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Effects of the Wine Polyphenolics Quercetin and Resveratrol on Pro-inflammatory Cytokine Expression in RAW 264.7 Macrophages

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ABSTRACT. The beneficial effects of moderate red wine consumption have been attributed, in part, to the presence of antioxidant components. Oxidant stress is an activating stimulus for the NF (nuclear factor)-κB/Rel family of transcription factors, which have binding sites in the promoter regions of many genes involved in inflammatory and immune responses. The effect of lipopolysaccharide (LPS)-stimulated activation of NF-κB and the subsequent production of tumor necrosis factor alpha (TNF-α) and NO was determined in the macrophage cell line RAW 264.7. Unexpectedly, the wine polyphenolics quercetin and resveratrol and the antioxidant *N*-acetylcysteine (NAC) did not inhibit LPS-induced activation of the NF-κB complex p50/65, as determined by mobility shift. Quercetin inhibited LPS-induced p50/50. Northern blot analysis indicated that quercetin (0.1 and 0.2 mM) inhibited LPS-dependent production of inducible nitric oxide synthase (iNOS) mRNA and decreased NO release, as measured by the Griess reaction. This flavonoid had no effect on LPS-induced TNF-α mRNA, but decreased LPS-stimulated TNF-α release, as measured by ELISA. Resveratrol (0.05 and 0.1 mM) posttranscriptionally decreased LPS-induced nitrite release. It increased basal levels of TNF-α mRNA and protein and enhanced LPS-induced TNF-α mRNA and cytokine release. Our results do not support the view that wine antioxidants inhibit LPS-induced NF-κB activation but instead that they have a more selective action on genes activated by LPS. BIOCHEM PHARMACOL **57**;8:941–949, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. macrophages; NF-κB; nitric oxide; quercetin; resveratrol; tumor necrosis factor alpha

A growing body of literature indicates that polyphenolics are the active ingredients in dietary plants and traditional medicines used for the treatment of disorders related to oxidative stress and inflammation [1–3]. Inflammation involves a complex web of intracellular and intercellular cytokine signals. Activated monocytes and/or macrophages release a variety of inflammatory mediators such as TNF-α†, IL-1, IL-6, ROIs, and NO [4]. Curcumin, a polyphenolic derived from turmeric and used for centuries in Asia as an anti-inflammatory remedy, suppresses the activation of the transcription factor NF-κB, thereby reducing the production of TNF-α and IL-1 in human macrophages [5]. Green tea, attributed with numerous biological activities, including antioxidant [6] and anti-carcinogenic properties [7, 8], contains the flavonoid epigallocatechin gallate, which inhibits LPS-induced nitrite production in mouse peritoneal macrophages [9].

Phenolics present in red wine have been shown to exhibit cancer preventative properties [10–12], stimulate endothelial vasorelaxation [13], and inhibit oxidation of human low-density lipoproteins [14, 15], platelet aggregation, and the synthesis of pro-atherogenic eicosanoids [16]. As antioxidants, these phytochemicals exhibit protective effects against biological free radicals in oxidative injury models by inhibiting lipid peroxidation [1, 17–20]. Epidemiological studies linking moderate wine consumption to a reduced risk of coronary heart disease [21–24] partially attribute reduced mortality rates to the antioxidant properties of wine phenolics, but the mechanism by which these phytochemicals exert such effects is not elucidated fully.

To obtain insight into the biological effects of quercetin and resveratrol (Fig. 1) on the inflammatory response of macrophages, the influence of these compounds on the activity of the transcription factor NF-κB and the NF-κB responsive genes TNF-α and iNOS was determined. Although NF-κB is induced by a wide variety of agents, in RAW 264.7 macrophages LPS is the most widely studied stimulant. Macrophage sensitivity to LPS is mediated by three major classes of transcription factors: the C/EBP, *fos/jun*, and Rel homology families [25].

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† *Abbreviations*: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide from *Escherichia coli*; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; PDTC, pyrrolidinedithiocarbamate; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; ROIs, reactive oxygen intermediates; SNP, sodium nitroprusside; SSC, sodium chloride/sodium citrate buffer; SSPE, sodium chloride/sodium phosphate/EDTA buffer; and TNF-α, tumor necrosis factor-α.

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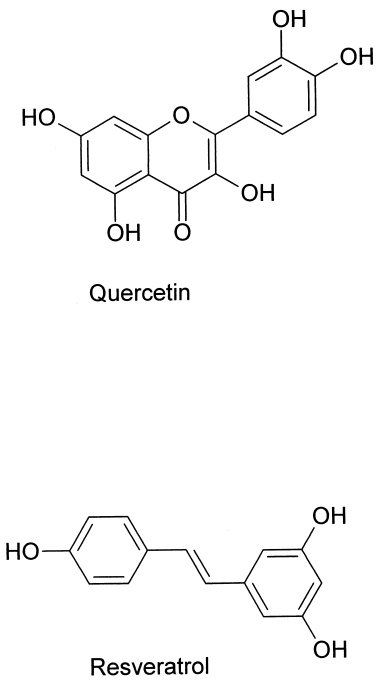

FIG. 1. Chemical structures of two wine polyphenolics, quercetin and resveratrol.

