Oxidative stress activates anion exchange protein 2 and AP-1 in airway epithelial cells

JENNIFER L. TURI,1 ILONA JASPERS,2 LISA A. DAILEY,3 MICHAEL C. MADDEN,3 LUISA E. BRIGHTON,2 JACQUELINE D. CARTER,3 EVA NOZIK-GRAYCK,1 CLAUDE A. PIANTADOSI,4 AND ANDREW J. GHIO3

1Department of Pediatrics, Duke University Medical Center; 4Department of Internal Medicine, Duke University Medical Center, Durham 27710; 2Center for Environmental Medicine and Lung Biology, University of North Carolina, Chapel Hill 27599; and 3National Health and Environmental Effects Research Laboratory, Office of Research and Development, Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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Oxidative stress activates anion exchange protein 2 and AP-1 in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 283: L791–L798, 2002. First published June 5, 2002; 10.1152/ajplung.00398.2001.—Anion exchange protein 2 (AE2) is a membrane-bound protein that mediates chloride-bicarbonate exchange. In addition to regulating intracellular pH and cell volume, AE2 exports superoxide (O2·-) to the extracellular matrix in an HCO3-dependent process. Given this ability to export O2·-, we hypothesized that expression of AE2 in the lung is regulated by oxidative stress. AE2 mRNA and protein expression was measured by RT-PCR and Western blot analysis, respectively, in differentiated human bronchial epithelial cells exposed to H2O2 (100 μM). Alterations in in vivo AE2 protein expression were evaluated in lung tissue of rats exposed to 70% O2. The role of transcription factor activator protein (AP)-1 in oxidant regulation of AE2 was evaluated by EMSA and by immunoblotting of nuclear phospho-c-jun. Results show increased AE2 mRNA and protein expression after oxidant exposure. This was preceded by transient increases in DNA binding of AE2-specific AP-1 and phosphorylation of c-jun. This study demonstrates that AE2 expression is regulated by oxidative stress in airway epithelial cells and that this regulation correlates with activation of AP-1.

superoxide; ion transport; gene regulation; activator protein-1

E X P E R I M E N T A L

Oxidative tissue injury by increased levels of reactive oxygen species (ROS) can be produced during inflammation, ischemia/reperfusion (13, 27), hyperoxia, and hypoxia (5). Normally, ROS generated as by-products of aerobic metabolism are contained by an elaborate system of antioxidant defenses. When the level of ROS generated is greatly increased, these defenses can be overwhelmed, causing a state of oxidative stress.

The most readily produced ROS is superoxide anion (O2·-), which is produced by the single electron reduction of oxygen. The majority of O2·- is generated intracellularly through the activity of such systems as the electron transport chain, cytochrome P-450 (24), and xanthine oxidase (25, 27). Increased generation of O2·- during periods of oxidative stress can lead to cellular injury by lipid peroxidation, protein oxidation, and DNA damage. Increased levels of intracellular O2·- can result in the release of free iron, thus accelerating macromolecule damage through the formation of hydroxyl radicals (•OH) (14). Superoxide has also been shown to cross the cell membrane to mediate injury in the extracellular environment (13). Because O2·- is an anion, a transport system is required for intracellular O2·- to move through the cell membrane (19). Extracellular transport of superoxide in the lung appears to be mediated primarily by anion exchange protein 2 (AE2) (23).

AE2 is a membrane-bound electroneutral chloride-bicarbonate (Cl−/HCO3−) exchanger. AE2s have been localized in virtually all cell types and primarily serve to regulate intracellular pH and cell volume (1). In addition to these regulatory functions, AE has also been implicated in the transport of O2·- in exchange for extracellular HCO3 in a number of cell types (19). AE2 has been localized to airway epithelial cells and vessels, where its association with extracellular O2·- has been shown to contribute to oxidative injury in an isolated perfused lung model of hyperoxia (23). Blocking the function of AE2 either by DIDS, a stilbene inhibitor that acts as a specific inhibitor of AE transporters, or by use of a bicarbonate-free buffer markedly decreased the degree of injury seen as shown by the formation of edema and pulmonary hypertension.

