Prolonged Hypoxia Differentially Regulates Hypoxia-inducible Factor (HIF)-1α and HIF-2α Expression in Lung Epithelial Cells

IMPLICATION OF NATURAL ANTISENSE HIF-1α*

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Transcriptional adaptations to hypoxia are mediated by hypoxia-inducible factor (HIF)-1, a heterodimer of HIF-α and aryl hydrocarbon receptor nuclear translocator subunits. The HIF-1α and HIF-2α subunits both undergo rapid hypoxia-induced protein stabilization and bind identical target DNA sequences. When coexpressed in similar cell types, discriminating control mechanisms may exist for their regulation, explaining why HIF-1α and HIF-2α do not substitute during embryogenesis. We report that, in a human lung epithelial cell line (A549), HIF-1α and HIF-2α proteins were similarly induced by acute hypoxia (4 h, 0.5% O2) at the translational or post-translational level. However, HIF-1α and HIF-2α were differentially regulated by prolonged hypoxia (12 h, 0.5% O2) since HIF-1α protein stimulation disappeared because of a reduction in its mRNA stability, whereas HIF-2α protein stimulation remained high and stable. Prolonged hypoxia also induced an increase in the quantity of natural antisense HIF-1α (aHIF), whose gene promoter contains several putative hypoxia response elements to which (as we confirm here) the HIF-1α protein can bind. Finally, transient transfection of A549 cells by dominant-negative HIF-2α, also acting as a dominant-negative for HIF-1α, prevented both the decrease in the HIF-1α protein and the increase in the aHIF transcript. Taken together, these data indicate that, during prolonged hypoxia, HIF-α proteins negatively regulate HIF-1α expression through an increase in aHIF and destabilization of HIF-1α mRNA. This trans-regulation between HIF-1α and HIF-2α during hypoxia likely conveys target gene specificity.

Maintaining oxygen homeostasis is essential to the development, growth, and the preservation of structural integrity of tissues. Lung hypoxia may be the consequence of some environments, e.g. high altitude, or may result from inadequate alveolar ventilation, e.g. chronic obstructive disease or pulmonary edema from acute lung injury or heart failure. Previous studies have reported lung alveolar epithelial cell adaptation to reduced O2 availability through an increase in glucose transport (1), glycolysis (2, 3), and vascular endothelial growth factor expression (4). In most cell types, activation of genes essential for cell survival is mediated by hypoxia-inducible factor (HIF)-1, a transcriptional complex that binds to the specific hypoxia response element (HRE). HIF-1 is a heterodimer composed of the HIF-1α and HIF-1β subunits, also known as the aryl hydrocarbon receptor nuclear translocator. HIF-1β is found generally to be constitutively expressed and insensitive to changes in O2 availability, whereas HIF-1α is acutely regulated in response to hypoxia. The predominant mode of HIF-1α regulation occurs post-transcriptionally (5). During normoxia, HIF prolyl hydroxylase-1–3 hydroxylate two proline residues of the oxygen-dependent degradation domain located in the central region of the protein (6–10). The von Hippel-Lindau (VHL) tumor suppressor protein, a component of a multisubunit ubiquitin ligase protein complex, binds to the hydroxylated oxygen-dependent degradation domain of the α-subunit, resulting in the ubiquitination and proteasomal degradation of HIF-1α (11, 12). Under hypoxic conditions, the VHL tumor suppressor protein fails to recognize the HIF-1α subunit, allowing HIF-1 to accumulate (6, 7). However, using mitochondrial depleted cells and a number of mitochondrial electron transport inhibitors, several groups have demonstrated that mitochondria may also act as the proximal O2 sensor during hypoxia via increased production of reactive oxygen species to stabilize HIF-α proteins (13, 14).

