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TOPICAL REVIEW

Molecular mechanisms of hypoxia-inducible factor-induced pulmonary arterial smooth muscle cell alterations in pulmonary hypertension

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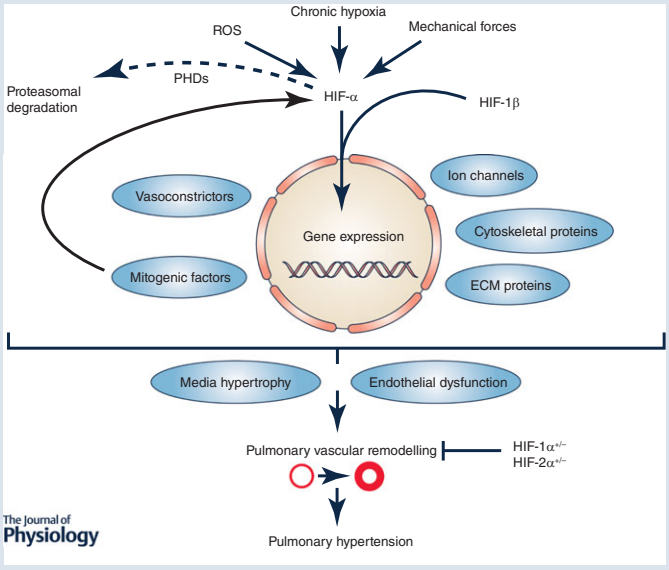

Abstract Oxygen (O_2) is essential for the viability and function of most metazoan organisms and thus is closely monitored at both the organismal and the cellular levels. However, alveoli often encounter decreased O_2 levels (hypoxia), leading to activation of physiological or pathophysiological responses in the pulmonary arteries. Such changes are achieved by activation of transcription factors. The hypoxia-inducible factors (HIFs) are the most prominent hypoxia-regulated transcription factors in this regard. HIFs bind to hypoxia-response elements (HREs) in the

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promoter region of target genes, whose expression and translation allows the organism, amongst other factors, to cope with decreased environmental O_2 partial pressure (pO_2). However, prolonged HIF activation can contribute to major structural alterations, especially in the lung, resulting in the development of pulmonary hypertension (PH). PH is characterized by a rise in pulmonary arterial pressure associated with pulmonary arterial remodelling, concomitant with a reduced intravascular lumen area. Patients with PH develop right heart hypertrophy and eventually die from right heart failure. Thus, understanding the molecular mechanisms of HIF regulation in PH is critical for the identification of novel therapeutic strategies. This review addresses the relationship of hypoxia and the HIF system with pulmonary arterial dysfunction in PH. We particularly focus on the cellular and molecular mechanisms underlying the HIF-driven pathophysiological processes.

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Abstract figure legend Schematic review summary. Hypoxia, reactive oxygen species (ROS) and mechanical forces can lead to hypoxia-inducible factor (HIF) stabilisation, its dimerisation with HIF-1β and nuclear translocation. Binding of HIF to the promoter region of target genes initiates altered expression of such genes, including vasoconstrictors, mitogenic factors, cytoskeletal proteins, extracellular matrix (ECM) proteins and ion channels. Among them mitogenic factors can themself influence HIF expression. Imbalance between vasoconstrictors/vasodilators and pro-/anti-proliferative mitogenic factors characterises endothelial dysfunction concomitant with media hypertrophy, pulmonary vascular remodelling and finally pulmonary hypertension. However, mice with partial HIF-1α or HIF-2α deficiency (HIF-1$\alpha^{+/-}$; HIF-2$\alpha^{+/-}$) possess reduced pulmonary vascular remodelling and pulmonary hypertension in comparison to controls. Under normoxic conditions HIF-α can be hydroxylated by prolyl hydroxylase domain proteins (PHDs) marking HIF-α for proteasomal degradation.

Biological role of oxygen in the lung

Ambient air contains 21% O_2 at sea level, which corresponds to approximately 160 Torr. The O_2 concentration drops to ~14% O_2 (100 Torr) in the pulmonary alveoli (Semenza, 2001). Finally, the partial pressure of O_2 (pO_2) in arterial and venous blood is ~100 Torr (~14% O_2) and ~40 Torr (6% O_2), respectively (Prabhakar & Semenza, 2012). Moderate hypoxia (10% O_2; simulated altitude of 5000 m) corresponds to 75–82 Torr (Suzuki *et al.* 1999). Following hypoxic exposure alveolar O_2 concentration drops to ~40 Torr, and thus the pO_2 in arterial blood is ~40 Torr (Bland *et al.* 1977).

