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Inhibition of Nuclear Factor κB by Phenolic Antioxidants: Interplay between Antioxidant Signaling and Inflammatory Cytokine Expression

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ABSTRACT

Phenolic antioxidants inhibit the induction of inflammatory cytokines by inflammatory stimuli. Here, we analyzed the mechanism by which the antioxidants inhibit LPS-induced expression of tumor necrosis factor α (TNFα) in macrophages. Hydroquinone and *tert*-butyl hydroquinone, prototypes of phenolic antioxidants, block lipopolysaccharide (LPS)-induced transcription of TNFα and a nuclear factor (NF)-κB–mediated reporter gene expression, suggesting NF-κB as a target in the inhibition. Analyses of the NF-κB activation pathway revealed that the antioxidants do not inhibit LPS-induced activation of the IκB kinase activity, degradation of IκBα, or translocation of activated NF-κB into the nucleus, but they do block the formation of NF-κB/DNA binding complexes. In vitro experiments showed that the antioxidants do not directly interfere with DNA binding of NF-κB. Structure-activity analyses suggest that inhibition of NF-κB function involves the redox cycling property of the antioxidants. These findings implicate a redox-sensitive factor important for the binding of NF-κB to its DNA recognition sequence as a target molecule in the inhibition of NF-κB function and inflammatory cytokine expression by phenolic antioxidants.

Phenolic antioxidants exhibit anti-inflammatory, anticarcinogenic, and antidiabetic activities in animals (Wattenberg et al., 1980; Talalay et al., 1988; Bjorkhem et al., 1991; Nagakawa et al., 1993; Futakuchi et al., 1998; Nishizono et al., 2000). Human beings consume appreciable amounts of the antioxidants from dietary sources, either as natural components or as synthetic food additives (Nunn, 1991; Yang et al., 2001). The broad spectrum of the functions of phenolic antioxidants suggests multiple targets of the antioxidants, through which the antioxidants interfere with various cellular functions and protect against pathological lesions such as cancer and inflammatory diseases.

The mechanism of action of phenolic antioxidants is best understood for the induction of phase 2 drug metabolizing enzymes, including NAD(P)H:quinone oxidoreductase and glutathione *S*-transferase. Induction of the genes involves an Nrf2-mediated transcriptional process (Itoh et al., 1997; Nguyen et al., 2000). Nrf2 is localized in the cytoplasm in a complex with Keap1 (Itoh et al., 1999). Upon exposure to phenolic antioxidants, Nrf2 dissociates from Keap1, translocates into the nucleus, and dimerizes with a Maf-like factor. The heterodimer binds to the antioxidant-response element located in the enhancer regions and transcribes the genes. Phase 2 enzymes are often known as detoxification enzymes, because they catalyze the metabolic conversions of many endogenous and exogenous chemicals through reduction and conjugation reactions. Loss of Nrf2 by targeted gene knockout increases the sensitivity of mice to carcinogenesis by polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene or toxicity by toxicants such as acetaminophen (Chan et al., 2001; Ramos-Gomez et al., 2001), which are attributed to diminished metabolic clearance of the chemicals.

Inflammation plays pivotal roles in the pathogenesis and development of inflammatory diseases as well as certain cancers, neurodegenerative lesions, and chemical toxicity (Laskin and Pendino, 1995; Luster, 1998; Wills-Karp, 1999). Inhibition of inflammation by antioxidants thus constitutes an important mechanism of chemoprotection by antioxidants

ABBREVIATIONS: TNFα, tumor necrosis factor α; IL, interleukin; LPS, lipopolysaccharide; tBHQ, *tert*-butylhydroquinone; HQ, 1,4-dihydroquinone; BNF, β-naphthoflavone; NAC, *N*-acetylcysteine; ELISA, enzyme-linked immunosorbent assay; IKK, IκBα kinase complex; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DIG, digoxigenin; GST, glutathione *S*-transferase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; DTT, 1,4-dithiothreitol; EMSA, electrophoretic mobility shift assay; AP-1, activator protein 1; PAGE, polyacrylamide gel electrophoresis; PDTC, pyrrolidinedithiocarbamate; E3330, (*E*)-3-(2-(5,6-dimethoxy-3-methyl-1,4-benzoquinonyl))-2-nonylpropanoic acid; HO-1, heme oxygenase-1; Nrf2, NfE2-related factor 2; Ref-1, Redox factor-1.

(Ma and Kinneer, 2002). Many of the inflammatory processes are mediated by inflammatory cytokines, which are produced and released by inflammatory cells in a sequential and concerted manner. TNFα, which is produced in the early stage of inflammation, controls the production of other cytokines, such as IL1β and IL6, and thus is a major cytokine in inflammatory responses (Locksley et al., 2001). Intervention of TNFα function by knocking out TNFα or its receptors or by using neutralizing agents implicates TNFα in a number of disease processes, including endotoxin sepsis, rheumatoid arthritis, asthma, and Parkinson's disease (Luster et al., 1999; Feldmann and Maini, 2001; Locksley et al., 2001; Sriram et al., 2002).

We have previously shown that phenolic antioxidants potently inhibit the expression of TNFα in LPS-stimulated macrophages (Ma and Kinneer, 2002). In this study, we analyzed the signaling pathway through which the antioxidants inhibit TNFα transcription. The findings reveal that phenolic antioxidants block LPS-induced NF-κB activation and suggest a redox-sensitive factor, which is important for DNA-binding of NF-κB, as a molecular target of the antioxidants in the inhibition of TNFα induction by LPS.

Materials and Methods

Materials. Restriction endonucleases and other general molecular biology reagents were purchased from New England Biolabs (Beverly, MA), Roche Applied Science (Indianapolis, IN), and Promega (Madison, WI). Radioactive compounds were from Amersham Biosciences (Piscataway, NJ). LPS, tBHQ, HQ, catechol, resorcinol, benzene, phenol, *para*-benzoquinone, BNF, and NAC were purchased from Sigma (St. Louis, MO). TCDD was from AccuStandard (New Haven, CT). Cell culture materials were from Invitrogen (Carlsbad, CA). Reagents for Northern blotting, ELISA, and IKK kinase assay are as described below.

Cell Culture. The mouse macrophage RAW 264.7 cell line was purchased from the American Type Culture Collection (Manassas, VA). The macrophage cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum at 5% CO_2 and 37°C. The cells were treated with LPS, phenolic antioxidants, or other chemicals as described in the figure legends; DMSO or water was used as the solvent control for the antioxidants and LPS, respectively.

