Hyperoxia synergistically increases TNF-α-induced interleukin-8 gene expression in A549 cells

GEOFFREY L. ALLEN,1 INGRID Y. MENENDEZ,1 MARNIE A. RYAN,1 ROBERT L. MAZOR,1 JONATHAN R. WISPÉ,2 MICHAEL A. FIEDLER,3 AND HECTOR R. WONG1

Divisions of 1Critical Care Medicine, 2Pulmonary Biology, and 3Pulmonary Medicine, Children's Hospital Research Foundation, Cincinnati, Ohio 45229

Allen, Geoffrey L., Ingrid Y. Menendez, Marnie A. Ryan, Robert L. Mazor, Jonathan R. Wispé, Michael A. Fiedler, and Hector R. Wong. Hyperoxia synergistically increases TNF-α-induced interleukin-8 gene expression in A549 cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L253–L260, 2000.—Interleukin (IL)-8 is an important mediator of acute lung injury. Hyperoxia induces IL-8 production in some cell types, but its effect on IL-8 gene expression in respiratory epithelium is not well described. In addition, IL-8 gene expression resulting from the combined effects of hyperoxia and proinflammatory cytokines has not been well characterized. We treated cultured respiratory epithelial-like cells (A549 cells) with hyperoxia alone, tumor necrosis factor (TNF)-α alone, or the combination of TNF-α and hyperoxia and evaluated IL-8 gene expression. Hyperoxia alone had a minimal effect on IL-8 gene expression, and TNF-α alone increased IL-8 gene expression in a time-dependent manner. In contrast, the combination of TNF-α and hyperoxia synergistically increased IL-8 gene expression as measured by ELISA (TNF-α alone for 24 h = 769 ± 89 pg/ml vs. hyperoxia + TNF-α for 24 h = 1,189 ± 89 pg/ml) and Northern blot analyses. Experiments involving IL-8 promoter-reporter assays, electromobility shift assays, and Western blot analyses demonstrated that hyperoxia augmented TNF-α-mediated activation of the IL-8 promoter by a nuclear factor (NF)-κB-dependent mechanism and increased the duration of NF-κB nuclear translocation after concomitant treatment with TNF-α. Additional reporter gene assays demonstrated, however, that increased activation of NF-κB does not fully account for the synergistic effect of hyperoxia and that the NF-IL-6 site in the IL-8 promoter is also required for the synergistic effect of hyperoxia. We conclude that low levels of oxygen (hyperoxia) alone have a minimal effect on IL-8 gene expression but synergistically increases IL-8 gene expression in the presence of TNF-α by a mechanism involving cooperative interaction between the transcription factors NF-κB and NF-IL-6.

ACUTE LUNG INJURY (ALI) remains a significant cause of morbidity and mortality in critically ill patients. Although oxygen is a key supportive therapy for these patients, high levels of oxygen (hyperoxia) have been shown to directly cause or exacerbate ALI (18, 34). Many factors appear to be involved in hyperoxia-induced ALI. Hyperoxia can directly cause lung injury by generation of reactive oxygen species. These species directly cause cellular damage by mechanisms such as lipid peroxidation, oxidation of cellular proteins, DNA damage, and mitochondrial damage. Other important mechanisms in hyperoxia-induced ALI include the apparent ability of hyperoxia to cause lung inflammation. In rodent models of hyperoxia-induced ALI, neutrophil chemotactic activity of bronchoalveolar lavage (BAL) fluid was significantly increased after exposure to hyperoxia (6, 15). These data suggest that hyperoxia contributes to lung inflammation by modulating chemokine gene expression.

Interleukin (IL)-8 is an 8-kDa protein belonging to the C-X-C family of chemokines (37). The primary function of IL-8 is to promote recruitment and activation of neutrophils to areas of inflammation (19, 22). In various forms of human ALI, neutrophil infiltration is an early and important pathophysiological event, and IL-8 appears to have an important role in mediating this process (5, 31, 32). Clinical studies demonstrated increased IL-8 levels in serum and BAL fluid of patients with ALI (11, 16, 17, 27). Increased BAL fluid levels of IL-8 predicted the development of ALI in at-risk patient populations and were associated with increased mortality in patients with ALI (2, 10, 25). In animal models of ALI, administration of IL-8 antibody conferred protection (4, 14, 30). Collectively, these data demonstrate the importance of IL-8 in the pathophysiology of ALI.