Cells rely on O_2 for energy production via oxidative phosphorylation in the mitochondrial respiratory chain. However, precise regulation of cellular pO_2 is necessary to minimize the production of reactive oxygen species (ROS), which are associated with cellular damage (Semenza, 2000*a*). Under acute hypoxic conditions (ranging from seconds to minutes) homeostatic mechanisms in the cardiovascular and respiratory systems (Semenza, 2004) are altered, primarily causing changes in cellular redox state, signalling, protein configuration and/or phosphorylation (Semenza, 2000*b*). These alterations also lead to hypoxic pulmonary vasoconstriction (HPV), an essential mechanism used to adapt pulmonary blood flow to the local alveolar ventilation situation. During regional alveolar hypoxia, HPV shifts blood flow from less ventilated and thus hypoxic areas of the lung to better oxygenated areas, optimizing and maintaining gas exchange. However, under generalized hypoxia, HPV results in constriction of precapillary pulmonary arteries, increasing pulmonary vascular resistance and thus contributing to PH. In addition, if hypoxia is chronic (ranging from hours to days), gene expression is changed (Semenza, 2004), leading to pulmonary arterial remodelling and PH. This remodelling process is mainly characterized by hypertrophy of the artery media caused by proliferation of the vascular smooth muscle cells starting around day 4 of hypoxia (Paddenberg *et al.* 2007) and is established after 3 weeks of hypoxic exposure (Semenza, 2001; Taraseviciene-Stewart *et al.* 2001). Prominent mediators of pulmonary vascular remodelling are the hypoxia-inducible factors (HIFs).

The HIF system

The HIFs are heterodimeric transcription factors, consisting of an α and a β subunit. The β subunit is identical to the aryl hydrocarbon nuclear translocator (ARNT). Both subunits are continuously transcribed and translated and belong to the PAS family of basic helix–loop–helix (bHLH) transcription factors (Wang *et al.* 1995). Changes in pO_2 show no influence on either HIF-α or HIF-β mRNA expression (Huang *et al.* 1996). Moreover, at the protein level, HIF-β is constitutively

expressed and not affected by pO_2. In contrast, the HIF-α protein is the regulatory subunit and remarkably unstable in normoxic (21% O_2 at sea level) cells (Wang *et al.* 1995). The half-life is about 5 min (Huang *et al.* 1996) under normoxic conditions. However, under hypoxia the abundance of HIF-α protein is increased.

Under normoxic conditions HIF-α is hydroxylated by prolyl hydroxylase domain proteins (PHDs) at conserved prolines 402 and 564 (Epstein *et al.* 2001; Ivan *et al.* 2001) in the oxygen-dependent degradation (ODD) domain (Huang *et al.* 1998). Removal of the entire ODD domain renders HIF-α stable even in normoxic cells, resulting in heterodimerization, DNA binding and transactivation, independent of hypoxia (Huang *et al.* 1998). PHDs utilize molecular O_2, Fe(II) and 2-oxoglutarate as co-substrate for hydroxylation (Epstein *et al.* 2001). HIF-α hydroxylation is recognized by the von Hippel-Lindau tumour suppressor protein (pVHL), a component of the multi-subunit E3 ubiquitin ligase complex (Ivan *et al.* 2001), which marks the HIF-α complex for proteasomal degradation (Huang *et al.* 1998). Under hypoxic conditions, however, HIF-α hydroxylation by PHD is inhibited (Epstein *et al.* 2001). HIF-α accumulates and translocates to the cell nucleus where it dimerizes with the HIF-β subunit, forming with transcriptional co-activators (CREB-binding protein (CBP) and p300) a functional transcription factor (Martin *et al.* 2010). Binding of the transcription factor via its bHLH domain to the HREs initiates expression of hypoxia-specific genes (Fig. 1). To date, several thousand human HIF target genes have been identified (Semenza, 2014). Amongst others they ensure sufficient O_2 delivery (angiogenesis), metabolic adaptations (genes involved in glycolytic pathways) and erythropoiesis (O_2 transport), cell proliferation and vascular remodelling (Semenza, 2000*b*, 2011).