Besides the well established HIF-1, two other members of the basic helix-loop-helix/PAS (PER/ARNT/SIM) superfamily have also been described: HIF-2α, referred also as to endothelial PAS domain protein-1 or HIF-1α-like factor, which bears functional resemblance to HIF-1α regarding hypoxic stabilization and binding to HIF-1α (15), and recently HIF-3α (16). The HIF-1α protein is ubiquitously expressed, whereas its HIF-2α and HIF-3α homologs have a more restricted pattern of expression (17, 18). There has been long-term interest in distinguishing the roles of HIF-1α and HIF-2α. Despite strong sequence and mechanistic similarities, targeted disruption of their genes in mice results in embryonic lethalties with startling differences in the phenotype (19). HIF-1α-null mice exhibit midgestation lethality and severe blood vessel defects, whereas HIF-2α-null mice also exhibit embryonic lethality with abnormal lung maturation and blood vessel defects and sometimes

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‡ The abbreviations used are: HIF, hypoxia-inducible factor; HRE, hypoxia response element; VHL, von Hippel-Lindau; aHIF, natural antisense hypoxia-inducible factor-1α; DTT, dithiothreitol; DRB, 5,6-dichlorobenzimidazole 1-β-d-ribofuranoside; UTR, untranslated region.
survive post-natally (19–22). These results establish that HIF-1α and HIF-2α perform distinct functions during embryogenesis and indicate that they stimulate different target genes and/or undergo undiscovered differences in their in vivo mechanisms of activation. More recently, specific inactivation of HIF-1α or HIF-2α by short interfering RNAs showed that, in cells expressing both HIF-α isoforms, HIF-2α does not substitute in regulating any of the hypoxia-inducible genes when HIF-1α is inactivated (23, 24).

The presence of HIF-1α and HIF-2α in lung alveolar epithelial cells has been reported in separate studies, suggesting that the two isoforms are coexpressed (25, 26). The hypoxic induction of HIF-2α has been demonstrated in vivo and in vitro whatever the cell background (15, 27), whereas the induction of HIF-1α is controversial. The HIF-1α protein is rapidly induced by acute exposure hypoxia in ferret lung and in the alveolar epithelial cell line A549 (25), but remains undetectable in the lungs of mice exposed to mild hypoxia (28) or in A549 cells exposed to 12 h of hypoxia (26). These findings raise important questions as to what extent HIF-1α and HIF-2α are differentially regulated by the severity and duration of hypoxia in alveolar epithelial cells. Moreover, the mechanisms by which alveolar epithelial cells sense hypoxia have not been yet elucidated.

The aim of the study was to investigate whether alveolar epithelial cells coexpress HIF-1α and HIF-2α and to determine the mechanisms of their hypoxic induction. Our results indicate that, in lung alveolar epithelial cells, acute hypoxia regulates HIF-1α and HIF-2α at the post-transcriptional level and involves several mechanisms such as production of mitochondrial reactive oxygen species and probably the inhibition of an enzyme that requires O2 and iron such as prolyl hydroxylase. One of the major findings was that, during sustained hypoxia, HIF-1α protein stimulation disappeared, whereas HIF-2α protein stimulation remained stable. Several lines of evidence suggest that the decrease in the HIF-1α protein was the consequence of negative feedback regulation. The initial rise in HIF-α proteins increased natural antisense HIF-1α (aHIF), which in turn destabilized HIF-1α mRNA and finally decreased HIF-1α protein expression. These results identify a novel post-transcriptional pathway that regulates the level of HIF-1α protein under hypoxic conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A549 human lung adenocarcinoma cells were cultured as recommended by the American Type Culture Collection. Cells were grown in Kaighn’s modified Ham’s F-12 medium supplemented with 2 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. Human lung microvascular endothelial cells were purchased from BioWhittaker, Inc. (Walkersville, MD) and cultured according to the manufacturer’s recommendations in EBM-2 medium (BioWhittaker, Inc.).

**Exposure to Hypoxia**—On the day of the experiment, the medium was replaced with a thin layer of fresh medium (0.15 ml/cm2) with 10% fetal calf serum to decrease the diffusion distance of the gas. Culture dishes were then placed in a humidified airtight incubator with inflow and outflow valves, and the hypoxic gas mixture (0.5 % O2, 5 % CO2, and balance N2) was delivered at 10 liters/min for 25 min. The airtight incubator was kept at 37 °C for 4, 6, or 12 h, whereas normoxic cells were placed at 37 °C in a 21% O2, 5% CO2, and 74% N2 humidified incubator until harvest.

