We previously demonstrated that tumor necrosis factor-α (TNFα) and H₂O₂ differentially regulate interleukin-8 (IL-8) and intercellular adhesion molecule (ICAM-1) gene expression in endothelial and epithelial cells. H₂O₂ induced IL-8 expression in the A549 and BEAS-2B epithelial cell lines, but not in the human microvessel endothelial cell line, HMEC-1 or human umbilical vein endothelial cells. In contrast, H₂O₂ induced ICAM-1 only in endothelial cells. Unlike H₂O₂, the proinflammatory cytokine TNFα induced IL-8 and ICAM-1 in both cell types. In this study, we examined the role of the redox-responsive transcription factors AP-1 and nuclear factor-κB (NF-κB) in the differential expression of IL-8. DNA binding studies using nuclear protein extracts from HMEC-1 and A549 cells stimulated with H₂O₂ or TNFα demonstrated differential activation and promoter binding of AP-1 and NF-κB. H₂O₂ activated AP-1 but not NF-κB in A549, whereas TNFα activated AP-1 as well as NF-κB. In HMEC-1, TNFα activated NF-κB but not AP-1, while H₂O₂ did not activate either transcription factor. The differential activation of the factors was also reflected in their differential binding to the IL-8 promoter. Moreover, the H₂O₂ concentration dependent increase in epithelial IL-8 mRNA expression directly corresponded to the H₂O₂ concentration dependent binding of AP-1 to the IL-8 promoter. Supershift analysis revealed H₂O₂ as well as TNFα induced AP-1 complexes containing c-Fos and JunD. TNFα induced NF-κB complexes containing Rel A (p65). Immunohistochemical staining of HMEC-1 and A549 cells revealed TNFα stimulated nuclear localization of Rel A, whereas no translocation of Rel A was detected in either cell type stimulated by H₂O₂. These data indicate that the cell type-specific induction of IL-8 gene expression by H₂O₂ and TNFα in HMEC-1 and A549 cells can be explained by the differential binding of AP-1 and NF-κB to the IL-8 promoter.

The chemotactic factor, interleukin-8 (IL-8), a member of the CXC chemokine family (1), is a potent activator and chemoattractant of neutrophils (2) and is secreted as a 72- or 77-amino acid protein by a wide variety of cell types including endothelial and epithelial cells (3, 4). IL-8 is induced by the proinflammatory cytokines TNFα, IL-1, IL-6, and interferon-γ (3, 5–7), and inhibited by the anti-inflammatory cytokine IL-10 (8, 9). IL-8 is regulated primarily at the level of gene transcription (10–13) and its promoter region contains functional binding sites for the transcription factors NF-κB, C/EBP, and AP-1 (14–16). TNFα activates the IL-8 and ICAM-1 genes through a cooperative interaction between NF-κB and C/EBP binding to a composite enhancer element within the proximal promoter (7, 17–19).

IL-8 is induced by oxidant stress (20, 21), and antioxidants have been shown to inhibit IL-8 expression (22, 23). H₂O₂ induces IL-8 expression in epithelial cell lines, fibroblasts, and whole blood (20, 21), and hypoxia followed by reoxygenation increases IL-8 expression in mononuclear and endothelial cells (24, 25) and in the lung and myocardium in vivo (26, 27). Nitric oxide, a reactive nitrogen species, has been shown to induce IL-8 through the activation of NF-κB (28).

We recently reported that H₂O₂ and TNFα differentially regulate IL-8 and ICAM-1 gene expression in epithelial and endothelial cells (20). IL-8 was induced by H₂O₂ in epithelial cells but not in endothelial cells. In contrast, H₂O₂ induced ICAM-1 in endothelial cells but not in epithelial cells. TNFα has also been shown to generate oxidant stress (29, 30) and induce IL-8 and ICAM-1 expression in both cell types (20). In addition, TNFα and H₂O₂ induce a differential pattern of CC chemokine expression in epithelial cells. While TNFα induced both RANTES and MCP-1, H₂O₂ only induced MCP-1 in A549 cells (20). These studies suggest that oxidant stress constitute cell type- and gene type-specific activation signals in epithelial and endothelial cells that may critically influence the site-specific recruitment of leukocyte subsets in inflammatory reactions.

