Pulmonary Perspective

HIF and the Lung

Role of Hypoxia-inducible Factors in Pulmonary Development and Disease

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The ability to sense and respond to changes in oxygen concentration is a fundamental requirement for the survival of all organisms. Throughout evolution, control of oxygen homeostasis has developed into a complex system, requiring both rapid adjustment to acute changes in oxygen and durable adaptation when the hypoxic stimulus is prolonged. There are numerous instances, in both physiologic and pathophysiologic conditions, during which the lung experiences localized or global hypoxia. It has become increasingly appreciated that adaptation to hypoxia requires the coordinated regulation of a large battery of genes, and that this collective response is controlled, to a large extent, at the level of transcription. In particular, the hypoxia-inducible factors (HIFs), have been identified as key mediators of adaptation to hypoxia. In this review, we will describe the HIF system and its role in a variety of developmental, physiologic, and pathogenic processes within the lung.

THE HIF SYSTEM

Originally identified as a protein that bound, under hypoxic conditions, to the hypoxia response element of the *EPO* gene (which encodes erythropoietin, the hormone controlling red blood cell production) (1), hypoxia-inducible factor 1 (HIF-1) is a highly conserved transcription factor that is now known to be present in almost all cell types, is tightly regulated by O₂ availability, and regulates the expression of hundreds of genes. HIF-1 exists as a heterodimer, consisting of HIF-1 α and HIF-1 β subunits. HIF-1 β is ubiquitously expressed, whereas HIF-1 α is found at very low levels under normoxic conditions. In mouse lung, HIF-1 α mRNA levels increase within 30 minutes of exposure to 7% O₂ (2). Under normoxic conditions, HIF-1 α protein is ubiquitinated and subjected to proteasomal degradation; however, acute exposure of pulmonary arterial smooth muscle cells (PASMCs) or endothelial cells (ECs) to hypoxia (1% O₂) causes increased HIF-1 α protein levels and HIF-1 DNA-binding activity (3). Thus, HIF-1 α confers sensitivity and specificity for hypoxic induction of HIF-1 transcriptional activity.

The mechanism by which hypoxia is transduced into an increase in HIF-1 activity (and, consequently, induction of hypoxia-inducible gene expression) was unclear until the discovery that HIF-1 α ubiquitination required hydroxylation at two proline residues, which are Pro-402 and Pro-564 in human HIF-1 α (4–7). Under normoxic conditions, hydroxylation of HIF-1 α is catalyzed by prolyl hydroxylase domain proteins (PHDs) using molecular O₂ as a substrate (4, 5, 8). To date, four PHD isoforms have been identified, although only PHD1–3 appear to hydroxylate HIF-1, with evidence suggesting that PHD2 is the primary isoform responsible for HIF-1 α hydroxylation *in vivo* (9–11).

At reduced O₂ concentrations, PHD activity decreases (Figure 1). Consequently, HIF-1 α hydroxylation at the proline residues decreases, resulting in protein stabilization. HIF-1 α then translocates into the nucleus, where it binds HIF-1 β and recruits coactivator proteins to the HIF binding site within the hypoxia response element, activating the transcription of various target genes. Increased HIF-1 α protein generally correlates with increased transcriptional activity, although HIF-1 transactivation is regulated by hydroxylation of HIF-1 α at an asparagine residue within the C-terminal transactivation domain via factor inhibiting HIF-1 (FIH-1), which blocks the binding of the transcriptional co-activators CBP and p300 (12). Since PHD and FIH-1 activity require O₂, hypoxia reduces the activity of both enzymes, leading to HIF-1 α stabilization and transactivation of HIF-1 target genes.

Several years after the discovery of HIF-1 α , a closely related protein was identified based on its sequence similarity and subsequently named HIF-2 α (13, 14). Like HIF-1 α , HIF-2 α is subjected to the same PHD-dependent degradation machinery, and dimerizes with HIF-1 β ; however, unlike HIF-1 α , which is found in all nucleated cells, HIF-2 α exhibits a much more restricted pattern of expression.