**Protein Extraction and Immunoblot Analysis**—Whole cell extracts and nuclear cell extracts were prepared as described previously (1). Cells were disrupted with a Dounce homogenizer in 10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl2, 10 mM KCl, 2 mM dithiothreitil (DTT), 0.4 mM phenylmethylsulfonil fluoride, and 1 mM Na3VO4. Nuclei were pelleted by centrifugation at 10,000 x g for 10 min and then resuspended in 0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM MgCl2, 2 mM DTT, 0.4 mM phenylmethylsulfonil fluoride, and 1 mM Na3VO4. The suspension was rotated for 30 min at 4 °C to extract nuclear proteins, which were then dialyzed in 25 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, 100 mM KCl, 20% glycerol, 2 mM DTT, 0.4 mM phenylmethylsulfonil fluoride, and 1 mM Na3VO4. Total cellular protein was precipitated by homogenizing lung tissue in 20 mM HEPES (pH 7.5), 1.5 mM MgCl2, 0.2 mM EDTA, 0.1 mM NaCl, 5 mM DTT, 1 mM phenylmethylsulfonil fluoride, and 1.2 mM Na2VO4. NaCl was added to a final concentration of 0.45 mM. Cellular debris was pelleted by centrifugation at 10,000 × g for 30 min, and the supernatant was collected as a sample. Nuclear extract and total cellular protein concentrations were determined with a method of Bradford (41). For immunoblot assays, 30 μg of samples were fractioned by 7.5% SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were blocked with 5% milk in 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (pH 7.6) and subsequently probed for HIF-1α or HIF-2α. Mouse anti-human HIF-1α (NB100-123) and rabbit antihuman HIF-2α (NB100-129) polyclonal antibodies (Novus Biological Science) were used at 1:1000 dilution, and mouse anti-β-actin monoclonal antibody (Sigma) was used at 1:6000 dilution. The anti-mouse or anti-rabbit IgG secondary antibody (Amersham Biosciences) was used at 1:2000 dilution, and the signal was analyzed by enhanced chemiluminescence (Amersham Biosciences). The abundance of HIF-1α and HIF-2α proteins was normalized to β-actin using NIH Image software.

**Ribonuclease Protection Assay**—Total RNA was extracted using Nucleospin nucleic acid purification kits (Clontech). The abundance of HIF-α mRNA was evaluated by ribonuclease protection assay as described previously (4). 32P-Labeled riboprobe were generated using Sp6 transcriptase. The RNA polymerase transcripts were labeled with 221 bp for HIF-2α (nucleotides 2542–2762; GenBank™/EMB accession number U81894; a gift from Dr. P. J. Ratcliffe, John Radcliffe Hospital, Oxford, United Kingdom), 255 bp for HIF-1α (nucleotides 764–1018; accession number U22431; a gift from Dr. P. J. Ratcliffe), and 187 bp for cyclophilin (nucleotides 437–624; accession number BC032158) (29). Ten micrograms of total RNA were co-hybridized overnight with 5 × 106 cpm for HIF-2α, 1 × 106 cpm for HIF-1α, and 5 × 105 cpm for cyclophilin probes in 80% formamide, 40 mM Pipes (pH 7.4), 400 mM NaCl, and 1 mM EDTA at 50 °C. RNA digestion (40 mg/ml RNase A and 2 mg/ml RNase T1; Roche Diagnostics) was performed at 30 °C for 60 min, and then digestion with 12.5 mg/ml proteinase K and Roche Digestase was carried out at 50 °C after phenol extraction and ethanol precipitation, protected fragments were separated by urea-PAGE. Gels were fixed with 10% acetic acid and vacuum-dried before exposure to Kodak X-Omat AR5 film, and the signal was quantitated from the gel using direct radioactivity measurement with an Instaimage (Packard Instrument Co.). Cyclophilin expression was used as an internal standard. Results are expressed as the ratio of expression of the mRNA of interest to cyclophilin mRNA.

**SYBR Green-based Real-time Quantitative PCR**—Reverse transcription was performed at 42 °C for 50 min in a volume of 100 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 800 μM dNTPs, 10 units of RNasin ribonuclease inhibitor, 0.5 μg of oligo(dT)15 primer, and 500 units of Moloney murine leukemia virus reverse transcriptase. RNase H (+Promega). The enzyme was inactivated at 95 °C for 5 min. The cDNAs were used for real-time PCR measurements.

