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Hypoxia and Pulmonary Hypertension

Nicoletta Charolidi and Veronica A. Carroll

Abstract
Vasoconstriction in response to low oxygen tension (hypoxia) in pulmonary arteries is an important physiological adaptation to reroute blood flow to areas of higher oxygenation for effective gaseous exchange. However, chronic hypoxia is a common feature of lung disease, such as chronic obstructive pulmonary disease (COPD). Hypoxic stress triggers cellular phenotypic alterations including increased proliferation and migration of vascular smooth muscle cells (VSMCs), as well as synthesis of extracellular matrix (ECM) proteins that remodel lung vasculature. Remodelling of vessels increases the risk of pulmonary hypertension (PH)—elevated pulmonary arterial pressure—and eventually right heart failure. This chapter will summarise the major pathways and mechanisms involved in hypoxia-driven pulmonary hypertension (PH).

Keywords: hypoxia, pulmonary hypertension, HIF-1α, HIF-2α, mTOR, VHL

1. Introduction
The main function of the cardiovascular system is to circulate and deliver oxygen to metabolically active tissues of the body. At physiologically normal oxygen levels, the pulmonary vasculature of healthy individuals is highly distensible, allowing the cardiac output to adjust to levels of activity. In varying degrees of oxygen availability, as in different altitudes, adaptive cardiovascular responses are employed. In acute hypoxia (short, transient reduction in oxygen tension), the pulmonary vascular bed constricts rapidly [1]. When oxygen levels are restored, it dilates again in a swift and reversible manner. With a sustained hypoxic exposure (hours to days), the response is different. There is a loss of pulmonary distensibility, increased arterial pressure, tachycardia and increased workload for the right cardiac ventricle. In return to normoxic conditions, there is, at least in the short term, a limited reversibility of these effects. The Operation Everest II study [2] demonstrated this phenomenon by monitoring the pulmonary vascular pressure of healthy individuals who were exposed to progressive...
partially pressured oxygen over a period of a few weeks. However, for high-altitude populations, such as the Tibetans, this is not the case. Due to natural selection and adaptation over many thousands of years living under low oxygen conditions, Tibetans have altered oxygen-sensing mechanisms and pulmonary vascular resistance to sustained hypoxia (discussed later in this chapter) [3].

Healthy, native sea-level dwellers, who move to high altitude, develop high pulmonary arterial pressure, but with time, in the majority of cases, it stabilises and becomes well tolerated [4]. By contrast, people with pre-existing lung pathologies, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, idiopathic pulmonary fibrosis, bronchiectasis or restrictive chest wall abnormalities, are at risk of developing pulmonary hypertension (PH). Chronic PH lowers quality of life and decreases life expectancy for the affected individuals [5–8].

The pathophysiology of hypoxia-associated PH is characterised by extensive vascular remodelling that leads to arterial narrowing rather than reversible vessel vasoconstriction (Figure 1). Processes that take place include endothelial cell dysfunction, muscularisation of normally non-muscular arteries, phenotypic switching and proliferation of vascular smooth muscle cells (VSMCs), increased extracellular matrix deposition and erythrocytosis [7, 9, 10]. In this chapter, recent developments in mechanistic aspects underlying hypoxia-induced pathophysiological changes in PH will be briefly summarised.

**Figure 1.** Schematic representation of pulmonary arterial responses to normoxia, acute hypoxia and chronic hypoxia. With acute and chronic hypoxia, the pulmonary artery undergoes vasoconstriction. In the case of acute hypoxia, the artery can reversibly dilate. But in chronic hypoxia, the artery undergoes non-reversible vascular remodelling characterised by intimal thickening due to VSMC dedifferentiation (loss of contractility, hypertrophy and hyperplasia). Additionally, there is distal muscularisation of non-muscular vessels, a settled-in endothelial cell dysfunction and erythrocytosis. Activation of HIF-1α and HIF-2α as well as over-activation of mTORC1 contributes to VSMC dedifferentiation and the establishment of hypoxic PH. Abbreviations: HIF-1α, hypoxia-inducible factor 1α; HIF-2α, hypoxia-inducible factor 2α; mTORC1, mechanistic target of rapamycin complex 1; PH, pulmonary hypertension.
2. Role of endothelial cell dysfunction

Endothelial cells in pulmonary vessels first sense hypoxic stress. Having a role in maintaining homeostasis, endothelial cells contribute to reducing the vascular tone in order for vasoconstriction to take place and regulate vessel adaptation to increased blood flow [11]. In healthy individuals, the endothelium is responsible for the balanced expression of vasoactive mediators that have either vasodilator ability, such as nitric oxide (NO) and prostacyclin (PGI\textsubscript{2}), or vasoconstrictive properties, such as endothelin-1 (ET-1) [11–14]. ET-1 is released abuminally and triggers vasoconstriction through binding to its VSMC receptors ET\textsubscript{A} and ET\textsubscript{B} [15]. However, when ET-1 binds to its endothelial ET\textsubscript{B} receptor, it can induce vasodilation through NO and PGI\textsubscript{2} recruitment [15], while this route also serves for ET-1 clearance from the lung [16].