NF-κB, a member of the Rel family, is a common regulatory element in the promoter region of many pro-inflammatory cytokines. In activated macrophages, NF-κB in synergy with other transcriptional activators plays a central role in coordinating the expression of genes encoding TNF-α, IL-1, IL-6, and iNOS [26]. The Rel family of transcription factors are dimeric proteins that reside in the cytoplasm bound to the inhibitory subunit I-κB. In response to a variety of stimuli, including TNF-α, IL-1, oxygen radicals, viruses, LPS, or UV light, I-κB is phosphorylated and degraded by the cytosolic proteosome. Active Rel dimers translocate to the nucleus and activate genes containing κB regulatory elements [27, 28]. The diverse signal transduction pathways that converge on I-κB phosphorylation and the specificities of the multiple DNA binding and inhibitory subunits of the Rel family are yet to be elucidated fully.

Since a variety of structurally diverse antioxidants inhibit NF-κB activation [29], we hypothesized that quercetin and resveratrol would inhibit the LPS-induced nuclear localization of this transcription factor in RAW 264.7 macrophages. Here we report the effects of quercetin, resveratrol, and the antioxidants NAC and PDTC on NF-κB activation and the expression of TNF-α and iNOS, which have NF-κB responsive elements in their promoters.

MATERIALS AND METHODS

Reagents

LPS, quercetin, resveratrol, penicillin/streptomycin, Denhardt's reagent, Griess reagent, deionized formamide, E-Toxate®, sodium nitroprusside, and DMSO were from the Sigma Chemical Co. Antibodies to p50, p65, and c-Rel were from Santa Cruz Biotechnology. NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and single base pair mutant (5′-AGT TGA GGC GAC TTT CCC AGG C- 3′) were from Promega. DMEM was from Gibco BRL. Fetal bovine serum was from Research Sera. Tissue culture plates were from Nalge Nunc International. [^{32}P]ATP and GeneScreen Plus® were from DuPont NEN. Bradford protein dye reagent was from Bio-Rad. The TNF-α ELISA kit was from Endogen. High purity neutral red was from Molecular Probes. The RiboQuant® Multi-Probe RNase Protection Assay System was from PharMingen. Bioxytech® GSH-400 was from Oxis International, Inc. TRI® Reagent was from the Molecular Research Center. The mouse macrophage-like cell line RAW 264.7 and the plasmid clone of mouse TNF-α were from ATCC. The cDNA for iNOS was a gift from Yoichi Osawa, University of Michigan.

Cell Culture

Cells were cultured in phenol red-free DMEM containing 50 U/mL of penicillin, 50 μg/mL of streptomycin, 44 mM sodium bicarbonate, and 10% fetal bovine serum at 37° in humidified air containing 5% CO_2. For preparation of RNA or nuclear extracts, cells were plated in 2.5 mL of medium in 5.5-cm dishes, cultured for 2 days until the cells reached 80% confluency (approximately 2×10^7 cells/dish), and then treated as described in the text. When nitrite and TNF-α were determined, cells were plated in 500 μL of medium in 24-well plates, cultured for 2 days (approximately 2×10^6 cells/well), and then treated. Cells were washed, and fresh complete medium without phenol red was added 2 hr before the indicated stimuli. Stock solutions of quercetin and resveratrol were prepared in DMSO and added to the medium. Concentrations of quercetin and resveratrol were similar to those used in studies reporting antioxidant and antitumor effects [1, 10, 20, 30]. The final concentration of DMSO did not exceed 0.2%.

Northern Blot Analysis and Ribonuclease Protection Assay

After 6 hr of treatment, total RNA was isolated with TRI® Reagent as specified by the manufacturer. For northern blot analysis, total RNA (20 μg) was electrophoresed in 1% agarose/15% formaldehyde gels, transferred overnight to GeneScreen Plus® membranes, and cross-linked to the membrane by UV irradiation. For iNOS mRNA detection, the membrane was prehybridized for 4 hr at 37° in hybridization buffer (50% deionized formaldehyde, 5× Denhardt's reagent, 5× SSC, 0.1% SDS, and 100 μg/mL of denatured salmon sperm DNA). For TNF-α mRNA detection, the membrane was prehybridized for 4 hr at 37° in 50% deionized formaldehyde, 5× Denhardt's reagent, 5× SSPE, 0.5% SDS, and 100 μg/mL of denatured salmon sperm

DNA, and was hybridized overnight in the same buffer containing 1–2 × 10^6 cpm/mL of denatured cDNA probe. For iNOS, following hybridization, the filters were washed twice for 30 min in 2× SSC, 0.1% SDS at room temperature, and twice for 30 min in 0.5× SSC, 0.1% SDS at 60°. The cDNA probe for iNOS was a 781 bp *Bam*HI fragment excised from full-length murine iNOS cDNA. For TNF-α, 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°. A portion (10 μg) of total RNA was analyzed for the mRNA of TNF-α using the RiboQuant® Multi-Probe RNase Protection Assay System as described by the manufacturer. Signals for northern blot analysis and RNase protection 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.

