases, cells were washed, and fresh complete medium was added before the indicated treatments. Stock solutions of EGb 761, quercetin, and MAPK inhibitors were prepared in DMSO; the final concentration of DMSO did not exceed 0.5%. Crystal violet uptake assays confirmed that results were not due to general cellular toxicity, and the E-toxate® test verified that the culture medium was not contaminated with bacterial endotoxin.

2.5. Measurement of TNF-α

Following 24 hr of LPS treatment, supernatants from RAW 264.7 macrophages were collected and centrifuged at 3500 *g* for 30 sec at 4°. Mouse serum was collected 1 hr following LPS treatment. Medium (50 μL) and serum (50 μL) were analyzed for TNF-α by ELISA (as described by the manufacturer) or with TNF-sensitive L929 fibroblasts as previously described [17]. Cell viability was determined by the crystal violet cytotoxicity assay [18].

2.6. Preparation of cytosolic and nuclear extracts

Nuclear extracts were prepared by a modified method of Dignam *et al.* [19]. Treated cells were washed, then scraped into 1.5 mL of ice-cold Tris-buffered saline (pH 7.9), and pelleted at 12,000 *g* for 30 sec at 4°. The pellets were suspended in 10 mM HEPES (pH 7.9), with 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/mL of leupeptin, aprotinin, and pepstatin, incubated on ice for 15 min, and then vortexed for 10 sec with 0.6% Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 *g* for 60 sec at 4°. The supernatants (cytosolic fraction) were removed, and the pellets were suspended in 20–50 μL of 20 mM HEPES (pH 7.9), with 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/mL of leupeptin, aprotinin, and pepstatin. The samples were incubated with rocking at 4° for 15 min, and then were centrifuged for 5 min at 12,000 *g* at 4°. Protein concentrations were determined by the Bradford method.

2.7. EMSA

To assay DNA–NF-κB binding, mixtures containing 5 μg of nuclear protein extract were incubated for 15 min at 4° in 20 μL of total reaction volume containing 10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, and 1 μg of sonicated salmon sperm DNA. Radiolabeled NF-κB consensus oligonucleotide (100,000–150,000 cpm of ^{32}P) was added, and the complete mixture was incubated for an additional 20 min at room temperature. Complexes were resolved on 7% native polyacrylamide gels containing 0.5× TBE (0.045 M Tris-borate/0.001 M EDTA) with 5 mM Tris/38 mM glycine running buffer. Gels were loaded while running at 50 V and then were run at 400 V until the bromophenol blue dye front reached the bottom. The gel was dried, and complexes were detected by autoradiography. The identity of the complexes was established with excess unlabeled oligonucleotide and antibody supershifts using rabbit polyclonal antibodies to p50 (nuclear localization signal), and goat antibodies to p65 (C-20) and c-Rel (N-terminus). For DNA binding to AP-1 and CRE, mixtures containing 5 μg of nuclear protein extract, 0.01 mg/mL of poly(dI-dC)-poly(dI-dC), 5 mM DTT, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, and 10 mM Tris–HCl (pH 7.5) were incubated at room temperature for 10 min. Radiolabeled oligonucleotide (100,000–150,000 cpm) was added, and the complete mixture was incubated at room temperature for 20 min. The mixtures were loaded onto 6% nondenaturing acrylamide gels while running at 50 V in 0.5× TBE running buffer. The voltage was increased to 350 V, and the gels were run until the bromophenol blue dye front reached the bottom. Gels were dried, and complexes were detected by autoradiography. The identity of the AP-1 and CRE complexes was established with excess unlabeled AP-1 or CRE nucleotide and antibody supershift using rabbit polyclonal antibodies to c-Jun/AP-1 and/or ATF-2. For all supershift analyses, 2 μg of antibody was added, and the reaction mixtures were incubated for 1 hr at 4° before the addition of ^{32}P.

2.8. Western blot analyses

Phosphorylation of MAPK (Erk1/2), p38 MAPK, and JNK/SAPK was analyzed with a commercially available

phospho-MAPK antibody kit according to the instructions of the manufacturer. Briefly, after indicated treatments, cells were lysed in SDS sample buffer, and proteins were separated by 8% SDS–PAGE. Separated proteins were electrophoretically transferred to nitrocellulose membranes, blotted with specific MAPK antibodies, and analyzed by ECL. To determine p65 and p50 levels, nuclear (20 μg protein) and cytoplasmic (40 μg protein) extracts were resolved on 8% SDS–PAGE, electrophoretically transferred to nitrocellulose membranes, probed with rabbit polyclonal antibodies against p50 or p65 (1:4000 dilution of TransCruz Gel Supershift reagents), and detected by ECL. For IκB analysis, treated cells were lysed in RIPA buffer [150 mM NaCl, 1% (w/v) deoxycholate, 0.1% (w/v) SDS in 50 mM Tris (pH 8.0)] containing 10 μg/mL of PMSF, 10 μg/mL of aprotinin, 100 μg/mL of leupeptin, 100 μg/mL of E-64, and 1 mM sodium orthovanadate. Proteins (20 μg) were resolved on 10% SDS–PAGE, transferred to PVDF membranes, probed with rabbit polyclonal antibodies to IκBα or IκBβ (1:1000 dilution), and detected by ECL. A time course to determine optimal LPS-induced phosphorylation was performed for each MAPK (data not shown). When indicated, signal intensity was quantified with Bio-Rad Molecular Analyst Software.

2.9. p38 MAPK immunoprecipitation-kinase assay

The activity of p38 MAPK was analyzed with a commercially available kit according to the instructions of the manufacturer. Briefly, RAW 264.7 macrophages were untreated (control) or treated with LPS for 15 min. Cells were harvested and lysed, and phosphorylated p38 MAPK was immunoprecipitated with a monoclonal phospho-specific antibody to p38 MAPK. The immunoprecipitate was incubated with ATF-2 fusion protein (a substrate of phosphorylated p38 MAPK), ATP, and either EGb 761, quercetin, SB203580, or DMSO. Samples were separated by 10% SDS–PAGE, transferred to nitrocellose, immunoblotted with anti-phospho-ATF-2, and analyzed by ECL.

