The role of inflammation in hypoxic pulmonary hypertension: from cellular mechanisms to clinical phenotypes

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1Developmental Lung Biology, Cardiovascular Pulmonary Research Laboratories, Division of Pulmonary Sciences and Critical Care Medicine, Division of Pediatrics-Critical Care, Departments of Medicine and Pediatrics, University of Colorado, Anschutz Medical Campus, Aurora, Colorado; 2Ludwig Boltzmann Institute for Lung Vascular Research, Graz, Austria; and 3Department of Pediatrics, Division of Gastroenterology, Hepatology, and Nutrition, University of Colorado Denver, School of Medicine, Anschutz Medical Campus, Aurora, Colorado

Submitted 25 August 2014; accepted in final form 19 November 2014

Pugliese SC, Poth JM, Fini MA, Olschewski A, El Kasmi KC, Stenmark KR. The role of inflammation in hypoxic pulmonary hypertension: from cellular mechanisms to clinical phenotypes. Am J Physiol Lung Cell Mol Physiol 308: L229–L252, 2015. First published November 21, 2014; doi:10.1152/ajplung.00238.2014.—Hypoxic pulmonary hypertension (PH) comprises a heterogeneous group of diseases sharing the common feature of chronic hypoxia-induced pulmonary vascular remodeling. The disease is usually characterized by mild to moderate pulmonary vascular remodeling that is largely thought to be reversible compared with the progressive irreversible disease seen in World Health Organization (WHO) group I disease. However, in these patients, the presence of PH significantly worsens morbidity and mortality. In addition, a small subset of patients with hypoxic PH develop “out-of-proportion” severe pulmonary hypertension characterized by pulmonary vascular remodeling that is irreversible and similar to that in WHO group I disease. In all cases of hypoxia-related vascular remodeling and PH, inflammation, particularly persistent inflammation, is thought to play a role. This review focuses on the effects of hypoxia on pulmonary vascular cells and the signaling pathways involved in the initiation and perpetuation of vascular inflammation, especially as they relate to vascular remodeling and transition to chronic irreversible PH.

We hypothesize that the combination of hypoxia and local tissue factors/cytokines (“second hit”) antagonizes tissue homeostatic cellular interactions between mesenchymal cells (fibroblasts and/or smooth muscle cells) and macrophages and arrests these cells in an epigenetically locked and permanently activated proremodeling and proinflammatory phenotype. This aberrant cellular cross-talk between mesenchymal cells and macrophages promotes transition to chronic nonresolving inflammation and vascular remodeling, perpetuating PH. A better understanding of these signaling pathways may lead to the development of specific therapeutic targets, as none are currently available for WHO group III disease.

chronic nonresolving inflammation; fibroblasts; hypoxia; inflammation; hypoxic pulmonary hypertension; macrophages

PULMONARY HYPERTENSION (PH) is not a disease per se but rather a pathophysiological parameter defined by a mean pulmonary artery (PA) pressure (mPAP) exceeding the upper limits of normal (i.e., ≥25 mmHg at rest) (158). PH occurs in a variety of clinical conditions and is associated with a broad spectrum of pathological abnormalities in the PAs of affected patients. However, all patients with PH suffer from exertional dyspnea, marked exercise limitation, and in severe cases right heart failure and death. Because of the diverse causes and mechanisms contributing, PH has been classified into five categories related to common clinical parameters, potential etiological mechanisms, and pathological, pathophysiological, and therapeutic characteristics (229).

The World Health Organization (WHO) five categories of pulmonary hypertension classification are as follows:

• pulmonary arterial hypertension (PAH) (group I)
• pulmonary hypertension due to left heart disease (group II)
• pulmonary hypertension due to lung diseases and/or hypoxia (group III)
• chronic thromboembolic pulmonary hypertension (group IV)
• pulmonary hypertension with unclear multifactorial mechanisms (group V)

The focus of this review will be specifically on the cellular and molecular mechanisms leading to the develop-
ment of PH and pulmonary vascular remodeling in the context of hypoxia and chronic lung disease, herein known as hypoxic PH or WHO group III PH. Although we will briefly highlight the longstanding work in the field demonstrating the effect of hypoxia on resident pulmonary vascular cells, our primary goal is to elucidate the new concepts involving the intertwining roles of hypoxia, inflammation, and their effects on recruited immune and progenitor cells in the setting of hypoxic PH. It is our belief that a better understanding of disease mechanisms in this group of patients will lead to improved targeted therapies.

Clinical Relevance

Over the past 30 years, basic and translational research in the PH field has led to the development of medications that have significantly decreased patient morbidity and also extended life expectancy in patients with WHO group I disease (i.e., PAH, including idiopathic PH, scleroderma PH, HIV PH, etc.) (140). Unfortunately, none of these drugs have been shown, in a randomized controlled trial, to benefit patients with WHO group III PH (218). A potential explanation for this may be that all current therapeutics function, in part, by causing diffuse PA vasodilatation, which can lead to worsening ventilation/perfusion mismatch and hypoxemia especially in patients with group III PH. Furthermore, whereas group I PAH is a relatively rare disease with a prevalence of about 12 patients per million adults, patients with WHO group III disease are much more common, making up the second largest group (behind WHO group II PH, i.e., PH from left-sided heart disease) of patients with PH (22). An insight into the potential scale of the problem is provided by the fact that there are an estimated 12 million patients with chronic obstructive pulmonary disease (COPD) in the United States (US) (47a). Even if only 5% had PH, that would mean greater than a half million patients in the US have WHO group III PH while not even considering the PH in this group arising from diseases such as idiopathic pulmonary fibrosis (IPF), sleep apnea, and chronic altitude exposure. In contrast, there are only a few thousand patients with WHO group I disease. Furthermore, PH in these patients represents an important marker for morbidity and mortality (183). Surveillance data based on hospitalizations in the United States suggest that as much as 26% of the mortality in patients with PH occurs in the setting of chronic respiratory disease (122). Furthermore, there exists a subset of patients (as high as 5–10%) with COPD and IPF who have severe PH (defined by mPAP >35–40 mmHg) and a drastically higher mortality rate (218). Previously termed “out-of-proportion PH,” these patients tend to develop right heart failure and die of their PH despite oxygen and pharmacological therapy similar to those given to patients with WHO group I disease. The degree of pulmonary vascular remodeling and irreversible fibrosis likely differentiates those with severe hypoxic PH from those with mild PH. This is illustrated by a recent pathological study by Carlsen et al. (43) that correlates pathological findings to severity of PH in explanted lungs from patients with COPD undergoing lung transplantation (43). They described severe pulmonary vascular remodeling with high-grade intimal lesions (one patient with plexiform lesions) in patients with out-of-proportion hypoxic PH (mean PAP >40 mmHg). This degree of vascular remodeling had not previously been described in “traditional hypoxic PH” in humans or animal models. These patients clearly have a subset of disease whereby the underlying mechanisms go beyond hypoxemia, are multifactorial in nature, and require independent study to develop novel therapeutics. We will examine some of the mechanisms below that may help to differentiate reversible hypoxic PH from irreversible disease.

The Effect of Acute and Chronic Hypoxia on Resident Pulmonary Vascular Cells

In an effort to better understand the pathophysiological mechanisms involved in forms of PH related to lung disease and/or global hypoxia (WHO group III PH), several animal models have been used, namely the hypoxic mouse, rat, and calf as well as lamb and pig models (239). Although there are differences between humans with group III PH and animal models, several key conserved pathological features exist that characterize the changes observed in the three compartments of the vascular wall (intima, media, adventitia) and their respective resident cells (endothelial, smooth muscle, and fibroblast). It should be noted that the degree of pulmonary vascular remodeling in response to hypoxia increases with phylogenetic order of species (cow > rat > mouse) and directly correlates to the amount of perivascular inflammation (241). We discuss below the effects of chronic hypoxia on the pathology of the vascular wall as well as on resident cells both in vivo and in vitro and underscore the importance of these cells with respect to the initiation and perpetuation of remodeling and inflammation.

The Intima and Endothelial Cells

The innermost intimal layer houses the PA endothelial cell (PAEC), which is in immediate contact with the blood supply and able to detect changes in oxygenation, circulating factors, flow, and pressure. In response to chronic hypoxia, the intimal layer develops the least amount of remodeling of the three layers of the vessel wall (109, 110, 160, 212, 239). The changes are characterized by PAEC hypertrophy, subendothelial edema and fibrosis, characterized by collagen and elastin deposition, and the development, at least in some humans, of a longitudinal muscle layer (109, 110, 160, 239). Importantly, hypoxic PH in most animals and humans does not lead to high-grade vascular remodeling with intimal obliteration and plexiform lesions as seen in PAH (107, 110). In animal models, chronic hypoxia induces intimal thickening as a result of hypertrophy and hyperplasia of the resident endothelial cell and thickening of the subendothelial space (26, 160, 212, 238, 243). While there are conflicting in vivo data regarding the extent of PAEC proliferation based on methodology and differing animal models, the degree of proliferation in most animal models of hypoxic PH overall is quite low (26, 160, 238, 243, 305). The proliferative response of endothelial cells observed in hypoxic PH is not likely an effect of hypoxia alone, as in vitro studies demonstrate growth inhibition with acute (24 h) or chronic hypoxia (5 days) compared with normoxia (256, 305). Conflicting data showing human PAEC proliferation in serum containing media at 1% hypoxia for 72 h have been reported, but growth was inhibited at earlier time points; there were no data at later time points (193).
It is possible that endothelial progenitor cells (EPCs), a heterogeneous population of resident and circulating progenitor cells that have the ability to either secrete proangiogenic factors or differentiate into mature endothelial cells, exhibit distinct responses to hypoxia and thus contribute to abnormal PAEC proliferation and function under hypoxic conditions (77). Similarly, EPCs appear to be important in the dramatic expansion of the vasal vasoconstriction, the vascular network derived from the systemic circulation, that takes place in chronic hypoxic PH in large animal species, where there is a large bronchial circulation supplying vessels even to the resistance level (68, 170). It thus seems possible and even likely that the pulmonary and bronchial circulations comprise endothelial cells (ECs) with very distinct responses to chronic hypoxia that are important in the development of PH. This possibly deserves further study although difficulties may be encountered because the mouse does not have an intrapulmonary bronchial circulation.

