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Antiapoptotic action of hypoxia-inducible factor-1α in human endothelial cells

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Hypoxia-inducible factor-1 (HIF-1) is the major transcription factor involved in the adaptive response to hypoxia and consists of HIF-1α and HIF-1β subunits. Indirect evidence suggests that HIF-1α may exert both proapoptotic and antiapoptotic actions in response to hypoxia. In this study, we evaluated the effects of RNA interference (RNAi) targeting HIF-1α messenger RNA (mRNA) on apoptosis in primary cultured human umbilical vascular endothelial cells (HUVECs) exposed to anoxia and reoxygenation (A/R). HUVECs were transfected with specific 21-nt small interfering RNA (siRNA) duplexes targeting HIF-1α mRNA sequences or scrambled RNA duplexes and subjected either to normoxia for 25 1/2 h or to anoxia for 1 1/2 h, and subsequently normoxia for 24 h (A/R). Control samples were subjected to A/R but not transfected. HUVECs apoptosis was evaluated by Tdt-mediated dUTP nick end-labeling (TUNEL) assay and by activated caspase-3 immunostaining and immunoblotting. The efficacy of RNAi was assessed by knockdown of HIF-1α mRNA and protein expression via in situ hybridization, real-time quantitative PCR, immunohistochemistry, and Western blotting. When compared with normoxic cultures, A/R significantly upregulated HIF-1α mRNA and protein expression in HUVECs, but did not appreciably alter the percentage of apoptotic cells. In contrast, a significantly greater proportion of HUVECs transfected with specific siRNA duplexes and exposed to A/R demonstrated evidence of apoptosis when compared with nontransfected cells. Transfection with specific siRNA duplexes knocked down HIF-1α mRNA and protein expression in A/R-treated cells by approximately 60%, whereas transfection with scrambled siRNA duplexes had no noticeable effect on HIF-1α expression. These findings strongly suggest that HIF-1α exerts an antiapoptotic role in HUVECs stressed by anoxia.
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Vascular endothelial cells can undergo apoptosis in response to a number of pathophysiological stimuli including hypoxia, ischemia–reperfusion, hypertension, endotoxin, proinflammatory cytokines, reactive nitrogen species, atherogenic risk factors, and ionizing radiation.[1] The induction of endothelial apoptosis, in turn, has been implicated in the pathogenesis of diverse diseases such as primary pulmonary hypertension,[2] atherosclerosis,[3,4] and allograft vasculopathy.[5] Moreover, endothelial apoptosis may play a regulatory role in neovascularization of tumors.[6]

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that functions as a major regulator of O_2 homeostasis and is a heterodimer composed of two basic–helix–loop–helix Per-AhR/Arnt-Sim (PAS) homology sequence proteins, HIF-1α and HIF-1β.[7,8]

Although it is well recognized that systemic hypoxia or tissue ischemia greatly upregulates HIF-1α expression compared with normoxic conditions, there is a variation in the extent and time course of HIF-1α induction between tissues.[9,10] The precise role of HIF-1α in either promoting or protecting against hypoxia-induced injury has not, however, been established, and the few studies attempting to address this question have been performed on nonendothelial-transformed cells.

The present study was undertaken to evaluate the role of HIF-1α in anoxia-induced apoptosis in primary cultured human endothelial cells. Using RNA interference (RNAi) targeting HIF-1α messenger RNA (mRNA) in a model of anoxia and reoxygenation (A/R), our findings strongly suggest that HIF-1α had a protective effect against apoptosis in these cells.

Materials and methods

Cell Cultures

Human umbilical vascular endothelial cells (HUVECs) were purchased from Cambrex (Gaithersburg,

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MD, USA) and cultured in EGM-2 medium (Cambrex) with 10% fetal bovine serum at 37°C in a humidified environment of 5% CO_2. Cells were regularly passaged to maintain exponential growth. All experiments were carried out between passages 2 and 5. The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and transferred to culture plates or chamber slides. For six-well plates, $4-5 \times 10^5$ cells were seeded in 2 ml medium per well. For eight-chamber slides, 0.25×10^5 cells were seeded in 0.2 ml medium per chamber. After 24 h incubation, the cells were 90–95% confluent and suitable for small interfering RNA (siRNA) transfection.

