ANTIOXIDANTS & REDOX SIGNALING
Volume 1, Number 2, 1999
Mary Ann Liebert, Inc.

Original Research Communication

Antioxidants Differentially Regulate Activation of Nuclear Factor-κB, Activator Protein-1, c-Jun Amino-Terminal Kinases, and Apoptosis Induced by Tumor Necrosis Factor: Evidence that JNK and NF-κB Activation Are Not Linked to Apoptosis

ANJU SHRIVASTAVA and BHARAT B. AGGARWAL

ABSTRACT

Tumor necrosis factor (TNF) is known to mediate its signaling through generation of reactive oxygen species (ROS), but the type of TNF signal regulated by ROS and the nature of the ROS species involved are not fully understood. In this report, we investigated the effect of various superoxide radical quenchers—pyrrolidine dithiocarbamate (PDTC), *N*-acetyl-L-cysteine (NAC), and glutathione (GSH)—an hydroxyl radical quencher (mannitol), and lipid peroxide quenchers—butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)–on TNF-induced activation of nuclear transcription factors-κB (NF-κB) and activator protein-1 (AP-1), c-jun amino-terminal kinase (JNK), and apoptosis in human monocytic U937 cells. TNF-induced NF-κB activation was inhibited by both superoxide and lipid peroxide quenchers but potentiated by an hydroxyl radical quencher. In contrast, none of the radical quenchers had any significant effect on TNF-induced AP-1 activation. TNF-induced JNK activation, similar to NF-κB, was inhibited by both superoxide and lipid peroxide quenchers but potentiated by hydroxyl radical quencher. TNF-induced activation of caspase activity was blocked by all three types of quenchers. TNF cytotoxicity, however, was potentiated by superoxide radical quenchers and suppressed by hydroxyl radical and lipid peroxide quenchers. Overall, these results suggest that hydroxyl radicals mediate TNF-induced apoptosis but not activation of NF-κB, AP-1, and JNK; superoxide radicals mediate NF-κB and JNK activation but potentiate apoptosis; and lipid peroxides are required for all the signals induced by TNF. Antiox. Redox Signal. 1, 181–191, 1999.

INTRODUCTION

DURING THE LAST 5 YEARS, researchers have identified over 15 different proteins that associate with the cytoplasmic domain of the tumor necrosis factor (TNF) receptor and mediate activation of nuclear factor κB (NF-κB), c-jun amino-terminal kinase (JNK), and caspases (Karin, 1995; Tewari *et al.*, 1995; Beg and Baltimore, 1996; Liu *et al.*, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996; Baeuerle and Baichwal, 1997; Cai *et al.*, 1997; Darnay and Aggarwal, 1997; Guo *et al.*, 1998; Hehner *et al.*, 1998; Roulston *et al.*, 1998). But none of these proteins ac-

Cytokine Research Laboratory, Department of Molecular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030.

count for the role of reactive oxygen intermediates/species (ROS) in TNF signaling. Several studies do indicate that ROS play a critical role in TNF-induced cytotoxicity (Wong and Goeddel, 1988; Wong *et al.*, 1989; Goossens *et al.*, 1995; Li and Oberley, 1997), most of which used either metabolic inhibitors that quench ROS, the overexpression of antioxidant enzymes, or TNF signaling in respiration-deficient cells to make their observations. Similarly TNF-induced NF-κB activation has been shown to be blocked by the superoxide radical quenchers pyrrolidine dithiocarbamate (PDTC) and *N*-acetyl-L-cysteine (NAC) (Schreck *et al.*, 1992; Anderson *et al.*, 1994). How these agent affect TNF-induced activation of JNK and caspases, is not known, however.

It has been demonstrated that NF-κB activation blocks TNF-induced apoptosis (Beg and Baltimore, 1996; Wang *et al.*, 1996; Van Antwerp *et al.*, 1996) while JNK activation mediates it (Guo *et al.*, 1998; Roulston *et al.*, 1998). Other studies, however, dispute the linkage of NF-κB activation with TNF-induced apoptosis (Cai *et al.*, 1997; Hehner *et al.*, 1998). Recently, we have shown that overexpression of superoxide dismutase blocks TNF-induced activation of NF-κB, activator protein-1 (AP-1), JNK, and caspases and of TNF cytotoxicity (Manna *et al.*, 1998). Several mechanisms account for these effects. For instance, TNF induces superoxide anion generation in the mitochondria (Schulz-Osthoff *et al.*, 1992), which in turn mediates TNF-induced cytotoxicity (Goossens *et al.*, 1995). In addition, depletion of mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF (Schulz-Osthoff *et al.*, 1993). More recent studies have, however, indicated that the respiration-deficient cells were resistant to TNF-induced capase-3 activation but sensitive to TNF-induced cytotoxicity and differentiation, suggesting a selective role of ROS (Higuchi *et al.*, 1997).

Although several studies suggest the role of mitochondria-derived ROS in TNF signaling, it is unclear which reactive oxygen species is involved in activation of which signal transduced by TNF under comparable conditions. Besides the superoxide radical, whether the hydroxyl radical or lipid peroxide radical plays any role in TNF-mediated signaling is not known. As a result, we investigated the effect of different quenchers of superoxide radicals, hydroxyl radicals, and lipid peroxides on TNF-induced activation of NF-κB, AP-1, JNK, and caspase-3 and on TNF cytotoxicity. Our results show that these various TNF signals are differentially regulated by different ROS and that TNF activates these signals independently of each other.

MATERIALS AND METHODS

Materials

The antioxidants PDTC, NAC, glutathione (GSH), butylated hydroxyanisole (BHA), mannitol, and butylated hydroxy toluene (BHT) were obtained from Sigma Chemical Co. (St. Louis, MO). BHA is a synthetic phenolic antioxidant that acts by its chain-breaking action during the autoxidation of lipids. GSH, the most abundant intracellular thiol compound in mammalian cells, is a scavenger of many free radicals and a substrate of glutathione peroxidase, which degrades intracellular hydrogen peroxide (H_2O_2). NAC is a precursor of GSH, and it can also directly scavenge many different types of free radicals because of its low reduction potential. PDTC is an iron chelator and ROS scavenger. Both mannitol and dimethyl sulfoxide (DMSO) are potent hydroxyl radical scavengers.

Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Antibody against JNK, and double-stranded oligonucleotides having NF-κB and AP-1 consensus sequences were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Poly(ADP) ribose polymerase (PARP) antibody was purchased from PharMingen (San Diego, CA).

Cell culture and treatments

We used U-937 cells for all studies because these cells were well characterized in our laboratory for various signals induced by TNF. U937 cells were cultured in RPMI 1640 medium

containing 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Cells were pretreated with test compounds or left untreated before the addition of TNF. All experiments were carried out in complete medium at 37°C. PDTC was dissolved in phosphate-buffered saline (PBS); GSH was dissolved in water; BHT and BHA were dissolved in absolute ethanol; NAC was dissolved in 25 m*M* Tris-HCl and adjusted to pH 7.4 with 1 *N* NaOH; mannitol was dissolved in medium without serum.