AE2 mRNA and protein expression and localization have been demonstrated in a number of tissues (15),
including the lung (7, 17, 18, 23). The relatively ubiquitous distribution of AE2 among epithelial cells demonstrates some tissue specificity through the expression of at least three isoforms (21). The DNA sequence of AE2 has been identified in the human (20) and the mouse (16) and has been shown to contain several response elements in its promoter region (20). Although the regulation of AE2 has been investigated in the kidney (8) and intestine (6), little is known about the regulation of AE2 expression in the lung.

Given the involvement of AE2 in superoxide exchange, we hypothesized that the expression of AE2 in the lung is regulated by oxidative stress. We tested this hypothesis in vitro by evaluating AE2 mRNA and protein expression in differentiated human bronchial epithelial (HBE) cells after exposure to hydrogen peroxide (H2O2). The regulation of AE2 by oxidative stress was further tested in vivo by measuring AE2 protein expression in the lungs of rats exposed to hyperoxia. Finally, we evaluated the role of activator protein-1 (AP-1), a redox-sensitive transcription factor, in regulating the expression of this protein.

METHODS

Cell culture and in vitro exposure. Primary HBE cells were obtained from healthy, nonsmoking adult volunteers after consent was obtained. The protocol and consent form were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Cells were obtained by cytologic brushing at bronchoscopy and expanded to passage 3 in bronchial epithelial growth medium. They were plated on collagen-coated filters supports for a 0.4-µm pore size (Trans-CLR; Costar, Cambridge, MA) at a density of 1 × 106 and inserted into 12-well culture plates. Cells were maintained in a 1:1 mixture of bronchial epithelial cell basic medium (BEBM) and DMEM-H with SingleQuot supplements, bovine pituitary extracts (13 mg/ml), bovine serum albumin (BSA, 1.5 µg/ml), and nystatin (20 units) with 0.5 ml in the apical chamber and 1.5 ml in the basolateral chamber. Medium was replaced every 48 h. Retinoic acid was added on day 2 to promote differentiation. Air-liquid interface (ALI) was created on day 6 by removing the apical medium. The cells were maintained in the above media until they had achieved uniform differentiation in ciliated, mucus-producing cells, ~9–10 days after creation of ALI.

Differentiated cells were exposed at the apical surface to a single application of H2O2 (100 µM) or to media consisting of a 1:1 mixture of BEBM and DMEM-H without supplements. Placing the oxidant in the apical chamber more closely mimics the in vivo situation and minimizes direct exposure of the transporter to the oxidant, which is located primarily at the basolateral plasma membrane (17, 18). This concentration of H2O2 was chosen because it is typically not associated with alterations in cell viability and replication competence (28). Cells were harvested for measurement of RNA expression after exposure for 0, 4, 12, and 24 h; protein expression after 0, 4, 12, 24, and 36 h; and AP-1 activation after 0, 30, 60, and 120 min.