Subendothelial thickening is observed in response to chronic hypoxic exposure. Hypoxia induces an increase in PAEC permeability, the influx of vasoactive mediators, and plasma proteins and subsequent activation of vascular wall proteases, which all contribute to remodeling (33, 200, 235). Changes in EC barrier function, induced by hypoxia, have been associated with alterations in actin stress fiber formation, increased cell stiffness, and contraction, all of which appear to be mediated by activation of MAP kinase and/or Rho kinase (12, 61, 132, 285). These hypoxia-induced alterations in cell shape and structure, resulting in increases in permeability, could result in exposure of smooth muscle cells (SMCs) to circulating vasoactive substances. They also contribute to the development of subendothelial edema, which is commonly observed. It is also interesting that, whereas EC contraction causes intracellular gap formation in a cultured monolayer in vivo, this response may actually reduce capillary diameter and thereby contribute to acute and chronic hypoxic vasoconstriction. In support of this are direct observations of contraction in PAs and veins 30–50 μm in diameter that have very little smooth muscle using confocal microscopy (27, 250, 290). It is also important to note that hypoxia can change plasma lemmal structure and function in PAECs. For instance, hypoxia has been shown to increase fluidity and transport of serotonin and polyamines and to decrease phospholipid concentration and transport of l-arginine in plasma membranes of PAECs (28–30). These changes could account for some of the alterations in the synthesis, release, or uptake of vasoactive factors by PAECs, which have an important role in acute and chronic responses of the pulmonary circulation to hypoxia.

Furthermore, ECs, in response to hypoxia, have been shown to produce more laminin, fibronectin, and elastin, all of which affect EC as well as SMC function and can contribute to the remodeling and fibrosis observed in chronic hypoxic PH (33).

Hypoxia has significant effects on the regulation of synthesis and secretion of vasoactive factors and inflammatory cytokines in PAECs. Hypoxia causes PAECs to induce a vasoconstrictive environment through decreased production and/or activity of prostacyclin and nitric oxide (NO) as well as increased production of endothelin (ET), serotonin, leukotrienes, and other mediators (1, 21, 53, 82, 113, 136, 195). In vitro studies also demonstrate that exposure of PAECs to hypoxia causes the synthesis and release of proinflammatory (IL-1, IL-6, IL-8), promitogenic (VEGF-1, ET-1, thromboxane, PDGF-B, CX3CL1), and antithrombic mediators (increased tissue factor, decreased thrombomodulin), as well as increased expression of inflammatory cell adhesion molecules (vascular cell adhesion molecule, intercellular adhesion molecule, P-selectin) (1, 10, 235, 246, 254, 311). These findings support a PAEC contribution to the observation that hypoxic forms of PH have a consistent and important inflammatory component and that the endothelium likely plays a role. However, they do not fully explain the observations that inflammatory cells aggregate mostly in the adventitia of chronically hypoxic animals (239, 241). It is possible that ECs of the vasa vasorum exhibit specific upregulation of adhesion molecules and cytokines that could facilitate it acting as a conduit for leukocyte delivery to the hypoxic vessel. This possibly should be explored as emerging evidence points to distinct EC-inflammatory cell interactions in the different organs of EC (42, 213)

Little is known regarding the mechanisms by which PAECs detect decreases in Po2 and transduce these signals into altered release of vasoactive and inflammatory factors. It is likely that a variety of ion channels are involved in these responses. It is known that PAECs depolarize in response to hypoxia (242). This depolarization is similar to those observed in PASMCs (see descriptions below). However, the ion channels involved in generating this depolarization probably differ. Work in PAECs show that membrane potential (Em) is controlled mainly by potassium K+ conductance (249, 266). In fact, vascular ECs possess several types of K+ channels including KCa, KV, KATP, Kir (171). Among these channels, Kir has been suggested to be the main contributor to the regulation of Em (171). Exactly how these channels control the aforementioned changes in EC function under hypoxic conditions remains unclear. Alteration of [Ca2+]i, is, on the other hand, a primary mechanism of EC signaling. While in PASMCs, hypoxic depolarization causes activation of L-type voltage-operated calcium channels (VOCC) and Ca2+ influx (see below); this does not occur in PAECs, which lack L-type VOCC. The major pathways for Ca2+ influx in PAECs are thought to be receptor-operated calcium channels and store-operated calcium channels. For example, the synthesis and release of several substances, including NO, ET-1, and prostacyclin are directly correlated with an increase in [Ca2+]i (249). However, the effects of acute hypoxia on [Ca2+]i, appear currently unclear (249). At present, it is felt that hypoxia may cause an initial transient increase in [Ca2+]i, attributable to calcium release from internal stores followed by a progressive decrease attributable to depolarization and reduced Ca2+ influx in PAECs (249). It is pointed out by Sylvester et al. (249) that more investigation is needed to determine the underlying mechanisms regarding the role of calcium and vasoactive/inflammatory mediator release in PAECs.

Thus, whereas hypoxia induces important EC changes that can directly lead to intimal thickening and remodeling, probably equally important are the indirect signals capable of recruiting circulating cells and inducing local cellular proliferation and smooth muscle contraction, hypertrophy, and even hyperplasia.

The Media and SMCs

The sine qua non of vessel remodeling in hypoxic PH is thickening of the media at all levels of the pulmonary arterial bed and the appearance of cells expressing α-smooth muscle
actin (α-SMA) in partially muscular or nonmuscular small PAs. Because the small PAs account for the majority of total cross-sectional area in the pulmonary vascular bed, it is these changes in the distal PAs that account for the greatest changes in pulmonary vascular resistance (PVR) (19, 20, 109, 260, 279). The thickening of muscular and large elastic vessels can include both hyperplastic and hyperproliferative responses as well as changes in extracellular matrix (ECM) composition. These changes likely contribute to decreases in compliance of the large vessels, a functional change increasingly thought to be important in PH disease progression (121).

The effect of hypoxia on PASMC proliferation is complex and somewhat controversial given the differences between animal models and location of SMCs within the PA that are examined. In vivo studies looking at proximal PAs in rat and mice demonstrate minimal smooth muscle proliferation in response to hypoxia with the majority of medial thickening secondary to SMC hypertrophy and deposition of elastin and collagen (161, 184, 235). Conversely, in large animal models and probably in humans, SMC proliferation during hypoxia exposure in vivo does occur in the large proximal PAs, probably owing to the existence of multiple phenotypically distinct SMC populations (91, 94, 96, 235, 283). When these distinct SMC populations are isolated and cultured, even from control animals not exposed to hypoxia, there is a spectrum of responses in which well differentiated SMCs do not proliferate in response to hypoxia, whereas the least differentiated SMCs show the greatest capability of proliferation in response to hypoxia (70, 71, 91). Hypoxia-induced proliferation coincided with increased responsiveness to G protein-coupled receptor agonists and to stimulation of protein kinase C (69, 91). Similar detailed analysis of human PASMC populations has not been reported to confirm findings in neonatal cows although multiple studies have reported the proliferative potential (to hypoxia and mitogens) of human PASMCs derived from proximal arteries (7, 51, 202, 307). On the basis of the proliferative potential of proximal PASMCs from humans in vitro, we believe that there are SMC populations in adult human PAs similar to those observed in calves and pigs (36, 108).

As opposed to proximal SMCs, normal distal PASMCs, at least from the bovine species, represent a uniform population of well-differentiated cells. Using bromodeoxyuridine staining, we have demonstrated that distal SMCs have low proliferative indices during normoxia in vivo and, when isolated and cultured, do not proliferate in response to hypoxia or traditional growth-promoting stimuli (26, 244). However, when neonatal calves are exposed to hypoxia, we find the development of early (days 4–7) but not late (day 14) distal PA medial compartment cell proliferation (26). When these distal medial SMCs from hypoxic calves are isolated and cultured, two populations emerge: a well-differentiated SMC [α-SMA+, smooth muscle-myosin heavy chain (SM-MHC) +], which is resistant to proliferation, and a second population of smooth muscle-like-cells (defined by low α-SMA expression, no SM-MHC), which are highly proliferative, apoptosis resistant, and release factors (S100A4, PDGF-β) that are involved in the continued stimulation of prolifereative and prosignaling pathways (95). These cells, in very early ex vivo passage, express hematopoietic (CD45, CD11b, CD14) and progenitor cell (c-kit, CD133, CD73, CD34) markers consistent with a circulating (nonresident) origin. These markers are lost with time in culture, and α-SMA expression is maintained. Similar smooth muscle-like cells that are hyperproliferative in response to hypoxia have been isolated from distal PAs of patients with COPD (119). In this report, distal PAs with higher proliferative and apoptotic indices correlated with more severe vascular remodeling. Cumulatively, these data raise the possibility that, in response to hypoxia, nonresident smooth muscle-like cells are recruited to the distal medial compartment, proliferate, and then potentially differentiate into “mature SMCs”.

To summarize, there is evidence of a modest increase in SMC proliferation in rodents and large animals in response to chronic hypoxia. In vivo studies in large animal models suggest that there are subsets of undifferentiated resident cells with high proliferative potential in proximal PAs and undifferentiated resident and perhaps nonresident cells in distal PAs that proliferate in response to hypoxia. Regarding in vitro studies, our work in large animal models demonstrates that, in proximal PAs, there are different SMC populations, with a continuum of proliferative potential, whereby the least differentiated cells have the highest proliferative potential. In SMCs isolated from distal PAs of normal animals, there exists a uniform population of well-differentiated nonproliferative cells, while a second population of highly proliferative nonresident cells can be isolated from hypoxic animals. Regarding the vast literature of whether SMCs proliferate in response to hypoxia in vitro, it becomes nearly impossible to be concrete in the answer to this question. This is because the differences in any of the following factors can influence the results of the experiment: species, location of PA from which cells are isolated, cell population within specific location of PA used, severity of hypoxia, seeding density, presence or absence of comitogens (e.g., presence of serum or even low concentrations of growth factors), and duration of hypoxia (185). A review by Pak et al. (185) notes conflicting reports on 16 studies that try to answer this very question and elucidates how each of these conditions can alter the results of the experiment. Although SMC proliferation cannot be studied in vivo in humans, more recent work has demonstrated distal well-differentiated PASMCs, characterized by α-SMA and SM-MHC expression, isolated from humans with PAH appear to be hyperproliferative at baseline compared with SMCs from control patients (81). Questions remain regarding whether in vitro culture selects for a subset of cells that are hyperproliferative and may only represent a small number of cells in vivo. Nonetheless, these data suggest that there are important differences between SMCs isolated from hypoxic animal models and humans with PAH with regard to their ability to proliferate without exogenous growth factors and mitogens.