Northern Blot Analysis. The cDNA fragments for mouse TNFα and HO-1 were synthesized by polymerase chain reaction amplification of the corresponding cDNA templates from BD Biosciences Clontech (Palo Alto, CA) using the primer sets specific for each mouse gene from the same company. The cDNAs were subcloned into the pCRII TA cloning vector (Invitrogen), verified by sequencing, and used to generate riboprobes for the corresponding mRNA species. A mouse actin cDNA fragment (~500 bp) was used to generate a riboprobe for actin. The riboprobes were synthesized in the presence of DIG-UTP using a DIG labeling Kit (Roche Applied Science). Total RNA was isolated from cells using the RNeasy kit (QIAGEN, Valencia, CA). Total RNA (5 μg in each lane) was fractionated on a 1% agarose-formaldehyde gel and transferred to a Nytran membrane by capillary action. After UV cross-linking, the membrane was hybridized overnight with a DIG-labeled riboprobe at 68°C. Signals were visualized by chemiluminescence using a DIG RNA detection kit with CDP-Star (Roche Applied Science) as a substrate.

Luciferase Reporter Construction and Assay. The mouse TNFα enhancer/promoter sequence (−1043 to +143) was subcloned into pGL3-Basic vector (Promega) at *Xho*I and *Hin*dIII sites to generate a TNFα-luciferase reporter construct. The NF-κB-luciferase vector, which contains five copies of NF-κB binding sites, was from Stratagene (La Jolla, CA). The reporter plasmids were transiently transfected into macrophage cells using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. For stable transfection, cells were selected in G-418–containing medium for 2 weeks and a clone with high inducible luciferase activity was selected for the study. For luciferase assay, cells were lysed in a lysis buffer (Promega), and cell lysate was assayed for luciferase activity using the luciferase kit (Promega) and Microplate Luminometer LB 96 V (Berthold Technologies USA LLC, Oak Ridge, TN). Statistical analysis was performed using Excel (Microsoft, Redmond, WA) and standard deviation was used to represent variations among triplicate samples. Similar results were obtained in separate experiments.

ELISA for p65. Macrophage cells were grown in 60-mm dishes in DMEM with 5% fetal bovine serum at 37°C until confluent. Cells were treated with the antioxidants for 1 h followed by stimulation with LPS for 3 h. Whole-cell extract was harvested using the lysis buffer provided in the Trans-Am NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA). The assay was performed according to the protocol provided in the kit using 20 μg of whole-cell extract per well; activities of p65 were measured using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm (corrected for absorbance at 655 nm).

Preparation of Nuclear Extracts. Nuclear extracts were prepared with a three-step procedure as described elsewhere (Ye et al., 1999). Briefly, cells were grown in a 100-mm dish to near-confluence. After treatment, cells were collected with a rubber policeman, washed with 1× phosphate-buffered saline (PBS), and lysed in 500 μl of lysis buffer on ice for 4 min. The lysis buffer contains 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μM 1,4-dithiothreitol (DTT). Cell lysate was centrifuged at 14,000 rpm for 1 min in a microcentrifuge. In the second step, the pellet (the nuclei fraction) was washed once in washing buffer, which is the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at −80°C, then thawed on ice, and centrifuged at 14,000 rpm for 5 min. Supernatant was collected as nuclear extract and stored at −80°C for further use.

EMSA. A NF-κB DNA-binding sequence (5′-GATTTTCCCAT-GAGTCT-3′) was used to synthesize oligonucleotides as the NF-κB binding probe (Ye et al., 1999). The complementary single strand oligomers were denatured at 80°C for 5 min and annealed at room temperature. An activator protein-1 (AP-1) DNA-binding sequence derived from the AP-1 binding site in the collagenase enhancer was used as a nonspecific competitor probe (Ye et al., 1999). Double-stranded oligonucleotide probes were labeled with [^{32}P]ATP using T4 kinase (New England Biolabs). The DNA-protein binding reaction was conducted in a 24-μl reaction mixture containing 1 μg of poly(dI-dC) (Sigma), 3 μg of nuclear extract, 3 μg of BSA, and 12 μl of reaction buffer (12% glycerol, 24 mM HEPES, pH7.9, 8 mM Tris-HCl, 2 mM EDTA, and 1 mM DTT). The mixture was incubated on ice for 10 to 20 min, followed by addition of 4×10^4 counts/min of a ^{32}P-labeled oligonucleotide probe. Incubation was continued at room temperature for 20 min. For competition experiments, 100 ng of either unlabeled double stranded NF-κB or AP-1 probe was added to the reaction mixture for competition with labeled NF-κB probe. For supershift assays, antibodies specific for the p50 or p65 subunit of NF-κB, c-Jun, nuclear factor of activated T cells, or c-Fos were added to the reaction mixture, respectively (2 μg/reaction). The antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The DNA/protein complexes were resolved in a 5% acrylamide gel (prerun at 170 V for 30 min with 0.5× Tris-borate/EDTA buffer) at 200 V for 90 min and visualized by exposure to films.

Immunoprecipitation and IKK Kinase Assay. Macrophage cells grown in six-well plates were transfected with pIKKβ-FLAG (kindly provided by Dr. C. M. Crews, Yale University, New Haven, CT), which expresses IKKβ with a FLAG tag, using the LipofectAMINE PLUS reagent as described above. Twenty-four hours

later, the cells were treated with DMSO, HQ (100 μM), or tBHQ (100 μM) for 1 or 3 h as indicated in the figures, followed by stimulation with LPS for 30 min. The cells were washed with ice-cold PBS, collected by scraping, and lysed in 100 μl of ice-cold AT buffer (20 mM HEPES, pH 7.9, containing 20% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM Na_3VO_4, 1 μg/ml PMSF, and 1 μg/ml leupeptin). The lysate was passed five times through a 27-gauge needle. Sodium chloride was added to 150 mM. The lysate was centrifuged in a microcentrifuge at 13,000*g* for 30 min at 4°C. Supernatant was collected for immunoprecipitation described below.

The IKK complex containing expressed IKKβ-FLAG was immunoprecipitated using an anti-FLAG M2 gel suspension (Sigma) according to the manufacturer's instructions. IKK kinase assay was carried out as described by others (DiDonato, 2000; Kwok et al., 2001). Briefly, the IKK complex precipitate was resuspended in a kinase reaction buffer (25 mM Tris, pH 7.5, containing 10 mM $MgCl_2$, 2 mM DTT, 50 μM ATP, 10 mM NaF, 0.5 mM Na_3VO_4, and 20 mM β-glycerophosphate). Kinase reaction was performed by incubating the precipitate with 5 μCi of [γ-^{32}P]ATP and 2 μg of GST-IκBα_{1-54} or its mutant with substitutions S32A and S36A. The GST-IκBα_{1-54} or its mutant recombinant protein was expressed and purified from *Escherichia coli* transformed with plasmid pGST-IκBα_{1-54} or pGST-IκBα_{1-54} mutant S32A/S36A (kindly provided by Dr. J. DiDonato, Lerner Research Institute, Cleveland, OH). The kinase reaction was carried out for 30 min at 30°C and was stopped by boiling in SDS-PAGE loading buffer. The proteins were resolved on a 12% SDS-PAGE. Phosphorylated proteins were visualized by exposure to X-ray films.