RNA Interference

RNAi was performed with siRNA duplexes targeting HIF-1α mRNA for degradation, as described previously.[11,12] The HIF-1α sequence for siRNA targeting, 5′-CCTACTGCAGGGTGAAGAA-3′, was 2433–2451 bases downstream of the first nucleotides of the start codon of human HIF-1α complementary DNA (cDNA) (GenBank Accession # AF304431). The order of HIF-1α nucleotides was scrambled to generate the control sequence 5′-GGGTGAACTCACGTCAGAA-3′. The siRNA duplexes were synthesized, purified, and annealed by Proligo (Boulder, CO, USA). HUVEC were transfected at a final siRNA duplex concentration of 100 nM by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in either 24- or six-well culture plates or eight-well Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL, USA) immediately before being subjected to anoxia. The cells were assayed 251/2 h after siRNA transfection by immunocytochemistry, TUNEL assay, in situ hybridization, Western blotting, and real-time quantitative PCR (Q-PCR).

Anoxia-Reoxygenation (A/R)

A/R was performed as described previously.[13] Immediately after transfection, HUVEC in either six-well culture plates or eight-chamber slides were subjected to anoxia by incubation at 37°C in a Plexiglas chamber (Modular Incubator Chamber, Billups-Rothenberg Inc., Del Mar, CA, USA) that was continuously purged at 1 l/min with an anoxic gas mixture (2% H_2, 5% CO_2, 93% N_2) for 90 min. Reoxygenation was performed by incubating the cells at 37°C for 24 h under normoxic conditions (21% O_2, 74% N_2, and 5% CO_2). Control samples were incubated under normoxic conditions at 37°C for 251/2 h. After incubation, the HUVECs were assayed for HIF-1α mRNA and protein expression.

Immunocytochemistry

For immunocytochemical detection of HIF-1α, a primary monoclonal anti-human HIF-1α (Santa Cruz Biotechnology, CA, USA) was used at a 1:50 dilution. A primary polyclonal antibody against activated caspase-3 (Cell Signaling Technology, MA, USA) was employed at a 1:50 dilution to detect immunoreactivity for activated caspase-3. In brief, chamber slides were blocked with 3% normal goat serum and incubated with primary antibody overnight at 4°C. After being washed, the slides were incubated with a biotinylated secondary antibody for 1 h at room temperature. Thereafter, the slides were incubated with Vectastain Elite ABC Reagent (Vector Labs., Burlingame, CA, USA), visualized using 3-3′-diaminobenzidine (DAB Kit, Vector Labs.) as a substrate, and counterstained with AutoHematoxylin (Invitrogen).

TUNEL Assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique was performed to detect and quantitate apoptotic cell death using the In situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN, USA), as reported previously.[14] Briefly, 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 slides were incubated with TUNEL reaction mixture for 1 h at 37°C. After washing, the slides were incubated with alkaline-phosphatase-conjugated anti-fluorescein antibody for 30 min at 37°C. Slides were developed with Fast Red (DAKO, Carpenteria, CA, USA) and lightly counterstained with hematoxylin.

In situ Hybridization

The in situ hybridization technique used was a modification of the method of Emson and Gait,[15] as described previously.[16] Chamber slides were permeabilized with proteinase K digestion (2.5 μg/ml) followed by acetylation. Hybridization was performed overnight at 37°C with a human HIF-1α-biotinylated DNA probe that is a 28-mer oligonucleotide (5′-CTTCACCCTGCAGTAGGTTTCTGC-TGCC-3′, GenBank Accession # BC012527), which was designed by us and chemically synthesized, purified, and biotinylated by GenSet Corp. (La Jolla, CA, USA). After posthybridization washes, the signals were detected immunochemically by subsequent incubation with a streptavidin–alkaline phosphatase (AP) conjugate (DAKO, Carpinteria, CA, USA) and developed on a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate. Image processing and analysis for in situ hybridization were performed, as reported previously.[16]

Q-PCR with LUX Primers

RNA extraction and reverse transcription were performed, as described previously.[17] Q-PCR with fluorogenic LUX primers was performed to detect mRNA levels of HIF-1α gene in HUVEC cells according to a previously published method.[18] A LUX primer pair for human HIF-1α was designed for Q-PCR using the LUX Designer Software (Invitrogen) and synthesized by Invitrogen. The LUX primer pair included one unlabeled primer and one labeled with the single fluorophore, FAM. The labeled primer possessed a fluorescent quenching hairpin structure that enabled real-time detection of the gene of interest without a separate quencher or probe. The sequences of the LUX primer pair for HIF-1α were cacgttTCATCCAAGAAGCCC-TAACG{FAM}G (labeled forward primer) and TCGCTTTCTCTGAGCATTCTGC (unlabeled reverse primer). The PCR product was 70 base pair long for HIF-1α. The 18S ribosomal RNA (rRNA) was 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 $50\,\mu l$ containing $25\,\mu l$ Platinum Quantitative PCR SuperMix-UDG (Invitrogen), $1\,\mu l$ ROX Reference dye (Invitrogen), $10\,\mu m$ of each primer, and cDNA generated from $100\,ng$ of total RNA as the PCR template. Q-PCR was performed 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 $30\,s$, followed by $4°C$ for $5\,min$. Fluorescence was monitored during every PCR cycle. Cycle threshold (C_t) values were used to determine the amount of HIF-1α mRNA and 18S rRNA for all groups. The mean C_t values of triplicate samples from each group were computed to determine the ratio of HIF-1α mRNA per 18S rRNA in each sample. All groups subjected to A/R were normalized to the normoxia group. The fold change in HIF-1α mRNA expression was calculated using a previously published formula.[19] All experiments were performed in triplicate.