In pathological PH, as in COPD, endothelial cell dysfunction is one of the major contributing factors for the progression of the condition. It has been found that endothelial NO synthase (eNOS), the enzyme responsible for NO production, as well as prostacyclin synthase, the enzyme responsible for PGI\textsubscript{2} production, is markedly diminished in patients with COPD [12, 17]. Furthermore, ET-1 has been reported to have an increased expression in the lungs of patients with PH and is a therapeutic target [14]. ET-1, as well as being a potent vasoconstrictor, is also a VSMC mitogen, acting through smooth muscle ET\textsubscript{A} and ET\textsubscript{B} receptors [15]. So in effect, during hypoxic endothelial dysregulation, the pathogenic excess of ET-1 maintains vessel constriction and VSMC proliferation.

3. Phenotypic switching of vascular smooth muscle cells

In hypoxia, the highly plastic VSMCs switch from a contractile to a synthetic phenotype, which is characterised by increased proliferation and extracellular matrix deposition [18]. Differentiated smooth muscle cells express a repertoire of contractile proteins, signalling molecules and receptors for their primary function of vessel contraction. These contractile VSMCs have little capacity for proliferation, protein synthesis or migration [18]. However, pulmonary VSMCs, under chronic hypoxic stimulation, switch to a synthetic state exhibiting hypertrophy, hyperplasia, loss of contractility and migration, contributing to the enlargement of the arterial intimal layer (Figure 1) and in the muscularisation of non-muscular pulmonary vessels [9]. Additionally, there is a deposition of collagen and elastic fibres. In extreme cases, the excessive VSMC proliferation can progress from vascular lesions to calcification. These phenomena seem to correlate with the degree of PH extent and COPD severity [19–21].

The endothelial dysfunction that takes place in PH may also contribute to the dedifferentiation and proliferation of VSMCs [22]. Specifically, dysregulated endothelial cells can cause alterations in AKT signalling in VSMCs, which in turn triggers their phenotypic switch [23]. This pathway is also affected by aberrant regulation of the mechanistic target of rapamycin (mTOR) pathway (discussed later in this chapter).
4. Hypoxia and pulmonary hypertension

The major cellular oxygen-sensing mechanism implicated in hypoxia-induced pulmonary hypertension is the hypoxia-inducible factor (HIF) pathway. HIFs are transcription factors that induce the activation of some several hundred genes in response to hypoxia [24]. Initially identified as regulators of erythropoietin (EPO), the hormone responsible for increased red blood cells in response to low oxygen levels, HIFs have since been found to regulate expression of genes that are important for angiogenesis, cellular metabolism, cardiovascular development and cardiovascular control [24–26].

In low oxygen conditions, HIFs bind DNA as heterodimeric complexes of alpha (HIF-α) and beta (HIF-β) subunits, with HIF-α being the subunit regulated by oxygen tension [27]. Higher animals have a series of isoforms for each of the HIF subunits as a result of gene evolutionary duplications [24]. In humans, there are three paralogs of HIF-α—HIF-1α, HIF-2α and HIF-3α—with the first two members being the best characterised [24, 25]. The expression of HIF-1α and HIF-2α is differentially regulated, while their balance is believed to be important for tissue-specific differences in oxygen sensing [25]. They both bind to the same DNA consensus (RCGTG) in hypoxia-response elements of the genome, but they only induce partially overlapping sets of genes [27, 28].

In normoxic conditions, the HIF-α subunit is hydroxylated by Fe(II) prolyl hydroxylase domain (PHD) enzymes (PHD1, PHD2 and PHD3 or otherwise known as Egln2, Egln1 and Egln3) that use 2-oxoglutarate and Fe$^{2+}$ as substrates [29]. After hydroxylation by PHDs, HIF-α is recognised and bound by the von Hippel-Lindau (VHL) protein, a ubiquitin E3 ligase, which marks HIF-α for proteasomal degradation. In hypoxia, PHD enzymes are inactive allowing HIF-α subunits to translocate to the nucleus and activate HIF target genes. HIFs are further regulated by factor-inhibiting HIF (FIH)-mediated asparaginyl hydroxylation, which impairs their recruitment to transcriptional complexes [30].