2.10. Northern blot analysis

After treatment, total RNA was isolated with the TRIzol® Reagent as specified by the manufacturer. Total RNA (20 μg) was electrophoresed in 1% agarose/15% formaldehyde gels, transferred overnight to GeneScreen Plus® membranes, and cross-linked to the membranes by UV irradiation. The membranes were prehybridized for 4 hr at 37° in 50% deionized formaldehyde, 5× Denhardt's reagent, 5× saline–sodium phosphate–EDTA buffer (SSPE), 0.5% SDS, and 100 μg/mL of denatured salmon sperm DNA, and hybridized overnight in the same buffer containing 1–2 × 10^6 cpm/mL of denatured ^{32}P-labeled cDNA probe. Following hybridization, the membrane was washed once with 2× SSPE, 0.1% SDS for 10 min at room temperature, and twice in 1× SSPE, 0.1% SDS for 15 min at 60°. Signals were detected with a GS-363 Bio-Rad Molecular Imager with a BI imaging screen, and signal intensity was quantified with Bio-Rad Molecular Analyst software.

2.11. Transient transfections

Endotoxin-free plasmid DNA was prepared with the EndoFree Plasmid Maxi Kit (Qiagen) followed by purification with DNA Etox resin (Sterogene) according to the instructions of the manufacturers. For each transfection, 1 × 10^6 RAW cells were distributed in 6-well plates and incubated for 16–24 hr. Transfections were performed according to the guidelines of the manufacturer, using 5 μL of SuperFect reagent and 2 μg of plasmid DNA per well. Reagent/DNA was incubated with the cells for 2 hr at 37°, and then was washed three times in 2 mL of serum-free DMEM. Wells were replenished with 2 mL of complete medium and incubated at 37° for 24 hr from the start of transfection. Transfected cells were stimulated for 4 hr (for pNF-κB-luc and pAP-1-luc) and 4.5 hr (for pCRE-luc) with 1 μg/mL of LPS, and then were solubilized in Passive Lysis Buffer (Promega) and assayed for luciferase activity. Stimulation with an LPS concentration of 1 μg/mL was based on studies by Thompson *et al.* [20] describing optimal methods for transient transfection of RAW 264.7 macrophages. We found no changes in LPS-induced TNF-α levels at 100 ng/mL of LPS compared with 1 μg/mL of LPS in RAW 264.7 cells (data not shown). After normalization to protein concentration with the Bio-Rad D_c protein assay reagent, data were expressed as fold-activation.

2.12. Statistical analysis

The statistical significance of the results was analyzed by Student's *t*-test for unpaired observations.

3. Results

3.1. Effect of EGb 761 on LPS-induced TNF-α in vivo

Animal studies suggested that EGb 761 protects against free radical formation and lipid peroxidation *in vivo* [21,22]. With dose and duration of treatment based on these studies, we hypothesized that EGb 761 would inhibit the release of LPS-induced TNF-α in C57BL/6 mice. Consistent with our hypothesis, intraperitoneal injection of LPS caused increased serum levels of TNF-α, and pretreatment with EGb 761 protected against LPS-induced increases of this cytokine (Fig. 1).

3.2. Effects of EGb 761, quercetin, and MAPK inhibitors on LPS-induced TNF-α in vitro

In vitro studies with the murine macrophage cell line RAW 264.7 were utilized to probe the mechanism by which

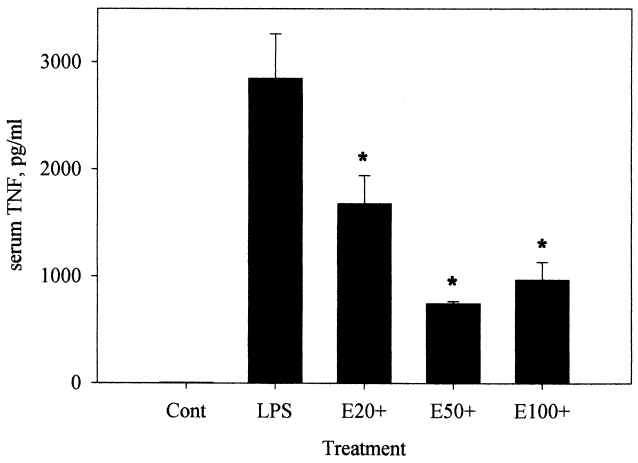

Fig. 1. Effect of EGb 761 on LPS-induced TNF-α *in vivo*. C56BL/6 mice were pretreated with EGb 761 or saline for 7 days. On day 7, immediately following EGb 761 treatment, mice were injected with LPS (1.3 mg/kg) or saline and killed 1 hr later for TNF-α analysis by ELISA. Cont, saline injections only (N = 2); LPS, pretreatment with saline, injection with LPS (N = 5); E20+ (N = 5), E50+ (N = 4), E100+ (N = 4), pretreatment with 20, 50, or 100 mg/kg of EGb 761 and LPS-injected. Data represent means ± SEM. Key: (*) significantly different from mice treated with LPS only, $P < 0.05$.

EGb 761 and quercetin inhibit TNF-α. As shown in Fig. 2, EGb 761 (400 μg/mL) and quercetin (200 μM) alone had no effect on culture supernatant levels of TNF-α, as measured by the L929 fibroblast assay. LPS increased TNF-α levels. Pretreatment with EGb 761 (400 μg/mL), quercetin (200 μM), UO126 (20 μM), or SB203580 (30 μM) inhibited LPS-induced TNF-α. Similar results were observed in independent experiments using ELISA to measure TNF-α in culture supernatants (data not shown). Initial concentration–response studies using either ELISA or the L929 fibroblast assay established that 400 μg/mL of EGb 761 inhibited 30–50% of LPS-induced TNF-α (data not shown). This concentration is approximately equivalent to 73 μM quercetin glycoside and 160 μM total flavonoid glycoside.