Western Blot Analyses

Western blotting was performed, as described previously.[20] HUVECs were washed with phosphate-buffered saline and lysed in ice-cold lysis buffer.[20] The lysates were clarified by centrifugation at $20\,800\,g$ for $5\,min$ at $4°C$. The supernatants were collected and their protein concentrations were determined by a Bio-Rad D_C Protein Assay (Bio-Rad Labs., Hercules, CA, USA). A total of $20\,\mu g$ of protein per sample were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel under reduced conditions and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). After transfer, the membrane was blocked with 10% nonfat dry milk in $50\,mM$ Tris-HCl, $150\,mM$ NaCl, 0.1% Tween-20 (TBS-T) overnight at $4°C$. Membranes were washed three times with TBS-T and then incubated for $11/2\,h$ at room temperature either with a goat polyclonal antibody against human HIF-1α (Santa Cruz Biotechnology, CA, USA) or a rabbit polyclonal antibody against activated caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA). After incubation with a relevant horseradish peroxidase-conjugated polyclonal secondary antibody for $1\,h$, the membrane was detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ), followed by exposure to Kodak XAR-5 film. Specific protein expression was quantified by a Fujifilm Luminescent Image Analyzer LAS-1000 Plus (Fujifilm Co.) and data were analyzed using ImageGauge 4.0 image analysis software (Fujifilm). The results were expressed as density values normalized to β-tubulin. Individual Western blot analyses were repeated at least three times.

Statistics and Data Analysis

The results of immunocytochemistry and TUNEL assay experiments were expressed as percentages of positive cells in a total of 2000 consecutively counted cells from 10 chamber slides ($n = 2$–3 replicates for each experiment). The results of Western blot and Q-PCR analyses were expressed as mean $\pm$ s.e.m. Statistical significance was evaluated by one-way ANOVA and the Tukey *post hoc* test was used to identify significant differences between individual groups. For these purposes, SigmaStat software version 2.03 (SPSS Inc., Chicago, IL, USA) was used. A value of $P < 0.05$ by the Tukey test was interpreted to denote statistical significance.