The aim of the present study was to investigate the transcriptional mechanism by which H₂O₂ and TNFα differentially regulate IL-8 gene expression in endothelial and epithelial cells. We demonstrate that the redox-sensitive transcription factors AP-1 and NF-κB are differentially activated by H₂O₂ and TNFα in HMEC-1 and A549 cells. Our findings suggest that the discordant binding of the redox-responsive transcription factors AP-1 and NF-κB to the IL-8 gene promoter mediates a transcriptional mechanism that discriminates between these cell types.

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1 The abbreviations used are: IL, interleukin; TNFα, tumor necrosis factor-α; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; HMEC, human microvessel endothelial cell; EMSA, electromophoretic mobility shift assay; NIC, nonspecific complexes; ICAM-1, intercellular adhesion molecule (CD54); ARE, antioxidant responsive elements.
the distinct pattern of IL-8 observed in epithelial and endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—30% H2O2, MOPS, fibronectin, fetal bovine serum, dexamethasone, and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide were purchased from Sigma. Dulbecco’s modified Eagle’s medium, 10 × phosphate-buffered saline (PBS), F-12K media, MCDB-131 media, and 1% trypsin-EDTA were purchased from Life Technologies, Inc. (Grand Island, NY). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P]ATP (3000 Ci/mmole) was purchased from NEN Life Science Products Inc. (Boston, MA). The IL-8 enzyme-linked immunosorbent assay kit was purchased from BioSource (Camarillo, CA). The A549 cell line was obtained from American Type Culture Collection (Rockville, MD). The human microvessel endothelial cell line (HMEC-1) was obtained from the Center for Disease Control (Atlanta, GA). Human epidermal growth factor was purchased from Becton Dickenson (San Jose, CA).

Cell Culture and Treatments—The A549 human type II lung carcinoma cell line was grown and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% gentamycin to 90% confluence. HMEC-1 was cultured in MCDB-131 media (Life Technologies, Inc.) with 10% fetal calf serum, 1% penicillin/streptomycin, 1% gentamycin, 1 μg/ml hydrocortisone, and 0.01 μg/ml epidermal growth factor to 90% confluence in 24-well dishes. Cells were washed twice with 1 × PBS and covered with serum-free, phenol red-free and growth factor-free media for 24 h prior to agonist treatments. A549 and HMEC-1 were grown on coverslips to 80% confluence. Cells were washed twice in PBS and fixed in 3.7% formaldehyde (diluted in PBS containing 0.2% Triton X-100) for 10 min. The coverslips were washed three times in PBS and covered with 0.2% Triton X-100 for 10 min. The blocking buffer was removed by draining and 4–8 μg of anti-human NF-κB (Rel A, p50, c-Rel) subunits (Santa Cruz Biotechnology, Santa Cruz CA) were included in the binding reaction. Protein-DNA and protein-DNA-antibody complexes were resolved in 5% polyacrylamide gels pre-electrophoresed for 30 min at room temperature in 10 or 20 × TBE buffer (22.5 mM Tris borate, 0.5 mM Na2EDTA, pH 8.3). Gels were dried at room temperature in 10 or 20 × TBE buffer and exposed to radiographic film with an intensifying screen at 70 °C.

DNA Binding Studies—Electrophoretic mobility shift assays (EMSA) were performed essentially as described (13). Briefly, nuclear protein extracts (3–6 μg of protein) prepared from A549 or HMEC-1 cells by the method of Osborn et al. (31) were incubated with 50,000 cpm (~0.1 ng) of 32P-end labeled oligonucleotide probes listed in Table I for 20–30 min at room temperature in 10 or 20 μl reaction volumes containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM MgCl2, 4 mM Tris-Cl (pH 7.9), 0.6 mM EDTA (pH 7.9), 0.6 mM dithiothreitol, and 0.25 μg of poly(dI-dC). To demonstrate binding specificity, 100-fold molar excess (10 ng) of a specific or nonspecific oligonucleotide as indicated in the figure legends was included in the binding reaction. For supershift analysis, antibodies (1–3 μg) against AP-1 (c-Jun, c-Fos, JunD) or NF-κB (Rel A, p50, c-Rel) subunits (Santa Cruz Biotechnology, Santa Cruz CA) were included in the binding reaction. Protein-DNA and protein-DNA-antibody complexes were resolved in 5% polyacrylamide gels pre-electrophoresed for 30 min at room temperature in 0.25 × TBE buffer (22.5 mM Tris borate and 0.5 mM Na2EDTA, pH 8.3). Gels were dried and exposed to radiographic film with an intensifying screen at ~70 °C. Gel shifts were performed at least twice with nuclear extract prepared from different batches of cells. Similar results were obtained and a representative gel is shown in the figures.