Semiquantitative determination of aHIF transcript concentrations was performed by real-time RT-PCR with β2-microglobulin as an internal control sequence as described (30). Briefly, cDNAs (4 μl of a 10-fold dilution for β2-microglobulin or 4 μl of undiluted cDNA for aHIF) were added to 16 μl of PCR mixture containing 2 μl of 10× SYBR Green I master mixture (Roche Diagnostics), 5 mM MgCl2, and 600 nm gene-specific primers. A melting curve was used to identify a temperature at which only the amplicon and not primer dimers accounted for the SYBR Green-bound fluorescence. Assays were performed with a thermocycler equipped with a thermal cycling module incorporating capabilities of the aHIF gene (Roche Diagnostics). The relative expression levels were determined by comparison with a serially diluted standard using thermocycler software. All data were normalized to the internal standard β2-microglobulin.

**Measurements of mRNA Half-life**—A549 cells were grown in medium with and without CoCl2 (250 μM) for 4 h. 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; 65 μM) was then added to block transcription. In the CoCl2-treated cells, supplemental CoCl2 was added with the transcription inhibitor to keep the CoCl2 concentration at 250 μM. Cells were harvested for RNA at 0, 0.5, 1.5, 2.0, and 4.0 h after the addition of the transcription inhibitor.

**Quantitation of Ongoing Transcription and Ongoing Protein Synthesis**—To verify whether ongoing transcription is essential for the hypoxic regulation mechanism of HIF-α mRNA, the medium was replaced with DRB (65 μM)-containing medium. Two hours after this treatment, cells were incubated in gas containing 0.5 or 21% O2 for 6 h. To elucidate the
necessity of ongoing protein synthesis for HIF-α mRNA regulation, the medium was replaced with cycloheximide (100 µM)-containing medium and incubated in gas containing 0.5 or 21% O₂ for 6 h.

Plasmid Construct and Transient Transfection Assay—The dominant-negative HIF-2α mutant expression vector phEP-1-(1–485), which contains the coding sequences corresponding to amino acids 1–485 of the HIF-2α cDNA insert in the eukaryotic expression vector pcDNA3 (Invitrogen), was a gift from Dr. K. Maemura (University of Tokyo, Tokyo, Japan) (31). A549 cells were seeded on 100-mm Petri dishes at 1.0 × 10⁶ cells/plate on the day before transfection and transfected with 2–20 µg of phEP-1-(1–485) using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. Twenty-two hours after transfection, cells were exposed to 0.5 or 21% O₂ for 6 h and harvested for RNA analysis. The expression of the dominant-negative HIF-2α mutant was evaluated by Northern blot analysis using a 32P-labeled randomly primed cDNA probe of dominant-negative HIF-2α (phEP-1-(1–485)).

HIF-1 DNA Binding—A TransFactor enzyme-linked immunosorbent assay kit (BD Mercury™, Clontech) for HIF-1 was used according to the manufacturer's instructions. A549 cell nuclear extracts were incubated in wells coated with the consensus binding sequence of HIF-1 (HRE). Bound HIF-1 and HIF-2 were detected by specific primary antibodies: two mouse monoclonal antibodies raised against human HIF-1α (from the kit) and human HIF-2α (sc-13596) from Santa Cruz Biotechnology, Inc.). A horseradish peroxidase-conjugated secondary antibody was used to detect the bound primary antibody. The blue enzyme product quantity was proportional to the amount of transcription factor bound to the DNA in the well. These experiments were also performed in the presence of 500 ng of an oligonucleotide sequence containing the putative HRE found in the human aHIF promoter (5′-GGTG CGC CGC AGC AGC TAC TGG ACG GCC GCC GCC-3′) (50) or a mismatch of this putative HRE sequence (5′-GGTG CGC CGC AGC AGC TAC TGG GCC GCC GCC-3′).

Statistical Analysis—All data are presented as the means ± S.D. with n = 3–5 in separate culture and are expressed as the ratio compared with the conditions of the normoxic control. For intergroup comparison, one-way analyses of variance were performed, and the difference was analyzed with Scheffe's test as a post-hoc analysis. To evaluate whether HIF-α mRNA regulation is dependent on ongoing transcription or ongoing protein synthesis, two-way analyses of variance were performed. Statistical significance was defined as p < 0.05.

