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Redox-Dependent Regulation of Interleukin-8 by Tumor Necrosis Factor-α in Lung Epithelial Cells

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SUMMARY: Increasing evidence supports a major role for interleukin-8 (IL-8), a potent neutrophil chemoattractant, in the chronic progression of inflammatory lung diseases. The present studies were designed to characterize the molecular events involved in IL-8 induction in pulmonary epithelial cells in response to tumor necrosis factor-α (TNF-α). IL-8 induction by TNF-α was redox sensitive, as indicated by electron spin resonance analysis and inhibition with membrane permeable hydroxyl scavengers. Furthermore using cell transfection and mobility shift assays, it was found that transcriptional activation of the IL-8 gene required TNF-α–induced activation and binding of nuclear factor-κB (NF-κB)- and NF–IL-6, nuclear transcription factors to regulatory elements in the IL-8 promoter. Activation of the IL-8 promoter by these transcription factors was also redox-sensitive. This response was mediated through the TNF-R1 receptor (p55), and not the TNF-R2 (p75) receptor, although both receptors can be found on pulmonary epithelial cells. Taken together these studies indicate that TNF-α–induced redox changes in lung epithelial cells are responsible for the transcriptional activation of IL-8 and that coordinate activation of NF-κB and NF–IL-6 mediate the response. (*Lab Invest 1999, 79:1027–1037*).

Tumor necrosis factor-α (TNF-α) seems to be a central element in pulmonary inflammatory diseases because many aspects of lung damage can be directly or indirectly ascribed to its synthesis and administration of neutralizing antibodies to TNF or soluble TNF receptors prevent inflammation and development of fibrosis in experimental models (Piguet, 1994; Smart and Casale, 1994; Tosi et al, 1992). In addition to its cytotoxic activity for some transformed cell lines, TNF-α can enhance microvascular permeability, stimulate the expression of adhesion molecules on endothelial cells, and signal the expression of members of the chemokine gene superfamily which are responsible for leukocyte recruitment to inflammatory sites (Smart and Casale, 1994; Tosi et al, 1992). In respect to the latter, TNF-α induces the expression and release of interleukin-8 (IL-8) from alveolar macrophages and pulmonary epithelial cells (Kunkel et al, 1991). IL-8, a member of the α-chemokine family, is one of the most potent neutrophil chemoattractants and activators identified (Kunkel et al, 1991; Matsushima and Oppenheim, 1989). Neutrophil accumulation is believed to play a major role in lung tissue damage through the release of reactive oxygen species (ROS), which are generated during the respiratory burst, as well as the release of neutral proteases and neutrophil elastases. Thus neutrophils have been shown to be important effector cells in adult respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic bronchitis, cystic fibrosis, asbestosis, and some forms of asthma (Carre et al, 1991; McElvaney et al, 1992; Miller et al, 1992).

Cytokine production in the lung historically has been associated with activated alveolar macrophages. However recently it was demonstrated that type II pulmonary epithelial cells are not only a physical barrier for inhaled pollutants but are also able to synthesize and secrete cytokines. Type II cells have a distinct cytokine profile from alveolar macrophages because they can secrete significant quantities of IL-8 and other chemokines in the absence of the ability to produce TNF-α (Rosenthal et al, 1994; Standiford et al, 1990). Recent evidence has indicated that IL-8 induction in lung epithelial cells also can be mediated directly by intracellular redox changes (DeForge et al, 1992; Driscoll et al, 1993; Simeonova and Luster, 1996). For example fibrogenic fibers, such as asbestos or silicate, generate ROS in cells through Fenton-type reactions involving iron present as a silicate-iron coordination complex on the fiber surface (Ghio et al, 1992). ROS, in turn, activate the nuclear factor-κB (NF-κB) and NF–IL-6 transcription factors which help regulate IL-8 gene transcription (Meyer et al, 1994; Simeonova et al, 1997). Lung epithelial cells can also express IL-8 in response to TNF-α (Standiford et al, 1990). Because NF-κB is also a part of the TNF-α signaling pathways (Collart et al, 1990), and TNF-α generates ROS in many cell types (Hennent et al,

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1993; Schulze–Osthoff et al, 1992), we hypothesized that TNF-α can influence the intracellular redox state resulting in IL-8 transcription and expression in lung epithelial cells. Hence multiple signaling pathways may exist leading to the generation of ROS which control the progression and severity of inflammatory lung diseases.

Results

Both normal human bronchial epithelial (NHBE) and A549 cells readily produced immunoreactive IL-8 when cultured in the presence of TNF-α (Fig. 1). The TNF-α dose-response for IL-8 secretion was comparable in both cell types, although constitutive levels in NHBE cells were relatively higher than in A549 cells. The IL-8 response was not accompanied by cytotoxicity, measured by lactate dehydrogenase (LDH) release (Fig. 1, insert). The biologic activities of TNF-α are mediated by two structurally related, but distinct, receptors designated TNF-R1 (p55) and TNF-R2 (p75). Using reverse transcriptase-polymerase chain reaction (RT-PCR), both NHBE and A549 cells were found to constitutively express TNF-R1, but not TNF-R2 (Fig. 2). However TNF-R2 mRNA was moderately, but reproducibly, induced when the cells were stimulated with TNF-α. To determine which of these receptors were responsible for mediating IL-8 secretion, A549 and NHBE cells were cultured in the presence of mutated TNF binding proteins that recognize either the TNF-R1 or TNF-R2 receptor. Incubation of the cells with the TNF-R1 binding protein (m55) resulted in IL-8 secretion at levels equal to that after treatment with wild-type TNF-α (Fig. 3). In contrast incubation with the TNF-R2 binding protein (m75) failed to stimulate IL-8 secretion, indicating that the response was mediated through the TNF-R1.

To determine the IL-8 promoter region responsible for TNF-α activation, A549 cells were transfected with chloramphenicol acetyltransferase (CAT) constructs containing deleted or mutated IL-8 promoter regions and the cells stimulated with TNF-α (Table 1). The addition of TNF-α induced a 14-fold increase in CAT expression in cells transfected with the −97/−69 IL-8 construct which contains binding sites for both the NF-κB and NF–IL-6 transcription factors. Mutation of either the NF-κB or NF-IL-6 binding sites completely abolished TNF-α responsiveness. DNA mobility shift assays were conducted to help characterize IL-8 gene regulation by TNF-α (Fig. 4). The −99/−66 fragment of the IL-8 promoter region, containing the adjacent NF–IL-6- and NF-κB–like binding sites, was used to probe nuclear extracts isolated from A549 cells. Whereas low levels of DNA binding activity were present constitutively in A549 cells, a marked increase occurred after incubation of the cells with TNF-α. The binding activity was almost completely prevented by the addition of excess unlabeled oligonucleotide competitor containing the NF–IL-6/NF-κB–like sites and partially inhibited when the nuclear extracts were preincubated with a competitor which recognizes either the NF-κB or NF–IL-6 binding site. Binding was not affected after incubation with an unrelated se-