Electrophoretic mobility shift assays

Nuclear extracts were prepared from treated cells, and electrophoretic mobility shift assay (EMSA) was carried out as previously described in detail (Chaturvedi *et al.*, 1994, and references therein). Briefly, nuclear extracts were prepared from 2×10^6 cells after different treatments and then either used immediately or stored at −70°C. EMSA were performed by incubating 8 μg of nuclear extract, with 8 fmol of ^{32}P-end-labeled, 45-mer double-stranded NF-κB oligonucleotide from the (HIV-LTR), 5′-TTGTTACAAGGGACTTTC-CGCTGGGGACTTTCCAGGGAGGCGTGG-3′, (underlined is the NF-κB consensus sequence) for 15 min at 37°C. The incubation mixture included 2–3 μg of poly(dI-dC) in a binding buffer: 25 m*M* HEPES, pH 7.9, 0.5 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 1% NP-40, 5% glycerol, and 50 m*M* NaCl). The DNA–protein complex formed was separated from free oligonucleotide on 7.5% native polyacrylamide gel using buffer containing 50 m*M* Tris, 200 m*M* glycine, pH 8.5, and 1 m*M* EDTA, and then the gel was dried. The specificity of binding was examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either p50 or p65 subunits of NF-κB for 30 min at 37°C before complex was analyzed by EMSA. Antibodies against cyclin D1 or c-Rel and preimmune serum were included as negative control. The EMSA for AP-1 was performed as described for NF-κB using ^{32}P-end-labeled double-stranded oligonucleotides (Manna *et al.*, 1998). Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition as described earlier (Chaturvedi *et al.*, 1994). Visualization and quantitation of radioactive bands was carried out by a phosphorimager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

c-Jun kinase assay

The c-Jun kinase assay was performed with slight modification by a method described earlier (Karin, 1995; Liu *et al.*, 1996; Haridas *et al.*, 1988). Briefly, after treatment of cells (3×10^6/ml) with TNF for different time periods, cells were lysed in buffer containing 20 m*M* HEPES, pH 7.4, 2 m*M* EDTA, 250 m*M* NaCl), 1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 m*M* PMSF, 0.5 μg/ml benzamidine, and 1 m*M* DTT. Cell extracts (150–250 μg/sample) were subjected to immunoprecipitation with 0.03 μg of anti-JNK antibody for 60 min at 4°C. Immune complex was collected by incubation with protein A/G Sepharose beads for 45 min at 4°C. The beads were then extensively washed with lysis buffer (4×400 μl) and kinase buffer (2×400 μl: 20 m*M* HEPES, pH 7.4, 1 m*M* DTT, 25 m*M* NaCl). *In vitro* kinase assays were performed for 15 min at 30°C with GST-Jun(1–79) as substrate in 20 m*M* HEPES, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* DTT, and 10 μCi [γ-^{32}P]ATP. The reaction was stopped by adding 15μl of 2× SDS sample buffer and boiling for 5 min, and the samples were subjected to SDS-PAGE (9%). GST-Jun(1–79) was detected by staining with Coomassie Blue. The dried gel was analyzed and quantified by Personal Densitometer Scan v1.30 using Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, CA).

TNF cytotoxicity assay

The sensitivity of U937 cells to TNF was determined by the modified tetrazolium salt (3-(4,5-dihydro-6-(4-(3,4-dimethoxybenzoil)-1-piperazinyl-2 (1*H*)-quinolinone, MTT) assay as described earlier (Hansen *et al.*, 1989; Haridas *et al.*, 1998). Briefly, cells (5,000/well) were incubated in the presence or absence of different concentrations of antioxidants for 30 min and then treated with 1 n*M* TNF in a final volume of 0.2 ml for 24, 48, and 72 hr. Then 0.02 ml of

a 5 mg/ml solution of MTT was added to each well. After a 2-hr incubation at 37°C, 0.1 ml of extraction buffer, consisting of 20% sodium dodecyl sulfate (SDS) in 50% dimethyl formamide, was added. After overnight incubation at 37°C, optical density (OD) was measured at 570 nm using a 96-well multiscanner autoreader (Dynatech MR 5000; Dynatech Laboratories; Chantilly, VA), with the extraction buffer serving as a blank.

Immunoblot analysis of PARP degradation

TNF-induced apoptosis was examined by proteolytic cleavage of PARP (Tewari *et al.*, 1995; Haridas *et al.*, 1998). Briefly, cells (2×10^6/ml) were treated with TNF (1 n*M*) for different times at 37°C. After treatment, cell extracts were prepared by incubating the cells for 30 min on ice in 0.05 ml of buffer containing 20 m*M* HEPES, pH 7.4, 2 m*M* EDTA, 250 m*M* NaCl, 0.1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml benzamidine, and 1 mM DTT. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved in 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-PARP antibody, and then detected by enhanced chemiluminescence (ECL; Amersham). Apoptosis was represented by the cleavage of 116-kDa PARP into an 85-kDa peptide product (Tewari *et al.*, 1995).

RESULTS

In the present study, we investigated the effect of quenchers of superoxide radicals, hydroxyl radicals, and lipid peroxides on TNF-in-

