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Hypoxia signaling during acute lung injury

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ACUTE LUNG INJURY (ALI) was first described in 1967 as “adult respiratory distress syndrome” (ARDS) because of the perception that ARDS is the “adult” version of the respiratory distress syndrome known in premature infants suffering from surfactant deficiency (5). Since then, the clinical definition of ALI has undergone several modifications and its name has subsequently been changed to “acute respiratory distress syndrome” (ARDS), reflecting the notion that ARDS can affect adults and children and is not necessarily related to surfactant deficiency (4). According to the modified definition of the Berlin Consensus conference in 2011, ARDS consists of acute onset of mild to severe hypoxemia, pulmonary edema not explained entirely by fluid overload or cardiac disease, and radiographic findings of bilateral opacities (53) (Fig. 1). The term “ALI,” which was used in the American-European Consensus Conference on ARDS in 1994 (7), now mainly refers to ALI in an experimental setting, as the complete Berlin definition is not fulfilled in all experimental animal models (48). From a pathophysiological perspective, it could be argued that it is somewhat surprising that the lungs become edematous and inflamed, where in untreated patients ventilation would be minimal, pulmonary blood flow may be reduced by hypoxic pulmonary vasoconstriction, while microthrombi attenuate metabolite supply and oxygen consumption is increased because of cellular metabolism. Data about lactate differences across the lung would support the notion that metabolism is increased (17, 40, 54). Importantly, breakdown in the alveolar-capillary barrier and alveolar epithelial injury play a critical role in initiating this type of injury (71).

ARDS is among the leading causes of death in critically ill patients (33). With an incidence of approximately 200,000 patients annually in the United States alone, ARDS carries a significant disease burden in regard to both morbidity and mortality (31, 55, 63). Furthermore, studies have revealed significant long-term disabilities in ARDS survivors, such as decreased exercise tolerance and increased utilization of health care services and associated escalation of health care costs (37). Despite an improved understanding of ARDS pathogenesis (35), current therapeutic interventions such as low-tidal volume ventilation (1) or prone positioning (34) are mainly supportive or aim toward avoiding additional injury of the lungs by mechanical ventilation. The fact that currently no specific treatment options are available to ARDS patients is thought to account for the plateauing of mortality since the mid-1990s despite considerable efforts (6, 31). Moreover, because of the ongoing threat of new influenza pandemics, ARDS could have an even greater impact on public health in the years to come (69).

The lungs are among the best “oxygenated” organs in the human body, as they receive oxygen supply by three independent pathways: 1) systemic well-saturated blood from the
Hypoxia-Inducible Transcription Factors

HIFs comprise a set of transcription factors that are activated during normoxic conditions but become transcriptionally active when oxygen levels fall (13, 67). For example, the transcription factor HIF1A is targeted for proteasomal degradation by a set of oxygen-sensing prolyl hydroxylases (PHDs) (26, 27). Hydroxylation of a conserved prolyl residue within the HIF1A oxygen-dependent degradation domain causes polyubiquitination and subsequent proteasomal degradation of HIF1A (41). Because of the fact that oxygen is required as a cofactor for PHD-mediated hydroxylation of HIF1A, hypoxia causes a functional inhibition of PHDs, leading to stabilization of HIF1A. Once stabilized, HIF1A forms a heterodimer with HIF1B, binds to coactivators, and attaches to the promoter region of hypoxia-responsive genes. In most instances, binding of HIF to a promoter that contains a “hypoxia-response element” (HRE) causes transcriptional alteration of the gene product (72). In most instances, binding of HIFs to an HRE results in the transcriptional induction of the gene product. Famous examples include HIF-driven induction of erythropoietin (EPO) or vascular endothelial growth factor (VEGF) (27). However, there are also examples described where binding of HIFs to an HRE causes inhibition of translation of the gene product (24, 49). Alternatively, HIFs can also function to inhibit the induction of a specific gene product by the transcriptional induction of inhibitory microRNAs (29). The number of estimated HIF target genes keeps growing rapidly. For example, a microarray study in hypoxic vascular endothelial cells indicates that a minimum of 570 (2.6%) of 22,283 of all gene probes are regulated by hypoxia in a HIF-1-dependent manner. Interestingly, this study identified 245 gene probes with increased expression and 325 gene probes with decreased expression based on HIF transcriptional activity during hypoxia (47). Among the gene products that have been identified as HIF target genes are well-known proteins that are induced during conditions of hypoxia, such as EPO or VEGF.

