THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 4, Issue of January 25, pp. 2477–2484, 2002
Printed in U.S.A.

Chemoprotection by Phenolic Antioxidants

INHIBITION OF TUMOR NECROSIS FACTOR α INDUCTION IN MACROPHAGES*

Received for publication, July 16, 2001, and in revised form, September 5, 2001
Published, JBC Papers in Press, November 2, 2001, DOI 10.1074/jbc.M106685200

Qiang Ma‡ and Krista Kinneer

From the Receptor Biology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, West Virginia 26505

Phenolic antioxidants exhibit anti-inflammatory activity in protection against chemical toxicity and cancer. To investigate the molecular mechanism of anti-inflammation, we analyzed the regulation of tumor necrosis factor α (TNF-α) expression in macrophages, a key step in inflammation, by the antioxidants. Whereas lipopolysaccharide (LPS), an inflammatory inducer, stimulates rapid synthesis of TNF-α protein, phenolic antioxidants, exemplified by *tert*-butyl hydroquinone and 1,4-dihydroquinone, block LPS-induced production of TNF-α protein in a time- and dose-dependent manner. Inhibition of TNF-α induction correlates with the capacity of the antioxidants to undergo oxidation-reduction cycling, implicating oxidative signaling in the inhibition. The antioxidants blocked LPS-induced increase of the steady-state mRNA of TNF-α but did not affect the half-life of the mRNA. Electrophoretic mobility shift assay reveals a total inhibition of LPS-induced formation of nuclear factor κB·DNA binding complexes by phenolic antioxidants. Finally, 1,4-dihydroquinone blocks the induction of TNF-α target genes interleukin 1β and interleukin 6 at both mRNA and protein levels. Our findings demonstrate that phenolic antioxidants potently inhibit signal-induced TNF-α transcription and suggest a mechanism of anti-inflammation by the antioxidants through control of cytokine induction during inflammation.

A variety of chemicals can protect animals against the toxic and carcinogenic effects of chemicals and microbes (1, 2), a phenomenon termed chemoprotection. Many protective chemicals manifest antioxidant activities (3, 4). Synthetic phenolic antioxidants, such as tBHQ,[1] are commonly used as food preservatives due to their potent anti-lipid peroxidation activity (5, 6), whereas natural phenolic antioxidants exist in a wide range of edible plants and animal tissues (7). As such, human beings consume appreciable amounts of phenolic antioxidants from dietary sources. Phenolic antioxidants exhibit anticarcinogenic, anti-inflammatory, antiatherosclerotic, and antidiabetic functions in animals (8–14). The broad spectrum of the biological functions of the antioxidants suggests the existence of multiple molecular targets that mediate diverse responses to the chemicals in cells and whole animals. Identifying the target molecules can facilitate the design of better preventive and/or therapeutic agents for protection against pathological responses to various occupational, environmental, or therapeutic chemicals as well as against certain conditions associated with aging, such as cancer and chronic inflammatory and cardiovascular diseases.

Current understanding of the mechanism of action by phenolic antioxidants comes mostly from studies on the induction of phase II detoxification enzymes by the antioxidants. Phase II enzymes, such as NAD(P)H:quinone oxidoreductase (15, 16) and glutathione *S*-transferase (17), metabolize chemicals to water-soluble products through reduction and conjugation reactions. Induction of phase II enzymes alters the metabolic fate of chemicals by enhancing their metabolism and excretion. Induction of glutathione *S*-transferase A1 and NAD(P)H:quinone oxidoreductase by phenolic antioxidants requires an ARE located in the enhancers of the genes (18, 19) and is mediated through an Nrf2-dependent signal transduction (20). Nrf2 is a member of the Cap'n Colar bZip family of transcription factors (21) that forms a cytoplasmic complex with Keap1 (22). Upon exposure to phenolic chemicals, Nrf2 dissociates from Keap1, translocates into the nucleus, dimerizes with an as-yet-unidentified partner transcription factor, and mediates the transcription of target genes through ARE-dependent transcription. Studies on Nrf2-null mice revealed that loss of expression of Nrf2 markedly enhances the susceptibility of the mice to toxicity by acetaminophen (23) or cancer by benzo(*a*)pyrene (24), which is attributable to diminished detoxification of the chemicals in the Nrf2-null mice. Thus, induction of phase II enzymes through Nrf2 can account for chemoprotection by phenolic antioxidants against certain carcinogens and toxins by altering the pharmacokinetic fate of the chemicals. However, such mechanisms do not readily explain the anti-inflammatory function of the antioxidants, which is largely unaddressed at present.

The importance of inflammation in the pathological responses to chemicals of endogenous or foreign origin is well recognized because more diseases, ranging from silicosis, asthma, and chronic hepatitis to idiopathic pulmonary fibrosis, Alzheimer's disease, and certain forms of neoplasia, manifest an inflammatory component that either causes or increases the severity of the diseases (25–29). Inflammation, in general, is a directed tissue response to tissue damage caused by noxious and injurious stimuli. Inflammation serves to isolate the injured tissue, inactivate the toxic stimuli, and repair the tissue damage. In many cases, however, inflammation can lead to

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: CDC/NIOSH, Mailstop 3014, 1095 Willowdale Rd., Morgantown, WV 26505. Fax: 304-285-5708; E-mail: qam1@cdc.gov.

[1] The abbreviations used are: tBHQ, *tert*-butylhydroquinone; HQ, 1,4-dihydroquinone; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; TNF-α, tumor necrosis factor α; IL, interleukin; HO-1, heme oxygenase-1; Nrf, NfE2-related factor; ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor κB; AP, activator protein; ROS, reactive oxygen species; ARE, antioxidant response element; NF-AT, nuclear factor of activated T cells; TAK, TGF-β-activating kinase; MEKK, MAPK kinase kinase.

further tissue damage and the development of inflammatory diseases. Thus, inhibition of inflammation constitutes an effective mechanism of chemoprotection against chemical-induced inflammatory lesions or disease conditions.

Inflammatory stimuli induce cytokines, which mediate tissue responses in different phases of inflammation in a sequential and concerted manner (28). Regulation of cytokine induction serves as a key mechanism of inflammation control by endogenous or exogenous chemicals. TNF-α is produced in the early phase of inflammation in cells of reticuloendothelial origin, such as macrophages. TNF-α mediates early-stage responses of inflammation by regulating the production of other cytokines, including IL-1β and IL-6. Cumulative evidence indicates that abnormalities in the production or function of TNF-α play essential roles in many inflammatory lesions (25, 29, 30). For instance, exposure to the bacterial endotoxin LPS can cause inflammatory liver damage and septic shock. Induction of TNF-α by LPS is a key step in the response; administration of TNF-α mimics the response, whereas blocking the function of TNF-α by using neutralizing agents of TNF-α protects animals from the LPS-induced lesions.