Immunoblotting. Macrophage cells cultured in six-well plates were treated with antioxidants as described in the figure legends. Whole-cell lysates were prepared with a lysis buffer (Promega) and centrifuged at 13,000*g* for 10 min to remove cell debris. Nuclear extracts were prepared as described above. Cell lysates or nuclear extracts of 5 μg were run on SDS-PAGE, blotted with anti-IκBα or anti-NF-κB p65 antibodies (Santa Cruz Biotechnology), and visualized by chemiluminescence using the ECL kit (Amersham Biosciences).

Immunofluorescent Microscopy. Macrophage cells were plated at a density of 4×10^5 in six-well plates containing sterile cover slips and grown at 37°C for 24 h. The medium was then replaced by a serum-free medium, and the cells were allowed to grow for another 24 h before treatment. Cells were treated with the antioxidants for 1 h followed by stimulation with LPS for 30 min. After treatment, the cells were washed twice with PBS prewarmed to 37°C and fixed to the cover slips by incubating in 3.7% formaldehyde for 10 min. Cells were then washed three times with PBS and permeated by incubating in 100% methanol for 6 min at −20°C. The cover slips were blocked in 1% BSA overnight at 4°C with shaking. Antibodies against NF-κB p65 subunit (1:500 dilution; Santa Cruz Biotechnology) were added in 1% BSA and incubated for 1 h with shaking at room temperature. For nuclear staining, YO-PRO 3 (Molecular Probes, Eugene, OR) was then added at a final concentration of 2 μM and incubated for 1 h in the dark. Cover slips were then washed three times each for 10 min with PBS. Fluorescein isothiocyanate-conjugated secondary antibodies were then added (1:20 dilution; Chemicon International, Temecula, CA) in 1% BSA and incubated for 1 h with shaking at room temperature. Cover slips were washed three times with PBS and mounted onto slides using Prolong antifade mounting reagent (Molecular Probes). Slides were examined using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY) fitted with an argon-ion laser for fluorescein isothiocyanate or He-Ne laser for YO-PRO 3, respectively. Slides were viewed and scanned at 63× under water immersion for best resolution.

Results

Inhibition of NF-κB–Mediated TNFα Transcription. Phenolic antioxidants potently inhibit signal-induced expression of inflammatory cytokines. As shown in Fig. 1, HQ blocks LPS-induced mRNA expression of TNFα in macrophage cells. The inhibition occurs at a concentration at which it induces HO-1, a stress-inducible enzyme important in the catabolism and detoxification of heme; thus, inhibition of TNFα expression is not caused by antioxidant toxicity to the cells. These results suggest inhibition of cytokine expression as a mechanism by which the antioxidants protect against chemical toxicity and cancer in addition to induction of detoxification enzymes. To identify the molecular target(s) of the antioxidants in TNFα inhibition, we examined TNFα transcription. The TNFα enhancer/promoter region (−1043 to +143) of the mouse TNFα gene was linked to luciferase to generate the TNFα luciferase chimeric reporter construct. The results revealed that HQ completely blocks LPS-induced luciferase activity in both transient and stable transfection experiments (Fig. 2), similar to the inhibition of TNFα mRNA expression by HQ. tBHQ inhibits the reporter expression induced by LPS at the same concentration as for HQ (data not shown).

NF-κB is a major transcription factor mediating LPS-induced TNFα expression through the NF-κB binding sites located in the enhancer region of the TNFα gene. We analyzed whether NF-κB serves as a target in the inhibition of TNFα expression by the antioxidants. Transfection experiments using a NF-κB binding site-luciferase reporter showed that HQ blocks the induction of luciferase expression by LPS (Fig. 3). Together, these results indicate that inhibition of TNFα expression by phenolic antioxidants occurs at a transcriptional level and is mediated by inhibiting NF-κB function.

Effect of Phenolic Antioxidants on NF-kB Activation. NF-κB is activated by LPS and other inflammatory or apoptotic signals through sequential signaling events. Recent studies reveal that the IκBα kinase complex, the 26S proteasome-mediated degradation of IκBα, and the cytoplasmic-nuclear transport of NF-κB represent major targets of

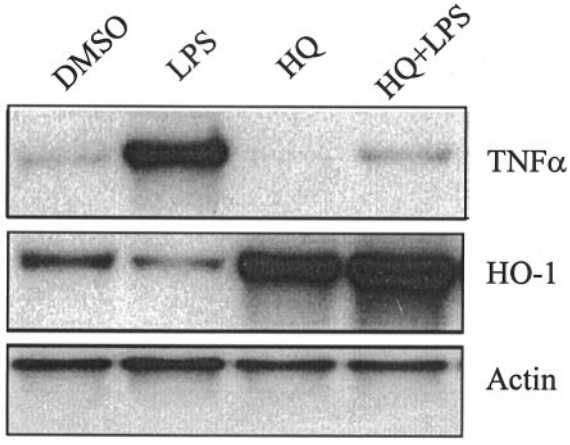

Fig. 1. Northern blot of TNFα and HO-1 expression. Macrophage cells were grown in 100-mm plates in DMEM with 5% fetal bovine serum to near-confluence. Cells were treated with HQ (100 μM) for 1 h, followed by LPS (1 μg/ml) stimulation for 5 h. Total RNA (5 μg/lane) was blotted for expression of TNFα, HO-1, and actin as described under *Materials and Methods*.

regulation of NF-κB functions by a range of endogenous and exogenous regulators, including oxidants/antioxidants (Karin et al., 2001; Adams, 2002; Ghosh and Karin, 2002). Thus, we examined if the antioxidants affect these steps of NF-κB activation by LPS. Macrophage cells were treated with HQ or tBHQ followed by stimulation with LPS for 30 min as indicated in Fig. 4. The IKK complex was immunoprecipitated and assayed for kinase activity with IκBα or its mutant (S32A/S36A) as a substrate. IKK from cells treated with LPS only catalyzes phosphorylation of IκBα (lane 1) but not its mutant (lane 2). Pretreatment with DMSO for 1 h has no effect on LPS-induced IKK activity (lane 3), whereas DMSO for 3 h slightly decreases the activity (lane 4). Treatment with HQ or tBHQ for 1 or 3 h exhibits effects on IKK activity (lanes 5 to 8) that are comparable with those of DMSO (lanes 3 and 4), suggesting that IKK is not a target of the antioxidants.

We examined whether HQ or tBHQ affects LPS-induced IκBα degradation, which is necessary for the release of NF-κB from the cytoplasmic NF-κB/IκBα complex and subsequent nuclear translocation. As shown in Fig. 5, LPS induces the degradation of IκBα. HQ or tBHQ does not affect the protein level of IκBα when treated alone, or LPS-induced IκBα degradation when cotreated with LPS. The results suggest that the antioxidants do not inhibit LPS-induced IκBα degradation and the signaling steps before it.