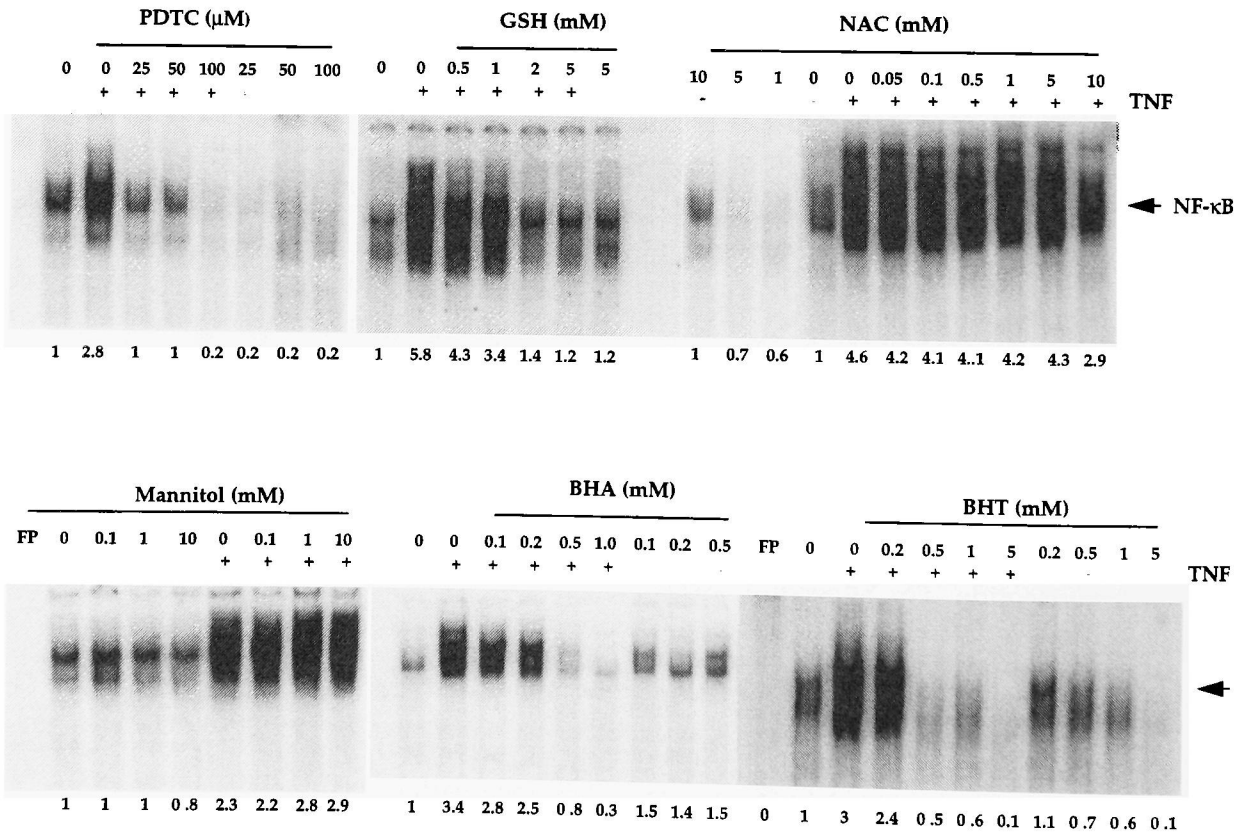

FIG. 1. Effect of various antioxidants on TNF-induced NF-κB activation in U937 cells. Cells (2×10^6 cell/ml) were preincubated at 37°C with different concentrations of various antioxidants for 30 min and then activated with TNF (100 p*M*) for 30 min. After treatment, nuclear extracts were prepared and assayed for NF-κB activation as described in Materials and Methods. Units at the bottom of the figure show fold increase in the TNF-induced NF-κB activation as compared with untreated control cells.

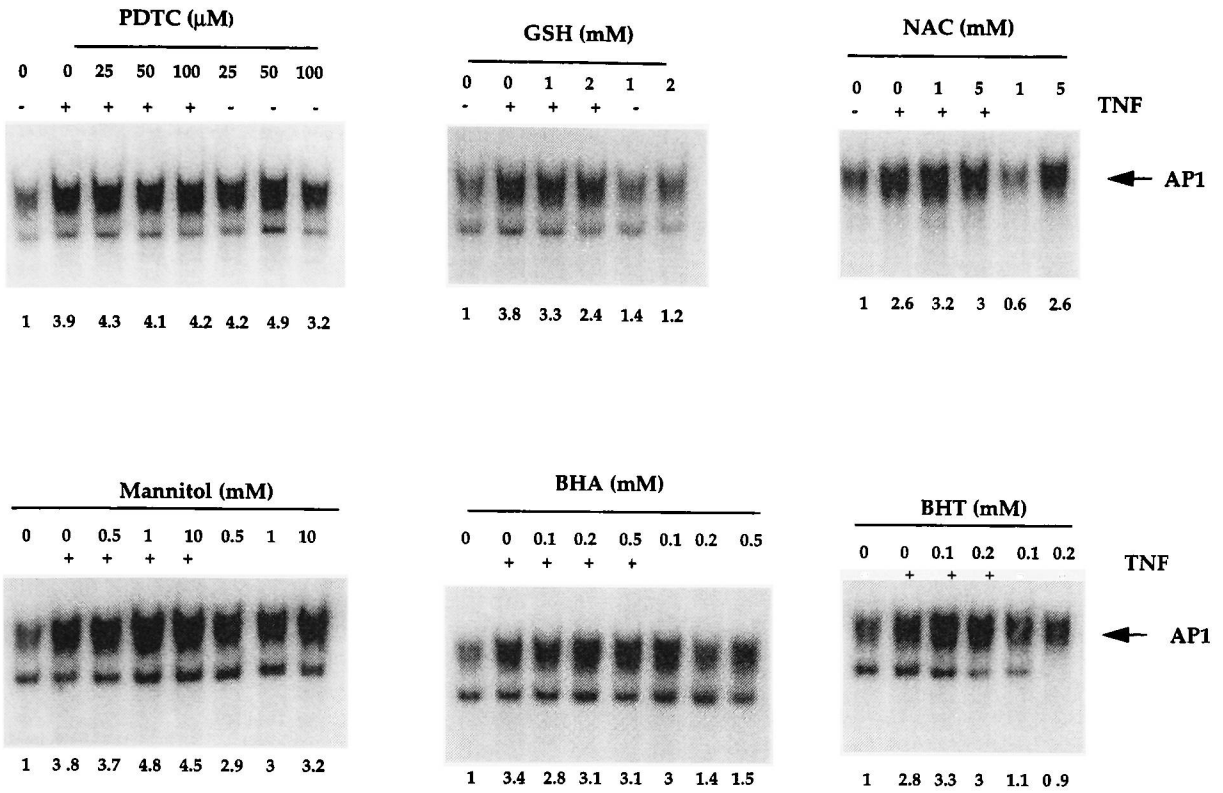

FIG. 2 Effect of various antioxidants on TNF-induced AP-1 activation in U937 cells. Cells (2×10^6 cell/ml) were preincubated at 37°C with different concentrations of various antioxidants for 30 min and then activated with TNF (100 p*M*) for 120 min. After treatment, nuclear extracts were prepared and assayed for AP-1 activation as described in Materials and Methods. Units at the bottom of the figure show fold increase in the TNF-induced AP-1 activation as compared with untreated control cells.

duced activation of NF-κB, AP-1, JNK, and caspase-3 and on TNF cytotoxicity. To quench superoxide radicals, PDTC, NAC, and GSH were used. Mannitol was used to quench hydroxyl radicals. BHT and BHA were used to abolish lipid peroxidation. The dose of these inhibitors for the time of exposure were not toxic to cells by themselves, nor were the vehicles in which they were dissolved (data not shown).

Both superoxide radical quenchers and lipid peroxidation inhibitors suppress and the hydroxyl radical quencher potentiates TNF-mediated NF-κB activation

U937 cells were pretreated with different concentrations of antioxidants for 30 min and then exposed to TNF (100 p*M*) for another 30 min. The results in Fig. 1 show that TNF-induced NF-κB is inhibited by PDTC, GSH, and NAC in a dose-dependent manner, with maximum effect at 25 μ*M*, 2 m*M*, and 10 m*M* respectively, suggesting that superoxide radical is needed for TNF-mediated NF-κB activation. NAC, a precursor of GSH, was least effective, inhibiting TNF-induced NF-κB at 10 m*M* by less than 50%. BHA and BHT also abolished activation in a dose-dependent manner, indicating the role of lipid peroxidation. Mannitol, the hydroxyl radical scavenger, did not inhibit TNF-induced NF-κB activation but potentiated it rather significantly. These observations suggest that the hydroxyl radical probably has no role in NF-κB activation in response to TNF in U937 cells, whereas superoxide anion and lipid peroxidation seem to play a significant role.

Specificity of the NF-κB band was confirmed by the observation that this band was supershifted by antibodies to either the p50 or p65 subunits of NF-κB, that it disappeared when unlabeled probe was used, and that it did not bind to oligonucleotide with mutated NF-κB sites (data not shown).

Antioxidants do not block TNF-induced AP-1 activation

TNF is also known to activate another transcription factor, AP-1 (Karin, 1995). The requirements for AP-1 activation by TNF differ from those of NF-κB (Meyer *et al.*, 1993). Therefore, we examined the effect of various antioxidants on activation of AP-1 induced by TNF. The results shown in Fig. 2 indicate that unlike their inhibition of TNF-induced NF-κB activity, none of the antioxidants inhibited TNF-induced AP-1 activation. The antioxidants PDTC, mannitol, and BHA by themselves upregulated AP-1 activity. The complete disappearance of the AP-1 band in competition assays with unlabeled AP-1-specific oligonucleotide confirmed the specificity of AP-1 activation (data not shown).