Immunostaining—A549 and HMEC-1 were grown on coverslips to 80% confluence. Cells were washed twice in PBS and fixed in 3.7% formaldehyde (diluted in PBS containing 0.2% Triton X-100) for 10 min. The coverslips were washed three times in PBS and covered with 0.2% bovine serum albumin diluted in PBS (blocking buffer) for 10 min. The blocking buffer was removed by draining and 4–8 μg of anti-human NF-κB (Rel A/p50) antibody (rabbit polyclonal IgG purchased from Upstate Biotechnology, NY) diluted in 100 μl of blocking buffer added to the cells for 60 min. The antibody was removed and the cells washed three times in PBS. The cells were then incubated with the secondary antibody fluorescein-tagged goat anti-rabbit IgG. After 60 min, the secondary antibody was removed and the cells washed three times in PBS. The coverslips were mounted to slides containing Citifluor. Immunofluorescent Rel A staining was detected using a fluorescent microscope.

RESULTS

H2O2 and TNFα Differentially Induce AP-1 and NF-κB Binding Activity in HMEC-1 and A549—To investigate the role of the redox-sensitive transcription factors AP-1 and NF-κB in the cell type-specific expression of IL-8 gene expression, we first performed a series of DNA binding studies using consensus binding sites for AP-1 and NF-κB (Table I). Nuclear protein extracts were prepared from A549 epithelial cells and HMEC-1 endothelial cells exposed for 15, 30, or 60 min to concentrations of H2O2 or TNFα shown previously to activate

| Name | Source | Sequence |
|------|--------|----------|
| cAP-1 | Consensus | 5′-CCCTTATAGATCTAAGGGA-3′ |
| cNF-κB | Consensus | 5′-AGTTGAGGAGACTTCCCGAC-3′ |
| Sp-1 | Consensus | 5′-ATGCCATGCGGGGCGAGC-3′ |
| AP-1 | IL-8 | 5′-GTGATGACTCAGTTGG-3′ |
| NF-κB | IL-8 | 5′-ATCGTGATCTCTGGA-3′ |
| AP-1m | IL-8 | 5′-GCTATGCTTTGCTGTTG-3′ |
| NF-κBm | IL-8 | 5′-ATGTTAAGCCCTTTCTCGGA-3′ |

Mutations of the wild type sequence is indicated by lower case letters and the core binding sites are indicated by underlines. Consensus oligonucleotides were purchased from Promega (Madison, WI). The IL-8 promoter sequences were synthesized by Integrated DNA technologies (Corvalis, IA). Mutant IL-8 oligonucleotides correspond to mutations introduced into the IL-8 promoter by Mukaida et al. (32).

IL-8 gene expression. DNA binding activity was determined by the EMSA. As shown in Fig. 1, H2O2 increased AP-1 binding activity in A549 (Fig. 1A) but not NF-κB binding activity (Fig.

![Fig. 1. H2O2 and TNFα induce different patterns of AP-1 and NF-κB binding activity in A549. Nuclear protein extracts from A549 cells treated with H2O2 (800 μm) or TNFα (100 units/ml) for 15, 30, and 60 min were incubated with 32P-end labeled consensus AP-1-binding site oligonucleotide (A) or consensus NF-κB-binding site oligonucleotide (B). Gel shift complexes were resolved by electrophoresis and visualized by autoradiography. Specificity of the induced complexes was determined by competition with a 100-fold molar excess of unlabeled cAP-1 or cNF-κB oligonucleotide. Arrows indicate migration of the induced DNA binding complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown.](http://www.jbc.org/Downloadedfrom)
AP-1 binding activity was detected as early as 15 min and was increased at 60 min (Fig. 1A). In contrast, TNFα increased both AP-1 and NF-κB binding activity in A549 cells, although their kinetics of induction differed. AP-1 binding activity induced by TNFα peaked at 30 min (Fig. 1A), whereas NF-κB binding activity continued to increase over the 60-min time course (Fig. 1B).