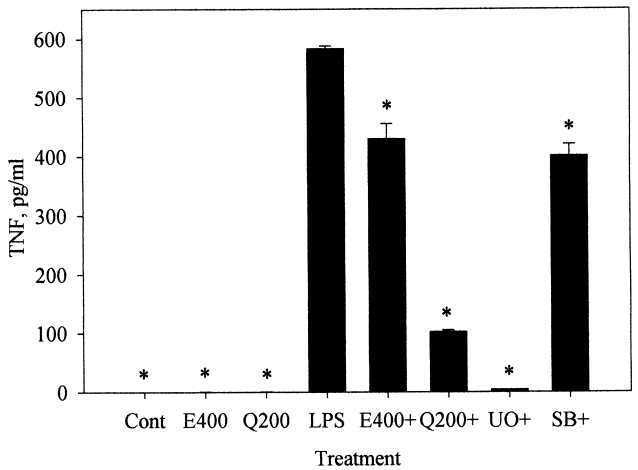

Fig. 2. Effects of EGb 761, quercetin, and MAPK inhibitors on LPS-induced TNF-α *in vitro*. RAW 264.7 macrophages were pretreated with vehicle only (Cont), EGb 761 only (E400, 400 μg/mL), or quercetin only (Q200, 200 μM), or were pretreated with DMSO (LPS), EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM), or SB203580 (SB+, 30 μM) for 1 hr, followed by treatment with LPS (100 ng/mL, 18 hr). Supernatants were collected and analyzed for TNF-α using the L929 fibroblast assay. Data represent means ± SEM, N = 3. Similar results were observed in three independent experiments. Key: (*) significantly different from treatment with LPS only, $P < 0.05$.

3.3. Effect of quercetin on LPS-induced TNF-α transcription

The suppression of LPS-induced TNF-α protein by EGb 761, quercetin, and MAPK inhibitors could result from an inhibition of gene transcription. To test this possibility, TNF-α mRNA was assessed by northern blot analysis (Fig. 3a). EGb 761, UO126, and SB203580 did not inhibit, while quercetin decreased LPS-induced up-regulation of TNF-α mRNA. Since quercetin had no effect on the half-life of TNF-α mRNA (Fig. 3b), inhibition of TNF-α secretion by quercetin most likely occurs at the transcriptional level.

3.4. Effects of EGb 761 and quercetin on LPS-induced IκB degradation and of EGb 761 on NF-κB–DNA binding

Because NF-κB is important in mediating transcriptional control of TNF-α, we analyzed the effects of EGb 761 and quercetin on the LPS-induced NF-κB signaling cascade. In unstimulated cells, NF-κB is retained in the cytoplasm by binding to a member of the inhibitory protein family, IκB. Activation by LPS requires sequential phosphorylation of IκB, ubiquitination and degradation by the proteasome, followed by translocation of NF-κB to the nucleus. Western blot analysis indicates that while the proteasome inhibitor MG132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal) protected IκBα and IκBβ from LPS-induced degradation, neither quercetin nor EGb 761 prevented this phenomenon (Fig. 4a).

Mobility shift analysis was utilized to determine the effects of EGb 761 on LPS-induced NF-κB–DNA binding. Our previous studies [14] demonstrated that quercetin (0.1 and 0.2 mM) decreased LPS-stimulated binding of p50/50, but had no effect on p50/65. These studies established that maximal LPS-induced NF-κB–DNA binding occurred at 1–2 hr. Figure 4b illustrates that pretreatment with EGb 761 diminished the binding of LPS-induced p50/50 and p50/65. The NF-κB–DNA binding complexes, p50/65 and p50/50, were identified with supershift analysis, using antibodies to Rel homology family members, p50 and p65, and correspond to complexes identified in our previous study [14].

3.5. Effects of EGb 761 and quercetin on LPS-induced nuclear localization of the p50 and p65 subunits of NF-κB

EMSA revealed a decrease in p50/50–and p50/65–DNA binding by EGb 761 but showed no substantial differences in the levels of LPS-induced p50/65–DNA binding with

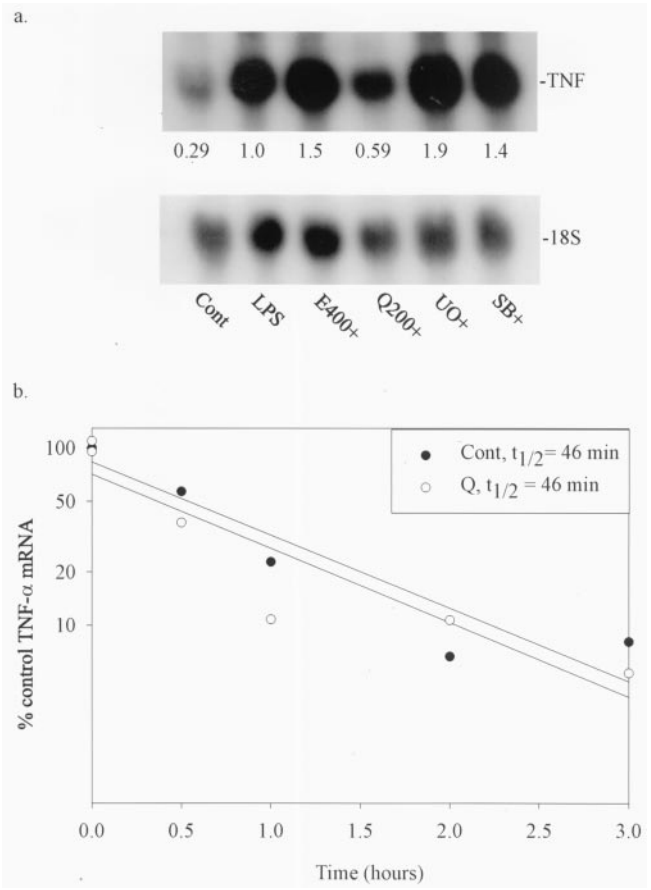

Fig. 3. Effect of quercetin on LPS-induced TNF-α transcription. (a) RAW 264.7 cells were treated with vehicle only (Cont) or were pretreated for 1 hr with DMSO (LPS), EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM), or SB203580 (SB+, 30 μM) prior to treatment with LPS (100 ng/mL, 6 hr). Total cellular RNA was isolated, and TNF-α and 18S RNA were assessed by northern analysis. The relative intensity of the signal after normalization to 18S RNA is shown below each lane. (b) RAW 264.7 macrophages were stimulated with LPS (100 ng/mL) for 6 hr, and then were treated with quercetin (200 μM) and actinomycin D (2.5 μg/mL) (Q), or actinomycin alone (Cont). Total cellular RNA was isolated at the indicated times and assessed for TNF-α and 18S RNA by northern blot analysis and the intensity of the TNF-α signal was normalized to the 18S signal. Results represent data from two independent experiments.