Mouse models of HIF-1α and HIF-2α have illustrated that the HIF pathway is critically important for the pulmonary hypoxic response and the development of PH. Heterozygous deficiency of either HIF-1α or HIF-2α allele in mice does not affect their life span, and these animals are largely normal in unstressed, normal oxygen conditions. In response to chronic hypoxia (10% for 3 weeks), HIF-1α$^{+/−}$ mice exhibit an attenuated PH with a low rise in right ventricular pressure and right ventricular hypertrophy [31]. Interestingly, heterozygous HIF-2α$^{+/−}$ mice, exposed to 10% oxygen for 10 weeks, showed a complete lack of any PH manifestation [32]. Of note, animals with hetero- or homozygous mutations in stabilising HIF-2α spontaneously developed progressive PH [33]. These studies all indicate a pathological role of both HIF-α subunits in PH development.

Cell-type-specific inactivation of HIF-α with the use of a variety of promoters has also been studied but with some variable results, which may be due to the method of HIF-α manipulations and/or the use of different mouse strains [34–36]. Nevertheless, there seems to be a clear link between HIFs and PH, since studies from human genetics, including several populations that have adapted to different altitudes, have demonstrated the importance of HIF-2α in pulmonary response to hypoxia and PH pathophysiology [37].
The Tibetans, who have lived for at least 25,000 years in 4000 m elevation and continuously inspired partially pressured oxygen (~80 mmHg), have been identified to have a number of single-nucleotide polymorphisms in close-to-one-another loci near the gene EPAS1, which encodes HIF-2α [38]. HIF-2α is the subunit responsible for EPO regulation and in turn erythropoiesis. Tibetans manifest blunted PH and reduced erythropoiesis at high altitude. At sea level, they manifest a lower pulmonary arterial pressure in response to hypoxia when compared with other populations [39, 40]. Recently, a missense mutation in PHD2 (EGLN1) was identified which allows for increased PHD2 activity under hypoxic conditions, thereby decreasing HIF-α stabilisation and reducing erythropoiesis at altitude [41].

Further evidence for a role for HIF-2α in PH comes from another human genetic study, which showed that an activating HIF-2α mutation (G→A substitution in position 2097) caused erythrocytosis with elevated total red cell volume and PH in an affected family [42].

5. VHL and pulmonary hypertension

The VHL protein is a tumour suppressor and an essential component for the clearance of HIF-α through the ubiquitin-proteasomal degradation pathway [24, 43]. A number of VHL mutations have been described that result in aberrant induction of HIF target genes, due to the loss of function of VHL and in turn to the loss of HIF-α regulation. VHL mutations are associated with VHL syndrome, which is a hereditary condition, characterised by highly vascularised tumours within specific tissues, including the renal, retinal and central nervous system [44]. However, a small number of VHL mutations (R200W, D126N, S183L, D126E) are associated with development of Chuvash polycythemia (CP) [45–47]. CP is a rare autosomal recessive condition that is endemic to the population in Chuvashia, Russia and in the island of Ischia, Italy [46, 48]. Chuvash patients manifest increased haemoglobin and haematocrit with elevated levels of EPO, as well as increased expression of vascular endothelial growth factor (VEGF) and ET-1, which are HIF-α target genes [45–49]. In addition, these patients are highly susceptible to both arterial and venous thrombosis and can develop mild to severe PH [45–49].

The importance of HIF-2α isoform in the regulation of pulmonary vascular control has also been demonstrated by the use of a mouse model of CP [50]. This model carries a hypomorphic VHL allele (with an R200W substitution) and recapitulates all symptoms of the human CP phenotype. Interestingly, when these mice are crossed with HIF-2α+/− or HIF-1α+/− strains for heterozygous deficiency in either of the two HIF-α, they manifest an ameliorated PH phenotype for suppressed HIF-2α, but not for HIF-1α.