JNK activation by TNF is inhibited by both superoxide and lipid peroxidation quenchers but potentiated by hydroxyl radical quencher

c-Jun is one of the components of heterodimeric AP-1. The phosphorylation of c-Jun at the amino-terminus leads to activation of its transcriptional activity and is mediated by a MAP kinase, JNK. Earlier studies showed that besides NF-κB and AP-1, TNF is also a potent inducer of JNK activation (Karin, 1995). Therefore, we examined the effect of antioxidants on TNF-induced JNK activation. JNK activity peaked within 15 min of TNF treatment of U-937 cells (data not shown). Thus, in subsequent experiments, cells were preincubated for 30 min with different antioxidants and then activated for JNK by exposure to TNF for 15 min. As shown in Fig. 3, most of the antioxidants had a biphasic dose effect on TNF-induced JNK activity. Both PDTC and GSH enhanced TNF-induced JNK activation at lower doses, but inhibited it at higher doses. NAC inhibited JNK activation at 10 m*M* and above concentration. Mannitol on the other hand appeared to be stimulatory: 100 m*M* mannitol by itself activated JNK about six-fold compared to untreated control. At this dose, mannitol showed an additive effect on TNF-induced JNK activity in these cells. BHA at the highest doses (0.5 and 1 m*M*) significantly inhibited TNF-induced JNK activation, whereas at 0.2 m*M* it could activate JNK activity, although not by much. On the other hand, BHT inhibited TNF-induced JNK activity at the lowest with two doses only: at the highest dose, activated JNK activity and, in the presence of TNF, showed an additive effect.

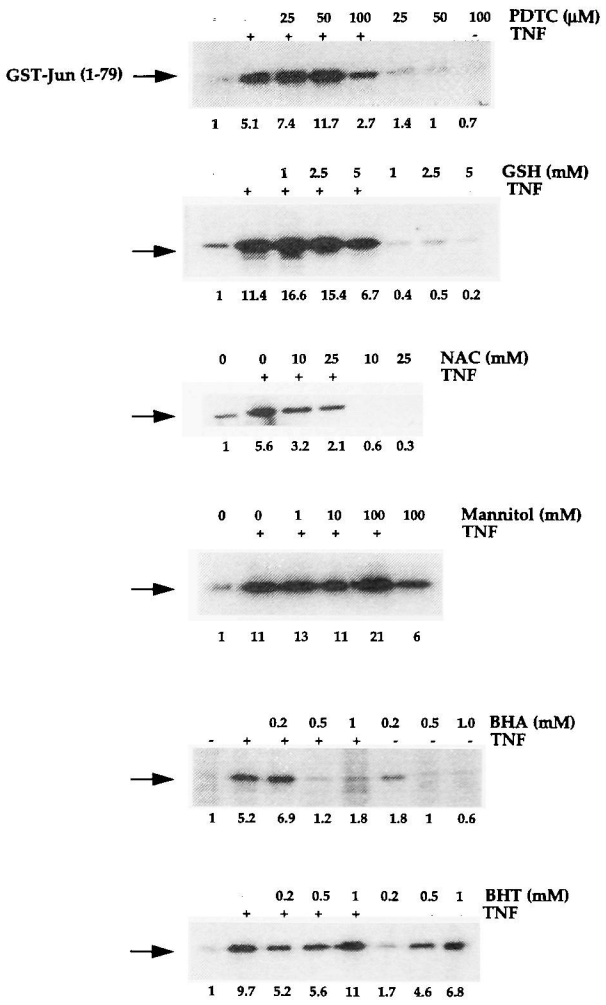

FIG. 3. Effect of various antioxidants on TNF-induced JNK activation in U-937 cells. Cells (3×10^6 cell/ml) were preincubated at 37°C with different concentrations of various antioxidants for 30 min and then activated with TNF (100 p*M*) for 15 min. After treatment, nuclear extracts were prepared and assayed for JNK activation as described in Materials and Methods. Units at the bottom of the figure show the fold increase in the TNF-induced JNK activation as compared with untreated control cells.

Activation of caspases by TNF is blocked by most antioxidants

TNF exhibits its cytotoxic effects by inducing either necrosis or apoptosis. One of the hallmarks of apoptosis is activation of caspases, a

protease that cleaves PARP substrate, reducing it from 116 kDa to 85 kDa (Tewari *et al.*, 1995). When U937 cells were treated with 1 n*M* TNF in the presence of 10 μg/ml cycloheximide, cleavage of PARP occurred within 2 hr (data not shown). In cells that were pretreated with different concentrations of antioxidants followed by TNF (1 n*M*) treatment for 2 hr, TNF-induced PARP cleavage was unaffected by PDTC, whereas GSH and NAC both inhibited caspase activation (Fig. 4). Higher doses of NAC appeared to be less protective. Mannitol, a hydroxyl radical scavenger, completely blocked caspase-3 activation. The lipid peroxidation (LPO) inhibitor BHA suppressed caspase activation. In contrast BHT, another LPO inhibitor was not effective in blocking TNF-induced PARP cleavage, suggesting that the two LPO inhibitors act differently.

Hydroxyl radical quencher blocks TNF-induced cytotoxicity

It has been previously shown that manganese superoxide dismutase (MnSOD) blocks the anti-proliferative effects of TNF (Wong *et al.*, 1989; Li and Oberley, 1997). Overexpression of MnSOD in MCF-7 cells protected them from TNF-mediated killing (Manna et al., 1998). We determined whether superoxide radical quenchers block the antiproliferative effects of TNF. PDTC, NAC, and GSH, all three superoxide radical quenchers, did not inhibit the antiproliferative effects of TNF (Fig. 5). Instead,

