Chapter 12

Stage-Specific Effects of Hypoxia on Interstitial Lung Disease

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Abstract

Interstitial lung disease (ILD) comprises a group of lung diseases principally affecting the pulmonary interstitium, for example, pulmonary fibrosis. Following acute lung injury (ALI), the fate of an injured lung progressing towards either injury resolution or pulmonary fibrosis is dictated by hypoxia at various stages during the disease progression. Hypoxia that is tissue destructive at one stage of lung injury becomes beneficial at a different stage, with each hypoxic stage involving a different scheme of molecular pathways, cellular interplay and tissue remodeling. In this chapter, we provide a detailed account of hypoxia during the different stages of lung injury in ILDs, delineate the cellular and molecular mechanisms mediating tissue remodeling in the hypoxic lungs as well as the basic and clinical findings in this field with an emphasis on future therapeutics to modulate hypoxia to treat ILD.

Keywords: acute lung injury, wound resolution, hypoxia, interstitial lung disease, PAH

1. Introduction

Interstitial lung disease (ILD) comprises a group of lung diseases principally affecting the pulmonary interstitium, for example, pulmonary fibrosis [1]. An injured lung as a result of infection, inhalation of chemical, and other harmful substances either resolves over time or progresses into irreversible damage and fibrosis. Therefore, lung injury as in acute respiratory distress syndrome (ARDS), due to conditions like hypoxia can progress to interstitial lung damage or fibrosis similar to ILD-associated pulmonary fibrosis. Yet, another important pulmonary pathological condition associated with hypoxia is the pulmonary arterial hypertension (PAH) [2]. The ARDS is a devastating clinical syndrome of acute lung injury (ALI) that affects both medical and surgical patients [3]. The official definition of ARDS was first published in 1994 by

American-European Consensus conference (AECC), according to which ARDS is characterized by arterial partial pressure of oxygen to fraction of inspired oxygen [PaO2/FIO2] ≤200 mm Hg with bilateral infiltrates on frontal chest radiograph, with no evidence of left atrial hypertension. A new entity—ALI was also introduced as a condition of less severe hypoxemia [PaO2/FIO2] ≤300 mm Hg. Arterial hypoxemia that is refractory to treatment with supplemental oxygen is a characteristic feature of acute lung injury. ALI is characterized by alveolar-capillary injury, inflammation with neutrophil accumulation and release of pro-inflammatory cytokines leading to alveolar edema [3]. Patients with ALI develop hypoxia. The term ALI was eventually removed in 2011 in the updated Berlin definition of ARDS. According to Berlin definition, ARDS was classified into three mutually exclusive categories based on the degree of hypoxemia; mild (200 mm Hg < PaO2/FIO2 ≤ 300 mm Hg), moderate (100 mm Hg < PaO2/FIO2 ≤ 200 mm Hg) and severe (PaO2/FIO2 ≤ 100 mm Hg) [4]. Hypoxia may be a consequence of ALI leading to deviation in lung function and preventing repair. Hypoxia induces destructive exudative changes within the lung parenchyma, which include the following: (1) increased alveolar paracellular permeability due to hypoxia disrupted alveolar epithelial cell (AEC) cytoskeleton and tight junction (TJ) protein organization; (2) Prolonged hypoxia induces loss of stress fibers such as actin (including breakdown of spectrin), internalization of TJ protein occludin and a decrease in zona occludens-1 (ZO-1) protein levels that are associated with trans-epithelial permeability; (3) reduced efficacy of AEC to clear alveolar edema fluid as a result of decreased expression of two major proteins, the apical epithelial sodium channel (ENaC) and the basolateral Na/K-ATPase channel which are involved in transcellular sodium (Na) transport. Thus, hypoxia-mediated effects not only enhance alveolar edema but also impair alveolar edema clearance contributing to reduced alveolar gaseous exchange capacity in ALI [5].

2. Hypoxia in alveolar edema and fluid clearance in the lungs

The mechanism by which hypoxia promotes pulmonary edema is not completely understood and is still under scrutiny. Alveolar edema accumulation is a result of enhanced pulmonary vascular permeability. Vascular endothelial growth factor (VEGF) is a potent inducer of endothelial dysfunction and thus can play a crucial role in vascular permeability [6]. Since VEGF is induced in hypoxic conditions and recovery from hypoxia, its role in pulmonary vascular remodeling and enhanced alveolar edema is prominent [7]. The source of VEGF in the inflammatory milieu of lung injury includes monocytes, eosinophils and aggregated platelets. Research on hypoxia-induced VEGF expression as a cause for pathological conditions has been carried out for more than two decades now. Studies have shown that both acute and chronic hypoxia induce an upregulation in the gene expression of VEGF, and its receptors (KDR/Flk and Flt) in the animal models of prolonged hypoxia-induced pulmonary hypertension [8]. In fact, the increase in the VEGF gene expression was seen as early as 2 h upon hypoxic challenge in isolated and perfused rat lungs while chronic hypoxia resulted in greater upregulation of the VEGF receptor genes. These studies also scrutinized the mechanism by which hypoxia induces VEGF expression by examining the role of nitric oxide synthase (NOS) and hypoxia inducible factors (HIFs) as the downstream regulators [8, 9]. Studies on transcriptional regulation of VEGF by hypoxia have revealed a functional HIF-1 binding

site on the rat VEGF 5′-flanking region as a possible transcriptional activator of VEGF gene by hypoxia [9]. Further studies have shown the involvement of specific regions in 3′-untranslated region (UTR) of VEGF gene in the stability of VEGF mRNA induced by hypoxia [10]. This has led to investigation of proteins that bind to this specific region to control the posttranscriptional regulation of VEGF expression. One such protein is HuR, a member of Elav-like protein family (Elav is a *Drosophila* RNA-binding protein required for neuronal differentiation). HuR was found to post-transcriptionally regulate VEGF expression by binding within four nucleotides of a canonical nonameric instability element in the VEGF AU-rich element [10]. Thus, hypoxia regulates VEGF at both transcriptional and posttranscriptional levels. Transcriptional regulation is by the hypoxia-induced transcription factor HIF-1 which activates VEGF transcription by binding to specific promoter sequences. A study exploring possible mechanisms involved in securing efficient translation of VEGF during hypoxic stress showed that internal ribosome entry site (IRES) present in the 5′-UTR of VEGF gene functions as an alternative to cap-dependent translation during such stressful conditions [11].