The pivotal role of TNF-α in inflammation and the potent anti-inflammatory activity of phenolic antioxidants raise the question of whether the induction of TNF-α during inflammation serves as a target of anti-inflammation by phenolic antioxidants. In this study, we tested this hypothesis by examining the effect of phenolic antioxidants on the induction of TNF-α by LPS in macrophage cells. Our data reveal that phenolic antioxidants block LPS-induced expression of TNF-α both time- and dose-dependently; the inhibition occurs at a transcriptional level and involves inhibition of NF-κB activation, the major regulator of TNF-α transcription in macrophage cells. To our knowledge, this study is the first report of inhibition of signal-induced TNF-α production by phenolic chemicals. Our findings provide new insights into the mechanism of chemoprotection against inflammatory diseases by phenolic antioxidants.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and other general molecular biology reagents were purchased from New England Biolabs (Beverly, MA), Roche Molecular Biochemicals, and Promega (Madison, WI). Radioactive compounds were from Amersham Biosciences, Inc. LPS, tBHQ, HQ, catechol, resorcinol, *para*-benzoquinone, α-naphthoflavone, and NAC were purchased from Sigma. Cell culture materials were from Invitrogen. Reagents for Northern blotting and ELISA are as described below.

Cell Culture—The mouse monocyte-macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA). The macrophage cells were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum at 5% CO_2 and 37 °C. The cells were treated with LPS, phenolic antioxidants, or other reagents as described in the figure legends; Me_2SO or water was used as the solvent control for the antioxidants and LPS, respectively.

Northern Blot Analysis—cDNA fragments for mouse TNF-α, IL-1β, IL-6, and HO-1 were synthesized by PCR amplification of the corresponding cDNA templates from CLONTECH 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 digoxygenin-UTP using a digoxygenin labeling kit (Roche Molecular Biochemicals). Total RNA was isolated from cells using the RNeasy kit (Qiagen, Valencia, CA). Total RNA (5 μg 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 digoxygenin-labeled riboprobe at 68 °C. Signals were visualized by chemiluminescence using a digoxygenin RNA detection kit with CDP star as a substrate (Roche Molecular Biochemicals). Quantitation of the blotting result was performed by using the ImageQuaNT program (Molecular Dynamics, San Jose, CA). All data were corrected for loading variations by comparing the amount of actin of each sample analyzed.

ELISA—The macrophage cells were plated in a 48-well cell culture dish at a density of 2×10^5 cells/well in 500 μl of medium. The cells were grown at 37 °C for 24 h before treatment. In a typical experiment, the cells were treated with a phenolic antioxidant or Me_2SO for 1 h, followed by stimulation with LPS for 5 h. The medium was collected and assayed for TNF-α, IL-1β, or IL-6 by using ELISA kits specific for each of the murine cytokines from R&D Systems (Minneapolis, MN). For TNF-α and IL-6, the medium was diluted with water at a 1:20 or 1:10 ratio, respectively; 50 μl of each diluted sample was used for ELISA. Quantitation of the ELISA results was performed using a Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA) set to 450 nm and corrected for absorbance at 570 nm according to the manufacturer's instructions.

Preparation of Nuclear Extracts—The nuclear extracts were prepared with a three-step procedure as described elsewhere (31). Briefly, the cells were grown in a 100-mm dish to near confluence. After treatment, the cells were collected with a rubber policeman, washed with 1× phosphate-buffered saline, and lysed in 500 μl of a 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. The 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 (lysis buffer without Nonidet P-40). In the final step, the nuclei were treated with an extraction buffer, which contains 500 mM KCl, 10% glycerol, and several other reagents as in the lysis buffer. The nuclei/extraction buffer mixture was frozen at −80 °C and then thawed on ice and centrifuged at 14,000 rpm for 5 min. The supernatant was collected as the nuclear extract and stored at −80 °C for further use.

Electrophoretic Mobility Shift Assay—A NF-κB DNA-binding sequence (5′-GATTTTCCCATGAGTCT-3′) was used to synthesize oligonucleotides as the NF-κB binding probe (31). The complementary single-strand oligomers were denatured at 80 °C for 5 min and annealed at room temperature. An activator protein (AP)-1 DNA-binding sequence derived from the AP-1 binding site in the collagenase enhancer was used as a nonspecific competitor probe (31). 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 bovine serum albumin, and 12 μl of a reaction buffer (12% glycerol, 24 mM HEPES, pH 7.9, 8 mM Tris-HCl, 2 mM EDTA, and 1 mM 1,4-dithiothreitol). The mixture was incubated on ice for 10–20 min, followed by the addition of 4×10^4 counts/min of a ^{32}P-labeled oligonucleotide probe; the incubation was continued at room temperature for 20 min. For competition experiments, 100 ng of cold (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, NF-AT, 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 (pre-run at 170 V for 30 min with 0.5× Tris-boric acid-EDTA buffer) at 200 V for 90 min and visualized by exposure to films.

H_2O_2 Detection—Macrophage cells were plated in a 6-well dish at 5×10^5 cells/well 16 h before treatment. 2′,7′-Dichlorofluorescin diacetate (5 μM; Sigma) was used to stain cells for H_2O_2 for 45 min before the treatment was completed. The cells were washed in phosphate-buffered saline and harvested in 1 ml of phosphate-buffered saline using a rubber policeman for quantitation of H_2O_2 by fluorescence-activated cell-sorting analysis (32). 2′,7′-Dichlorofluorescin diacetate was excited at 488 nm and detected at 525 nm.

Other Methods—Lactate dehydrogenase activity in cell culture medium was measured by using a lactate dehydrogenase kit from Roche Diagnostics Corp. (Indianapolis, IN) according to the manufacturer's instructions. The lactate dehydrogenase activity was measured spectrophotometrically by using the COBAS chemistry system FARA (Roche Diagnostics Corp.) and expressed in units/liter. Protein concentration was determined by using the Bradford method (33) with reagents from Bio-Rad.

RESULTS

Phenolic Antioxidants Block LPS-induced TNF-α Production—To analyze the mechanism of anti-inflammation by phe-