Comparison of CP and HIF-2α gain-of-function mutation human phenotypes has additionally shown that the latter condition somehow manifests more moderate symptoms than the first. The explanation for this may be that, in CP, both HIF-α subunits are upregulated, and therefore, there may be an additive effect [51]. Furthermore, VHL has a number of HIF-α-independent functions that may also play a role in the CP phenotype.
6. New advances: hypoxic induction of zinc transporters

Zinc, an essential dietary element, plays an important cytoprotective role for the lung by sheltering the pulmonary epithelium from extrinsic activation of apoptotic pathways following acute lung injury [52]. Zinc transporters are responsible for zinc cellular uptake and homeostasis [53]. A recent linkage analysis study that compared a PH-resistant rat strain, Fisher 344 (F344), with the Wistar Kyoto (WKY) strain identified the gene Slc39a12, which encodes the ZIP12 zinc transporter, as a major regulator of hypoxia-induced pulmonary vascular remodelling [53]. In the F344 strain, this gene lacks a crucial thymidine, which leads to a frameshift mutation in exon 11 and renders translation of the protein redundant. ZIP12 is normally expressed in endothelial, interstitial and VSMCs, but its expression increases in remodelled pulmonary vessels following hypoxia-induced PH [53]. ZIP12 is likely a HIF target gene since both HIF-1α and HIF-2α were detected bound to ZIP12 hypoxia-response element. The investigators of this study further generated a ZIP12−/− rat model for comparison with the original F344 and WKY strains and found that genetic disruption of ZIP12 recapitulates the phenotype of the PH-resistant F344 strain under conditions of hypoxia.

Zinc-binding motifs have been considered as potential PH drug-therapeutic targets with phosphodiesterase type 5 (PDE5) and histone deacetylases as examples [54, 55]. Zinc is a structural component of a number of intracellular enzymes, transcription factors, other proteins and cofactors and is a putative drug target for PH.

7. Role of hypoxia-inducible microRNAs in pulmonary hypertension

MicroRNAs (miRNAs) are small non-coding RNA molecules (about 21 nucleotides long) that regulate gene expression post-transcriptionally. Hypoxic stimulation of a variety of human cell types has shown induction of more than 90 miRNAs [56], with altered expression of some of these miRNAs involved in VSMC remodelling and endothelial cell dysfunction in PH [57].

MiRNAs that have been causally implicated in PH include miR-204, miR-138, miR-21 and miR-130/miR-301, among others (Table 1). MiR-204 has been shown to be downregulated in VSMCs of patients suffering from PH, as well as in mouse models of the disease [58, 59]. The degree of miR-204 suppression has been found to be inversely proportional to the degree of pulmonary artery resistance and pressure, while compensating for the loss of miR-204 through nebulisation in PH patients has been shown to reverse the VSMC proliferative and anti-apoptotic phenotype [59]. MiR-204 is involved in the activation of the nuclear factor of activated T cell (NFAT) pathway, the Rho pathway, VSMC proliferation and resistance to apoptosis, as well as downregulation of transcripts such as bone morphogenetic protein receptor type II (BMPR2) and interleukin-6 (IL-6) [60–62]. Also, miR-204 regulates the expression of the Runt-related transcription factor 2 (RUNX2), which has been shown to stabilise HIF-1α in chondrocytes by competing with VHL [20, 63]. In the context of hypoxia, RUNX2 is upregulated, since miR-204 is downregulated, and therefore sustains HIF-1α activation,
which in turn contributes to aberrant VSMC proliferation, resistance to apoptosis and their transdifferentiation to osteoblast-like cells [20].

MiR-138 is upregulated by hypoxia and suppresses HIF-1α [64]. However, its upregulation also contributes to endothelial cell dysfunction in PH by downregulating the small EF-hand Ca\(^{2+}\)-binding protein S100A1 that relays Ca\(^{2+}\) oscillations, controlling vascular tone responses [64].

MiR-21 expression has been found to be upregulated in both pulmonary VSMC and endothelial cells during hypoxic conditions [61, 65]. This upregulation, in turn, leads to downregulation of programmed cell death protein 4 (PDCD4), sprouty homolog 2 (SPRY2) and peroxisome proliferator-activated receptor-α (PPARα), which when dysregulated play a role in the increased proliferation and resistance to apoptosis [65–67]. Treatment of mice with anti-miR-21 during hypoxia showed an improvement in distal pulmonary artery muscularisation [69]. However, miR-21 has also been shown to have a protective effect during PH [61]. Using VHL-null mice, IL-6 transgenic mice, pulmonary vessels from patients with PH as well as deficient (miR-21\(^{−/−}\)) or miR-21 overexpression (miR-21\(^{+/+}\)) mouse models, it has been demonstrated that miR-21 loss of function causes onset of PH [61]. Specifically, miR-21 deletion showed exaggerated pulmonary vascular remodelling, whereas in mice overexpressing miR-21, these disease-associated phenotypes were abolished [61].