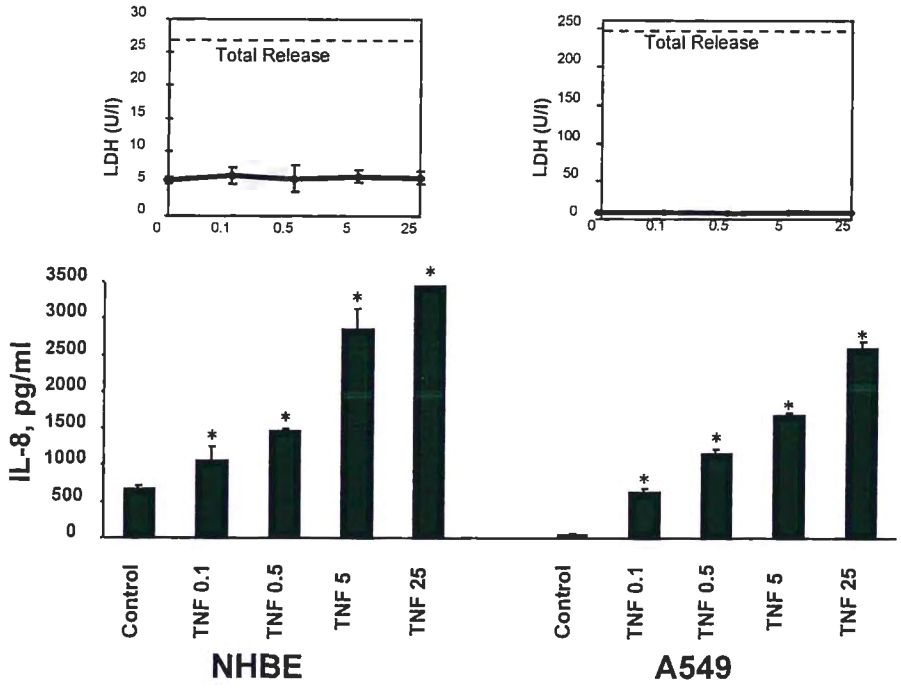

Figure 1.
Interleukin-8 (IL-8)secretion by tumor necrosis factor-α (TNF-α) in normal human bronchial epithelial (NHBE) or A549 cells. Cell cultures were incubated with TNF-α (0 to 25 ng/ml) for 18 hours and IL-8 was quantified in supernatants as indicated in "Material and Methods." Lactate dehydrogenase release was used as a measure of cell damage. Values represent the mean ± SE of three separate experiments. The *asterisk* represents a significantly different ($p < 0.05$) response from that of the control group.

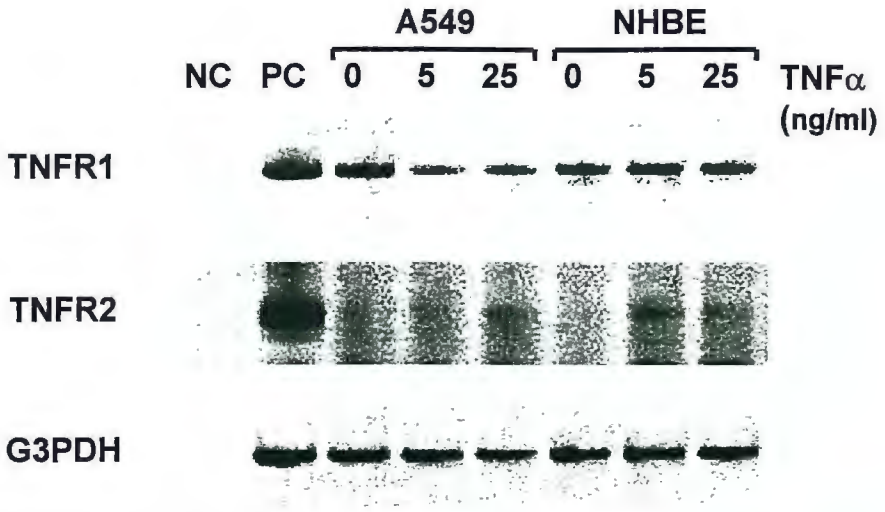

Figure 2.
Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of TNF-R1, TNF-R2, and glyceraldehyde 3-phosphate-dehydrogenase (G3PDH) mRNA transcripts from A549 and NHBE cells cultured for 2 hours in the presence of 0, 5, or 25 ng/ml TNF-α. cDNA equivalents of 0.1 μg of RNA were amplified for 30, 35, or 25 cycles for TNF-R1, TNF-R2, or G3PDH, respectively. *PC*, positive control; *NC*, negative control.

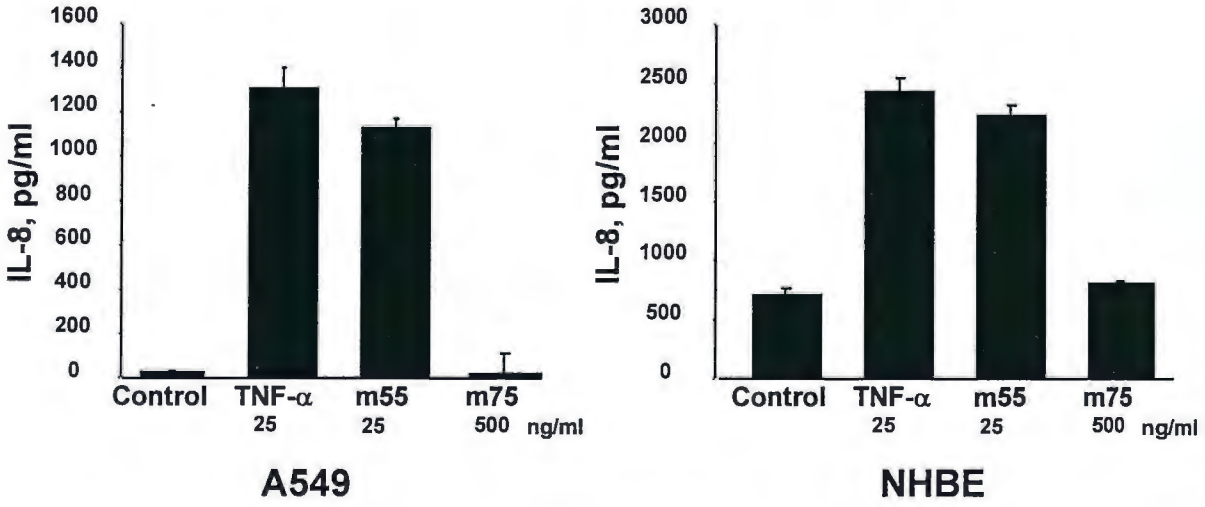

Figure 3.
IL-8 secretion by wild-type or mutated TNF-α proteins capable of binding either the TNF-R1(m55) or TNF-R2(m75) receptors. Cell cultures were treated for 18 hours and IL-8 secretion was quantified in supernatants by ELISA. Values represent the mean ± SE of three separate experiments.