LPS induces the accumulation of NF-κB in the nucleus as revealed by immunoblotting the protein in the nucleus (Fig. 6). HQ or tBHQ at 100 μM does not inhibit the LPS-induced accumulation of NF-κB in the nucleus. Similar results were obtained by using immunofluorescent microscopy. As shown in Fig. 7, NF-κB is translocated from the cytoplasm into the nucleus upon treatment with LPS. HQ or tBHQ does not inhibit the translocation. Taken together, these findings suggest that inhibition of NF-κB by phenolic antioxidants takes place after NF-κB is translocated into the nucleus.

Inhibition of NF-κB DNA Binding. The function of NF-κB in the nucleus was analyzed by an ELISA-based assay, which measures the binding activity of NF-κB to its DNA recognition sequence. As shown in Fig. 8A, LPS stimulates NF-κB binding to DNA (compare lanes 1 and 2); the activity is blocked by a wild-type oligonucleotide probe, but not a mutant oligonucleotide, showing that the binding is specific for the NF-κB binding sequence. Cotreatment with HQ and LPS blocks LPS-induced binding. Resorcinol, a HQ analog inactive in the induction of detoxification enzymes (Prochaska et al., 1985), fails to inhibit LPS-induced binding. To further characterize the inhibition of NF-κB DNA binding by HQ, EMSA was performed. Figure 8B showed that LPS induces the formation of the NF-κB p65/p50 heterodimer and p50 homodimer bound to DNA. The binding can be blocked by excess amounts of unlabeled NF-κB but not AP1-binding sequences. Furthermore, the binding can be supershifted by antibodies against NF-κB p50 subunit, whereas anti-cJun and anti-Sp1 antibodies have no effect on the binding, indicating that the binding is specific for NF-κB and its DNA-binding sequence. HQ at 100 μM blocks the formation of NF-κB-DNA complexes, whereas resorcinol does not. These results imply that phenolic antioxidants inhibit LPS-induced TNFα expression by blocking DNA-binding of NF-κB.

To examine if phenolic antioxidants directly interfere with the DNA-binding of NF-κB, we performed EMSA in which phenolic antioxidants were incubated with nuclear extracts from DMSO or LPS-stimulated macrophages. As shown in Fig. 9, LPS induces the formation of the p65/p50 and p50/p50 DNA complexes. Incubation with DMSO slightly reduces the complex formation. HQ or tBHQ at 100 or 400 μM does not affect the formation of the complexes from either DMSO- or LPS-stimulated cells, suggesting that HQ or tBHQ does not

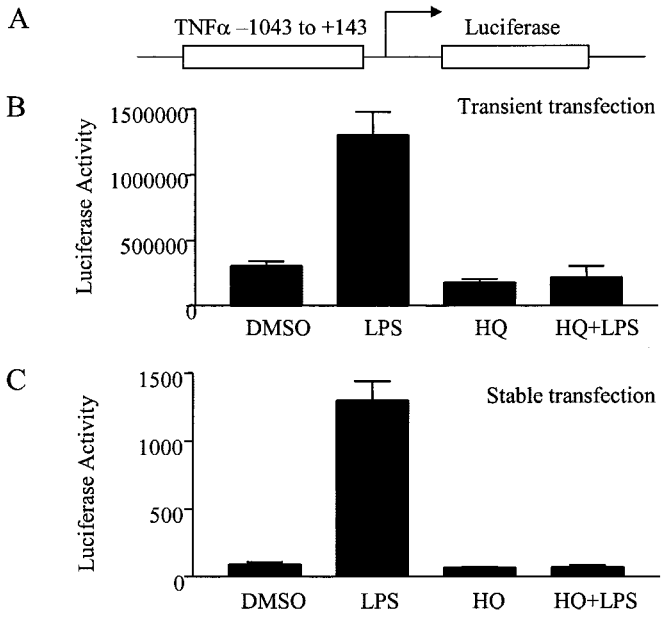

Fig. 2. Inhibition of TNFα enhancer/promoter activity. A, schematic presentation of the TNFα enhancer/promoter-luciferase reporter construct. B, transient transfection. Cells grown in 24-well plates were transfected with the TNFα luciferase reporter plasmid. Forty-eight hours later, the cells were treated with HQ, LPS, or both as described for Fig. 1. Cell lysate was prepared and measured for luciferase activities as described under *Materials and Methods*. C, macrophage cells stably transfected with the TNFα luciferase reporter plasmid were selected for high inducibility of luciferase activity. The cells were grown in 48-well plates, treated as indicated, and harvested for measuring luciferase activity as described for B.

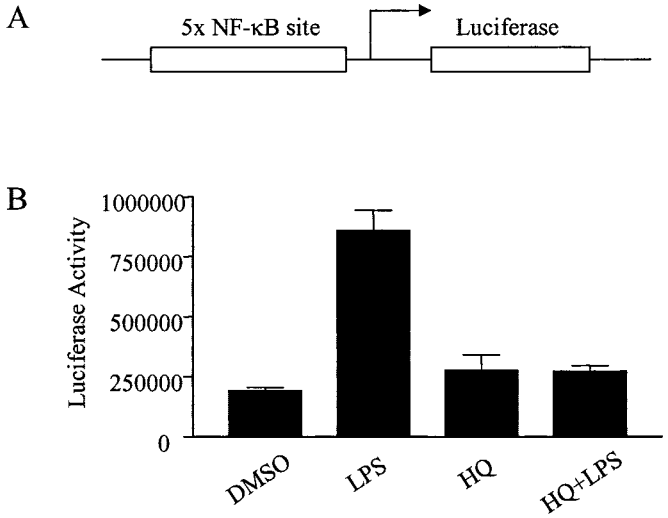

Fig. 3. Inhibition of NF-κB reporter expression. A, schematic presentation of the NF-κB luciferase reporter. B, transient transfection. Macrophage cells grown in 24-well plates were transfected with the NF-κB reporter plasmid, treated with antioxidants and/or LPS, and assayed for luciferase activity as described for Fig. 2.

directly interfere with the binding of NF-κB with its DNA binding sequence.

Inhibition of NF-κB by Phenolic Antioxidants Involves a Redox-Sensitive Factor in NF-κB DNA-Binding. Phenolic antioxidants exhibit certain structure-activity relationships in antioxidant responses, such as the induction of detoxification enzymes. Structural analogs that can easily undergo oxidation-reduction cycling often exhibit high induction activities, whereas analogs with low redox capabilities are inactive (Prochaska et al., 1985; Rushmore et al., 1991). We examined if inhibition of NF-κB by the antioxidants involves redox cycling of the antioxidants. Figure 10A shows that HQ, catechol, and *para*-benzoquinone, which can undergo redox cycling, strongly inhibit LPS-induced TNFα transcription (measured as the TNFα-luciferase reporter activity), whereas benzene, phenol, and resorcinol, which do not readily cycle between reduction and oxidation, are inactive in inhibiting the induction. BNF, which can be metabolized to products with redox capabilities and exhibits certain antioxidant functions, strongly inhibits TNFα transcription similarly to tBHQ in both 6- and 24-h treatments (Fig. 10B). TCDD, which is metabolically stable, does not exhibit observable inhibitory activity toward the induction. These results suggest that inhibition of NF-κB by phenolic antioxidants involves the redox reactions of the chemicals. To further test the role of redox reactions in NF-κB inhibition, we examined whether other antioxidants inhibit TNFα expression. NAC, an antioxidant and a precursor of intracellular glutathione, inhibits TNFα induction by LPS similarly to HQ in our experimental system, confirming a role of reduction-oxidation cycling in the inhibition of TNFα induction by antioxidants (Fig. 11).