IL-8 was initially discovered as a secreted product of monocytes (37) and is now known to be produced by several cell types, including the respiratory epithelium (31). Previous work (7, 24) characterizing the effects of hyperoxia on IL-8 gene expression involved alveolar macrophages and isolated peripheral blood monocytes. Relatively less is known about the effects of hyperoxia on IL-8 gene expression in the respiratory epithelium. Because the respiratory epithelium is primarily exposed to hyperoxia during treatment of ALI, understanding the effect of hyperoxia on IL-8 gene expression on the respiratory epithelium could provide further insight into the pathogenesis of hyperoxia-mediated ALI. Most studies (7, 24) investigating the effect of hyperoxia on IL-8 gene expression focused on hyperoxia as an isolated stimulus. This scenario is uncommon in clinical medicine. Patients who require high levels of oxy-
gen typically have a significant degree of underlying lung inflammation characterized by increased alveolar levels of cytokines such as tumor necrosis factor (TNF-\(\alpha\)) and IL-1\(\beta\). Therefore, understanding the complex interaction of hyperoxia with coexistent proinflammatory stimuli is clinically relevant to patients with ALI.

In this study, we designed a model that attempts to partially mimic the alveolar milieu of patients with ALI. Respiratory epithelial cells were simultaneously exposed to hyperoxia and TNF-\(\alpha\). In this context, we evaluated IL-8 gene expression and investigated the effect of hyperoxia on IL-8 promoter regulation, specifically evaluating the role of nuclear factor (NF)-\(\kappa\)B and NF-IL-6.

**MATERIALS AND METHODS**

Cell culture and reagents. A549 respiratory epithelial cells (American Type Culture Collection, Manassas, VA) were used for all experiments. These cells, best described as “epithelium-like,” are derived from a human lung adenocarcinoma, retain several features of type II pneumocytes, and have been used successfully as a model to evaluate IL-8 gene regulation in vitro (13, 31). Cells were maintained in a room air-5% CO\(_2\) incubator at 37°C in DMEM (GIBCO BRL) containing 8% FBS and penicillin-streptomycin (GIBCO BRL).

**ExPERIMENTAL CONDITIONS.** In all experiments, the following conditions were used: serum-free medium plus room air (control), TNF-\(\alpha\) (2 ng/ml) plus room air, TNF-\(\alpha\) (2 ng/ml) plus hyperoxia (95% \(\text{O}_2\)), and hyperoxia alone. Hyperoxia was achieved by placing cells in sealed modular chambers (Billups-Rothenberg, Del Mar, CA) and flushing the chambers with a gas mixture of 95% \(\text{O}_2\)-5% \(\text{CO}_2\) at 1 l/min for 30 min. Entry and exit ports were subsequently clamped, and the cells were returned to a 37°C incubator.

**IL-8 ELISA.** Cells were exposed to the experimental conditions, and supernatants were harvested 8 and 24 h after treatment. Immunoactive IL-8 levels were determined with a commercially available human IL-8 ELISA kit (Biosource International, Camarillo, CA). All procedures were performed according to the manufacturer’s instructions.

**Northern blot analysis.** Cells were treated with the experimental conditions and harvested 2, 4, and 24 h after treatment. Total RNA was isolated with the TRIzol Reagent (GIBCO BRL). RNA concentrations were determined by spectrophotometry (260 nm), and 15 μg of RNA from each sample underwent electrophoresis in gels containing 1% agarose and 3% formaldehyde. Integrity of RNA was confirmed visually by ethidium bromide staining and brief ultraviolet light illumination. RNAs were transferred to nylon membranes (Micron Separations, Westboro, MA) and ultraviolet cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA). Membranes were prehybridized for 4 h at 42°C and subsequently hybridized overnight with a radiolabeled IL-8 cDNA probe (13). The cDNA probe was labeled with \(\text{[}\text{\text{32P}}\text{]}\text{dCTP}\) (specific activity 3,000 Ci/mM, NEN Research Products, Boston, MA) by random priming (Pharmacia, Piscataway, NJ). Membranes were subsequently washed twice with 2× saline-sodium citrate-0.1% SDS at 53°C, developed with a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), and analyzed with ImageQuant Software. To normalize for loading differences, membranes were stripped by boiling in 5 mM EDTA and rehybridized with an end-labeled \(\text{[}\text{\text{32P}}\text{]}\text{ATP oligonucleotide probe for 18S rRNA.}