RESULTS

Acute Hypoxia Similarly Regulates HIF-1α and HIF-2α Expression in Alveolar Epithelial Cells—HIF-1α and HIF-2α proteins were determined in whole cell and nuclear extracts of A549 cells. During normoxia, HIF-1α was almost undetectable in both whole cell and nuclear extracts, whereas HIF-2α was detectable in whole cell extracts, but not in nuclear extracts. Exposure to hypoxia (0.5% O₂) for 4 h induced HIF-1α and HIF-2α protein accumulation in both whole cell and nuclear extracts (Fig. 1A). The hypoxic induction of HIF-1α and HIF-2α proteins was dependent on the following: (i) cell density, with a reduced hypoxic response when the cells were cultured at low density (data not shown), and (ii) O₂ concentration, with the increase seen for O₂ concentrations below 3%, with maximal stimulation at 0.5% O₂ (Fig. 1B). Interestingly, the response to graded hypoxia was strikingly similar for HIF-1α and HIF-2α proteins.

To investigate whether increases in HIF-α protein levels are related to post-transcriptional or post-translational changes, HIF mRNA levels were also evaluated after a 4-h incubation at different O₂ concentrations. Four hours of hypoxia did not change HIF-1α and HIF-2α mRNA levels (Fig. 1C), suggesting that hypoxia-induced increases in HIF-1α and HIF-2α proteins are likely due to translational or post-translational changes.

Important insights into the mechanism of oxygen sensing and/or signal transduction underlying HIF-1α activation have been gained using pharmacological interventions that perturb the response to HIF-1α. To compare the regulation of HIF-1α and HIF-2α, we measured the response of each protein to several of these interventions. CoCl₂ and iron chelators mimic hypoxia inactivation of target genes and induce HIF-1α and HIF-2α at the protein levels (7, 32). When A549 cells were exposed to CoCl₂ or desferrioxamine, a marked and similar increase in HIF-1α and HIF-2α was observed, and this effect was dependent on the concentration of hypoxia-mimetic com-
HIF-1α and HIF-2α protein levels in A549 cells. A, cells exposed to normoxia were incubated with 100 or 250 μM CoCl2 or desferrioxamine (DFO) for 4 h prior to whole cell extraction and immunoblot analysis. B, shown are the effects of mitochondrial reactive oxygen species inhibitors on hypoxic induction of HIF-1α and HIF-2α proteins. Cells were exposed to normoxia (N; 21% O2) or hypoxia (H; 0.5% O2) for 4 h in the absence or presence of 0.5 μM diphenyleneiodonium (DPI), 1 μM rotenone, or 0.1 μM myxothiazol. Each experiment was repeated three times with similar results. Representative blots are shown.

Implication of Transcriptional Activity in the Hypoxic Regulation of HIF-1α and HIF-2α mRNAs—The dependence of hypoxic regulation of HIF-1α and HIF-2α mRNA levels on ongoing transcription was evaluated by pretreating the cells with DRB for 2 h before hypoxic exposure for 4 h. Following this treatment, the decrease in HIF-1α mRNA levels (Fig. 6a) and the stimulation of HIF-2α mRNA (data not shown) induced by hypoxia were completely abolished.

Implication of Translational Activity in the Hypoxic Regulation of HIF-1α and HIF-2α mRNAs—To investigate whether hypoxia-induced regulation of HIF-1α and HIF-2α requires protein synthesis, cells were pretreated with cycloheximide, an inhibitor of translation, before hypoxic exposure. Cycloheximide completely prevented the decrease in HIF-1α mRNA (Fig. 6b) and the increase in HIF-2α mRNA (data not shown) by hypoxia. This result implicates ongoing protein synthesis in the regulation of HIF-α mRNA.

Hypoxia Increases the Antisense HIF-1α Transcript—Recently, aHIF, which is complementary to 1027 bp of the 3’-untranslated region (UTR) of HIF-1α mRNA, has been reported to be constitutively expressed in many human fetal tissues and tumors (30). Binding of aHIF to the HIF-1α mRNA 3’-UTR could expose AU-rich elements present in the HIF-1α mRNA 3’-UTR and thus possibly increase the degradation speed of HIF-1α mRNA (30, 33). Fig. 7a shows that aHIF was barely detectable in A549 cells under normoxic conditions, dramatically increased after 4 h of either hypoxia or CoCl2 treatment, and remained sustained until 12 h. The maximal induction of the aHIF transcript by hypoxia occurred before any decrease in HIF-1α mRNA levels.