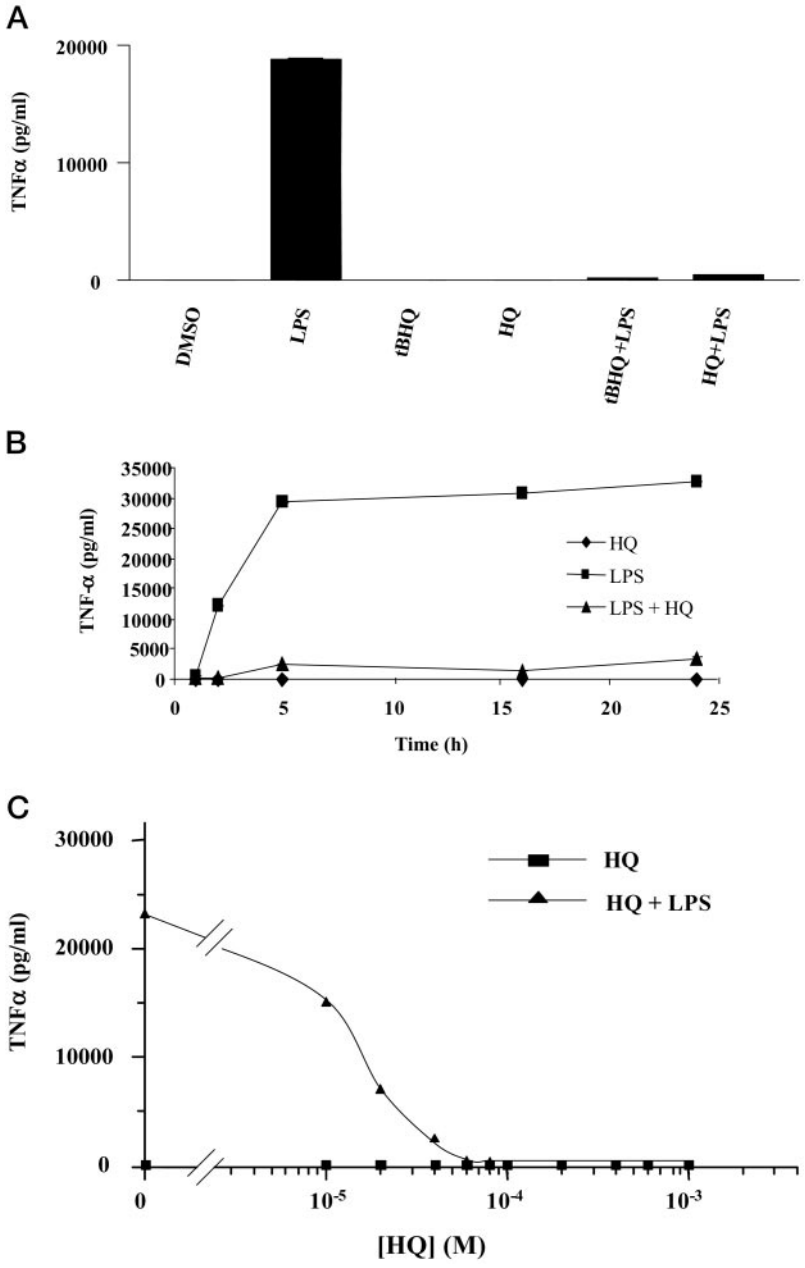

FIG. 1. **Inhibition of TNF-α induction by tBHQ and HQ.** Macrophage cells were grown overnight in a 48-well dish. *A*, inhibition by tBHQ or HQ. Cells were treated with tBHQ or HQ at 90 μM for 1 h and then treated with LPS (1 μg/ml) for 5 h. *B*, time curve of inhibition. The cells were treated with HQ (90 μM) for 1 h and then stimulated with LPS for the indicated time periods. *C*, dose curve of inhibition. Cells were treated with HQ at the indicated concentrations for 1 h, followed by LPS stimulation for 5 h. The culture medium was collected and assayed for the TNF-α protein by ELISA as described under "Experimental Procedures." The results represent the means and S.D. of three treatments.

nolic antioxidants, we examined the effect of the chemicals on signal-induced production of TNF-α in macrophage cells. tBHQ and HQ, two prototypical phenolic antioxidants, were chosen as testing agents in the study. Little TNF-α protein was detected by ELISA specific for mouse TNF-α in controls (Fig. 1*A*, *DMSO*). tBHQ or HQ alone does not affect TNF-α production (Fig. 1*A*, *tBHQ* and *HQ*). Upon exposure to LPS, large quantities of TNF-α were produced, and the induction was inhibited by tBHQ or HQ (Fig. 1*A*, compare *LPS* with *tBHQ+LPS* and *HQ+LPS*). Thus, tBHQ or HQ totally blocks LPS-induced production of TNF-α protein in macrophages.

Next, we analyzed the time- and concentration-response curves. As shown in Fig. 1*B*, LPS induces a 5-fold increase in TNF-α production within 1 h after treatment, reaching a maximum of ~300-fold at 5 h. The maximum induction is maintained for over 24 h. HQ blocks the induction of TNF-α by LPS throughout the testing time course. These results suggest that inhibition of TNF-α production by HQ occurs early and is therefore likely to be a primary response; furthermore, the inhibition is complete and long-lasting. HQ inhibits LPS-induced TNF-α production dose-dependently (Fig. 1*C*). The IC_{50} value of the inhibition by HQ is ~15 μM, which is similar to the potency of HQ for other biological responses, such as the induction of phase II drug-metabolizing enzymes (34). Therefore, the inhibition by HQ is potent and may be mediated through a mechanism analogous to the induction of phase II enzymes by phenolic antioxidants. tBHQ inhibits LPS-induced TNF-α production both time- and dose-dependently in a manner similar to that of HQ (data not shown).

Role of Oxidative Signaling—Certain phenolic chemicals undergo oxidation-reduction cycling in cells. We tested whether the inhibition of TNF-α production by phenolic chemicals involves redox cycling of the chemicals. HQ, catechol, and resorcinol are structural analogs of diphenols, which differ with regard to the positions of hydroxyl groups on the aromatic ring. HQ and catechol undergo facile reversible oxidations to the corresponding quinones, whereas resorcinol can not. HQ and catechol strongly inhibit LPS-induced TNF-α production; in contrast, resorcinol is inactive for the inhibition (Fig. 2*A*). To exclude cell toxicity as a possible cause of the inhibition, we

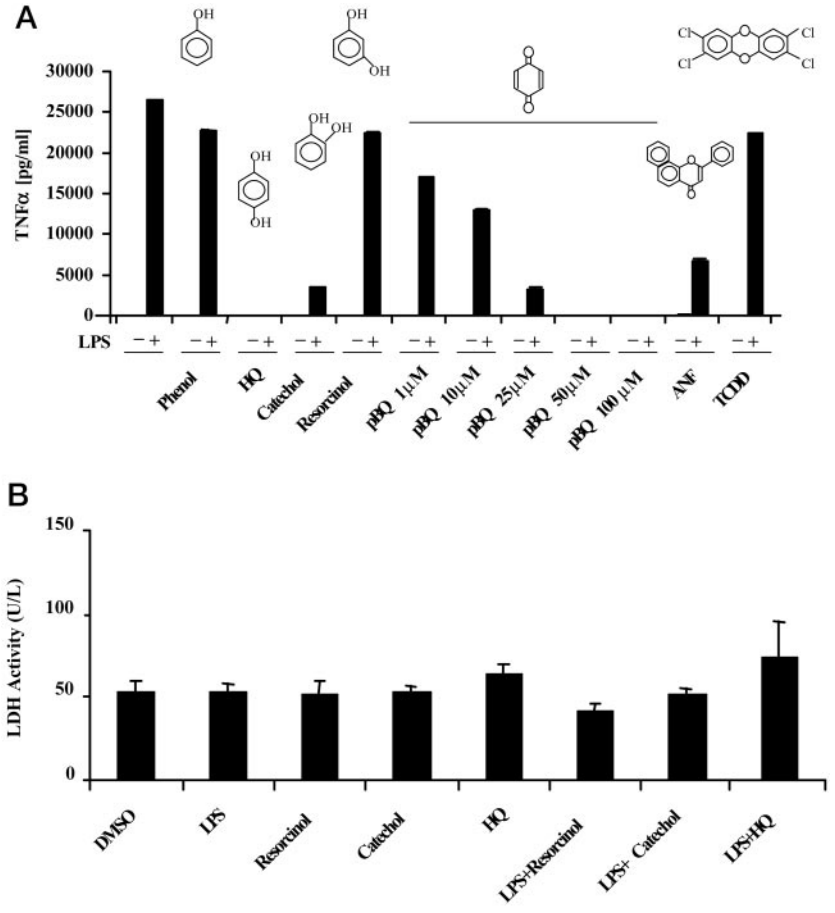

FIG. 2. **Inhibition of TNF-α induction by HQ analogs.** Macrophage cells were pretreated with HQ, catechol, and resorcinol at 100 μM or with phenol (100 μM), *para*-benzoquinone (at the concentrations indicated), α-naphthoflavone (10 μM), or TCDD (1 nM) for 1 h and then stimulated with LPS (1 μg/ml) for 5 h. The culture medium was assayed for TNF-α by using ELISA (*A*) or assayed for lactate dehydrogenase by using the lactate dehydrogenase kit from Roche Diagnostics Corp. (*B*) as described under "Experimental Procedures." The means and S.D. of three treatments are presented.