Discussion

The potent inhibition of signal-induced cytokine expression during inflammation by phenolic antioxidants suggests a molecular target(s) of the antioxidants in the regulation of inflammatory cytokine expression. Here, we provide evidence to show that phenolic antioxidants block induction of TNFα by LPS via inhibition of the function of NF-κB in macrophage cells. The antioxidants inhibit LPS-induced accumulation of TNFα mRNA, transcriptional activity of TNFα enhancer/promoter, expression of an NF-κB-controlled luciferase reporter, and DNA binding of NF-κB. NF-κB represents a group of structurally related proteins, including c-Rel, RelA(p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), which form hetero- or

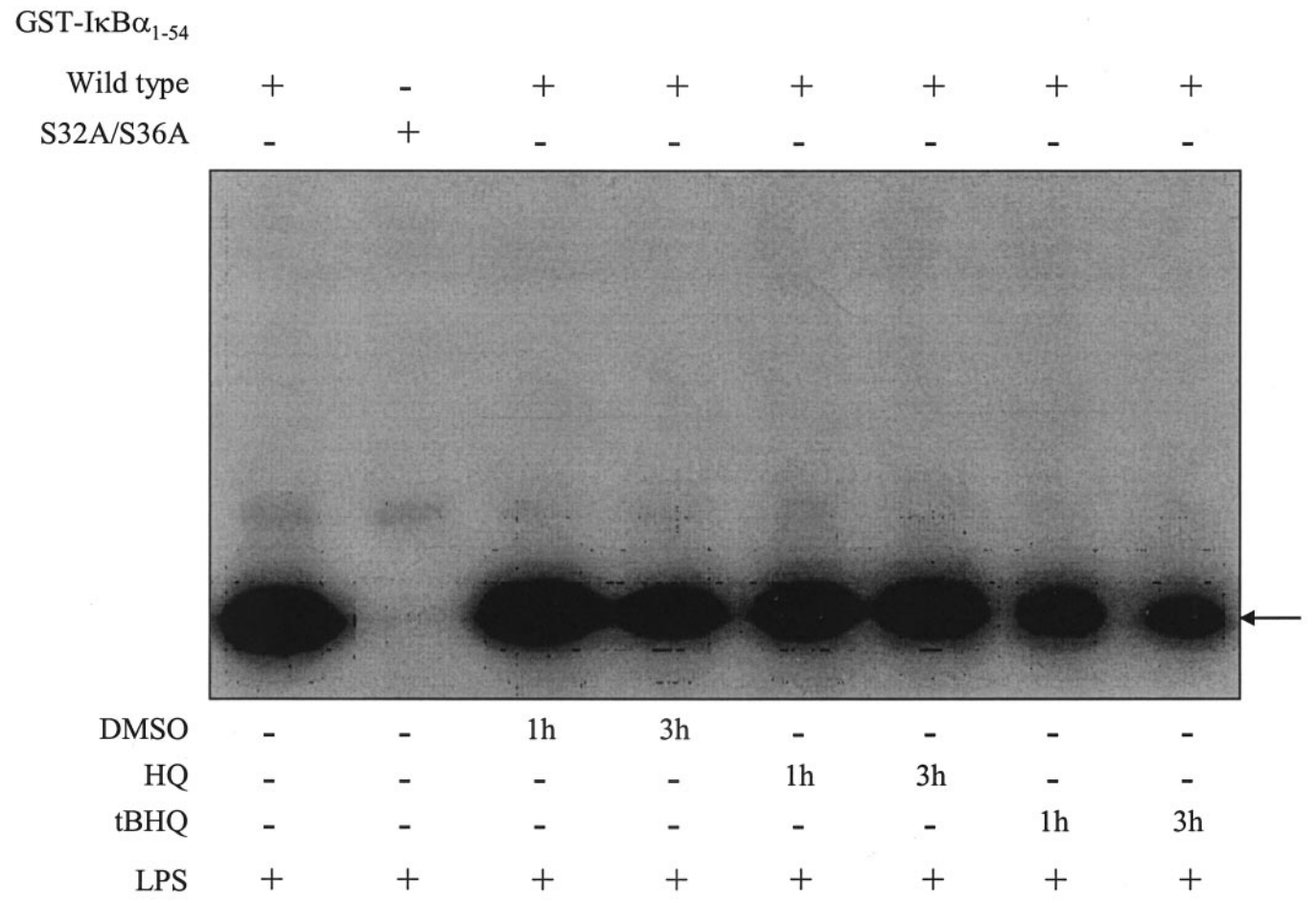

Fig. 4. Efffect of HQ and tBHQ on IKK activity. Macrophage cells were transfected with pIKKβ-FLAG. Twenty-four hours later, the cells were treated with DMSO, HQ (100 μM), or tBHQ (100 μM), followed by stimulation with LPS (10 μg/ml) for 30 min as shown in the figure. Cell lysate was collected and IKK complex containing IKKβ-FLAG was immunoprecipitated with anti-FLAG as described under *Materials and Methods*. IKK kinase activity was measured according to procedures described by DiDonato (2000) with purified GST- IκBα$_{1–54}$ or its mutant S32A/S36A recombinant protein as a substrate. The proteins were resolved by SDS-PAGE and phosphorylated proteins were visualized by exposure to X-ray films. Arrow indicates phosphorylated GST-IκBα$_{1–54}$.

homo-dimers as DNA-binding forms (Ghosh and Karin, 2002; Karin and Lin, 2002; Pomerantz and Baltimore, 2002). The NF-κB transcription factors play evolutionarily conserved and critical roles in the triggering and coordination of both innate and adaptive immune responses by controlling the expression of a wide variety of genes important for immune functions, including pro-inflammatory cytokines. In addition, NF-κB activates genes coding for regulators of apoptosis and cell proliferation and thus is critical for apoptotic processes. Our findings of inhibition of NF-κB by phenolic antioxidants provide an explanation for the anti-inflammatory function of the antioxidants: the antioxidants block DNA-binding of NF-κB and the expression of TNFα and other pro-inflammatory cytokines, thereby blocking the cascade of inflammatory responses.

