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HIF-1α has an Anti-Apoptotic Effect in Human Airway Epithelium That Is Mediated via Mcl-1 Gene Expression

Xiu-Huai Liu,* Erik Z. Yu, Ying-Yue Li, and Elliott Kagan

Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

Abstract Hypoxia-inducible factor-1α (HIF-1α) and myeloid cell leukemia-1 (Mcl-1) proteins have been shown to regulate apoptosis in some cell systems but have not been studied in this context in airway epithelium. Using a model of anoxia/reoxygenation (A/R), the present study employed RNA interference (RNAi) targeting HIF-1α and Mcl-1 to evaluate their possible anti-apoptotic effects on HBE1 cells, an immortalized human bronchial epithelial cell line. The cells were either cultured under normoxic conditions or were transfected with small interfering RNA (siRNA) duplexes targeting HIF-1α or Mcl-1 mRNA and then immediately exposed to A/R. As controls, non-transfected HBE1 cells and cells transfected with scrambled RNA duplexes were subjected to A/R. Apoptosis was evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay and RNAi was assessed by knockdown of HIF-1α and Mcl-1 mRNA and protein expression using real-time quantitative RT-PCR (Q-PCR), immunohistochemistry, and Western blots. HBE1 cells transfected with siRNA duplexes targeting either HIF-1α or Mcl-1 and subjected to A/R manifested considerable apoptosis, a finding not observed in either non-transfected cells or cells transfected with scrambled RNA duplexes. Specific knockdown of mRNA and protein expression by RNAi in HBE1 cells after A/R was shown for siRNA duplexes targeting either HIF-1α or Mcl-1. Unexpectedly, knockdown of HIF-1α induced parallel knockdown of Mcl-1 mRNA and protein expression, whereas Mcl-1 knockdown had no noticeable effect on HIF-1α expression. Thus, although both of these proteins were shown to be anti-apoptotic, the action of HIF-1α appeared to be mediated in part via Mcl-1. J. Cell. Biochem. 97: 755–765, 2006.

Key words: anoxia; apoptosis; HBE1; RNAi

The airway epithelium is a specialized physical barrier whose integrity is vital to normal lung development and innate host defense. Maintenance of airway epithelial barrier function is critical for ion and water transport, mucociliary clearance of microbes, and modulation of the host response to environmental agents [Thompson et al., 1995]. The bronchial epithelium is, however, susceptible to injury from inhaled environmental pollutants and allergens and is highly sensitive to the damaging effects of oxygen free radicals [Mills et al., 1999]. Notably, airway epithelial denudation and shedding are prominent features of asthma [Trautmann et al., 2002] and are associated with bronchial epithelial cell apoptosis induced by oxidative stress [Bucchieri et al., 2002; Truong-Tran et al., 2003]. Apoptosis of the airway epithelium also has been shown to be a consequence of ischemia-reperfusion injury associated with lung allograft transplantation [Shaw et al., 2000], a procedure known to induce oxidative damage [Kozower et al., 2003; Naidu et al., 2003].

Hypoxia-inducible factor-1 (HIF-1) is a redox-sensitive transcriptional factor that mediates changes in gene expression in response to alterations in oxygen concentration [Semenza, 2000; Tacchini et al., 2002]. HIF-1 is a heterodimer composed of two proteins with basic-helix- loop-helix (bHLH)/PER-ARNT-SIM (PAS), HIF-1α and HIF-1β [Semenza, 2000]. While HIF-1β is constitutively expressed in cells, HIF-1α

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*Correspondence to: Dr. Xiu-Huai Liu, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799. E-mail: xliu@usuhs.mil

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expression is induced by systemic hypoxia or tissue ischemia [Semenza, 2000; Tacchini et al., 2002] and this protein has been found to exert either pro-apoptotic or anti-apoptotic effects on different cells under different experimental conditions [Piret et al., 2002; Yu et al., 2004]. Myeloid cell factor-1 (Mcl-1), an anti-apoptotic protein of the Bcl-2 family [Kozopas et al., 1993], also has been shown to be overexpressed in response to hypoxia in polymorphonuclear leukocytes [Leuenroth et al., 2000a,b]. However, it is not known whether either HIF-1α or Mcl-1 plays any role in modulating airway epithelial apoptosis.

In the present study, we employed the technique of RNA interference (RNAi) targeting HIF-1α mRNA and Mcl-1 mRNA and a model of anoxia/reoxygenation (A/R) to study apoptosis in HBE1 cells, a papilloma virus-transformed tracheobronchial epithelial cell line. We utilized A/R, the in vitro counterpart of ischemia-reperfusion injury, for these studies, since A/R has proven to be a useful in vitro model for studying the effects of ischemia-reperfusion injury [Homma et al., 1995]. We observed that although both of these proteins exerted anti-apoptotic activity, the effect of HIF-1α appeared to be mediated, at least in part, via Mcl-1.

MATERIALS AND METHODS

Cell Culture

The HBE1 cells were a gift from Dr. Reen Wu (Center for Respiratory Biology and Medicine, University of California at Davis). The cells were cultured at 37°C in 5% CO_2 in serum-free Ham's F-12/DMEM (1:1) medium supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml epidermal growth factor (EGF), 0.1 μM dexamethasone, 20 ng/ml cholera toxin, 65 ng/ml bovine pituitary extract, 30 nM retinoic acid, and 1.5 μg/ml bovine serum albumin (BSA). The day before transfection, cells were trypsinized, diluted with fresh medium, and plated into T25 flasks at 2.5×10^6 cells in 4.5 ml medium per flask or into 8-chamber glass slides (Nalge Nunc, Naperville, IL) at 0.6×10^5 cells in 0.4 ml each well. Before plating cells, 8-chamber slides were thin-coated with rat tail type I collagen (BD Biosciences, Bedford, MA). After incubation for 24 h, the cells were 80–90% confluent and ready for transfection with small interfering RNA (siRNA).