Thus far, three ubiquitously expressed HIF-α subunits are known with variable tissue expression and abundance, but similar O_2-dependent regulation, at least for short hypoxic exposure (Heidbreder *et al.* 2003; Clerici & Planes, 2009). HIF-1α is present in all nucleated cell types, HIF-2α expression is restricted to the vascular endothelium and type II pneumocytes (Wiesener *et al.* 2003), whereas HIF-3α can be found in the cortex, hippocampus, lung, heart, liver and kidney (Heidbreder *et al.* 2003). HIF-3α expression is induced by HIF-1 in hypoxic cells, suggesting that HIF-3α may act as a negative-feedback factor attenuating HIF-1 activity during prolonged hypoxia to prevent overshoot phenomena (Makino *et al.* 2007; Augstein *et al.* 2011). In addition, over-expression of HIF-3α decreases the hypoxia-induced increase in the HIF-1α target gene VEGF (Augstein *et al.* 2011).

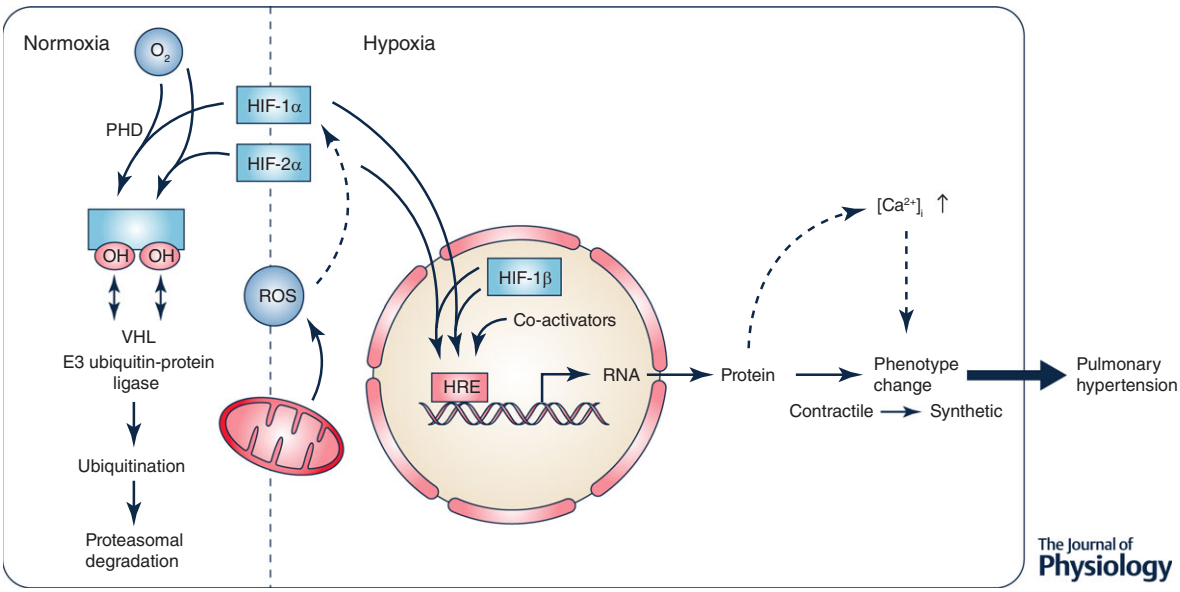

Figure 1. Schematic diagram displaying the regulatory mechanisms of HIF activation in pulmonary hypertension
Under hypoxic conditions the HIF-1α and HIF-2α subunits are stabilized, among others by reactive oxygen species (ROS). They translocate from the cytoplasm to the nucleus where they dimerize with the β-subunit forming, together with co-activators, an active transcription factor. HIF binding to the hypoxia response elements (HREs) allows the expression of HIF target genes, contributing to the pathogenesis of pulmonary hypertension. During normoxia, HIF hydroxylation carried out by prolyl-hydroxylase (PHD) targets the α-subunit for proteasomal degradation through the von Hippel–Lindau (VHL) protein, a member of the E3 ubiquitin proteasome ligase family. However, HIFs might also be stabilized by ROS under normoxic conditions leading to dimerization translocation and expression of target genes.

HIF-1α and HIF-2α share high sequence homology, whereas the HIF-3α sequence is quite different. HIF-3α, produces multiple splice variants that contain extra DNA binding elements and protein–protein interaction motifs (Maynard *et al.* 2005). Nevertheless, all HIF-α subunits are subjected to the same PHD-dependent degradation machinery.