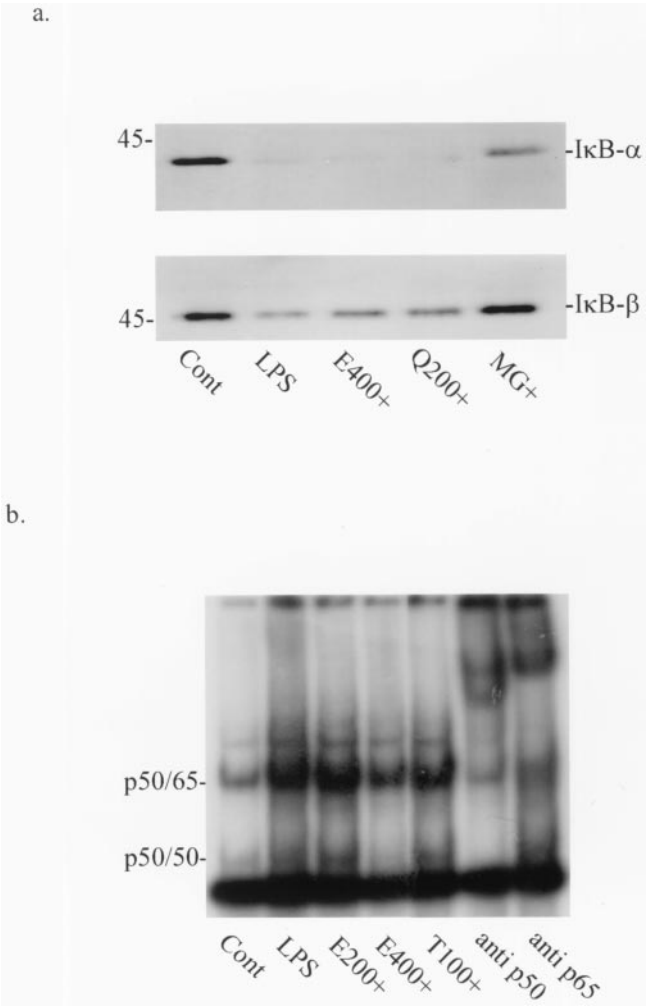

Fig. 4. Effects of EGb 761 and quercetin on LPS-induced IκB degradation and of EGb 761 on NF-κB–DNA binding. (a) RAW 264.7 cells were pretreated with DMSO only (Cont) or pretreated for 1 hr with DMSO (LPS), EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), or MG132 (MG+, 25 μM) prior to treatment with LPS (100 ng/mL, 20 min). Cell lysates were immunoblotted with specific antibodies to IκBα or IκBβ and were analyzed by western blot. (b) Mixtures containing 5 μg of nuclear protein were assayed for NF-κB–DNA binding by EMSA using ^{32}P-labeled NF-κB consensus oligonucleotides. Cells were treated with DMSO only (Cont) or were pretreated for 1 hr with DMSO (LPS), EGb 761 (E200+, 200 μg/mL; E400+, 400 μg/mL), or the non-specific tyrosine kinase inhibitor tyrophostin (T100+, 100 μM), and then were stimulated with LPS (100 ng/mL for 60 min). Supershift analysis was performed on nuclear extracts from LPS-treated cells that were preincubated for 30 min with the indicated antibodies against members of the NF-κB/Rel family. Complex identity is in agreement with previous results [14].

quercetin treatment. Cytoplasmic and nuclear pools of the p50 (Fig. 5, a and b) and p65 (Fig. 5, c and d) subunits were therefore examined by western blot analysis to determine if nuclear translocation of these subunits was altered by EGb 761 or quercetin. Treatment of cells with LPS induced the movement of p50 and p65 from the cytoplasm (Fig. 5, a and c) to the nucleus (Fig. 5, b and d). Pretreatment of cells with EGb 761 diminished LPS-induced nuclear levels of p50 by 25% when signal intensities were quantified, as described in "Materials and methods." Quercetin had no effect on nuclear translocation of this subunit. EGb 761 and quercetin had no effect on cytoplasmic or nuclear levels of p65. The proteasome inhibitor MG132 blocked nuclear translocation of both p50 and p65 subunits.

3.6. Effects of EGB 761 and quercetin on NF-κB-dependent transcriptional activity

Because quercetin and EGb 761 diminished LPS-induced NF-κB–DNA binding, we examined the effects of these compounds on NF-κB-dependent transcriptional activation. RAW 264.7 macrophages were transiently transfected with the pNF-κB-luc plasmid, containing the luciferase reporter gene driven by a basic promoter element

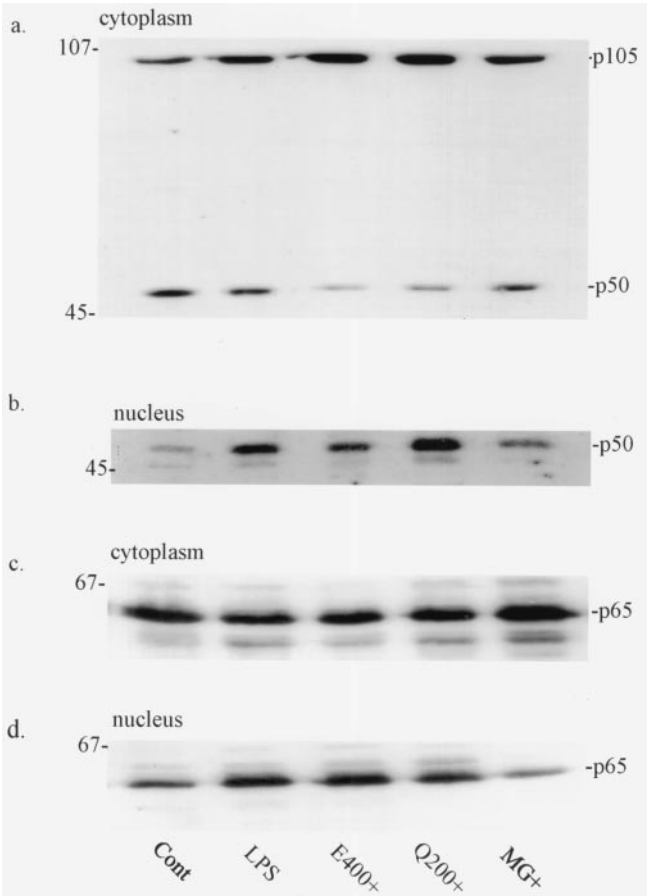

Fig. 5. Effect of EGb 761 on the LPS-induced nuclear localization of the p50 subunit of NF-κB. Cell extracts from RAW 264.7 cells were treated with DMSO only (Cont) or were pretreated for 1 hr with DMSO (LPS), EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), or MG132 (MG+, 25 μM) prior to treatment with LPS (100 ng/mL, 15 min). p50 (a and b) and p65 (c and d) were analyzed by western blot; a and c represent cytoplasmic extracts, and b and d nuclear extracts. Two independent experiments gave similar results.