ROLE OF HIFS IN LUNG DEVELOPMENT

Given that development occurs in a hypoxic environment, it is not surprising that HIFs would be important contributors to embryogenesis. Indeed, HIF mRNA and protein levels are quite high in the fetal lung (15–17), with the entire HIF system in place as early as 8 weeks of gestation in the human (15). HIF-1 α protein expression is induced by hypoxia in cultured cells derived from all cell types found in the adult lung (3), whereas HIF-2 α protein expression is restricted to the vascular endothelium and type II pneumocytes (18). During fetal development, the lung of the normal embryo is hypoxic (19), and analysis of the spatial pattern of expression in human lungs during initial development of the pulmonary vasculature revealed high levels of HIF-1 α protein localized predominantly in branching epithelium (15). HIF-2 α protein was also present in epithelium, as well as in mesenchymal structures, which give rise to the vascular endothelium (15). Given the distinct expression pattern observed in the developing lung, HIF-1 α and HIF-2 α may serve different, and specific, functions in epithelial and vascular morphogenesis. This possibility is supported by results from studies examining HIF-1 α and HIF-2 α loss-of-function models. Genetic deletion of HIF-1 α or HIF-1 β results in fetal death at approximately Embryonic Day 10, with severe cardiovascular malformations (20–24). In contrast, loss of HIF-2 α leads to fetal death in approximately 50% of embryos, with the remaining offspring exhibiting impaired lung development, reduced production of surfactant, postnatal respiratory distress, and neonatal lethality (23, 25). Although homozygous deletion

of HIF-1 α or HIF-2 α is lethal, animals heterozygous for either factor develop normally and survive to adulthood, with the lung architecture and function appearing outwardly normal under normoxic conditions.

In the embryonic lung of sheep and primates, levels of both HIF-1 α and HIF-2 α protein levels are high in the third trimester, although HIF-1 α protein levels quickly decrease upon delivery (16, 17). HIF-1 α and HIF-2 α protein levels are also markedly reduced in the lungs of mechanically ventilated preterm animals (16, 17), where the decline in HIF levels may have severe consequences for lung development, leading to vascular and alveolar hypoplasia, neonatal respiratory distress, and bronchopulmonary dysplasia. Consistent with this possibility, *in vivo* use of PHD inhibitors to increase HIF protein levels improved lung growth and function in a baboon model of prematurity (26, 27). Moreover, *in vitro* assays demonstrated that induction of HIF-1 protein in fetal lung buds was sufficient to induce lung development even under non-hypoxic conditions (28), whereas depletion of HIF-1 α using antisense oligonucleotides reduced lung branching morphogenesis and vascularization (29). These results indicate that the HIF system plays a critical role in pulmonary development, and suggest the possibility that therapies aimed at increasing HIF levels might enhance lung angiogenesis and may be promising candidates for the treatment of bronchopulmonary dysplasia.

ROLE OF HIFs IN PULMONARY HYPERTENSION

Prolonged exposure to alveolar hypoxia, due to chronic lung disease or residence at high altitude, is a significant cause of pulmonary hypertension (World Health Organization Class 3). The role of HIF in the development of pulmonary hypertension associated with hypoxemia has been explored using murine models, where partial deficiency of either HIF-1 α (*Hif1a*^{+/-} mice) or HIF-2 α (*Hif2a*^{+/-} mice) markedly attenuated the increase in pulmonary arterial pressure and right ventricular

hypertrophy that are induced by chronic hypoxic exposure of wild-type mice (25, 30).

The reduction in pulmonary hypertension was due, at least in part, to the reduced pulmonary vascular remodeling observed in these animals (25, 30). The components of hypoxia-induced pulmonary vascular remodeling include PASMC proliferation, migration, and hypertrophy. While the effects of HIF deficiency on pulmonary vascular smooth muscle migration and proliferation during hypoxic exposure have not been reported, electrophysiologic measurements revealed that PASMCs from wild-type (*Hif1a*^{+/+}) mice exposed to 10% O₂ for 3 weeks exhibited an increase in cell capacitance, which is a measure of cell size (31). In contrast, PASMCs isolated from normoxic and chronically hypoxic *Hif1a*^{+/-} mice were not different, suggesting that HIF-1-dependent smooth muscle cell hypertrophy contributes to pulmonary vascular remodeling during hypoxia. In *Hif1a*^{+/+} mice, exposure to chronic hypoxia is associated with PASMC depolarization, reductions in K⁺ channel expression and activity, and elevated intracellular calcium concentration and pH, due to up-regulation of transient potential receptor proteins and Na⁺/H⁺ exchanger isoform 1, respectively (31–34). These alterations in PASMC ion homeostasis, which are associated with a more contractile, apoptosis-resistant, proliferative, and migratory phenotype, were significantly attenuated or absent in PASMCs from chronically hypoxic *Hif1a*^{+/-} mice (31–34). Whether reduction in HIF-2 α results in similar alterations in PASMC responses to hypoxia is presently unknown.