The HIF-1 subunit has been most extensively studied. It was identified in 1991 as a protein that bound, under hypoxic conditions, to the HRE of the erythropoietin (EPO) gene (Semenza *et al.* 1991), which is critically involved in red blood cell production.

HIF-induced pulmonary arterial smooth muscle cell dysfunction in pulmonary hypertension

PH is a life threatening disease with multifactorial causes. It is well established that prolonged exposure to hypoxia due to chronic lung diseases or residence at high altitude leads to PH development (Arias-Stella & Saldana, 1963; Stenmark *et al.* 2006); however, short exposure to hypoxia induces a tremendous change in gene expression (Kwapiszewska *et al.* 2005). PH is characterized by a pronounced pulmonary arterial remodelling process, leading in the case of chronic hypoxia to muscularization of previously non-muscularized arteries and an increase in the degree of muscularization of already muscularized pulmonary arteries. In this regard, pulmonary arterial smooth muscle cell (PASMC) hyper-proliferation in the media layer of the pulmonary artery is suggested to be the key event in vascular remodelling and the main determinant of elevated pulmonary vascular resistance in hypoxia-induced PH (Pak *et al.* 2007).

Recently, considerable work has been done to investigate the molecular and cellular responses to alveolar hypoxia. The direct impact of the HIF system on PH pathogenesis has been confirmed *in vivo*, using haplo-insufficient HIF-1α and HIF-2α knockout mice. Homozygous HIF-1α and HIF-1β mice are not viable due to cardiac and vascular defects (Iyer *et al.* 1998). Loss of HIF-2α causes fetal death in 50% of embryos (Compernolle *et al.* 2002; Brusselmans *et al.* 2003). However, mice with partial HIF-1α or HIF-2α deficiency are viable and develop normally. When exposed to chronic hypoxia (10% O_2) the outcome of these mice is improved due to a smaller increase in pulmonary arterial pressure, right ventricular hypertrophy and pulmonary vascular remodelling, as compared to respective controls (Yu *et al.* 1999; Brusselmans *et al.* 2003), suggesting a role of the HIF system in the impaired development of PH (Yu *et al.* 1999). Recent data in PASMCs also indicate that HIF-1α regulates the expression of an ion channel subunit that mitigates vasoconstriction (Ahn *et al.* 2012). Finally, the role of HIF-1α in modulating pulmonary vascular tone and pulmonary vascular remodelling was addressed in more detail using smooth muscle cell specific inducible HIF-1α knockout mice. This study showed that selective deletion of HIF-1α leads to attenuated pulmonary vascular remodelling and pulmonary hypertension in chronic hypoxic mice. However, right ventricular hypertrophy was unchanged despite attenuated pulmonary pressures (Ball *et al.* 2014), suggesting that right heart hypertrophy in the absence of PH may be caused by a direct cardiac HIF induction, independent of elevated pulmonary artery pressure. However, others observed that a complete loss of HIF-1α in smooth muscle cells raised right ventricular systolic pressure in normoxic and hypoxic mice, compared to respective wild-type littermates. The number of muscularized arteries in murine lungs was not changed. Moreover, myosin light chain (MLC) phosphorylation, which determines vascular tone, was higher in PASMCs isolated from smooth muscle specific HIF-1α knockout mice compared to control, during both normoxia and after acute hypoxia. Thus, results from this group indicate a primary role for smooth muscle HIF-1α in modulating vascular tone specifically, and not vascular remodelling (Kim *et al.* 2013). By using HIF-2α overexpressing mice, caused by a single missense mutation in HIF-2α, a clear association of HIF-2α with the development of PH in mice was shown (Tan *et al.* 2013).

In addition, there are studies investigating the indirect effect of HIF on PH pathogenesis. In this regard, mice with mutation of the von Hippel–Lindau (VHL) tumour suppressor protein, a Chuvash polycythaemia disease model, possess elevated HIF-2α levels and increased pulmonary arterial pressure and pulmonary vessel muscularization (Hickey *et al.* 2010). The role of HIF in PH is also strengthened by (1) elevated HIF-2α protein expression in Fawn-hooded rats, (2) enhanced HIF-1α levels in plexiform lesions of PH patients, and (3) increased HIF-1α expression in the pulmonary vasculature of chronic hypoxic mice (Tuder *et al.* 2001; Bonnet *et al.* 2006; Mizuno *et al.* 2011). However, in rat pulmonary arteries HIF-1α protein levels decline with increasing time of hypoxia (Jiang *et al.* 2007), indicating that there might be species-dependent differences in HIF stability in chronic hypoxia or that other factors than hypoxia contribute to HIF stabilization.