Many investigators have sought to define the mechanisms involved in hypoxia-induced PASMC proliferation. A hallmark of hypoxic exposure in PASMCs is an increase in intracellular \([Ca^{2+}]_i\) concentration (179, 249). This is, of course, unique to the pulmonary circulation, as systemic SMCs respond to hypoxia with no change or a decrease in intracellular \([Ca^{2+}]_i\) (179, 249). This increase in intracellular \([Ca^{2+}]_i\) promotes both vasoconstriction and, in the cases of cells proliferating, proliferation. Changes in the expression and/or function of several \([Ca^{2+}]_i\) channels, transporters, and \([Ca^{2+}]_i\)-handling proteins have been reported to be involved in PH pathogenesis and have been summarized in a recent paper by Olschewski et al. as well as by Sylvester et al. (179, 249).
Intracellular Ca\(^{2+}\) is regulated by store depletion from endoplasmic and sarcoplasmic reticulum and/or by calcium entering the cell from the extracellular space. Extracellular Ca\(^{2+}\) entry is mostly regulated by voltage-dependent Ca\(^{2+}\) channels and voltage-independent nonselective cation channels, such as store-operated calcium channels and receptor-operated calcium channels. Classical transient receptor potential (TRPC) proteins build a group of nonselective cation channels, which consist of seven members based on their amino-acid homology (73). With regard to hypoxic PH, it has been shown that TRPC6 and TRPC6 are upregulated by hypoxia-inducible factor (HIF)-1α (273). There may also well be a positive feedback, as the increase in intracellular Ca\(^{2+}\) may activate Ca\(^{2+}\) transcription factors like nuclear factor of activated T-cells or activator protein 1 to regulate gene expression, including channel and transporter genes, and further increase the Ca\(^{2+}\) influx/elimination ratio, thereby creating a vicious circle (179). In very interesting experiments, the Weissmann group has shown that TRPC6 is essential for acute hypoxic vasoconstriction but not for chronic hypoxia-induced PH, as their promoter analysis detected no HIF-1 binding sites in the murine TRPC1 promoter. Additional recent studies support the role of [Ca\(^{2+}\)]\(_i\) in PASMCs in the medial remodeling observed under hypoxic conditions. The Shimoda group (141) has shown that increases in intracellular calcium in PASMCs but not aortic SMCs increase aquaporin-1 (AQ1), and the increased AQ1 protein levels mediate migration of PASMCs, as evidenced by the fact that silencing AQ1 via siRNA prevented hypoxia-induced migration of PASMCs (141). These studies extend the role that intracellular [Ca\(^{2+}\)]\(_i\) plays in regulating SMC proliferation as well as migration (141).

Changes in K\(^+\) channel activity are also consistently observed in response to chronic hypoxia. It is now well described that, in response to prolonged hypoxia, PASMCs exhibit decreased Kv channel gene and protein expression (179). The consequences of Kv downregulation by chronic hypoxia are reduced whole-cell K\(^+\) current and depolarization of PASMCs. The resultant membrane depolarization opens L-type voltage-gated calcium channels and augments calcium influx, thereby increasing [Ca\(^{2+}\)]\(_i\), leading to vasoconstriction and proliferation (179, 249). The pathological relevance of K\(^+\) channel downregulation in hypoxic vascular remodeling is supported by the findings that therapies upregulating K\(^+\) channels, such as Kv1.5 gene therapy, have shown therapeutic benefit (179). Recent studies by the Yuan group (268) also identify voltage-dependent calcium channels as being important in chronic hypoxia-induced PH (268). Table 1 summarizes changes in ion channel activity in PASMCs known to be induced by hypoxia. For both, regulators of the Ca\(^{2+}\) homeostasis and K\(^+\) channel activity, transcriptional regulation (e.g., due to transcription factors), HIF, or cAMP response element-binding protein (CREB) binding to the promoter region and/or posttranslational modification including nitrosylation represent potential mechanisms (180). For further review of this important topic, the reader is referred to recent reviews from the Sylvester (249), Olschewski (above) (249), Schumacker (276), and Shimoda (226, 227) groups. Although the reports described above clearly demonstrate the role of ion channels in medial vessel remodeling and distal vessel muscularization in the context of chronic hypoxic PH, we are not aware of any studies that link this process with inflammation. However, on the basis of the emerging data involving the effects of inflammation on SMC proliferation and migration (see below), there may be an important interplay between ion channels and inflammation, at

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**Monocrotaline-induced PH rat model**

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**Sugen plus chronic hypoxia-induced PH rat model**

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**Newborn lambs with high altitude-induced PH**

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**Persistent PH of the newborn lamb model**

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</table>

**Human PAH tissue**

<table>
<thead>
<tr>
<th>Channel/Transporter</th>
<th>mRNA</th>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.5</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>(308, 309)</td>
</tr>
<tr>
<td>TASK-1 (KCNK3)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>(152, 178)</td>
</tr>
<tr>
<td>CLIC4</td>
<td>n/a</td>
<td>↑</td>
<td>n/a</td>
<td>(284)</td>
</tr>
<tr>
<td>CaSR</td>
<td>n/a</td>
<td>↑</td>
<td>↑</td>
<td>(293)</td>
</tr>
</tbody>
</table>

Upward and downward arrows show increase and decrease, respectively; n/a means no available information. Kv, voltage-gated K\(^+\) channel; BKCa, large-conductance Ca\(^{2+}\)-activated K\(^+\) channel; PH, pulmonary hypertension; PAH, pulmonary arterial hypertension; TASK, TWIK-related acid-sensitive K\(^+\) channel; TRPC, transient receptor potential canonical; TRPV, transient receptor potential vanilloid; CLIC, CIC intracellular channel; CIC, voltage-gated Cl\(^-\) channel; TMEM16A, Ca\(^{2+}\)-activated Cl\(^-\) channel; ASIC, acid-sensing ion channel; CaSR, Ca\(^{2+}\)-sensing receptor; NHE, Na\(^+\)/H\(^+\) exchanger.
Review

least in SMCs. One potential candidate for mediating the cytokine effects is perhaps the c-Src kinase. Because basal levels of c-Src kinase are known to regulate smooth muscle Ca<sup>2+</sup> and K<sup>+</sup> channels and c-Src is sensitive to cytokines, changes in c-Src activity may result in changes in ion fluxes (179). Investigation of the interaction of inflammation and ion channel gating, especially the significance of S-glutathionylation, S-nitrosylation, and protein kinase-mediated phosphorylation, will result in a better understanding of the physiological and pathophysiological importance of these mediators for the ion channels in the pulmonary circulation.

Medial SMCs may also have important functions with regard to recruitment of circulating inflammatory and progenitor cells, as well as in the activation of resident progenitor cells. Horita et al. (117) reported mice with an inducible smooth muscle-specific phosphatase and tensin homolog deleted on chromosome 10 (PTEN) deletion develop severe PH with intimal obliteration after 4 wk of chronic hypoxia (117). Furthermore, there was increased whole-lung expression of inflammatory mediators [stromal cell-derived factor 1 (SDF-1) and IL-6] and increased perivascular macrophage recruitment. Interestingly, hypoxia-induced perivascular proliferation in the SMC-specific PTEN knockout mice was most dramatically increased in α-SMA-negative cells. Although the authors show decreased PASMC PTEN expression in patients with idiopathic PAH (IPAH), we do not know whether this leads to increased perivascular inflammation in humans, especially in those with hypoxic PH. It is, therefore, possible that in patients with hypoxic PH, the combination of hypoxia plus a “second hit” (PTEN deletion) can transform resident SMCs into cells that are capable of inflammatory cell recruitment, local EC proliferation and proliferation, and migration to distal previously nonmuscularized vessels. Also of possible relevance are studies showing that decreases in myocardin-related transcription factor-A (MRTF-A) in SMCs, which can occur when bone morphogenetic protein receptor type 2 (BMPR-2) signaling is impaired, as has been reported to occur in response to chronic hypoxia, may lead to a proinflammatory SMC (269). Interestingly, MRTF-A inhibits RelA/p65 in a BMP-dependent manner (269). Similarly Yeager et al. (297) have shown that ET-1 stimulated PASMCs, via the unfolded protein response (UPR), release proinflammatory and chemotactic mediators. Although this experiment was not performed in a hypoxic model of PH, given local ET-1 production in response to hypoxia by ECs, this process likely occurs in hypoxic models of PH as well. There is evidence that hypoxia induces PASM ET-1 in a HIF-1-dependent manner, which induces a feed-forward loop, whereby ET-1 further stimulates HIF-1α protein and HIF-1 gene expression (192). On the other hand, at least in hypoxic animal models, there is in vitro evidence that recruited proinflammatory cells exert mitogenic effects on medial PASMCs (95, 263). The data above raise the possibility that resident SMCs have the potential to induce a feed-forward loop, whereby they induce a proinflammatory and promitogenic environment and in turn respond with increased proliferation.