**Transient transfections and luciferase assays.** Four separate promoter-luciferase reporter plasmids were used for these studies. The first plasmid (wild type) contained a 200-bp segment (−97 to +103 bp) of the IL-8 promoter immediately 5′ to the firefly luciferase gene (13). The second plasmid (mutant NF-\(\kappa\)B) had four base substitution mutations in the NF-\(\kappa\)B binding site of the IL-8 promoter, as indicated by lowercase letters: −82 to −72 bp, GTCGAGTTTCC → cTGCAGATTCG (12). The third plasmid (mutant NF-IL-6) had five base substitution mutations in the NF-IL-6 binding site of the IL-8 promoter, as indicated by lowercase letters: −92 to −81 bp, GTCGAAATCGT → GCTaGcAgCGT (12). The fourth plasmid (NF-\(\kappa\)B dependent) was a synthetic construct in which the luciferase gene was driven by three tandem NF-\(\kappa\)B binding motifs, followed by a minimal interferon-\(\beta\) promoter (a kind gift from Dr. Roland M. Schmid, University of Ulm, Ulm, Germany). This plasmid was previously demonstrated to be a sensitive tool to specifically evaluate NF-\(\kappa\)B activation (33).

Cells were transfected in duplicate by incubation with cationic liposomes (Lipofectin, GIBCO BRL) suspended in Opti-MEM (GIBCO BRL) for 4 h. For all four plasmids, the liposome-to-DNA ratio was 10:2 μg. After transfection, cells were washed with PBS and allowed to recover overnight. After treatment with the experimental conditions for 4 h, cellular proteins were extracted and analyzed for luciferase activity according to the manufacturer’s instructions (Promega) with a Berthold AutoLumat LB953 luminometer. Total protein was measured by the Bradford assay (Bio-Rad) and stored at −70°C until used for electrophoretic mobility shift assay (EMSA).

**Nuclear protein extraction.** Nuclear protein extracts were prepared from treated cells grown to 80% confluence in 100-mm² dishes. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed twice with PBS, harvested by scraping into 1 ml of PBS, and pelleted at 6,000 rpm for 5 min. The pellet was washed twice with PBS, resuspended in one packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% (vol/vol) Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)), and incubated for 5 min with occasional vortexing. After centrifugation at 6,000 rpm, one cell pellet volume of extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 M EDTA, 1.5 mM MgCl₂, 25% (vol/vol) glycerol, 1 mM DTT, and 0.5 mM PMSF) was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. The nuclear proteins were isolated by centrifugation at 14,000 rpm for 15 min. Protein concentrations were determined by Bradford assay (Bio-Rad) and stored at −70°C until used for electrophoretic mobility shift assay (EMSA).

**EMSA.** The NF-\(\kappa\)B oligonucleotide probe used for EMSA (5′-GTGAAATTCTCTGTA-3′) corresponds to the NF-\(\kappa\)B site in the IL-8 promoter and was synthesized at the University of Cincinnati DNA Core Facility. The probe was labeled with \(\text{[}\text{\text{32P}}\text{]}\text{ATP using T4 polynucleotide kinase (GIBCO BRL) and purified in Bio-Spin chromatography columns (Bio-Rad).}

For EMSA, 10 μg of nuclear proteins were preincubated with EMSA buffer (12 mM HEPES, pH 7.9, 4 mM Tris·HCl, pH 7.9, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly(dⅢ-dC), 12% (vol/vol) glycerol, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide probe for an additional 10 min. Protein-nucleic
acid complexes were resolved with a nondenaturating polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide to bis-acrylamide) and run in 0.5× Tris-borate-EDTA buffer (45 mM Tris·HCl, 45 mM boric acid, and 1 mM EDTA) for 1 h at constant current (30 mA). Gels were transferred to Whatman 3M paper, dried under a vacuum at 80°C for 1 h, and exposed to photographic film at −70°C with an intensifying screen.

Western blot analysis. Nuclear proteins were boiled in equal volumes of loading buffer (125 mM Tris·HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol), and 20 µg of protein were loaded per lane on 8–16% Tris-glycine gradient gels (Novex, San Diego, CA). Proteins were separated electrophoretically and transferred to nitrocellulose membranes (Novex) with the Novex Xcell Mini-Gel system. For immunoblotting, membranes were blocked with 10% nonfat dried milk in Tris-buffered saline (TBS) for 1 h. Primary antibody against the p65 subunit of NF-κB (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:100 for 1 h. After two washes in TBS-0.05% Tween 20, secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, Sigma) was applied at 1:10,000 for 1 h. Blots were washed in TBS-Tween 20 two times over 30 min, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham), and exposed to photographic film.