The family of miR-130/301 is also upregulated in pulmonary VSMCs and the endothelium in hypoxia, as well as in the lungs of mice with PH due to chronic hypoxic exposure [68]. This upregulation is mediated by HIF-2α and Oct-4. MiR-130/301 is a master regulator miRNA subordinating other miRNA pathways, and, for instance, it suppresses miR-204 [68].

miR-223, miR-17, miR-130, miR-145, miR-424 and miR503 are also involved in the pathophysiology of PH (reviewed in Ref. [70]). So far, PH animal models have helped greatly in these studies, but the exact role and balance for each of these miRNAs in human PH have not been fully elucidated.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Change in PH</th>
<th>Target transcripts</th>
<th>Cellular function, process or pathway affected</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-204</td>
<td>↓</td>
<td>BMPR2, IL-6, RUNX2 among others</td>
<td>Activation of NFAT pathway, VSMC proliferation, resistance to apoptosis, Rho pathway, HIF-1α pathway</td>
<td>[20, 58–63]</td>
</tr>
<tr>
<td>miR-138</td>
<td>↑</td>
<td>HIF-1α, S100A1</td>
<td>HIF-1α pathway, endothelial regulation of vasomotor tone</td>
<td>[64]</td>
</tr>
<tr>
<td>miR-21</td>
<td>↑</td>
<td>PDCD4, SPRY2, PPARα</td>
<td>VSMC proliferation, resistance to apoptosis</td>
<td>[61, 65–67]</td>
</tr>
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8. mTOR signalling in hypoxia-induced pulmonary hypertension

Mechanistic target of rapamycin (mTOR) is a cellular hub that controls growth factor signalling and nutrient sensing to regulate cell growth, proliferation, metabolism and survival [71]. mTOR is a protein kinase that is the catalytic component of two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [72, 73]. mTORC1 is composed of mTOR, Raptor, LST8/GβL, PRAS40 and DEP domain containing mTOR-interacting protein (DEPTOR), and its activity is stimulated by growth factor signals to regulate protein synthesis through 4E-BP1/BP2 and the S6 kinases, S6K1 and S6K2 [74, 75]. By contrast, mTORC2, which comprises mTOR, Rictor, LST8/GβL, DEPTOR, SIN1 and PRR5, regulates cytoskeletal organisation [76, 77] and has a role in phosphorylation of protein kinase C (PKC), protein kinase B (PKB) and serum- and glucocorticoid-induced protein kinase (SGK) to promote cell survival and cell cycle progression [78–80].

Aberrant mTOR activity has a well-characterised role in promoting proliferative diseases including cancer and smooth muscle cell pathologies [71]. mTORC1 signalling is activated following vascular injury promoting Vinhibitor, rapamycin, promotes smooth muscle cell (SMC) remodelling. Accordingly, mTOR inhibitors are widely used in drug-eluting stents to prevent restenosis. In addition, mTOR also regulates the differentiation state of VSMCs since the mTOR inhibitor, rapamycin, promotes SMC differentiation and expression of contractile proteins [81]. mTORC1 activity is low in differentiated contractile VSMCs but becomes activated by growth factors and is thought to contribute to the change towards a synthetic phenotype that is characterised by increased SMC proliferation and migration. As such, rapamycin analogues may have therapeutic potential for treating PH.

The relationship between hypoxic conditions and mTOR is complex and depends, in part, on cellular context. Many cell types respond to prolonged periods of hypoxia by inactivating energy-intensive processes such as protein synthesis and proliferation, and accordingly mTOR is downregulated [82]. By contrast, the vasculature responds to long-term hypoxia by promoting new blood vessel growth—angiogenesis, which in turn, restores O₂ to deprived tissues. Hypoxic stress is a key driving force in the vascular remodelling observed in pulmonary hypertension, and HIFs activate pulmonary artery endothelial and smooth muscle cell proliferation, which is mediated by both mTORC1 and mTORC2 [83–85]. Currently, the mechanisms by which hypoxia/HIFs signal to activate mTOR in ECs and VSMCs are poorly understood [86–90].

9. Conclusion

Severe PH associated with hypoxic lung disease is a life-threatening condition with poor survival rates. Despite significant advances in targeted therapeutics for PH, randomised clinical trial data for this particular group of patients are scarce, and it is not clear whether endothelin receptor antagonists will benefit patients with hypoxia-associated PH. Importantly, recent genetic studies identifying mutations in the oxygen-sensing machinery have provided new mechanistic insights into the aetiology of PH. Further studies are required to determine whether specific targeting of HIF-2α will provide additional therapeutic benefit for this complex disease.
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