Because of the central role of NF-κB in immune, inflammatory, and apoptotic responses, there is an increasing interest in modulating NF-κB activity/function as a potentially effective preventive/therapeutic strategy for combating certain immune-, inflammation-, or apoptosis-related diseases, including cancer and chemical toxicity. Activation of NF-κB by a variety of stimuli involves complex signal transduction pathways that ultimately result in the activation of a specific IKK and translocation of NF-κB from cytoplasm to the nucleus. In the case of LPS stimulation, LPS binds to the LPS-binding protein and CD14 (Aderem and Ulevitch, 2000; Ghosh and Karin, 2002). The complex is presented to LPS receptor TLR4, a member of the Toll receptors, which play essential roles in discriminating "self" from pathogen-derived ligands. Several kinase-mediated pathways are activated and converge upon the IKK complex, which contains IKKα, IKKβ, and IKKγ/NEMO subunits. Upon activation, IKK phosphorylates IκBα, a component of the cytoplasmic NF-κB complex, leading to degradation of IκBα through the ubiquitin-26S proteasome pathway (Zandi et al., 1997). NF-κB is subsequently translocated into the nucleus where it binds to corresponding DNA recognition sequences located in the enhancer region of TNFα and mediates gene transcription. Several steps of NF-κB activation are noticeably susceptible to regulators of NF-κB and serve as potential targets of anti–NF-κB drugs and environmental chemicals. It is known that IKK is both essential and highly regulated; furthermore, many xenochemicals including oxidants/antioxidants modulate IKK activation/activity (Karin et al., 2001; Kwok et al., 2001). Exposure to As^{3+} inhibits NF-κB activation through inhibition of IKK activity toward IκBα; the inhibition involves interaction of As^{3+} with a specific cysteine residue in IKKβ (Karin et al., 2001). Degradation of IκBα through the ubiquitin-26S proteasome pathway has been explored for developing chemical or peptide inhibitors of the 26S proteasomes as anti–NF-κB drugs in the therapy of certain cancers (Adams, 2002). Lastly, nuclear translocation of NF-κB can be inhibited by various stimuli (Ghosh and Karin, 2002). In this article, we examined whether the antioxidants affect the activation of NF-κB at these steps. The data reveal that phenolic antioxidants do not inhibit the activation/kinase

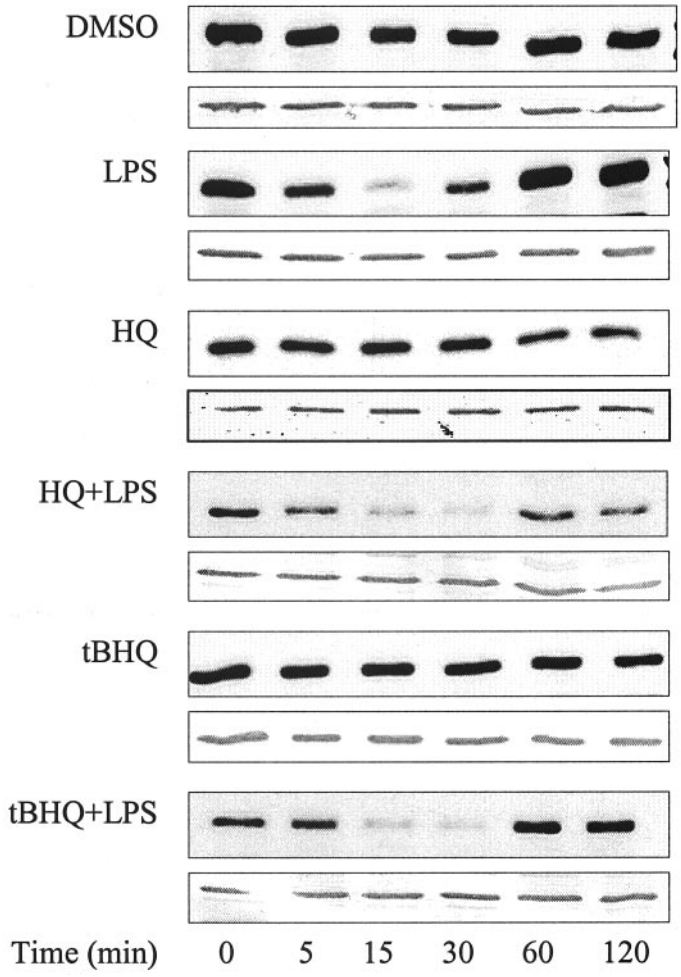

Fig. 5. Effect on IκBα degradation. Macrophage cells were grown to confluence in six-well plates. The cells were incubated in DMEM without serum for ~18 h for synchronization and were then treated with HQ (100 μM) or tBHQ (100 μM) for 1 h, followed by LPS (1 μg/ml) for 5, 15, 30, 60, or 120 min. Cell lysate was prepared and analyzed by immunoblotting with antibodies specific for IκBα. The actin level in each sample was used to control sample loading variation.

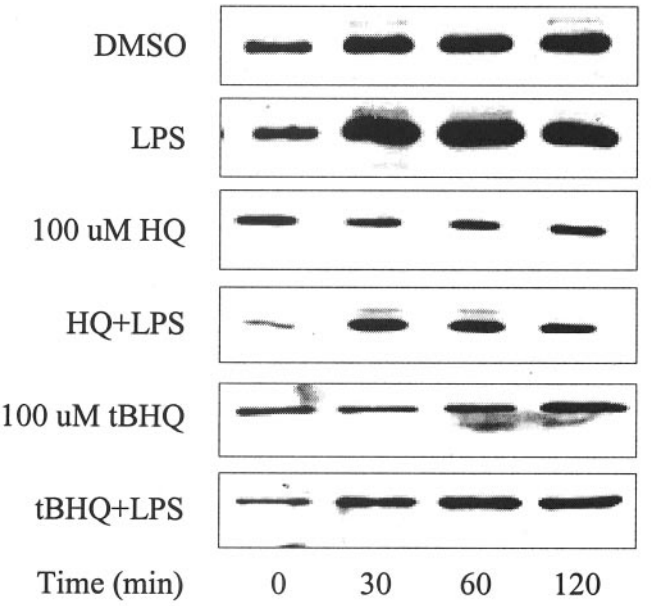

Fig. 6. Effect on NF-κB nuclear translocation. Macrophage cells in 100-mm plates were treated with HQ or tBHQ at 100 μM for 1 h, followed by LPS (1 μg/ml) for 30, 60, or 120 min as described under *Materials and Methods*. Nuclear extracts were prepared and analyzed by immunoblotting for nuclear accumulation of the NF-κB p65 subunit.