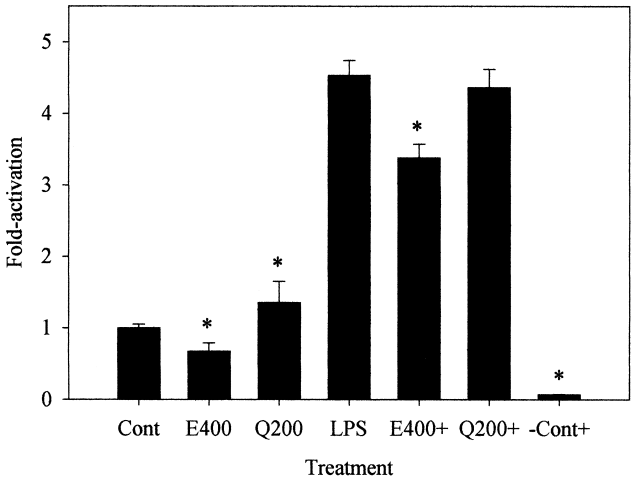

Fig. 6. Effects of quercetin and EGb 761 on NF-κB-dependent transcriptional activity. Twenty-four hours after transient transfection with the pNF-κB-luc reporter plasmid, RAW 264.7 cells were treated with vehicle only (Cont), EGb 761 only (E400, 400 μg/mL), or quercetin only (Q200, 200 μM) or with vehicle (LPS), EGb 761 (E400+, 400 μg/mL), or quercetin (Q200+, 200 μM) for 1 hr followed by a 4-hr treatment with LPS (1000 ng/mL). -Cont+ represents cells transiently transfected with the negative control plasmid pCIS-CK and treated with vehicle for 1 hr followed by treatment with LPS (1000 ng/mL for 4 hr). Cells were harvested for luciferase activity and protein determination. Data, representative of two separate experiments, N = 3 for each experiment, are expressed relative to control luciferase activity, normalized to protein, and plotted as means ± SEM, N = 6. Key: (*) significantly different from control treatment, $P < 0.05$.

(TATA box) plus five repeats of the NF-κB/Rel binding site. LPS treatment caused a 4.5-fold increase in luciferase activity compared with the control (Fig. 6). EGb 761 diminished LPS-induced luciferase activity by approximately 25%, while quercetin had no effect. Cells transfected with the negative control pCIS-CK plasmid (-Cont+, which does not contain *cis*-acting elements) did not respond to LPS. Treatment with EGb 761 or quercetin in the absence of LPS had no effect on cells transfected with the pNF-κB-luc plasmid.

3.7. Selective effects of EGB 761 and quercetin on p44/42 MAPK and JNK/SAPK

The MAPK pathway is important in the post-transcriptional regulation of TNF-α. Transcription factors that bind to AP-1 and CRE are activated by MAPKs. We therefore investigated the effects of EGb 761 and quercetin on LPS-stimulated activation of the MAPK cascade. As shown in Fig. 7a, LPS induced phosphorylation of p44/42 MAPK. EGb 761 and quercetin partially inhibited and UO126, a specific MEK 1/2 inhibitor, completely inhibited LPS-induced phosphorylation. In contrast, SB203580, a specific inhibitor of p38 MAPK, was without effect. EGb 761 had no effect on and quercetin inhibited LPS-induced phosphorylation of JNK/SAPK (Fig. 7b) and its substrates, c-Jun (Fig. 7c) and ATF-2 (Fig. 7d). UO126 had no effect and SB 203580 showed slight inhibition of LPS-induced ATF-2 phosphorylation (Fig. 7d). ATF-2 is a substrate for both p38 MAPK and JNK/SAPK; therefore, the *in situ* inhibition of phosphorylation of this protein by quercetin but not EGb 761 (Fig. 7c) stresses the importance of JNK/SAPK in the activation of this transcription factor. The MEK/ERK inhibitor PD98059 (2′-amino-3′-methoxyflavone), at a concentration of 50 μM, caused inhibition of LPS-induced phosphorylation of JNK/SAPK and c-Jun (data not shown). Compared with the control, treatment with EGb 761 or quercetin in the absence of LPS had no effect on ERK1/2, JNK/SAPK, or p38 MAPK phosphorylation (data not shown). Preliminary time–course studies using the general antiphosphotyrosine antibody 4G10 established that maximal MAPK phosphorylation occurred 15–30 min following LPS stimulation (data not shown).

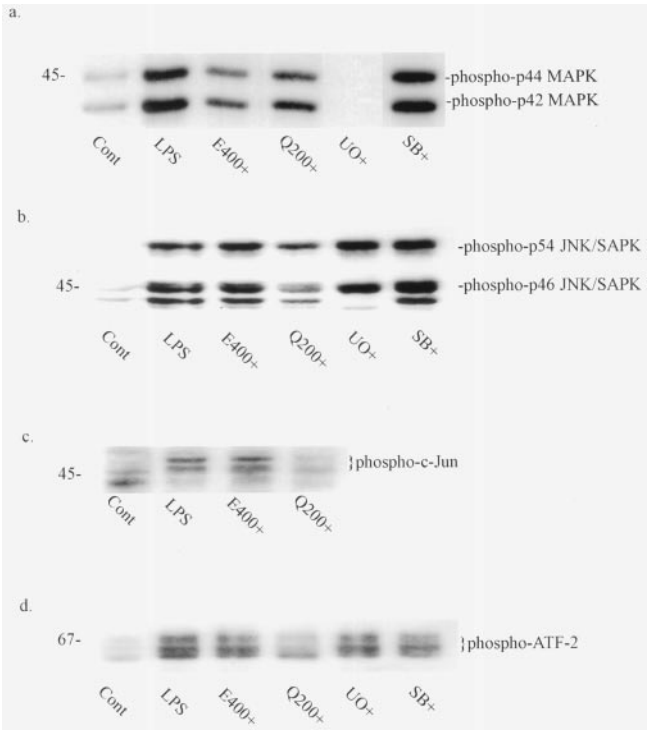

Fig. 7. Effects of EGb 761 and quercetin on LPS-induced phosphorylation of p44/42 MAPK (ERK 1/2), JNK/SAPK, c-Jun, and ATF-2. RAW 264.7 cells were unstimulated (Cont) or were stimulated with LPS (100 ng/mL) for 15 min. Prior to LPS treatment, cells were pretreated for 1 hr with EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM), or SB203580 (SB+, 30 μM). Cell lysates were immunoblotted with a specific antibody to (a) phospho-p44/42 MAPK, (b) phospho-JNK/SAPK, (c) phospho-c-Jun, or (d) phospho-ATF-2. In each case, at least two independently performed western analyses gave similar results.