Results

TUNEL Assays

When apoptosis of HUVECs was evaluated by the TUNEL technique, $<2\%$ of cells cultured under normoxic conditions manifested evidence of apoptotic change after $251/2\,h$ in culture (Figure 2). Notably, both nontransfected HUVECs as well as cells transfected with siRNA duplexes under normoxic conditions demonstrated minimal evidence of apoptosis by the TUNEL technique. Nontransfected HUVECs that were subjected to A/R ($11/2\,h$ of anoxia and $24\,h$ of normoxia) exhibited only slightly more (approximately 8%) apoptotic cells (Figures 1a and 2). In contrast, a significantly greater proportion (approximately 48%) of HUVECs transfected with siRNA duplexes targeting HIF-1α mRNA prior to A/R were TUNEL positive (Figures 1b and 2).
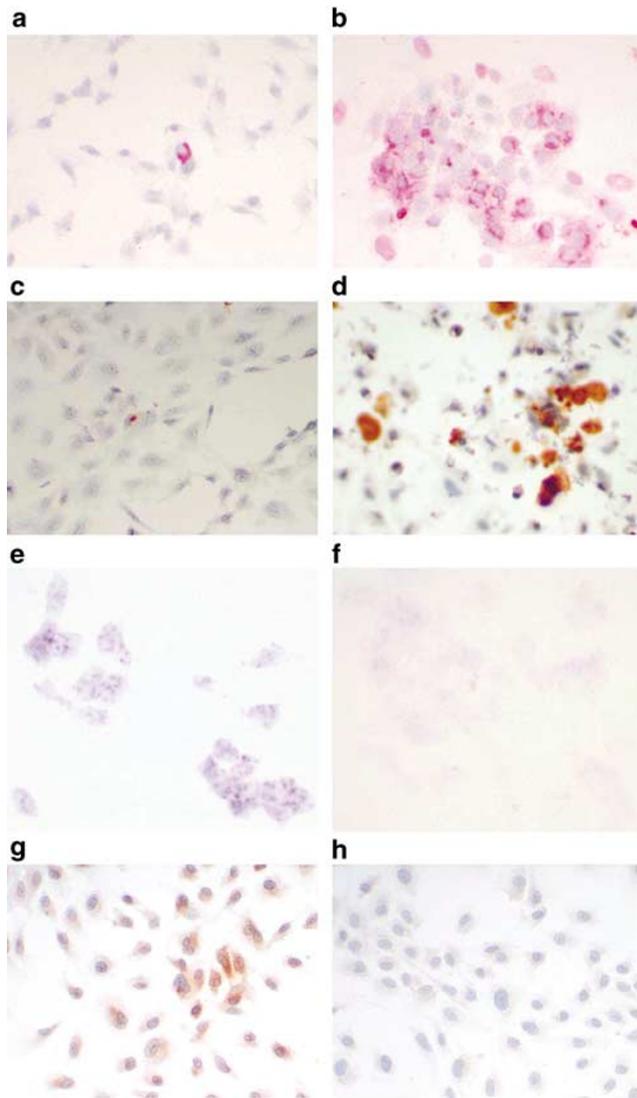
Figure 1 (a–d) Representative photomicrographs showing apoptotic changes in HUVECs subjected to A/R. Only occasional nontransfected cells show positive TUNEL staining (a) and immunoreactivity for activated caspase-3 (c). In contrast, the majority of cells transfected with HIF-1α siRNA duplexes demonstrate positive TUNEL staining (b) and immunoreactivity for activated caspase-3 associated with morphologic evidence of apoptotic damage (d). (e–h) Representative photomicrographs demonstrating knockdown of HIF-1α mRNA and protein expression by HIF-1α siRNA duplexes in HUVECs subjected to A/R. Many nontransfected cells demonstrate strong HIF-1α mRNA expression by in situ hybridization (e), whereas HIF-1α mRNA is barely detectable in cells transfected with siRNA duplexes (f). Strong immunoperoxidase reactivity for HIF-1α is observed in most nontransfected cells, (g) but only an occasional cell transfected with siRNA shows HIF-1α immunoreactivity (h).

The fact that TUNEL staining in transfected HUVECs cultured under normoxic conditions and in HUVECs transfected with scrambled RNA duplexes prior to A/R was not a notable feature and was comparable to that seen in equivalent nontransfected cultures (Figure 2) indicates that transfection, per se, was not a significant factor for inducing apoptotic change in HUVECs.
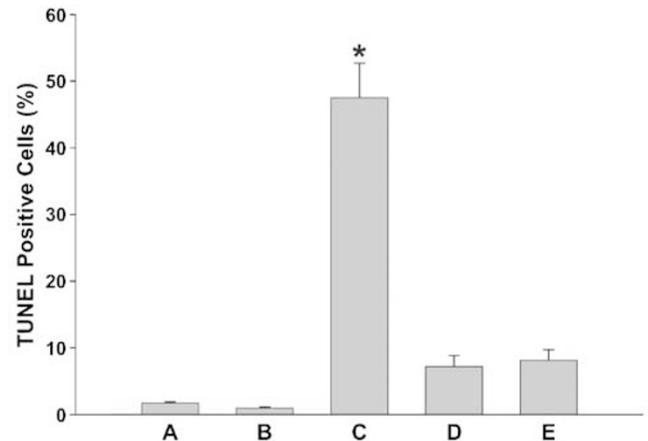

Figure 2 Group comparisons of TUNEL assays illustrating the effects of normoxia and A/R on cultured HUVECs. Transfection with HIF-1α siRNA greatly increased the number of TUNEL-positive cells subjected to anoxic stress. Results in each category represent mean ± s.e.m. of three experiments. (A) Nontransfected normoxic cultures. (B) Normoxic cultures transfected with HIF-1α siRNA duplexes. (C) Cultures transfected with siRNA duplexes and subjected to A/R. (D) Cultures transfected with scrambled RNA duplexes and subjected to A/R. (E) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with the other four groups.