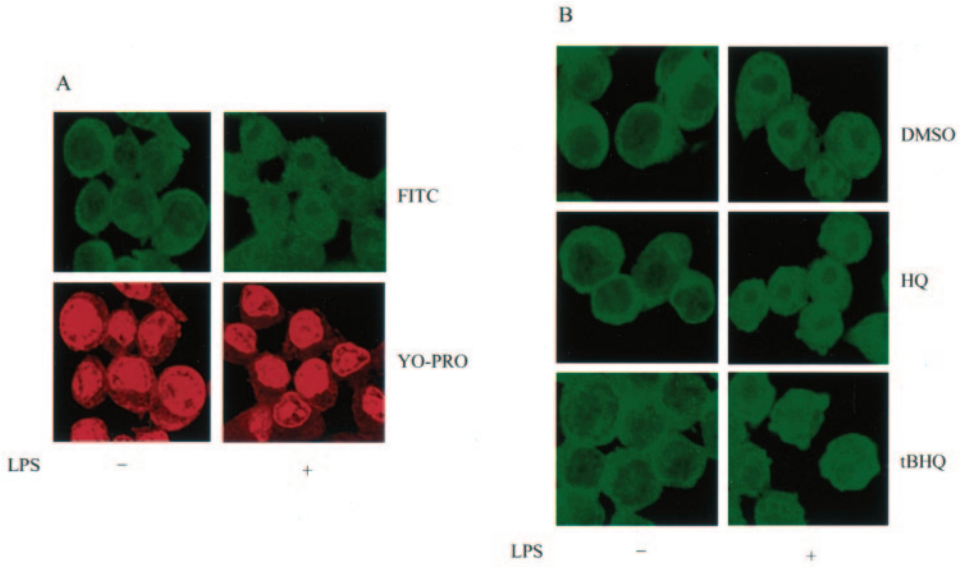

Fig. 7. Immunofluorescent microscopy. Macrophage cells grown on cover slips were treated as indicated. Cells were treated with HQ or tBHQ at 100 μM for 1 h, followed by LPS for 1 h. The cells were fixed with methanol, stained with antibodies against the p65 subunit of NF-κB, and visualized with fluorescent confocal microscopy as described under *Materials and Methods*. A, LPS-induced nuclear translocation of NF-κB. YO-PRO, which stains for the nucleus, was used to show the nucleus. B, effect of antioxidants on NF-κB nuclear translocation.

activity of IKK or interfere with the degradation of IκBα through the 26S proteasome pathway, and the antioxidants do not affect the subsequent translocation of NF-κB into the nucleus. These findings strongly suggest that phenolic antioxidants do not block the cytoplasmic signaling events of NF-κB activation; instead, the antioxidants may affect the functions of NF-κB in the nucleus.

Certain antioxidants are known to exhibit inhibitory activities toward NF-κB; moreover, inhibition by antioxidants seems to involve different target of NF-κB signal transduction. For example, the antioxidants PDTC and NAC inhibits NF-κB activation by TNF, LPS, and UV in Hela, Jurkat, and primary neuron cells through blocking signal-induced degradation of IκBα without inhibiting IKK (Li and Karin, 1999; Ma and Kinneer, 2002); whereas As^{3+} inhibits phosphorylation of IκBα by IKK (Karin et al., 2001). Evidence suggesting that antioxidants or quinoid compounds regulate nuclear NF-κB is provided in this and a few recent studies. Our data reveal that blocking of LPS-induced NF-κB functions by phenolic antioxidants is caused by inhibition of the binding of NF-κB to DNA recognition sequences; furthermore, the inhibition does not involve direct interference with DNA-binding of NF-κB as revealed by in vitro experiments. These results implicate a protein target of the phenolic antioxidants in the binding of NF-κB to DNA. Shimizu et al. (2000) have shown that a novel quinoid derivative (E3330), an anti-inflammatory drug, suppresses NF-κB function without affecting the degradation of IκBα and the nuclear translocation of NF-κB (Hiramoto et al., 1998). By using high-performance affinity beads coupled with E3330, the investigators were able to demonstrate that E3330 binds to Ref-1 (Shimizu et al., 2000), a redox-sensitive nuclear factor that interacts with NF-κB and enhances its DNA binding activity through redox reactions. E3330 does not interfere with the interaction between Ref-1 and NF-κB directly but may inhibit the redox reactions of Ref-1, particularly the reduction of Cys-62 of nuclear p50 of NF-κB (Nishi et al., 2002). Whether Ref-1 or a related factor(s) serves as the target protein of phenolic antioxidants and mediates the inhibition of NF-κB DNA binding by phenolic antioxidants requires substantial biochemical and genetic analyses of Ref-1 and NF-κB functions in the presence of phenolic antioxidants in future studies. It was shown that an Nrf1 homolog can bind to the E3 NF-κB binding site of TNFα enhancer and influence NF-κB function (Novotny et al., 1998; Prieschl et al., 1998). Thus, it is possible that phenolic antioxidants activate an Nrf-like factor analogous to

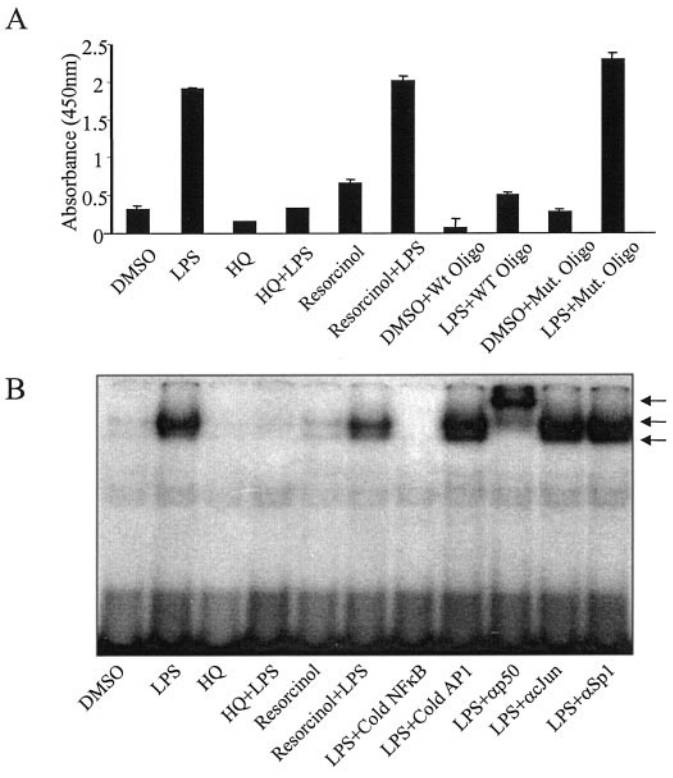

Fig. 8. Inhibition of NF-κB DNA binding. A, ELISA for p65. Cells were grown in 60-mm plates and were treated with HQ or resorcinol at 100 μM for 1 h, followed by LPS (1 μg/ml) for 3 h. Cell lysate was prepared and assayed for binding of p65 to DNA using the Trans-Am NF-κB p65 transcription factor assay kit as described under *Materials and Methods*. WT, wild type. B, EMSA. Cells were grown in 100-mm plates and treated as indicated. Nuclear extracts were prepared and assayed by EMSA as described under *Materials and Methods*. The arrows (from top to bottom) indicate the supershifted, the p65/p50, and the p50/p50 shifted bands, respectively.