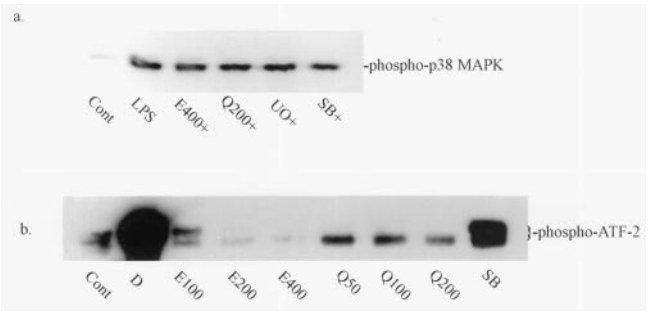

Fig. 8. Effects of EGb 761, quercetin, and SB203580 on p38 MAPK activity. (a) RAW 264.7 cells were unstimulated (Cont) or were stimulated with LPS (100 ng/mL) for 15 min. Prior to LPS treatment, cells were pretreated for 1 hr with EGb 761 (E400+, 400 μg/mL), quercetin (Q200+, 200 μM), UO126 (UO+, 20 μM), or SB203580 (SB+, 30 μM). Cell lysates were immunoblotted with a specific antibody to phospho-p38 MAPK. Three identical experiments independently performed gave similar results. (b) *In vitro* p38 MAPK activity was analyzed by an immunoprecipitation kinase assay. The kinase assay solution contained immunoprecipitated p38 MAPK from untreated cells (Cont) or LPS-treated cells and DMSO (D), EGb 761 (E100, 100 μg/mL; E200, 200 μg/mL; E400, 400 μg/mL), quercetin (Q50, 50 μM; Q100, 100 μM; Q200, 200 μM), or SB203580 (SB, 30 μM). A second, independent experiment gave similar results.

3.8. Inhibitory effects of EGB 761 and quercetin on p38 MAPK activity

Immunoblot analysis of p38 MAPK revealed that only SB203580 diminished LPS-induced phosphorylation of p38 MAPK (Fig. 8a). p38 MAPK activity, analyzed by an *in vitro* kinase assay after specific immunoprecipitation with anti-p38 MAPK antibody, demonstrated that LPS induced p38 MAPK activity as shown by the increased phosphorylation of the p38 MAPK substrate ATF-2 (Fig. 8b). EGb 761 and quercetin concentration-dependently inhibited ATF-2 phosphorylation. The p38 MAPK inhibitor SB203580, at a concentration of 30 μM, inhibited *in vitro* p38 MAPK activity by more than 50%. Similar concentrations of SB203580 have been used to inhibit *in vitro* p38 MAPK activity from RAW 264.7 macrophages [23].

3.9. Inhibitory effects of EGB 761 and quercetin on LPS-induced AP-1–DNA binding

Because the activation of JNK/SAPK and/or ERK1/2 results in AP-1 activation, we examined the effects of EGb 761 and quercetin on LPS-induced AP-1–DNA binding by EMSA (Fig. 9). Consistent with our results from western analysis, which demonstrated that quercetin inhibited the activation of JNK/SAPK and that both EGb 761 and quercetin diminished p44/42 MAPK phosphorylation, quercetin dramatically decreased and EGb 761 diminished the binding of nuclear extract proteins to the AP-1 complex. The identity of the AP-1 complex was confirmed using antibodies to

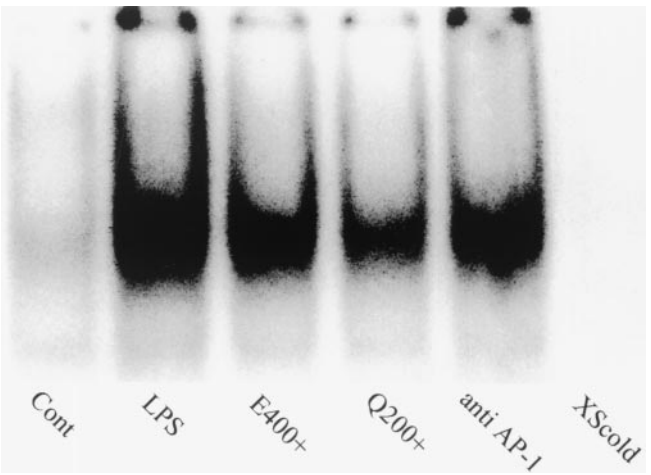

Fig. 9. Effects of EGb 761 and quercetin on LPS-induced AP-1 binding. Nuclear extracts were prepared and assayed for AP-1 induction by EMSA as described in "Materials and methods." RAW 264.7 cells were treated with DMSO (Cont), or were pretreated for 1 hr with DMSO (LPS), EGb 761 (E400+, 400 μg/mL), or quercetin (Q200+, 200 μM) prior to stimulation with LPS (100 ng/mL for 60 min). Antibody and competition analysis was performed on nuclear extracts from cells treated with LPS. Anti AP-1: antibody to c-Jun/AP-1; XS cold: 100-fold excess unlabeled AP-1 consensus oligonucleotide. Similar results were obtained in three independent experiments.