Immunocytochemical Staining and Immunoblotting for Activated Caspase-3

To determine whether HIF-1α abrogated apoptosis via the caspase cascade, cultured HUVECs were evaluated further by immunocytochemical staining (Figures 1c, 1d, and 3) and by Western blot analysis (Figure 4) for the effector caspase protein, activated caspase-3. The results supported this contention and largely paralleled those of TUNEL staining, with approximately 2% apoptotic cells being noted in both transfected and nontransfected normoxic cultures and about 11% of nontransfected cells showing apoptotic changes after A/R (Figure 3). As was observed by the TUNEL technique, significantly greater percentages (approximately 45%) of HUVECs transfected with the siRNA duplexes targeting HIF-1α mRNA manifested immunoreactivity for activated caspase-3 after A/R (Figure 3). Administration of siRNA to A/R-exposed cells also induced significantly greater amounts of immunodetectable activated caspase-3 protein in HUVECs lysates by Western blots (Figure 4).

HIF-1α mRNA and Protein Expression

To assess the efficacy of RNAi targeting HIF-1α in HUVECs, HIF-1α mRNA and protein expression were compared in transfected and nontransfected cells under different exposure conditions. Whereas HIF-1α mRNA was detectable in HUVECs cultured under normoxic conditions, mRNA expression
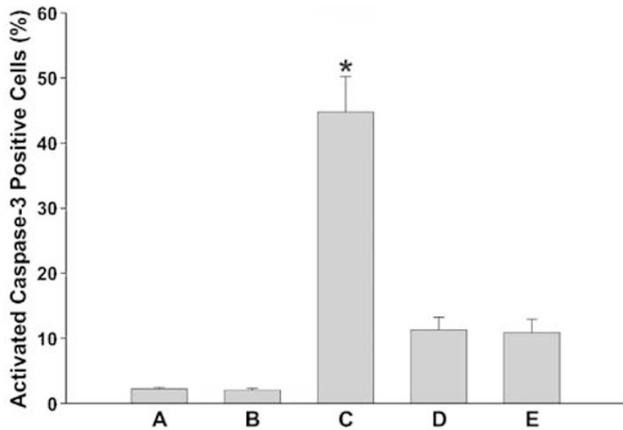
Figure 3 Group comparisons of immunoperoxidase staining for activated caspase-3 showing effects of normoxia, A/R, and transfection with scrambled RNA duplexes on cultured HUVEC. Transfection with HIF-1α siRNA (but not scrambled RNA) duplexes prior to A/R markedly increased the number of cells manifesting immunoreactivity for activated caspase-3. Results in each category represent mean±s.e.m. of three experiments. (A) Nontransfected normoxic cultures. (B) Normoxic cultures transfected with HIF-1α siRNA duplexes. (C) Cultures transfected with siRNA duplexes and subjected to A/R. (D) Cultures transfected with scrambled RNA duplexes and subjected to A/R. (E) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with the other four groups.

quantified by Q-PCR was significantly greater after A/R in nontransfected cells (Figure 5). When, however, cells were transfected with siRNA duplexes prior to A/R, HIF-1α mRNA expression was largely abrogated (Figure 5). Cultures examined by *in situ* hybridization also demonstrated knockdown of HIF-1α mRNA expression in transfected HUVECs subjected to anoxic stress (Figures 1e, 1f, and 6).

The effect of the siRNA duplexes on HIF-1α protein expression induced by A/R was evaluated by both immunocytochemistry and Western blot analyses. Significant reductions in the proportion of HUVECs manifesting immunoreactivity for HIF-1α were seen after transfection with siRNA (Figures 1g, 1h, and 7). A similar reduction in immunodetectable HIF-1α protein was observed in lysates from cells transfected with siRNA (Figure 4). Collectively, these findings indicate significant knockdown of HIF-1α mRNA and protein expression by siRNA transfection.