Assessment of cellular injury by carbonyl analysis and lactate dehydrogenase release. HBE cells grown at ALI were exposed to a single application of H2O2 at varying concentrations (100 µM, 1 mM, 5 mM) for 4 h. After treatment with H2O2, medium was removed, and cells were scraped off the filters into 500 µl of methanol and kept on ice. The cells were incubated briefly with 2,4-dinitrophenylhydrazine (DNPH in methanol), which reacts selectively with carbonyls. Samples were analyzed for carbonyl content as an end product of lipid peroxidation after separation from protein and nucleic acids using a 2690 Separation Module HPLC. Separations were done on a Waters’ Xterra C18 column (2.1 × 15 mm; 3.5 µm). Carbonyl analysis was performed using a 2487 dual wavelength absorbance detector (at 365 nm) and a ZMD mass spectrometer (Waters’ Associates, Milford, MA). The solvent system consisted of water-methanol (9:1, solvent A) and acetonitrile-methanol (9:1, solvent B) both containing 0.01% formic acid. The solvent gradient used was 0–10 min at 44.4% solvent A-55.6% solvent B and then a linear gradient to 11% solvent A-89% solvent B over 30 min at 0.25 ml/min. The mass spectrometry was run in electrospray ionization negative mode with a capillary voltage of 2 kV, cone voltage of −18 V, extractor voltage of −3 V, source block temperature of 150°C, desolvation temperature of 350°C, and radio frequencies of 0.3. Selected ion monitoring was done at m/z 223, 237, 251, 265, 279, 283, 307, 321, 335, 349, 363, and 417 ± 2; these values correspond to the derivatized parent ions for C2-C21, respectively. The DNPH derivative of cis-11-hexadecenal (m/z 417; Aldrich Chemical, Milwaukee, WI) was added as an internal standard in some runs. Data (e.g., peak area) were analyzed using Waters’ MassLynx software (version 3.2).

Cytotoxicity was measured by lactate dehydrogenase (LDH) release into the basolateral media using a commercial kit (Sigma Diagnostics, St. Louis, MO) modified for automated measurement (Cobas Fara II, centrifugal analyzer). Additional LDH measurements were made in HBE cells exposed to a single application of H2O2 (100 µM) for variable duration (0, 4, 12, and 24 h).

Evaluation of AE2 localization by immunofluorescence. HBE cells were grown and differentiated at ALI. The filter supports for the cells were excised and embedded in paraffin. Sections of 8 µm were deparaffinized in xylene and hydrated to 70% alcohol. Nonspecific binding sites were blocked with 20% normal goat serum in Tris-buffered saline (TBS). Antigen-antibody complexes were stained with a FITC-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR; 1:200 dilution in Tris-buffered saline (TBS)-Triton X-100 (0.25%) for 30 min. The slides were incubated overnight at 4°C with a mouse polyclonal antibody against the COOH-terminal amino acids of AE2 [1:10 dilution with Tris-Triton X-100 (0.25%)]. The primary AE2 antibody was developed in mouse ascites fluid against the rat AE2 COOH-terminal amino acids 1,224–1,237 (CEGVDEYNEMPMPV-COOH) (30) by established methods (22). Antigen-antibody complexes were stained with a FITC-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR; 1:200 dilution in Tris-Triton X-100) at pH 7.4 for 1 h at room temperature. Control sections were incubated with only the FITC-conjugated antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The sections were dehydrated using a 1:1 mixture of glycerol in phosphate-buffered saline (PBS).

Western blot analysis of AE2 protein expression. Cells were washed with ice-cold PBS and lysed with buffer containing 1% Nonidet P (NP)-40, 0.5% deoxycholate, and 0.1% SDS, and protease inhibitors [Cocktail Set III: 100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 µM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, and 1 mM pepstatin A; Calbiochem, La Jolla, CA]. Cells were harvested and sheared through a 22-gauge needle, and cellular debris was pelleted by centrifugation at 500 g for 5 min. The supernatant was removed. Protein content of the lysate was determined using the Bradford assay (Bio-Rad, Hercules, CA), and the sample was mixed with 4× sample loading buffer (0.5 M
Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 0.7 M β-mercaptoethanol, and 0.05% bromphenol blue) at a 3:1 ratio.

Equal quantities of whole cell lysate protein were separated by SDS-polyacrylamide gel electrophoresis (7.5%) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 3% casein in PBS-Tween 20 (PBS-T) for 1 h at room temperature. This was followed by immunoblotting using the polyclonal AE2 antibody (1:1,000 dilution in PBS-T) overnight at 4°C. Antibody-antigen complexes were stained with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and developed using enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech). Bands were quantified using GeneTools Image Analysis Software (Syngene, Frederick, MD).