siRNAs and Transfection

RNA interference was performed with siRNA duplexes for degrading HIF-1α mRNA or Mcl-1 mRNA as previously described [Harborth et al., 2001; Elbashir et al., 2002]. The sense-strand sequence of siRNA targeting HIF-1α mRNA, 5′-CCUACUGCAGGGUGAAGAA-3′, is 2,433–2,451 bases downstream of the start codon of human *HIF-1α* gene (GenBank Accession # AF304431), which was designed by us and synthesized by Proligo (Boulder, CO) and has been proved having an anti-apoptotic effect in human endothelial cells stressed by anoxia/reoxygenation [Yu et al., 2004]. The sense-strand sequence of siRNA targeting human Mcl-1 mRNA is 5′-AAUCGUUGUCUCGAGUGAU-3′, 5,055–5,073 bases of *Mcl-1* gene (GenBank Accession # AF147742.1), which was designed by using Dharmacon siDESIGN Center software and synthesized by Dharmacon, Inc. (Lafayette, CO). The sequences of scrambled RNA duplexes for HIF-1α and Mcl-1 are 5′-GGGUGAACUCACGUCAGAA-3′ and 5′-UUGAGAGCUAUACGCUGUU-3′, respectively. HBE1 cells in T25 flasks or 8-chamber slides were transfected with 100 nM siRNA duplexes or scrambled RNA duplexes with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) immediately before A/R.

Anoxia/Reoxygenation (A/R)

A/R was performed according to a previously described protocol [Yu et al., 2004], a modification of the method developed by Kokura et al. [1999]. In brief, immediately after transfection with HIF-1α siRNA, Mcl-1 siRNA, or scrambled RNA duplexes, the HBE1 cells were exposed to anoxia by incubation at 37°C in a Plexiglas chamber (Modular Incubator Chamber, Billups-Rothenberg, Del Mar, CA) that was continuously purged (1 L/min) with an anoxia gas mixture (2% H_2/5% CO_2/93% N_2) for 90 min. Immediately thereafter, reoxygenation was performed by exposing the cells to normoxic conditions (21% O_2/74% N_2/5% CO_2) at 37°C for 24 h. Control cells were either transfected or not transfected, and exposed to the normoxic conditions at 37°C for 25.5 h. The cells were then evaluated for apoptosis by TUNEL assay, for mRNA expression by real-time reverse transcription-PCR (Q-PCR), and for protein expression by immunocytochemistry and Western blot analysis.

Immunocytochemistry

Monoclonal anti-human HIF-1α (Santa Cruz Biotechnology, CA) was used at a dilution of 1:50 and polyclonal antibody against human Mcl-1 (Santa Cruz Biotechnology) was employed at a dilution of 1:80. Briefly, cells in 8-chamber slides were blocked with 3% normal goat serum for 30 min and incubated with primary antibody at 4°C overnight. After washing, the cells were incubated with a relevant biotinylated secondary antibody for 1 h at ambient temperature and visualized by using a peroxidase substrate 3-3′-diaminobenzidine (DAB) kit (Vector, Burlingame, CA). Thereafter, the cells were counterstained with Auto Hematoxylin (Invitrogen).

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique was performed to quantify apoptotic cell death by using In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as previously reported [Rollwagen et al., 1998]. Briefly, cells in chamber slides were fixed with 4% paraformaldehyde for 1 h and permeabilized in 0.1% Triton-100/0.1% sodium citrate at 4°C for 2 min. The cells were incubated with TUNEL reaction mixture at 37°C for 1 h. After washing, the cells were incubated with an alkaline-phosphatase-conjugated anti-fluorescein antibody at 37°C for 30 min. The cells were stained with Fast Red (DAKO, Carpeteria, CA) and lightly counterstained with Auto Hematoxylin.

Western Blotting

Western blotting was conducted as described previously [Yu et al., 2004]. HBE1 cells were washed with phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer (M-PER Mammalian Protein Extract Reagent, PIERCE, Rockford, IL). The lysates were cleared by centrifugation at 27,000*g* for 15 min at 4°C. The supernatants were collected and their protein concentrations were determined with Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA). A total of 20 μg protein per sample were resolved by 12% SDS–PAGE under reduced conditions and transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). After transfer, the membranes were blocked with 10% non-fat dry milk in TBS-T (50 mM Tris-HCl/150 mM NaCl/0.1% Tween-20) at 4°C overnight, washed three times with TBS-T, and then incubated either with a polyclonal antibody against human HIF-1α (Santa Cruz Biotechnology) or with a polyclonal antibody against Mcl-1 (Santa Cruz Biotechnology) at ambient temperature for 1.5 h. After incubation with a relevant horse radish peroxidase (HRP)-conjugated polyclonal secondary antibody for 1 h, the membranes were detected by chemiluminescent reaction (Amersham Biosciences, Piscataway, NJ), followed by exposure to Kodak XAR-5 film. The levels of specific protein bands were determined by a Fujifilm Luminescent Image analyzer LAS-1000 Plus (Fujifilm Co.) and the data were analyzed by an image analysis software (Fujifilm Image Gauge 4.0). A monoclonal antibody against β-tubulin (Santa Cruz Biotechnology) was used as background control. The results were expressed as density values in each square mm. Western blot analyses were repeated at least three times.

Real-Time Quantitative RT-PCR Using LUX primers

Real-time quantitative RT-PCR (Q-PCR) using LUX primers was performed to determine the HIF-1α and Mcl-1 mRNA levels. RNA extraction and reverse transcription were performed as reported previously [Yu et al., 2004]. Briefly, total RNA from HBE1 was isolated with TRIZOL (Invitrogen), followed by extraction with chloroform and isopropyl alcohol. Subsequently, the RNA solution was treated with RQ1 DNase, followed by extraction with phenol/chloroform. Reverse transcription was performed with oligo(dT)18 primer and SuperScript II RNase H^- reverse transcriptase (Invitrogen). RNase H (Invitrogen) was used to eliminate RNA in the cDNA solution. Q-PCR with fluorogenic LUX primers was performed, as described previously [Yu et al., 2004]. LUX primer pairs of human HIF-1α or Mcl-1 for real-time Q-PCR were designed using the LUX™ Designer Software (Invitrogen) and synthesized by Invitrogen. The sequences of the LUX primer pair for human HIF-1α are cacgttTCATCCAAG AAGCCCTAACG[FAM]G (labeled forward primer) and TCGCTTTCTCTGAGCATTCTGC (unlabeled reverse primer) and the sequences of the LUX primer pair for human Mcl-1 are gtacccAAGAGGAGCTGGACGGG [FAM]AC (labeled forward primer) and CGGCGTCGAGGGTAGTGA (unlabeled reverse primer). The PCR product was 70 bp long for