Specificity of siRNA Transfection

Since transfection of HUVECs with double-stranded RNA might, conceivably, have downregulated HIF-1α mRNA and protein expression nonspecifically, it was necessary to determine whether or not the duplexes used specifically targeted *HIF-1α*. Accordingly, HUVECs were transfected with scrambled RNA duplexes containing 21-nt sequences that demonstrated no homology with any human gene by a BLAST search. Transfection of HUVECs with scrambled RNA duplexes prior to A/R had no
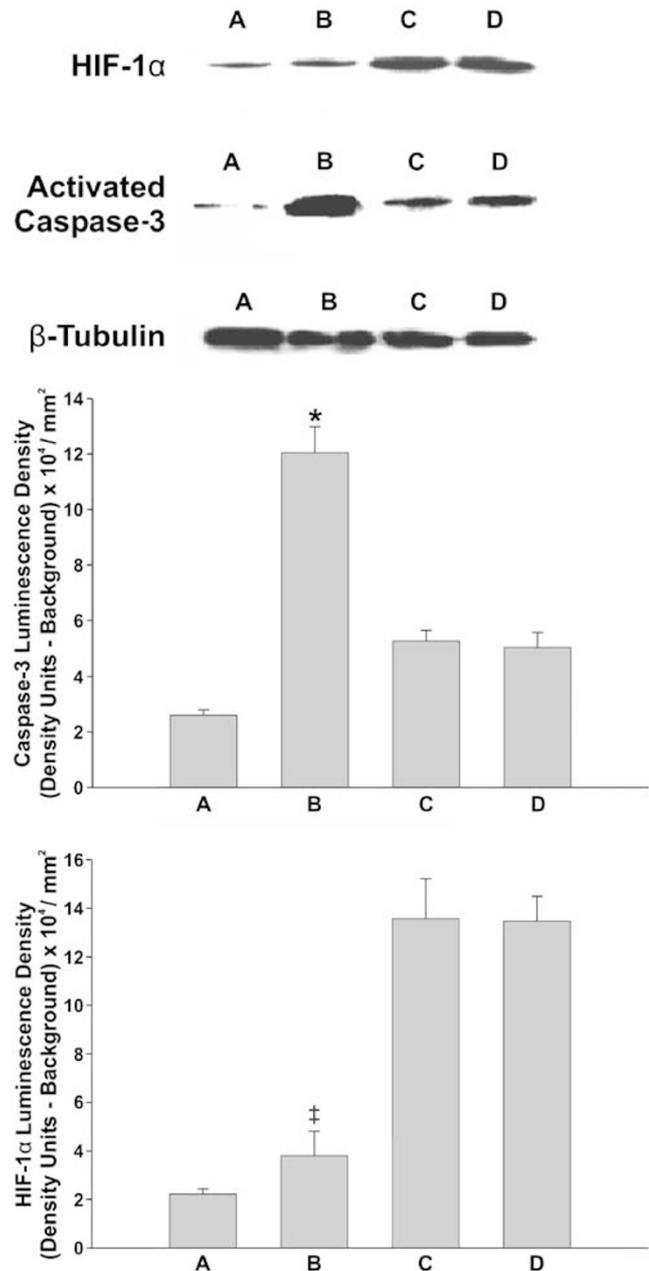

Figure 4 Western blot analyses of HUVECs lysates for HIF-1α and activated caspase-3 showing the effects of normoxia, A/R, and transfection with scrambled RNA duplexes on cultured HUVECs. Transfection with HIF-1α siRNA duplexes prior to A/R largely attenuated HIF-1α expression, but greatly increased activated caspase-3 expression. The upper panel depicts representative immunoblots demonstrating HIF-1α, activated caspase-3, and β-tubulin protein expression. The middle panel depicts group comparisons of activated caspase-3 immunoblots. The lower panel depicts group comparisons of HIF-1α immunoblots. Results in each category represent mean±s.e.m. of three experiments. (A) Normoxic cultures. (B) Cultures transfected with HIF-1α siRNA duplexes and subjected to A/R. (C) Cultures transfected with scrambled RNA duplexes and subjected to A/R. (D) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with the other three groups. ‡Significantly different ($P < 0.05$) compared with groups C and D.
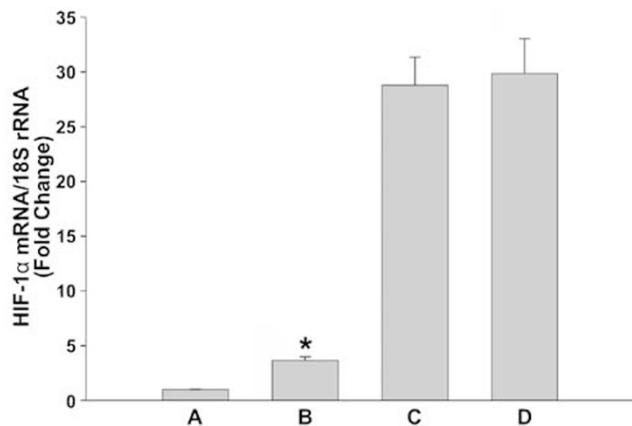
Figure 5 Group comparisons of Q-PCR analyses of HIF-1α mRNA expression showing the effects of normoxia, A/R, and transfection with scrambled RNA duplexes on cultured HUVEC. Transfection with HIF-1α siRNA (but not scrambled RNA) duplexes prior to A/R inhibited HIF-1α mRNA expression. Results in each group category (other than group a) represent mean±s.e.m. of three experiments. (A) Normoxic cultures. (B) Cultures transfected with HIF-1α siRNA duplexes and subjected to A/R. (C) Cultures transfected with scrambled RNA duplexes and subjected to A/R. (D) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with groups C and D.
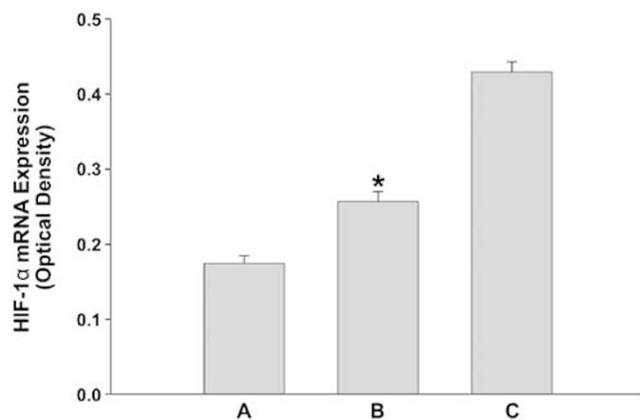