Analysis of AE2 mRNA expression by RT-PCR. RNA was prepared by lysing cells in buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 10 mM DTT. After shearing through a 22-gauge needle, the lysate was layered over an equal volume of 5.7 M CsCl with 0.1% RNase-free RNAase. The RNA was precipitation for 2 h at 80,000 rpm. Reverse transcription and DNA amplification were performed as previously described (4).

Oligonucleotide sequences used were synthesized using an Applied Biosystems 391 DNA synthesizer (Perkin-Elmer, Foster City, CA) based on the sequences published in GenBank. The following sequences were used: GAPDH sense, 5′-CATCTGGAGAAGCCGAGG-3′; GAPDH antisense, 5′-CAGCAACCACCTGGAGTG-3′; AE2 sense, 5′-TGTAGGCAGAACCCAGGTTG-3′; AE2 antisense, 5′-GCAGGAAGAGGCGTAAAGA-3′. cDNA was amplified for 26 and 36 cycles for GAPDH and AE2, respectively. PCR products were analyzed by gel electrophoresis through a 2% agarose gel in 0.5× Tris-borate-EDTA buffer. The gels were stained with 1 μg/ml ethidium bromide and photographed under ultraviolet illumination with Polaroid type 66P/N film. Bands were quantified using the Kodak 1D Image Analysis software (Eastman Kodak, Rochester, NY). The optical densities for AE2 mRNA bands were normalized against those of GAPDH bands.

Evaluation of AE2 protein induction in vivo after O2 exposure. Animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Sprague-Dawley rats were exposed to 70% O2 for up to 7 days. After euthanasia, the trachea was cannulated and the lungs inflation fixed at 20 cmH2O pressure in 4% paraformaldehyde for immunohistochemistry or flash-frozen for Western blot analysis. AE2 protein expression was analyzed by immunohistochemistry using an established protocol (23). Briefly, paraform-embedded tissue sections were incubated with the primary AE2 antibody (1:100) followed by a biotinylated goat anti-mouse IgG antibody and labeled with horseradish peroxidase-conjugated streptavidin (Innogenex). Slides were developed with 3,3′-diaminobenzidine and counterstained with hematoxylin. Negative controls were performed with normal mouse serum and antibody co-incubated with AE2 peptide. Tissue sections were examined by light microscopy and photographed at ×132. Western blot analysis was performed as described in Western blot analysis of AE2 protein expression using membrane fractions of the lung protein, isolated by ultracentrifugation at 100,000 g for 1 h at 4°C to enhance detection of AE2 (23).

Isolation of nuclear protein. After cells were washed with ice-cold PBS, cold cytoplasmic extraction buffer (CEB; 10 mM Tris·HCl, pH 7.9; 60 mM, 1 mM EDTA, and 1 mM DTT) with protease inhibitors (Cocktail Set III; Calbiochem) was added to apical chamber. Cells were harvested and transferred to microcentrifuge tube. They were then allowed to swell on ice for 15 min. NP-40 (Sigma) was added to a final concentration of 0.1% and the tube vortexed for 10 s. The nuclei were pelleted by centrifugation at 14,000 g for 40 s. The nuclei were washed with CEB and centrifuged again at 14,000 g for 30 s. The supernatant was discarded, and the nuclei were incubated for 10 min on ice in nuclear extraction buffer (20 mM Tris·HCl, pH 8.0, 400 mM NaCl, 1.5 mM MgCl2, 1.5 mM EDTA, 1 mM DTT, and 25% glycerol) with protease inhibitors. The sample was briefly centrifuged and the supernatant removed. An aliquot was stored at −80°C for analysis by electrophoretic mobility shift assay (EMSA). The remainder was processed as described above for Western blot analysis.