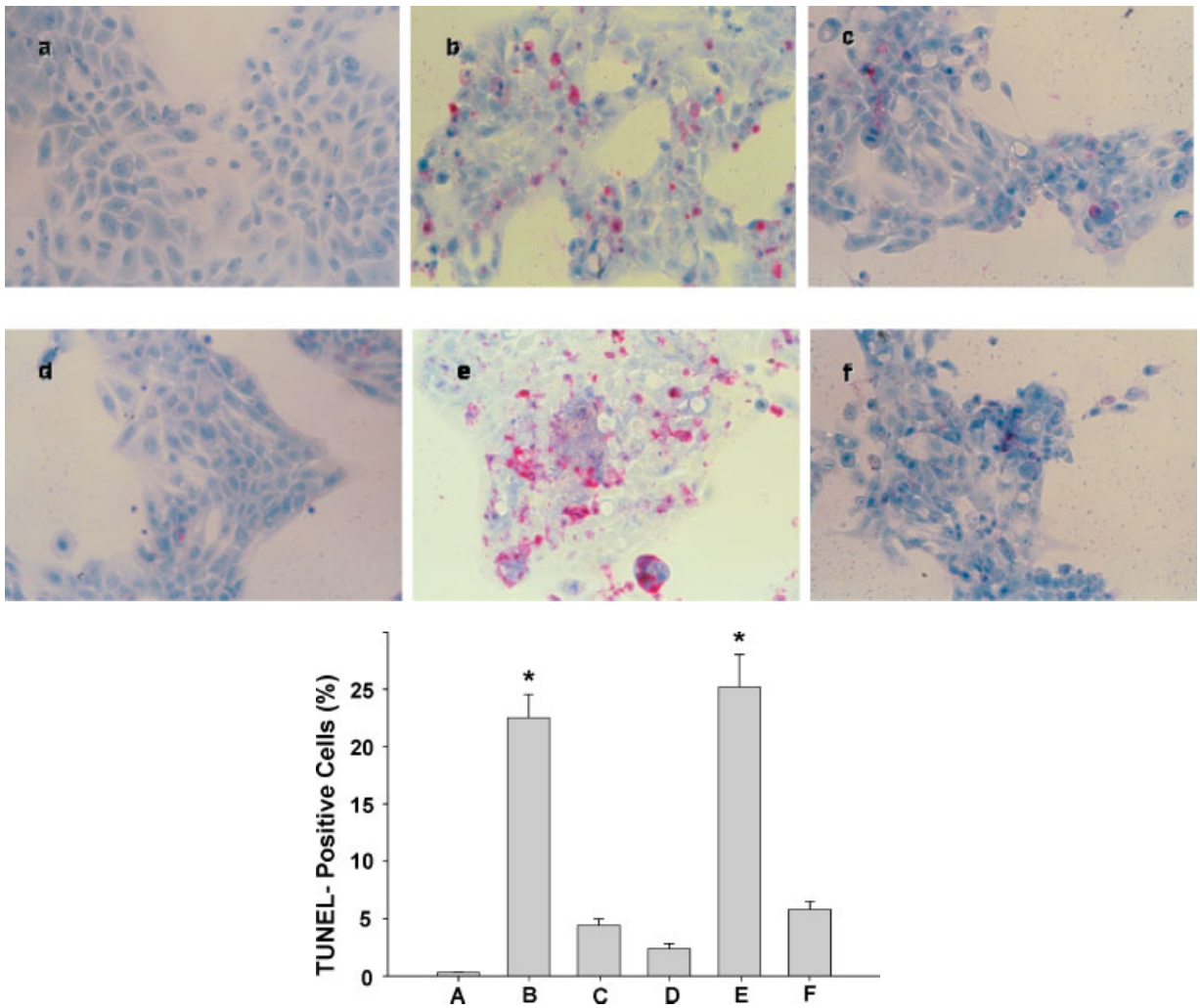

Fig. 1. TUNEL staining (**top panel**) and group comparisons of TUNEL staining (**bottom panel**) in HBE1 cells either cultured under normoxic conditions (**a**, **A**) or subjected to A/R (**b**–**f**, **B**–**F**). Virtually no apoptotic changes were seen in normoxic (a, A) or non-transfected (d, D) cultures. However, transfection with either HIF-1α siRNA (b, B) or Mcl-1 siRNA (e, E) greatly increased the number of TUNEL-positive cells subjected to oxidative stress. No differences were noted between non-transfected cultures (d, D) and cells transfected with HIF-1α (c, C) or Mcl-1 (f, F) scrambled RNA duplexes. *$P < 0.05$ compared with groups A, C, D, and F.

HIF-1α or 89 bp long for Mcl-1. 18S rRNA was also measured as an endogenous reference to control differences in harvested RNA samples across all experimental groups. The LUX primer pair with JOE labeling for 18S rRNA was purchased from Invitrogen. The total mixture for each reaction was 50 μl containing 25 μl Platinum quantitative PCR SuperMix-UDG (Invitrogen), 1 μl ROX Reference dye (Invitrogen), 10 μM each primer and cDNA generated from 100 ng of total RNA as the PCR template. Q-PCR was carried out with an ABI Prism 7700 Sequence Detector. The reaction mixtures were incubated at 50°C for 2 min and 95°C for 2 min and then cycled for 45 times using 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s (three steps), followed by 4°C for 5 min. Fluorescence was monitored during every PCR cycle. Cycle threshold (Ct) values were used to determine the amount of HIF-1α mRNA, Mcl-1 mRNA, and 18S rRNA for all groups. The mean Ct values of triplicate samples from each group were computed to determine the ratio of HIF-1α mRNA per 18S rRNA or the ratio of Mcl-1 mRNA per 18S rRNA in the samples. All groups subjected to A/R were normalized to the normoxia group. The fold changes in HIF-1α mRNA or Mcl-1 mRNA expression were calculated using a previously