From a pharmacological perspective, modifying the PHD-HIF pathway has been an intriguing target for many years (26). For example, studies from the cancer field have suggested that inhibiting HIF stabilization or HIF transcriptional activity could be an important target for preventing tumor growth or metastasis (58, 61). Other studies indicate an anti-inflammatory role of the PHD-HIF pathway during intestinal inflammation and provide evidence that HIF activators can be used in the treatment of inflammatory bowel disease or ischemia and reperfusion injury of different organs (12, 16, 28, 29, 66). Moreover, experimental evidence implicates a functional role of HIFs in attenuating neutrophil apoptosis (70) and in improving bacterial killing (14). Again, other studies provide evidence that transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion, concomitant with stabilization of HIFs via “imprinting” on intestinal epithelial cells, thereby promoting the resolution of intestinal inflammation (9). Several pharmacological compounds have been shown to function as HIF activators. Most of them function through a pharmacological mechanism involving inhibition of PHDs. For example, some of these compounds have already been tested in patients; FG-2216, an orally active PHD inhibitor, was found to stabilize HIF1A in patients with end-stage renal disease and resulted in increased EPO levels independently from the presence of hypoxia (8, 26). Because of concerns with regard to the safety of FG-2216—one patient treated with this compound reportedly developed fatal hepatic necrosis—this compound was discontinued (46). However, a second-generation PHD inhibitor from FibroGen (FG-4592) is currently being examined in phase III clinical trials (26, 32).

In the present review, we discuss evidence that supports the use of HIF activators in the treatment of ALI.

Inflammatory Lung Injury Is Exacerbated by Hyperoxygenation

Important experimental studies provide an indirect indication for a functional role of HIFs in ALI by examining mice during endotoxin-induced lung injury (68). In these studies, the authors subjected mice to inhalation of combined toxins from Gram-positive and Gram-negative bacteria as a model of polymicrobial lung infection where lipopolysaccharide (LPS) and staphylococcal enterotoxin B strongly potentiate their toxicities (68). To test the impact of oxygenation on the outcome of ALI, mice were subsequently maintained at different oxygen concentrations (Fig. 2). Intriguingly, the authors found that five times more mice with inflamed lungs died after exposure to 100% oxygen than those left at 21% ambient oxygen tension. These studies suggest that hyperoxygenation—such as is frequently encountered in intensive care settings—potentiates lung injury during endotoxin-induced lung injury (68).
siRNA approaches to knock down the important steps in thereby implying "normoxic" HIF stabilization. Utilizing natant of stretch cells revealed normal oxygen concentrations, interestingly, measurements of the oxygen content in the supernatant during stretch conditions of alveolar epithelial cells (18). In-
to pro- ceeding their studies to show that lower levels of oxygen, and concomitant activation of hypoxia-dependent signaling pathways, dampen lung inflammation (68). Subsequent studies confirmed a detri-
mental role of oxygenation in other models of ALI (2, 50). Together, such studies highlight an anti-inflammatory role of hypoxia-dependent signaling pathways during ALI and have introduced the idea of utilizing either HIF activators or activa-
tors of HIF target genes in lung protection.

Functional Role of HIF1A in Acute Lung Injury

The above studies show that mice kept at higher oxygen levels during inflammatory lung injury are prone to increased mortality levels (68). While the authors elegantly demonstrate a causal relationship between their findings and an alteration of the hypoxia-elicited adenosine signaling pathway, these find-
ings could also be related to alterations in the production of reactive oxygen species (ROS). For example, it is conceivable that higher levels of ROS due to more substrate availability could contribute to the detrimental effects of hyperoxegenation in this model. However, a subsequent study performed ALI experiments in genetic models for HIF and found that HIF deletion was associated with elevated ROS levels, thereby directly implicating HIFs in lung protection during ALI (see below) (18).

Stretch injury of pulmonary epithelial cells. In this study, cyclic mechanical stretch of pulmonary epithelial cells was used as a model to study ALI in vitro. Indeed, plating pulmonary epithelia or alveolar epithelial cells on a rubber membrane and subsequently exposing them to conditions of cyclic me-
chanical stretch represents an in vitro approach to study basic mechanisms of injurious mechanical ventilation. A microarray performed in pulmonary epithelial cells exposed to cyclic mechanical stretch conditions showed that a large number of known HIF target genes were transcriptionally induced (18). Similarly, primary alveolar epithelial cells exposed to stretch showed time- and stretch dose-dependent HIF1A stabilization. Together, these studies indicate that HIF1A can be stabilized during stretch conditions of alveolar epithelial cells (18). Interes-
tingly, measurements of the oxygen content in the supernatant of stretch cells revealed normal oxygen concentrations, thereby implying "normoxic" HIF stabilization. Utilizing siRNA approaches to knock down the important steps in cellular succinate generation, the authors were able to imply stretch-induced repression of succinate dehydrogenase (SDH) and concomitant elevations of succinate levels (a previously described PHD inhibitor) in normoxic stabilization of HIFs during cyclic mechanical stretch (see also Mechanism of HIF Stabilization During ALI).