measured lactate dehydrogenase activity in the medium, which reflects cellular damage. As shown in Fig. 2*B*, LPS, HQ, catechol, and resorcinol did not cause a significant increase in lactate dehydrogenase activity, given either alone or in combination. These data demonstrate that the antioxidants do not cause marked damage to the cells at the concentrations tested. Several related chemicals were similarly tested for inhibition of TNF-α induction (Fig. 2*A*). Phenol, which has a single hydroxyl group, does not inhibit TNF-α production at 100 μM. *para*-Benzoquinone, which is reduced to hydroquinone through a number of reductive pathways (15), exhibits dose-dependent inhibition with a potency similar to that of HQ (IC_{50} = ~15 μM). α-Naphthoflavone (ANF), a polycyclic aromatic hydrocarbon that is metabolized to oxidizable products in cells (34), inhibits TNF-α production, whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is metabolically stable, does not. These data demonstrate that inhibition of LPS-induced TNF-α production by phenolic antioxidants correlates with the oxidation-reduction capability of the chemicals.

LPS stimulates the generation of reactive oxygen species (ROS), such as H_2O_2, in macrophages (35). We tested whether phenolic antioxidants affect LPS-induced ROS production. As shown in Fig. 3*A*, LPS induces a marked increase in H_2O_2; tBHQ or HQ blocks LPS-induced H_2O_2 production. Resorcinol does not affect H_2O_2 induction. This observation can be explained in two ways: first, the LPS-induced ROS production and its inhibition by the antioxidants are unrelated to TNF-α induction and inhibition; alternatively, they are integral to the LPS response and the antioxidant action. To test the possibilities, we examined whether ROS inhibitors block TNF-α induction. NAC, which inhibits LPS-induced H_2O_2 production in macrophages (35), inhibits TNF-α induction in a manner similar to that of HQ (Fig. 3*B*). Together, these findings support the notion that oxidative signaling contributes to the inhibition of LPS responses by phenolic antioxidants.

Inhibition of TNF-α Production Is Transcriptional—To analyze the mechanism of the inhibition, we measured the mRNA expression of TNF-α. The macrophage cells express a barely detectable level of TNF-α mRNA in the absence of LPS stimulation (Fig. 4, *A* and *B*, *lane 1*). LPS induces a ~50-fold increase in the expression of TNF-α mRNA (Fig. 4, *A* and *B*, *lane 2*). HQ (100 μM) alone does not affect TNF-α mRNA expression (Fig. 4, *A* and *B*, *lane 3*), but it blocks LPS-induced expression of TNF-α mRNA dose-dependently, with an IC_{50} of ~15–20 μM (Fig. 4, *A* and *B*, *lanes 4–7*), which is similar to the IC_{50} measured by ELISA (Fig. 1*C*). On the contrary, resorcinol, given either alone or with LPS (Fig. 4, *A* and *B*, *lanes 8* and *9*), does not affect the expression of TNF-α mRNA. Thus, inhibition of TNF-α mRNA expression by phenolic antioxidants correlates with the inhibition of TNF-α protein production. To exclude the possibility that HQ inhibits gene transcription in general, we tested whether HQ induces HO-1, a known inducible gene by phenolic antioxidants (36), under the same condition used for TNF-α analysis. HO-1 is expressed at a low level in macrophage cells (Fig. 4, *A* and *C*, *lane 1*). HQ induces HO-1 to a level ~6-fold higher than control at 1 μM (Fig. 4, *A* and *C*, *lane 4*) and to a level >10-fold higher than control at 25, 50, and 100 μM (Fig. 4, *A* and *C*, *lanes 3* and *5–7*). LPS does not induce HO-1 or affect HO-1 induction by HQ. Resorcinol induces HO-1 to a minor extent (Fig. 4, *A* and *C*, *lanes 8* and *9*). Thus, HQ maintains its capacity to induce HO-1 under the same condition it inhibits TNF-α induction by LPS.

Inhibition of LPS-induced increase in TNF-α mRNA can be due to a decrease in the synthesis of TNF-α mRNA, an increase in the degradation of TNF-α mRNA, or both. In Fig. 5, we measured the half-life ($t_{1/2}$) of LPS-induced TNF-α mRNA in

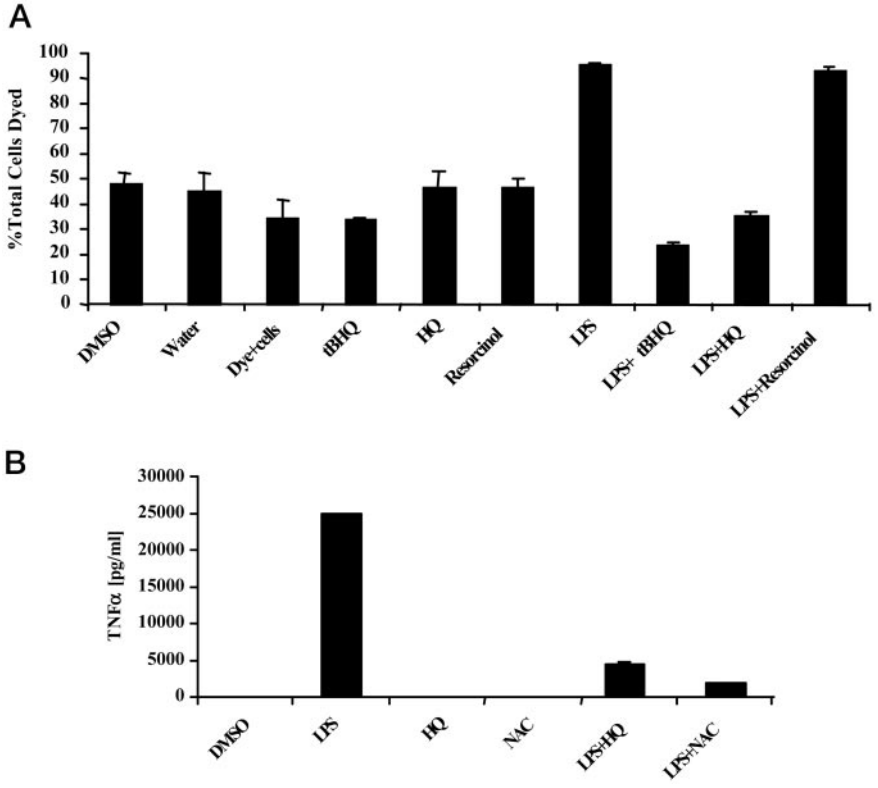

FIG. 3. **Role of ROS.** *A*, quantitation of H_2O_2. Cells were plated in 6-well dishes, treated with antioxidants (100 μM each) and LPS (1 μg/ml), stained with 2′,7′-dichlorofluorescin diacetate, and analyzed for H_2O_2 by fluorescence-activated cell-sorting analysis as described under "Experimental Procedures." *B*, inhibition by NAC. Cells were plated in 48-well dishes, treated with chemicals (NAC, 20 μM; HQ, 100 μM; LPS, 1 μg/ml), and analyzed for TNF-α production by ELISA. The means and S.D. of three treatments are presented.

the absence or presence of HQ. The data indicate that HQ does not affect the stability of LPS-induced TNF-α mRNA in either the absence or presence of actinomycin D, an inhibitor of RNA synthesis (Fig. 5*A*). The $t_{1/2}$ of LPS-induced TNF-α mRNA is ~1.2 h, whereas the $t_{1/2}$ in the presence of HQ is ~1.4 h (Fig. 5*B*). These data indicate that HQ inhibits LPS-induced expression of TNF-α through inhibition of the transcription of the gene.