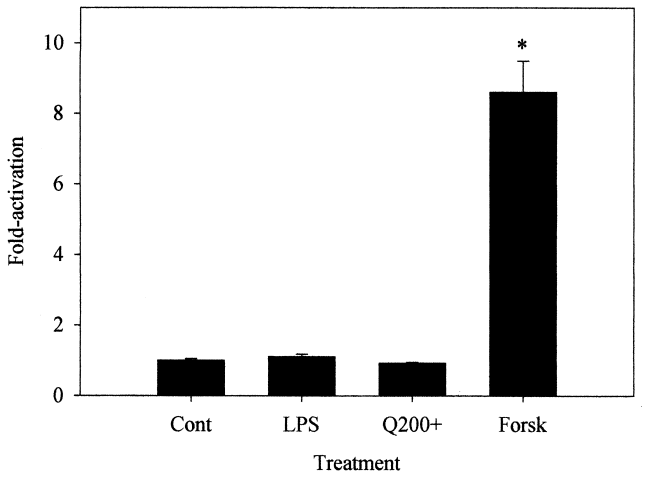

Fig. 10. Effect of LPS on CRE-dependent transcriptional activity in RAW 264.7 macrophages. Twenty-four hours after transient transfection with pCRE-luc, cells were treated with vehicle only (Cont, N = 6), were pretreated for 1 hr with DMSO (LPS, N = 6) or quercetin (Q200+, 200 μM, N = 3) followed by treatment with LPS (1000 ng/mL for 4.5 hr), or were pretreated with vehicle for 1 hr, followed by treatment with forskolin (Forsk, 10 μM, N = 3) for 4.5 hr. Cells were harvested for luciferase activity and protein determination. Data, representative of two separate experiments, are expressed relative to control luciferase activity normalized to protein and plotted as means ± SEM, N = 6. Key: (*) significantly different from control, $P < 0.05$.

c-Jun/AP-1, which did not result in a supershift, but did decrease AP-1–DNA binding. Excess unlabeled AP-1 oligonucleotide inhibited AP-1–DNA binding.

3.10. Effects of LPS on ATF/CRE binding and transcriptional activity in RAW 264.7 macrophages

The TNF-α promoter contains the CRE sequence, which binds Jun–ATF-2 heterodimers. Since quercetin inhibits LPS-induced c-Jun and ATF phosphorylation, we examined whether transcriptional inhibition of TNF-α by quercetin may be due to inhibition of binding to the CRE. Transient transfection of RAW 264.7 macrophages with the pCRE-luc reporter plasmid (Fig. 10) revealed that LPS did not stimulate transcriptional activity. In contrast, forskolin treatment, which activates CRE via activation of adenylate cyclase and CREB, resulted in a 9-fold enhancement of luciferase activity. EMSA did not reveal a dramatic difference in nucleoprotein binding between control and LPS-treated macrophages, and quercetin had no effect on c-Jun/ATF heterodimer levels (data not shown).

4. Discussion

Many cell types respond to oxidative stress by up-regulating the Rel/NF-κB family of transcription factors, which play a central role in coordinating the expression of genes involved in the inflammatory process. NF-κB is inhibited by a variety of structurally diverse antioxidants and the overexpression of antioxidant enzymes [24]. Polyphenolic phytochemicals with antioxidant properties, such as curcumin and epigallocatechin gallate, have been shown to inhibit LPS-induced activation of NF-κB and κB-responsive cytokines [25,26]. Here, we investigated whether EGb 761 and quercetin, natural product antioxidants, inhibit LPS-induced release of TNF-α by blocking activation of NF-κB.

The precise mechanism by which LPS stimulation induces NF-κB has yet to be elucidated. The most abundant form of the transcription factor is a heterodimer of p50 and p65 subunits, in which the p65 subunit contains the transcriptional activation domain. In unstimulated cells, NF-κB is retained in the cytoplasm by the binding of a family of inhibitors (IκBα, IκBβ, IκBγ). Activation by LPS requires sequential phosphorylation of IκB by an IκB-kinase (IKK1 or IKK2), ubiquitination and degradation by the proteasome, followed by translocation of NF-κB/Rel proteins to the nucleus [27]. Several studies have advanced our understanding of the role of protein kinases in LPS-mediated NF-κB activation. Tyrosine kinase inhibitors inhibit the activation of NF-κB and the subsequent release of proinflammatory cytokines [28]. IKK1 and IKK2 appear to be differentially regulated. In LPS-stimulated monocytes, prolonged NF-κB activity correlates with IKK2-dependent IκBβ phosphorylation and degradation. MEKK-1, the upstream activator of JNK/SAPK and p38 MAP kinase pathways, preferentially stimulates IKK2 activity [29]. p38 MAP kinase regulates NF-κB-dependent gene expression, in part, by phosphorylating the transcription factor TFIID (TBP), which is important for transcriptional activation of NF-κB [30]. The role of ROIs in the regulation of NF-κB is controversial. To date, data suggest that IκB phosphorylation and degradation may be the steps that are sensitive to oxidative stress and antioxidants [24].

Based on our results from western analysis of IκB degradation and nuclear translocation of the p50 and p65 subunits of NF-κB, mobility shift analysis of NF-κB–DNA binding, and transient transfections with the pNF-κB-luc reporter, we conclude that although quercetin inhibits LPS-induced TNF-α mRNA, it has no effect on LPS-induced binding of the p50/65 heterodimer or NF-κB-dependent transcriptional activity. As described in the Introduction, the 5′ flanking region of the TNF-α promoter contains binding sites for numerous transcription factors, including NF-κB, AP-1, CRE, and C/EBPβ. A reporter plasmid containing the promoter region of murine TNF-α may help elucidate the mechanism by which quercetin inhibits LPS-induced TNF-α transcription. Most studies examining the *in vitro* effects of quercetin on cultured cells use quercetin concentrations ranging between 1 and 100 μM [15,31–34]. Although we used a higher concentration of quercetin (200 μM) to evaluate the inhibition of LPS-induced TNF-α, crystal violet uptake assays confirmed that this concentration does not exhibit cytotoxicity.

EGb 761 decreased LPS-induced p50/50 binding, nu-

clear translocation of the p50 subunit, and NF-κB transcriptional activity in the pNF-κB-luc reporter construct, but this does not appear to be significant in TNF-α transcriptional inhibition since EGb 761 does not diminish LPS-induced TNF-α mRNA. Components other than quercetin are most likely responsible for the effects of EGb 761 on NF-κB.

Since quercetin and EGb 761 can act as radical scavengers, the mechanism by which LPS induces NF-κB in RAW 264.7 macrophages may not involve reactive oxygen species. TNF-α inhibition by EGb 761 and quercetin cannot be explained merely by their antioxidant properties.