Evaluation of DNA binding by EMSA assay. AE2-specific AP-1 oligonucleotide sequences were synthesized using an Applied Biosystems 391 DNA synthesizer (Perkin-Elmer). The sequences were chosen to contain the core AP-1 binding site, GAGTCA, as well as a number of consecutive nucleotides flanking the potential binding sites within the AE2 promoter (−AGCCACCGCATGGGGTGCAG-3′). The probes were labeled by incubating 15 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 100 μCi of adenosine 5′-[γ-32P]triphosphate (ICN, Irvine, CA) with 100 ng of double-stranded probe at 37°C for 30 min. The mixture was passed through a desalting column (Nuc Trap, Stratagene, San Diego, CA) to remove unincorporated 32P. The DNA binding reaction, consisting of 4 μg nuclear extract, 1.5 μl labeled probe, and 10 μl running buffer (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA, 1 mM DTT, and 5% glycerol), and 2 μg poly(dI-dC) (Roche Molecular Biochemical), was performed at room temperature for 25 min. The samples were separated by electrophoresis on a 4.5% nondenaturing polyacrylamide gel containing 0.5× Tris-borate-EDTA. The gels were dried and autoradiographed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Separate experiments were performed to further characterize the AP-1 DNA-binding complex. A commercially available c-Jun gel shift kit was used (c-Jun Nushift kit; Geneka Biotechnology) according to the supplier’s instructions. Briefly, an AP-1 consensus sequence was labeled as above (5′-CGCTTGATGATCGCCGGAAA-3′). The DNA binding reaction was performed using 6 μg of nuclear protein in the presence of 2 μg of phospho-c-Jun (p-c-Jun Ser63), 200 μg/0.1 ml; Santa Cruz Biotechnology) or c-Jun antibody (c-Jun/AP-1, 200 μg/0.1 ml; Santa Cruz Biotechnology). In addition, the specificity of the DNA binding was determined using competition with cold, wild-type or mutated AP-1 oligonucleotides (5′-CGCTTGATGATCGCCGGAAA-3′) added to the reaction mixture at 100-fold excess. The samples were separated by electrophoresis on a 4.5% nondenaturing polyacrylamide gel containing 0.5× Tris-glycine-EDTA and processed as described above.

Evaluation of nuclear levels of phosphorylated c-Jun by Western blot analysis. Equal quantities of nuclear protein were loaded into each lane and separated by SDS-polyacrylamide gel electrophoresis (12%) and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 1% BSA and 1% casein in Tris-buffered saline-Tween 20 (TBS-T) for 1 h at room temperature. This was followed by immunoblotting using a mouse monoclonal antibody against phospho-c-Jun (Ser63) antibody (diluted 1:10,000 in 1% BSA plus 1% casein-TBS-T; p-c-Jun, 200 μg/0.1 ml; Santa Cruz Biotechnology). Antigen-antibody complexes were stained with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:1,000, Santa Cruz Biotechnology) for 1 h at room temperature and developed using enhanced chemilumines-
Bands were quantified using GeneTools Image Analysis software (Syngene). Protein levels were expressed as percent induction over control.

Statistical analysis. Data are expressed as means ± SE. A minimum of at least three separate experiments was performed for each measurement. Data were compared using one-way analysis of variance followed by the Fisher's protected least square difference test. Significance was assumed at \( P < 0.05 \).

RESULTS

AE2 is expressed in differentiated HBE cells. The constitutive expression of AE2 was confirmed in our differentiated cell culture model. HBE cells grown at ALI until differentiated into a mucociliary epithelium expressed AE2 by immunofluorescence. Significant labeling of the plasma membrane with the AE2 antibody was noted with a tendency for protein localization at the basolateral plasma membrane in most of the cells (Fig. 1). Counterstaining of the nuclei with DAPI suggested significant perinuclear localization of the AE2 protein as well. Negative controls produced without the addition of the primary antibody, showed no fluorescence staining (data not shown).