Statistical analysis. Differences in immunoreactive IL-8 levels and luciferase activity between the experimental groups were evaluated by one-way analysis of variance and the Student-Newman-Keuls test. P < 0.05 was considered significant.

RESULTS

Hyperoxia augments TNF-α-mediated immunoreactive IL-8 production. We first determined if hyperoxia would modulate TNF-α-induced production of immunoreactive IL-8. A549 cells were treated with the experimental conditions, and immunoreactive IL-8 levels were determined by ELISA. Treatment with hyperoxia alone minimally increased immunoreactive IL-8 levels compared with those in unstimulated control cells (Fig. 1). Treatment with TNF-α alone significantly increased immunoreactive IL-8 levels at 8 and 24 h compared with those in unstimulated control cells. Concomitant treatment with TNF-α and hyperoxia increased immunoreactive IL-8 levels compared with those in cells treated with TNF-α alone. The amount of IL-8 generated after treatment with the combination of TNF-α and hyperoxia was greater than would be anticipated if the effects of TNF-α and hyperoxia were simply additive. These data demonstrate that the combination of TNF-α and hyperoxia synergistically increases production of immunoreactive IL-8.

Hyperoxia augments TNF-α-mediated expression of IL-8 mRNA. Having demonstrated that hyperoxia augments production of immunoreactive IL-8 in the presence of TNF-α, we next investigated the effects of hyperoxia on steady-state IL-8 mRNA levels. Cells were treated with the experimental conditions, and IL-8 mRNA was measured by Northern blot analyses. Treatment with hyperoxia alone did not detectably affect IL-8 mRNA expression (data not shown). Treatment with TNF-α alone increased IL-8 mRNA expression in a time-dependent manner (Fig. 2). Concomitant treatment with TNF-α and hyperoxia increased IL-8 mRNA expression compared with that in cells treated with TNF-α alone. As in the ELISA-related experiments, the amount of IL-8 mRNA expression after treatment with the combination of TNF-α and hyper-
oxygen was greater than would be anticipated if the effects of TNF-α and hyperoxia were simply additive. These data indicate that hyperoxia augments TNF-α-induced IL-8 production by mechanisms that increase IL-8 mRNA expression.

Hyperoxia augments TNF-α-induced IL-8 promoter activity. Transcriptional control of the IL-8 promoter is known to have an important role in IL-8 gene expression (20, 23, 26). Therefore, we investigated the effect of hyperoxia on IL-8 promoter activation. Cells were transiently transfected with an IL-8 promoter-luciferase reporter plasmid (13) and treated as indicated in MATERIALS AND METHODS. Treatment with hyperoxia alone did not significantly increase luciferase activity compared with that in control cells (Fig. 3). Treatment with TNF-α alone caused an ~2.2-fold induction of luciferase activity. The combination of TNF-α and hyperoxia caused an ~4.2-fold induction of luciferase activity, a greater induction of luciferase activity than would be expected if the effects of TNF-α and hyperoxia were simply additive. These data demonstrate that hyperoxia augments TNF-α-mediated activation of the IL-8 promoter.

Mutation in the NF-κB binding site of the IL-8 promoter abolishes TNF-α- and hyperoxia-mediated activation of the IL-8 promoter. The transcription factor NF-κB is known to be involved in IL-8 gene regulation (20, 23, 26). To determine the role of NF-κB in our experimental system, we transfected cells with an IL-8 promoter-luciferase plasmid containing a mutated NF-κB binding site (12). Treatment with hyperoxia alone, TNF-α alone, or the combination of TNF-α and hyperoxia did not induce luciferase activity in cells transfected with the mutant NF-κB promoter-luciferase reporter plasmid (Fig. 4). From these results, we conclude that induction of the IL-8 gene by TNF-α and the combination of TNF-α and hyperoxia are NF-κB dependent in A549 cells.