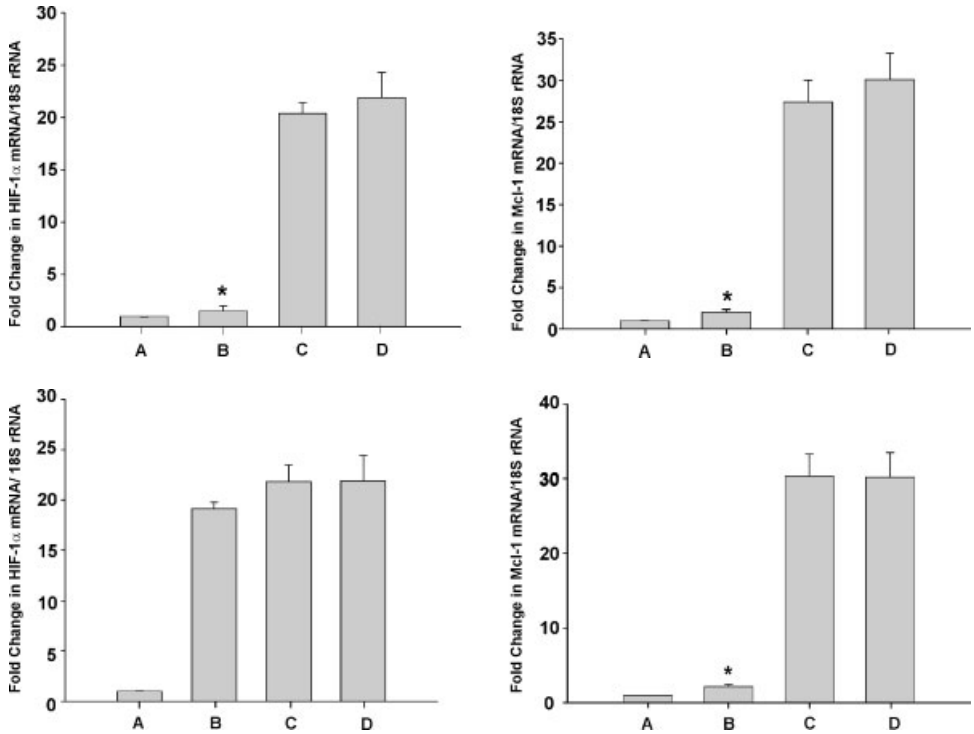

Fig. 2. Q-PCR group comparisons of HBE1 cells cultured under normoxic conditions (**A**) or after exposure to A/R (**B**–**D**), showing the effects of HIF-1α siRNA (**top panel**) or Mcl-1 siRNA (**bottom panel**) on expression of HIF-1α (**top graphs**) and Mcl-1 (**bottom graphs**) mRNA. Left panel: HBE1 cells transfected with HIF-1α siRNA (B) downregulated both HIF-1α (left graph) and Mcl-1 (right graph) mRNA expression. No such effect was seen with non-transfected cultures (D) or cultures transfected with scrambled RNA duplexes (C). Normoxia induced minimal HIF-1α and Mcl-1 mRNA expression. *Significantly different ($P < 0.05$) compared with groups C and D. Bottom panel: HBE1 cells transfected with Mcl-1 siRNA (B) downregulated Mcl-1 mRNA expression (right graph) but had no noticeable effect on HIF-1α mRNA expression (left graph). No effect was seen with non-transfected cultures (D) or cultures transfected with scrambled RNA duplexes (C). Normoxia induced minimal HIF-1α and Mcl-1 mRNA expression. *Significantly different ($P < 0.05$) compared with groups C and D.

published formula [Pfaffl, 2001]. A minimum of triplicate experiments were performed ($n = 3$).

Statistical Analysis

Results from TUNEL assays and immunocytochemistry were expressed as percentages of positive cells versus total cells. HIF-1α or Mcl-1 immunoreactive cells and TUNEL-positive (apoptotic) cells in a total of 2,000 cells from 10 chambers of three experiments were counted. Results of Western blot and Q-PCR analyses were expressed as mean ± SEM. Statistical significance was evaluated by one-way ANOVA and followed by the Turkey post-hoc test to identify significant differences between individual groups. For this purpose, SigmaStat software 2.03 (SPSS Science, Chicago, IL) was employed. A value of $P < 0.05$ by the Turkey test was interpreted to denote statistical significance.

RESULTS

A/R Induced Upregulation of HIF-1α and Mcl-1 and had a Minimal Apoptotic Effect

When apoptosis of HBE1 cells was evaluated by the TUNEL assay, approximately 0.5% of cells cultured under normoxic conditions exhibited apoptotic changes (Fig. 1a,A). Conditions of A/R also induced negligible (2.4%) apoptosis in HBE1 cells (Fig. 1d,D).

The expression level of HIF-1α and Mcl-1 mRNA and proteins was significantly increased in HBE1 cells after A/R. Thus, A/R induced upregulation of HIF-1α and Mcl-1 mRNA levels (as detected by Q-PCR) by approximately 20-fold to 30-fold (Fig. 2D, all graphs) from the levels under normoxic conditions. Moreover, the percentages of HBE1 cells that were immunoreactive for HIF-1α (Fig. 3d,D) and Mcl-1 (Fig. 4d,D) were increased by 30-fold and 20-fold, respectively. Similarly, Western blot