Oxidative stress regulates expression of AE2 in airway epithelial cells. \( \text{H}_2\text{O}_2 \), although a relatively weak oxidant, can rapidly cross cell membranes to react with other ROS and with metal ions to cause significant cellular injury (11). To determine whether \( \text{H}_2\text{O}_2 \)-mediated oxidative stress regulates the expression of AE2, we exposed the apical surface of differentiated HBE grown at ALI to \( \text{H}_2\text{O}_2 \) (100 \( \mu \)M). Western blot analysis revealed a single band at 95 kDa (Fig. 2), consistent with the size of the AE2 isoform reported in lung tissue of other species (23). Constitutive AE2 protein expression was noted before exposure to \( \text{H}_2\text{O}_2 \). An increase in protein expression was seen within 12 h of exposure, with peak expression at 24 h and return to baseline by 36 h.

Cellular injury after exposure to \( \text{H}_2\text{O}_2 \) was measured by carbonyl content as an end product of lipid peroxidation and by LDH release. Cells that were exposed to 5 mM \( \text{H}_2\text{O}_2 \) had an increase in a DNPH-reactive product (based on ultraviolet absorbance at 365 nm) that eluted from the HPLC at \( \sim 9.9 \) min. This product was not observed in cells incubated with smaller concentrations of \( \text{H}_2\text{O}_2 \) or in unexposed cells. The peak did not have the elution time of other straight-chain aldehyde standards and had an m/z value of 267, which is consistent with the value of a hydroxylated four-carbon carbonyl. Butanal, a four-carbon straight-chain aldehyde, eluted at \( \sim 12.3 \) min. On the basis of the elution time and m/z, we tentatively identified the carbonyl product as a hydroxylated four-carbon carbonyl. Butanal eluted sooner in a reverse phase separation than the complimentary aldehyde (26). No differences were observed in the amounts of other aldehydes (from C2–C12) in the cell extracts among the treatment groups.

Cytotoxicity was measured by LDH release after addition of \( \text{H}_2\text{O}_2 \). There was no increase in LDH until incubation with 1 mM, and the increase was only statistically significant after exposure to 5 mM \( \text{H}_2\text{O}_2 \) (Fig. 3). No toxicity was evident after a single application of 100 \( \mu \)M \( \text{H}_2\text{O}_2 \), either after 4 h (Fig. 3) or up to 24 h after exposure (data not shown).

We exposed Sprague-Dawley rats to 70% \( \text{O}_2 \) to determine whether AE2 protein expression is regulated by oxidative stress in vivo. Western blot analysis performed on lung tissue of exposed rats demonstrated increased AE2 protein expression 1 day after exposure to hyperoxia. This increase in expression was sustained throughout the 7-day exposure period (Fig. 4A). Immunohistochemistry displayed a similar increase in
increased within 4 h of exposure to H$_2$O$_2$. This increase amplified to 36 cycles. This expression significantly persisted 12 h after exposure with return to near baseline levels of DNA binding within 120 min. The migration of the AP-1 complex was supershifted with the addition of the phosphorylated c-Jun antibody (Fig. 7). Migration of the AP-1 complex with the nonspecific c-Jun antibody abrogated the protein-DNA complex, as expected for an antibody directed against the DNA binding region. The overall decrease in intensity of the nonshifted AP-1 complex was similar with both the phosphorylated c-Jun and the nonspecific c-Jun antibodies. This suggests that the majority of c-jun comprising the bound AP-1 is in the phosphorylated form.

To evaluate the specificity of the AP-1 DNA binding, we performed competition assays, adding 100-fold excess unlabeled AP-1 oligonucleotide to the DNA-protein reaction mixture. Figure 7 shows that wild-type AP-1 oligonucleotides successfully competed for DNA binding, whereas mutant AP-1 oligonucleotides do not, confirming the specificity of the AP-1 DNA-binding complex.