Hyperoxia prolongs TNF-α-mediated nuclear translocation of NF-κB. In these experiments, we directly examined the effect of hyperoxia on TNF-α-mediated nuclear translocation of NF-κB. Hyperoxia alone for 1–4 h did not detectably increase nuclear translocation of NF-κB compared with that in control cells as measured by EMSA (Fig. 5, lanes 3–6). Treatment with TNF-α alone for 1 or 2 h increased nuclear translocation of NF-κB compared with that in control cells (Fig. 6, lanes 1, 2, and 4). The specificity of the shifted band was demonstrated in a previous report (36). The amount of NF-κB nuclear translocation was greatest after 1 h of TNF-α alone (Fig. 6, lane 2) and was decreased by 2 h (Fig. 6, lane 4). Concomitant treatment with TNF-α and hyperoxia for 1 h did not significantly alter NF-κB nuclear translocation compared with that in cells treated with TNF-α alone for 1 h (Fig. 6, lanes 2 and 3). In contrast, cells that were concomitantly treated with TNF-α and hyperoxia for 2 h demonstrated relatively more NF-κB nuclear translocation compared with that in cells treated with TNF-α alone for 1 h (Fig. 6, lanes 4 and 5). The amount of NF-κB nuclear translocation after 2 h of treatment with TNF-α and hyperoxia was similar to that of cells treated with TNF-α alone for 1 h (Fig. 6, lanes 2 and 5).

To confirm our EMSA data, we next performed Western blot analyses for the NF-κB subunit p65 using nuclear proteins of cells treated with TNF-α with and without hyperoxia. Treatment with TNF-α alone increased nuclear levels of p65 compared with those in control cells (Fig. 7, lanes 1, 2, and 4). As with our EMSA data, the amount of nuclear p65 was greatest after 1 h of TNF-α (Fig. 7, lane 2) and was decreased by 2 h (Fig. 7, lane 4). Concomitant treatment with TNF-α and hyperoxia for 1 h did not significantly alter nuclear p65 levels compared with those in cells treated...
with TNF-α alone (Fig. 7, lanes 2 and 3). In contrast, cells that were concomitantly treated with TNF-α and hyperoxia for 2 h demonstrated increased nuclear p65 levels compared with cells treated with TNF-α alone for 2 h (Fig. 7, lanes 4 and 5). Collectively, the EMSA and Western blot data suggest that hyperoxia increases the duration of TNF-α-mediated nuclear translocation of NF-κB.

Hyperoxia does not augment TNF-α-mediated luciferase activity in cells transfected with a NF-κB-dependent promoter-luciferase reporter plasmid. Having demonstrated that induction of the IL-8 gene by TNF-α is a NF-κB-dependent process and that hyperoxia increases the duration of TNF-α-mediated NF-κB nuclear translocation in our model, we hypothesized that augmentation of TNF-α-mediated IL-8 production by hyperoxia results from increased NF-κB activity. To test this hypothesis and to measure NF-κB activity, we performed transient transfections with a synthetic promoter-luciferase reporter plasmid containing three NF-κB sites in tandem. This plasmid was previously reported as an effective tool to specifically evaluate in vitro NF-κB activation (33). Treatment with hyperoxia alone did not activate NF-κB (Fig. 8). Treatment with TNF-α alone resulted in a ~3-fold induction of luciferase activity, and the addition of hyperoxia to TNF-α did not increase luciferase activity compared with those in cells treated with TNF-α alone. From these data, we conclude that hyperoxia does not augment TNF-α-induced activation of NF-κB in A549 cells.

The NF-IL-6 site in the IL-8 promoter is also required for synergistic activation by hyperoxia and TNF-α. Data derived from transient transfection assays indicate that the NF-κB site in the IL-8 promoter is required for the synergistic effect of hyperoxia in the presence of TNF-α. Data derived from EMSA and Western blot analyses indicate that hyperoxia increases the duration of TNF-α-mediated nuclear translocation of NF-κB. NF-κB-dependent reporter gene assays, however, indicate that increased activation of NF-κB alone does not fully account for the mechanism by which hyperoxia augments TNF-α-mediated expression of the IL-8 gene. Collectively, these data suggest that other IL-8

![Fig. 5. Electrophoretic mobility shift assay (EMSA) demonstrating that hyperoxia alone does not induce NF-κB nuclear translocation. Cells were treated as indicated for indicated times. Control cells (lane 1) were treated in room air and basal growth medium. As a positive control, one group of cells was treated with TNF-α alone (2 ng/ml) for 1 h (lane 2). EMSAs were performed using an oligonucleotide corresponding to NF-κB site in IL-8 promoter. Data represent 1 of 3 identical experiments.](image1)