Inhibition of NF-κB Activation—Previous studies have established that NF-κB is the major transcription factor that mediates the induction of TNF-α by LPS in macrophage cells (37). Therefore, we tested whether phenolic antioxidants inhibit the LPS-induced activation of NF-κB by using an electrophoretic mobility shift assay, which measures the formation of nuclear NF-κB dimers that bind to the NF-κB DNA response element, as a mechanism of inhibition of TNF-α transcription. As shown in Fig. 6, LPS induces the formation of two NF-κB·DNA complexes (compare *lanes 1* and *2*). The LPS-induced NF-κB bands are specific for NF-κB binding element because unlabeled NF-κB DNA probe can compete off the binding of NF-κB to labeled NF-κB DNA probe (compare *lanes 7* and *8*), whereas the AP-1 DNA probe, which is the DNA binding element for AP-1 proteins, fails to compete with the labeled NF-κB probe for binding (*lane 9*). The two NF-κB/DNA bands represent the p65/p50 heterodimer and the p50 homodimer of NF-κB, respectively, because antibodies against p50 supershift both bands (*lane 10*), whereas antibodies against p65 disrupt the upper band (p65/p50; *lane 11*), but not the p50 homodimer band. However, antibodies against c-Jun, NF-AT, or c-Fos have no effect on NF-κB band formation (*lanes 12–14*). Treatment with HQ alone does not induce NF-κB activation (*lane 3*), but it completely blocks LPS-induced formation of NF-κB dimer·DNA complexes (*lane 4*). In contrast, resorcinol exhibits no obvious effect on NF-κB activation in the absence or presence of LPS (*lanes 5* and *6*). These data suggest that HQ inhibits LPS-induced activation of NF-κB as a mechanism to inhibit TNF-α induction by LPS.

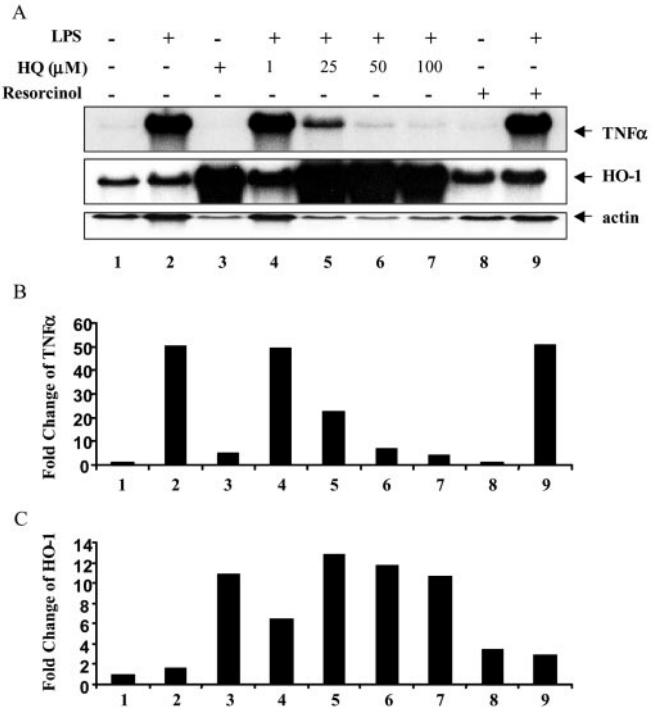

FIG. 4. **Inhibition of TNF-α mRNA induction.** Macrophage cells grown in 100-mm dishes were treated with HQ for 1 h, followed by stimulation with LPS (1 μg/ml) for 3 h. The concentrations of HQ used for *lanes 3–7* were: 100, 1, 25, 50, and 100 μM, respectively. Resorcinol (100 μM) was used as a control. *A*, total RNA was prepared, and the blot was analyzed with riboprobes for TNF-α, HO-1, and actin as described under "Experimental Procedures." Actin was used as a control for equal loading, whereas HO-1 was used to show the inductive activities of HQ and resorcinol. *B*, quantitation of Northern blot results for TNF-α. *C*, quantitation of Northern blot results for HO-1. The results were quantitated by using ImageQuaNT software (Molecular Dynamics) and corrected for loading variations by comparing the amount of actin in each sample.

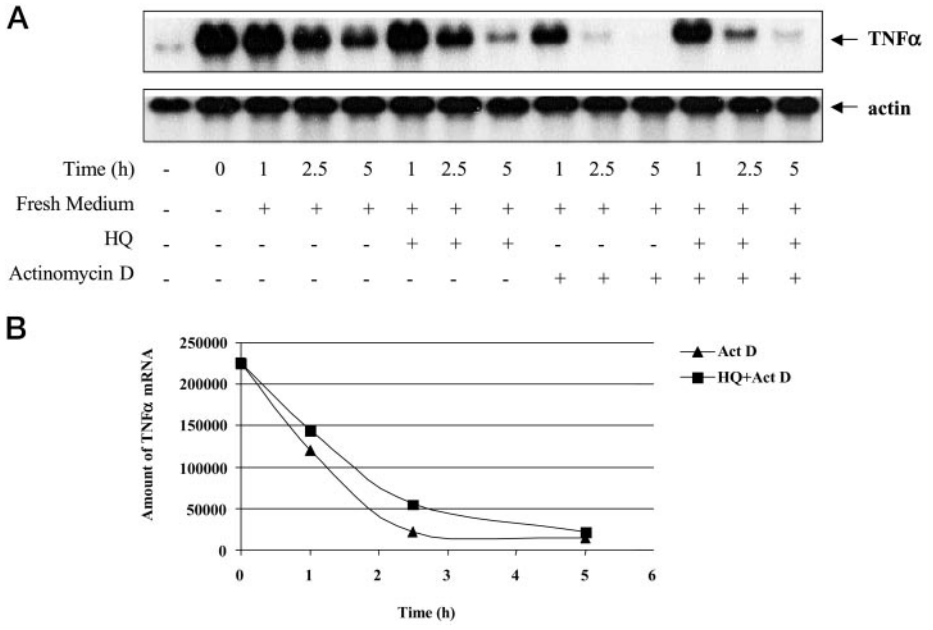

FIG. 5. **Effect of HQ on TNF-α mRNA stability.** Macrophage cells were treated with LPS (1 μg/ml) for 3 h. The cells were washed three times with phosphate-buffered saline and then cultured in fresh medium in the absence or presence of HQ (100 μM), actinomycin D (2 μg/ml), or both for the indicated time periods. *A*, total RNA was prepared, and the blot was analyzed for TNF-α and actin mRNAs (5 μg/lane). *B*, quantitation of Northern blotting. The results were quantitated by using ImageQuaNT software (Molecular Dynamics) and corrected for loading variations by comparing the amount of actin in each sample.