Nitrite Assay

After 24 hr of treatment, 100 μL of medium from the 24-well plates was incubated with an equal volume of Griess reagent for 15 min at room temperature. The absorbance at 550 nm was measured using an Anthos htIII microplate reader. Nitrite concentration was determined using dilutions of sodium nitrite in phenol red-free DMEM as a standard. Additions of quercetin and resveratrol to standard solutions of sodium nitrite confirmed that the wine components did not interfere with the nitrite assay.

TNF-α

Cells were plated and grown to confluency in 24-well tissue culture dishes. Following 24 hr of treatment in 0.5 mL of medium, supernatants were collected and centrifuged for 30 sec at 3500 *g*. Conditioned medium (50 μL) was analyzed for TNF-α by ELISA as per the manufacturer's instructions. For samples containing LPS, the supernatant was diluted 50% with culture medium prior to analysis.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by a modified method of Dignam *et al.* [31]. 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. The pellet was 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. The supernatant was removed, and the pellet was suspended in 50–100 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, 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*. Protein concentration of the supernatant was determined by the method of Bradford.

EMSA

The activation of NF-κB was assayed by gel mobility shift assays using nuclear extracts from control and treated cells. Mixtures containing 10 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 (about 150,000 cpm of ^{32}P) was added, and the complete mixture was incubated for an additional 20 min at room temperature. Complexes were separated on a 7% native polyacrylamide gel containing 0.5× Tris, borate, ethylenediamine tetraacetic acid with a 5 mM Tris/38 mM glycine running buffer. The gel was dried, and complexes were detected by autoradiography. The identity of the complexes was established with excess cold (unlabeled) NF-κB oligonucleotide and antibody supershifts obtained with rabbit polyclonal antibodies to p50 (nuclear localization signal), and goat polyclonal antibodies to p65 (C-20) and c-Rel (N-terminus).

Cell Viability Assay

Uptake of the dye neutral red was used as a measure of cell viability [32]. Cells were plated in 24-well plates, and samples (N = 3) were treated for various times in 0.5 mL of medium. Following treatment, the medium was removed and replaced with 500 μL of medium containing 50 μg/mL of neutral red for 90 min at 37°. Following neutral red treatment, the medium was removed, and the wells were washed three times with 500 μL of 37° PBS. The neutral red was extracted with 500 μL of 50% ethanol, 50 mM sodium citrate, pH 4.2. Duplicate 200-μL samples from each well were transferred to a 96-well plate, and the absorbance at 510 nm was measured with an Anthos htIII microplate reader.

Endotoxin Assay

The E-Toxate® (Limulus Amebocyte Lysate) test was used, following the manufacturer's instructions, to test all solutions for the presence of gram-negative bacterial endotoxin.

Statistical Analysis

Student's unpaired *t*-test was used to assess the statistical significance of differences.

RESULTS

Effect of Antioxidants on NF-κB Binding Activities and Characterization of Binding Complexes

Induction of pro-inflammatory cytokines is modulated, in part, through the activation of NF-κB. As a result, we

sought to determine what effects the antioxidants in wine had on basal NF-κB levels and whether they could prevent LPS-induced nuclear localization of this transcription factor. Following a 1-hr pretreatment with quercetin (0.1 or 0.2 mM), resveratrol (0.05 or 0.1 mM), NAC (30 mM), or PDTC (0.1 mM), RAW 264.7 cells were treated with 100 ng/mL of LPS or 0.5 mM H_2O_2 for 2 hr. Neutral red uptake assays and the E-Toxate® test verified that cellular responses to the various treatments were not due to general cellular toxicity or bacterial endotoxin contamination (results not shown).

LPS-induced NF-κB binding activities exhibited time dependence and sequence motif specificity (Fig. 2A). Distinct NF-κB binding complexes were detected and identified using antibodies to three members of the Rel homology family, p50, p65, and c-Rel, and excess cold (unlabeled) NF-κB consensus oligonucleotide. Antibodies to p50 completely shifted all complexes, whereas antibodies to p65 shifted the larger complex (labeled C3). This suggests that C2 consists of the p50/50 homodimer, and the uppermost complex (labeled C3) consists of classic NF-κB (p50/65). Antibodies to c-Rel shifted the slow-migrating complex above C3, suggesting that this consists of p50 and c-Rel. Complex identification is in agreement with results previously reported for this cell line [33, 34]. C1 may be a complex that results from proteolysis of p50/50. This complex was observed in LPS-activated murine macrophages [35]. Our analysis focused on the well characterized Rel dimers p50/50 (C2) and p50/65 (C3).