![Fig. 6. EMSA demonstrating that hyperoxia increases duration of NF-κB nuclear translocation in presence of TNF-α. Cells were treated as indicated for indicated times. Control cells (lane 1) were treated in room air and basal growth medium. EMSAs were performed using an oligonucleotide corresponding to NF-κB site in IL-8 promoter. Data represent 1 of 3 identical experiments.](image2)

![Fig. 7. Western blot analysis of nuclear proteins demonstrating that hyperoxia increases duration of p65 nuclear translocation in presence of TNF-α. Cells were treated as indicated for indicated times. Control cells (lane 1) were treated in room air and basal growth medium. Western blot analyses were performed using a polyclonal anti-p65 antibody. Data represent 1 of 3 identical experiments.](image3)
promoter elements may be involved. Accordingly, we investigated the role of NF-IL-6 because this transcription factor was previously reported to play a cooperative role with NF-κB in the regulation of IL-8 (20, 23, 26).

The role of NF-IL-6 was investigated by transiently transfecting A549 cells with an IL-8 promoter-luciferase reporter plasmid containing base substitution mutations in the NF-IL-6 site. Treatment with hyperoxia alone did not increase luciferase activity in cells transiently transfected with the mutant NF-IL-6 promoter-luciferase plasmid (Fig. 9). Treatment with TNF-α alone significantly increased luciferase activity, but the addition of hyperoxia did not augment TNF-α-mediated luciferase activity. These data indicate that an intact NF-IL-6 site is required for the mechanism by which hyperoxia augments TNF-α-mediated activation of the IL-8 promoter.

DISCUSSION

Previous studies involving alveolar macrophages, U937 cells, isolated peripheral blood monocytes, and human whole blood demonstrated that hyperoxia modulates IL-8 gene expression (7, 8, 24). Oxidant stress other than hyperoxia was previously described to induce IL-8 expression in respiratory epithelial cells. DeForge et al. (9) and Lakshminarayanan et al. (21) demonstrated that A549 cells exposed to H₂O₂ produced IL-8. In our current study, we used hyperoxia as an oxidant stress model because hyperoxia is a clinically relevant form of oxidant stress. In addition, we used a respiratory epithelial cell model because the respiratory epithelium is primarily exposed to hyperoxia in patients with ALI.

We evaluated how the interaction between hyperoxia and TNF-α affects IL-8 gene expression in A549 cells. In these experiments, hyperoxia alone had a minimal effect on IL-8 gene expression. In contrast, the combination of hyperoxia and TNF-α synergistically increased IL-8 gene expression. Synergy was evident by increased IL-8 peptide expression (ELISA) and increased IL-8 mRNA expression (Northern blot analysis) in cells treated with the combination of hyperoxia and TNF-α compared with cells treated with TNF-α alone. To provide more mechanistic insight into these findings, we performed transient transfections using an IL-8 promoter-luciferase reporter plasmid. These experiments demonstrated that the augmentation of TNF-α-mediated IL-8 gene expression by hyperoxia is secondary to increased activation of the IL-8 promoter.

The amount of induced luciferase activity measured in transiently transfected cells was proportionally less compared with induced IL-8 levels measured by ELISA. This discrepancy most likely represents differences in assay sensitivities. Alternatively, this discrepancy may indicate that the observed effects on IL-8 gene expression are not solely transcriptional but may also involve posttranscriptional regulation of the IL-8 gene. Future studies are needed to establish whether changes in mRNA stability are relevant in our experimental model.

Previous studies demonstrated the importance of NF-κB in the regulation of IL-8 gene expression (12, 13, 20, 23, 26). Our current results are partially consistent with these data. Site-directed mutagenesis of the NF-κB binding site in the IL-8 promoter abolished the ability of TNF-α to induce luciferase activity. Furthermore, hyperoxia had no intrinsic ability to induce luciferase activity in cells transfected with this mutant NF-κB promoter and did not interact with TNF-α to increase luciferase activity.

Oxidant stress is known to induce NF-κB activity in certain experimental models (1, 3, 29). Therefore, we evaluated the effect of hyperoxia on NF-κB nuclear

---

**Fig. 8.** Luciferase assay from cells transfected with NF-κB-dependent promoter-luciferase plasmid. Cells were treated as indicated and harvested to determine luciferase activity after 4 h. Control cells were transfected and treated with basal growth medium and room air. Values are means ± SE of 5 separate experiments, each in duplicate. *P < 0.05 vs. control.