Becker et al. studied hypoxia-induced VEGF's role in enhancing pulmonary vascular permeability. They showed that ischemia/hypoxia-induced upregulation of VEGF mRNA and protein was associated with increased pulmonary vascular permeability [12]. Their study was also supported by several other studies which have reported an increase in vascular permeability due to exogenously administered VEGF in skin, muscle, GI tract and airways. In their study, hypoxic ischemia-enhanced VEGF expression, which was associated with increased HIF-1α protein expression and redistribution of VEGF protein to alveolar septae as demonstrated by immunohistochemical staining. This distribution of VEGF protein in the alveolar septae was further associated with increased pulmonary vascular permeability, suggesting its role in acute lung injury and alveolar edema [12]. The enhanced pulmonary vascular permeability effect of VEGF was also confirmed by another study in a sepsis-induced lung injury model, which showed that enhanced plasma VEGF level was accompanied by increased expression of vascular permeability-mediating VEGF receptor, Flt-1 and not the angiogenic-mediating receptor, Flk-1. As a result, enhanced lung edema was observed confirming the role of VEGF in causing alveolar edema [13].

Na,K-ATPase channels present in the alveolar epithelial cells play a major role in edema clearance from the alveoli [14]. Hypoxia-induced pulmonary edema also disrupts their function and inhibits edema clearance. Studies have shown that hypoxia generated reactive oxygen species (ROS) activates PKCζ (Protein Kinase C Zeta is a key regulator of critical intracellular signaling pathways induced by various extracellular stimuli), which in turn, phosphorylates the α1-subunit of Na,K-ATPase at Ser-18 site leading to its endocytosis through a clathrin-dependent mechanism and eventually to lysosomal degradation. With the loss of Na,K-ATPase, edema reabsorption is impaired and thus hypoxia not only promotes pulmonary edema but also inhibits its clearance as observed in conditions like ALI [14].

3. Hypoxia in pulmonary aquaporin's expression and edema

Aquaporins (AQPs) comprise a group of cell membrane water-transporting proteins that are involved in physiological as well as pathological fluid transport. They have been identified

in the lung and are believed to play a major role in pulmonary edema [15]. AQPs can bidirectionally transport fluid across the alveolar epithelium and hence are involved in both edema formation and clearance of edema from alveoli (thus injury resolution). About 6 (AQP-1, -3, -4, -5, -8 and -9) of the 13 different AQPs are distributed in lung tissue, and it is very interesting to study how hypoxia regulates the expression of these AQPs and thus pulmonary edema formation or clearance of edema. AQPs expression could play a major role in the pathological condition of hypoxia-induced enhanced pulmonary edema and ALI [15]. Several studies have scrutinized the role of aquaporins in pulmonary edema, and the results are controversial, yet intriguing. For example, Wu et al. studied the role of AQP-1 [expressed on pulmonary endothelial cells (ECs) and alveolar type II cells] and AQP-4 (expressed throughout the airways epithelial cells) in relation to high-altitude hypoxia lung injury. They found that hypoxia-induced pulmonary edema was associated with a decreased expression of AQP-1 and no change in the expression of AQP-4 [16]. They went on to reason that hypoxia resulted in pulmonary edema as a consequence of decreased function of AQP-1, which plays a regulatory role in water clearance around the bronchi and vessels. However, the relation of AQP-1 expression and pulmonary edema, as a result of hypoxia was only correlative and the study did not use knockout models to confirm the relationship between these effects of hypoxia. On the contrary, Su et al. showed that depletion of AQP-1 does not affect isosmolar fluid clearance and had no effect on lung edema. Nevertheless, depletion of AQP-1 resulted in a 10-fold decrease in the alveolar-capillary osmotic water permeability. They concluded that depletion of AQP-1 did not have any effect on lung edema formation and resolution [17]. Several other reports have also ruled out the role of AQP-1, -4 and -5 in physiological clearance of water in the lung or the accumulation of edema in the injured lung. Another report using gene knockout mouse model of AQP5 in hypoxic conditions showed a significant increase in pulmonary edema with the loss of AQP-5 [18]. As aforementioned, a few other reports also demonstrated that upregulation and downregulation of AQPs expression is related to pulmonary edema in different kinds of lung injuries. AQP-1 has also been shown to facilitate stabilization of HIF and has been speculated that besides its role as water transporter, it could also be involved in oxygen transport [19]. Therefore, the effect of hypoxia on AQPs expression especially in the lung and its effect on pulmonary edema warrants further studies before arriving at a conclusion [16–20].

4. Hypoxia in pulmonary arterial hypertension (PAH)

Prolonged lung injury can lead to lung fibrosis as well as PAH. Hypoxia is a well-studied trigger for pulmonary vascular remodeling and PAH development [2]. In fact, hypoxia-induced PAH is an established animal model for studying the pathophysiology and therapeutic management of PAH. PAH is a refractory disease characterized by uncontrolled vascular remodeling involving enhanced proliferation and differentiation of pulmonary vascular ECs and pulmonary vascular smooth muscle cells [2]. This vascular remodeling ensues enhanced pulmonary arterial pressure (≥25 mm Hg on right heart catheterization) due to increased pulmonary vasoconstriction and increased pulmonary vascular resistance and eventually right ventricular failure [2]. Chronic hypoxia is a well-known trigger