In contrast to A549, HMEC-1 displayed a very different pattern of binding activity for the two transcription factors. As shown in Fig. 2, H2O2 and TNFα did not increase AP-1 binding activity over the constitutive binding activity in HMEC-1 and in fact slightly reduced this constitutive binding activity at 15 and 30 min (Fig. 2A). However, like A549, TNFα induced NF-κB binding activity in HMEC-1 and the kinetics were similar in the two cell types (Fig. 2B). NF-κB binding activity was detected at 15 min and continued to increase at 30 and 60 min. These data indicate that H2O2 and TNFα differentially activate the redox-responsive transcription factors AP-1 and NF-κB in A549 and HMEC-1 providing a potential mechanism for the cell type-specific expression of IL-8 and ICAM-1 in epithelial and endothelial cells we previously reported (13, 20).
binding of AP-1 and NF-κB to the consensus sites was observed on the IL-8 promoter sites. The correspondence between the consensus and IL-8-binding sites suggests that the distinct expression patterns of IL-8 induced by H2O2 and TNFα in epithelial and endothelial cells is mediated by the differential activation of AP-1 and NF-κB binding to the IL-8 promoter. 

**TNFα Induce Distinct NF-κB Binding Complexes in HMEC-1 and A549**—To demonstrate that the IL-8 and consensus oligonucleotides were indeed binding similar AP-1 and NF-κB proteins, we performed a series of competition experiments. As shown in Fig. 5, the AP-1 and NF-κB binding complexes formed on the IL-8 promoter sites were competed by a 100-fold molar excess of unlabeled consensus cAP-1 or cNF-κB oligonucleotide. Arrows indicate migration of the induced DNA binding complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown.

**Mutation of the IL-8 Promoter Binding Sites Abrogate NF-κB and AP-1 Binding Activity**—To determine if mutations of the AP-1 and NF-κB-binding sites in the IL-8 promoter alter AP-1 and NF-κB binding activity in A549 and HMEC-1, we introduced point mutations in the IL-8 promoter oligonucleotides (Table I) that were previously reported to reduce IL-8 promoter

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**FIG. 4.** H2O2 and TNFα induce different patterns of AP-1 and NF-κB binding activity on the IL-8 promoter in HMEC-1. Nuclear protein extracts from HMEC-1 cells treated with H2O2 (100 μM) or TNFα (100 units/ml) for 15, 30, and 60 min were incubated with 32P-end-labeled oligonucleotides from the AP-1-binding site in the IL-8 promoter (A) or the NF-κB-binding site in the IL-8 promoter (B). Gel shift complexes were resolved by electrophoresis and visualized by autoradiography. Specificity of the induced complexes was determined by competition with a 100-fold molar excess of unlabeled AP-1, NF-κB, or Sp1 oligonucleotide. Arrows indicate migration of the induced DNA binding complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown.

**FIG. 5.** TNFα induce distinct NF-κB binding complexes in HMEC-1 and A549. Nuclear protein extracts from A549 and HMEC-1 cells treated with H2O2 (800 and 100 μM, respectively) or TNFα (100 units/ml) for 60 min were incubated with 32P-end-labeled oligonucleotides from the AP-1-binding site in the IL-8 promoter (A) or the NF-κB-binding site in the IL-8 promoter (B). Gel shift complexes were resolved by electrophoresis and visualized by autoradiography. Specificity of the induced complexes was determined by competition with a 100-fold molar excess of unlabeled consensus cAP-1 or consensus cNF-κB oligonucleotide. Arrows indicate migration of the induced DNA binding complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown.
As shown in Fig. 6A, mutation of the AP-1 or NF-κB-binding sites disabled their ability to compete with their respective wild type binding sites. In addition, as shown in Fig. 6B and C, no induced DNA binding activity was detected on the mutant oligonucleotide probes. These data demonstrate that mutation of the AP-1 and NF-κB-binding sites in the IL-8 promoter that affect function also prevent H₂O₂ and TNFα induced DNA binding activity.

**Composition of the AP-1 and NF-κB Binding Complexes Induced by H₂O₂ and TNFα**

To identify the AP-1 and NF-κB components induced in A549 and HMEC-1, we used antibodies to AP-1 and NF-κB subunits in supershift assays. As shown in Fig. 7A, TNFα and H₂O₂ induced the same AP-1 components in A549 cells. Antibodies to c-Jun, c-Fos, and JunD, and antibodies against NF-κB components Rel A (p65), p50, and cRel were added to the binding reaction as indicated above each lane. Gel supershift complexes were resolved by electrophoresis and visualized by autoradiography. SS indicates the migration of the antibody supershift complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown. Note that antibodies to JunD, c-Fos, and Rel A (p65) generated supershift complexes.

