Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung

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Yu, Aimee Y., Maria G. Frid, Larissa A. Shimoda, Charles M. Wiener, Kurt Stenmark and Gregg L. Semenza. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L818–L826, 1998.—Hypoxia-inducible factor (HIF)-1 is a basic helix-loop-helix transcription factor that transactivates genes encoding proteins that participate in homeostatic responses to hypoxia. Several of these downstream gene products, such as erythropoietin, vascular endothelial growth factor, heme oxygenase-1, and inducible nitric oxide synthase, may contribute to the pathogenesis of pulmonary hypertension. Previous studies demonstrated increased HIF-1 mRNA levels in rats and mice subjected to hypoxia. In this study, we have demonstrated pulmonary expression of HIF-1 in vivo. HIF-1 protein induction was measured maximally when lungs were ventilated with 0 or 1% O2 for 4 h. On reoxygenation, HIF-1 protein expression was induced maximally when lungs were ventilated with 0 or 1% O2 for 4 h. Reoxygenation, HIF-1 was rapidly degraded, with a half-life of <1 min. These findings demonstrate that HIF-1 expression is tightly coupled to O2 concentration in vivo and are consistent with the involvement of HIF-1 in the physiological and pathophysiological responses to hypoxia in the lung.

pulmonary cells; gene transcription

MAINTENANCE OF HOMEOSTATIC MECHANISMS in response to hypoxia is essential for survival. The lung exhibits unique mechanisms for adaptation to conditions of low O2 tension. Acute exposure to hypoxia results in vasoconstriction of resistance-sized pulmonary arteries, increased pulmonary arterial pressure, and a redistribution of blood flow from the basal to the apical portion of the lung (13, 20, 52). These changes are designed to correct imbalances between ventilation and perfusion. Chronic hypoxia occurs in such diverse conditions as chronic obstructive pulmonary disease, interstitial fibrosis, hypoventilation syndromes, thromboembolic disease, cystic fibrosis, and residency at high altitude and can lead to maladaptive vascular remodeling of pulmonary arteries and, eventually, cor pulmonale resulting from chronic elevation of pulmonary arterial pressure. The histopathological hallmarks of chronic hypertensive changes include disruption of the internal elastic membrane, medial thickening of normally muscular arteries, extension of smooth muscle into peripheral arteries (neomuscularization), and reduction in artery number (37, 49).

Several factors may play a role in mediating the pathophysiological changes associated with chronic hypoxia. Studies (24, 32, 47) have demonstrated that polycythemia is a contributing factor to the development of pulmonary hypertension and cor pulmonale. Mitogens and vasoactive mediators have also been implicated in the process. Thromboxane, vascular endothelial growth factor (VEGF), platelet-derived growth factor B, endothelin-1, and angiotensin-converting enzyme mRNA and protein levels increase in chronic pulmonary hypertension (5, 7, 9, 31, 33, 44). Conversely, prostacyclin levels decrease (50). The role of nitric oxide is controversial because both increased and decreased levels have been found (1, 8, 19, 34). The regulatory mechanisms controlling expression of these factors have not been determined.

Hypoxia-inducible factor (HIF)-1, a transcription factor that is expressed in response to hypoxia and that activates expression of the genes involved in erythropoiesis, angiogenesis, glycolysis, and modulation of vascular tone, is a potential mediator of pulmonary responses to hypoxia. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits, which are both members of the basic helix-loop-helix-PAS family of proteins (53). HIF-1α is an 826-amino acid protein unique to HIF-1, whereas HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator, which can heterodimerize with several other proteins including the aryl hydrocarbon receptor (21) and HIF-2α (also known as EPAS1, HLF, HRF, and MOP2) (12, 15, 22, 48). HIF-1 was originally identified by its binding to the hypoxia response element located in the 3′-flanking region of the erythropoietin (EPO) gene (42). Expression of HIF-1 is tightly regulated by O2 availability because both HIF-1α and HIF-1 DNA binding activity increased exponentially when cultured cells were exposed to decreasing O2 concentrations (26). HIF-1α expression is the limiting factor for HIF-1 DNA binding activity and transcriptional activity in cultured cells (26, 40). HIF-1 also regulates the expression of genes encoding VEGF...
using both cultured cells and an isolated perfused lung multiple pulmonary cell types under hypoxic condi-
tions would be induced in the lung in response to acute or chronic hypoxia. Based on these results, we hypothesized that HIF-1 in the lung remain unclear.