Compared with untreated cells, all complexes were enhanced in cells stimulated with LPS for 2 hr (Fig. 2B). Pretreatment with the antioxidant NAC (30 mM) did not protect against LPS-induced complex formation. PDTC pretreatment (0.1 mM) and quercetin (0.1 and 0.2 mM) decreased LPS-stimulated binding of p50/50 (C2). Resveratrol, like NAC, did not protect against LPS-induced nuclear localization of NF-κB complexes. Quercetin (0.2 mM) and resveratrol (0.05 and 0.1 mM) alone appeared to slightly enhance the signals associated with p50/50 (C2) and p50/65 (C3).

H_2O_2 is known to activate NF-κB in a redox-sensitive manner [26, 29]. Therefore, we investigated the effect of antioxidants on H_2O_2-stimulated NF-κB activation (Fig. 2C). Relatively high concentrations (0.5 mM) of H_2O_2 were required to observe NF-κB activation in this cell line. Unlike results with LPS, pretreatment with NAC decreased H_2O_2-stimulated binding of p50/65 (C3), while PDTC inhibited H_2O_2-induced binding of all complexes. Glutathione (30 mM), quercetin (0.2 mM), and resveratrol (0.1 mM) did not inhibit and may have enhanced H_2O_2-induced formation of all complexes.

NF-κB analysis by mobility shift assay is difficult to quantify. We, therefore, examined the regulation of the NF-κB inducible genes iNOS and TNF-α, which may reflect the activation or inhibition of NF-κB complexes by antioxidants.

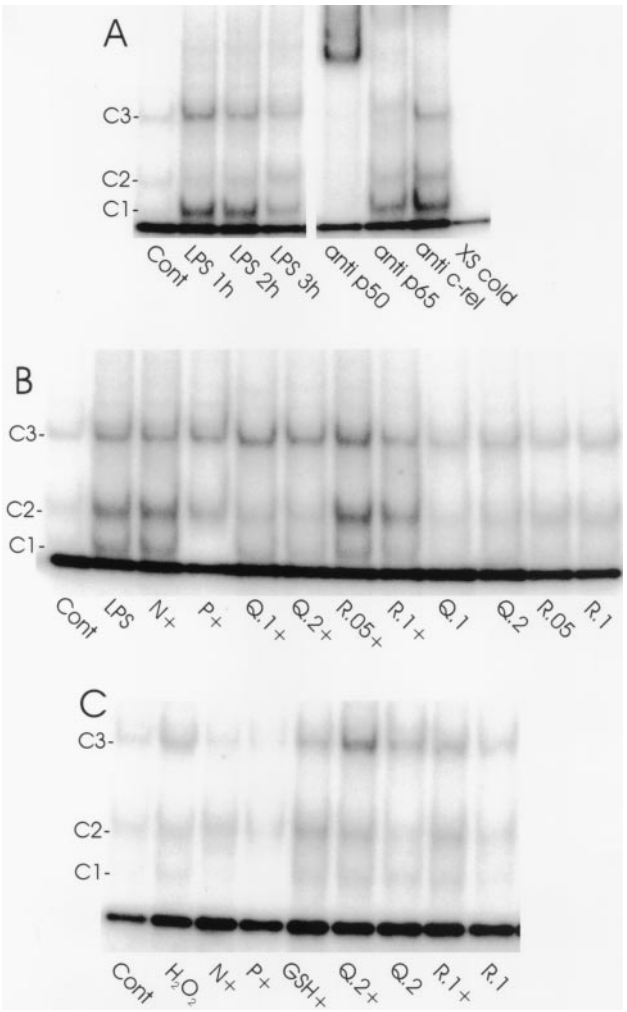

FIG. 2. Effect of antioxidants and wine polyphenolics on NF-κB binding activities in EMSA. Binding activity was assayed using ^{32}P-labeled NF-κB consensus oligonucleotide in the presence or absence of indicated antibodies and assessed by EMSA. Dried gels were analyzed by autoradiography. The results shown are from a single experiment and are representative of what was observed in two or more additional experiments. (A) Time course for LPS activation and characterization of binding complexes using anti-Rel family antibodies. RAW 264.7 cells were either untreated (Cont), or treated with LPS (100 ng/mL) for 1, 2, or 3 hr prior to the preparation of nuclear extracts. Antibody analysis was performed on nuclear extracts obtained after 2 hr of LPS treatment. Anti c-Rel: antibody to N-terminus; XS cold: 100-fold excess cold (unlabeled) NF-κB oligonucleotide. (B) Effect of antioxidants on LPS-induced NF-κB binding activities. Cells were either untreated (Cont), treated with LPS (100 ng/mL) for 2 hr, treated with quercetin (Q.1, 0.1 mM; Q.2, 0.2 mM) or resveratrol (R.05, 0.05 mM; R.1, 0.1 mM) for 3 hr, or pretreated for 1 hr with antioxidants and then treated with LPS 2 hr prior to the preparation of nuclear extracts (N+, 30 mM NAC; P+, 0.1 mM PDTC; Q.1+, 0.1 mM quercetin; Q.2+, 0.2 mM quercetin; R.05+, 0.05 mM resveratrol; R.1+, 0.1 mM resveratrol). (C) Effect of antioxidants on H_2O_2-induced NF-κB binding activities. Cells were either untreated (Cont), treated with H_2O_2 (0.5 mM) for 2 hr, treated with resveratrol (R.1, 0.1 mM) or quercetin (Q.2, 0.2 mM) for 3 hr or pretreated for 1 hr with antioxidants, then H_2O_2 (0.5 mM) 2 hr prior to the preparation of nuclear extracts (N+, 30 mM NAC; P+, 0.1 mM PDTC; GSH+, 30 mM glutathione; Q.2+, 0.2 mM quercetin; R.1+, 0.1 mM resveratrol).