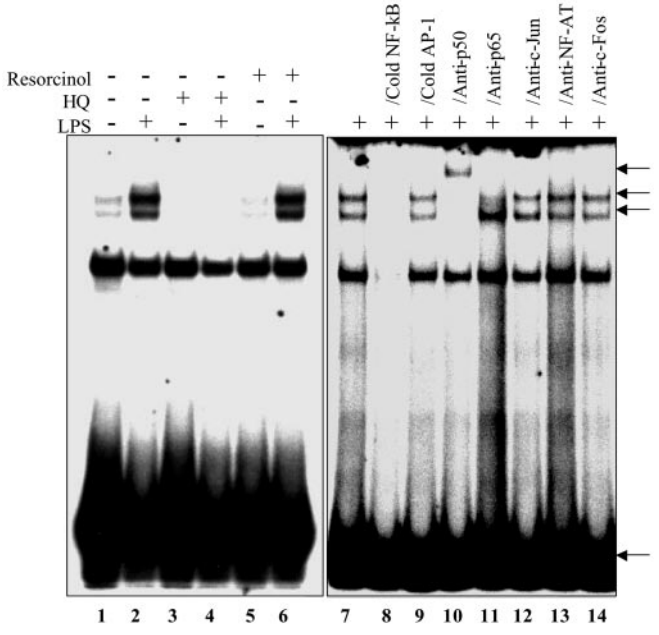

FIG. 6. **Inhibition of NF-κB·DNA binding complex formation.** Macrophage cells grown in 100-mm dishes were treated with HQ (100 μM) or resorcinol (100 μM) for 1 h, followed by stimulation with LPS (1 μg/ml) for 3 h. Nuclear extracts were prepared from the cells and analyzed by electrophoretic mobility shift assay for (*A*) the formation of NF-κB·DNA binding complexes and (*B*) the specificity of NF-κB/DNA binding as described under "Experimental Procedures." The specificities of the NF-κB·DNA binding complexes were tested by (1) competition with cold NF-κB DNA probe (100 ng) or AP-1 DNA probe (100 ng) and (2) supershift with antibodies specific for p50 or p65 of NF-κB or c-Jun, NF-AT, or c-Fos, as indicated (2 μg antibodies/reaction). The *arrows* (from *top* to *bottom*) indicate the supershifted bands, the p65/p50 heterodimer·DNA complex, the p50 homodimer·DNA complex, and the labeled free probes at the bottom of the gels.

Inhibition of TNF-α Target Gene Expression—Because TNF-α mediates the production of many other cytokines during inflammation (29), in particular, the production of IL-1β and IL-6, we tested whether the blocking of TNF-α production by phenolic antioxidants has an effect on the production of TNF-α target genes. Fig. 7 shows that both IL-1β (Fig. 7*A*) and IL-6 (Fig. 7*B*) are expressed at very low levels in control cells. LPS induces large increases in the production of both proteins at 5 h after treatment, which continues to increase for up to 24 h during the testing period. HQ alone does not affect the production of both proteins but totally blocks LPS-induced production of IL-1β and IL-6 at both the 5 and 24 h time points.

We tested whether the inhibition of IL-1β and IL-6 is due to the inhibition of transcription of the genes. As shown in Fig. 8, LPS induces a marked increase in the mRNA levels of IL-1β and IL-6. HQ blocks the LPS-induced expression of IL-1β and IL-6 mRNAs, whereas resorcinol has no effect on LPS-induced mRNA expression of the genes. The inhibition of IL-1β and IL-6 mRNA expression is not due to inhibition of gene transcription in general because HQ induces HO-1 mRNA expression under the same condition in which it inhibits IL-1β and IL-6 mRNA expression. Together, these data suggest that HQ inhibits LPS-induced gene transcription downstream of TNF-α by blocking TNF-α expression and thereby suppresses broad aspects of the inflammatory process.

DISCUSSION

Protection against chemical-induced toxicity and cancer by using synthetic or natural chemicals, which is termed chemoprotection, constitutes an economical and promising means of disease control and prevention (2). The best-studied examples of chemoprotection exploit the anticancer functions of various protective chemicals (also termed chemoprevention) (1, 2). It is known that certain chemicals manifest anti-inflammatory activities in chemoprotection. Because of the prominent roles of inflammation in the etiology and pathogenesis of many disease conditions and chemical toxicities, anti-inflammation by chemoprotective agents represents new opportunities for chemoprotection. The mechanism of anti-inflammation by chemoprotective antioxidants is largely unclear at present. In this study, we examined the regulation of TNF-α induction, a key event in inflammatory responses, by phenolic antioxidants as a mechanism of anti-inflammation. Our data revealed that phenolic antioxidants, such as tBHQ and HQ, potently inhibit signal-induced production of TNF-α and its target genes IL-1β and IL-6 in macrophage cells.

TNF-α was initially identified as a product of lymphocytes and macrophages that caused the lysis of certain types of cells, especially tumor cells. Studies on TNF-α and its receptors have unveiled important aspects of host defense, inflammation, apoptosis, autoimmunity, cancer, organogenesis, and chemical tox-

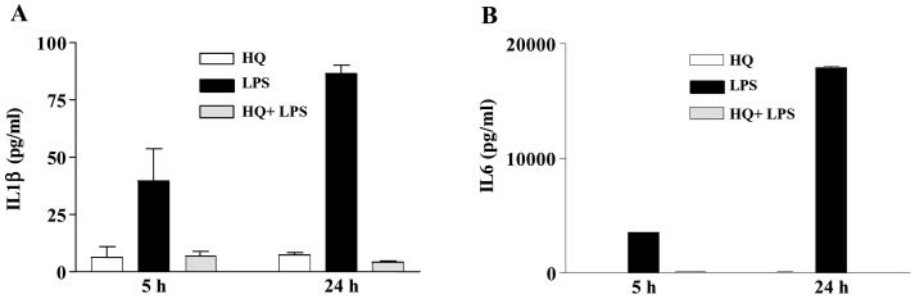

FIG. 7. **Inhibition of IL-1β and IL-6 protein production.** Macrophage cells were grown in 48-well dishes and treated with HQ (100 μM) for 1 h, followed by stimulation with LPS (1 μg/ml) for 5 or 24 h. The culture medium was collected and assayed for IL-1β (*A*) or IL-6 (*B*) using ELISA kits specific for murine IL-1β or IL-6, respectively. The results represent the means and S.D. of three treatments.

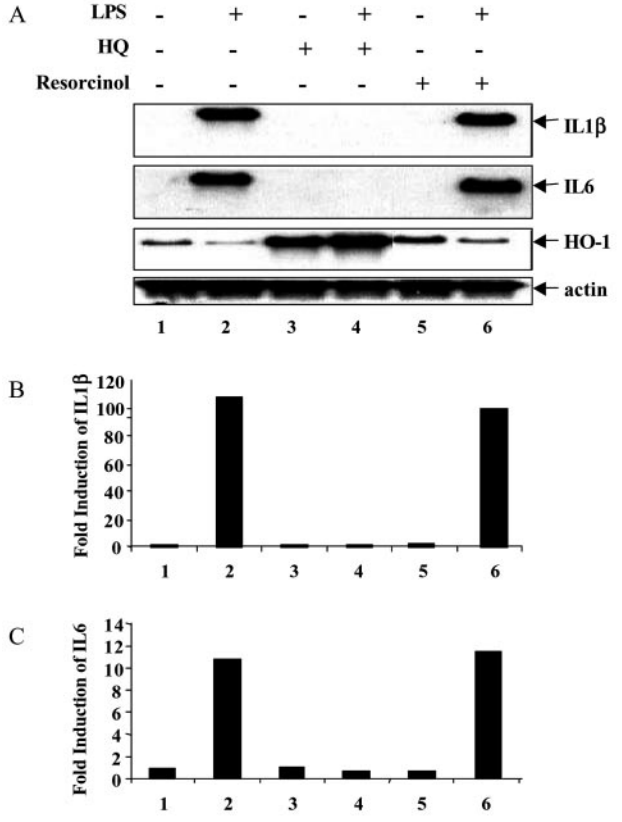

FIG. 8. **Inhibition of IL-1β and IL-6 mRNA induction.** Macrophage cells were treated with HQ or resorcinol at 100 μM for 1 h and then stimulated with LPS (1 μg/ml) for 5 h. *A*, total RNA was prepared and analyzed by Northern blotting for IL-1β, IL-6, HO-1, or actin, respectively (5 μg/lane). *B*, quantitation of Northern blot results for IL-1β. *C*, quantitation of Northern blot results for IL-6. Northern blot results were quantitated as described in the Fig. 4 legend.