Literature suggests that components of the MAPK pathway are important in the regulation of TNF-α [12,35]. p44/42 (ERK1/2), p38, and JNK/SAPK play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stress. MAPK cascades are usually organized in a three-kinase architecture consisting of a MAPK/extracellular-signal related kinase (ERK) kinase kinase (MEKK), which activates a MAPK/ERK kinase (MEK), which, in turn, activates a MAPK/ERK. The MEKs are dual specificity kinases, phosphorylating MAPKs on threonine and tyrosine on specific TXY sequences. Phosphorylation induces dimerization and translocation of the MAPK from the cytoplasm to the nucleus. In RAW 264.7 macrophages, p44/42 MAPK appears to be downstream of PKC, Raf, and MEK1/2 [36]. ERK 1/2 activation can be inhibited by the noncompetetive MEK1/2 inhibitor UO126 [37]. The upstream members of the p38 MAP kinase family are MEKK-1 and MEK3/6. p38 MAPK can be selectively inhibited by SB203580, which binds to its ATP-binding site [38]. The JNK/SAPK pathway consists of MEKK-1 and MKK4/7. c-Jun, a component of the transcription factor AP-1, is a major downstream target of the JNK/SAPK signaling pathway [39]. To the best of our knowledge, no specific inhibitor of JNK/SAPK has been identified. Signals downstream of the three distinct MAPK cascades do not operate in a purely linear fashion, and may ultimately converge. For instance, the transcription factor Elk-1 can be activated by ERK1/2, JNK/SAPK, and p38, whereas ATF-2 is regulated by JNK/SAPK and p38. The challenge is to understand how the coordination and regulation of these pathways result in a specific biological response.

Our results from western blot analysis indicate that quercetin inhibits LPS-induced phosphorylation of JNK/SAPK and its downstream substrates, c-Jun and ATF-2. Both quercetin and EGb 761 diminished phosphorylation of ERK1/2 and p38 MAPK activity. Inhibition occurred downstream of TRAF6 and MEKK1, as phosphorylation of NIK (data not shown) and IκB was not affected by EGb 761 or quercetin. ERK1/2 and p38 MAPK are not required for TNF-α transcription, as evidenced by northern blots illustrating that the specific inhibitors UO126 and SB203580 have no effect on LPS-induced TNF-α mRNA. The unique effect of quercetin on TNF-α transcription, therefore, is not due to its effects on ERK1/2 or p38 MAPK.

We propose that inhibition of TNF-α transcription by quercetin is due to inhibition of JNK/SAPK via effects on the transcription factor AP-1. AP-1 was first defined as a DNA binding activity specific for positive regulatory elements in the SV40 early promoter. It is activated in response to a wide array of stimuli, including LPS, UV and ionizing irradiation, peptide growth factors, and cytokines. AP-1 is not a single transcription factor, but a series of related dimeric complexes of Fos and Jun family proteins that bind to the DNA sequence 5′-TGAGTCA-3′ [40]. In addition to forming Jun–Jun and Jun–Fos dimers, Jun proteins can form heterodimeric Jun–ATF-2 complexes, which recognize the CRE binding sequence. Activation of ERK and/or JNK/SAPK results in AP-1 activation. Both c-Jun and c-Fos are activated by JNK/SAPK, c-Jun by direct phosphorylation, and c-Fos by activation of Elk-1, a transcriptional element on the c-Fos promoter [41]. AP-1 and NF-κB may form synergistic complexes that enhance transcription [42]. Consistent with our hypothesis, EMSA results demonstrated that quercetin dramatically decreases AP-1–DNA binding.

Transient transfections with the pCRE-luc reporter and EMSAs demonstrated that LPS does not stimulate transcriptional activity or ATF/CRE binding in RAW 264.7 macrophages. A lack of an obvious difference between control and LPS-stimulated RAW 264.7 nuclear extracts in EMSAs has been reported previously [43]. These results suggest that ATF/CRE is not involved in the LPS-induced transcription of TNF-α.

The post-transcriptional effects of EGb 761 and quercetin on TNF-α may be explained by inhibition of ERK1/2 phosphorylation and p38 MAPK activity. In our system, both UO126 and SB203580 block LPS-induced release of TNF-α, yet have no effect on steady-state levels of TNF-α mRNA. Several studies have illustrated the role of MAPKs in the post-transcriptional regulation of TNF-α biosynthesis. Upstream components in the ERK pathway, Ras and Raf, are required for TNF-α production at the level of both transcription and translation in RAW 264.7 macrophages [13]. The use of specific p38 inhibitors and kinase defective mutants of JNK/SAPK established that these kinases are necessary for LPS-induced translation of TNF-α mRNA [12,38]. MAPKAP kinase 2, a substrate of p38 MAPK, is essential for LPS-induced TNF-α translation [44]. We were not able to monitor the phosphorylation of MAPKAP kinase 2 directly; however, the inhibition of p38 MAPK *in vitro* with ATF as a substrate suggests that this is a likely site for EGb 761 and quercetin action. Quercetin may exhibit more profound effects than EGb 761 on TNF-α translation by concomitantly inhibiting JNK/SAPK. The proteins involved in translational control are yet to be fully elucidated, but two protein complexes that bind to TNF-α mRNA are implicated. Protein complex 1, containing an RNA-binding protein, binds to clustered AUUUA pentamers independently of LPS, and may be responsible for translational repression [4]. Complex 2, composed of a 55-kDa protein, binds to UUAUUUAUU sequences following LPS stimulation and

may mediate translational de-repression [45]. It will be necessary to evaluate the effect of EGb 761 and quercetin on the 3′-UTR of TNF-α in order to understand the effects of these natural products on post-transcriptional regulation of TNF-α.

In conclusion, EGb 761 and its aglycone component, quercetin, have selective effects on TNF-α and the MAPK cascade. Although both EGb 761 and quercetin inhibit TNF-α secretion in LPS-stimulated RAW 264.7 macrophages, our results suggest that quercetin is unique in its ability to inhibit TNF-α transcription by inhibiting phosphorylation and activation of JNK/SAPK, and, therefore, suppressing activation of the transcription factor AP-1. EGb 761 diminished LPS-induced NF-κB transcriptional activity slightly but had no effect on TNF-α transcription. Both EGb 761 and quercetin can also inhibit TNF-α production at a post-transcriptional level. ERK1/2 and p38 MAPK activities, which are important in the post-transcriptional regulation of TNF-α mRNA, were inhibited by EGb 761 and quercetin.

Acknowledgments

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