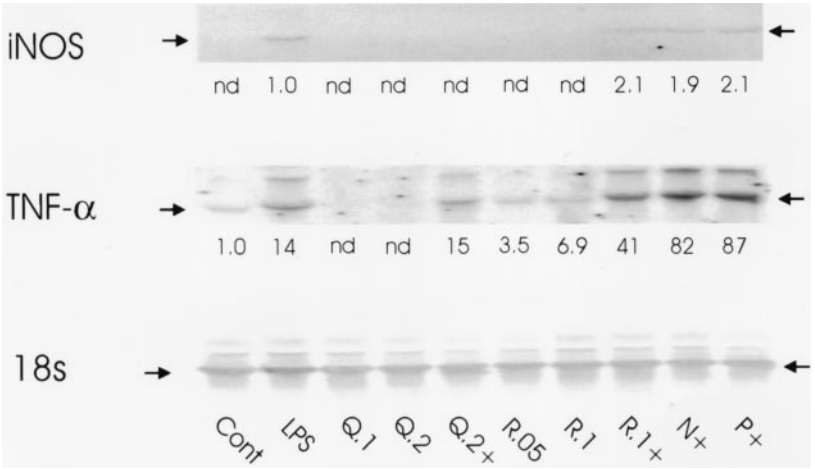

FIG. 3. Northern blot analysis of iNOS and TNF-α mRNA expression in RAW 264.7 macrophages. Cells were cultured for 6 hr with medium only (Cont), quercetin (Q.1, 0.1 mM; Q.2, 0.2 mM) or resveratrol (R.05, 0.05 mM; R.1, 0.1 mM) or for 5 hr with LPS (100 ng/mL), or pretreated with NAC (N+, 30 mM), PDTC (P+, 0.1 mM), quercetin (Q.2+, 0.2 mM), or resveratrol (R.1+, 0.1 mM) for 1 hr, then LPS for 5 hr prior to the isolation of total RNA. Following cell lysis and RNA purification, iNOS and TNF-α mRNA and 18S rRNA were assessed in 20 μg of total cellular RNA by northern blot analysis as described in Materials and Methods. The relative intensity of the signal after normalization to the 18S rRNA is shown below each lane.

Effect of Antioxidants on Expression of iNOS and TNF-α mRNA

RAW 264.7 cells were pretreated with quercetin or resveratrol for 1 hr, then LPS was added, and the cells were incubated for an additional 5 hr before mRNA for TNF-α and iNOS was analyzed (Fig. 3). The intensity of mRNA signals was normalized to 18S rRNA and compared with either LPS treatment (iNOS) or untreated cells (TNF-α). Northern blot analysis using a cDNA probe for iNOS revealed a 4.4 kb iNOS mRNA transcript in cells treated with LPS. The mRNA was not detectable in untreated cells or those treated with quercetin (0.1 and 0.2 mM) or resveratrol (0.05 and 0.1 mM) alone. Quercetin (0.2 mM) inhibited LPS-induced expression of iNOS mRNA. Resveratrol (0.1 mM), NAC (30 mM), and PDTC (0.1 mM) caused a small increase in the levels of LPS-induced iNOS mRNA.

Quercetin (0.1 and 0.2 mM) inhibited constitutive expression of TNF-α mRNA but had no effect on LPS-dependent increases in TNF-α mRNA. Resveratrol (0.05 and 0.1 mM) increased basal levels of TNF-α mRNA by 3.5- and 6.9-fold, respectively. There was a 2.9-fold enhancement of LPS-induced TNF-α mRNA by resveratrol (0.1 mM). NAC and PDTC enhanced LPS-induced TNF-α mRNA by about 6-fold. These findings were confirmed by RNase protection assays (results not shown).