Recent *in vitro* findings from our group indicate enhanced PASMC proliferation following hypoxic exposure or HIF-1α over-expression, compared to control (Malczyk *et al.* 2013; Veith *et al.* 2014). Moreover, our data indicate that the contractile PASMC phenotype is replaced by a synthetic phenotype during hypoxia (Veith *et al.* 2014).

Thus, while a global decrease of HIF-1α may mitigate against the development of pulmonary hypertension, the specific role in PASMCs needs further investigation, and HIF-1α stabilization in cell types other than PASMCs should also be considered as potential contributors to

hypoxia-induced remodelling/muscularization, e.g. via HIF-1α mediated induction of growth factors.

HIF-regulated ion channel expression in pulmonary hypertension

Both pulmonary vascular remodelling and active PASMC contraction can cause a decrease in lumen diameter, and an increase in pulmonary vascular resistance and pulmonary arterial pressure, resulting in PH. Several studies indicate the involvement of the HIF system in altered ion channel and ion transporter expression leading to contraction of the pulmonary vasculature.

Hypoxia and HIF-dependent regulation is proposed for classical transient receptor potential (TRPC) 1 and TRPC6 proteins in murine and rat PASMCs (Wang *et al.* 2006; Malczyk *et al.* 2013). TRPCs are non-selective cation channels, permeable to Ca^{2+} ions. An increase in cytosolic free Ca^{2+} concentration is suggested as a major trigger for PASMC proliferation in PH. In this regard, TRPC1 silencing by specific siRNA transfection or TRPC1 knockout impaired hypoxia-induced PASMC proliferation *in vitro*. In contrast, TRPC6 has no effect on PASMC hyperplasia and pulmonary vascular remodelling, but is necessary for acute hypoxic responses (pulmonary vasoconstriction) (Weissmann *et al.* 2006; Malczyk *et al.* 2013).

Previous studies indicate that alterations in PASMC pH homeostasis are crucial for the development of hypoxia-induced pulmonary hypertension. In line with this hypothesis, hypoxia and HIF-1α-dependent regulation of the Na^+/H^+ exchanger isoform 1 (NHE1) was observed (Shimoda *et al.* 2006), leading to an alkaline shift in pH. Both activation of the Na^+/H^+ exchanger and an alkaline shift in pH are suggested to be required for PASMC proliferation in response to growth factors (Quinn *et al.* 1996).

In addition to TRPC and NHE, the family of voltage-gated K^+ (K_v) channels $K_v1.5$ and $K_v2.1$ have altered expression following hypoxic exposure. Chronic hypoxia reduces K^+ currents in PASMCs (Wang *et al.* 1997), leading to membrane depolarization, activation of voltage-dependent Ca^{2+} channels (VDCCs) and Ca^{2+} influx. However, in PASMCs isolated from heterozygous HIF-1α mice, the reduction in voltage-gated K^+ currents following chronic hypoxia was absent (Shimoda *et al.* 2001), indicating a role of HIF-1α activation in regulating both pulmonary vascular remodelling and vascular tone.

The HIF system in endothelial dysfunction underlying PH

Plexiform lesions are a hallmark of severe PH in humans. They are characterized by a dysregulated proliferation of pulmonary arterial endothelial cells and/or decreased cell death (Fig. 2), leading to vascular occlusions. HIF-1α and HIF-1β are highly expressed in endothelial cells of plexiform lesions in patients with severe PH (Tuder *et al.* 2001). Thus, they might play a critical role in the expression of mitogenic factors and vasoconstrictors and their receptors such as vascular endothelial growth factor (VEGF), placental growth factor (PGF), platelet-derived growth factor (PDGF), angiopoietin-1 and -2 (ANGPT1 and -2) and serotonin (5-HT) (Kelly *et al.* 2003; Esteve *et al.* 2007; Haugen *et al.* 2012). Similar results were recently obtained with cultured endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension. These cells have greater proliferation rate and decreased apoptosis, higher levels of phosphorylated STAT3 and increased expression of its downstream pro-survival target, Mcl-1 (Masri *et al.* 2007).