**Fig. 6.** Mutation of the IL-8 promoter binding sites abrogate NF-κB and AP-1 binding activity. Nuclear protein extracts from A549 cells treated with H₂O₂ (800 μM) or TNFα (100 units/ml) for 60 min were incubated with 32P-end-labeled oligonucleotides from the AP-1 or NF-κB-binding site in the IL-8 promoter (A) or oligonucleotides containing mutations in the AP-1 (B) or NF-κB-binding sites (C) as indicated in Table I. Gel shift complexes were resolved by electrophoresis and visualized by autoradiography. Specificity of the induced complexes was determined by competition with a 100-fold molar excess of unlabeled AP-1, AP-1m, NF-κB, or NF-κBm oligonucleotide. Arrows indicate migration of the induced DNA binding complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown.

**Fig. 7.** Composition of the AP-1 binding complexes induced by H₂O₂ and TNFα. Nuclear protein extracts from A549 and HMEC-1 cells treated with H₂O₂ (800 and 100 μM, respectively) or TNFα (100 units/ml) for 60 min were incubated with 32P-end-labeled oligonucleotides from the AP-1-binding site in the IL-8 promoter (A) or the NF-κB-binding site in the IL-8 promoter (B). Antibodies against AP-1 components c-Jun, c-Fos, and JunD, and antibodies against NF-κB components Rel A (p65), p50, and cRel were added to the binding reaction as indicated above each lane. Gel supershift complexes were resolved by electrophoresis and visualized by autoradiography. SS indicates the migration of the antibody supershift complexes. NIC indicates the migration of the non-induced nonspecific gel shift complexes. The migration of the free probe is not shown. Note that antibodies to JunD, c-Fos, and Rel A (p65) generated supershift complexes.

Activity (18, 32). As shown in Fig. 6A, mutation of the AP-1 or NF-κB-binding sites disabled their ability to compete with their respective wild type binding sites. In addition, as shown in Fig. 6, B and C, no induced DNA binding activity was detected on the mutant oligonucleotide probes. These data demonstrate that mutation of the AP-1 and NF-κB-binding sites in the IL-8 promoter that affect function also prevent H₂O₂ and TNFα induced DNA binding activity.
and HMEC-1 were treated with H$_2$O$_2$ or TNFα for 1 h and assessed for nuclear translocation of Rel A by immunostaining using an anti-Rel A antibody and a fluorescein isothiocyanate-conjugated secondary antibody. Staining was observed under a fluorescent microscope. Note the prominent nuclear staining of the TNFα-treated cells.

**FIG. 8.** TNFα but not H$_2$O$_2$ induces translocation of Rel A. A549 and HMEC-1 were treated with H$_2$O$_2$ or TNFα for 1 h and assessed for nuclear translocation of Rel A by immunostaining using an anti-Rel A antibody and a fluorescein isothiocyanate-conjugated secondary antibody. Staining was observed under a fluorescent microscope. Note the prominent nuclear staining of the TNFα-treated cells.

Although TNFα induced multiple gel shift complexes, we were only able to detect Rel A (p65) as a component of the NF-κB binding complexes in A549 and HMEC-1. As shown in Fig. 7B, Rel A (p65) antibodies supershifted NF-κB complexes induced by TNFα in both cell types. In contrast, no supershifts were detected with p50 or cRel antibodies. In control experiments, the anti-p50 antibody did supershift NF-κB binding complexes derived from monocytic cells, indicating that the p50 antibody was functional (data not shown).

To directly demonstrate that H$_2$O$_2$ did not activate Rel A in HMEC-1 and A549 cells, we examined nuclear translocation of Rel A by immunohistochemistry. In resting cells, NF-κB resides primarily in the cytoplasm complexed with an inhibitor protein IκB (33). Cellular activation leads to the phosphorylation of IκB, which targets the inhibitor protein for ubiquitination and then proteolysis by the proteasome (34). Proteolytic degradation of IκB permits NF-κB to translocate to the nucleus and bind its recognition site in the promoter of IL-8 (35). To examine NF-κB activation and nuclear translocation, HMEC-1 and A549 cells were treated with H$_2$O$_2$ or TNFα for 60 min and stained with a rabbit anti-Rel A (p65) antibody. Localization of Rel A was detected using a fluorescein-tagged goat anti-rabbit secondary antibody. As shown in Fig. 8, TNFα dramatically increased the nuclear staining of the two cell types, whereas little or no increase in nuclear fluorescence was observed in either HMEC-1 or A549 cells stimulated with H$_2$O$_2$. These data support the DNA binding studies demonstrating that TNFα but not H$_2$O$_2$ activates NF-κB in HMEC-1 and A549 cells.