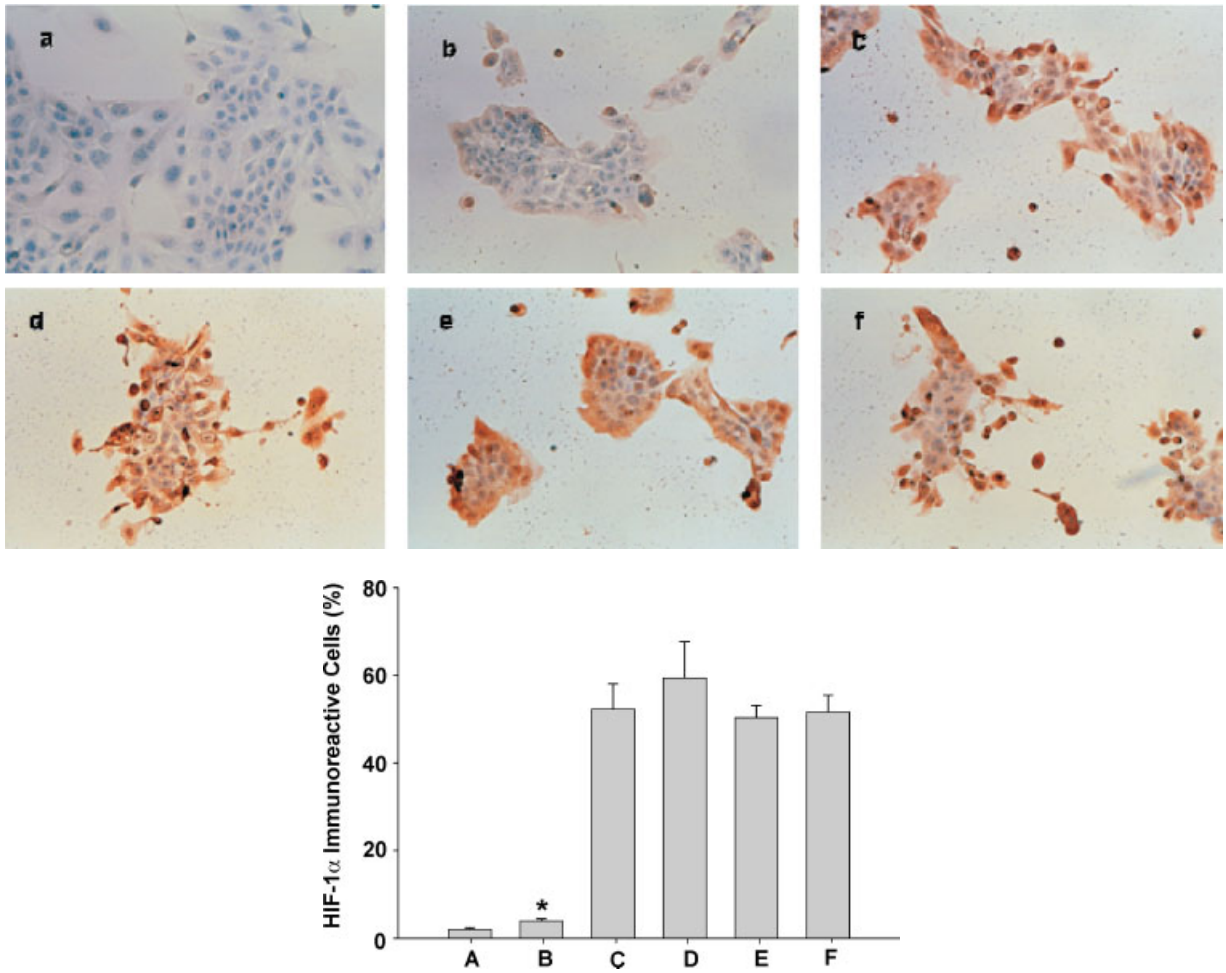

Fig. 3. Immunostaining (**top panel**) and group comparisons of immunostaining (**bottom panel**) for HIF-1α in HBE1 cells cultured either under normoxic conditions (**a**, **A**) or subjected to A/R (**b**–**f**, **B**–**F**). Normoxia induced minimal HIF-1α immunoreactivity (a, A), whereas A/R upregulated HIF-1α expression in non-transfected cells (d, D), an effect that was abrogated by HIF-1α siRNA (b, B) but not by Mcl-1 siRNA (e, E). Cultures transfected with HIF-1α (c, C) or Mcl-1 (f, F) scrambled RNA duplexes did not inhibit HIF-1α immunoreactivity. $^*P < 0.05$ compared with groups C, D, E, and F.

analyses revealed that HIF-1α (D in upper right panel of Fig. 5) and Mcl-1 protein levels (D in lower right panel of Fig. 6) were significantly increased after A/R by threefold and twofold, respectively.

siRNA Targeting Either HIF-1α or Mcl-1 Induced Significant Apoptotic Changes in HBE1 Cells Under A/R Conditions

A/R induced significantly greater apoptotic change detectable by TUNEL staining ($P < 0.05$) after transfection with siRNA duplexes targeting either HIF-1α mRNA (Fig. 1b,B) or Mcl-1 mRNA (Fig. 1e,E), when compared with non-transfected HBE1 cells (Fig. 1d,D). In this regard, no noticeable difference was observed in the severity of apoptosis induced by siRNA duplexes targeting either HIF-1α or Mcl-1 in HBE1 cells. In contrast, transfection of the cells with scrambled RNA duplexes under conditions of A/R had no obvious apoptotic effect (Fig. 1c,f,C,F), suggesting that transfection, per se, was not a significant factor for inducing apoptosis in HBE1 cells. These findings also indicated that the actions of both HIF-1α and Mcl-1 in airway epithelial cells were anti-apoptotic under conditions of oxidative stress.

siRNA Targeting HIF-1α Knocked Down MCl-1 Expression Whereas siRNA Targeting Mcl-1 did Not Affect HIF-1α Expression After A/R

The effectiveness of RNAi targeting HIF-1α and Mcl-1 under conditions of A/R was evaluated by assessing knockdown of mRNA and protein expression of the targeted genes in