**Fig. 9.** Luciferase assay from cells transiently transfected with "mutant NF-IL-6" IL-8 promoter-luciferase plasmid. Cells were treated as indicated and harvested to determine luciferase activity after 4 h. Control cells were transfected and treated with basal growth medium and room air. Values are means ± SE of 5 separate experiments, each in duplicate. *P < 0.05 vs. control.
translocation (EMSA and Western blot analyses) and NF-κB activation (reporter gene assays). Hyperoxia alone did not detectably affect NF-κB nuclear translocation as measured by EMSA and Western blot analyses. In contrast, hyperoxia modestly prolonged the time during which NF-κB was present in the nucleus after concomitant treatment with TNF-α. Thus increased activation of NF-κB could account for the mechanism by which hyperoxia augments TNF-α-mediated IL-8 gene expression.

To test this hypothesis, we performed transient transfections with a synthetic NF-κB-dependent promoter-luciferase reporter plasmid, which allowed us to specifically evaluate NF-κB activity. These experiments demonstrated that hyperoxia did not augment TNF-α-mediated NF-κB activity. Also, treatment with hyperoxia alone did not increase NF-κB activity. These results provide functional evidence suggesting that hyperoxia does not augment TNF-α-mediated IL-8 gene expression by simply augmenting NF-κB activation in A549 cells. These data further indicate that other promoter elements may be involved in the mechanism by which hyperoxia augments TNF-α-mediated expression of the IL-8 gene. Indeed, transient transfections with an IL-8 promoter-luciferase plasmid containing a mutant NF-IL-6 site indicated that NF-IL-6 is also required for the synergistic effect of hyperoxia. In combination, our data demonstrate that NF-κB is required for induction of IL-8 gene expression by TNF-α and by the combination of TNF-α and hyperoxia. Increased activation of NF-κB alone, however, is not sufficient to fully account for the synergistic effect of hyperoxia in the presence of TNF-α. The NF-IL-6 site is also required for the synergistic effect of hyperoxia. Thus the mechanism by which hyperoxia augments IL-8 gene expression appears to involve interactions between NF-κB and NF-IL-6. This assertion is well supported by previous studies demonstrating cooperative interactions between NF-κB and NF-IL-6 in other experimental models (20, 23, 26).

The pathophysiology of ALI is a complex process involving multiple stimuli. Hyperoxia can directly cause ALI by generating cytotoxic reactive oxygen species. Hyperoxia is also known to cause lung inflammation, but the mechanisms of this effect are not fully understood. Rats exposed to 95% O₂ alone demonstrated pulmonary infiltration with neutrophils within 48 h of exposure (28). BAL fluid from rats exposed to 95% O₂ had increased chemotactic properties, and the degree of chemotactic activity was highly correlated with the degree of lung injury (6, 15). In combination with our current data, these data indicate that hyperoxia may also cause ALI, indirectly, by modulating chemokine gene expression.

The scenario of exposing patients without lung disease to hyperoxia alone is clinically uncommon. Virtually all patients with ALI severe enough to require high levels of O₂ have a significant degree of underlying inflammation. Levels of cytokines such as TNF-α are increased in ALI, and the interaction of these mediators with hyperoxia may influence gene expression in parenchymal lung cells. It is becoming increasingly apparent that interactions between simultaneous or sequential stimuli significantly impact the course of ALI and other forms of organ injury during proinflammatory states. The term "multiple hits" has been used to describe this concept in which there are important additive, synergistic, or unexpected effects of exposing cells or whole organs to multiple stimuli (35). The results of our current study suggest that cytokines, such as TNF-α, may have important interactions with hyperoxia that influence IL-8 gene expression. Our data also suggest that the respiratory epithelium, previously thought to be a passive target in hyperoxia-induced ALI, is actually an active participant in this process. Further elucidation of the mechanisms by which hyperoxia modulates IL-8 gene expression in the respiratory epithelium may allow for the development of more rational therapeutic interventions for hyperoxia-induced ALI.

This work was supported in part by the Children's Hospital Research Foundation and National Heart, Lung, and Blood Institute Grant K08-HL-03725 (to H. R. Wong).

Address for reprint requests and other correspondence: H. R. Wong, Division of Critical Care Medicine OSB5, Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH 45229 (E-mail: wonghr@chmcc.org).

Received 11 August 1999; accepted in final form 15 September 1999.

REFERENCES