Effects of Antioxidants on Cytokine Expression and Enzyme Activity

Consistent with an enhanced expression of TNF-α mRNA in the presence of resveratrol, 0.05 and 0.1 mM concentrations of this phytoalexin increased basal levels of TNF-α protein by 2- and 8-fold, respectively (Fig. 4A). Resveratrol also caused a 1.6-fold enhancement of LPS-induced levels of TNF-α. Quercetin (0.2 mM), while having no effect on mRNA, caused an 85% decrease in LPS-stimulated release of TNF-α. A lower concentration of quercetin (0.1 mM) caused a small but significant increase in LPS-stimulated TNF-α secretion. NAC (30 mM) had no effect on LPS-induced TNF-α release, while PDTC (0.1 mM) enhanced LPS-induced TNF-α secretion by 3.6-fold.

Treatment of cells with resveratrol, quercetin, NAC, and PDTC had no effect on basal nitrite formation, as measured by the Griess reaction (Fig. 4B). However, resveratrol (0.05 and 0.1 mM), which enhanced iNOS mRNA, decreased LPS-induced nitrite release 16 and 34%. Quercetin, which inhibited LPS-induced iNOS mRNA, decreased LPS-induced nitrite release in a concentration-dependent fashion. Nitrite release was inhibited 24 and 74% by 0.1 and 0.2 mM quercetin. NAC (30 mM) and PDTC (0.1 mM) inhibited the formation of nitrite 83 and 99%, respectively. PDTC is a known inhibitor of NO mRNA translation [36, 37].

To confirm that inhibition of nitrite formation was not due to an effect on the ability of the Griess reaction to detect nitrite, the reaction was repeated with the artificial nitrite donor SNP (Fig. 5). Only PDTC (0.1 mM) inhibited nitrite formation from SNP, while resveratrol (0.05 and 0.1 mM), quercetin (0.1 and 0.2 mM), and NAC (30 mM) all increased the amount detected. Enhancement of nitrite production from donor molecules such as SNP in the presence of NAC has been reported previously [38].

DISCUSSION

Effects on NF-κB Activation

Activity of the transcription factor NF-κB is induced by a wide variety of agents, including ROIs, TNF-α, LPS, viral infection, UV irradiation, phorbol esters, and NO. Although no common second messenger has been identified, most NF-κB activating signals can be inhibited by antioxidants [26, 29]. LPS-induced NF-κB activation in other cell lines appears to be mediated through its ability to stimulate the production of superoxide, H_2O_2, and other ROIs [39]. Our results suggest that LPS and H_2O_2 exhibit differential effects on Rel transcription factors in RAW 264.7 cells. Activation of p50/65 by LPS was resistant to PDTC, NAC, resveratrol, and quercetin. H_2O_2-induced p50/65 activation, on the other hand, was inhibited by NAC and PDTC