for the abovementioned events. The mechanism by which hypoxia induces PAH has been extensively studied and involves several molecular signaling pathways. Leptin, a non-glycosylated protein, synthesized and secreted by adipocytes is encoded by obese (ob) gene, which is hypoxia sensitive. HIF-1 induces the expression of ob gene in adipocytes, and clinical studies have suggested an association between plasma leptin levels and severity of PAH [21]. Results of studies scrutinizing the role of leptin signaling in hypoxia-induced PAH show that hypoxia-induced leptin expression results in pulmonary arterial smooth muscle cells (PASMCs) proliferation through ERK, STAT and AKT pathways [21]. These results were further confirmed in ob/ob mice. Obese gene knockout mice subjected to hypoxia showed an attenuated hypoxia-induced PAH that was gauged in terms of reduced right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) when compared to wild-type (WT) mice. Thus, leptin signaling could be a potential therapeutic target to treat hypoxia-induced PAH [21]. In hypoxia-induced pulmonary hypertension, iron supplementation has been found to be beneficial [22]. A study involving human subjects in an acute model of mountain sickness has shown that iron supplementation was associated with a decrease in pulmonary arterial systolic pressure (PASP) while progressive development of iron deficiency correlated with worsening of pulmonary arterial pressure determined by echocardiography, thus suggesting a causal relationship between iron deficiency and acute hypoxic PAH [23]. Recent studies speculate that iron deficiency may worsen hypoxic pulmonary hypertension through HIFs signaling [24].

HIFs are transcription factors comprising of an O_2-sensitive α-subunit, mainly HIF-1α and HIF-2α and a constitutively expressed β-subunit which are responsible for mediating adaptive responses to hypoxia and ischemia [25]. HIF-α and HIF-β form heterodimer and induce the transcription of over 100 genes that affect cellular functions ranging from metabolism, survival, proliferation, migration and angiogenesis among several others [25]. While HIF-1α is more ubiquitously expressed, HIF-2α expression is predominant in the lung tissue [25]. Several studies have shown the mechanistic role of HIF-2α in hypoxia-induced PAH. In hypoxia-induced PAH studies, even partial deficiency of either HIF1α (HIF1$\alpha^{+/-}$) or HIF2α (HIF2$\alpha^{+/-}$), achieved using murine models, significantly decreased pulmonary arterial pressure and right ventricular hypertrophy induced by chronic hypoxia in comparison with wild-type mice that did not have any alteration in HIF1α or HIF2α expression [26]. The role of HIFs in hypoxia-induced PAH was further scrutinized and deficiency in HIFs-related beneficial effects in PAH was at least partly due to the reduced pulmonary vascular remodeling observed in these animals. Further *in vitro* analysis on PASMCs showed that HIF-1-dependent smooth muscle hypertrophy contributed to pulmonary vascular remodeling during hypoxia [26]. HIF1α is involved in hypoxia-induced PASMC depolarization, reduction in K^+ channel expression and activity and elevated intracellular calcium concentration and pH. This eventually results in altered PASMC ion homeostasis contributing to a more contractile, apoptosis resistant, proliferative and migratory phenotype [26]. Furthermore, in human PAH patients and mouse models of PAH, dysregulation of HIF pathway was reported and it has been associated with HIF-2α mutations, which was confirmed by studies where loss of one copy of HIF-2α gene was sufficient to attenuate hypoxia-induced PAH in these animal models [27]. On the other hand, HIF-2α gain of functions is associated with PAH. Studies scrutinizing the

mechanism by which HIF-2α regulates hypoxic PAH have found several ways by which it mediates the hypoxic effects. In human PASMC, hypoxia increases expression of transcription factor forkhead box M1 (FoxM1), through HIF-2α, to promote PASMC proliferation [27]. Secreted matricellular protein thrombospondin-1 (TSP-1) is believed to play an important role in vascular health and disease via inhibition of vasodilation in part by limiting NO production and signaling [28]. Vascular remodeling in PAH involves the proliferation of both pulmonary artery smooth muscle cells (PASMCs) and fibroblasts apart from endothelial dysfunction. In a recent study published from our laboratory, we showed that hypoxia-induced pulmonary rarefaction and fibrosis in mice lung, and mechanistically, we found that hypoxia-induced Akt1 expression in fibroblasts was associated with enhanced TSP-1 expression resulting in fibroproliferation and fibrosis [29]. Another study has shown that hypoxia, in a HIF-2α-dependent manner, increases the expression of TSP-1 in pulmonary tissue and pulmonary artery cells which in turn contributes to enhanced endothelial permeability (mediated in part by changes in cell-cell adhesion) and accompanied by increased fibroblast and PASMC proliferation which is at least partially due to restricted adhesion of these cells in their mouse model of hypoxia-induced PAH. Also it was speculated that TSP-1 could promote hypoxic pulmonary artery contraction through enhanced TSP-1–induced endothelin-1 expression [28].

Prolyl hydroxylase domain-containing enzymes (PHDs) use molecular O_2 as a substrate to hydroxylate-specific proline residues of HIF-α which subsequently promotes HIF-α binding to von Hippel-Lindau (VHL protein) and ubiquitin E3 ligase, resulting in ubiquitination and proteasomal degradation [27]. In patients with idiopathic pulmonary fibrosis (IPF), PHD2 expression is diminished in ECs of obliterative pulmonary vessels [27]. A study using mouse model of endothelial and hematopoietic cells-specific knockdown of gene encoding PHD2 has shown that these mice spontaneously develop PAH with obliterative vascular remodeling as seen in human PAH [27]. They found that PHD2 deficiency in ECs promoted HIF-2α-mediated (and not HIF-1α) expression of CXCL12 (also known as stromal cell-derived factor 1α) that had a paracrine effect on PASMC proliferation contributing to the pathogenesis of severe PAH in this mouse model. PHD2 deficiency in ECs also promoted endothelin-1 expression that resulted in pulmonary artery-vasoconstriction. Thus, HIF-2α-mediated vascular remodeling and plexiform-like lesions formation (due to PASMC proliferation) resulted in PAH in this mouse model [27, 28]. As discussed above, prevention of PASMC apoptosis along with enhanced proliferation is an important pathological event in hypoxic PAH. Another study showed the mechanism by which hypoxia mediates this effect. In PASMCs, hypoxia induces opening of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}), which results in calcium-dependent increase in mitochondrial permeability or mitochondrial membrane transition (MPT). MPT eventually leads to loss of mitochondrial membrane potential (denoted by $\Delta\Psi m$), thus preventing the cytochrome C release from mitochondria and inhibition of cytochrome C–caspase 9 pathway induced PASMC apoptosis [30]. The involvement of mitoK_{ATP} channels in hypoxia-induced PASMC apoptosis resistance was further confirmed by administering 5-hydroxydecanoate (5-HD), a compound that prevents opening of mitoK_{ATP} channels abolishes these effects of hypoxia to a certain extent and prevents mitoK_{ATP} channels opening and PASMC apoptosis. Hypoxia-induced opening of mitoK_{ATP} was not only associated with prevention of PASMC apoptosis but also increased the production of H_2O_2 in