Fig. 3. Cytotoxicity in differentiated HBE cells exposed to H$_2$O$_2$. Differentiated HBE cells grown at ALI were exposed to media (Ct) or to a single application of varying concentrations of H$_2$O$_2$ (100 μM, 1 mM, or 5 mM) for 4 h. The presence of cytotoxicity was measured by lactate dehydrogenase (LDH) release into the basolateral media using a commercial kit (Sigma Diagnostics) modified for automated measurement (Cobas Fara II, centrifugal analyzer). No evidence of LDH release is seen after exposure to 100 μM H$_2$O$_2$. Significant toxicity is noted only after exposure to 5 mM H$_2$O$_2$ (*$P < 0.001$).
Phosphorylation of c-jun is required for AP-1 transcriptional activity. Therefore, we evaluated whether nuclear levels of phosphorylated c-jun were increased in HBE cells exposed to H2O2. Western blot analysis using nuclear protein from cells exposed to H2O2 consistently showed an increase in phosphorylation of c-jun. The response was seen typically within 30 min of exposure to H2O2 with a decrease by 120 min (Fig. 8). Although the overall response was consistent, there was some variability in the timing of onset and duration of the increased nuclear translocation of phosphorylated c-jun. On the basis of the DNA binding of AE2-specific AP-1 and the phosphorylation of c-jun with exposure to H2O2, we concluded that AP-1 activation correlates with oxidative stress induced upregulation of AE2 expression in HBE cells.

**DISCUSSION**

This study demonstrates that the expression of AE2 in airway epithelial cells is regulated by oxidative stress and that this regulation appears to be mediated in part by transcription factor AP-1. Increased protein expression is seen both in HBE cells exposed to H2O2 and in airway epithelium in rats exposed to hyperoxia. In the HBE cells this is preceded by a dramatic increase in the transcription of mRNA as demonstrated by RT-PCR. Evidence that AP-1 activity correlates with this regulation is demonstrated by increased binding of the AE2-specific AP-1 sequence after exposure to H2O2, as well as by the increased nuclear translocation of activated c-jun.

AE2 protein expression has been demonstrated previously in lung tissue (7, 17, 23) and in immortalized cell lines (17), although not in primary bronchial epi-

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**Fig. 4.** AE2 protein expression in vivo after exposure to hyperoxia. Rats were exposed to 70% O2 for 0, 1, 3, and 7 days (d). Western blot analysis (A) was performed using membrane fractions of lung protein separated by SDS-polyacrylamide gel electrophoresis (8%) and then immunoblotted with the AE2 antibody (1:1,000). Paraffin-embedded tissue was analyzed for AE2 protein expression by immunohistochemistry (B) using the primary AE2 antibody (1:100). Slides were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. Tissue sections were examined by light microscopy and photographed at ×132.

**Fig. 5.** AE2 mRNA expression after stimulation with H2O2. Differentiated HBE cells grown at ALI were treated with a single application of H2O2 (100 μM). Cells were harvested after 4, 12, or 24 h and compared with Ct. Total RNA was isolated, and AE2 mRNA levels were analyzed by RT-PCR. Representative ethidium bromide-stained amplification products for AE2 from 3 separate experiments are shown (A) along with densitometric analysis (B). Data are expressed as the relative quantity of AE2 mRNA normalized against GAPDH (± SE). A marked transient increase in AE2 mRNA expression is evident 4 h after stimulation with H2O2 (*P < 0.05 vs. control).