Analysis of the aHIF promoter showed that it contains several putative HREs that could bind HIF-1α and HIF-2α proteins. We postulated that the hypoxia-induced increase in HIF-1α and HIF-2α protein levels might be involved in aHIF overexpression. Several lines of evidence support such hypothesis. (i) Cycloheximide, a protein synthesis inhibitor that inhibited hypoxia-induced HIF-1α protein increases, also completely abolished the augmentation of aHIF RNA levels during prolonged hypoxia is related mainly to decreased mRNA stability, and (ii) the hypoxic induction of HIF-2α mRNA increase is due to transcriptional activation rather than to a change in mRNA stability.

Distinct HIF-1α and HIF-2α Regulation by Hypoxia

Fig. 2. Effects of cobalt chloride, desferrioxamine, and inhibitors of reactive oxygen species on HIF-1α and HIF-2α protein levels in A549 cells. A, cells exposed to normoxia were incubated with 100 or 250 μM CoCl2 or desferrioxamine (DFO) for 4 h prior to whole cell extraction and immunoblot analysis. B, shown are the effects of mitochondrial reactive oxygen species inhibitors on hypoxic induction of HIF-1α and HIF-2α proteins. Cells were exposed to normoxia (N; 21% O2) or hypoxia (H; 0.5% O2) for 4 h in the absence or presence of 0.5 μM diphenyleneiodonium (DPI), 1 μM rotenone, or 0.1 μM myxothiazol. Each experiment was repeated three times with similar results. Representative blots are shown.

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prolonged hypoxia (Fig. 7B). (ii) HIF-1α and HIF-2α proteins that were present in the nuclear extracts of A549 cells bound to a putative HRE sequence and were displaced in the presence of an oligonucleotide sequence containing the human putative HRE found in the human aHIF gene, but not in the presence of a mismatch of this putative HRE sequence (Fig. 8).

**Inhibition of Hypoxic Induction of aHIF in A549 Cells Transfected with Dominant-negative HIF-2α**—The truncated HIF-2α protein lacking the transactivation domain at amino acids 486–639 functions as a dominant-negative mutant. The dominant-negative HIF-2α protein (phEP-1-(1–485)) retains the ability to form heterodimers and, by sequestering HIF-1α, acts as a dominant-negative for both HIF-2α and HIF-1α subunits (31). To investigate whether binding of HIF-α proteins is involved in the hypoxic induction of the aHIF transcript, A549 cells were transfected with the dominant-negative HIF-2α plasmid (phEP-1-(1–485)). Transfected cells exhibited much lower aHIF RNA hypoxic induction and concomitantly prevented the hypoxic decrease in HIF-1α mRNA (Fig. 9).

**DISCUSSION**

**Similar Regulation of HIF-1α and HIF-2α by Acute Hypoxia**—This study demonstrates that, in alveolar epithelial cells, both HIF-1α and HIF-2α were expressed and concomitantly increased by acute hypoxia. This result differs from that of Sato et al. (26), who reported the presence of HIF-2α (but not HIF-1α) in A549 cells. Our finding that prolonged exposure to hypoxia induced down-regulation of HIF-1α expression likely explains the discrepancy between the two studies since the experiment of Sato et al. was performed after 12 h of hypoxia. Our study also demonstrates that inductions of HIF-1α and HIF-2α show very similar characteristics of regulation in A549 cells. The acute hypoxic...
induction of HIF-1α and HIF-2α proteins occurred without change in mRNA abundance, consistent with post-translational regulation of HIF-α through an increase in protein stability as reported in many cell lines (34). In this study, the effects of hypoxia were mimicked by the iron chelator desferrioxamine, suggesting that the O₂ sensor would be the prolyl hydroxylase enzymes involved in HIF-α degradation and requiring O₂ and iron for their activity (6, 7). In the same line, cobalt chloride, which has been reported to inhibit the hydroxylation of proline residues in the oxygen-dependent degradation domain of HIF-α (32), induced HIF-1α and HIF-2α protein accumulation. In this study, mitochondrial reactive oxygen species inhibitors partially prevented HIF-1α and HIF-2α induction, suggesting that the mitochondrial respiratory chain is also required for HIF-α protein stabilization. These results are in line with a recent report showing that, in A549 cells, hypoxic (but not anoxic) stabilization of HIF-1α requires mitochondrial oxygen species (14). Thus, across a range of experimental conditions, we observed a concordance between HIF-1α and HIF-2α expression in A549 cells. Not only do both molecules respond to hypoxia, but it appears that the same or strikingly similar oxygen-sensing and signal transduction mechanisms regulate the abundance of both transcription factors in these cells.