mitochondria. The effect of this ROS production was an increased transcriptional activity of AP-1, which is responsible for the proliferation of PASMCs. Thus, hypoxia through mitoK_{ATP} opening prevented apoptosis and enhanced proliferation of PASMCs. As discussed, apart from proliferation of PASMCs, hypoxia-induced prevention of PASMC apoptosis also plays a major role in PAH. Another mechanism involves inhibition of the mitochondrial pro-apoptotic Bax protein expression and induction of the anti-apoptotic Bcl-2 expression, thus preventing the release of mitochondrial cytochrome C into cytoplasm and eventually inhibiting cleavage of caspase 9 resulting in PASMC apoptosis [31]. Therefore, hypoxia-HIF signaling is a potential therapeutic target to treat PAH, and several in vivo studies have demonstrated this [30–32].

5. Hypoxia and alveolar epithelial-to-mesenchymal transition (EMT)

Several groups have studied the role of hypoxia in disease progression and pathogenesis of ILDs such as pulmonary fibrosis [33, 34]. Activated myofibroblasts play an important role in the production of collagen and ECM proteins during pulmonary fibrosis. The source of these myofibroblasts are numerous, which include resident stromal fibroblasts, bone marrow-derived fibroblasts, and mesenchymal transition of epithelial and ECs [33]. Epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial cells lose many of their epithelial characteristics such as cell-cell interaction and apicobasal polarity and acquire properties typical to mesenchymal cells. EMT is driven by a cytokine, transforming growth factor-β1 (TGF-β1) and is characterized by changes in cell morphology and acquisition of mesenchymal markers including α-smooth muscle actin (α-SMA) and vimentin as well as loss of epithelial markers such as E-cadherin [33, 34]. Active TGF-β1 binds to its receptors (transmembrane serine-threonine kinase receptor I and II), which leads to a downstream activation of the transcription factor Smad, whose target genes include α-SMA and vimentin [33]. Increasing evidence over the years has highlighted the critical role of EMT in pathological conditions such as fibrosis apart from its well-known involvement in tissue development during embryogenesis. Exposure to hypoxia during ALI could promote phenotypic changes in AEC consistent with EMT. In vitro studies on rat AEC cultured on semipermeable filters showed that prolonged hypoxic exposure (1.5% O2 for up to 12 days) induced profound changes in AEC phenotype consistent with EMT including change in cell morphology, decrease in transepithelial resistance and in the expression of epithelial markers such as zona occludens (ZO-1), E-cadherin, AQP-5, TTF-1, together with an increase in mesenchymal markers such as vimentin and α-SMA. Supporting this phenotypical switch, expression of transcription factors driving EMT such as SNAIL1, ZEB1 and TWIST1 increased after 2, 24 and 48 h of hypoxia, respectively. Hypoxia also induced expression and secretion of two EMT inducers TGF-β1 and connective tissue growth factor (CTGF) [35].

Similarly, Zhou et al. investigated the effect of hypoxia on the induction of EMT in AEC. Results from this study suggest that hypoxia induces EMT in transformed human, rat and mouse AEC lines, and freshly isolated rat type II AECs [36]. They also scrutinized the mechanism by which hypoxia induces EMT in AEC and showed the involvement of

hypoxia-induced mitochondrial ROS production and HIF-1α stabilization in TGF-β1 production, resulting in EMT [37]. Treatment of cells with ROS scavenger Euk-134 or using mitochondria-deficient cells prevented hypoxia-induced EMT illustrating their importance in this cellular process. Moreover, although ROS is known to stabilize HIF-1α, their results showed that normoxic stabilization of HIF-1α failed to induce α-SMA expression, suggesting that HIF alone is not sufficient to induce EMT in AEC. Their data suggest that ROS and HIF-1α stabilization are upstream of TGF-β1 production in hypoxia-induced EMT in AEC. However, TGF-β1 can also increase ROS production and HIF-1α stabilization. TGF-β1 can either directly activate NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase or upregulate gene expression of Nox4 NADPH oxidase to generate ROS [38, 39]. TGF-β1 decreases mitochondrial complex IV activity resulting in disruption of mitochondrial membrane potential and ROS production [40]. TGF-β1 was reported to stabilize HIF-1α through selective inhibition of PHD2 (a HIF-1α prolyl hydroxylase) expression thus reducing HIF-1α prolyl hydroxylation leading to its stabilization [41]. Therefore, TGF-β1 and ROS/HIF may form a feedback loop to maintain a prolonged signaling cascade initiated by either ROS/HIF or TGF-β1 leading to hypoxia-induced EMT in AECs [36].

In one interesting study, investigators evaluated the possible role of tissue hypoxia in the development of fibrotic lesions in lung fibrosis [42]. In this study, they used animal models of ALI/ARDS, in which severe inflammation progresses into the early (exudative) phase of ALI and sequentially fibrosis develops as the late (fibrotic) phase of ALI. They found intriguing effects of acute versus persistent hypoxia as seen in exudative and fibrotic phases of ALI, respectively. Acute hypoxia induced de novo Surfactant Protein-D (SP-D) expression in AECs followed by stabilization of HIF-1α expression [42]. Contrastingly, persistent hypoxia-induced HIF-1α stabilization repressed SP-D expression and enhanced the mRNA levels of an EMT-driving transcription factor TWIST, but not SNAIL. This was accompanied by phenotypic switch in the AECs exposed to persistent hypoxia (72-h hypoxia for in vitro studies) as seen by decreased E-cadherin expression and enhanced vimentin expression. SP-D is mainly derived from alveolar epithelial cells and therefore loss of its expression during persistent hypoxia along with enhanced EMT transcription factor expression clearly indicates phenotypic switch of these alveolar epithelial cells to more proliferative phenotype contributing to lung fibrosis [42].