**H$_2$O$_2$ Induction of IL-8 mRNA Expression Correlates with AP-1 Activation**—H$_2$O$_2$ induces IL-8 mRNA expression and protein secretion in a dose-dependent fashion in A549 with optimal induction between 400 and 800 μM H$_2$O$_2$ (20, 21). To relate AP-1 binding activity to IL-8 gene expression, we compared the induction of AP-1 binding activity on the IL-8 promoter and IL-8 mRNA expression in response to increasing concentrations of H$_2$O$_2$. As shown in Fig. 9, H$_2$O$_2$ increased IL-8 mRNA expression and AP-1 binding activity in a concentration-dependent manner. The concentration-dependent increase in IL-8 mRNA expression coincided with the concentration-dependent increase in AP-1 binding activity. These data suggest that H$_2$O$_2$ induction of IL-8 gene expression in A549 cells is mediated by AP-1 binding to the IL-8 promoter.

**Thiol Oxidation Inhibits H$_2$O$_2$ Induction of AP-1 Activity and IL-8 Expression**—AP-1 and NF-κB binding activity is mediated by a conserved redox-sensitive cysteine residue within the DNA-binding domain (36, 37). Thiol oxidation agents such as diamide can inhibit AP-1 and NF-κB binding activity (38). To determine whether thiol oxidation of AP-1 or NF-κB could modulate IL-8 gene expression, we examined the effect of diamide on H$_2$O$_2$ induction of AP-1 activity and IL-8 protein secretion. Nuclear protein extracts from A549 cells pretreated for 60 min with increasing concentrations of diamide followed by stimulation with 800 μM H$_2$O$_2$ for 60 min were assessed for AP-1 binding activity. As shown in Fig. 10, diamide dose dependently inhibited H$_2$O$_2$ induced AP-1 binding activity to the IL-8 promoter with complete inhibition at 800 μM diamide. These data demonstrate that diamide-mediated thiol oxidation inhibits H$_2$O$_2$ induction of AP-1 binding to the IL-8 promoter in A549 cells.

To relate the diamide effect on AP-1 binding activity with IL-8 expression, we measured culture supernatants at 24 h for IL-8 protein secretion. As shown in Fig. 11, diamide dose dependently inhibited the H$_2$O$_2$ induction of IL-8 protein secretion, consistent with the close association between AP-1 binding activity and IL-8 gene expression in A549 cells. However, in contrast to the effect of diamide on AP-1 binding activity, IL-8 secretion was inhibited by much lower concentrations of diamide, suggesting that thiol oxidation can also effect IL-8 expression independently of AP-1 binding activity. Moreover, the H$_2$O$_2$ induced IL-8 secretion was more sensitive to diamide than the spontaneous secretion of IL-8, suggesting that the constitutive and induced mechanisms of IL-8 expression may differ in A549 epithelial cells. Taken together, these data indicate that H$_2$O$_2$ induction of IL-8 in A549 cells is mediated by...
**H$_2$O$_2$ and TNF$\alpha$ Differentially Induce AP-1 and NF-$\kappa$B**

**DISCUSSION**

IL-8 and ICAM-1 are critical protein factors in the recruitment of leukocytes to sites of inflammation and oxidant stress is an important regulator of their expression (13, 20). The up-regulation of ICAM-1 on the surface of endothelium is required for the firm adhesion of rolling neutrophils and a chemotactic gradient of IL-8 is critical for the adherent neutrophils to migrate across the alveolar-capillary membrane during lung inflammation and injury (39). In this study, we investigated the mechanism by which IL-8 and ICAM-1 are differentially regulated by oxidant stress in epithelial and endothelial cells. As summarized in Table II, we demonstrate that H$_2$O$_2$ differentially activates the redox-responsive transcription factors AP-1 and NF-$\kappa$B in epithelial and endothelial cells. H$_2$O$_2$ selectively induced AP-1 in A549 cells, whereas TNF$\alpha$ induced both AP-1 and NF-$\kappa$B (Table II). Moreover, H$_2$O$_2$ induction of AP-1 binding to the IL-8 promoter was closely associated with H$_2$O$_2$ induction of IL-8 mRNA expression, suggesting that H$_2$O$_2$ induces IL-8 in epithelial cells through the action of the transcription factor AP-1. In contrast, in HMEC-1, AP-1 binding activity on the IL-8 promoter was constitutive and H$_2$O$_2$ did not stimulate an increase with this basal AP-1 binding activity. H$_2$O$_2$ also did not stimulate NF-$\kappa$B binding activity in HMEC-1, whereas TNF$\alpha$ increased only NF-$\kappa$B binding activity (Table II). Consistent with the DNA binding studies, we recently showed that TNF$\alpha$ but not H$_2$O$_2$ could induce IL-8 promoter activity in HMEC-1.\(^2\)