Recent studies (35, 56) have demonstrated increased HIF-1α and HIF-1β mRNA levels in the lungs of mice exposed to acute or chronic hypoxia. Based on these results, we hypothesized that HIF-1α protein expression would be induced in the lung in response to acute hypoxia. Furthermore, based on the involvement of HIF-1 in a variety of homeostatic responses to hypoxia, we hypothesized that HIF-1 would be expressed in multiple pulmonary cell types under hypoxic conditions. In this study, we have tested these hypotheses using both cultured cells and an isolated perfused lung preparation.

MATERIALS AND METHODS

Animal protocols. All procedures were reviewed and approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine (Baltimore, MD). Adult male ferrets were anesthetized with pentobarbital sodium (30–40 mg/kg ip). The animals were intubated and ventilated with 16% O2 for 30 min, followed by 0, 1, 4, 7, 10, or 16% O2 for up to 8 h. All gas mixtures contained 5% CO2 and 0.4 mM PMSF, and 1 mM Na3VO4. The suspension was disrupted with a Dounce homogenizer in 10 mM Tris·HCl (pH 7.6), 1.5 mM MgCl2, 100 µg/ml of streptomycin. Hep3B cells were cultured as previously described (42). All cultures were maintained at 37°C in 5% CO2-95% air. Cells were exposed to 1% O2-5% CO2-balanced N2 in tightly sealed modular incubator chambers (Billups-Rothenberg, Del Mar, CA) for 6 h at 37°C.

Cell culture. Primary sheep pulmonary arterial endothelial (PAE) and aortic endothelial (AoE) cells (provided by S. Sanders, Johns Hopkins University) were maintained in medium 199 supplemented with 10% fetal bovine serum (FBS), 10% NuSerum IV (Collaborative Research, Bedford, MA), 50 U/ml of penicillin, 50 µg/ml of streptomycin, 25 µg/ml of fungizone (all three from Gibco BRL, Grand Island, NY), 2 mM l-glutamine, 2.5 µg/ml of thymidine, and 0.3% NaHCO3. Human bronchial epithelial (HBE) cells (provided by A. Choi, Johns Hopkins University) were cultured in MEM with Earle’s salts, 10% FBS, 2 mM glutamine, and 25 µg/ml of fungizone. Rat aortic smooth muscle (AoSM) cells (also provided by A. Choi) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 50 µg/ml of gentamicin. Human A549 alveolar type II epithelial cells (provided by D. Jacoby, Johns Hopkins University) were maintained in MEM, 10% FBS, 2 mM glutamine, and 25 µg/ml of fungizone. Mouse alveolar macrophages (MH-S; provided by P. Noble, Johns Hopkins University) were cultured in RPMI 1640 medium, 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Rat microvascular endothelial (MVE) cells (provided by U. Ryan, T Cell Sciences) were cultured as previously described (10). Primary human pulmonary arterial smooth muscle (PASM) cells (provided by J. Sham, Johns Hopkins University) obtained from Clonetics (Walkersville, MD) were cultured in Clonetics smooth muscle basal medium supplemented with 5% FBS, 4 ng/ml of epidermal growth factor (EGF), 2 ng/ml of fibroblast growth factor (FGF)-2, 4 µg/ml of insulin, 40 µg/ml of gentamicin, and 60 ng/ml of amphotericin B. Other human and rat PASM cells were isolated from intralobar pulmonary arteries with the method of Smirnov et al. (43) and cultured in Ham’s F-12 medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Hep3B cells were cultured at 37°C in 5% CO2-95% air. Cells were exposed to 1% O2-5% CO2-balanced N2 in tightly sealed modular incubator chambers (Billups-Rothenberg, Del Mar, CA) for 6 h at 37°C.

RNA extraction and analysis. Total RNA was isolated from lung tissue by acid guanidinium thiocyanate-phenol-chloroform extraction (6). Aliquots (15 µg) of RNA were fractionated by 1.4% agarose-2.2 M formaldehyde gel electrophoresis, transferred to a nylon membrane by capillary action, and cross-linked to the membrane by ultraviolet irradiation. The nylon membrane was incubated in Quik-Hyb (Stratagene, La Jolla, CA) containing 32P-labeled HIF-1α cDNA at 65°C for 2 h (16, 56). The blots were washed in 0.1× saline-sodium citrate (1× saline-sodium citrate is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)-0.1% SDS twice for 15 min at room temperature and twice for 10 min at 50–55°C. The blots were then exposed to film for autoradiography. To control for loading variation, the blots were stripped and rehybridized with a 32P-labeled oligonucleotide complementary to 18S rRNA (16). Autoradiographic signals were quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