Ventilator-induced lung injury. Subsequently, the authors continued their studies in an in vivo model of ALI induced by injurious mechanical ventilation (Fig. 3) (19, 20). For example, they exposed previously described HIF-reporter mice to ALI (57) and observed HIF stabilization after injurious mechanical ventilation of the lungs (18). While pharmacological approaches to inhibit the transcriptional activity of HIFs were associated with increased susceptibility to lung injury, mice that were pretreated with a HIF activator were protected during ALI (18). Because mice with homozygote deletion of Hif1a die during embryogenesis (10, 56, 73), the authors utilized trans-
genic mice with a “floxed” Hif1a allele to perform genetic studies on the functional role of HIF1A during ALI. For this purpose, they took advantage of the so-called “Cre-lox mecha-

Fig. 2. Moderate hypoxia improves outcome in polymicrobial ALI. Animals were treated with intratracheal lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB) to mimic polymicrobial lung injury. High oxygen concentrations led to decreased survival within 48–60 h after intratracheal injection of toxins as hypoxia suppresses adenosine A2A receptor-mediated activation of anti-inflammatory pathways.

Fig. 3. Experimental setup for a murine model of ventilator-induced lung injury. Anesthetized animals are intubated or tracheotomized and then connected to the ventilator. ECG, SpO2, and temperature can be monitored continuously. Additionally, a catheter is placed in the carotid artery for blood pressure and blood gas monitoring. Ventilator-induced lung injury is induced by pressure-controlled ventilation (PCV) with a pmax of 45 cmH2O for various time periods.
of the lungs implicated alveolar epithelial HIF1A signaling in lung protection during ALI. Together, these genetic and pharmacological studies implicate HIF1A in dampening lung inflammation and pulmonary edema in ALI induced by mechanical ventilation (18).

**Mechanism of HIF Stabilization During ALI**

Several studies have examined different mechanisms of how HIFs can be stabilized during mucosal inflammation. One important mechanism implicated in HIF stabilization during inflammation or infection is referred to as “inflammatory hypoxia.” As such, because of the increasing demand for oxygen in inflamed, activated tissues, shifts in metabolic supply and demand ratios account for inflamed tissues to become severely hypoxic. Several factors contribute to such changes in supply and demand of metabolites. On one hand, metabolic demand in an inflamed tissue is dramatically increased. Such increases in demand can involve both resident tissues (such as the epithelial surface lining the mucosa) and invading inflammatory cells. At the same time, metabolic supply from the bloodstream is diminished because of vascular thrombosis or compression (12). For instance, studies utilizing nitroimidazole compounds for staining hypoxic conditions in vivo (e.g., during mucosal inflammation) reveal profound tissue hypoxia once an inflammatory disease has established itself (42). Inflammatory hypoxia is thought to occur because of increased oxygen demands during inflammation and simultaneously occurring decreased oxygen supply—for example, due to vascular compression of inflammation. For example, inflammatory cells—such as neutrophils—can consume prodigious amounts of oxygen, thereby significantly contributing to inflammatory hypoxia and subsequent HIF stabilization (9). As such, transmigrating neutrophils are thought to shape the mucosal microenvironment through localized depletion of oxygen, subsequent stabilization of HIFs, and concomitant promotion of the resolution of inflammation (9). Other studies have shown that LPS increases HIF1A levels in a TLR4-dependent fashion (52). Further studies have demonstrated that HIFs can be stabilized during bacterial infections via the bacterial release of siderophores, which promote local depletion of the mucosal microenvironment of iron—an essential cofactor for PHDs (36).

It is likely that during conditions of ALI several factors contribute to pulmonary HIF stabilization, including shifts in oxygen supply and demand (“inflammatory hypoxia”), inflammatory-cell-driven oxygen depletion, and HIF stabilization through inflammatory mediators such as LPS or other bacterial compounds. However, because the lungs are more highly oxygenated than other organs, some studies have examined alternative pathways of hypoxia-independent HIF stabilization during ALI (18). Importantly, previous studies from the cancer field had suggested that normoxic stabilization of HIFs in cancers involves the inhibition or mutation of the metabolic enzyme SDH (43, 44, 60). Concomitant elevations in succinate levels can subsequently function as PHD inhibitors and thereby promote HIF stabilization. Therefore, mutations of SDH can lead to an inhibition of PHDs and subsequent normoxic stabilization of HIFs (43, 44, 60). Consistent with these studies, recent studies in models of ALI suggest that stretch conditions—such as occur during ALI induced by mechanical ventilation—are associated with decreased SDH activity. These studies indicate that a contributing factor to hypoxia-independent HIF stabilization during ALI could be stress-driven SDH inhibition, increased succinate levels, PHD inhibition, and concomitant normoxic HIF stabilization (18).