In contrast to H$_2$O$_2$, TNF$\alpha$ appears to induce IL-8 in epithelial and endothelial cells mostly through the activation of NF-$\kappa$B. As with AP-1, a redox mechanism appears to be involved since diamide, which is known to target a conserved cysteine residue in the DNA-binding domain of NF-$\kappa$B, inhibited TNF$\alpha$ induced IL-8 expression.\(^2\) This conclusion is consistent with several studies demonstrating that TNF$\alpha$ activates IL-8 and ICAM-1 transcription through a cooperative interaction between NF-$\kappa$B and NF-IL-6 (C/EBP-$\beta$) (17, 19). Indeed, we found little or no binding activity on the IL-8 NF-IL-6 binding site,\(^2\) consistent with the binding requirement of NF-$\kappa$B for NF-IL-6 to also bind to the IL-8 promoter (17). In agreement with our findings, several studies have shown that H$_2$O$_2$ also does not activate NF-$\kappa$B in primary endothelial cells (13, 40, 41). However, H$_2$O$_2$ has been reported to activate NF-$\kappa$B in porcine aortic endothelial cells and transformed endothelial cell lines (40, 42, 43), indicating H$_2$O$_2$ activation of NF-$\kappa$B is complex and may depend on the endothelial cell type. In contrast to endothelial cells, in A549 epithelial cells, TNF$\alpha$ induced, in addition to NF-$\kappa$B, AP-1 binding activity on the IL-8 promoter, suggesting AP-1 may contribute to the TNF$\alpha$ induction of IL-8 in epithelial cells. Indeed, the dual binding of AP-1 and NF-$\kappa$B to the IL-8 promoter may account for the greater induction of IL-8 expression by TNF$\alpha$ than H$_2$O$_2$ in A549 cells.

Oxidant stress has been reported to affect AP-1 and NF-$\kappa$B differently, suggesting distinct mechanisms of redox regulation of these transcription factors (44, 45). Our studies suggest that H$_2$O$_2$ and TNF$\alpha$ regulate IL-8 and ICAM-1 gene expression

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\(^2\) V. Lakshminarayanan and K. A. Roebuck, unpublished data.
through distinct signal transduction pathways, and are consistent with the data of Das \textit{et al.} demonstrating differential redox regulation of AP-1 and NF-\kappaB in A549 cells (46). They found that thiols induced NF-\kappaB but not AP-1, while oxidants induced AP-1 but not NF-\kappaB. Oxidants such as ozone have also been shown to activate IL-8 expression through AP-1 and NF-\kappaB in A549 cells (47). AP-1-like proteins have also been associated with the transcriptional regulation of \gamma-glutamylcysteine synthetase-heavy subunit by oxidants in A549 cells (48). The differential activation of AP-1 and NF-\kappaB has also been shown in other cell systems (44, 45, 49–53). We conclude that the redox regulation of AP-1 and NF-\kappaB contribute to the distinct patterns of gene expression induced in epithelial and endothelial cells by H$_2$O$_2$ and TNF\alpha. A similar finding has been reported for the manganese superoxide dismutase gene in pulmonary epithelial cells (54). However, in contrast to A549 cells, the H441 pulmonary epithelial cell line did not mediate H$_2$O$_2$ or TNF\alpha induction of AP-1, suggesting H$_2$O$_2$ and TNF\alpha induce manganese superoxide dismutase expression independent of AP-1 activity. Thus, H$_2$O$_2$ and TNF\alpha can activate gene expression in epithelial cells via both AP-1 dependent and independent mechanisms. This is consistent with our diamide studies demonstrating that the thiol oxidation sensitivity of IL-8 expression was considerably greater than that of the AP-1 binding activity.

AP-1 is composed of heterodimers of Jun and Fos proteins and oxidant stress increases the transcription of \textit{c-jun} and \textit{c-fos} gene expression (55). Although \textit{c-jun} has been demonstrated to be activated by H$_2$O$_2$ in epithelial cells (56), we did not detect any \textit{c-Jun} protein in the H$_2$O$_2$-induced AP-1 complexes in A549 cells. Instead, another member of the Jun family, JunD, was induced by H$_2$O$_2$. H$_2$O$_2$ also rapidly activates \textit{c-fos} gene expression (57–60), and indeed we detected increased \textit{c-Fos} protein activity in A549 cells (62).