Endothelial-to-mesenchymal transition (EndMT) is similar to EMT, which is characterized by a loss of endothelial cell-cell junctions, the acquisition of migratory properties, and phenotypic switch involving loss of endothelial-specific markers such as CD31 and vascular endothelial (VE)-cadherin expression, and the acquisition of mesenchymal markers α-SMA, and vimentin [43]. EndMT also contributes to fibrosis. The role of EndMT in pulmonary fibrosis involves phenotypic switch in the pulmonary EC lining the pulmonary capillaries. Radiation-induced pulmonary fibrosis (RIPF) may involve hypoxia-mediated EndMT as an initial pathological insult leading to fibrosis [13]. Fleckenstein and colleagues have shown that radiation during thoracic radiotherapy for lung cancer induces tissue hypoxia, in part, due to enhanced oxygen consumption by Macrophages. These macrophages are activated because of radiation-induced reduction in blood perfusion in the lungs contributing to lung injury [44]. This suggests

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that hypoxia plays a major role in the radiation-induced lung injury. Fleckenstein et al. also reported that hypoxia is important in triggering continuous production of fibrogenic cytokines and perpetuation of late lung tissue injury [44]. However, the precise mechanism by which hypoxia affects radiation-induced fibrosis remains elusive. EndMT of the pulmonary ECs was shown as a possible consequence of radiation-induced hypoxia resulting in lung fibrosis and injury by Choi et al. [43]. They investigated the reason behind fibrotic effects of radiation in a mouse model of RIPF and in *in vitro* studies on human pulmonary ECs. Since fibrosis is a long-term event, their investigation aimed at elucidating the mechanisms behind the early damage to ECs by radiation and its link to the later observed fibrosis. Their results indicate ECs specifically expressing hypoxic marker, CA9, just prior to the substantial fibrogenesis. They went on to show that radiation-induced vascular hypoxia-triggered EndMT in vascular ECs, and in fact, this was observed prior to the onset of alveolar EMT and thus could be a trigger to EMT as well. Thus, EndMT contributed to chronic tissue fibrosis and targeting EndMT was speculated to be a potential therapeutic target to treat RIPF [43, 44].

In conclusion, current evidences suggest that the pathogenesis of human pulmonary fibrosis might involve the recruitment of fibroblasts derived from AECs through hypoxia-induced EMT as well as fibroblasts derived from pulmonary ECs through hypoxia-induced EndMT, apart from the bone marrow-derived precursors forming the fibrotic lesions. Thus, hypoxia could contribute to the formation of fibrotic lesions in the lung and hence the pathogenesis of pulmonary fibrosis (see **Figure 1**).

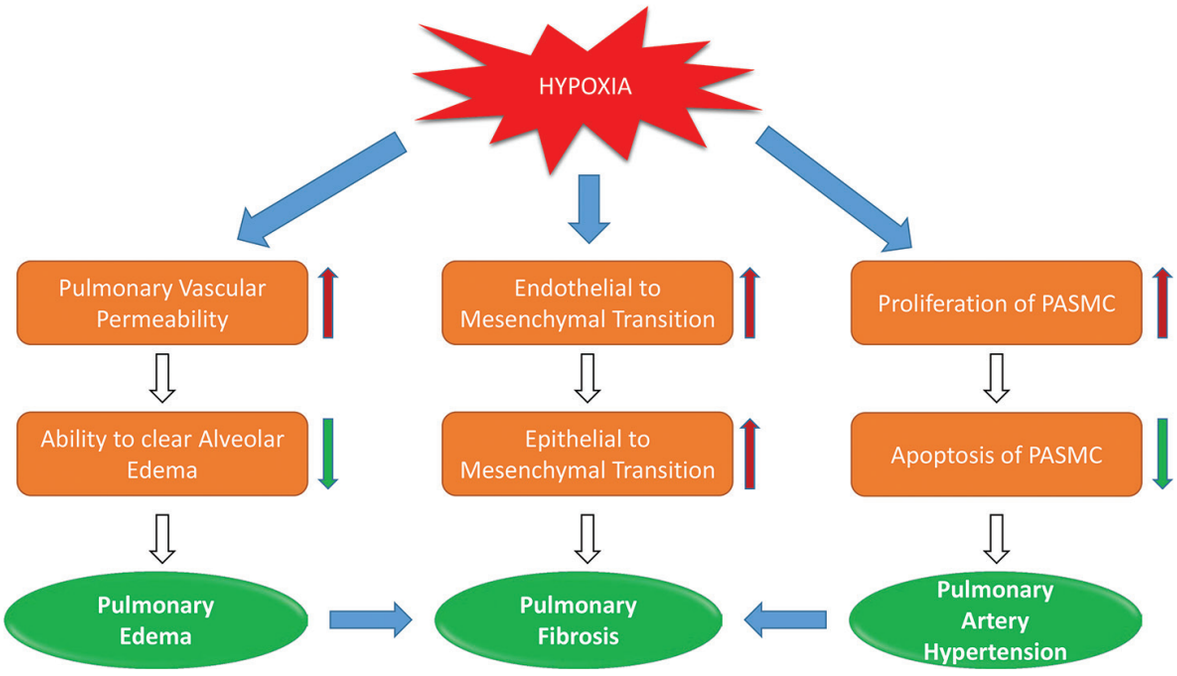

Figure 1. Summary of the effect of hypoxia on pulmonary tissue and vasculature. Hypoxia induces pulmonary edema by enhancing vascular permeability and decreasing the ability of alveolar fluid clearance. Hypoxia induces pulmonary vascular EndMT and alveolar EMT that result in myofibroblast proliferation ensuing pulmonary fibrosis. Hypoxia-induced PAH is a result of enhanced proliferation and survival of PASMCs. ALI and PAH can eventually progress to pulmonary fibrosis.