Table 1. TNF-α–Induced CAT Activity in A549 Cells Transfected with IL-8 Promoter Constructs

Construct	Fold increase
−546	7
−97/69	14
−97/69 with mNF-κB*	0
−97/69 with mNF–IL-6*	0

TNF-α, tumor necrosis factor α; CAT, chloramphenicol acetyltransferase; IL-8, interleukin-8; NF-κB, nuclear factor-κB.
* m, mutated binding site.

quence containing the AP-1 binding site (data not shown).

Previous studies from our laboratory demonstrated that ROS are proximal mediators of IL-8 gene expression and secretion in pulmonary epithelial cells (Simeonova and Luster, 1996). To help evaluate whether oxidative stress is involved in IL-8 induction by TNF-α, an electron spin resonance (ESR) spin trapping technique was used to determine the ability of TNF-α to stimulate the formation of ROS in lung epithelial cell cultures. The technique measures radical generation by determining 5,5-dimethyl-1-Pyroline (DMPO)/OH· spin adduct formation. Rat pulmonary macrophages, which possess a marked ability to generate ROS under appropriate conditions, were also included. As shown in Figure 5, treatment of A594 cells with TNF-α induced a time-dependent increase in radical formation. The maximum peak height formed was comparable to that induced by phorbol myristate acetate (PMA), although less than that produced by rat alve-

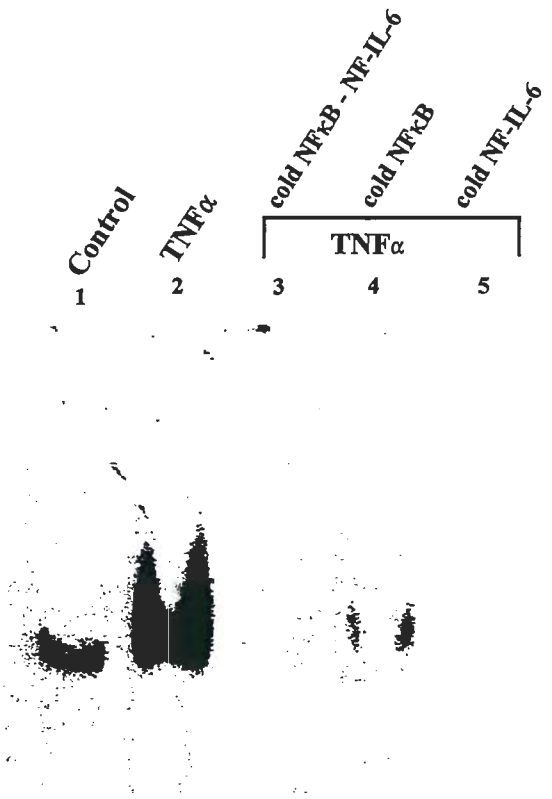

Figure 4.
Characterization of TNF-α–induced DNA binding activity to the nuclear factor (NF)–IL-6 and NF-κB–like binding sites. Nuclear extracts from control or TNF-α (10 ng/ml; 15 minutes) stimulated (*2*) A549 cells were analyzed by electrophoresis mobility shift assay. The ^{32}P -labeled −99/−66 sequence of the IL-8 promoter, which contains binding sites for NF-κB and NF–IL-6 was used as a probe. One hundred-fold excess of the unlabeled oligonucleotides, NF-κB/NF–IL-6 (*3*), NF-κB (*4*), or NF–IL-6 (*5*) were added as competitors.

olar macrophages. The spin adducts generated are DMPO/OH·. A DMPO/OH· signal indicates the formation of OH/O_2^-, because cellular enzymes can metabolize the DMPO/O_2^- adduct to DMPO/OH·. Further characterization of the DMPO adducts was conducted by adding specific antioxidants to the reaction mixture. Superoxide dismutase (SOD) inhibited DMPO/OH· formation only by 50%, demonstrating that the DMPO/OH· adducts observed partially involve O_2^- (data not shown). The ROS in TNF-α–induced IL-8 expression was further characterized using 1,1,3,3-tetramethyl-2-thiourea (TMTU), which is assumed as a derivative of thiourea to be a scavenger of OH· radicals. Preliminary studies were conducted to characterize the ability of TMTU to scavenge ROS in a cell free OH·-radical generating system using indirect ESR analysis. As shown in Figure 6, a mixture of H_2O_2 and $FeSO_4$ yielded an ESR spectrum consisting of a 1:2:2:1 quartet with splittings of $a_n = a_h = 149$ G, where $a_n = a_h$ denote the hyperfine splittings of the nitroxyl and α-hydrogen, respectively. Based on these splitting constants and the 1:2:2:1 line shape, this spectrum was assigned to the DMPO/OH· spin adduct. The addition of TMTU dose dependently reduced the relative peak. The DMPO/OH· spin adduct also were dose dependently reduced by 1,3-dimethyl-2-thiourea (DMTU), another derivative of thiourea, or N-acetylcysteine (NAC), a well characterized precursor of glutathione but with slightly weaker effect compared with TMTU (data not shown). In subsequent experiments TNF-α–stimulated A549 cells were cultured in the presence or absence of TMTU and the IL-8 secretion as well as the ESR spectra examined. As shown in Figure 7, pretreatment of A549 cells with TMTU dose-dependently and concomitantly inhibited both the TNF-α–induced formation of DMPO/OH· adducts and IL-8 secretion. Similar results were observed when NHBE cells were tested (data not shown). To examine whether inhibition of IL-8 secretion was reflected at the level of gene expression, RNA was isolated from A549 cells that had been incubated with TNF-α in the presence or absence of TMTU and the relative number of IL-8 and glyceraldehyde 3-phosphate-dehydrogenase (G3PDH) mRNA transcripts were determined by competitive RT-PCR (Fig. 8). The constitutively expressed gene, G3PDH, was used to adjust for equal cDNA concentrations. Based on linear regression analysis, it was estimated that TNF-α treatment produced an increase in IL-8 mRNA transcripts several orders in magnitude and TMTU reduced the response close to the control levels.

To determine the role of ROS in NF-κB and NF–IL-6 activation by TNF-α, A549 cells were transfected with the −97/−69 IL-8 construct and stimulated with TNF-α in the presence or absence of TMTU, DMTU, or NAC (Fig. 9). All of the antioxidants tested significantly reduced CAT expression by almost 50% or greater. Actin-luciferase construct activity, used to normalize for transfection efficiency, was not altered by treatment with TNF-α or the antioxidants and the antioxidants had no effect in the absence of TNF-α (data not shown).