The redox regulation of AP-1 and NF-\kappaB may also be involved in the differential expression of CC chemokines. We reported previously that H$_2$O$_2$ induces MCP-1 but not RANTES in A549 cells, whereas TNF\alpha induced both chemokines (20). MCP-1 has been reported to be regulated by redox mechanisms (63–65), involving NF-\kappaB and AP-1 (66–70), and RANTES has recently been shown to be regulated by NF-\kappaB (71). We propose that the differential activation of redox-responsive transcription factors like AP-1 and NF-\kappaB set up distinct patterns of gene expression in epithelial and endothelial cells that may critically influence the site-specific recruitment of leukocyte subsets during inflammatory responses.

The differential activation of AP-1 and NF-\kappaB by H$_2$O$_2$ and TNF\alpha are consistent with our previous results demonstrating that H$_2$O$_2$ and TNF\alpha activate ICAM-1 gene expression through distinct cis-acting elements in the ICAM-1 promoter (13). H$_2$O$_2$ activated cis-acting ICAM-1 transcription through an element of the promoter that contain antioxidant responsive elements (ARE), whereas TNF\alpha targeted the proximal promoter containing a composite binding site for NF-\kappaB and NF-IL-6 (13). It was previously demonstrated that TNF\alpha activates ICAM-1 and IL-8 through the cooperative interaction of the NF-\kappaB and NF-IL-6-binding sites (7, 17–19). H$_2$O$_2$ induces ICAM-1 expression in HMEC-1 (20), even though H$_2$O$_2$ does not induce AP-1 or NF-\kappaB in these endothelial cells. In contrast, H$_2$O$_2$ does not induce ICAM-1 in A549 cells even though AP-1 is activated. These data suggest that H$_2$O$_2$ induction of ICAM-1 in endothelial cells is not mediated by AP-1 or NF-\kappaB, but more likely by a novel redox-responsive transcription factor.

In EAHy926, an epithelial/endothelial hybrid cell line generated from the fusion of A549 and human umbilical vein endothelial cells, H$_2$O$_2$ induces both ICAM-1 and IL-8, indicating that fusion of endothelial and epithelial cells abrogates their ability to differentially regulate the two genes (13, 20). The dominance of the H$_2$O$_2$ gene induction suggests the involvement of positive trans-acting factors in the discordant oxidant regulation of ICAM-1 and IL-8 in epithelial and endothelial cells. ICAM-1 expression was induced through a H$_2$O$_2$ responsive region of the promoter containing tandem 16-base pair AP-1/Ets composite sites (13). These AP-1/Ets repeats also have homology to known antioxidant responsive elements (ARE) first identified as redox-responsive elements in GST Ya subunit gene (72–76). AP-1 can physically interact with Ets at AP-1/Ets composite sites to functionally activate gene transcription in response to H$_2$O$_2$ (77–79). H$_2$O$_2$ increased binding activity on the ICAM-1 ARE sites and mutation of either the AP-1 or Ets motif abrogated the H$_2$O$_2$ induced binding activity (13). However, in A549 cells, ICAM-1 expression is not stimulated by H$_2$O$_2$, whereas H$_2$O$_2$ induced ICAM-1 in HMEC-1 (20). Since H$_2$O$_2$ did not induce AP-1 or NF-\kappaB in HMEC-1, it appears that H$_2$O$_2$ induction of ICAM-1 in endothelial cells is mediated by novel redox responsive transcription factors. ARE-binding proteins capable of binding the ICAM-1 AP-1/Ets sites have recently been identified providing a potential mechanism by which H$_2$O$_2$ could activate ICAM-1 in endothelial cells independently of AP-1 or NF-\kappaB (80–82). Indeed, oxidant stress can differentially induce other transcription factors in epithelial and endothelial cells. For example, we previously demonstrated that H$_2$O$_2$ differentially induces the winged-helix transcription factor, HFH-11 in A549 and HMEC-1 (83). H$_2$O$_2$ increased HFH-11 expression in HMEC-1 but not in A549 cells (20).

In conclusion, we have demonstrated that H$_2$O$_2$ and TNF\alpha can differentially activate the redox-responsive transcription factors AP-1 and NF-\kappaB in epithelial and endothelial cells. We propose this differential regulation of AP-1 and NF-\kappaB leads to different patterns of gene expression in epithelial and endothelial cells that may be critical for their function during oxidant stress.

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