icity (29, 38). TNF-α plays a major role in regulating inflammation, mostly through the induction of inflammatory cytokines, including IL-1 (IL-1α and IL-1β), IL-6, IL-8, the macrophage inflammatory protein-2, granulocyte-macrophage colony-stimulating factor, and adhesion molecules. Because TNF-α is the main mediator for a number of inflammatory toxic responses to chemicals, it represents a promising target for the prevention of chemical-induced inflammatory toxicity. In congruence with this view, we found that phenolic antioxidants, which exhibit strong anti-inflammatory activities in animals, are potent inhibitors of signal-induced TNF-α production. These findings provide evidence that regulation of TNF-α production serves as an effective target of chemoprotection.

TNF-α is induced by many external stimuli, the strongest of which is the bacterial endotoxin LPS. Induction by LPS is mediated through complex signal transduction pathways involving both transcriptional and posttranscriptional mechanisms (39). LPS binds the soluble LPS-binding protein, and the complex binds CD14, a monocyte/macrophage receptor molecule. CD14 presents the LPS·LPS-binding protein complex to the LPS receptor TLR4, a member of the Toll family of receptors, which play important roles in innate immunity by discriminating "self" from pathogen-derived ligands (also termed pathogen-associated molecular patterns) (40). Signals originating in the LPS-triggered TLR4 receptor activate several signaling pathways, which involve the Toll adaptor MyD88 and the mitogen-activated protein 3-kinases TAK-1 and MEKK-1. TAK-1 and MEKK-1 activate IκB kinase, which phosphorylates IκBα, a component of the cytoplasmic NF-κB complex, leading to the degradation of IκBα through the ubiquitin-26S proteasome pathway and the activation of NF-κB. The activated NF-κB translocates into the nucleus, binds to the corresponding DNA element (k3) in the enhancer of TNF-α, and mediates transcription of the gene. The 3′-untranslated region of the TNF-α mRNA contains an AU-rich element that mediates posttranscriptional regulation of TNF-α production by LPS (41). In this scenario, LPS activates the c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase pathways, which relieve the AU-rich element-dependent posttranscriptional repression, resulting in enhanced TNF-α mRNA stability and translation. In addition, LPS activates Tpl2, a serine threonine kinase type of proto-oncogene, leading to activation of the extracellular signal-regulated kinase 1/2 pathway; the Tpl2/extracellular signal-regulated kinase-transduced signals specifically control TNF-α induction by regulating nucleocytoplasmic mRNA transport through a mechanism that targets the AU-rich element of the TNF-α mRNA (42). In this study, we found that LPS induces large increases in both the mRNA and protein of TNF-α, whereas phenolic antioxidants completely block the increases of TNF-α at both levels. Furthermore, the data revealed that phenolic antioxidants do not change the half-life of the TNF-α mRNA in the presence or absence of actinomycin D. These findings demonstrate that inhibition of LPS-induced TNF-α production by phenolic antioxidants occurs at a transcriptional level. Indeed, electrophoretic mobility shift assay experiments revealed that phenolic antioxidants block the formation of NF-κB·DNA binding complexes in the nucleus. Because NF-κB is the major mediator of LPS-induced TNF-α transcription, this finding suggests that phenolic antioxidants block the signal transduction of NF-κB as a main mechanism of inhibition of TNF-α transcription. However, this conclusion does not exclude other transcription factors that contribute to TNF-α regulation as a target of the antioxidants.

The mechanism of action of phenolic antioxidants is best understood for the induction of phase II detoxification enzymes, which is mediated through the Nrf2 and ARE-dependent signal transduction and transcription (19–22). It is currently unclear whether and how the Nrf2 pathway plays a role in the inhibition of NF-κB function by phenolic antioxidants. Because the inhibition of TNF-α production occurs early in the induction process, it is unlikely that the antioxidants exert the inhibition through Nrf2-mediated gene transcription. A recent study has revealed that a splice variant of Nrf1, a member of

the Cap'n Colar bZip proteins (43), is induced to mediate the induction of TNF-α in murine mast cells by immunoglobulin G plus antigen (IgG complexes) (44). The splice variant, after phosphorylation by casein kinase II, is capable of binding the k3 site of the TNF-α enhancer, which mediates the transcription of TNF-α in mast and macrophage cells. Heterologous expression of the variant protein reconstitutes the induction as revealed by reporter activity under the control of the TNF-α enhancer. By analogy with these findings, we envision that phenolic antioxidants activate Nrf2 or a related transcription factor that interacts with TNF-α enhancer in macrophages and thereby inhibits the function of NF-κB in TNF-α transcription. The observation that phenolic antioxidants block the formation of NF-κB·DNA complexes supports this notion. Alternatively, a non-Cap'n Colar bZip protein is activated by phenolic antioxidants and inhibits NF-κB function at k3 (45). Identification of protein factors that bind to the k3 sequence in the presence of both LPS and phenolic antioxidants may distinguish these possibilities. Finally, the antioxidants can block the signal transduction upstream of NF-κB activation and thereby inhibit NF-κB-mediated gene transcription. Biochemical analyses of NF-κB activation can lead to the identification of the molecular target(s) of phenolic antioxidants for inhibition of NF-κB activation.

Several lines of evidence support the notion that a common mechanism involving oxidative signaling mediates the activation of Nrf2 by phenolic antioxidants. First, phenolic chemicals are potent inhibitors of lipid peroxidation. Second, the antioxidants undergo redox cycling in cells; their capacity for enzyme induction correlates with the oxidation-reduction liability (46). Third, a variety of other chemicals with diverse structures can activate Nrf2/ARE-dependent induction of the enzymes (34). In this study, we found that inhibition of LPS-induced TNF-α transcription by phenolic antioxidants is also consistent with their capacity for oxidation-reduction cycling; thus, oxidizable tBHQ, HQ, and catechol are strong inhibitors, whereas resorcinol is inactive. Furthermore, tBHQ or HQ, but not resorcinol, inhibits LPS-stimulated production of H_2O_2. NAC, which inhibits ROS production by affecting the ratio of reduced *versus* oxidized glutathiones, blocks the TNF-α induction in a manner similar to that of HQ. The simplest explanation of these findings is that phenolic antioxidants activate an as-yet-unidentified Nrf2-like factor through a signaling molecule generated during the oxidation-reduction reactions of the antioxidants, which in turn inhibits NF-κB function. The interaction of the signaling molecule with its target proteins can be either direct, in which it binds to the target through thio groups, or indirect, in which it influences the functional groups of the protein by affecting the redox environment in cells. However, this conclusion does not exclude other signaling mechanisms in the action of phenolic antioxidants. Analyses of the molecular mechanism by which phenolic antioxidants activate target proteins will provide new insights into the mechanism of antioxidant action, regulation of TNF-α production, and control of NF-κB function in future studies.