The least understood change in hypoxia-induced pulmonary vascular remodeling, yet arguably the most consistent, is the muscularization of the normally partially or nonmuscular segments. Several mechanisms have been invoked to explain the distal muscularization process, from contribution/recruitment of pericytes and/or “intermediate cells” or interstitial fibroblasts with their subsequent differentiation toward a SM-like cell, to endothelial- and/or epithelial-to-mesenchymal transdifferentiation, to contribution of mesenchymal precursor cells or circulating mononuclear cells (68, 93, 263, 289). However, recently, a potentially more definitive answer to this issue has been generated using a Cre ER-Lox P lineage-tracing system (224). Using this system, the investigators found that preexisting SMCs are the major source of hypoxia-induced distal arterial muscularization. The studies indicate that α-SMA+, SM-MHC+, and PDGFR-B+ cells migrate along the axial direction of nonmuscularized EC tubes, contributing to 80% of cells contributing to the distal muscularization process. Interestingly, the investigators also demonstrate that the number of alveolar myofibroblasts originating from an α-SMA-negative population of cells increases significantly within 7 days of hypoxia and continues to do so over the next 2 wk. At least 75% of the alveolar myofibroblasts induced by hypoxia are green fluorescence protein-negative, proving their origin from SM actin-negative cells that were present before hypoxia treatment. In addition to the hypermuscularization of the pulmonary vasculature, the number of alveolar α-SMA myofibroblasts also progressively increases with hypoxia treatment. These alveolar α-SMA myofibroblasts also markedly upregulate SM-MHC with ~85% of the SMA-positive myofibroblasts also expressing SM-MHC by 21 days of hypoxia. In contrast to those cells observed around the newly muscularized vessels, PDGFR-β expression was not detected at any stage in myofibroblast accumulation. Importantly, the investigators demonstrated that the initial muscularization is driven primarily by migration. This is then followed by proliferation of the newly arrived distal arterial SMCs. The newly muscularized distal arterials form a single layer of SMCs, indicating that SMA-positive progenitors migrate axially along the EC tube, as in development, but, in contrast to large PA morphogenesis, they do not migrate radially outward. Proliferation had ceased at 21 days of hypoxic exposure. Similar to development, it seems that ECs participate in this directional axial PASMC migration. Importantly, it appears that in mice there is a pool of SM-MHC-positive PDGFR-β-positive cells adjacent to the arterial muscular-nonmuscular transition in normoxic animals. These cells appear primed to dedifferentiate, migrate, and proliferate in response to insults such as hypoxia. The role of inflammatory cells in this process needs to be elucidated. Furthermore, studies will need to be done to determine the existence and potential role of these cells in larger animals and humans.

The Adventitia and Resident Fibroblast

The outermost portion of the vessel wall, the adventitia, serves the vessel as an ECM scaffold that contains conduits for nutrient supply and removal, that is the vasa vasorum, lymphatic vessels, and trophic nerves, as well as resident cells such as fibroblasts, progenitor cells, and immune cells (macrophages and dendritic cells). Because of this cellular and structural complexity, the adventitia is the most heterogeneous compartment of the vessel wall. In response to hypoxia in humans and in animal models of PH, the adventitia undergoes substantial thickening attributable to a significant increase in collagen and ECM protein deposition, marked expansion of the vasa vasorum, proliferation of resident fibroblasts and possibly macrophages, activation of resident progenitor cells, as well as

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recruitment of circulating immune and progenitor cells (241). Accumulating experimental data suggest that, in response to vascular stresses, including hypoxia and overdistention, the resident adventitial fibroblast, in the function of a sentinel cell, is the first to become activated and to respond through proliferation, upregulation of contractile and ECM proteins, and to release factors that can directly affect medial SMC tone and growth, as well as stimulate the recruitment of inflammatory and progenitor cells (145, 181, 282). It is also possible that resident macrophages act in this capacity and cause subsequent but still early activation of fibroblasts. More recent data have provided evidence that these hypoxia-induced changes in the fibroblast, through epigenetic mechanisms, “lock” the adventitial fibroblast into an “activated” phenotype (78, 145, 270). These activated fibroblasts through secretion of a variety of cytokines, chemokines, growth factors, and matricellular proteins modulate either directly or indirectly overall vascular function and structure (65, 241), consistent with findings that adventitial fibroblasts exhibit the earliest and most significant increases in proliferation in hypoxic rats and calves.

In vitro experimental studies support the notion that adventitial fibroblasts have a greater propensity to proliferate under hypoxic conditions than do resident PA SMCs (80, 199, 245). In contrast to the SMC, adventitial fibroblasts isolated and cultured from the PA consistently demonstrate proliferative responses to decreased oxygen concentrations in the absence of exogenous mitogens (241). The responses observed occur in general in response to 3% O₂ exposure, which is far higher than the O₂ concentrations often used in many experiments in tissue outside the lung and in cancer (<1% O₂), where hypoxia is thought to decrease protein transcription and to turn off the AKT/mammalian target of rapamycin (mTOR) signaling. Hypoxia-induced signaling pathways involved in fibroblast proliferation include ERK 1/2, JNK1, and p38 MAPK, but not JNK2 (66, 280). Activation of these signaling pathways by hypoxia is largely dependent on Gα(i/o)-mediated signaling potentially in a ligand-independent manner (66, 92). These hypoxia-induced signaling pathways differ significantly from serum-induced signaling pathways. It is also important to note that hypoxia can directly induce differentiation of at least certain vascular fibroblast populations into myofibroblasts, which likely play an important pathophysiological role in contributing to abnormalities of tone and structure of the PA in PH (228). The hypoxia-induced differentiation pathway is different than the hypoxia-induced DNA signaling pathways and takes place through simultaneously activated signaling pathways (228). It should also be noted that chronic hypoxia leads to the emergence of fibroblasts with dramatically heightened proliferative capabilities, a phenotype that persists even under normoxic conditions in culture. In these cells, there is a dramatic change in the function of PKC-γ, whereby it no longer acts as it does in normal fibroblasts as a suppressor of replication but rather as a stimulant for proliferation (67). More recently, it has been shown that these cells also exhibit a vague dependence on JNK1 for proliferation (186). These changes are likely driven by epigenetic modifications in the proliferative machinery of the cell, which are discussed below. Also discussed below is the fact that a subset of fibroblasts from the hypoxic, hypertensive vessel are potent producers of cytokines capable of driving macrophage activation, PAEC and PASMC proliferation, and even activation of quiescent fibroblasts, thus serving to perpetuate the inflammatory remodeling process (65, 78, 269, 270).

Signalizing Pathways Involved in Hypoxia-Induced Inflammation and Remodeling

Because all animal organisms depend on oxygen, they developed evolutionarily conserved systems sensing oxygen and adapting cellular metabolism to oxygen availability (220 –223). One of those systems is the propyl-hydroxylase (PHD)-HIF system of proteins. Three known PHDs hydroxylate proline residues of the HIF-α subunits (HIF-1α-3α). This hydroxylation mediates their interaction with the von Hippel-Lindau (VHL) E3 ubiquitin-ligase complex targeting HIF proteins for proteasomal degradation (194). PHD activity depends on several cofactors, one of which is oxygen (124, 125, 217). Indeed, the Kₐ values of all three PHDs for O₂ (230 –250 μM) are above the concentration of oxygen in ambient air, indicating that these PHDs are capable of sensing small changes in oxygen concentration (114, 257). PHD activity is inhibited by, not only the lack of oxygen, but also intermediates of cell metabolism derived from the citric acid cycle, such as fumarate, malate, and succinate (135, 219). The result of PHD inhibition is the accumulation of HIF-α subunits, which now dimerize with HIF-1β. Additionally, a histone-acetyltransferase (p300 HAT) is recruited to the HIF-α subunits, a process regulated by the oxygen-dependent asparagine-hydroxylase factor inhibiting HIF-1 (Kₐ for O₂ ~ 100 μM) (134). The entire complex consisting of the α-subunit, HIF-1β, and p300 HAT binds to hypoxia-response elements (HREs) within DNA, initiating or enhancing the transcription of target genes (194).

A seminal study by Yu et al. (304) demonstrated that HIF-1α haplo deficiency delays but does not abrogate the onset of hypoxic PH in mice because of impaired hypoxia-induced vascular remodeling (304). Brusselsmans et al. (38) demonstrated that HIF-2α haplo deficiency protects mice from hypoxic PH over 4 wk (38). Again, the authors identified impaired vascular remodeling in the HIF-2α haplo-deficient mice as the main reason for protection, whereas acute hypoxia-induced vasoconstriction was preserved. Further studies utilizing acriflavine or digoxin to inhibit HIF-signaling confirmed the results from the genetic studies (3). Consistent with these studies, human patients with a HIF-2α gain-of-function mutation or with VHL mutations develop PH or are more susceptible for the disease (87, 99, 231). Of note, hematocrit and concentrations of circulating cytokines and neurotransmitters such as norepinephrine were modulated by the HIF genotype in many of the aforementioned studies, suggesting that organs other than the lung contribute to PH (e.g., adrenal glands). Consistent with this, SMC-specific deletion of HIF-1α ameliorated hypoxic PH in mice, but it did not abrogate it completely (e.g., right ventricular hypertrophy was preserved despite decreased PAP) (23). The role of HIF signaling specific to perivascular fibroblasts and perivascular resident/recruited innate immune cells in the pathogenesis of hypoxic PH remains to be determined.

In an effort to identify lung-specific patterns of gene regulation that are essential to the initiation of pulmonary hypoxic responses, Leonard et al. (143) analyzed the early gene expression profile in lungs of mice exposed to hypoxia (FIO₂ 0.10). Twenty transcription factor-binding sites were overrepresented...
within these hypoxia-regulated genes, most significantly the binding sites for the HIF-1α homodimer (CACGTG) and for CREB (248). Further studies demonstrated that hypoxia-induced CREB activation is lung selective, as neither activation of CREB nor induction of putative target genes could be observed in other major organs after whole-body hypoxia (143). It still has to be elucidated what the functional relevance of the activation of the CREB family of transcription factors is. Does it initiate lung inflammation or does it ameliorate it (e.g., CREB is required to prevent vascular permeability increase) (97)?

Recently, the role of additional oxygen-sensitive pathways has been explored in hypoxic PH. Goncharov et al. (104) demonstrated that the mTOR complex 2 contributes to PASMC proliferation and survival in hypoxic and nonhypoxic PH. Inhibition of a fourth oxygen-sensitive pathway, the UPR, also ameliorates hypoxic PH (137, 299).