In contrast to endothelial cells, hypoxic smooth muscle cells possess elevated VEGF levels, but decreased ANGPT2 expression and no changes in PDGF and ANGPT1 expression (Kelly *et al.* 2003), indicating that each cell type shows a different expression pattern of HIF target genes.

There is controversy as to whether angiogenesis increases or decreases in PH. Some groups report elevated hypoxia-induced angiogenesis in the pulmonary circulation (Howell *et al.* 2003), while others do not (Hislop & Reid, 1976; Rabinovitch *et al.* 1979; Partovian *et al.* 2000). However, it is well established that chronic hypoxia induces expression of VEGF, a potent inducer of angiogenesis, in a HIF-1α and/or HIF-2α dependent manner (Tuder *et al.* 1995; Clerici & Planes, 2009).

In addition to the HIF-dependent regulation of mitogens, growth factors (PDGF, epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2) and thrombin), cytokines and vasoactive substances (endothelin-1) themselves can induce HIF-1 target gene expression via the mitogen-activated protein kinase and/or phosphatidylinositol 3-kinase signal transduction pathways (Laughner *et al.* 2001; Fukuda *et al.* 2003; Schultz *et al.* 2006; Whitman *et al.* 2008), thus utilizing a positive feedback loop to amplify the HIF activity.

Crosstalk of the HIF system and reactive oxygen species

Currently, there is intense debate about whether there is an increase or a decrease in ROS in hypoxia-induced PH (Ward, 2006; Weir & Archer, 2006). We and others have evidence that reduced O_2 levels can lead to increased ROS levels in acute and chronic hypoxia (Chandel *et al.* 1998; Liu *et al.* 2006; Mittal *et al.* 2007; Nozik-Grayck & Stenmark, 2007; Sanders & Hoidal, 2007; Ismail *et al.* 2009; Nisbet *et al.* 2009; Waypa & Schumacker, 2010; Weissmann *et al.* 2012).

Accordingly, complex III in the mitochondrial electron transport chain, plasma membrane bound NADPH oxidases or cytochromes (Klimova & Chandel, 2008; Nisbet *et al.* 2009; Weissmann *et al.* 2012; Veit *et al.* 2013) are suggested as ROS sources. Superoxide radicals can be reduced to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide is much more stable than superoxide and can cross cell membranes, and may thus act as a potent signalling molecule. Recently, much literature has emerged concerning the possible role of ROS in HIF induction and PH development (Beckman & Koppenol, 1996; Liu *et al.* 2006; Mittal *et al.* 2007; Clerici & Planes, 2009).

There are several theories as to how ROS can lead to HIF-α stabilization. One theory suggests that hydrogen peroxide can act as a diffusible second messenger, activating redox-sensitive transcription factors, including HIF-1α and the activation and expression of voltage-gated K_v channels (Tuder *et al.* 2007). Another theory is that under hypoxia, mitochondria scavenge available O_2 and PHDs are then deprived of their co-substrate and cannot hydroxylate HIF-α, marking it for proteasomal degradation (Hagen *et al.* 2003). A third possibility is that ROS interfere with the regulation of PHDs. In this regard, hydrogen peroxide might inhibit PHDs by oxidizing non-haem-bound Fe^{2+} to Fe^{3+} (Hagen, 2012). However, other findings postulate low sensitivity of PHDs to hydrogen peroxide (Masson *et al.* 2012).

There is also evidence, that inhibitors of mitochondrial ROS or electron transport chain reduce HIF-1α and HIF-2α protein accumulation (Chandel *et al.* 1998, 2000; Mansfield *et al.* 2005), suggesting that the HIF-α subunit requires a functional electron transport chain for stabilization under hypoxic conditions but not under anoxic conditions (Schroedl *et al.* 2002).