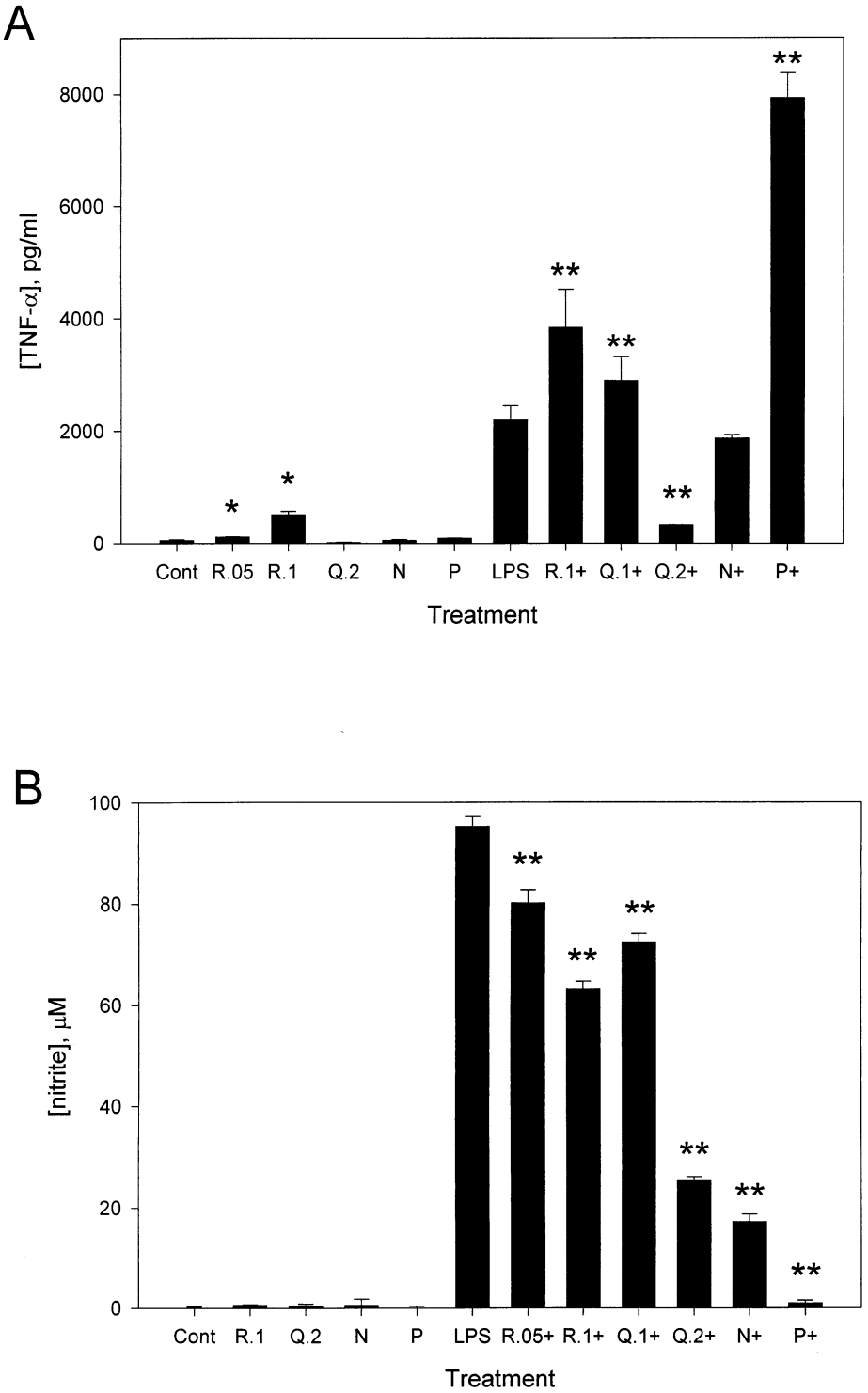

FIG. 4. Effect of wine phenolics on TNF-α and NO production. RAW 264.7 macrophages were cultured in the presence of resveratrol (R.05, 0.05 mM; R.1, 0.1 mM), quercetin (Q.2; 0.2 mM), NAC (N, 30 mM), or PDTC (P, 0.1 mM), LPS (100 ng/mL) or antioxidants plus LPS (R.05+, 0.05 mM resveratrol; R.1+, 0.1 mM resveratrol; Q.1+, 0.1 mM quercetin; Q.2+, 0.2 mM quercetin; N+, 30 mM NAC; P+, 0.1 mM PDTC). After 24 hr, supernatants were collected, and the concentrations of TNF-α and nitrite were determined. Values are means ± SD, N = 3. (A) TNF-α analysis by ELISA. Similar trends were observed in four separate experiments. Key: (*) $P < 0.01$ compared with control; and (**) $P < 0.05$ compared with LPS stimulation. (B) NO analysis by the Griess reaction. Similar results were obtained in a second independent experiment. Key: (**) $P < 0.01$ compared with LPS stimulation.

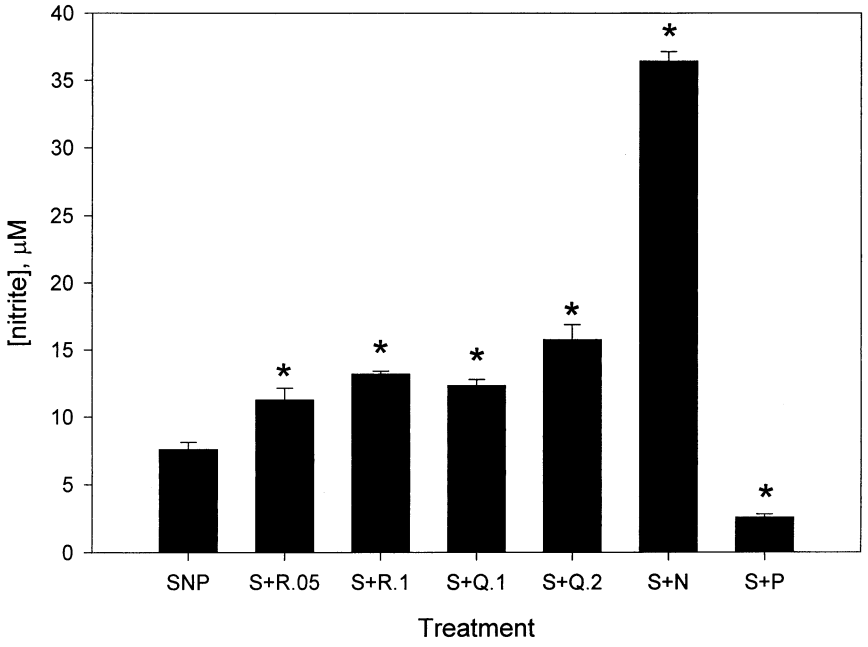

FIG. 5. Effect of antioxidants and wine polyphenolics on nitrite production by SNP. Nitrite levels were measured by the Griess reaction in culture medium incubated with SNP (1 mM), SNP + 0.05 mM resveratrol (S+R.05), SNP + 0.1 mM resveratrol (S+R.1), SNP + 0.1 mM quercetin (S+Q.1), SNP + 0.2 mM quercetin (S+Q.2), SNP + 30 mM NAC (S+N), or SNP + 0.1 mM PDTC (S+P), for 5 hr at 37°. All values represent means ± SD of six independent experiments. Key: (*) $P < 0.01$ compared with SNP treatment.

but not by quercetin or resveratrol. LPS-induced p50/50 activation was attenuated by PDTC and quercetin, with NAC and resveratrol having little effect. Only PDTC protected against H_2O_2-induced p50/50 activation. LPS, therefore, appears to induce p50/65 and p50/50 through unique mechanisms and via signaling pathways that differ from the activation triggered by H_2O_2.