While it is easy to understand how HIF would be involved in the pathogenesis of hypoxic pulmonary hypertension, it is possible that HIFs also play a role in other forms of pulmonary hypertension that are not due to alveolar hypoxia. For example, HIF-1 α levels are up-regulated in the Fawn-hooded rat, a genetic model of pulmonary arterial hypertension that exhibits several characteristics of the human disease, including reduced K⁺ channel expression, elevated intracellular calcium levels, and excessive PASMC proliferation even under normoxic conditions

Figure 1. Regulation of HIF-1 by hypoxia. Under normal conditions, hydroxylation of HIF-1 α by prolyl hydroxylase domain proteins (PHD), using molecular oxygen, leads to interaction with Von Hippel-Lindau (VHL) and addition of ubiquitin (Ub), targeting HIF-1 α for proteosomal degradation. When oxygen levels fall, HIF-1 α is not targeted for degradation, and translocates to the nucleus where it binds with HIF-1 β and recruits co-activators at the hypoxia response element (HRE) to initiate gene transcription.

(35). Furthermore, immunohistochemical examination of tissues and cells from patients with non-hypoxia-associated pulmonary arterial hypertension also revealed elevated expression of HIF-1 α (36). Although the mechanism by which HIF levels are elevated under these conditions is still incompletely understood, these studies would suggest that therapies aimed at reducing HIF-1 or HIF-2 levels and/or activity have clinical potential, even in cases of pulmonary hypertension not associated with hypoxemia.

ROLE OF HIFs IN LUNG INJURY

Hypoxia may be a consequence of acute lung injury, leading to aberrations in lung function and repair. Early events in acute lung injury include damage of the alveolar lining layer, apoptosis of alveolar epithelial cells, and lung edema, whereas at later phases, reactive hyperplasia of alveolar type II cells is predominant, leading to fibrosis. During acute lung injury, increased vascular permeability can result from hypoxia, ischemia, and/or inflammatory stimuli. The role of the HIF system in modulating pulmonary vascular leak is still unresolved, with only a handful of published studies. In a hypoxic ischemia/reperfusion model, up-regulation of HIF-1 protein levels was associated with increased vascular endothelial growth factor (VEGF) levels and augmented barrier disruption (37), whereas another study reported HIF-1-dependent down-regulation of adenosine kinase, the enzyme that converts adenosine to AMP, which attenuated hypoxia-induced pulmonary vascular leak (38). While neither study directly tested whether loss of HIF-1 altered the observed changes in vascular permeability, taken at face value these results imply that HIF-1 may play both barrier-protective and barrier-disruptive roles. With respect to epithelial cell damage and subsequent fibrotic lung disease, hypoxia has been reported to induce alveolar type II cell apoptosis via HIF-1 α (39). Furthermore, up-regulation of HIF-1 by inflammatory levels of NO may lead to suppression of epithelial cell wound repair (40), suggesting that increased HIF-1 levels may render the injured, hypoxic lung less able to mount an appropriate healing response after epithelial injury. Recently, epithelial-mesenchymal transition (EMT) has been proposed to contribute to pulmonary fibrosis in patients with acute lung injury (41). Emerging evidence indicates that this process is under the control of hypoxia-induced increases in mitochondrial-derived reactive oxygen species (ROS) (42), which serve to stabilize HIF-1 α in several cell types, including alveolar epithelial cells (43). While these studies provide circumstantial evidence for the involvement of HIF in the development of acute lung injury, direct testing of the role of HIF has only been performed in models of lung injury due to splanchnic ischemia-reperfusion and intestinal inflammation. In