Acknowledgments—We thank Dr. M. Luster for insightful suggestions, Dr. J. Ye for valuable comments, Drs. J. M. Matheson and S. Benkovic for internal review of the manuscript, and H. Michael for secretarial assistance.

REFERENCES

1. Wattenberg, L. W. (1985) *Cancer Res.* **45,** 1–8
2. Yang, C. S., Landau, J. M., Huang, M. T., and Newmark, H. L. (2001) *Annu. Rev. Nutr.* **21,** 381–406
3. Garewal, H. S. (ed) (1997) in *Antioxidants and Disease Prevention (Modern Nutrition Series)*, pp. 1–186, CRC Press, New York
4. Ahmad, S. (ed) (1995) *Oxidative Stress and Antioxidant Defenses in Biology*, pp. 1–457, Chapman & Hall, New York
5. Verhugen, H., Schilderman, P. A. E. L., and Kleinjans, J. C. S. (1991) *Chem. Biol. Interact.* **80,** 109–134
6. Nunn, C. J., Verhagen, H., and Kleinjans, J. C. S. (1991) *Food Chem. Toxicol.* **29,** 73–75
7. Bishop, A., Gallop, P. M., and Karnovsky, M. L. (1998) *Nutr. Rev.* **56,** 287–293
8. Wattenberg, L. W. (1972) *J. Natl. Cancer Inst.* **48,** 1425–1430
9. Ulland, B. M., Weisburger, J. H., Yammanoto, R. S., and Weisburger, E. K. (1973) *Food Cosmet. Toxicol.* **11,** 199–207
10. Hirose, M., Ito, T., Takahashi, S., Ozaki, M., Ogiso, T., Nihro, Y., Miki, T., and Shirai, T. (1998) *Eur. J. Cancer Prev.* **7,** 233–241
11. Nagakawa, J., Hirota, K., Hishinuma, I., Miyamoto, K., Sonoda, J., Yamanaka, T., Katayama, K., and Yamatsu, I. (1993) *J. Pharmacol. Exp. Ther.* **264,** 496–500
12. Nagakawa, J., Hishimuma, I., Miyamoto, K., Hirota, K., Abe, S., Yamanaka, T., Katayama, K., and Yamatsu, I. (1992) *J. Pharmacol. Exp. Ther.* **262,** 145–150
13. Bjorkhem, I., Henriksson-Freyschuss, A., Breuer, O., Diczfalusy, U., Berglund, L., and Henriksson, P. (1991) *Arterioscler. Thromb.* **11,** 15–22
14. Nishizono, S., Hayami, T., Ikeda, I., and Imaizumi, K. (2000) *Biosci. Biotechnol. Biochem.* **64,** 1153–1158
15. Ernster, L., Estabrook, R. W., Hochstein, P., and Orrenius, S. (eds) (1987) *DT-diaphorase: A Quinone Reductase with Special Functions in Cell Metabolism and Detoxication*, Vol. 27A, Chem. Scr., pp. 1–207, Cambridge University Press, New York
16. Ma, Q., Cui, K., Xiao, F., Lu, A. Y. H., and Yang, C. S. (1992) *J. Biol. Chem.* **267,** 22298–22304
17. Pickett, C. B., and Lu, A. Y. H. (1989) *Annu. Rev. Biochem.* **58,** 743–764
18. Favreau, L., and Pickett, C. (1991) *J. Biol. Chem.* **266,** 4556–4561
19. Rushmore, T. H., King, R. G., Paulson, K. E., and Pickett, C. B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3826–3830
20. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Htayama, I., Yamamoto, M., and Nabeshima, Y. (1997) *Biochem. Biophys. Res. Commun.* **236,** 313–322
21. Chan, K., and Kan, Y. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12731–12736
22. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) *Genes Dev.* **13,** 76–78
23. Chan, K., Han, X.-D., and Kan, Y. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4611–4616
24. Ramos-Gomez, M., Kwak, M.-K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kensler, T. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3410–3415
25. Luster, M. I. (1998) *Environ. Health Perspect.* **106,** A418–A419
26. Wills-Karp, M. (1999) *Annu. Rev. Immunol.* **17,** 255–281
27. Pohl, L. R., Pumford, N. R., and Martin, J. L. (1996) *Eur. J. Haematol.* **60,** 98–104
28. Laskin, D. L., and Pendino, K. J. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 655–677
29. Locksley, R. M., Killeen, N., and Lenardo, M., J. (2001) *Cell* **104,** 487–501
30. Feldmann, M., and Maini, R. N. (2001) *Annu. Rev. Immunol.* **19,** 163–196
31. Ye, J., Shi, X., Jones, W., Rojanasakul, Y., Cheng, N., Schwegler-Berry, D., Baron, P., Deye, G. J., Li, C., and Castronova, V. (1999) *Am. J. Physiol.* **276,** L426–L434
32. Ye, J., Wang, S., Leonard, S. S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castronova, V., and Shi, X. (1999) *J. Biol. Chem.* **274,** 34974–34980
33. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
34. Prestera, T., Holtzclaw, W. D., Zhang, Y., and Talalay, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2965–2969
35. Sanlioglu, S., Williams, C. M., Samavati, L., Butler, N. S., Wang, G., McCray, P. B., Jr., Ritchie, T. C., Hunninghake, G. W., Zandi, E., and Engelhardt, J. F. (2001) *J. Biol. Chem.* **276,** 30188–30198
36. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) *J. Biol. Chem.* **275,** 16023–16029
37. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Biochem.* **16,** 225–260
38. Aggarwal, B. B., and Natarajan, K. (1996) *Eur. Cytokine Netw.* **7,** 93–124
39. Aderem, A., and Ulevitch, R. J. (2000) *Nature* **406,** 782–787
40. Wagner, H. (2001) *Immunity* **14,** 499–502
41. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., and Kollias, G. (1999) *Immunity* **10,** 387–398
42. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kotoyiannis, D., Stamatakis, K., Lin, J.-H., Patriotis, C., Jenkings, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) *Cell* **103,** 1071–1083
43. Chan, J. Y., Kwong, M., Lu, R., Chang, J., Wang, B., Benedict, T. S., and Kan, Y. W. (1998) *EMBO J.* **17,** 1779–1787
44. Prieschl, E. E., Novotny, V., Csonga, R., Jaksche, D., Elbe-Burger, A., Thumb, W., Auer, M., Sting, G., and Baumruker, T. (1998) *Nucleic Acids Res.* **26,** 2291–2297
45. Hiramoto, M., Shmizu, N., Sugimoto, K., Tang, J., Kawakami, Y., Ito, M., Aizawa, S., Tanaka, H., Makino, I., and Handa, H. (1998) *J. Immunol.* **160,** 810–819
46. Prochaska, H. J., De Long, M. J., and Talalay, P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8232–8236

Chemoprotection by Phenolic Antioxidants: INHIBITION OF TUMOR NECROSIS FACTOR α INDUCTION IN MACROPHAGES

Qiang Ma and Krista Kinneer

J. Biol. Chem. 2002, 277:2477-2484.
doi: 10.1074/jbc.M106685200 originally published online November 2, 2001