As discussed above, inflammation is nearly universally observed in hypoxic PH. It remains elusive what exactly triggers inflammation during hypoxia in the absence of necrosis and cell death (such as during cardiac ischemia). It is known that the IκB kinases IKK-α and IKK-β are phosphorylated in hypoxia, which promotes degradation of IκB-α, allowing translocation of the NF-κB p65 subunit to the nucleus, where it initiates proinflammatory gene transcription, including transcription of HIF-1α (210). In addition, HIF-1α has been shown to cooperate with NF-κB to induce proinflammatory IL-1β (253). Interestingly, the accumulation of IKK-β occurs at higher oxygen levels (≤10%) than the accumulation of HIF-1α protein and is also negatively regulated by PHD1, one of the PHDs regulating HIF protein levels (60). Thus PHD inhibition regulates both NF-κB signaling and HIF signaling (210) and as such can fine tune inflammatory responses and control/coordinate both the induction and resolution of inflammation.

The link between NF-κB and hypoxia signaling is a prime example of how different signaling pathways converge to modulate and control cellular behavior. Intriguingly, HIF-1α plays critical roles in both hypoxia signaling and inflammatory signaling. Although hypoxia signaling on its own might be insufficient to initiate or maintain robust cellular responses, additional inflammatory stimuli (i.e., a second hit) could support and increase hypoxia signals and vice versa. In keeping with this, a clinical “two-hit-model” hypothesis has been put forth for hypoxic PH in humans, specifically for those conditions in which hypoxia and inflammation (elicited by a different trigger) occur simultaneously (e.g., interstitial lung disease, COPD, etc.). This is most evident in human chronic mountain sickness (CMS), a disease whereby long-term residents of high altitude develop excessive erythrocytosis, severe hypoxemia, and in some cases severe PH and right heart failure (142). Many patients with CMS actually have a secondary form characterized by having a concomitant inflammatory respiratory disorder such as interstitial lung disease, obstructive sleep apnea, and/or COPD/emphysema (190). Evidence for this synergism between hypoxia, inflammation, and severe PH can be found in multiple animal models. In each of the following animals, the genetic manipulation or treatment results in mild PH, but in combination with hypoxia the animals develop severe PH and pulmonary vascular remodeling: transgenic IL-6-overexpressing mouse, Sugan (vascular endothelial growth factor receptor inhibitor) plus hypoxia in mice and rats, the fawn-hooded rat plus hypoxia, cell-free hemoglobin plus hypoxia in rats and BMPR2 knockout mice plus hypoxia (2, 40, 166, 234, 274, 281). This synergism between hypoxia and inflammation is not limited to the pulmonary vasculature, as hypoxia exacerbates both LPS-induced and allergen-induced lung inflammation (6, 37). The studies cited thus far set a framework wherein hypoxia through a variety of signaling pathways (including HIFs), in conjunction with an inflammatory second hit, can be initiate and perpetuate a chronic inflammatory state. Furthermore, this raises the question of whether patients with severe out-of-proportion PH have a second hit that can potentially be diagnosed and treated. Finally, dissecting the signaling mechanisms that control synergism between hypoxia and inflammation with a focus on the role of HIF1 will provide novel therapeutic targets.

The Role of Mechanical Forces and Hypoxia on the Initiation and Perpetuation of Inflammatory Vascular Remodeling

It has become increasingly clear that hypoxia alone is not enough to initiate and perpetuate pulmonary vascular remodeling and that hypoxia-induced changes in flow and shear wall stress also play an important role. The first studies to examine the role of blood flow in hypoxia-induced PH used pulmonary vasodilators to prevent hypoxic pulmonary vasoconstriction (HPV) during hypoxic exposure. It was found that, whereas pulmonary vasodilators can attenuate HPV when administered before and after exposure to hypoxia, they were not shown to completely prevent PH from chronic hypoxia (233). Nifedipine has been shown to completely prevent the change in PVR with acute hypoxia in dogs but at the expense of nearly doubling the CO and therefore mPAP (303). To this end, nifedipine attenuated but did not prevent PH and pulmonary vascular remodeling during chronic intermittent hypoxia in rats (162). To circumvent the problems with only partial chemical attenuation of HPV and changes in cardiac output, Rabinovitch et al. (201) designed an experiment using rats exposed to hypoxia with a banded left PA. In the banded left PA, the pulmonary vasculature maintains low flow during hypoxia, while the right side experiences supranormal flow. In rats exposed to hypoxia, the banded PA was protected from pulmonary vascular remodeling (medial hypertrophy, distal muscularization), whereas there was more severe vascular remodeling in the unbanded vessel. This suggests that hypoxia alone is not sufficient to induce pulmonary vascular remodeling but is necessary in combination with flow to induce the pulmonary vascular remodeling typical of hypoxic PH. Further support of this concept is emphasized by only minimal increases in mPAP and PVR that occur with pneumonectomy (removal of one lung) in dogs but dramatic increases in mPAP and PVR in unilateral PA ligation plus altitude exposure vs. animals at sea level (112, 131, 267). Although these studies did not address the combination of hypoxia and flow as an inflammatory stimulus, augmenting flow with pneumonectomy or subclavian artery to PA anastomosis plus the inflammatory chemical monocrotaline in rats induced more severe pulmonary vascular remodeling and inflammatory cell recruitment compared with rats with intact lungs (75, 176, 252). Furthermore, a series of studies by the Tan laboratory (146–148, 251) have described the production and release of vasoactive and inflammatory mediators as well
as growth factors from pulmonary ECs in response to high pulsatility flow, which is known to be present in the setting of many forms of PH, including hypoxic, attributable to proximal PA stiffening even in the absence of hypoxia. Taken together, this work would suggest that hypoxia acts as an inflammatory stimulus in combination with changes in flow and pulsatility to initiate and perpetuate pulmonary vascular remodeling (see Fig. 1).

The Role of Chronic Nonresolving Inflammation in Hypoxic Pulmonary Hypertension

Although hypoxia has the ability to initiate inflammation and induce important effects on resident pulmonary vascular cells, these effects are minimal without the synergism between hypoxia and inflammatory signals/mediators, changes in flow/shear wall stress, and cell-cell interactions that shape the process of pulmonary vascular remodeling. It should be noted that, in animal models of PH involving hypoxia exposure up to 5 wk and most forms of human hypoxic PH, the pathological changes are mild compared with those found in patients with PAH and completely resolve or dramatically improve with achievement of relative normoxia. To our knowledge, none of the murine or rat models of hypoxia-induced PH develop occlusive intimal or plexiform lesions characteristic of IPAH and are thought to be completely reversible upon return to normoxia (103, 239). Intriguingly, in the mouse it appears that inflammatory responses occur early and then spontaneously resolve (263). Altitude-induced pulmonary hypertension in cattle, although as stated is associated with far greater inflammation and remodeling than the mouse or rat, is generally reversible upon return to sea level (103, 138, 239). A subset of cattle residing at altitude develop severe pulmonary hypertension and signs of right heart failure, so-called brisket disease, some of which develop nonreversible PH and die, despite relocation to lower altitude (111, 138, 206) (personal communication Joseph Neary, VetMB, and Timothy Holt, DVM, January 2014). These animals succumb to severe right heart failure, and their vessels exhibit marked intimal fibrosis along with marked medial thickening and adventitial fibrosis (111, 155, 169, 206) (See Fig. 2). It is possible that these animals represent a natural model of out-of-proportion PH, which may be associated with an as yet undefined second hit. We propose that, in both out-of-proportion PH involving humans and animals, there is an important interplay between hypoxia, inflammation, and epigenetic changes that lead to chronic nonresolving inflammation and irreversible pulmonary vascular remodeling (see Fig. 3).

It is increasingly clear that inflammation is observed in all described animal models of PH as well as in humans with PAH and other forms of chronic PH (239, 258, 298). Several recent

Fig. 1. Inflammatory mechanisms involved in the initiation of hypoxia-induced pulmonary vascular remodeling. In hypoxic pulmonary hypertension (PH), the pulmonary artery (PA) undergoes changes in all three layers, including adventitial thickening with fibroblast proliferation and immune cell recruitment, medial thickening attributable mostly to smooth muscle cell hypertrophy and hyperplasia, and intimal thickening attributable to subendothelial space thickening and endothelial cell hypertrophy and hyperplasia. These effects are mediated through the inflammatory cascade triggered by the combination of hypoxia and changes in flow/shear wall stress. These effects include generation of reactive oxygen species (ROS) through xanthine oxidoreductase (XOR), activation of NF-κB signaling, production of IL-6, and activation of hypoxia-inducible factor (HIF) and its downstream pathways. These pathways have important effects on each of the resident pulmonary vascular cells shown herein. PAEC, PA endothelial cell; SMC, smooth muscle cell; ECM, extracellular matrix.
Fig. 2. Pulmonary vascular remodeling in brisket disease. Severe “out-of-proportion” brisket disease-induced pulmonary vascular remodeling is shown. The depiction is of a distal PA stained with Pentachrome from yearling steer that died of brisket disease despite relocation to a lower altitude. Strikingly, the neointima (neoint) is dramatically thickened and fibrotic, as highlighted by the blue color (ground substance), and causes luminal obliteration similar to lesions seen in irreversible PA hypertension. Also notice the dramatic neovascularization of all 3 layers of the vessel wall by vasa vasorum, as depicted by the arrowheads. The adventitial layer is dramatically increased in size and also fibrotic, as evidenced by collagen deposition (yellow). Adv, adventitia; M, media. Scale = 100 μm.

Recent evidence shows that OPN is significantly upregulated in hypoxic PH and that fibroblasts both in vivo and ex vivo exhibit increased expression of OPN, as well as its cognate receptors, α(V)β3(3) and CD44, compared with control fibroblasts (15). Augmented OPN expression in PH fibroblasts corresponded to their high proliferative, migratory, invasive, and inflammatory properties and to constitutive activation of ERK1/2 and AKT signaling. OPN silencing via small-interfering RNA or sequestering OPN production by specific antibodies led to decreased proliferation, migration, invasion, and attenuated ERK1/2, AKT phosphorylation in fibroblasts from the chronically hypoxic vessel (15). Interestingly, OPN has been shown to be a biomarker of PH and is as consistently expressed in the plasma of patients with PH as IL-6 or monocyte chemoattractant protein-1 (MCP-1) (86). Collectively, these findings are consistent with the hypothesis that sustained hypoxia creates a PA-specific proinflammatory microenvironment that is shaped to recruit, retain, and activate/polarize mononuclear cell populations to promote vascular remodeling.