Figure 6 Group comparisons of HIF-1α *in situ* hybridization analyses illustrating the effects of normoxia and A/R on cultured HUVECs. Transfection with HIF-1α siRNA significantly decreased the amount of HIF-1α mRNA detectable in HUVECs subjected to anoxic stress. Results in each category represent mean±s.e.m. of three experiments. (A) Normoxic cultures. (B) Cultures transfected with HIF-1α siRNA duplexes and subjected to A/R. (C) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with the other two groups.

noticeable effect on HIF-1α mRNA (Figures 5 and 6) and protein expression (Figures 4 and 7). These findings indicate that the observed knockdown of HIF-1α mRNA and protein expression in HUVECs was due to specific targeting of *HIF-1α* by the siRNA duplexes. Since HUVECs transfected with scrambled RNA duplexes prior to A/R did not exhibit appreciable apoptotic changes (Figure 2), these findings indicate that the specific targeting of HIF-1α mRNA by siRNA duplexes induced a significant apoptotic effect (Figures 1b and 2).
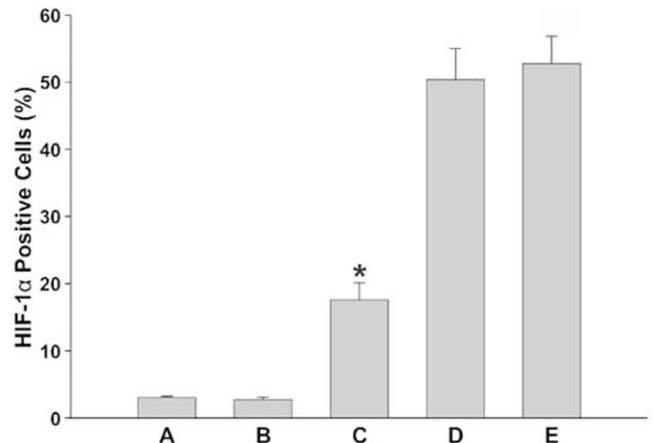

Figure 7 Group comparisons of immunoreactivity for HIF-1α showing the effects of normoxia A/R, and transfection with scrambled RNA duplexes on cultured HUVECs. Transfection with HIF-1α siRNA significantly decreased the percentages of cells manifesting immunoreactivity for HIF-1α in response to anoxic stress. Results in each category represent mean±s.e.m. of three experiments. (A) Nontransfected normoxic cultures. (B) Normoxic cultures transfected with HIF-1α siRNA duplexes. (C) Cultures transfected with siRNA duplexes and subjected to A/R. (D) Cultures transfected with scrambled RNA duplexes and subjected to A/R. (E) Nontransfected cultures subjected to A/R. *Significantly different ($P < 0.05$) compared with the other four groups.

Discussion

The issue of whether HIF-1α is a pro- or antiapoptotic protein is a matter of some debate[21] and there are published studies that are supportive of either viewpoint. It has been shown that HIF-1α may indirectly produce a proapoptotic effect either by upregulating the expression of proteins in the Bcl-2 family that are known to mediate cell death or by associating with and stabilizing these proteins. The tumor suppressor protein p53 can activate target genes that initiate cell death and, in one study, the accumulation of wild-type p53 in response to hypoxia was shown to be HIF-1α-dependent since p53 induction did not occur in a mutant hepatoma cell line that was incapable of synthesizing HIF-1α, whereas transfection with HIF-1α increased the amount of endogenous p53 in normoxic MCF7 cells.[22] Other investigators have demonstrated that hypoxic induction of the proapoptotic protein BNIP3 was mediated via HIF-1α in the RCC4 renal carcinoma and KA13 Chinese hamster ovary cell lines.[23]