Recently, HIF-1 regulation by non-hypoxia generated ROS has gained considerable interest. In this regard, $CoCl_2$ treatment of normoxic Hep3B cells generates ROS, leading to HIF-1 DNA binding and expression of HIF target genes (Chandel *et al.* 1998). Moreover, angiotensin-II

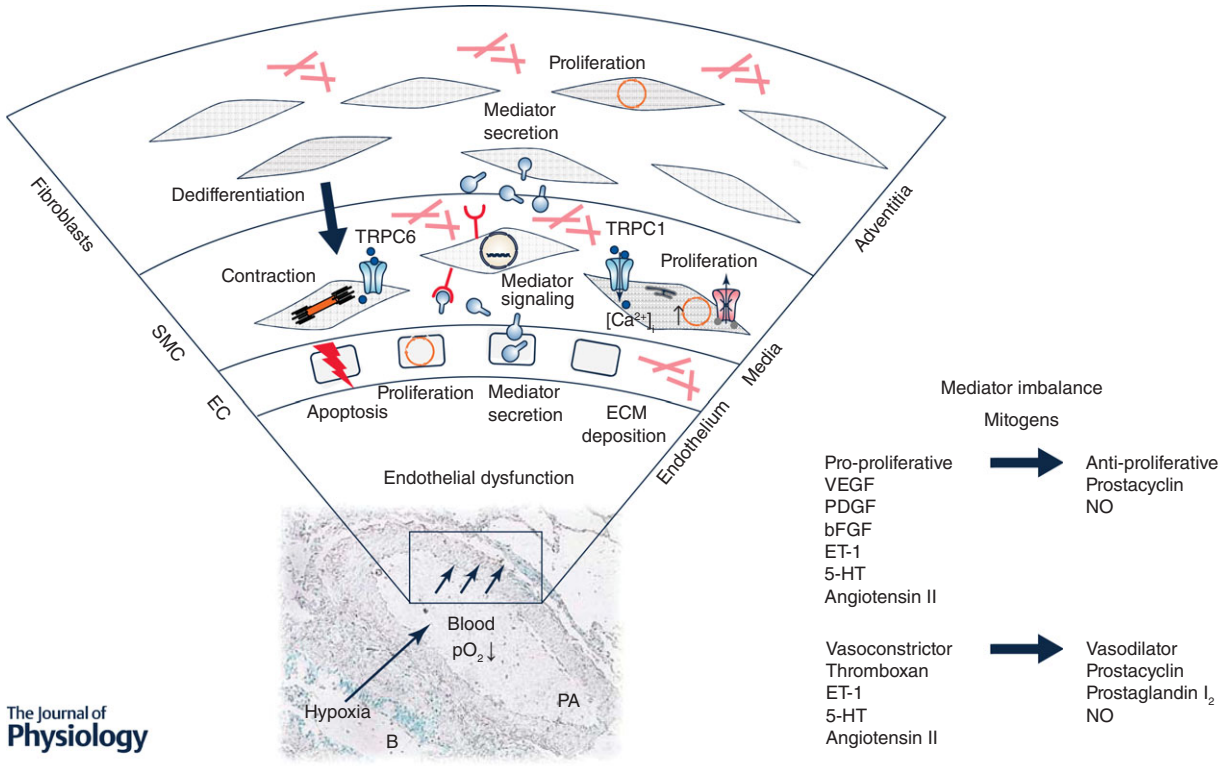

Figure 2. Schematic diagram displaying the molecular mechanisms of vascular alterations in pulmonary hypertension
A reduction in environmental O_2 partial pressure ($pO_2\downarrow$ = hypoxia) leads to less oxygenated blood. Hypoxia is sensed within the pulmonary artery leading to endothelial dysfunction, mediator secretion, changes in gene expression, deposition of extracellular matrix (ECM) proteins, membrane depolarization due to reduced outward K^+ currents and activation of inward Ca^{2+} currents and increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Elevated $[Ca^{2+}]_i$ positively affects contraction of smooth muscle cells (SMC) and as well as cell proliferation. The imbalance between pro- and anti-proliferative mitogens and vasodilators and vasoconstrictors contributes to pulmonary vascular remodelling and pulmonary hypertension. Abbreviations: EC, endothelial cells; B, bronchus; PA, pulmonary artery; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; ET-1, endothelin 1; 5-HT, 5-hydroxytryptamine; NO, nitric oxide.

treatment of vascular smooth muscle cells mediates hydrogen peroxide generation and HIF-1 stabilization under normoxic conditions (Page *et al.* 2002, 2008).