Discussion

Chronic inflammatory lung diseases, including idiopathic pulmonary fibrosis, chronic bronchitis, cystic fibrosis, asbestosis, silicosis, and some forms of asthma, are associated with marked neutrophil accumulation. Neutrophils are thought to contribute significantly to the pathophysiology of inflammatory lung diseases by causing tissue damage through the release of ROS, elastases, and neutral proteases. This influx of neutrophils into the lung is mediated by chemotactic factors released from pulmonary macrophages and epithelial cells including the C5a complement component, leukotriene B4, platelet activating factor, and chemokines (Bokoch, 1995). Regarding the latter, IL-8, a member of the α-chemokine family, represents one of the most potent and stable neutrophil chemotactic factors known (Kunkel et al, 1991) and a variety of experimental approaches have demonstrated its presence and importance in inflammatory lung diseases (Carre et al, 1991; McElvaney et al, 1992; Miller et al, 1992). The regulation of IL-8 gene expression seems to be relatively complex. Like most inflammatory mediators, IL-8 expression can be induced by inflammatory cytokines, particularly TNF-α,

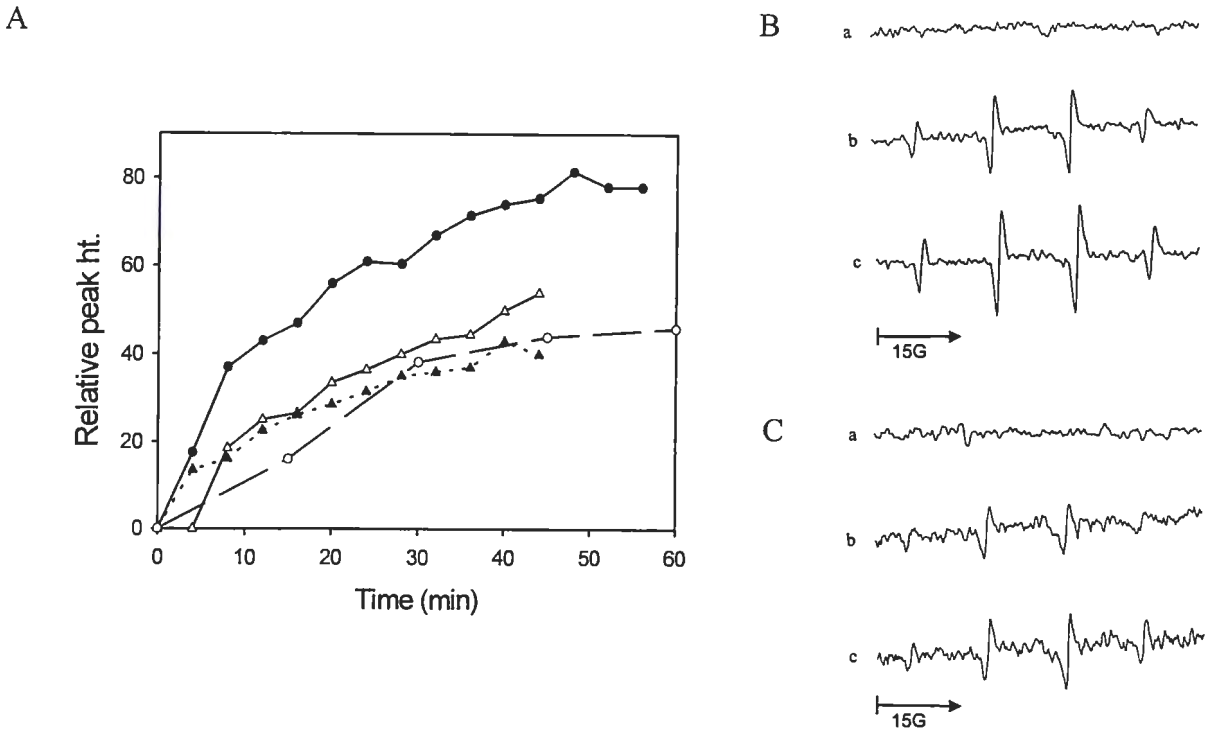

Figure 5.

Time-dependent quantitation of reactive oxygen species (ROS) generation in A549 cells or rat alveolar macrophages by electron spin resonance (ESR) analysis. A, The spin adducts were measured at 60 minutes by a Varian E9 ESR spectrometer and are presented as relative peak height. A549 cells plus 50 ng/ml TNF-α (○) or phorbol myristate acetate (PMA) (▲); alveolar macrophages plus 50 ng/ml TNF-α (●) or PMA (△) B, Spin adducts produced by alveolar macrophages in the presence of TNF-α: *a*, 0 minutes; *b*, 24 minutes; *c*, 48 minutes. C, Spin adducts produced by A549 cells in the presence of TNF-α: *a*, 0 minutes; *b*, 30 minutes; *c*, 60 minutes.

through the activation of specific transcription factors such as NF-κB, and their subsequent binding to DNA sites located in the gene's promoter region. The IL-8 gene also can be activated by oxidative stress (DeForge et al, 1992; Simeonova and Luster, 1996). Accordingly it has been shown that NF-κB is a redox/oxidative-responsive element (Meyer et al, 1994) and we have extended these observations to demonstrate that binding of the NF-κB and NF–IL-6 transcription factors to the IL-8 or IL-6 promoters is highly sensitive to changes in the intracellular redox state (Simeonova and Luster, 1996; Simeonova et al, 1997).

In the present studies, we examined TNF-α–mediated IL-8 signaling in lung epithelial cells and observed that the signaling events, including transcriptional regulation, are also redox sensitive. This process involves a TNF-mediated oxidative response resulting in the activation of NF-κB and NF–IL-6 and their subsequent binding to regulatory elements within the IL-8 promoter. ESR analysis further demonstrated that lung epithelial cells, like alveolar macrophages, generate ROS in response to TNF-α which consist primarily of OH· radicals and O_2^-. Additional evidence that IL-8 synthesis in lung epithelial cells is transcriptionally regulated was indicated by the ability of TNF-α to activate CAT expression in pulmonary epithelial cells transfected with the IL-8 promoter, CAT plasmid. These observations are consistent with those of Nakamura, et al (1991), who demonstrated that TNF-α stimulates IL-8 gene expression predominantly at the transcriptional level in bronchial epithelial cells. The 5′ flanking region of the IL-8 gene contains binding sites for several transcription factors, including AP-1, AP-2, hepatocyte nuclear factor, interferon regulatory factor 1, glucocorticoid response element, as well as NF-κB and NF–IL-6 (Mukaida et al, 1989). Experimental data suggests that different families of transcription factors and their interactions allow for the varied and coordinated regulation of IL-8 gene expression in a cell-specific manner (Mukaida et al, 1990). Using cell transfection and electrophoresis mobility shift assay (EMSA) methods, we demonstrated that the IL-8 promoter fragment, containing binding sites for NF-κB and NF–IL-6 transcription factors, is essential for TNF-α induction in pulmonary epithelial cells. Both transcription factors are necessary, as mutations in either one of the DNA binding sites abolish TNF-α–induced IL-8 activity. Studies with protein members of the C/EBP and NF-κB families of transcription factors indicated that functional, as well as direct protein-protein interactions occur between these factors as a result of the presence of a basic leucine-zipper region and Rel homology domain (Stein et al, 1993). The IL-8 promoter region that regulates TNF-α in the human bronchial squamous carcinoma cell line, HS-24, is associated with the −130/+44 IL-8 gene segment, which includes binding sites for AP-1, NF-κB, and NF–IL-6, and not to smaller fragments (Nakamura et al, 1991). The difference between these findings and ours probably reflects the differences in cell types