In contrast to these observations, other evidence suggests that HIF-1α may have a protective role in limiting hypoxia-induced apoptosis. In this regard, one study showed that pancreatic cancer cell lines that constitutively expressed HIF-1α were more resistant to apoptosis induced by hypoxia than were similar cell lines that lacked constitutive HIF-1α expression.[24] In another study, the induction of HIF-1α by hypoxia was shown to be protective

against the apoptotic effect of *tert*-butyl hydroperoxide in the HepG2 hepatoma cell line.[25] Further evidence supporting an antiapoptotic role for HIF-1α was demonstrated when neutralizing antibody against vascular endothelial growth factor (VEGF), the major target gene protein transactivated by HIF-1, was shown to block the antiapoptotic effect of hypoxia on HepG2 cells.[26] Caution should, however, be exercised when interpreting these studies since they were based on indirect evidence obtained from mutant cell lines,[24] from studies of downstream target proteins such as VEGF,[26] or from the use of chemical inducers of HIF-1α such as $CoCl_2$[25] that, by themselves, can induce apoptosis.[27,28]

In the current study, we used the technique of RNAi to evaluate the role of HIF-1α in modulating anoxia-induced apoptotic injury in primary cultured HUVECs. For this purpose, HUVECs were transfected with siRNA duplexes targeting HIF-1α mRNA prior to being subjected to conditions of A/R. The efficacy of transfection was assessed by the ability of the duplexes to knock down HIF-1α mRNA (as determined by *in situ* hybridization and Q-PCR) and protein expression (as evaluated by immunoperoxidase staining and Western blot analyses). As expected, A/R induced upregulation of HIF-1α mRNA expression in nontransfected cells by *in situ* hybridization and by Q-PCR, findings that were not seen in HUVECs incubated under normoxic conditions. A/R also induced HIF-1α protein expression in nontransfected cells, as detected by immunoperoxidase staining and by Western blotting. When, however, HUVECs were transfected with siRNA duplexes prior to anoxic stress, HIF-1α mRNA and protein induction were largely abrogated. The specificity of the siRNA duplexes for the *HIF-1α* target gene was demonstrated by the inability of scrambled siRNA duplexes to inhibit HIF-1α mRNA or protein expression in HUVECs subjected to A/R. These scrambled sequences showed no similarity with any known human gene by a BLAST search.

A/R did induce a mild degree of apoptosis in nontransfected HUVECs, as reflected by positive TUNEL staining and immunoreactivity for activated caspase-3 by immunocytochemistry and immunoblotting in approximately 7–11% of the cells. However, significantly greater proportions (approximately 45–48%) of HUVECs transfected with HIF-1α siRNA duplexes exhibited apoptotic changes that were evident by these assays. Our findings clearly underscore the role of HIF-1α as an antiapoptotic protein in the context of anoxic stress. Our studies also suggest that the protective action of HIF-1α appears to involve the caspase pathway via caspase-3, an effector molecule in the caspase-mediated cascade of apoptosis induction.[29] However, the data are not definitive, and, conceivably, HIF-1α may exert its effects via activation or downregulation of other pro- or antiapoptotic proteins. These observations are of special interest, given the current controversy as to whether HIF-1α should be regarded as a pro- or an antiapoptotic protein.[21]

Although this study has not addressed the mechanisms governing the antiapoptotic effect of HIF-1α in the context of hypoxic or A/R-mediated injury, other investigators have shown that the induction of HIF-1α protein and its transcriptional activation by hypoxia and oxidative stress are regulated by several signaling pathways including PI3K/AKT/FRAP[30–32] p38, and ERK kinase[30,32,33] in which the redox-sensitive factors Ref-1 and thioredoxin and the Rho family small GTPase Rac1 have been shown to play a role.[31,33,34] Moreover, protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators has been shown to relate to enhanced DNA binding of HIF-1α and ATF-1/CREB transcription factors and to increased expression of glycolytic enzymes.[35] In this respect, it is worth noting that HIF-1α can mediate crosstalk between hypoxia and glucose metabolism via glucose response elements.[36,37]

Since a significant proportion of transfected HUVECs did not manifest evidence of apoptotic changes by TUNEL assay and activated caspase-3 immunostaining after A/R in the current study, it is probable that other antiapoptotic proteins distinct from HIF-1α may also have conferred some cytoprotection against anoxic stress, as has been shown with respect to CD105[38] and Mcl-1.[39]

In summary, we have employed RNAi targeting the *HIF-1α* gene in primary cultured HUVECs and have demonstrated the role of HIF-1α as an antiapoptotic protein in a model of anoxic stress. This study underscores the utility of using RNAi as a loss-of-function approach that is currently believed to be more efficacious, selective, and specific than antisense technologies.[40,41]

Acknowledgements

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