Despite these findings, our understanding of the cellular and molecular mechanisms that contribute to nonresolving inflammation in PH is incomplete. Recent studies that have begun to address this question demonstrate that pulmonary arterial adventitial fibroblasts isolated and cultured from chronically hypoxic animals express a constitutively active proinflammatory phenotype characterized by expression of cytokines, chemokines, and adhesion molecules capable of recruiting, activating, and retaining mononuclear cells (145). Importantly, the proinflammatory activity of the fibroblasts far surpassed that of SMCs derived from the vascular media of the same animals (145) and persisted even when cells were removed from their chronically hypoxic inflammatory microenvironment, suggesting that these stable changes in gene expression are under epigenetic control (see below).

Macrophages in Hypoxic Pulmonary Hypertension

In rodent and large animal models of PH, acute exposure to hypoxia leads to increased expression of inflammatory mediators and increased numbers of inflammatory cells in both the alveolar space and around the PA (41, 139, 164, 265). Mice exposed to acute hypoxia (8–10% FoO2) show increased expression of MCP-1, macrophage inflammatory protein-2, IL-1α, and IL-6 in whole lung (164). Similarly, in rats exposed to acute hypoxia, there are increased numbers of alveolar macrophages and inflammatory mediators including HIF-1 in bronchoalveolar lavage (BAL) fluid (153). Importantly, depletion of alveolar macrophages using clodronate liposomes attenuates alveolar inflammation, suggesting a role in either the initiation or maintenance of hypoxia-induced inflammation (153). Wood and Gonzalez (50) have elegantly described the role of alveolar macrophages as hypoxia sensors that secrete MCP-1 and induce both local (alveolar compartment) and systemic inflam-
Fig. 3. Cellular mechanisms involved in chronic nonresolving perivascular inflammation and irreversible pulmonary vascular remodeling. The transition from reversible pulmonary vascular remodeling to the irreversible remodeling seen in severe hypoxic PH involves increased thickening of all 3 layers of the vascular wall with fibrosis seen in both adventitial and intimal layers, leading to intimal occlusion. We hypothesize that these changes are mediated by hypoxia plus an as yet undefined “second hit,” which may include inflammation, infection, genetic, or environmental factors. Central to this hypothesis is the sentinel adventitial fibroblast, which undergoes important epigenetic changes that “lock” this cell into a proinflammatory and promitogenic phenotype. This epigenetically locked in fibroblast is characterized by increased HIF and NF-κB signaling and a shift to aerobic glycolysis, which triggers changes in micro-RNAs (miRs), increased histone deacetylase (HDAC) activity, and increased DNA methylation. Collectively, these changes induce the fibroblast to recruit inflammatory monocytes, induce SMC hypertrophy and proliferation, and activate naïve monocytes into a unique proinflammatory and proremodeling phenotype via paracrine IL-6 and secreted metabolites, including lactate and succinate. This macrophage phenotype is characterized by signal transducer and activator of transcription 3 (STAT-3)-HIF-1-C/EBPβ signaling, activation of NF-κB, and a shift to aerobic glycolysis. In a feed-forward loop, this macrophage can then further induce monocyte recruitment, macrophage activation, and SMC proliferation and activate naïve fibroblasts through various paracrine factors. Together, the fibroblast-macrophage signaling unit is able to perpetuate increased vascular remodeling and fibrosis through a persistent state of chronic nonresolving inflammation. TIMPs, tissue inhibitors of metalloproteinases; MMPs, matrix metalloproteinases; SDF-1, stromal cell-derived factor 1; MCP-1, monocyte chemoattractant protein-1; CCR2, C-C chemokine receptor type 2; VWGF, vascular endothelial growth factor; ARG1, arginase 1.
mation in the mesentery and skeletal muscle through local mast cell degranulation. In hypoxic rats, by artificially maintaining normoxia in the mesentery and skeletal muscle (while the alveolar compartment is hypoxic), they demonstrate that these effects are mediated through MCP-1 and not regional hypoxemia. Although they did not determine the effect on mast cells in the pulmonary vasculature, many other groups have confirmed the importance of mast cells in hypoxic PH, IPAH, and other forms of PH (24, 25, 62, 84, 115).

Unfortunately, in the aforementioned experiments (153), the effect of alveolar macrophage depletion on hypoxia-induced PH was not examined, and thus the role of alveolar macrophages in the pathogenesis of hypoxic PH remains unclear. However, Vergadi et al. (263) showed that early recruitment and alternative activation of alveolar macrophages are important for the later development of hypoxic PH. Furthermore, soluble factors generated by alveolar macrophages were able to induce smooth muscle proliferation in vitro (263). Finally, transgenic and inducible overexpression of lung-specific heme oxygenase (HO)-1, an anti-inflammatory mediator, resulted in downregulation of alveolar compartment inflammatory cytokines and conversion of the alternatively activated macrophage phenotype into anti-inflammatory IL-10-producing phenotype (263). These studies suggest that HO-1 plays a critical role in regulating cellular phenotypes. Inflammatory cytokines/mediators, including leukotrienes in the alveolar compartment, have also been reported in the monocrotaline rat model of PH and in infants with persistent PH of the newborn (165, 237, 240). These studies suggest that the alveolar macrophage and mediators derived thereof affect cellular phenotypes in the pulmonary vascular wall thorough paracrine signaling pathways.

Resident and Recruited Perivascular Macrophages

The role of resident and recruited perivascular macrophages in shaping pulmonary vascular remodeling also remains largely unknown. Because of the inherent difficulty associated with isolating perivascular macrophages, very little is known regarding the importance of these cells in hypoxic PH. However, recent work supports the hypothesis that resident tissue macrophages play important roles in maintaining tissue homeostasis by communicating with the local parenchymal and nonparenchymal cells (i.e., “client” cells) (79). It is believed that, under tissue stress, resident macrophages instruct their client cells to mount an appropriate response, including the recruitment of blood monocytes to complement the resident macrophage pool (e.g., recruitment of CCR2+ cells) (79). Recruitment of CCR2+ inflammatory macrophages has been shown to be critical in a wide variety of acute and chronic inflammatory disease models (16, 54, 154, 182). Consistent with this hypothesis, Frid et al. (93) have demonstrated that ablation of circulating blood monocytes in a rat model of hypoxic PH prevented both remodeling and PH (93). It can further be hypothesized that client cells play a critical role in providing turn-off signals to both resident and recruited macrophages and resident cells, like fibroblasts, to promote resolution of inflammation. Thus intricate cross-talk between resident and recruited macrophages with their client cells is key in maintaining tissue homeostasis, coordinating an appropriate inflammatory response tailored to the inciting noxious agent, and finally providing signals that allow for resolution when the inflammatory trigger has been removed. Malfunctioning of this cross-talk is thus hypothesized to result in aberrant permanent activation of macrophages and client cells with subsequent progression to chronic nonresolving inflammation as the driver of pathological tissue remodeling. Consistent with this hypothesis, El Kasmi et al. (78) recently published that activated adventitial fibroblasts, derived from humans with IPAH and animal models of PH, polarize naïve macrophages via paracrine IL-6 and induce a proinflammatory, profibrotic phenotype regulated by signal transducer and activator of transcription 3 (STAT-3)-HIF-1-CCAAT/enhancer-binding protein-β (C/EBP-β) and independent of IL-4/IL-13-STAT-6 signaling. More importantly, this study brings to light important macrophage signaling pathways (IL-6-STAT-3-HIF-1-C/EBP-β), which have previously been implicated in PH and hypoxia-induced inflammation and challenge the current paradigm of IL-4/IL-13-STAT-6-mediated alternative activation of macrophages in hypoxic PH. It should be mentioned that this work was largely done using fibroblasts from a hypoxic calf model of PH and from patients with IPAH, establishing conserved signaling pathways between the two forms of PH. This study also raises important questions about the role of resident macrophages in instructing adventitial fibroblasts (i.e., client cell) into an activated phenotype. Evidence of macrophage stromal cell inflammatory cross-talk has been reported in adipose tissue, cancer, and rheumatoid arthritis (48, 57, 74).

In the study mentioned earlier, Vergadi et al. (263) show early alveolar macrophage recruitment (day 4) and elevated IL-4 and IL-13 in BAL fluid in hypoxic mice. This macrophage phenotype was characterized by expression of Arg1, Fizz1, Ym1, and CD206, canonical IL-4/IL-13-STAT-6 target genes. Overexpression of lung-specific HO-1, an anti-inflammatory antioxidant enzyme, resulted in attenuation of hypoxic pulmonary hypertension, downregulation of inflammatory cytokines, and a change in macrophage phenotype to an anti-inflammatory IL-10-producing cell. Although this paper supports the role of IL-4/IL-13/STAT-6 signaling in hypoxic PH, the target genes examined can also be induced by hypoxia/HIF signaling and metabolites involved in aerobic glycosylation such as lactate, and thus more rigorous characterization is warranted in future studies to define the functional phenotype of macrophages in mouse models of hypoxic PH (56). Nevertheless, this work and prior studies by the Kourembanas group (164, 300) highlight the therapeutic potential of HO-1 signaling as a novel anti-inflammatory pathway and specifically in modulating macrophage programming.