Total cellular and nuclear protein extraction. Nuclear extracts were prepared as previously described (55). Cells were disrupted with a Dounce homogenizer in 10 mM Tris-HCl (pH 7.6), 1.5 mM MgCl2, 10 mM KCl, 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na3VO4. Nuclei were pelleted by centrifugation at 10,000 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 PMSF, 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
Fig. 1. Immunoblot analysis of hypoxia-inducible factor (HIF)-1 expression in cultured pulmonary cells. Aliquots (15 µg) of nuclear extracts prepared from nonhypoxic (-) and hypoxic (+) human Hep3B (lanes 1 and 2), human bronchial epithelial (HBE; lanes 3 and 4), mouse alveolar macrophage (MH-S; lanes 5 and 6), human alveolar epithelial (A549; lanes 7 and 8), sheep pulmonary arterial endothelial (PAE; lanes 9 and 10), rat microvascular endothelial (MVE; lanes 11 and 12), sheep aortic endothelial (AoE; lanes 13 and 14), human pulmonary arterial smooth muscle (PASM; lanes 15 and 16), and rat aortic smooth muscle (AoSM; lanes 17 and 18) cells were analyzed with anti-HIF-1α (top) and anti-HIF-1β (bottom) antibodies. Nos. at left, migration of molecular-mass markers (in KDa).
more of these growth factors induced expression of HIF-1α at 20% O2, we isolated human and rat primary cells, which proliferated independently of growth factors other than those present in 10% FBS, and exposed them to 20 or 1% O2 for 6 h. Immunoblot assays with the nuclear extracts of these cells were performed. Again, a high level of HIF-1α expression was evident in the nonhypoxic cells, and hypoxic exposure resulted in increased protein levels (Fig. 3A).

To further verify that the addition of EGF, FGF-2, and insulin did not alter basal or induced expression of HIF-1, both rat and human PASM cells, initially cultured in Ham's F-12 medium, were rinsed with PBS and then cultured in either Ham's F-12 medium with 10% FBS again or the supplemented Clonetics medium for 48 h. Immunoblot analysis revealed that HIF-1α expression was not altered by the presence of additional growth factors at either 1 or 20% O2 (Fig. 3A). Moreover, EMSA demonstrated that HIF-1 DNA binding activity in rat or human PASM cells was not affected by the use of growth factor-supplemented Clonetics medium instead of Ham's F-12 medium with 10% FBS (Fig. 3B). Therefore, the high HIF-1α expression at 20% and the further induction of protein at 1% O2 was not affected by the presence of additional growth factors in the medium.

Kinetics of HIF-1 protein expression in hypoxic lung. Compared with other species, the ferret exhibits a vigorous hypoxic response (36, 46). A blood-perfused ferret lung model was utilized to determine the temporal characteristics of HIF-1 expression in vivo. The integrity of the preparation could only be ensured for 8 h because after this period, normal vasoconstrictive responses to hypoxia began to decline. Ferret lungs were ventilated with 16% O2-5% CO2 for 0.5 h, followed by 0% O2-5% CO2 for 0, 1, 2, 4, 6, or 8 h. Total protein was extracted from freeze-clamped lung samples obtained at these time points, and immunoblot assays were performed with affinity-purified anti-HIF-1 antibodies (Fig. 4). HIF-1α protein was not detectable at 0 h, increased at 1–2 h, peaked at 4 h, and remained elevated thereafter. HIF-1β protein levels did not change significantly with exposure to hypoxia in vivo.

Kinetics of HIF-1α mRNA induction. To determine whether HIF-1α mRNA expression was regulated by O2 concentration, ferret lungs were ventilated with 0% O2-5% CO2 for 0, 1, 2, 4, 6, and 8 h, and total RNA was isolated. Northern blot hybridization analysis revealed a 3-fold increase in HIF-1α mRNA levels after 1 h of hypoxia, and a maximal 10-fold induction occurred at 2 h (Fig. 5). mRNA levels declined to basal levels after 8 h of continuous hypoxia.
Posthypoxic decay of HIF-1α protein. To characterize the posthypoxic decay of HIF-1α, ferret lungs were ventilated with 0% O₂ for 4 h and then reoxygenated with 16% O₂. Freeze-clamped tissue was obtained after 0, 1, 3, 5, 15, 30, and 60 min of reoxygenation and subjected to immunoblot assay (Fig. 6). After a return to 16% O₂, HIF-1α protein levels were 40% of maximum after 1 min, 15% after 3 min, and 8% after 5 min. In contrast, HIF-1β protein levels remained constant under all conditions.