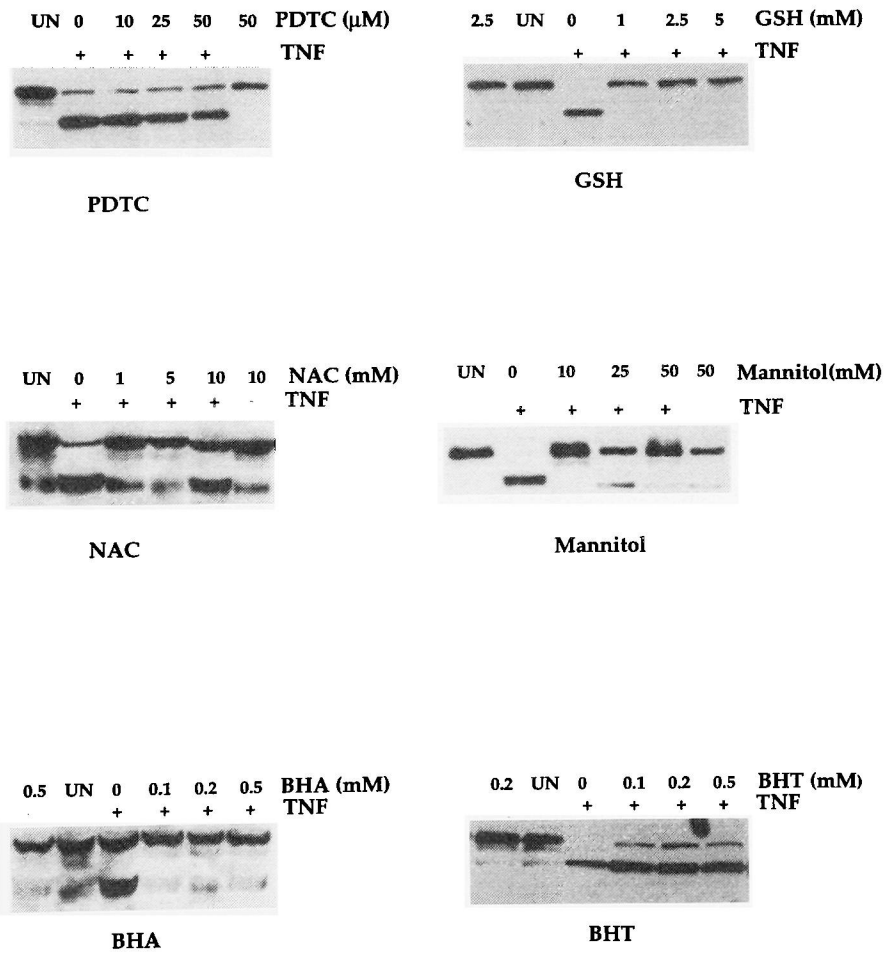

FIG. 4. Effect of various antioxidants on TNF-induced caspase-3 activation in U-937 cells. Cells (2×10^6 cell/ml) were preincubated at 37°C with different concentrations of various antioxidants for 30 min and then activated with TNF (1 n*M*) in the presence of 10 μg/ml cycloheximide for 2 hr. After treatment, cytoplasmic extracts were prepared and assayed for PARP cleavage by western blot as described in Materials and Methods.

they slightly potentiated the effects of TNF. BHT also enhanced the effects of TNF. Similar to caspase activation, however, BHA slightly suppressed the antiproliferative effects of TNF. In comparison to all antioxidants, mannitol was most effective in blocking the antiproliferative effects of TNF. These observations thus suggest that hydroxyl radical is most critical for the antiproliferative effects of TNF.

DISCUSSION

The aim of the present report was to investigate the role of ROS in TNF-induced activation of NF-κB, AP-1, JNK, and apoptosis. Different antioxidants known to scavenge/inhibit specific ROS or ROS-induced effects were used. Normally, extracellular signals lead to generation of superoxide radical that is quickly converted into H_2O_2 by dismutases. This H_2O_2 is converted either into water in the presence of GSH, NAC, or catalase or else into a hydroxyl radical in the presence of metal ions like Fe^{2+} released from (4Fe/4S) clusters of dehydrogenases upon superoxide attack by the Fenton or Herber-Weiss reaction (Halliwell and Cross, 1994). Alternatively, a hydroxyl radical can react with other molecules such as lipids, causing LPO in the presence of Fe^{2+} and disturbing the membrane fluidity and integrity of the plasma membrane.

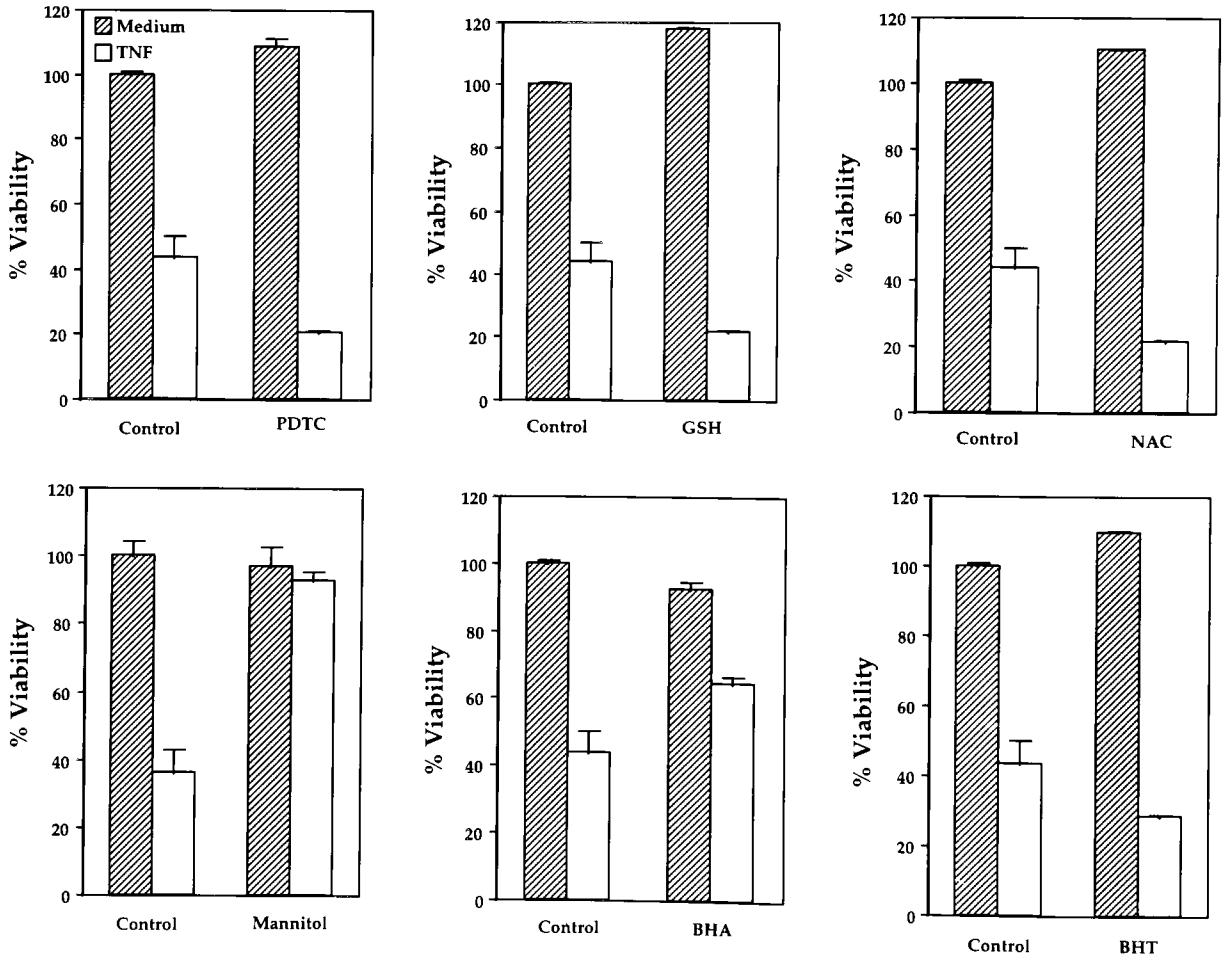

FIG. 5. **Effect of various antioxidants on the antiproliferative effects of TNF in U-937 cells.** Cells (5×10^3 cell/0.1 ml) were preincubated at 37°C with PDTC (20 m*M*), GSH (2 m*M*), NAC (2 m*M*), mannitol (10 m*M*), BHA (0.2 m*M*), or BHT (0.2 m*M*) for 30 min and then activated with TNF (1 n*M*) for 72 hr. After incubation, cell viability was determined by the MTT method, as described in Materials and Methods. Results are normalized to the untreated control and expressed as percent viability.

In our study, TNF-induced NF-κB activation was inhibited by PDTC, GSH, and NAC as well as by BHA and BHT, both inhibitors of LPO whereas mannitol, a hdyroxyl radical scavenger, showed no effect on TNF-induced activation of NF-κB. Thus, LPO may be an important event for NF-κB activation by TNF. Our results are in agreement with a report (Bowie *et al.*, 1997), which showed that LPO is important for NF-κB activation induced by TNF in endothelial cells. They also showed that TNF-induced NF-κB activation was not inhibited but rather slightly potentiated by the hydroxyl radical quencher mannitol. In addition, they showed no role for H_2O_2 in TNF-induced NF-κB activation (Bowie *et al.*, 1997).