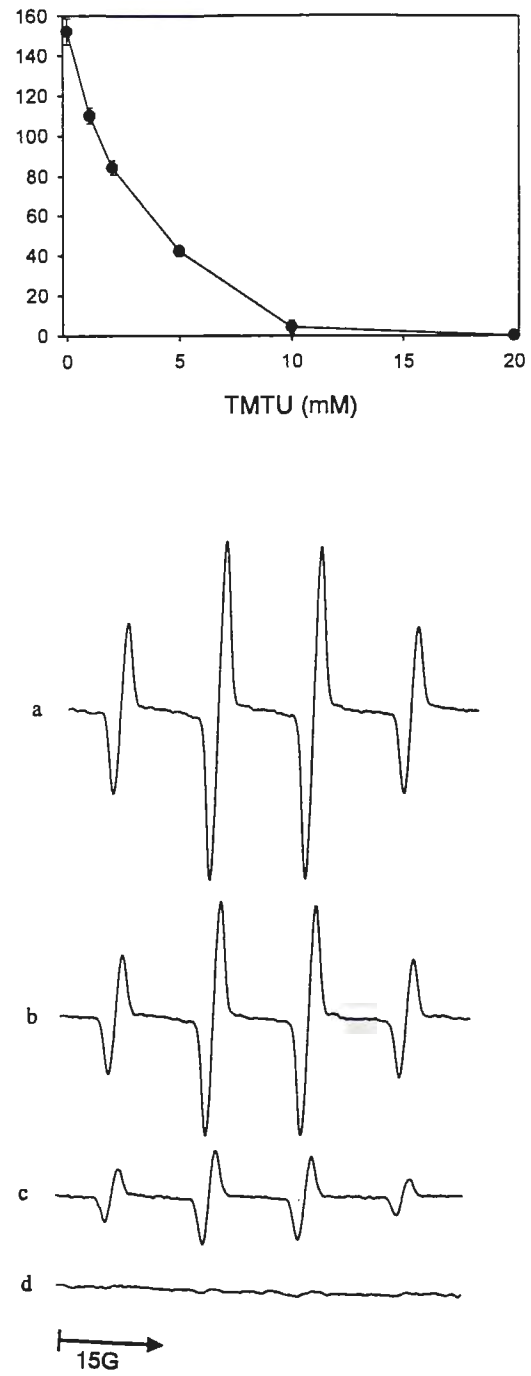

Figure 6.

ESR analysis of the OH· scavenging effect of 1,1,3,3-tetramethyl-2-thiourea (TMTU). A cell-free system containing $FeSO_4$ plus H_2O_2 was incubated with several concentrations of TMTU. ROS generation was measured as 5,5-dimethyl-1-Pyroline (DMPO)-adducts as described in "Materials and Methods." The spin adduct signals of: *a*, $FeSO_4$ plus H_2O_2; *b*, $FeSO_4$ plus H_2O_2 in the presence of 1 mM of TMTU; *c*, $FeSO_4$ plus H_2O_2 in the presence of 5 mM of TMTU; *d*, $FeSO_4$ plus H_2O_2 in the presence of 10 mM of TMTU; and *e*, spin adducts presented as relative peak height. Varian E9 ESR spectrometer was used.

studied. Mukaida, et al (1994), reviewing the data on IL-8 transcriptional regulation, suggested that although NF-κB is central for IL-8 transcription, cooperation with other transcription factors, particularly NF–IL-6 and AP-1, may be required for optimal and cell-specific expression.

TNF-α activities are mediated by two distinct cell surface receptors, designated TNF-R1 (p55) and TNF-R2 (p75), which belong to the TNF/nerve growth factor receptor superfamily. Although both receptors are coexpressed on the surface of most cell types, their degree of expression and function vary (Brockhaus et al, 1990; Darnay and Aggarwal, 1997; Hohmann et al, 1989; Tartaglia et al, 1991). We observed that TNF-R1 is constitutively expressed in lung epithelial cells, whereas TNF-R2 expression is inducible. Consistent with previous studies regarding the biologic role of TNF-R1, whereas NF-κB is activated after binding to either receptor, TNF-R1 alone is sufficient for IL-8 induction in lung epithelial cells. Even for the same receptor, multiple signaling pathways seem to exist for TNF-α mediated responses. Two distinct families of proteins, the TNF receptor associated factors and the death domain homologs help coordinate the cell- and tissue-specific responses in this complex signaling pathway (Darnay and Aggarwal, 1997). Increasing evidence suggests that the signal pathway used by TNF-α responsible for cytotoxicity involves the generation of ROS. Cytotoxic events by TNF-α, which occur independently of de novo transcription and translation, occurs through mitochondrial production of oxygen radicals generated primarily at the ubisemiquinone site (Garcia–Ruiz et al, 1997; Hennent et al, 1993; Slowik et al, 1996). This requires ceramide, a sphingolipid generated in cells after stimulation with TNF-α which generates H_2O_2 from the mitochondrial electron transport chain. In contrast to cytotoxicity, TNF-α regulates inflammatory processes through the induction of genes that code for mediators, such as IL-8. The extent ROS mediates these responses has not been established, although it is known that TNF-α can stimulate cytokine expression through the redox sensitive nuclear transcription factor, NF-κB (Miyamato et al, 1994). NF-κB, a dimer of the Rel family of proteins, becomes activated after dissociation of an inhibitor protein belonging to the IκB family. Events that occur before IκB dissociation are under intense investigation but involve protein kinases, proteases, protein phosphatases, sphingomyelinases or phospholipases, many of which are oxidant sensitive.