**HIF Target Genes Implicated in Lung Protection**

In light of studies showing a protective effect of HIFs in ALI, it became an important question to identify the mechanism of HIF-dependent lung protection, including the identification of HIF target genes that are responsible for its anti-inflammatory roles. Several studies that had identified detrimental effects of oxygenation during ALI implicate an alteration of purinergic signaling events. Purinergic signaling events involve the activation of ATP/ADP and adenosine receptors. For example, adenosine is generated in the extracellular compartment as a breakdown product of ATP or ADP (30, 38, 39). This pathway is under the enzymatic control of CD39 (conversion of ATP/ADP to AMP) and CD73 (conversion of AMP to adenosine). Adenosine signaling events involving the Adora2a and Adora2b adenosine receptors have been implicated in mediating lung protection in different models of ALI (15, 21, 22, 51, 59, 68). Interestingly, this pathway is under the control of hypoxia signaling, where hypoxia and concomitant HIF stabilization promote the extracellular conversion of ATP/ADP to adenosine. Moreover, signaling events through the Adora2a and Adora2b adenosine receptors are increased by HIFs (25, 27, 29). Importantly, the pacemaker enzyme for extracellular adenosine generation, CD73, and the Adora2a and Adora2b adenosine receptors are direct HIF target genes, where binding of HIF to a HRE contained within the promoter of these genes drives their transcription during conditions of hypoxia (3, 45, 59).
65). While CD73 and the Adora2b adenosine receptor have been shown to be driven by HIF1α, the Adora2a adenosine receptor appears to be a HIF2A target gene (3, 45, 65). Studies on the detrimental effects of high oxygen concentrations during ALI indicate a functional role of attenuated Adora2a signaling, as the detrimental effects of oxygenation can be overcome by utilizing a selective Adora2a agonist (68). Other studies implicate attenuated Adaroa2a signaling on macrophages in mediating oxygenation-driven exacerbation of ALI (2). Other studies implicate purinergic signaling events on natural killer T cells as essential in mediating hyperoxic ALI (50). Interestingly, a recent study demonstrates that Adora2b is induced via HIF during conditions of cyclic mechanical stretch or during injurious mechanical ventilation (23). Together, these studies indicate that hypoxia signaling may enhance the extracellular generation of adenosine and concomitant adenosine signaling events as a mechanism for lung protection provided by hypoxia signaling.

Other studies provide evidence for a direct role of HIFs in promoting alveolar epithelial carbohydrate metabolism. HIFs are known to transcriptionally induce multiple enzymes that are part of the glycolytic pathway (62). Indeed, alveolar epithelial cells are known to be highly dependent on their capacity to perform glycolysis. Studies following the injection of labeled glucose and subsequent measurements of glucose metabolites indicate that conditions of ALI are associated with increased pulmonary glycolysis. Interestingly, this response is completely abolished in mice with targeted deletion of Hif1α in alveolar epithelial cells, in conjunction with increased lung inflammation and pulmonary edema (18). Similarly, pharmacological inhibition of glycolysis is associated with exacerbated lung disease during ALI (18). Interestingly, extensions of these studies also implicate HIFs in optimizing mitochondrial respiration and ATP production (18). Taken together, these studies indicate a functional role of HIF1α in optimizing carbohydrate metabolism in alveolar epithelial cells during ALI, and thereby providing lung protection (Fig. 4).

Conclusions

Several studies have shown that during conditions of lung injury HIFs are stabilized and can contribute to lung protection during ALI. While some studies implicate purinergic signaling events in the lung protection provided by HIFs, other studies suggest that transcriptional optimization of alveolar epithelial carbohydrate metabolism could be critical for lung protection. While pharmacological HIF activators have been tested in patients—for example, for the treatment of renal anemia (26)—studies of HIF activators during ALI have not been performed in a clinical setting. While the preclinical data clearly demonstrate a beneficial effect of HIF activators during ventilator-induced ALI, systemic effects of HIF activators in patients suffering from ARDS and sepsis could be hard to predict. Indeed, HIF activators have been shown to have many implications for innate and adaptive immunity, such as promoting regulatory T cell (Treg) functions (11) or attenuating mucosal inflammation via PMN-epithelial cross talk pathways (9, 11, 14). In light of studies implicating a functional role of HIFs expressed in alveolar epithelial cells, it is conceivable that HIF activators could be given via an inhaled route. As such, systemic effects of HIF activators could be minimized while simultaneously achieving a sufficient concentration to stabilize HIFs in the target cell population. However, additional studies are required to test a potentially therapeutic effect of inhaled HIF activators during ALI.