In our study, although inhibitors of superoxide radicals and LPO blocked NF-κB activation, they had no effect on AP-1 activation. PDTC, which also has an iron-chelating property apart from its radical scavenging effect, could be responsible for down-regulation of ·OH and LPO, activates AP-1 by itself and upregulated TNF-induced AP-1 activity. BHA and mannitol had similar effects, whereas GSH had no effect on TNF-induced AP-1 activity, suggesting that the $\cdot O_2^-$ radical is mainly responsible for AP-1 activation. Because PDTC blocks the conversion of $\cdot O_2^-$ to ·OH or LPO, whereas mannitol and BHA scavenge ·OH and inhibit LPO, respectively, it is unlikely that either ·OH or LPO are important in activation of AP-1. H_2O_2 also seems to have no direct effect on AP-1 activity.

Similarly, these antioxidants had differential effects on TNF-induced JNK activation. PDTC, GSH, and NAC themselves did not activate JNK activity, but they did upregulate TNF-induced JNK activity, whereas mannitol itself activated JNK activity at higher concentration and had an additive effect on TNF-induced JNK activity. On the other hand, the LPO inhibitors, BHA at a lower dose and BHT at a higher dose, could activate JNK on their own. These results agree with a recent report of JNK activation by BHA (Yu *et al.*, 1997). These authors also showed that BHA-induced JNK activation is resistant to NAC and GSH. Doses of BHA and BHT that did not activate JNK inhibited TNF-induced JNK activation. These results indicate that H_2O_2 may be responsible for JNK activation, inasmuch as NAC, GSH, and PDTC at higher doses inhibited TNF-induced JNK activity.

Although the role of ROS in the antiproliferative effects of TNF was well documented, which oxygen species is involved was not known. Our results suggest that mannitol completely blocks the antiproliferative effects of TNF, suggesting the role of the hydroxyl radical. These results are in agreement with a recent report which showed that DMSO, another hydroxyl radical quencher, blocks Fas-mediated apoptois in Jurkat cells (Bauer *et al.*, 1998). PDTC has also been reported to block apoptosis in Jurkat cells induced by TNF (Giri and Aggarwal, 1998). In the present study, PDTC had no effect on TNF-induced antiproliferative effects in U937 cells, thus suggesting the effects were cell-type specific.

Our results show that BHA differs from BHT in that BHA suppresses the TNF-mediated antiproliferative effects. This difference between BHT and BHA is consistent with a previous report which showed that the cytotoxic effects of TNF in L-929 cells are suppressed by BHA but not BHT (Brekke *et al.*, 1992). We found that both mannitol and BHA also blocked caspase activation, consistent with its antiproliferative effects. With other antioxidants, however, no relationship was found between caspase-3 activation and the antiproliferative effects of TNF. For instance GSH and NAC blocked caspase activation but not the antiproliferative effects. This is consistent with our recent report with respiration-deficient cells in which these two activities of TNF were found not to be linked (Higuchi *et al.*, 1997). GSH and NAC was recently shown to block TNF-induced cell death through inhibition of neutral sphingomyelinase in human breast cancer MCF-7 cells (Liu *et al.*, 1998). This correlated with inhibition of caspase-3. The latter is in agreement with our results. Recently, we also found that overexpression of γ-glutamylcysteine synthetase, a rate-limiting enzyme in GSH synthesis, blocks TNF-induced activation of NF-κB, JNK, AP-1, and apoptosis (Manna *et al.*, 1999).

Our results show that mannitol inhibits TNF-induced cytotoxicity without inhibiting NF-κB activation, whereas PDTC blocks NF-κB with-

out blocking cytotoxicity, thus suggesting that the signal needed for NF-κB activation is different from that needed for cytotoxicity. Furthermore NF-κB activation occurs independent of cytotoxicity. These results are consistent with reports indicating that NF-κB activation is not linked with TNF-induced cytotoxicity (Cai *et al.*, 1997; Darnay and Aggarwal, 1997; Guo *et al.*, 1998; Hehner *et al.*, 1998; Roulston *et al.*, 1998). Our results also indicate that JNK activation plays no role in TNF-induced cytotoxicity, which is consistent with some (Liu *et al.*, 1996) but not other reports (Guo *et al.*, 1998; Roulston *et al.*, 1998). The differences may be due to the cell type used. Overall, our results suggest that ROS are needed for all signals transduced by TNF, but the nature of the ROS may vary depending on the signal.

ACKNOWLEDGMENT

This research was conducted by the Clayton Foundation for Research. This is CRL contribution number 215.

ABBREVIATIONS

AP-1, activator protein-1; BHA, butylated hydroxy anisole; BHT, butylated hydroxy toluene; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FCS, fetal calf serum; GSH, glutathione; H_2O_2, hydrogen peroxide; HIV, human immunodeficiency virus; JNK, c-Jun amino-terminal kinase; LPO, lipid peroxidation; LTR, long terminal repeat; Mn-SOD, manganese superoxide dismutase; MTT, 3-(4,5-dihydro-6-(4-(3,4-dimethoxybenzoil)-1-piperazinyl-2 (1*H*)-quinolone; NAC, *N*-acetyl-L-cysteine; OD, optical density; NF-κB, nuclear factor κB; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP) ribose polymerase; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PMSF, phenyl methyl sulfonyl fluoride; ROI, reactive oxygen intermediates; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor.

REFERENCES

ANDERSON, M.T., STAAL, F.J., GITLER, C., HERZENBERG, L.A., and HERZENBERG, L.A. (1994). Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway. Proc. Natl. Acad. Sci. USA **91,** 11527–11531.

BAEUERLE, P.A., and BAICHWAL, V.R. (1997). NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. Adv. Immunol. **65,** 111–137.

BAUER, M.K.A., VOGT, M., LOS, M., SIEGEL, J., WESSELBORG, S., and SCHULZE-OSTHOFF, K. (1998). Role of reactive oxygen intermediates in activation-induced CD95 (APO-1/Fas) ligand expression. J. Biol. Chem. **273,** 8048–8055.

BEG, A.A., and BALTIMORE, D. (1996). An essential role for NF-KappaB in preventing TNF-α-induced cell death Science **274,** 782–784.

BOWIE, A.G., MOYNAGH, P.N., and O'NEILL, L.A.J. (1997). Lipid peroxidation is involved in the activation of NF-κB by tumor necrosis factor but not interleukin-1 in the human endothelial cell line ECV304: Lack of involvement of H_2O_2 in NF-κB activation by either cytokine in both primary and transformed endothelial cells. J. Biol. Chem. **272** 25941–25950.

BREKKE, O.L., SHALABY, M.R., SUNDAN, A., ESPEVIK, T., and BJERVE, K.S. (1992). Butylated hydroxyanisole specifically inhibits tumor necrosis factor-induced cytotoxicity and growth enhancement. Cytokine **4,** 269–280.

CAI, Z., KORNER, M., TARANTINO, N., and CHOUAIB, S. (1997). IkB-a overexpression in human breast carcinoma MCF7 cells inhibits nuclear factor-κB activation but not tumor necrosis factor-alpha-induced apoptosis. J. Biol. Chem. **272,** 96–101.