Work from the John’s laboratory (291) has implicated hypoxia-induced mitogenic factor (HIMF, also known as FIZZ1 or RELM), a member of the resistin family of proteins, in the pathogenesis of hypoxic pulmonary hypertension. They have demonstrated both alveolar epithelium and perivascular expression of HIMF in the hypoxic mouse model of PH (291). Additionally, RELM-b, the closest human homolog to HIMF, is expressed in the endothelium and vascular smooth muscle of remodeled vessels, as well as in plexiform lesions, macrophages, T-cells, and myofibroblast-like cells in humans with scleroderma PH (13). Because HIMF expression in macrophages has been involved in canonical TH2 responses mediated through IL-4 and STAT-6, it is noteworthy that HIMF expression in hypoxic mice is not attenuated in IL-4 or STAT-6 knockout mice. These data suggest that expression of HIMF
can also be induced by other pathways, such as hypoxia/HIF1 signaling (291). Interestingly, intravenous injection of HIMF results in PH and pulmonary vascular remodeling in wild-type but not IL-4 knockout mice mediated, at least in part, by inducing EC apoptosis (292). Exactly how IL-4 facilitates HIMF-induced pulmonary vascular remodeling independent of the canonical IL-4-STAT-6 pathway remains to be determined. Intriguingly, recent work by Colegio et al. (56) shows that tumor-derived lactic acid can lead to FIZZ1 expression in tumor-associated macrophages in a HIF-1-dependent and IL-4/IL-13-independent manner. This work supports the idea that mediators produced downstream of glycolysis, which occurs in hypoxic PH and PAH, are able to directly affect pulmonary vascular remodeling (259).

NF-κB is important in the initiation of inflammation especially with regard to hypoxia, but little is known about NF-κB signaling in macrophages and fibroblasts in hypoxic PH. NF-κB expression is increased in the lungs in animal models of PH, including hypoxic PH, and chemical inhibitors attenuate experimental PH (120, 144, 211, 216). NF-κB is also activated in macrophages, perivascular lymphocytes, SMCs, and ECs in the vessel wall of patients with IPAH (197). Unpublished data from our laboratory using RNAseq in adventitial fibroblasts from calves with hypoxic PH show the NF-κB pathway to be significantly upregulated compared with control fibroblasts. This is consistent with increased fibroblast NF-κB signaling in other inflammatory diseases (39, 72). We have also demonstrated that these fibroblasts produce large amounts of MCP-1 and in turn can induce naïve THP-1 monocytes to produce MCP-1 (145). A similar feed-forward loop of human mesenchymal stromal cell (MSC) activation of naïve macrophages that in turn induce MSC migration has previously been described (14). MCP-1 has been shown to induce SMC proliferation and increased IL-6 production in an NF-κB-dependent fashion (264). We suspect that the NF-κB pathway is necessary for IL-6 production in adventitial fibroblasts and is also upregulated in activated perivascular macrophages in response to paracrine MCP-1 although more work is necessary to prove this hypothesis.

Although more work needs to be done regarding IL-6, STAT-3, and HIF-1 signaling in macrophage polarization and pulmonary vascular remodeling in PH, numerous recently published papers suggest that these pathways are essential for immune cell recruitment, hypoxia-induced inflammation, and chronic nonresolving inflammation in other diseases. Using a wire-induced femoral artery injury model in mice with macrophage-specific deletion (LysMcre) of HIF-1α, Nakayama et al. (167) found a reduction in vascular remodeling, macrophage recruitment, and perivascular inflammation (IL-6, TNF-α). Fielding et al. (85) recently used a mouse model of acute peritoneal inflammation to demonstrate that IL-6 was required to switch from acute inflammation to a chronic profibrotic state and the development of peritoneal fibrosis. Lastly, in human rheumatoid arthritis synovial fibroblasts, STAT-3 was required for hypoxia-induced inflammation (101). Although not yet demonstrated in macrophages with regard to inflammation, cooperative signaling between STAT-3 and HIF-1 modulates hypoxia-induced signaling in cancer cells (189). Future studies need to be designed to dissect and define the role of IL-6, STAT-3, and HIF-1 in regulating the transition from acute to chronic inflammation and promoting “arrest” of vascular cells in a profibrotic phenotype that propagates pulmonary vascular remodeling.

In summary, several signaling pathways (HIF-1, IL-6-STAT-3, NF-κB, HIMF, IL-4/IL-13-STAT-6) with regard to macrophage activation have been implicated in hypoxic PH. We believe that fibroblast-macrophage cross-talk is essential for chronic nonresolving inflammation in hypoxic PH and, through epigenetic mechanisms discussed in detail below, involves epigenetically “locked in” activated mesenchymal cells that stimulate macrophage activation. We acknowledge that macrophages constantly survey local tissue status and alter their phenotype based on the changing tissue microenvironment (175). Therefore, it is more than likely that an activated proinflammatory macrophage phenotype has a reversible transcriptional program that can be transformed into an anti-inflammatory proresolution macrophage based on local stimuli. This may have important therapeutic implications in reversing chronic nonresolving inflammatory processes.

Role of Reactive Oxygen Species in Chronic Hypoxia-Induced Inflammation and Pulmonary Hypertension

There is increasingly good evidence to support the idea that reactive oxygen species (ROS), including superoxide (O2·−), contribute to chronic hypoxic PH. ROS production is increased in the PA under hypoxic conditions (90, 118, 126, 129, 150, 173, 278). However, the source(s) and cell type(s) responsible for ROS production in the hypoxic PA have not been completely defined (5). Sources of ROS by cells within the vasculature include NADPH oxidase mitochondrial electron transport chain, cytochrome p450, nitric oxide synthase, and xanthine oxidase (5, 174). Although many cells may contribute to the generation of ROS in the hypoxic pulmonary circulation, including endothelium, SMCs, neutrophils, macrophages, and epithelial cells, there has been particular interest in their production by cells within the vascular adventitia, as it has been demonstrated above that this compartment of the vascular wall undergoes the earliest and greatest changes in response to hypoxic exposure. It is increasingly appreciated that ROS generated in the PA adventitia contribute to the so-called “outside-in” effects on pulmonary vasoconstriction and pulmonary vascular remodeling (9, 89, 241). In the vascular adventitia, activated adventitial fibroblasts produce ROS predominantly via NADPH oxidase (NOX) with both NOX2 and NOX4 having been identified as important fibroblast isoforms (9, 46, 89, 205). The production of ROS by the resident adventitial fibroblast, as mentioned above, promotes recruitment of circulating mononuclear cells/macrophages, which in turn generate more ROS through the inflammatory cell NOX and probably xanthine oxidase, greatly magnifying the impact of ROS (59). These different sources of ROS can result in oxidant production in, not only the adventitial compartment, but also other compartments within the vasculature, which determine the local redox state and specific targets of ROS (9, 174). Superoxide (O2·−) generated within any cellular compartment can directly modulate selected targets or indirectly impact signaling pathways through its rapid, spontaneous, or enzymatic dismutation to hydrogen peroxide. ROS can thus modulate the phenotype of, not only fibroblasts, but also other vascular walls cells by affecting proliferation, migration, differentiation, and matrix production (174). ROS can function as
signaling molecules by targeting HIF, NF-κB, Nrf2, MAPK, K+ channel regulation, and even BMPR2 signaling (5, 9, 174). Another source of ROS in the hypoxic pulmonary circulation is thought to be xanthine oxidoreductase (XOR) (9, 118). XOR-derived ROS has been implicated in hypoxic PH and other inflammatory diseases (127). Although the primary cellular sources of xanthine oxidase contributing to hypoxic PH remain undefined, activation of xanthine oxidase in leukocytes during hypoxia appears important in mediating pulmonary vascular dysfunction. Previous work shows that XOR regulates leukocyte adhesion in vivo (207, 208, 287), and its inhibition is protective in COPD airways (123), ischemia reperfusion injury (4), acute lung injury (225), and other respiratory disorders that exhibit a hypoxic-inflammatory component (34). Our recent work shows that XOR promotes the inflammatory state of pulmonary mononuclear phagocytes in part though effects on HIF-1α (102). Also a very recent report shows that XOR (and not the NOX)-derived ROS mediates HIF-2α degradation by intermittent hypoxia (168). Significantly, microarray analysis identified XOR as a prominent molecular signature of sepsis-induced systemic inflammation in many organs, including the lung (55). As a source of both ROS and reactive nitrogen species, XOR has been shown to promote DNA double-strand breaks and activates histone H2AX with resultant epigenetic modifications in the inflammatory microenvironment caused by cigarette smoke (133). Also, a significant role of XOR-derived ROS in epigenetic changes comes from studies showing its role during inflammation-induced colorectal cancer (156). These data strongly support the role of XOR as an inflammatory mediator that is likely involved in chronic hypoxic pulmonary vascular remodeling.

The importance of ROS, and particularly adventitial ROS, in regulating vascular structure and function under hypoxic conditions is further supported by the high expression of the key antioxidant enzyme, extracellular superoxide dismutase (extracellular SOD or SOD3) in the vascular adventitia (98). This is the dominant SOD isoform in the vasculature and is highly localized to the adventitia (261). Overexpression of SOD in the lung, which increases adventitial SOD3, protects against adventitial medial and intimal wall remodeling in hypoxic as well as other animal models of PH (8, 174, 262, 288). Conversely, SOD1 knockout mice demonstrate exaggerated PH in response to chronic hypoxia (203).

Collectively, these studies provide strong evidence for hypoxia-induced generation of ROS, which plays clinical roles in the activation and regulation of signaling pathways involved in chronic hypoxia-induced pulmonary vascular remodeling and pulmonary hypertension.

**Contribution of Epigenetics to Chronic Hypoxia-Induced Lung Vascular Inflammation and Remodeling in Pulmonary Hypertension**

The aforementioned studies are in complete agreement with the idea that chronic inflammatory microenvironments lead to stable heritable changes in gene expression and cell function without modification of the underlying DNA base composition; i.e., epigenetic change. There are at least three distinct mechanisms of epigenetic regulation, DNA methylation, histone modifications, and gene silencing mediated by microRNAs (miRs). These pathways of gene regulation are often altered in human diseases such as cancer and are well recognized to contribute to uncontrolled cell growth, migration, and invasion (157, 255, 301). Furthermore, it is also important to note that there are substantial interactions between these epigenetic pathways involved in gene regulation. Studies in a wide variety of cells, mostly cancerous, have demonstrated that hypoxia can regulate changes in all of these epigenetic regulatory pathways (35, 191).