The HIF system and cytoskeletal and extracellular matrix proteins

A growing body of evidence suggests the importance of cytoskeletal proteins in dysregulated PASMC function in PH. In this regard, we identified the cytoskeletal scaffold proteins four-and-a-half LIM domains 1 (FHL-1) and paxillin as novel hypoxia and HIF-1α target genes in primary human PASMCs. Both are up-regulated following hypoxic exposure and negatively affected by HIF-1α silencing (Kwapiszewska *et al.* 2008; Veith *et al.* 2012). However, HIF-2α knockdown affects FHL-1, but not paxillin, mRNA expression (Kwapiszewska *et al.* 2008; and unpublished observations). Along these lines, we observed enhanced paxillin levels following HIF-1α over-expression (Veith *et al.* 2014). In addition, phosphorylation of the cytoskeletal proteins cofilin and paxillin is hypoxia and HIF-1α dependent (Veith *et al.* 2013, 2014). All three of them are predominantly localized in the media of small intrapulmonary vessels, major sites of vascular remodelling in PH (Jeffery & Wanstall, 2001), suggesting them as effectors of HIF-induced PASMC dysfunction.

Cellular copper (Cu) plays an important role in extracellular matrix remodelling. Elevated Cu levels in vascular smooth muscle cells have been demonstrated to be associated with hypoxia-induced PH. In this context, it was shown that the Cu-uptake transporter 1 (CTR1) and the Cu-efflux pump (ATP7A) were both up-regulated in lung tissue and pulmonary arteries of mice with hypoxia-induced PH. The hypoxia-mediated up-regulation of CTR1 is mediated in a HIF-1α-dependent manner as HIF-1α silencing by siRNA transfection attenuated the hypoxia-mediated CTR1 up-regulation. Hypoxia also significantly increased expression and activity of lysyl oxidase (LOX), a Cu-dependent enzyme that crosslinks collagen and elastin in the extracellular matrix and is involved in PASMC proliferation. Crosslinking of extracellular matrix proteins leads to increased pulmonary arterial stiffness which, in combination with the increased PASMC proliferation and migration, further contributes to the development and progression of PH (Zimnicka *et al.* 2014).

Non-hypoxia-driven HIF activation

In addition to hypoxia-induced HIF activation, research is focusing on HIF-1 regulation by non-hypoxic stimuli as, for example, the endothelium is not only exposed to decreased pO_2, but also to mechanical forces due to vessel constriction. In this regard, elevated HIF-1α and HIF-2α levels were detected in stretch-induced but not shear-stress-induced angiogenesis in rat skeletal muscle (Milkiewicz *et al.* 2007). Next to mechanical forces, stimulation with mitogens such as angiotensin II, which is upregulated in PH, increases transcription and functional HIF-1α protein levels (Page *et al.* 2002) as well as increases VEGF expression in vascular smooth muscle cells (Page *et al.* 2002). In addition, it is well established that the non-hypoxia driven ROS generation may also interfere with HIF signalling (Page *et al.* 2002, 2008). Moreover, it was shown that dysfunction of the two Krebs cycle enzymes α-ketoglutarate dehydrogenase and succinate dehydrogenase leads to accumulation of succinate and fumarate which inactivate PHDs and thus stabilize HIF-1α (Isaacs *et al.* 2005; Selak *et al.* 2005).

Therapeutic intervention targeting the HIF–PHD axis

Recently, numerous pharmacological studies have emerged targeting the HIF–PHD axis. In this regard cardiac glycosides, such as digoxin, ouabain or proscillaridin A, inhibit HIF protein synthesis and HIF target gene expression (Zhang *et al.* 2008; Yoshida *et al.* 2010). *In vivo*, digoxin prevents and reverses the development of chronic hypoxia-induced PH in mice (Abud *et al.* 2012). Acriflavine, which is the most potent inhibitor of HIF subunit dimerization (Lee *et al.* 2009), leads to reduced hypoxia-induced PH in rats (Abud *et al.* 2012). Finally, the HIF–PHD axis can be directly targeted by iron supplementation, since PHD activity is iron sensitive (Smith *et al.* 2008). Interestingly, infusion of iron reduced, whereas iron chelation increased, elevated pulmonary arterial pressure in response to hypoxia (Smith *et al.* 2008, 2009).

Summary and conclusions

In summary, hypoxia-activated HIF signalling pathways in part drive the pathogenesis of PH, as they regulate the expression of several genes critical for pulmonary vascular remodelling and disease progression. Understanding the molecular mechanisms of the HIF signalling axis can help to develop new therapeutic approaches for PH treatment.

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Additional information

Competing interests

None declared.

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