We provide several lines of evidence to support a role of ROS in TNF-mediated cytokine signaling. ESR measurement of DMPO/OH· adducts demonstrated a time-dependent induction of ROS, including OH· and O_2^-, in lung epithelial cells treated with TNF-α. Additionally pretreatment of the cells with OH·-radical scavenger, TMTU, demonstrated an inhibition of ROS generation and a corresponding reduction in TNF-α–induced IL-8 gene expression. At the molecular level, IL-8 gene transcription is triggered through NF-κB/NF–IL-6 binding sites contained within the IL-8 promoter which represent reflecting redox-sensitive elements. Consistent with redox-sensitive regulation, antioxidants down-regulate NF-κB/NF–IL-6 dependent IL-8 transcriptional activation and IL-8 expres-

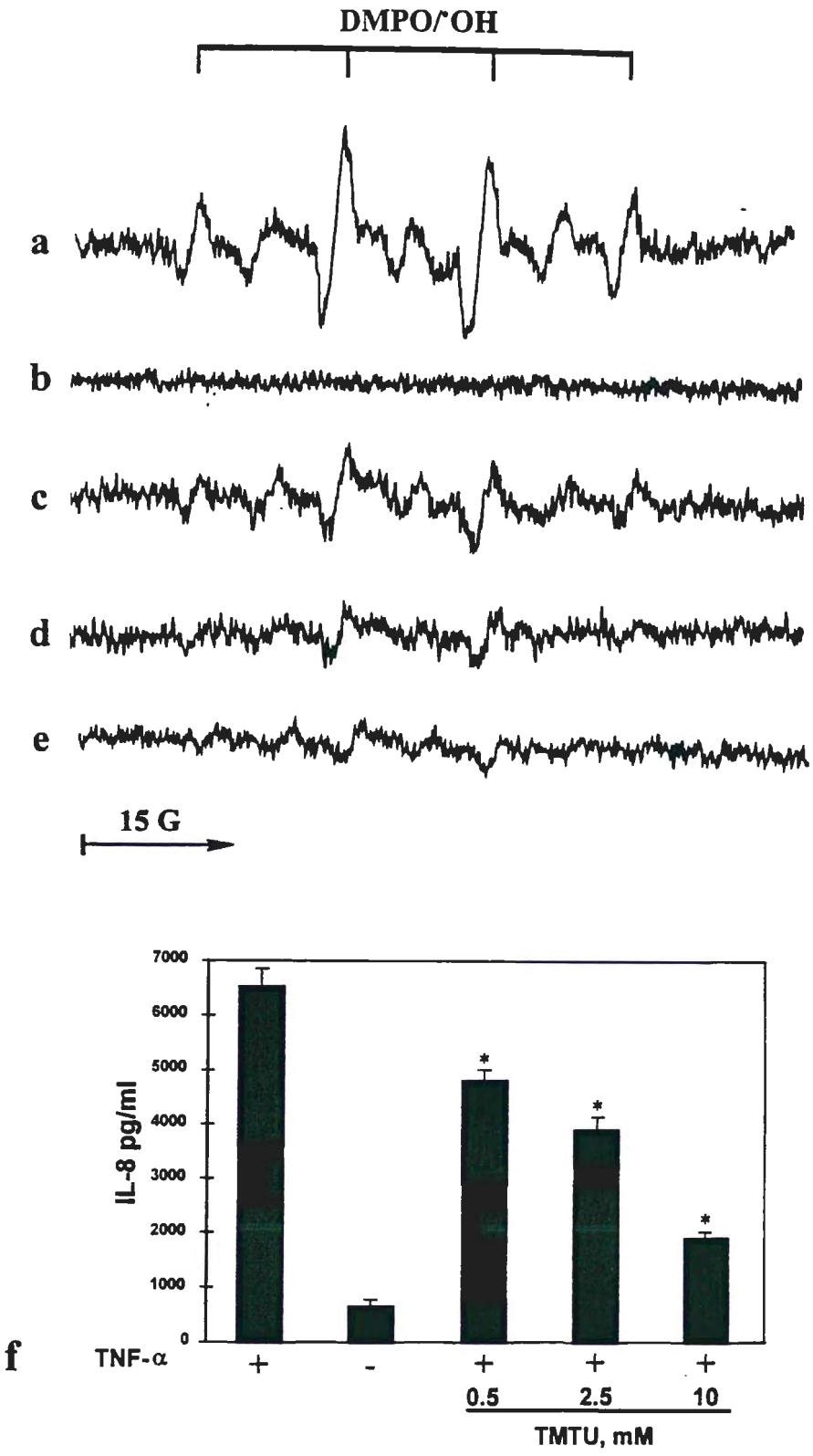

Figure 7.

TNF-α–induced DMPO/OH· adducts and IL-8 secretion in A549 lung epithelial cells in the presence of antioxidants. Cells (1×10^6) were pretreated for 0.5 hours with different concentrations of TMTU and exposed to 50 ng/ml of TNF-α. The spin adducts were measured by Bruker ER 300 ESR spectrometer at 40 minutes: *a*, A549 cells plus TNF-α; *b*, A549 cells; *c*, A549 cells plus 0.5 mM of TMTU and TNF-α; *d*, A549 cells plus 2.5 mM of TMTU and TNF-α; *e*, A549 cells plus 10 mM of TMTU and TNF-α; and *f*, IL-8 secretion of A549 cells measured by ELISA in 18 hours. The *asterisk* represents a significant ($p < 0.05$) decrease from cells treated with TNF-α alone. Values represent the mean ± SE of triplicate experiments.

