Hypoxia-Induced Pulmonary Vascular Remodeling
Cellular and Molecular Mechanisms

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Abstract—Chronic hypoxic exposure induces changes in the structure of pulmonary arteries, as well as in the biochemical and functional phenotypes of each of the vascular cell types, from the hilum of the lung to the most peripheral vessels in the alveolar wall. The magnitude and the specific profile of the changes depend on the species, sex, and the developmental stage at which the exposure to hypoxia occurred. Further, hypoxia-induced changes are site specific, such that the remodeling process in the large vessels differs from that in the smallest vessels. The cellular and molecular mechanisms vary and depend on the cellular composition of vessels at particular sites along the longitudinal axis of the pulmonary vasculature, as well as on local environmental factors. Each of the resident vascular cell types (ie, endothelial, smooth muscle, adventitial fibroblast) undergo site- and time-dependent alterations in proliferation, matrix protein production, expression of growth factors, cytokines, and receptors, and each resident cell type plays a specific role in the overall remodeling response. In addition, hypoxic exposure induces an inflammatory response within the vessel wall, and the recruited circulating progenitor cells contribute significantly to the structural remodeling and persistent vasoconstriction of the pulmonary circulation. The possibility exists that the lung or lung vessels also contain resident progenitor cells that participate in the remodeling process. Thus the hypoxia-induced remodeling of the pulmonary circulation is a highly complex process where numerous interactive events must be taken into account as we search for newer, more effective therapeutic interventions. This review provides perspectives on each of the aforementioned areas. (Circ Res. 2006;99:675-691.)

Key Words: pulmonary hypertension ■ pulmonary vasoconstriction ■ fibrocyte ■ inflammation ■ progenitor cell ■ adventitia

Pulmonary hypertension contributes to the morbidity and mortality of adult and pediatric patients with various lung and heart diseases.1–8 Importantly, many of these diseases or conditions are associated with persistent or intermittent hypoxia, either globally or regionally, within confined areas of the lung. These diseases include those associated with upper airway obstruction, such as obstructive sleep apnea, Pickwickian syndrome, obesity hypoventilation syndrome, and damage or injury to the respiratory center either as a primary disorder (Ondine’s curse) or secondary to trauma or other neurologic disease. A number of primary lung diseases are also associated with the presence of chronic hypoxia, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, diffuse interstitial fibrosis, bronchopulmonary dysplasia, radiation fibrosis, infiltrative lung tumors, and collagen vascular disease.1–7 Neuromuscular and skeletal disorders affecting the chest wall such as scoliosis, Duchenne muscular dystrophy, and poliomyelitis may also impair ventilation and be associated with hypoxia-induced pulmonary hypertension.1,4,8 In many of these conditions, parenchymal lung injury and inflammation contribute directly to the pulmonary hypertension, but there is little question that hypoxia also contributes to the vascular changes and ultimately the pulmonary hypertension observed in each of these conditions.

The idea that hypoxia alone can cause pulmonary hypertension and significant structural remodeling of pulmonary arteries (PAs) in humans is supported by observations that in persons living at high altitude there is chronic elevation of pulmonary artery (PA) pressure only, a small portion of which may be reversible with administration of oxygen.9 Furthermore, in high altitude residents, a far greater increase in PA pressure in response to exercise is observed than in sea-level dwellers.9 In the lungs of these persons, increased expression of α-smooth muscle actin (α-SM-actin) is observed in the walls of small PAs, which normally have little if any smooth muscle, and larger, more proximal vessels exhibit a thickened media and adventitia, findings collectively considered as hallmarks of hypoxia-induced pulmonary vascular remodeling and hypertension. In further support of the idea that chronic hypoxia alone can cause rapid and significant changes in human PAs are the results of a simulated climb of Mount Everest in studies termed “Oper-
Hypoxia-Induced Vascular Remodeling: Observations in Animal Models

Chronic hypoxic exposure induces changes in the structure of PAs, as well as in biochemical and functional phenotypes of each of the cell types composing the artery, from the hilum of the lung to the most peripheral vessels in the alveolar wall.

Morphological Changes of the Pulmonary Arteries in Response to Chronic Hypoxia

A number of features are observed nearly universally in the PAs of mammals who develop pulmonary hypertension on chronic exposure to hypoxia. (Importantly, there are species that have adapted to residing in chronically hypoxic conditions in which vascular remodeling is not observed, eg, pika, yak, snow pig, llama.12–16) The magnitude of the changes induced depends on the species studied, the sex of the animal, and the developmental stage at which the animal is exposed to hypoxia.16–19 Structural changes include the appearance of SM-like cells (as defined by the expression of α-SM-actin) in previously nonmuscularized vessels of the alveolar wall, so called “distal extension of smooth muscle,” an apparent sine qua non of hypoxia-induced pulmonary hypertension. In addition, there is medial and adventitial thickening of the muscular and elastic vessels. The medial thickening is believed to be attributable to hypertrophy and increased accumulation of smooth muscle cells as well as increased deposition of extracellular matrix proteins, predominantly collagen and elastin. The adventitial thickening is assumed to be caused by accumulation of fibroblasts and myofibroblasts and an often marked increase in extracellular matrix accumulation (collagens, elastin, fibronectin, tenascin).13,15,20 Intimal changes have been consistently observed in hypoxic rat models of pulmonary hypertension, yet they are usually minimal, at least from a morphological point of view.13,15,21 However, in hypoxic neonatal calves with extreme elevations of pulmonary arterial pressure (mean, ≥100 mm Hg), intimal thickening is more pronounced and perhaps more similar to the changes observed in humans.22,23 Lastly, many studies have also reported a reduction in the cross-sectional area of the pulmonary vascular bed resulting from loss of small blood vessels (sometimes termed rarefaction or pruning) following chronic hypoxic exposure.13,24–27 This, along with the remodeling described above, is thought to be responsible for the structural or nonvasoconstrictive component of chronic hypoxic pulmonary hypertension. However, this concept of vascular rarefaction has recently been challenged by reports demonstrating angiogenic responses in the pulmonary capillaries in response to hypoxia.28,29 “Angiogenesis” is thought to counteract pulmonary hypertension by lowering perfusion resistance in the pulmonary vascular bed. In support of this idea is the fact that lung overexpression of angiotatin, an antiangiogenic cleavage product of plasminogen, aggravates pulmonary hypertension in chronically hypoxic mice.30 In addition, overexpression of vascular endothelial growth factor (VEGF) in the lung has been shown to protect against hypoxic pulmonary hypertension.27