rodent models of traumatic hemorrhagic shock due to gut ischemia-reperfusion, there is a rapid and prolonged increase in nuclear HIF-1 α protein levels (44), with mice partially deficient for HIF-1 α exhibiting attenuated intestinal mucosal damage, bacterial translocation, and subsequent lung injury (45). Further experiments testing the effect of HIF-1 α or HIF-2 α loss-of-function in other models of lung injury will be required to delineate the exact role of HIF in acute lung injury and evaluate the suitability of therapies aimed at modulating HIF levels in this context.

ROLE OF HIFs IN LUNG CANCER

As solid tumors expand, lack of blood supply renders areas of the tumor hypoxic. Since hypoxia-induced angiogenesis is a requirement for tumor growth, hypoxia is both a consequence of, and contributor to, tumorigenesis. The degree of hypoxia within a tumor is associated with increased expression of angiogenic factors (46) and more aggressive tumors (47–49). Given the positive association between hypoxia and tumorigenesis, it is not surprising that HIF-1 α protein expression is correlated with more aggressive and radiation-resistant tumor cells (50), while inhibition of HIF by treatment with digoxin or acriflavine prevents tumor xenograft growth and vascularization in mice (51, 52). With respect to the lung, both small cell and non-small cell lung cancers exhibit high levels of HIF-1 α and HIF-2 α , both of which are associated with poor prognosis (46, 53–56). Overexpressing HIF-2 α leads to increased tumor size, invasion, and angiogenesis in the inducible *LSL-Kras^{G12D}* murine model of non-small cell lung cancer (57), resulting in decreased survival. Surprisingly, deletion of HIF-2 α in this model resulted in increased tumorigenesis (58), whereas in the A549 lung carcinoma cell line and in drug-resistant non-small cell lung cancer cells, silencing HIF-2 α prevented tumor growth (59). A small molecule inhibitor of HIF-1 α , PX-478, demonstrated effectiveness against tumor growth in an orthotopic mouse model of human lung cancer (54); however, in mice injected with A549 cells, silencing HIF-1 α impaired tumor vascularization and increased the necrotic area, but did not reduce tumor cell proliferation and only slightly impacted tumor growth (59). A similar lack of effect of HIF-1 α silencing on tumor growth was observed in *LSL-Kras^{G12D}* mice (58). In contrast, reduction of HIF-1 α levels markedly impaired metastasis in murine models of human lung and mammary cancer (54, 60). While these studies might imply a more prominent role for HIF-2 α than HIF-1 α in the growth of certain lung cancer tumors, the divergent effects of HIF-1 α and HIF-2 α silencing in the aforementioned studies suggest a complex regulation of tumor

Figure 2. Illustration of the role of HIF in various lung processes. E-M = endothelial-mesenchymal; SMC = smooth muscle cell; ROS = reactive oxygen species.

growth and invasion that is not controlled simply by the absence or presence of HIF-1 or HIF-2, but rather may be dependent on the absolute levels of HIF and/or the specific tumor cells involved. Nonetheless, addition of HIF inhibitors to current cancer therapies may prove beneficial in controlling tumor progression and/or metastasis.

CONCLUSIONS

Since its discovery nearly 20 years ago, HIF has emerged as a central component of a myriad of O₂-dependent physiologic and pathophysiologic processes and is now recognized as a master regulator of O₂ homeostasis. Understanding the precise role of the HIF system in lung development and in lung diseases such as pulmonary hypertension, acute lung injury, and cancer (Figure 2), and the identification and/or creation of tools to manipulate HIF levels *in vivo*, hold promise for better therapeutic options to treat lung dysfunction in both the neonate and adult.

Author Disclosure: L.A.S. has received sponsored grants from the National Institutes of Health (over \$100,000), and AHA (\$50,001–\$100,000). G.L.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: Work in the authors' laboratories is supported by Public Health Service grants from AHA, NCI, NHLBI, and NIGMS and by the Johns Hopkins Institute for Cell Engineering. G.L.S. is the C. Michael Armstrong Professor at The Johns Hopkins University School of Medicine.

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