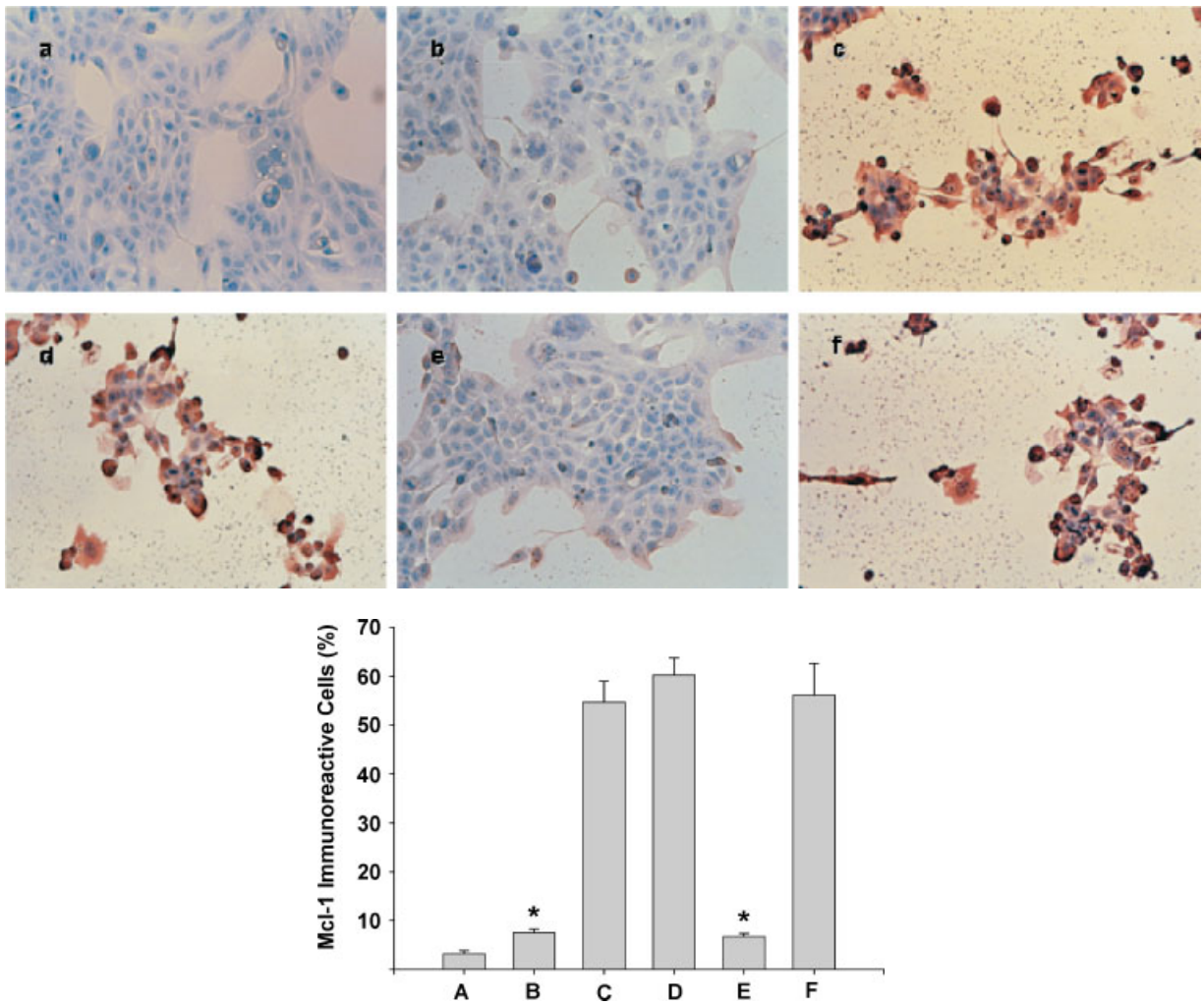

Fig. 4. Immunostaining (**top panel**) and group comparisons of immunostaining (**bottom panel**) for Mcl-1 in HBE1 cells cultured either under normoxic conditions (**a**, **A**) or subjected to A/R (**b**–**f**, **B**–**F**). Normoxia induced minimal Mcl-1 immunoreactivity (a, A), whereas A/R upregulated Mcl-1 expression in non-transfected cells (d, D), an effect that was abrogated by both HIF-1α siRNA (b, B) and Mcl-1 siRNA (e, E). Cultures transfected with scrambled HIF-1α (c, C) or scrambled Mcl-1 (f, F) RNA duplexes did not inhibit Mcl-1 immunoreactivity. $*P < 0.05$ compared with groups C, D, and F.

HBE1 cells using appropriate siRNA duplexes. For these studies, scrambled RNA duplexes were used as negative controls.

As shown in Figure 2 (group B, left graph in top panel), siRNA duplexes targeting HIF-1α largely abrogated HIF-1α mRNA expression in HBE1 cells subjected to A/R. A similar effect was noted with respect to Mcl-1 mRNA expression when Mcl-1 specific siRNA duplexes were used (Fig. 2B, right graph in bottom panel). siRNA duplexes against HIF-1α induced knockdown of HIF-1α protein expression, as reflected by the percentages of HIF-1α immunoreactive cells (Fig. 3b,B) and by Western blot analyses (B in right upper panel of Fig. 5). In like fashion, Mcl-1 protein expression was largely abolished by siRNA duplexes targeting Mcl-1 (Figs. 4e, E and 6, B in right lower panel).

The scrambled RNA duplex control for HIF-1α had no noticeable effect on either HIF-1α mRNA (Fig. 2C, left graph in top panel) or HIF-1α protein expression (Figs. 3c, C and 5, C in upper right panel). Similarly, the scrambled duplex control for Mcl-1 did not affect Mcl-1 mRNA (Fig. 2C, right graph in right panel) or protein expression (Figs. 4f, F and 6, C in lower right panel). Collectively, these findings indicate that the observed knockdown of HIF-1α and Mcl-1 mRNA and protein expression in HBE1 cells under conditions of oxidative stress was due to specific targeting of the affected genes by the their corresponding siRNA duplexes.