Changes in epigenetic modifications have also recently been associated with PH, a disease characterized, not only by chronic inflammation, but also by mesenchymal cell (SMC and fibroblast) proliferation, resistance to apoptosis, and fibrosis (232, 236). For instance, recent studies have demonstrated that SOD2 expression is decreased in PAs and plexiform lesions because of hypermethylation of CpG islands in the SOD2 gene (17). Reversal of the methylation was shown to rescue SOD2 expression, inhibit proliferation, and increase cell apoptosis of PASMCs from the fawn-hooded rat (17). Histone acetylation has also been shown to play an important role in the development of PH and specifically hypoxic PH. Increased histone deacetylase (HDAC) expression has been reported in lung tissues of patients with IPAH as well as in tissues from hypoxia-induced PH rats and calves (312). Specific increases in Class I HDACs were observed in the fibroblast from hypoxic animals as well as in cells from patients with IPAH. Treatment with Class I HDAC inhibitors markedly decreased cytokine/chemokine mRNA expression levels in fibroblasts as well as in their ability to induce monocyte migration and proinflammatory activation. Most interestingly, studies with several HDAC inhibitors, including the Class I-specific HDAC inhibitor, were shown to both suppress and reverse hypoxia-induced cardiopulmonary remodeling in rats (47, 312).

Increases in HDAC expression, and thus histone acetylation, have also shown to contribute to the abnormalities of mesenchymal cell proliferation in rats, calves, and sheep (47, 270, 294, 312). Studies in cells derived from the hypertensive pulmonary circulation of all three species have demonstrated that HDAC inhibition results in a decrease in proliferation mediated in part by regulation of cell-cycle regulatory genes, including p16ink, p21, and p27. In PASMCs of hypoxic sheep fetuses, HDAC inhibition also decreased PDGF-induced cell migration and ERK activation and modulated global DNA methylation, again consistent with the idea that interactions among the epigenetic mechanisms are important in controlling cell phenotypes (294, 312). It is also interesting to note that chromatin immunoprecipitation analysis experiments have shown that TGF-β, which is thought to play an important role in hypoxic and other forms of PH, increases binding of Smad2/3, Smad4, and the transcriptional corepressor HDAC-1 to the peroxisome proliferator-activated receptor (PPAR)-γ promoter. This reduces PPAR-γ, which has been shown to be associated with heightened proliferation and other abnormal activities in cells from the PH animals. Treatment with the PPAR-γ agonist, rosiglitazone, prevented this interaction, again implicating the role of HDACs in chronic hypoxia-induced remodeling (105).

Recent studies have also implicated miRs in the development of PH. miR204 expression has been shown to be decreased in animal models of PH and in human patient samples, and rescue of miR204 reverses PH in rats (58). miR17 has also been shown to be upregulated in hypoxia- and monocrotaline-
induction of miR210 is HIF-1α-dependent. Inhibition of miR210 in human PASMCs causes significant decrease in cell number under hypoxic conditions attributable to increased apoptosis probably via regulation of the transcription factor E2F3 (106). Another interesting study has demonstrated that hypoxia and miR210 increase proliferation in IPF fibroblasts (31). miR210 expression markedly increases in IPF fibroblasts in response to hypoxia, and knockdown of miR210 decreases hypoxia-induced fibroblast proliferation. Importantly, the investigators showed that silencing HIF-2α inhibits the hypoxia-mediated increase in miR210 expression, indicating that in certain cells HIF-2α is upstream of miR210 (31). Importantly, in situ analysis of IFP lung tissue demonstrated that miR210 expression was distributed similarly with HIF-2α and the hypoxic marker CA-1X in cells within the IFP fibrotic reticulum. Thus the authors raised the possibility that a pathological feed-forward loop could exist in fibrotic lungs, in which hypoxia promotes fibroblast proliferation via stimulation of miR210 expression, which in turn worsens hypoxia. Other miRs including miR145, 21, and 206 have been implicated in hypoxic forms of PH (44, 45, 214, 310). miR21 expression is increased in the distal PAs of hypoxia-exposed mice, and putative targets of miR21 including BMPR2 were increased (295). Sequestration of miR21 diminished chronic hypoxia-induced PH and vascular remodeling. miR145 is also of particular interest, as it was shown that miR145 was increased in hypoxic mouse lungs and that miR145 deficiency (knockout mice) and anti-miR145 both resulted in significant protection from hypoxia-induced PH.

Another recent study has demonstrated the complex relationship between gene regulation and gene expression with regard to epigenetic mechanisms. Wang et al. (270) demonstrated in adventitial fibroblasts from neonatal calves with severe hypoxia-induced PH that miR124 expression was decreased and that miR124 directly regulated MCP-1 expression and indirectly regulated proliferation through the alternative splicing factor PTBP1 (270). Further, it was shown that down-regulation of miR124 was mediated through Class I specific HDACs. The authors discovered that treatment of PH fibroblasts with HDAC inhibitors, including SAHA, apicidin, and OSU42 led to significant increases in miR124 while decreasing direct targets of miR124, including the alternative splicing factor PTBP1 and the proinflammatory cytokine MCP-1. Thus miR expression itself, under hypoxic conditions, is regulated by epigenetic modifications, specifically the removal of acetylation marks on histone, resulting in more condensed chromatin structure and inhibition of transcription. Collectively, these epigenetic changes, which occur in the setting of hypoxia and inflammation, begin to explain the constitutively activated phenotype of PH fibroblasts (270).

The aforementioned studies demonstrate that, although positive adaptive responses to acute hypoxia in the lung are probably crucial for maintaining homeostatic responses, long-term chronic hypoxia can result in responses that are detrimental to the lung and the lung vasculature. The classical adaptive responses to hypoxia, which aim to restore oxygen homeostasis in tissues including the lung, are regulated by the HIF family of proteins. It is thus not surprising that, in addition to work specifically in the lung, there is much current research which implicates epigenetic mechanisms in modulating the cellular response to hypoxic environments (35, 52, 275). There is increasing evidence supporting the idea that the activity of hypoxia-induced transcription factors, including HIF, is superimposed on a background of epigenetic changes that are essential for determining the cellular or tissue-specific hypoxic response. For instance, interesting work demonstrates that epigenetic modifications at the DNA and histone level have the ability to dictate HIF binding to target gene promoters and thus to regulate hypoxic gene expression. Furthermore, hypoxia itself is a potent inducer of chromatin remodeling via the regulation of enzymes that modulate DNA methylation and histone modifications. Long-term adaptation to chronic hypoxia involves significant modification of chromatin structure to maintain the hypoxic phenotype, even in the absence of HIF-1α. It is important to note that data in the pulmonary circulation, along with many other organs, suggest that chronic hypoxia is capable of inducing changes in gene expression that are independent of classical HIF pathway. Again, this is probably attributable to alterations in the methylation status of gene sequence or modification of the histone code, which are likely mediated through prolonged alterations in epigenetic-modifying enzymes.

There are four current opinions on the interactions of epigenetics and hypoxia: 1) HIF stabilization is influenced by the epigenetically controlled expression of VHL and PHD3. 2) Epigenetic mechanisms regulate HIF binding by maintaining a transcriptionally active chromatin confirmation within and around HIF binding site regions. This may occur through the action of the HIF-1α coactivation complex or through direct modifications of hypoxia-regulated rebinding sites, which prevent HIF binding. 3) A significant number of histone dimethylase enzymes are direct HIF-1 target genes and therefore play a role in the regulation of transcription during hypoxic responses. 4) Significant global changes in histone modifications and DNA methylation occur in response to hypoxic exposure.

Investigation of all these possibilities in the setting of chronic hypoxia in the lung will be important, as it will likely dictate new therapeutic approaches to ameliorate chronic hypoxia-induced lung tissue responses. At present, current data point to the possibility that HDAC inhibitors are important in controlling hypoxic-generated proliferative inflammatory and fibrotic responses. Clearly, other possibilities that will be aimed at DNA methylation and/or histone methylation will be tested in the not too distant future.

Conclusion

Hypoxic pulmonary hypertension or WHO group III PH comprises a heterogeneous group of diseases sharing the common feature of chronic hypoxia-induced pulmonary vascular remodeling, characterized by mild to moderate remodeling that
is largely reversible compared with the progressive irreversible disease seen in WHO group I disease. Despite the fact that patients with WHO III PH are much more likely to die as a result of their underlying lung disease than from complications of PH, the presence of PH in these patients is the most important marker of morbidity and mortality. Furthermore, this patient population is exponentially larger than that of patients with PAH, yet numerous clinical trials involving pulmonary vasodilators proven efficacious for PAH have either failed or shown harm in patients with hypoxic PH. There is a subset of patients with hypoxic PH who develop severe out-of-proportion PH characterized by pulmonary vascular remodeling that is irreversible and similar to that in WHO group I disease. We have highlighted the mechanisms involved in both the initiation and perpetuation of inflammation in the vessel wall as well as the interplay between hypoxia, inflammation, and their effects on resident pulmonary vascular cells and recruited immune and progenitor cells. We hypothesize that, in those patients with severe out-of-proportion irreversible hypoxic PH, they experience an as yet undefined second hit, whereby the mechanisms involved go beyond those related to hypoxia alone, leading to chronic nonresolving inflammation. We propose that important epigenetic changes, at least in mesenchymal cells and possibly in perivascular macrophages, are paramount in “locking” cells into a proremodeling, proinflammatory, and promitogenic phenotype. Furthermore, these fibroblast/macrophage-stromal cell interactions are necessary for the maintenance of chronic nonresolving inflammation and persistent pulmonary vascular remodeling. After nearly 20 yr of vasodilator therapies that have reduced both morbidity and mortality in patients with PAH, mortality remains unacceptably high, forcing the field to move toward treatments targeting the underlying pathogenesis of pulmonary vascular remodeling. Similarly, the persistent failure of pulmonary vasodilators in hypoxic PH urges us to shift away from recycling pulmonary vasodilator therapies proven to be beneficial in PAH to those that aim to disrupt and repair the basic pathological inflammatory mechanisms responsible for the initiation and perpetuation of disease.

GRANTS
This work was supported by the National Institutes of Health grants Program Project Grant HL-014985-39 (K. Stenmark) and Axis Grant HL-114887 (K. Stenmark).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: S.C.P. and K.R.S. edited and revised manuscript; S.C.P., K.C.E.K., and K.R.S. approved final version of manuscript.

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Hypoxic pulmonary hypertension

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