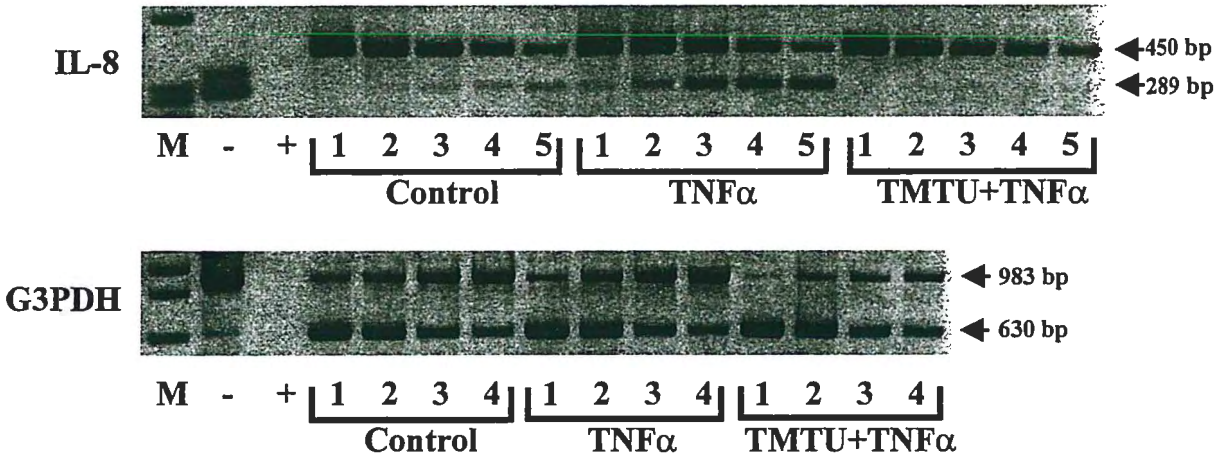

Figure 8.
Competitive RT-PCR analysis of IL-8 and G3PDH mRNA transcripts from A549 cells exposed for 30 minutes to 25 ng/ml TNF-α in the presence or absence of 10 mM of TMTU. cDNA equivalents of 0.1 μg of RNA were amplified for 28 or 25 cycles in the presence of 2- or 4-fold dilutions of competitors for IL-8 and G3PDH, respectively. For IL-8: top band, IL-8 competitor; bottom band, sample IL-8. Lane 1, 0.250 attomoles competitor; lane 2, 0.0625 attomoles; lane 3, 0.03 attomoles; lane 4, 0.015 attomoles; lane 5, 0.0075 attomoles. For G3PDH: top band, sample G3PDH; bottom band, G3PDH competitor. Lane 1, 10 attomoles; lane 2, 2.5 attomoles; lane 3, 0.6 attomoles; lane 4, 0.15 attomoles.

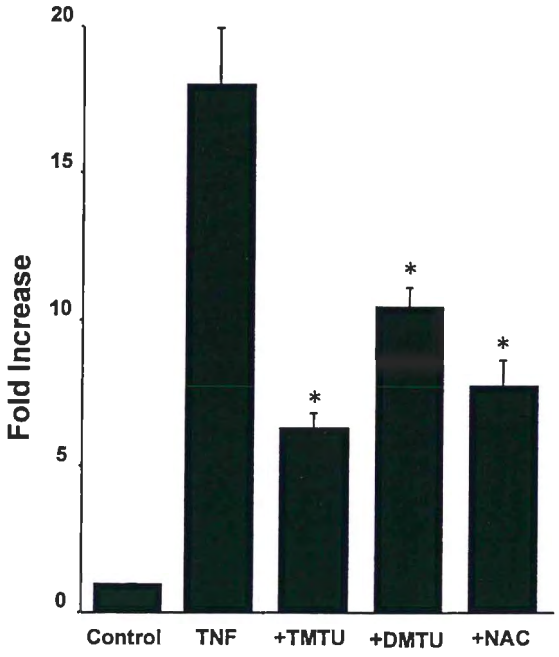

Figure 9.
Transfection studies showing inhibition of TNF-α–induced, NF-κB/NF–IL-6 dependent transcription by antioxidants. A549 cells were transfected with 1.5 μg of a −97/−69 IL-8 promoter construct linked to the chloramphenicol acetyltransferase (CAT) reporter plasmid and 0.5 μg of β-actin LUC control plasmid. The transfected cells were cultured with medium alone, TNF-α (25 ng/ml), or TNF-α in the presence of 10 mM of TMTU, DMTU, or NAC. Activity was determined by assaying the CAT concentration in cellular extracts prepared 24 hours after transfection. Transfection efficiencies were normalized by analyzing luciferase (LUC) activity. The results are presented relative to CAT levels in unstimulated cells. Experiments were repeated three times with representative data shown. The *asterisk* represents a significantly different ($p < 0.05$) response from that of the control.

sion. Further investigation of these molecular mechanisms should lead to a better understanding of the pathogenesis of chronic lung diseases and development of novel therapeutics.

Materials and Methods

Reagents

Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, Minnesota) or Endogen (Woburn, Massachusetts). The TNF-R1 (p55) specific binding mutant, Trp^{32} Thr^{86} TNF-α, and the TNF-R2 (p75) specific binding mutant, Asn ^{143} Arg^{145} TNF-α, were kindly provided by Dr. Loetscher (Hoffmann-La Roche Ltd., Basel, Switzerland). $FeSO_4$ TMTU, DMTU, NAC, and DMPO were purchased from Sigma Chemical Company (St. Louis, Missouri). Catalase (from bovine liver) was supplied from Calbiochem-Novabiochem Corporation (San Diego, California). H_2O_2 was from Fisher Scientific Company (Pittsburgh, Pennsylvania).

Cell Preparation

A549 cells, a pulmonary human type-II like epithelial cells, derived from an individual with alveolar cell carcinoma, were purchased from the American Type Culture Collection (Rockville, Maryland). These cells, which retain many of the characteristics of normal type II cells such as surfactant production, cytoplasmic multilamellar inclusion bodies, and cuboidal appearance, have been extensively used to assess type II pneumocyte effector cell function (Lieber et al, 1976). A549 cells were plated at 1×10^5 cells/well into 24-well microtiter plates (Corning Glass Works, Corning, New York) in F12 media (GIBCO BRL, Gaithersburg, Maryland) containing 10% fetal bovine serum (HyClone, Logan, Utah), 2 mM of L-glutamine, 50 μg/ml of gentamicin (GIBCO BRL).

Cryopreserved, primary NHBE cells were purchased from Clonetics Corporation (San Diego, California) and grown in 25-cm^2 tissue culture flasks in bronchial epithelial cell-growth medium (BEGM), supplied by Clonetics. The cultures were incubated at 37° C in a

humidified, 95% air/5% CO_2 atmosphere. After trypsinization the cells were subcultured into 24-well culture plates at seeding densities of 0.5×10^5 cells/well. When the cells were 50% to 60% confluent, the medium was changed to BEGM without hydrocortisone and the cells were allowed to grow for an additional 24 hours. Aliquots of cell culture supernatants were assayed immediately for the presence of LDH, a measure of cytoplasmic leakage, as previously described (Simeonova and Luster, 1995). Triton X-100 [0.5% in PBS] was added for 30 minutes to control cultures to assess total LDH release. Trypan blue exclusion was used to confirm the LDH results. Additional aliquots of supernatants were kept frozen at −70° C until tested for cytokines. TMTU, DMTU, and NAC were dissolved in culture media and added to the cells 1 hour before treatment. The pH of NAC solution was corrected to 7.2 by 2N NaOH. Fresh solutions of H_2O_2 were prepared in distilled water before each experiment.

Adult F344/N rats (Charles River, Portage, Michigan), were killed by CO_2 asphyxia using National Institute of Occupational Safety and Health (NIOSH) approved guidelines for the humane treatment of laboratory rodents, and alveolar macrophages were isolated as described previously (Simeonova and Luster, 1995). Briefly bronchoalveolar lavage was performed by infusing the lung six times with 8 ml of PBS. The bronchoalveolar lavage fluid was collected and centrifuged (240 $\times g$ for 10 minutes), and the pelleted cells were resuspended in PBS.