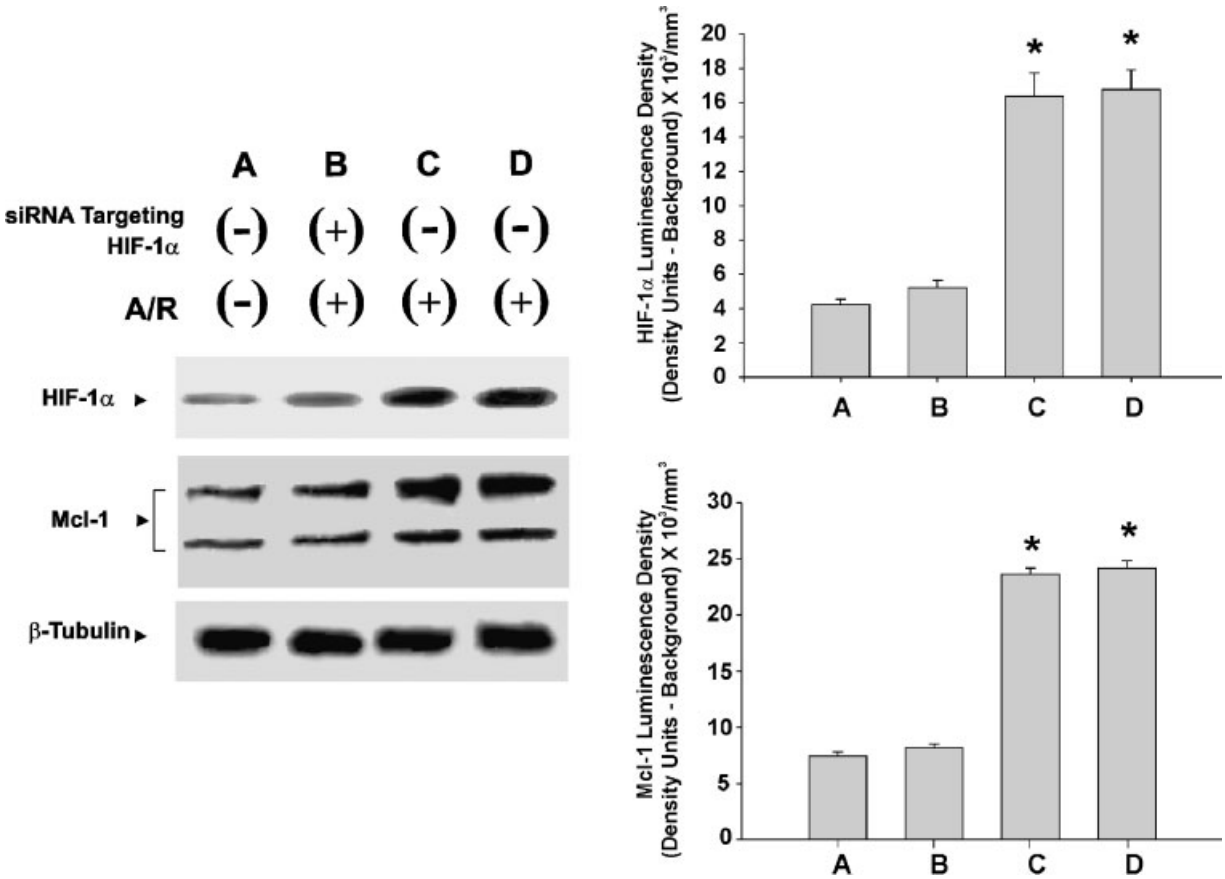

Fig. 5. Group comparisons of Western blots probed for HIF-1α (**right upper panel**) and Mcl-1 (**right lower panel**) after transfection with HIF-1α siRNA. Lysates were obtained from HBE1 cells cultured under normoxic conditions (**A**) or exposure to A/R (**B–D**). Transfection with HIF-1α siRNA largely attenuated both HIF-1α (B in upper right panel) and Mcl-1 (B in right lower panel) protein expression, when compared with non-transfected cultures (D) or cultures receiving appropriate scrambled RNA duplexes (C). The left panel depicts representative immunoblots demonstrating HIF-1α, Mcl-1 and β-tubulin protein expression after transfection with HIF-1α siRNA. Results in each category represent mean ± SEM. of three experiments. *Significantly different ($P < 0.05$) compared with groups A and B.

As expected, the siRNA duplexes against Mcl-1 had no effect on HIF-1α mRNA (Fig. 2B, left graph in bottom panel) or protein expression (Figs. 3e, E and 6, B in right upper panel). We were, however, surprised to observe that siRNA duplexes targeting HIF-1α induced significant knockdown of Mcl-1 mRNA (Fig. 2B, right graph in top panel) and protein expression (Figs. 4b, B and 5, B in right lower panel). These findings suggest that, at least under conditions of A/R, the anti-apoptotic effect of HIF-1α appears to be mediated via Mcl-1.

DISCUSSION

Hypoxia and oxidative stress are known to regulate the expression of a number of different apoptosis-related proteins in a variety of cell types. Thus, hypoxia has been shown to upregulate the expression of IAP2, a member of an apoptosis inhibitor protein family [Dong et al., 2002], as well as Bcl-2 family members including BNip3 [Bruick, 2000; Sowter et al., 2001], NIX [Bruick, 2000; Sowter et al., 2001], Noxa [Kim et al., 2004], Mcl-1 [Leuenroth et al., 2000a,b], and Bcl-XL [Dong and Wang, 2004]. It is also well-recognized that hypoxia and oxidative stress can induce the expression of HIF-1α [Semenza, 2000; Tacchini et al., 2002], a transcription factor with demonstrable pro-apoptotic or anti-apoptotic activity, depending on the nature of the cultured cells and the cultural conditions [Piret et al., 2002; Yu et al., 2004]. The ability of HIF-1α to regulate hypoxia-induced apoptosis has been demonstrated in diverse cell types in culture including cortical neurons [Zaman et al., 1999], pancreatic cancer