HIF-1α expression with varying O₂ concentration. To analyze the O₂ concentration-dependent expression of HIF-1α protein, ferret lungs were ventilated with 16% O₂ for 0.5 h followed by 0, 1.3, 4, 7, 10, or 16% O₂ for 4 h. Immunoblot assay of freeze-clamped tissue revealed markedly increased HIF-1α protein levels after 4 h of ventilation with 0 and 1.3% O₂ (Fig. 7). Modest HIF-1α expression was detected in lungs ventilated with 4% O₂. No protein was detected in lungs ventilated with 7, 10, or 16% O₂. HIF-1β was detectable in all tissue samples and did not vary with O₂ concentration.

Analysis of HIF-1 expression by immunohistochemistry. To localize HIF-1 expression in vivo, ferret lungs were ventilated with 0 or 16% O₂ for 4 h. Biopsies were obtained and frozen for subsequent sectioning. Immunohistochemistry of lung sections with anti-HIF-1α antibodies revealed no detectable HIF-1α expression in normoxic ferret lungs compared with control sections incubated without primary antibodies (Fig. 8), consistent with the immunoblot assay. In contrast, HIF-1α protein expression was detected throughout the hypoxic lung, with the highest expression occurring in the bronchial epithelium, bronchial smooth muscle, alveolar epithelium, and vascular endothelium. A lower level of HIF-1β expression was detected in the nuclei of vascular smooth muscle cells and chondrocytes. Compared with control sections, HIF-1β protein was detected throughout the normoxic lung, with high expression in the bronchial epithelium, bronchial smooth muscle, and vascular endothelium. Analysis of the hypoxic lung revealed high HIF-1β levels in the bronchial epithelium, bronchial smooth muscle, and vascular endothelium. There was a modest increase in HIF-1β expression in the alveolar epithelium and in chondrocytes. These results demonstrate that HIF-1α expression is induced by hypoxia in multiple pulmonary cell types in vivo.
DISCUSSION

The lung serves as the portal for O₂ delivery to the body, and the physiological mechanisms it adopts in response to hypoxia regulate the quantity of O₂ that is delivered to other organs. HIF-1, an essential mediator of homeostatic responses to hypoxia (26), may play important roles in these physiological adaptations but, before this study, the spatial, temporal, and O₂-dependent expression of HIF-1 in the lung had not been determined. The results of this study extend the observations of Wiener et al. (56), who reported increased HIF-1α mRNA expression in the lungs of mice and rats acutely subjected to 7% O₂, and of Palmer et al. (35), who demonstrated increased HIF-1α mRNA levels in hypoxic bovine PAE cells and in the lungs of chronically hypoxic rats. We have demonstrated hypoxia-induced expression of HIF-1α protein and HIF-1 DNA binding activity in all cultured pulmonary cell types examined, including alveolar epithelial and macrophage, bronchial epithelial, microvascular endothelial, and PAE and smooth muscle cells. Immunohistochemical analysis of hypoxic ferret lungs revealed that the highest expression of HIF-1α was in alveolar and bronchial epithelium and vascular endothelium. These results do not exclude the possibility that HIF-1α is induced in other pulmonary cell types in vivo at lower, but still physiologically relevant, levels of expression.

Most of the cultured pulmonary cells examined exhibited minimal or no HIF-1α expression under nonhypoxic conditions. However, cultured PASM cells manifested high constitutive levels of HIF-1α protein and HIF-1 DNA binding activity. In contrast, AoSM cells derived from the systemic circulation exhibited minimal levels of HIF-1α protein and HIF-1 DNA binding activity at 20% O₂. Expression of HIF-1α was evident in both human and rat PASM cells at 20% O₂ and in cells maintained in either Ham’s F-12 medium with 10% FBS or a proprietary medium supplemented with 5% FBS, EGF, FGF-2, and insulin. Therefore, constitutive HIF-1α expression by PASM cells is not due to species differences or the presence of additional growth factors. To our knowledge, this is the first report of constitutive
HIF-1α expression in cultures of differentiated primary cells. Previous work (23, 25) has demonstrated high constitutive levels of HIF-1α protein in J1 embryonic stem cells as well as in v-Src-transformed rat fibroblasts. Because HIF-1 regulates the expression of genes encoding glycolytic enzymes and glucose transporters, the high basal HIF-1α protein levels in these cells may be adaptive for the increased metabolic requirements of tumor cells or the proliferating blastocyst. The full significance of HIF-1α expression in cultured PASM cells under nonhypoxic conditions remains to be determined. However, these findings suggest that a signal transduction pathway that is not hypoxia driven can upregulate HIF-1 expression in a cell type-specific manner.