IL-8 ELISA

For IL-8 quantitation, 96-well microtiter plates (Dynatech Laboratories, Chantilly, Virginia) were coated overnight at 4° C with mouse anti-human IL-8 monoclonal antibody (R&D Systems). Nonspecific binding sites were blocked by treating the plates with 1% BSA in PBS for 2 hours. All samples were added in 0.1-ml aliquots and the plates were washed with PBS between each step. IL-8 standards or test samples were added for 2 hours preceded by addition of the detection antibody (swine anti-goat IgG, peroxidase conjugated) at a 1:7500 dilution for 1 hour at room temperature.

O-phenylenediamine dihydrochloride substrate (0.4 mg/ml), in phosphate-citrate buffer supplemented with 4.4 mM H_2O_2, was added for 10 minutes at room temperature and the enzymatic reaction was stopped by the addition of 50 μl of 4N HC1. The plates were read at 490-nm wave-length, using a UVmax kinetic microplate reader (Molecular Devices, Corp., Menlo Park, California). IL-8 concentrations in test samples were determined from a standard curve of human recombinant IL-8 using a curve-fitting program. Data shown are representative of at least three separate experiments. Statistical significance was determined by the RS/1 multicomparison procedure using Wilkes-Shapiro test for normality, one-way ANOVA, and Dunnett's test for multiple comparisons with a common control group. When variances were nonhomogeneous, multiple comparisons using the Bonferroni adjustment of the Student t test were performed. Statistically significant differences were reported when the p value was less than 0.05..

RT-PCR

Cellular RNA was extracted from 1×10^6 cells/sample using the RNeasy mini kit (Qigen Inc., Valencia, California) according to manufacturer's instructions. PCR amplification was performed as described previously using commercially available PCR primers (Clontech Laboratories, Inc., Palo Alto, California) for human IL-8, TNF-R1, TNF-R2, and G3PDH, the latter used an indicator of constitutive expression. The gels were scanned by computerized laser densitometer (Eagle Eye II Image Analysis System; Strategene, La Jolla, Califomia) and analyzed by National Institutes of Health (NIH) Image 1.54 analysis software package.

Quantitative analysis of cytokine message was conducted by means of competitive PCR using stepwise dilutions with a synthetic competitor (PCR MIMICS; Clontech Lab) as a template. The competitors are sequences complementary to the cytokine primers but with different size PCR products. Three-microliter aliquots of cDNA were amplified for 25 to 28 cycles with the cytokine primers in the presence of concentrations of the respective competitor ranging from 10^2 to 10^{-5} attomoles. The relative amount of mRNA transcripts was determined visually and by laser densitometry and values were plotted against the reciprocal of the molar amount of the competitor.

Nuclear Extracts and EMSA

Nuclear proteins were prepared from aliquots of 1×10^7 cells according to the method of Schreiber et al (1989). DNA binding reactions and EMSA were performed as described previously (Pennypacker et al, 1994). Briefly the 5′ ends of the double-stranded oligonucleotides were labeled with γ^{32}P-ATP (New England Nuclear/Dupont, Boston, Massachusetts), using 6 to 10 U of T4 polynucleotide kinase (USB/Amersham, Cleveland, Ohio). Binding reactions (30 μl) were performed on ice for 30 minutes in reaction mixtures containing 10 μg of nuclear proteins, 20 mM of Tris-HCl (pH 7.8), 100 mM of NaCl, 5 mM of $MgCl_2$, 1 mM of EDTA, 5 mM of dithiothreitol, 50 mg/ml of BSA, 2 μg of poly(dI-dC).poly(dI-dC), 10% glycerol, and 0.1 ng (2×10^5 cpm) of specified probe. For the detection of DNA-binding activity, DNA oligonucleotides containing specific transcription factor binding sites on the IL-6 promoter were used: NF-κB-NF-IL-6 binding site (−99/−66 from human IL-8 gene), 5′-CCATCAGTTGCAAATCGTGGAATTTCCTCTGACA-3′; NF-κB binding site (−85/−68 human IL-8 gene), 5′-TCGTGGAATTTCCTCTGA-3′ and NF-IL-6 binding site (−99/−78): 5′-CCATCAGTTGCAAATCGTGGAA-3′. Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel. Gels were electrophoresed at 125 volts in 50 mM of Tris-50 mM boric acid/1 mM of EDTA, dried and autoradiographed overnight. The autoradiograms were

scanned with an Eagle Eye II Image Analysis System (Stratagene). To characterize DNA binding activity, nuclear protein extracts were preincubated before the addition of labeled probe with a 200-fold excess of unlabeled oligomers for 30 minutes.

Cell Transfections and CAT Assay

The pUXCAT-546 IL-8 plasmid, was a gift from Dr. N. Mukaida, of the Cancer Research Institute, Kanazawa University, Japan (Mukaida et al, 1990). The −97/−69 IL-8 wild-type and mutated plasmids [mutations in the NF-κB or NF–IL-6 binding sites (Mukaida et al, 1990)] were gifts from Dr. B. Stein, Signal Pharmaceuticals, Inc. A549 cells (2×10^5 cells/well) were seeded into 35-mm tissue culture plates in F12 culture medium supplemented with 10% fetal bovine serum and 50 μg/ml of gentamicin and grown to 60% to 70% confluence. Transient transfections were performed with lipofectamine (GIBCO BRL), according to the manufacturer's instructions. The transfection mixture contained 2 μg of total plasmid DNA (reporter gene expression vector and internal control β-actin luciferase vector) and 6 μl of lipofectamine in serum- and antibiotic-free F12 medium containing insulin-transferrin-selenium supplement (GIBCO BRL) in a total volume of 0.2 ml. The cells were exposed to the test samples for 18 hours and CAT levels were determined by a commercial ELISA (Boehringer Mannheim, Indianapolis, Indiana). Cell extracts were prepared using a lysing buffer (Boehringer Mannheim) and luciferase activity was measured using a luciferase assay system (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Protein concentrations were determined using a commercially available protein assay system (BioRad, Hercules, California).

ESR Measurements

Short-lived radicals were detected with the use of DMPO, a spin trap that forms relatively long-lived free radical products, the so-called spin adducts, which can be detected by conventional ESR (Lo and Cruz, 1995). The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived trapped radical. ESR measurements were made using a Varian E9 (Varian Instruments Division, Palo Alto, California) or Bruker ER 300 ESR (Bruker Instruments Inc., Billerica, Massachusetts) spectrometer and a flat cell assembly. Reactants were mixed in test tubes in a total final volume of 0.5 ml. The reaction mixture was transferred to a flat cell for ESR measurement. The following spectrometer settings were used: microwave frequency 9.424; microwave power 50 mw; center field 3370 G; scan range 100 G; receiver gain 2.5×10^4; time constant 0.5 ms. All experiments were performed at room temperature and under ambient air. An EPRDAP 2.0 (U.S. EPR Inc., Clarksville, Maryland) computer program was used for data acquisitions and analysis.

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