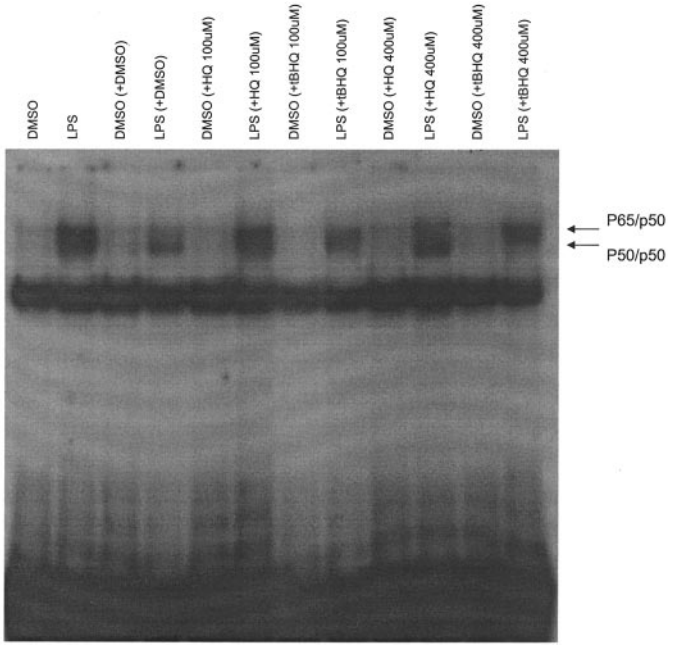

Fig. 9. In vitro effect on NF-κB DNA binding. Nuclear extracts were prepared from DMSO- or LPS- (1 μg/ml, 3 h) stimulated cells. Nuclear extracts were incubated with DMSO, HQ, or tBHQ as indicated for 30 min at room temperature and were then analyzed for NF-κB DNA binding by EMSA as described under *Materials and Methods*. The specificities of DNA-binding were established as described to Fig. 8B (data not shown).

the activation of Nrf2, which binds to the NF-κB sites of the TNFα gene and inhibits NF-κB in TNFα transcription. In vivo DNA footprinting and genetic studies in Nrf null cells may distinguish these possibilities.

The molecular events through which phenolic antioxidants activate target proteins are not clear at present. In the case

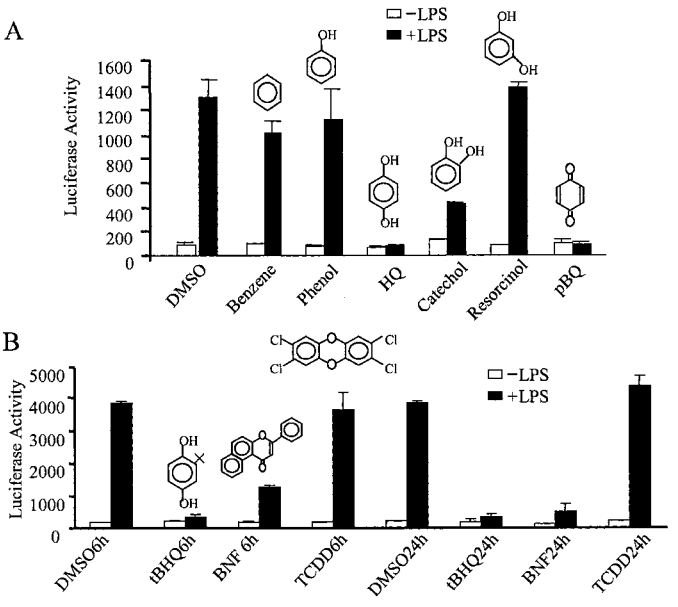

Fig. 10. Inhibition of TNFα transcription by HQ analogs. Macrophage cells stably transfected with the TNFα-luciferase reporter plasmid were grown in 48-well plates, treated with antioxidants or analogs for 1 h at a concentration of 100 μM [benzene, phenol, HQ, catechol, resorcinol, *para*-benzoquinone (pBQ)], and tBHQ), 10 μM (BNF), or 1 nM (TCDD) for 1 h, followed by LPS stimulation for 5 h. Cell lysate was prepared and luciferase activity was measured as described for Fig. 2.

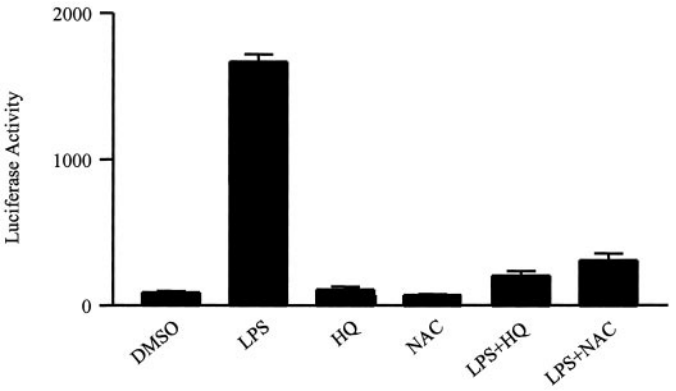

Fig. 11. Inhibition of TNFα transcription by NAC. Macrophage cells stably transfected with the TNFα luciferase reporter plasmid were grown in 48-well plates and treated with HQ (100 μM) or NAC (20 μM) for 1 h followed by LPS for 5 h. Cell lysate was prepared and assayed for luciferase activity as described for Fig. 2.

of Nrf2 activation, it was suggested that phenolic antioxidants bind to Keap1, a cytosolic protein partner of Nrf2, through binding to the thiol groups of Keap1 (Dinkova-Kostova et al., 2002). This antioxidant-thiol group binding triggers the release of Nrf2 from the Keap1/Nrf2 complex and subsequent translocation of Nrf2 into the nucleus. In the current study, we found that phenolic antioxidants do not interfere with NF-κB binding to its DNA sequence directly. Moreover, the antioxidants exhibit certain structure-activity relationships in the inhibition of NF-κB functions. Thus, HQ, tBHQ, catechol, and *p*-BQ, which can undergo reduction-oxidation cycling, are strong inhibitors of NF-κB, whereas resorcinol, benzene, and phenol, which do not readily undergo redox cycling, are inactive. Large molecules such as BNF that can be metabolized to HQ-like chemicals inhibit NF-κB, whereas metabolically stable TCDD fails to inhibit. Therefore, inhibition of NF-κB by phenolic antioxidants correlates with their redox capabilities and suggest a redox-sensitive protein factor as its target in the inhibition. It is possible that phenolic antioxidants activate an Nrf2-like factor through interaction with its thiol groups similarly to the activation of Nrf2, which then inhibits NF-κB function. Alternatively, the antioxidants activate a Ref-1 like factor through interaction with its redox functional groups and interfere with the redox reactions between Ref-1 and NF-κB (Nishi et al., 2002). Cloning of the target protein can reveal insights into the interaction of the antioxidants with its receptors and inhibition of inflammatory cytokine expression at a molecular level by the antioxidants. Furthermore, these studies may provide new approaches for developing effective preventive/therapeutic anti-inflammatory agents.

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