6. Hypoxia in lung injury resolution (fate of hypoxia as a consequence of pathological conditions)

While in the early stages of ALI, hypoxia plays a major role in the progression of lung injury, intriguingly in chronic pulmonary pathological conditions that ensue hypoxic milieu, and hypoxia has also been found to be involved in enhancing injury resolution. Studies indicate a protective and anti-inflammatory role of HIFs such as HIF-1α in lung protection during the early exudative phase of ALI [45–47]. As mentioned above, hypoxia inactivates PHDs and stabilizes HIF-1α [45–47]. During the acute stage of ALI, inflammation, including enhanced neutrophil activity within the alveoli, leads to an increased alveolar edema and decreased alveolar gaseous exchange capacity. HIF stabilization has been shown to have anti-inflammatory role in conditions like intestinal inflammation. The protective role of HIF activators in the treatment of inflammatory bowel disease or ischemia and reperfusion injury of several organs has been shown in several studies [48–50]. Interestingly, Eckle et al. showed the beneficial role of normoxic HIF1A stabilization in lung protection during ALI, where HIF-dependent control of alveolar-epithelial glucose metabolism function as an endogenous feedback loop to dampen lung inflammation [51]. In vivo HIF-1α increased glycolysis, lactate production and glucose flux rates in alveolar epithelium. Overall, this normoxic stabilization of HIF-1α in alveolar epithelium increased glycolytic capacity and TCA flux thus optimizing mitochondrial respiration to enhance ATP production. This HIF-dependent protection of mitochondrial function in ALI not only enhanced ATP production but also concomitantly prevented ROS accumulation and lung inflammation [51]. Hence, the role of hypoxia and subsequent HIF stabilization in reducing inflammation is prominent in resolution of ALI.

6.1. Hypoxia and adenosine signaling in lung injury resolution

Emigration of polymorphonucleated neutrophils (PMNs) through the endothelial barrier in an injured lung creates a potential for vascular fluid leakage leading to edema and decreased oxygenation [52]. The vascular endothelial adaptations to hypoxia include enhanced extracellular adenosine production during limited oxygen availability. In the vascular ECs, hypoxia induces enhanced expression of surface ectonucleotidases, CD39 that converts ATP/ADP to AMP (ectoapyrase), as well as CD73 that is involved in phosphohydrolysis of AMP to adenosine thus forming the source for extracellular adenosine production [52]. This enhanced extracellular adenosine can then signal through four different G-protein-coupled adenosine receptors, all of which are present on vascular endothelia thus enhancing adenosine signaling that is implicated in tissue protection in different models of injury including ALI. Several studies, notably couple of them from Eltzschig, H.K., et al. [52, 53], have shown the role of extracellular adenosine and its signaling in attenuating hypoxia-induced vascular leakage. They also showed that the source of ATP in hypoxic milieu is the PMNs. Hypoxia induces the production of ATP by PMNs, however, the exact mechanism by which ATP is produced still needs to be explored. This ATP is then phosphohydrolyzed as mentioned above to produce extracellular adenosine [53]. Enhanced adenosine concentrations activate adenosine receptor, (AdoRA_{2A}/A_{2B} on ECs, which when activated increases intracellular cyclic AMP (cAMP)

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and activates protein kinase A (PKA) to induce resealing of the endothelial-barrier [54]. The resealing of endothelial-barrier during PMN transmigration was obviated by inhibition of cAMP formation. This resealing effect is mediated by PKA-induced phosphorylation of vasodilator-stimulated phosphoprotein, a protein responsible for changes in the geometry of actin filaments and distribution of junctional proteins as a result affecting the characteristics of junctional proteins and increasing barrier function [54]. Intriguingly, adenosine not only activates the endothelial A_{2B} receptor, but also neutrophil A_2 adenosine receptor which has been shown to play an important role in limitation and termination of PMN mediated systemic inflammatory responses. Few others have also demonstrated that PMN A_2 adenosine receptor stimulation decreased leukocyte adherence and transmigration which might contribute to attenuated vascular leak associated with leukocyte accumulation [53–55]. Thus, hypoxia-induced adenosine signaling in vascular ECs and PMNs contributes to decreased vascular leak and inflammation, both of which are beneficial in inflammatory conditions such as ALI (see **Figure 2**).