Quercetin, while enhancing constitutive levels of p50/65, inhibited LPS-induced p50/50. This inhibition may be attributed to the free radical scavenging activity of quercetin. This seems unlikely, given that it did not diminish H_2O_2-induced p50/50 activation. The inhibition of NF-κB by PDTC does not necessarily implicate the involvement of ROIs. Although this metal chelator is often utilized to block NF-κB, its mechanism does not appear to involve its antioxidant properties but rather an inhibition of NF-κB-DNA binding [40]. The inability of NAC to attenuate LPS-induced p50/50 or p50/65 activation suggests that glutathione depletion is not the mechanism by which LPS induces NF-κB. Similarly, glutathione and NAC had no effect on H_2O_2-induced p50/50 binding. It should be noted that NAC inhibits p50/50 and p50/65 binding in RAW 264.7 cells activated at a 100-fold higher concentration of LPS than that used in our studies [41].

Effect of Quercetin on iNOS and TNF-α

In the present study, quercetin inhibited iNOS mRNA, the release of NO, and the production of TNF-α. These results are consistent with studies reporting that quercetin inhibits LPS-induced release of TNF-α from mouse peritoneal macrophages [42] and NO and TNF-α release from LPS-stimulated rat Kupffer cells [43]. Quercetin is a major component in *Ginkgo biloba* extract, which was shown to inhibit iNOS mRNA and NO production in LPS/IFN-γ-activated macrophages while having no effect on NF-κB activation [2].

It seems likely that quercetin inhibits NO production by several mechanisms. Quercetin inhibits various tyrosine and serine/threonine kinases, including PKC and mitogen-activated protein (MAP) kinase [30, 43–47]. Tyrosine kinases are known to attenuate LPS-induced increases in nitrite in murine macrophages and TNF-α and NO in LPS-treated rats [48]. PKC is involved directly in the induction of NOS in rat hepatocytes [49]. Quercetin may also inhibit LPS-induced iNOS mRNA expression through inhibition of transcription factors other than NF-κB. In addition to NF-κB, the promoter region for the iNOS gene is known to contain consensus sequences for binding IFN-γ and IL-6 regulatory factors [50–52]. The antioxidant PDTC, while inhibiting p50/50, did not suppress iNOS mRNA, ruling out inhibition of this heterodimer as a likely explanation. Suppression of NO release by quercetin may be attributed to direct free radical and NO scavenging activity; however, it did not decrease levels of nitrite when added to solutions of the NO generator SNP.

Transfection experiments with reporter constructs indicate that p50 exhibits negative effects on LPS-induced TNF-α transcription in murine macrophages [35]. The resistance of LPS-induced TNF-α mRNA to quercetin may therefore be attributed to removal of the transcriptional inhibition imparted by p50/50. Our results are similar to findings by Kawada *et al.* [43], who reported that quercetin inhibits NO and TNF-α production in LPS-stimulated rat Kupffer cells at a posttranscriptional level. Further studies are necessary to determine the exact mechanism by which quercetin inhibits TNF-α translation.

Effects of Resveratrol on iNOS and TNF-α

Resveratrol, although slightly enhancing iNOS mRNA, concentration-dependently decreased NO production in activated RAW 264.7 macrophages. It did not inhibit nitrite generated by SNP, abrogating the explanation that this phenolic lowers LPS-induced nitrite production by scavenging NO. Resveratrol also inhibits NO production in LPS-stimulated rat Kupffer cells at a posttranscriptional level [43].

A dramatic and unique effect in our study was a 3.5- to 6.9-fold increase in basal TNF-α mRNA expression with a concomitant increase in TNF-α secretion from undetectable levels to 100 and 500 pg/mL with 0.05 and 0.1 mM resveratrol, respectively. Addition of resveratrol to LPS-treated cells resulted in a 1.6-fold increase in TNF-α secretion. The biological effects of resveratrol were reviewed recently by Soleas *et al*. [53]. By acting as a cyclooxygenase-1 inhibitor [10], resveratrol may interfere with the negative feedback loop of prostaglandins, which down-regulate TNF-α production at the mRNA level [54]. The induction of TNF-α by activated macrophages can lead to cytostatic and cytotoxic activities on malignant cells [55]. Resveratrol-mediated release of TNF-α may, therefore, contribute to the antitumorigenic properties of this wine component.

In summary, while quercetin and resveratrol can act as antioxidants in some assays, our results do not support the view that their mechanism of action is via inhibition of LPS-induced NF-κB activation in this cell line. Instead, these compounds have more selective actions on genes activated by LPS.

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