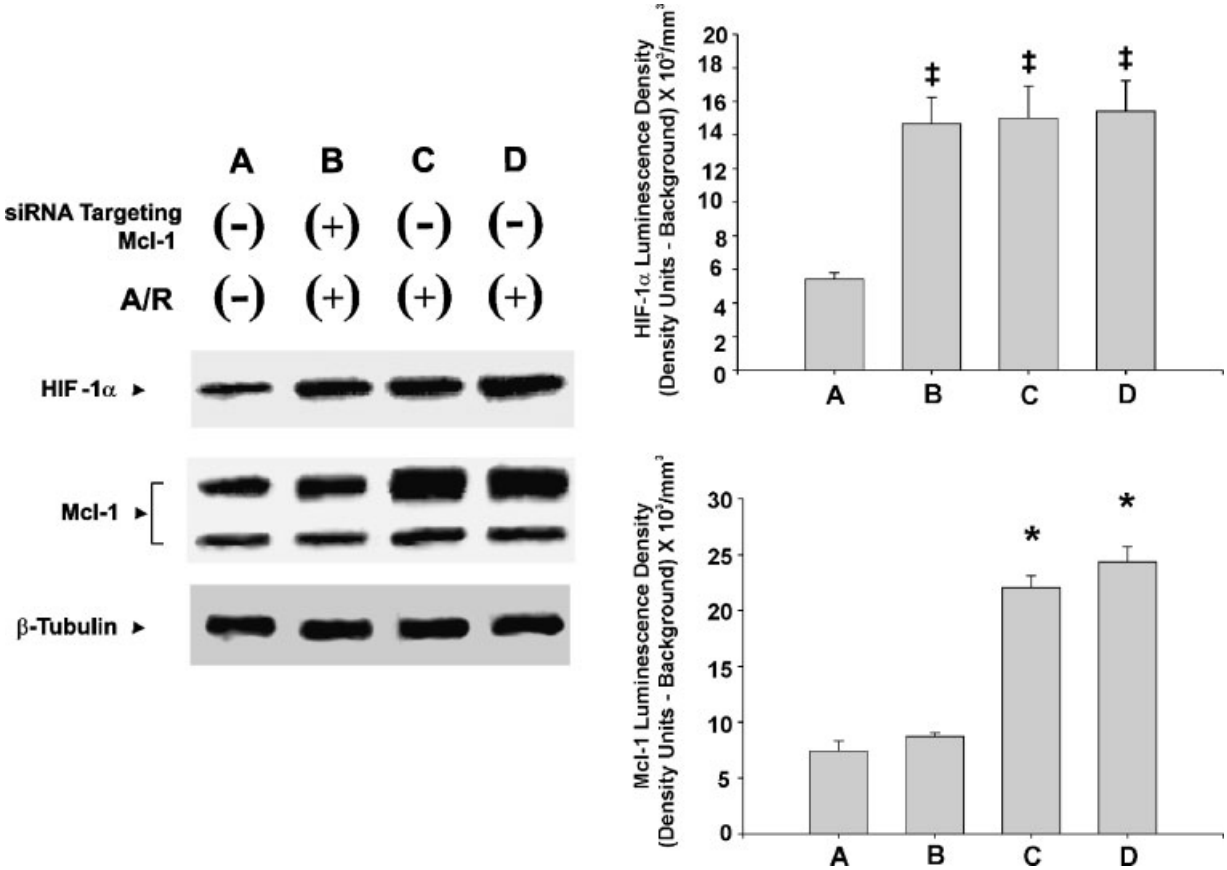

Fig. 6. Group comparisons of Western blots probed for HIF-1α (**right upper panel**) and Mcl-1 (**right lower panel**) after transfection with Mcl-1 siRNA. Lysates were obtained from HBE1 cells cultured under normoxic conditions (**A**) or exposure to A/R (**B**–**D**). Transfection with Mcl-1 siRNA largely attenuated Mcl-1 (B in right lower panel) but did not affect HIF-1α (B in right upper panel) protein expression, when compared with non-transfected cultures (D) or cultures receiving appropriate scrambled RNA duplexes (C). The left panel depicts representative immunoblots demonstrating HIF-1α, Mcl-1 and β-tubulin protein expression after transfection with Mcl-1 siRNA. Results in each category represent mean ± SEM. of three experiments. ‡Significantly different ($P < 0.05$) compared with group A. *Significantly different ($P < 0.05$) compared with groups A and B.

cells [Akakura et al., 2001], and human umbilical vein endothelial cells [Yu et al., 2004]. It seems probable that the aforementioned hypoxia-induced effects on apoptosis-related proteins may be mediated via HIF-1α. In this regard, it is noteworthy that several studies have shown that HIF-1α regulated the expression of BNip3 [Bruick, 2000; Sowter et al., 2001], NIX [Bruick, 2000; Sowter et al., 2001], Noxa [Kim et al., 2004], and Mcl-1 [Piret et al., 2005]. Furthermore, HIF-1α was reported to be involved in hypoxia-induced, p53-mediated apoptosis by physically binding to p53 [Schmid et al., 2004; Sanchez-Puig et al., 2005].

In the present study, we have evaluated the effects of A/R, a model of oxidative stress [Morin et al., 2003], on HIF-1α and Mcl-1 expression in human airway epithelial cells. We showed that A/R induced significant apoptotic activity, as reflected by TUNEL-positive staining, in HBE1 cells transfected with siRNA duplexes targeting either HIF-1α or Mcl-1 mRNA. In contrast, non-transfected HBE1 cells or HBE1 cells transfected with scrambled Mcl-1 or HIF-1α RNA duplexes manifested minimal apoptotic activity after A/R. These observations indicated that both Mcl-1 and HIF-1α exerted anti-apoptotic activity and that transfection, per se, was not responsible for the induction of apoptosis.

The efficacy of RNAi targeting HIF-1α and Mcl-1 was assessed by measurement of mRNA expression (by Q-PCR) and of protein expression (by Western analysis and by immunocytochemistry). Under conditions of A/R, siRNA duplexes targeting HIF-1α knocked down HIF-1α mRNA and protein expression whereas corresponding scrambled RNA duplexes administered under the same conditions had no

statistically significant silencing effect on *HIF-1α* gene expression. We had previously demonstrated similar findings in human umbilical vein endothelial cells [Yu et al., 2004].

In the current study, siRNA duplexes targeting Mcl-1 largely abrogated Mcl-1 mRNA and protein expression in the HBE1 cells after A/R but had no noticeable silencing effect on *HIF-1α* gene expression. An unexpected finding, however, was the fact that siRNA duplexes against HIF-1α not only knocked down HIF-1α mRNA and protein expression under these circumstances but also significantly downregulated expression of Mcl-1 mRNA and protein expression. The corresponding scrambled RNA duplexes for Mcl-1 had no noticeable effect on either *Mcl-1* or *HIF-1α* gene expression. These collective observations suggest that the anti-apoptotic effect of HIF-1α following oxidative stress in HBE1 cells appears to be mediated, at least in part, via Mcl-1. Although this study did not address the mechanism of how HIF-1α may modulate Mcl-1 function in this regard, it is conceivable that HIF-1α may have an enhancing effect on Mcl-1 expression. This assertion is supported by the recent demonstration that the *Mcl-1* gene promoter has a hypoxia responsive element (HRE) and can bind HIF-1α in vitro [Piret et al., 2005]. Furthermore, in that study, hypoxia-induced overexpression of Mcl-1 in HepG2 hepatoma cells was noted to be HIF-1α-dependent.

In summary, we have employed RNAi targeting both the *HIF-1α* and *Mcl-1* genes in human airway epithelial cells in an in vitro model of oxidative stress. Although both of these proteins were shown to be anti-apoptotic, the action of HIF-1α appeared to be mediated, at least in part, via Mcl-1. Therefore, *Mcl-1* functions as a target gene of HIF-1α in human airway epithelial cells, which may contribute to the further understanding of the control of oxygen homeostasis in human health.

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