In contrast to the analysis of cultured cells, immunohistochemical analysis of normoxic ferret lungs did not reveal detectable expression in PASM cells. This difference may be due to species variation because we did not examine cultured ferret PASM cells. Another potential explanation for this difference is that although isolated PASM cells constitutively express HIF-1α expression, these cells may be subjected to paracrine-negative regulatory mechanisms that preclude this expression in vivo. Alternatively, isolation of PASM cells may select for a small subpopulation of cells that constitutively express HIF-1α. There is mounting evidence indicating that the media of both systemic and pulmonary arteries is composed of heterogeneous subpopulations of PASM cells (3, 18). Frid et al. (17) isolated four distinct smooth muscle cell subpopulations from pulmonary arteries based on morphology, expression of muscle-specific markers, and proliferative properties. We may have assayed different PASM subtypes as well, based on their growth factor requirements. Unlike the rat and human PASM cells we isolated, the human PASM cells obtained from Clonetics required EGF, FGF-2, and insulin in the medium. Without these factors, the cells exhibited growth arrest and death (data not shown). Both human PASM primary cell cultures were spindle shaped and expressed α-smooth muscle actin (data not shown) and, on the basis of these characteristics, resemble the L2 and L3 subpopulations isolated by Frid et al. (17). Whether PASM subtypes that differ in their proliferative responses to hypoxia (17) differ in their expression of HIF-1α remains to be determined.

Expression of HIF-1α is dependent on O2 concentration and duration of the hypoxic stimulus. In ferret lungs ventilated with 0% O2, HIF-1α levels were maximal after 4 h. Induction of HIF-1α increased dramatically at O2 concentrations < 4%. In contrast, HIF-1β levels did not change with an increase in duration of hypoxia or a decrease in O2 concentration. Significant induction of HIF-1α, but not of HIF-1β, expression was also demonstrated in the hearts of anemic fetal sheep (29). HIF-1 levels are, therefore, determined by HIF-1α protein levels in vivo. These temporal characteristics and responses of HIF-1α protein expression in vivo to varying O2 concentrations were remarkably similar to those of tissue culture cells noted in previous studies (26, 53, 54). On termination of the hypoxic stimulus, HIF-1α protein levels decreased rapidly in Hep3B cells (53). In the reoxygenated ferret lungs, we found that the half-life of HIF-1 was < 1 min. The rapid degradation of HIF-1α in normoxia may be effected by ubiquitination of the protein and subsequent digestion by proteosomal enzymes (39). The extremely rapid degradation of HIF-1α under posthypoxic conditions provides further evidence that HIF-1α expression is tightly regulated by O2 concentration in vivo.

The physiological significance of HIF-1α expression in the lung remains to be established. HIF-1α expression was not detectable when ferrets were ventilated with O2 concentrations of 7% or higher, yet profound pulmonary hypertension develops in animals chronically exposed to 10% O2. These results suggest that either HIF-1 plays no role in the pathophysiology of pulmonary hypertension or the physiologically relevant induction of HIF-1α expression that occurs at 10% O2 is below the sensitivity of our assays. Recent studies support the latter interpretation. First, HIF-1α mRNA expression was modestly increased in lungs of rats exposed to 10% O2 for 3 wk (35). Second, whereas mice homozygous for a loss-of-function mutation at the Hif1a locus encoding HIF-1α die in midgestation due to cardiovascular malformations, heterozygous mice are viable and manifest no gross abnormalities (23). However, compared with wild-type mice, the heterozygotes manifest significantly lower right ventricular pressures and a lesser degree of right ventricular hypertrophy when exposed to 10% O2 for 3 wk (57). Thus the expression of HIF-1α in hypoxic pulmonary cells, as demonstrated in this study, is likely to have considerable physiological and clinical relevance.

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