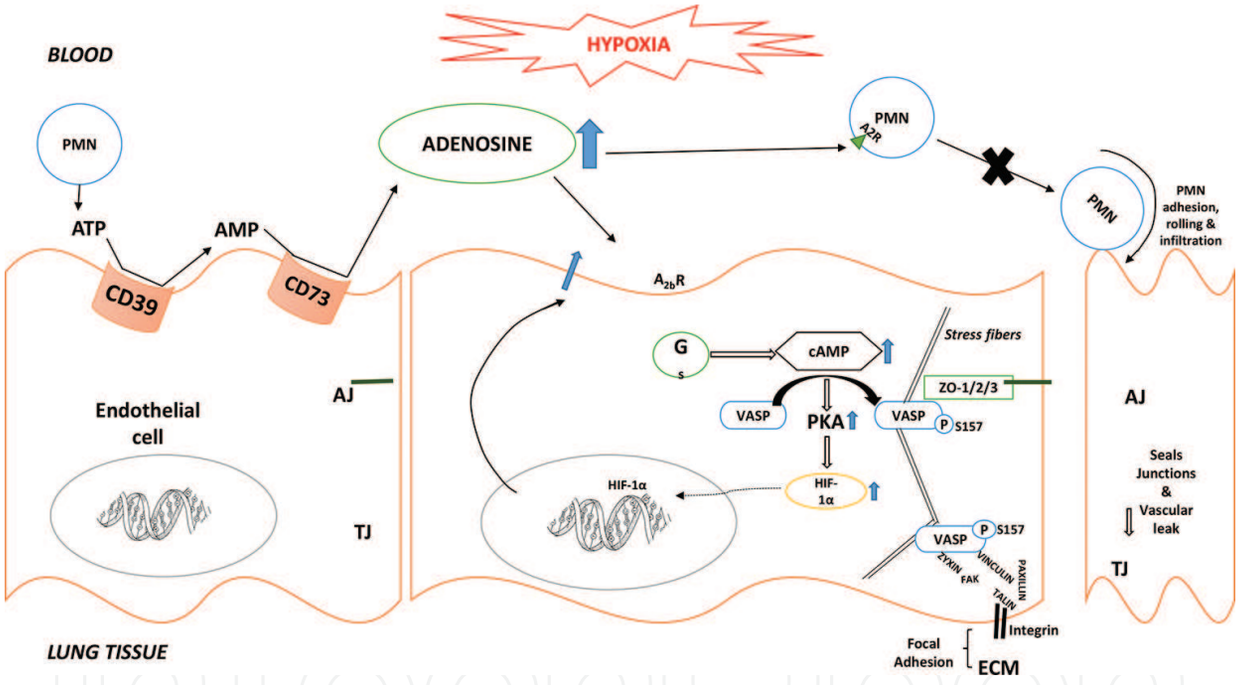

Figure 2. Hypoxia and adenosine signaling in the lungs. Hypoxia-induced extracellular adenosine production acts through adenosine receptors on ECs to enhance intracellular cAMP and PKA production. PKA catalyzes the phosphorylation of VASP, which integrates into stress fibers and helps seal the endothelial barrier by enhancing expression of AJs, TJs and also focal adhesion. PKA also enhances HIF-1A expression, which translocates into nucleus and enhances adenosine receptor transcription. Extracellular adenosine also acts on A2-receptors on PMNs and prevents their adhesion, rolling and infiltration into lung tissue. Thus, hypoxia-induced extracellular adenosine seals endothelial junctions, prevents PMN infiltration and protects lung tissue by preventing alveolar edema accumulation. PKA, protein kinase-A; PMN-polymorphonuclear neutrophils; ATP, adenosine triphosphate; AMP, adenosine monophosphate; $A_{2b}R$, adenosine 2b receptor; cAMP, cyclic AMP; VASP, vasodilator-stimulated phosphoprotein; AJ, adherent junction; TJ, tight junction and ECM, extracellular matrix.

When adenosine signaling was inhibited in transgenic mice with targeted disruption of CD73 that were subjected to hypoxia, fulminant vascular leakage, associated with severe edema

and inflammation was seen [56]. Recently, studies have shown three other mechanisms by which hypoxia enhances extracellular adenosine levels, including hypoxia-mediated repression of the equilibrative nucleoside transporters (ENT-1 and ENT-2) that are responsible for adenosine transport across the membrane into the cytoplasm; HIF-1α mediated inhibition of intracellular adenosine kinase that converts intracellular adenosine to AMP and transcriptional induction of $AdoRA_{2B}$ receptor [57]. These studies indicate the protective role of adenosine signaling during hypoxia, especially in the pulmonary tissue [37]. On the other hand, chronically increased adenosine levels are detrimental as seen in pathological conditions, such as asthma and chronic obstructive pulmonary disease (COPD), and they also correlate with degree of inflammation in COPD. In order to regulate excessive adenosine signaling, chronic exposure to hypoxia eventually induces endothelial CD26 and extracellular adenosine deaminase (ADA). CD26 on EC surface acts as the ADA-complexing protein and localizes ADA accumulation on EC surface limiting extracellular adenosine accumulation during prolonged hypoxia [55].

6.2. Hypoxia and lung inflammation

Uncontrolled inflammation is one of the major players in ALI and suppression of inflammation is beneficial for injury resolution [58, 59]. Interestingly, as mentioned above, hypoxia-induced, HIF-1–mediated enhanced expression of Adenosine A_2 receptor on different types of immune cells, along with enhanced extracellular adenosine levels, which activate these receptors, are responsible for anti-inflammatory and tissue-protecting effects of hypoxia [58, 59]. This anti-inflammatory effect is attributed to elevated intracellular cAMP levels through activation of adenylyl cyclase. Even pharmacological immunosuppressive molecules, such as catecholamines, neuropeptides, histamine and prostaglandins are known to have their effects through elevation of cAMP levels [59]. Therefore, this extracellular adenosine serves to report excessive collateral immune damage and prevents further damage by suppressing-activated immune cells. Adenosine triggers high-affinity A_{2A} adenosine receptors on activated immune cells resulting in enhanced intracellular cAMP levels to suppress these immune cells. Few studies also show that hypoxia inhibits adenosine kinase, an enzyme responsible for re-phosphorylation of adenosine to AMP, to maximize the anti-inflammatory effect [60].

6.3. Adenosine receptors in inflammation

Adenosine receptors are a family of heptahelical transmembrane G-protein-coupled purinergic receptors that are classified into four types based on the potency of agonists with respect to the intracellular production of cAMP [37]. They are A1, $A_{2A,}$ A_{2B} and A3 receptors. Extracellular agonists signal through these G protein receptors and can either stimulate (Gs) or inhibit (Gi) adenylyl cyclase, an enzyme that catalyzes the formation of cAMP. Cloning experiments show that high-affinity A_{2A} and low-affinity A_{2B} receptors activate adenylyl cyclase (Gs) enhancing the levels of intracellular cAMP, whereas high-affinity A1 and low-affinity A3 receptors inhibit (Gi) adenylyl cyclase [37].