**Differential Regulation of HIF-1α and HIF-2α by Prolonged Hypoxia**—An interesting finding is that HIF-1α and HIF-2α proteins, which displayed similar induction by acute hypoxia, were differentially regulated during prolonged hypoxia: HIF-1α protein levels decreased from 6 to 12 h, whereas HIF-2α protein levels did not change. These observations agree with some previous reports showing that, in different cell lines, hypoxia-induced HIF-1α protein may disappear with prolonged hypoxia (15, 35, 36).

The present results indicate that, in addition to the decrease in the HIF-1α protein, prolonged hypoxia also induced a decrease in HIF-1α mRNA. This down-regulation of HIF-1α mRNA was likely due to the induction of the HIF-1α and/or HIF-2α protein. This is supported by the following data. (i) Cycloheximide, an inhibitor of protein synthesis, ablated the HIF-1α mRNA decrease, and (ii) the transient transfection of A549 cells with the dominant-negative HIF-2α mutant, which also acts as a dominant-negative for HIF-1α, prevented the down-regulation of HIF-1α mRNA. Therefore, during prolonged hypoxia, the HIF-1α mRNA level is regulated by autonegative feedback by HIF-1α itself or by trans-regulation between HIF-1α and HIF-2α.

Thrash-Bingham and Tartof (37) have identified in renal clear cell carcinoma an endogenous aHIF transcript for the 3‘-untranslated region of HIF-1α. Interestingly, the expression of this antisense transcript is inversely related to VHL tumor suppressor gene expression, which regulates HIF-1α subunit expression. In the latter study, they demonstrated that the hypoxic induction of the aHIF transcript occurs concomitantly with the decrease in HIF-1α mRNA, suggesting that the accumulation of aHIF might represent an intermediate step in the negative feedback loop between the HIF-1α protein and mRNA. In our study, several lines of evidence indicate that aHIF could play an important role in the regulation of HIF-1α expression. First, aHIF mRNA was quietly expressed during normoxia and dramatically increased from 4 to 12 h of hypoxia in A549 cells.

**Fig. 5. Effect of cobalt chloride on HIF-1α and HIF-2α mRNA stability in A549 cells.** Cells were pretreated with or without 250 μM CoCl₂ for 4 h, followed by treatment with 65 μM DRB and harvesting at various times. HIF-1α and HIF-2α mRNA levels were normalized to the cyclophilin level at each time point and are expressed as the percentage of signal present at 0 h of DRB treatment. Data are presented as means ± S.D. of four different experiments.

**Fig. 6. Effects of inhibition of ongoing transcription and ongoing protein synthesis on HIF-1α mRNA levels in A549 cells.** A, cells were treated with 65 μM DRB for 2 h and then exposed to normoxia (N, 21% O₂) or hypoxia (H, 0.5% O₂) for 4 h. Data are presented as means ± S.D. of four different experiments. p < 0.001, significant interaction between the O₂ concentration and DRB. B, cells were treated with 100 μM cycloheximide (CHX), immediately followed by a 4-h exposure to normoxia (21% O₂) or hypoxia (0.5% O₂). Data are presented as means ± S.D. of four different experiments. p < 0.001, significant interaction between the O₂ concentration and cycloheximide treatment. *, p < 0.01 versus 21% O₂.
Second, the aHIF promoter possesses several functional putative HREs capable of binding HIF-1 and HIF-2 in vitro. Third, transfection with the dominant-negative mutant of HIF-2/H9251, which also acts as a dominant-negative for HIF-1/H9251, reduced the hypoxic increase in aHIF RNA and increased HIF-1/H9251 mRNA levels. Finally, a recent report suggests that aHIF could regulate HIF-1/H9251 mRNA by increasing HIF-1/H9251 mRNA instability via exposing AU-rich elements in the HIF-1/H9251 mRNA 3′-UTR (30). This latter hypothesis matched well with our observation of a hypoxia-induced HIF-1/H9251 mRNA half-life decrease.