**Fig. 6.** DNA binding of AP-1 in stimulated HBE cells. Nuclear protein was isolated from differentiated HBE cells exposed to a single application of H2O2 (100 μM) for 30, 60, or 120 min and compared with unstimulated Ct. DNA binding activity was evaluated by EMSA using a radiolabeled probe corresponding to the activator protein (AP)-1 consensus sequence from the AE2 promoter region. The DNA binding reaction was performed using 4 μg of nuclear protein. Exposure to H2O2 resulted in a transient increase in the extent of binding of the AE2-specific AP-1 sequence.
The transcription factor AP-1 is known to respond to intracellular concentrations of ROS, including H2O2, to coordinate the induction of a number of antioxidant defenses (3). DNA binding of AP-1 requires the dimerization of c-jun with itself or with a member of the Fos family of binding proteins. Activation of AP-1 then requires the phosphorylation of serine residues within the transactivation domain of c-jun. Given the increased expression of AE2 after stimulation with H2O2 and the presence of an AP-1 consensus binding sequences within the promoter region of AE2, we speculated that AP-1 may be one mechanism by which AE2 is regulated after exposure to oxidative stress. Our investigation revealed that the upregulation of AE2 is preceded by an increase in AP-1 DNA binding and phosphorylated c-jun levels. This result implicates AP-1 in the regulation of AE2, although the substantially more gradual increase in AE2 expression suggests the possibility that posttranscriptional or post-translational modification may also be involved.

We used primary human airway epithelial cells grown at ALI and allowed to differentiate into mucociliary epithelium as our in vitro model, because these cells display cytological and electrophysiological fea-

![Fig. 7](image)

**Fig. 7.** A: supershift of AP-1 complexes with phosphorylated c-Jun. Phosphorylation of the bound subunit c-Jun was evaluated by EMSA supershift using a radiolabeled probe corresponding to the AP-1 consensus sequence. The AP-1 complexes were supershifted using an antibody against phosphorylated c-Jun. Although a supershift is not seen with addition of the nonspecific c-Jun antibody, a decrease in DNA binding is seen. This is consistent with antibody specificity against the c-jun DNA binding region. B: specificity of AP-1 binding. Competition with 100-fold excess of cold, wild-type AP-1 results in the inhibition of complex formation (No comp.). This is not seen with the excess mutated AP-1 (mAP-1), indicating specificity of AP-1 binding.

![Fig. 8](image)

**Fig. 8.** Nuclear levels of phosphorylated c-Jun after exposure to H2O2. Differentiated HBE cells were exposed to H2O2 (100 μM) for 30, 60, and 120 min. Equal quantities of nuclear protein were separated by SDS-polyacrylamide gel electrophoresis (12%) followed by immunoblotting using a monoclonal antibody against phosphorylated c-Jun (1:10,000). A representative immunoblot is shown (A) along with analysis by densitometry (B). Data are expressed as percent induction over control (± SE). Increased levels of phosphorylated c-Jun are present in the nucleus after exposure to H2O2.
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tures that mimic native epithelium (29). This provides a more physiological rationale for studies of ion transport and regulation. Cells grown submerged on plastic lose polarity and varying degrees of their normal differentiated mucociliary features (10) and, therefore, may not respond to oxidant stress in a physiological manner. The use of differentiated cells in culture, however, may explain some of the observed variability in the duration of activation of AP-1. As these cells are obtained from healthy volunteers, it is difficult to know whether there was some degree of activation in the days preceding their collection or if there are differences in individual susceptibility. Despite these inherent problems associated with the use of primary cell cultures, the overall response of AE2 to \( \text{H}_2\text{O}_2 \) was found to be consistent among individuals.

In conclusion, we have shown that oxidative stress regulates the expression of AE2 protein in airway epithelial cells of the human and the rat. In HBE cells, this increase in AE2 expression is preceded by increases in mRNA, which occurs concurrently with an increase in activation of AP-1. This may well explain, in part, the mechanism of regulation by oxidative stress. Increased expression of the AE2 protein potentially provides the cell greater ability to transport \( \text{O}_2^- \) out of the cell during times of oxidative stress. This ability may result in increased intracellular protection. Conversely, it may lead to the generation of greater oxidative stress in the extracellular matrix. Further research will be required to determine the effect of this upregulation on cellular function and mechanisms of oxidative injury during times of cellular stress.

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