6.4. Hypoxia induced adenosine signaling in individual immune cells

a. *Polymorphonuclear Leukocytes (PML):* Pathological stimulation of inflammation can result in deleterious nonspecific PML bactericidal effector functions directed towards hosts' healthy tissue resulting in extensive collateral damage [54]. PMN toxic effects on microvascular endothelium are more prominent as they attach to ECs, easily because they use the same recepvtors (CR3, CD11b/CD18) that ensure PML attachment to pathogenic microorganisms [54]. Hypoxia-induced extracellular adenosine acts through adenosine receptor (high affinity A1 and A2 receptors) to mediate its anti-inflammatory effect. However, since both A1 and A2 are high affinity receptors, the overall effects of adenosine on PML might depend on the interplay between them and their expression on PML [61]. Studies show that the anti-inflammatory effects of A_{2A} receptor are to a certain extent prevented by A1 receptor, on the other hand, deleterious effects such as chemotaxis, adhesion and oxygen radical production stimulated by A1 were inhibited by A_{2A} [61, 62]. Overall, hypoxia-induced extracellular adenosine may protect the microvascular endothelium from PML by inhibiting the expression of β2-integrins and adhesion, ROS production, TNF-α production and degranulation, all of which without compromising the bactericidal function of PML such as production of bactericidal toxins and complement receptor type-3–mediated phagocytosis of bacteria [54, 63].

b. *Mononuclear phagocytes and dendritic cells:* In macrophages, activation of A1 receptor is stimulatory, while A2 receptor activation is inhibitory [54]. A_{2A} receptor activation in lipopolysaccharide (LPS)-stimulated macrophages was associated with the inhibition of IL-12 production but enhanced IL-10 secretion. In LPS-stimulated dendritic cells, adenosine enhanced A_{2A} receptor expression and intracellular cyclic AMP production along with inhibition of IL-12 production. In dendritic cells, except adenosine, other cAMP-elevating agents increase IL-10 and lower expression of MHC type II [64]. However, adenosine-mediated A_{2A} activation decreases the capacity of maturing dendritic cells to induce T-helper (Th1) polarization of native $CD4^+$ T-lymphocytes (possible anti-inflammatory effect). Upon LPS-induced differentiation of dendritic cells, A_{2A} activation favors production of CCL17 over CXCL10 chemokines [65]. Overall, these studies suggest that extracellular adenosine stimulation of adenosine receptors on antigen-presenting cells (macrophages and dendritic cells) might play an important role in the downregulation and polarization of immune response, modulation of MHC class I and II expression, and/or decrease in IL-12 and increase in IL-10 or IL-4 production to favor the initiation of a Th2 response over a Th1 response. This effect of adenosine on innate and adoptive immune system plays a crucial role in the modulation of inflammatory response [54, 64–66].

c. *Thymocytes:* The microenvironment of thymocytes is hypoxic even under normal physiological condition when compared to other lymphoid and non-lymphoid tissues [54]. Thus, the thymic environment favors increased adenosine levels and its signaling. Patients with severe combined immunodeficiency were found to be ADA deficient (enzyme responsible for decreased adenosine levels), where ADA deficient patients had developmental defects

in T- and B-cells [67, 68]. This enhanced extracellular adenosine signals through A_{2A} receptor and induces apoptosis in a subset of immature thymocytes through its cAMP elevating effects. In peripheral T-cells, activation of extracellular adenosine-mediated A_{2A} receptor inhibits TCR-triggered IL-2 receptor upregulation, thereby inhibiting T-cell proliferation [69]. Other effects of adenosine signaling in $CD8^+$ cytotoxic T-lymphocytes include inhibition of inflammatory cytokine production, lethal hit delivery by granule exocytosis, as well as FasL mRNA upregulation. It is interesting to note that in human blood peripheral leukocytes, more $CD4^+$ than $CD8^+$ T-cells express A_{2A} receptor, but on activation of T-cells increased A_{2A} receptor expression is predominantly observed in $CD8^+$ T-cells. These studies suggest the variable expression of A_{2A} receptors on T-cell subset and how they favor the production of anti-inflammatory cytokines over inflammatory cytokines. Compared to T-lymphocytes, not much is known about the effects of A_{2A} receptor signaling in B-cell development, activation, antibody-production and class switching, and cytokine secretion [70].

However, it is very important to note that all the above mentioned effects of extracellular adenosine on immune cells were mostly observed in pharmacological experiments and is yet to be explored whether there are sufficient levels of extracellular adenosine in vivo to signal through A_{2A} receptor on immune cells. So far, there is no evidence of physiological downregulation of immune cells by extracellular adenosine in vivo. However, hypoxia-induced extracellular adenosine may have anti-inflammatory effects even in in vivo similar to in vitro studies [67, 71, 72].

7. Conclusions and future directions

Hypoxia, either as a consequence of the pathological condition during ILDs or as an etiology for ILDs has several roles in modulating the severity of the disease condition. Most of the effects of hypoxia are regulated through HIFs. Interestingly, stabilization of HIFs at various stages of lung injury can have different consequences either favoring injury resolution or worsening the condition. This complicates to provide a potential therapeutic target against HIFs to treat ILDs. Targeting hypoxia signaling was speculated to have therapeutic importance in inflammatory and ischemic conditions, such as inflammatory bowel disease, myocardial ischemic-reperfusion injury, ALI and so on. However, most of the clinical trials for drug discovery examined HIF inhibitors in the context of cancer treatment. Some of the examples include pharmacological HIF inhibitors such as dutasteride152 (ClinicalTrials.gov identifier: NCT00880672), topotecan153 (ClinicalTrials.gov identifier: NCT00117013), PX-478 (ClinicalTrials.gov identifier: NCT00522652) or digoxin13 (ClinicalTrials.gov identifier: NCT01763931) or the antisense oligonucleotide HIF inhibitor EZN-2968 (ClinicalTrials.gov identifier: NCT01120288). Apart from HIF inhibitors, HIF-stabilizing agents such as PHD inhibitors are also being studied as potential therapeutic targets in conditions where HIF stabilization is beneficial, such as, conditions which require enhanced angiogenesis (HIF activates VEGF and enhances angiogenesis) like bronchopulmonary dysplasia, a chronic disease effecting preterm neonates in which enhanced angiogenesis improves lung growth and function. Favoring the plethora of evidence from preclinical studies, in future, we can expect more clinical trials targeting PHD-HIF pathway as a potential therapy for ILDs and several other ischemic conditions.

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