In contrast to HIF-1α, the hypoxic stimulation of the HIF-2α protein induced by short-term exposure was maintained unchanged during prolonged hypoxia, although HIF-2α mRNA increased significantly from 6 and 12 h of hypoxia. This hypoxic up-regulation of HIF-2α mRNA likely occurred at the transcriptional level because the mRNA half-life mRNA was unchanged. These results are consistent with a previous study showing that, in A549 cells, the HIF-2α protein increases its own promoter activity (26). Interestingly, analysis of the 3′-UTR of HIF-2α shows that it is short with no AU-rich elements despite the high sequence homology of HIF-2α to HIF-1α in its open reading frame (38). This 3′-UTR design could prevent HIF-2α mRNA from being destabilized by aHIF. Considering that aHIF mRNA expression remained high even after HIF-1α protein expression was decreased, it is likely that the HIF-2α protein is involved in the regulation of aHIF expression.

The trans-regulation between HIF-1α and HIF-2α was suggested by Krieg et al. (39), who demonstrated that, in human renal clear cell carcinoma without the VHL tumor suppressor gene, the increase in HIF-1α and HIF-2α proteins is associated with strong expression of HIF-2α mRNA and poor expression of HIF-1α mRNA, whereas in human renal clear cell carcinoma with the wild-type VHL tumor suppressor gene, reciprocal results are obtained. The physiological implication of this trans-

**Fig. 7. Effects of hypoxia and cobalt chloride on aHIF expression.** A, cells were exposed to normoxia in the absence or presence of 250 μM CoCl₂ or to hypoxia (0.5% O₂) for 4, 6, and 12 h. Total RNA were prepared, and aHIF RNA amounts were determined by real-time RT-PCR analysis and normalized to β₂-microglobulin (β2) levels. The amount of aHIF (in arbitrary units) found at the beginning of each experiment was taken as 1. Data are presented as means ± S.D. of three different experiments. B, cells were treated with 100 μM cycloheximide (CHX) and immediately exposed to hypoxia (H; 0.5% O₂) or to normoxia (N; 21% O₂) for 4 h. Total RNA was prepared and reverse-transcribed. Each bar (in arbitrary units) represents the mean ± S.D. of three different experiments. The amount of aHIF under normoxia was taken as 1. *, p < 0.05 versus 21% O₂; **, p < 0.01 versus 21% O₂.

**Fig. 8. Effect of hypoxia on HIF-1 and HIF-2 binding activities.** Nuclear extracts from A549 cells exposed to normoxia (N; 21% O₂) or to hypoxia (H; 0.5% O₂) for 4 h were prepared as described under “Experimental Procedures.” Fifteen micrograms of nuclear extract proteins obtained under each condition were incubated in wells coated with the consensus binding sequence of HIF-1 (HRE). Bound HIF-1 and HIF-2 were detected using specific antibodies raised against their respective α-subunits. The same experiments were performed in the presence of 500 ng of a double-stranded oligonucleotide containing the putative HRE sequence encountered in the human aHIF (ahif) gene promoter or 500 ng of the mismatch sequence (see “Experimental Procedures”). The binding activity of each transcription factor was normalized to that measured during normoxia (taken as 1). All assays were done in duplicate.
regulation between HIF-1α and HIF-2α is unknown since several recent reports indicate that there is no redundancy between HIF-1α and HIF-2α functions (22, 24, 40). However, the time-dependent trans-regulation mechanism between HIF-1α and HIF-2α observed in this study implicates different characteristics of regulation in the acute and prolonged phases of hypoxia. Our previous studies indicated that the hypoxia-sensitive GLUT1 and vascular endothelial growth factor genes are up-regulated during prolonged hypoxia (1, 4), suggesting that these genes are transactivated by HIF-2α rather than by HIF-1α. The importance of HIF-2α versus HIF-1α in the adaptation of alveolar epithelial cells to hypoxia and the trans-regulation of the two isoforms need to be further investigated by specifically inactivating HIF-1α and HIF-2α. These findings indicate that, although the oxygen-sensing mechanism involving oxygen-dependent hydroxylation of the HIF-α subunits is probably a universal mechanism in cells and highly conserved during evolution, additional regulatory steps appear to operate that determine which of the alternative subunits is induced. It is tempting to speculate that the differential expression of HIF-1α and HIF-2α during prolonged hypoxia conveys target specificity.

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**Prolonged Hypoxia Differentially Regulates Hypoxia-inducible Factor (HIF)-1α and HIF-2α Expression in Lung Epithelial Cells: IMPLICATION OF NATURAL ANTI-SENSE HIF-1α**

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