CHATURVEDI, M.M., LAPUSHIN, R., and AGGARWAL, B.B. (1994). Tumor necrosis factor and lymphotoxin. Qualitative and quantitative differences in the mediation of early and late cellular response. J. Biol. Chem. **269,** 14575–14583.

DARNAY, B.G., and AGGARWAL, B.B. (1997). Early events in TNF signaling: A story of associations and dissociations. J. Leukocyte Biol. **61,** 559–566.

GIRI, D.K., and AGGARWAL, B.B. (1998). Constitutive activation of NF-kappaB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. J. Biol. Chem. **273,** 14008–14014.

GOOSSENS, V., GROOTEN, J., DE VOS, K., and FIERS, W. (1995). Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc. Natl. Acad. Sci. USA **92,** 8115–8119.

GUO, Y.L., BAYASAL, K., KANG, B., YANG, L.J., and WILLIAMSON, J.R. (1998). Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-alpha in rat mesangial cells. J. Biol. Chem. **273,** 4027–4034.

HALLIWELL, B., and CROSS, C.E. (1994). Oxygen-de-

rived species: their relation to human disease and environmental stress. Environ. Health Persp. **102**(Suppl. 10), 5–12.

HANSEN, M.B., NIELSEN, S.E., and BERG, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods **119,** 203.

HARIDAS, V., DARNAY, B., NATARAJAN, K., HELLER, R., and AGGARWAL, B.B. (1998). Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, NF-κB activation, and c-Jun kinase activation, J. Immunol. **160,** 3152–3162.

HEHNER, S.P., HOFMANN, T.G., RATTER, F., DUMONT, A., DROGE, W., and SCHMITZ, M.L. (1998). Tumor necrosis factor-alpha-induced cell killing and activation of transcription factor NF-kappaB are uncoupled in L929 cells. J. Biol. Chem. **273,** 18117–81121.

HIGUCHI, M., AGGARWAL, B.B., and YEH, E.T. (1997). Activation of CPP32-like protease in tumor necrosis factor-induced apoptosis is dependent on mitochondrial function. J. Clin. Invest. **99,** 1751–1758.

KARIN, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. **270,** 16483.

LI, J.J., and OBERLEY, L.W. (1997). Overexpression of manganese-containing superoxide dismutase confers resistance to the cytotoxicity of tumor necrosis factor α and/or hyperthermia. Cancer Res. **57,** 1991–1998.

LIU, Z-G., HSU, H., GOEDDEL, D.V., and KARIN, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87,** 565–576.

LIU, B., ANDRIEU-ABADIE, N., LEVADE, T., ZHANG, P., OBEID, L.M., and HANNUN, Y.A. (1998). Glutathione regulation of neutral sphingomyelinase in tumor necrosis factor-alpha-induced cell death. J. Biol. Chem. **273,** 11313–11320.

MANNA, S.K., KUO, M.T., and AGGARWAL, B.B. (1999). Overexpression of γ-glutamylcysteine synthetase abolishes tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappa B and activator protein-1. Oncogene (in press).

MANNA, S.K., ZHANG, H.J., YAN, T., OBERLEY, L.W., and AGGARWAL, B.B. (1998). Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. J. Biol. Chem. **273,** 13245–13254.

MEYER, M., SCHRECK, R., and BAEUERLE, P.A. (1993). H_2O_2 and antioxidants have opposite effects on activation of NF-kappa B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J. **12,** 2005–2015.

ROULSTON, A., REINHARD, C., AMIRI, P., and WILLIAMS, L.T. (1998). Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor-α. J. Biol. Chem. **273,** 10232–10239.

SCHRECK, R., 1992. SCHRECK, R., MEIER, B., MANNEL, D.N., DROGE, W., and BAEUERLE, P.A. (1992). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J. Exp. Med. **175,** 1181–1194.

SCHULZE-OSTHOFF, K., BAKKER, A.C., VANHAESEBROECK, B., BEYAERT, R., JACOB, W.A., and FIERS, W. (1992). Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J. Biol. Chem. **267,** 5317–5323.

SCHULZE-OSTHOFF, K., BEYAERT, R., VANDEVOORDE, V., HAEGEMAN, G., and FIERS, W. (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J. **12,** 3095–3104.

TEWARI, M., QUAN, L.T., O'ROURKE, K., DESNOYERS, S., ZENG, Z., BEIDLER, D.R., POIRIER, G.G., SALVESEN, G.S., and DIXIT, V.M. (1995). Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. Cell **81,** 801–809.

VAN ANTWERP, D.J., MARTIN, S.J., KAFRI, T., GREEN, D.R., and VERMA, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science **724,** 787–789.

WANG, C.Y., MAYO, M.W., and BALDWIN, A.S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science **274,** 784–787.

WONG, G.H.W., and GOEDDEL, D.V. (1988). Induction of manganese superoxide dismutase by tumor necrosis factor: Possible protection mechanism. Science **242,** 941–944.

WONG, G.H.W., ELWELL, J.H., OBERLEY, L.W., and GOEDDEL, D.V. (1989). Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell **58,** 923–931.

YU, R., TAN, T.H., and KONG, A.T. (1997). Butylated hydroxyanisole and its metabolite tert-butylhydroquinone differentially regulate mitogen-activated protein kinases. The role of oxidative stress in the activation of mitogen-activated protein kinases by phenolic antioxidants. J. Biol. Chem. **272,** 28962–28970.

Address reprint requests to:
Dr. Bharat B. Aggarwal
Cytokine Research Laboratory
Department of Molecular Oncology
The University of Texas M.D. Anderson Cancer Center
Box 143
1515 Holcombe Boulevard
Houston, TX 77030

This article has been cited by:

1. Christopher Runchel , Atsushi Matsuzawa , Hidenori Ichijo . 2011. Mitogen-Activated Protein Kinases in Mammalian Oxidative Stress Responses. *Antioxidants & Redox Signaling* **15**:1, 205-218. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
2. Chuntao Yang, Hongzhong Ling, Meifen Zhang, Zhanli Yang, Xiuyu Wang, Fanqin Zeng, Chuhuai Wang, Jianqiang Feng. 2011. Oxidative stress mediates chemical hypoxia-induced injury and inflammation by activating NF-#b-COX-2 pathway in HaCaT cells. *Molecules and Cells* **31**:6, 531-538. [CrossRef]
3. Amol V. Janorkar, Lacey M. Harris, Beau S. Murphey, Brittany L. Sowell. 2011. Use of three-dimensional spheroids of hepatocyte-derived reporter cells to study the effects of intracellular fat accumulation and subsequent cytokine exposure. *Biotechnology and Bioengineering* **108**:5, 1171-1180. [CrossRef]
4. Younes Smani, Fernando Docobo-Pérez, Michael J. McConnell, Jerónimo Pachón. 2011. Acinetobacter baumannii-induced lung cell death: Role of inflammation, oxidative stress and cytosolic calcium. *Microbial Pathogenesis* **50**:5, 224-232. [CrossRef]
5. Hwa Jin Moon, Woo Hyun Park. 2011. Butylated hydroxyanisole inhibits the growth of HeLa cervical cancer cells via caspase-dependent apoptosis and GSH depletion. *Molecular and Cellular Biochemistry* **349**:1-2, 179-186. [CrossRef]
6. Dong-Oh Moon, Mun-Ock Kim, Sang-Hyuck Kang, Yung Hyun Choi, Sung Yong Park, Gi-Young Kim. 2010. HA14-1 sensitizes TNF-#-induced apoptosis via inhibition of the NF-#B signaling pathway: Involvement of reactive oxygen species and JNK. *Cancer Letters* **292**:1, 111-118. [CrossRef]
7. Dong-Oh Moon, Mun-Ock Kim, Jae-Dong Lee, Yung Hyun Choi, Gi-Young Kim. 2010. Rosmarinic acid sensitizes cell death through suppression of TNF-#-induced NF-#B activation and ROS generation in human leukemia U937 cells. *Cancer Letters* **288**:2, 183-191. [CrossRef]
8. Derick Han , Maria D. Ybanez , Sara Ahmadi , Kelvin Yeh , Neil Kaplowitz . 2009. Redox Regulation of Tumor Necrosis Factor Signaling. *Antioxidants & Redox Signaling* **11**:9, 2245-2263. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
9. U RESCH, Y SCHICHL, S SATTLER, R DEMARTIN. 2008. XIAP regulates intracellular ROS by enhancing antioxidant gene expression. *Biochemical and Biophysical Research Communications* **375**:1, 156-161. [CrossRef]
10. Margret S. Rodrigues , Mamatha M. Reddy , Martin Sattler . 2008. Cell Cycle Regulation by Oncogenic Tyrosine Kinases in Myeloid Neoplasias: From Molecular Redox Mechanisms to Health Implications. *Antioxidants & Redox Signaling* **10**:10, 1813-1848. [Abstract] [Full Text PDF] [Full Text PDF with Links]
11. Johan Bylund, Kelly L. MacDonald, Kelly L. Brown, Piotr Mydel, L. Vincent Collins, Robert E. W. Hancock, David P. Speert. 2007. Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-independent activation of NF-#B. *European Journal of Immunology* **37**:4, 1087-1096. [CrossRef]
12. Val Vallyathan, Donna Pack, Steve Leonard, Robert Lawson, Marc Schenker, Vince Castranova. 2007. Comparative in Vitro Toxicity of Grape- and Citrus-Farm Dusts*. *Journal of Toxicology and Environmental Health, Part A* **70**:2, 95-106. [CrossRef]
13. D ICHIKAWA, M FUNAKOSHITAGO, E AIZUYOKOTA, Y SONODA, J INOUE, T KASAHARA. 2006. TNF-receptor associated factor 6-deficient fibroblast is sensitive to the TNF-#-induced cell death: Involvement of reactive oxygen species. *Biochemical and Biophysical Research Communications* **351**:1, 93-98. [CrossRef]
14. Jian Zou, Fulton Crews. 2006. CREB and NF-#B Transcription Factors Regulate Sensitivity to Excitotoxic and Oxidative Stress Induced Neuronal Cell Death. *Cellular and Molecular Neurobiology* **26**:4-6, 383-403. [CrossRef]
15. C Bubici, S Papa, K Dean, G Franzoso. 2006. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene* **25**:51, 6731-6748. [CrossRef]
16. James A. McCubrey , Michelle M. LaHair , Richard A. Franklin . 2006. Reactive Oxygen Species-Induced Activation of the MAP Kinase Signaling Pathways. *Antioxidants & Redox Signaling* **8**:9-10, 1775-1789. [Abstract] [Full Text PDF] [Full Text PDF with Links]

17. Janet V. Cross , Dennis J. Templeton . 2006. Regulation of Signal Transduction Through Protein Cysteine Oxidation. *Antioxidants & Redox Signaling* **8**:9-10, 1819-1827. [Abstract] [Full Text PDF] [Full Text PDF with Links]
18. Yukio Murakami, Masao Shoji, Atsushi Hirata, Shoji Tanaka, Shigemasa Hanazawa, Ichiro Yokoe, Seiichiro Fujisawa. 2006. An ortho dimer of butylated hydroxyanisole inhibits nuclear factor kappa B activation and gene expression of inflammatory cytokines in macrophages stimulated by Porphyromonas gingivalis fimbriae. *Archives of Biochemistry and Biophysics* **449**:1-2, 171-177. [CrossRef]
19. Dr. Irfan Rahman , Se-Ran Yang , Saibal K. Biswas . 2006. Current Concepts of Redox Signaling in the Lungs. *Antioxidants & Redox Signaling* **8**:3-4, 681-689. [Abstract] [Full Text PDF] [Full Text PDF with Links]
20. Mehdi Shakibaei , Gundula Schulze-Tanzil , Yasunari Takada , Bharat B. Aggarwal . 2005. Redox Regulation of Apoptosis by Members of the TNF Superfamily. *Antioxidants & Redox Signaling* **7**:3-4, 482-496. [Abstract] [Full Text PDF] [Full Text PDF with Links]
21. Prashanth Kenchappa, Ajay Yadav, Gurvinder Singh, Srinivas Nandana, Kakoli Banerjee. 2004. Rescue of TNF?-inhibited neuronal cells by IGF-1 involves Akt and c-Jun N-terminal kinases. *Journal of Neuroscience Research* **76**:4, 466-474. [CrossRef]
22. Suhua Han, Luis A Espinoza, Hongling Liao, A Hamid Boulares, Mark E Smulson. 2004. Protection by antioxidants against toxicity and apoptosis induced by the sulphur mustard analog 2-chloroethylethyl sulphide (CEES) in Jurkat T cells and normal human lymphocytes. *British Journal of Pharmacology* **141**:5, 795-802. [CrossRef]
23. R Uzarski. 2003. Potentiation of trichothecene-induced leukocyte cytotoxicity and apoptosis by TNF-# and Fas activation. *Chemico-Biological Interactions* **146**:2, 105-119. [CrossRef]
24. Ae Kyung Lee, Sang Hyun Sung, Young Choong Kim, Sang Geon Kim. 2003. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF- # and COX-2 expression by sauchinone effects on I-# B # phosphorylation, C/EBP and AP-1 activation. *British Journal of Pharmacology* **139**:1, 11-20. [CrossRef]
25. K Ramana. 2003. Aldose reductase mediates the mitogenic signals of cytokines. *Chemico-Biological Interactions* **143-144**, 587-596. [CrossRef]
26. A Garg. 2002. Reactive oxygen intermediates in TNF signaling. *Molecular Immunology* **39**:9, 509-517. [CrossRef]
27. John J Haddad. 2002. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cellular Signalling* **14**:11, 879-897. [CrossRef]
28. N Singh. 2002. Insights into the molecular mechanism of apoptosis induced by TNF-# in mouse epidermal JB6-derived RT-101 cells. *Biochemical and Biophysical Research Communications* **295**:1, 24-30. [CrossRef]
29. M Cho. 2002. Potent inhibition of lipopolysaccharide-inducible nitric oxide synthase expression by dibenzylbutyrolactone lignans through inhibition of I-#B# phosphorylation and of p65 nuclear translocation in macrophages. *International Immunopharmacology* **2**:1, 105-116. [CrossRef]
30. Mingqing Song , Nalini Santanam . 2001. Increased Myeloperoxidase and Lipid Peroxide-Modified Protein in Gynecological Malignancies. *Antioxidants & Redox Signaling* **3**:6, 1139-1146. [Abstract] [Full Text PDF] [Full Text PDF with Links]
31. Jean-Yves Matroule, Jacques Piette. 2000. Photosensitization and Redox Signaling. *Antioxidants & Redox Signaling* **2**:2, 301-315. [Abstract] [Full Text PDF] [Full Text PDF with Links]