Site-Specific Changes in Pulmonary Vascular Structure: Cellular Mechanisms

The cellular and molecular mechanisms used to effect cellular and structural changes at specific locations within the pulmonary circulation are different. This is because the cellular composition of the artery changes along the longitudinal axis of the pulmonary circulation. Although it has been said that pulmonary hypertension is a disease of the distal lung circulation, it is increasingly appreciated that structural change in larger vessels may contribute directly to right ventricular work and failure and to changing flow dynamics and thus distal vascular remodeling. An understanding of the vascular remodeling induced by chronic hypoxia thus requires an evaluation of the cells participating and the potential mechanisms through which these cellular changes are effected at different vascular sites.

Vascular Remodeling in Large Proximal Pulmonary Arteries

In the large PAs (conducting or elastic), the media and adventitia both increase in thickness in response to chronic hypoxic exposure. In the rat, adventitial thickening is early and dramatic, whereas thickening of the media lags behind.13,15 Adventitial fibroblasts demonstrate earlier and more significant increases in DNA synthesis compared with smooth muscle cells (SMCs). In fact, medial SMCs were not observed to demonstrate an increase in DNA synthesis until at least day 3, and, at this point in time, the labeling index increased from less than 0.5 to only approximately 1 to 1.5. The findings in the mouse are similar, with a brief rise in the medial cell labeling index in the main PA at days 4 to 6, followed by a rapid decline in medial SMC labeling and total SMC number compared with controls at 3 weeks. This
suggests that the medial thickening is largely attributable to increased matrix (elastin and collagen) deposition and cell hypertrophy. The mouse adventitia also undergoes thickening with an accumulation of cells and matrix proteins (predominately collagen).31,32

In large animals (eg, calf and pig), the responses are different, with early and dramatic medial thickening predominating.33,34 The differences may be explained by the fact that the cellular composition of the proximal pulmonary and systemic vessels of larger mammalian species (including the cow, lamb, pig, and human) is more complex than that of the rodent species (Figure 1).14,35–39 The media of large conducting pulmonary and systemic vessels in various large mammalian species, including the human, is composed of multiple phenotypically distinct SMC populations.14,35–38 These different SMC populations appear to serve different functions based on observations of distinct ion channel expression and proliferative and matrix-producing capabilities in response to many stimuli including hypoxia.40–46 There is evidence to support the argument that these cells are derived from distinct lineages and are not simply a common cell exhibiting different states of differentiation.35–37,47 Although it is evident that these phenotypically distinct cell populations serve different functions in health and disease, at present, the embryonic origin of distinct cell populations, the ratio of different cells at a given vascular site in a given species, and the mechanisms directing the developmental assembly of these cells types into a functioning, mature blood vessel wall remain to be determined.

It has been shown in larger mammalian species that the medial thickening of proximal PAs in response to chronic hypoxia can be accounted for, in large part, by the proliferation of a distinct SM-like subpopulation residing within the media.37,48 This SM-like population exists in a relatively "undifferentiated state" (as assessed by SM-specific cytoskeletal and contractile protein marker expression) in comparison with other SMCs within the vessel wall. The in situ observations are consistent with cell culture studies demonstrating that only less differentiated SM-like cells demonstrated increases in proliferation in response to hypoxic exposure.42,43,49 SMCs that maintain a more differentiated phenotype in culture exhibited growth inhibition under hypoxic conditions.42 The adventitia of the large PA of calves underwent less thickening than in rats or mice, perhaps because of the fact that the PA media in larger mammals contains cells capable of rapidly responding to hypoxia and increases in wall tension by proliferating and secreting matrix proteins (Figure 1). Because thickening serves to decrease wall stress, the existence of such a cell population within the media of large vessels may provide the vascular wall with a mechanism to adapt rapidly to increased wall tension. Thus, highly differentiated SMCs are spared from dedifferentiating and proliferating and may maintain their normal homeostatic functions, such as contraction in these vessels.

Little is known regarding the mechanisms that confer unique proliferative characteristics to specific cell populations that exist in the large PAs. It has been demonstrated that less differentiated and more proliferation-prone medial cells are characterized by exuberant responses to G protein-coupled receptor (GPCR) agonists, compared with the differentiated medial SMCs that do not exhibit proliferative responses to hypoxia.37,42,43,49 These observations suggest that there may be differences in receptor expression and/or intracellular signaling among various medial cell types. Differences in endothelin production, receptor expression, and responses have been described in distinct cell populations derived from the ovine PA.50,51

Medial SM-like cells, that respond with increased proliferation to hypoxia both in vivo and in vitro, have been demonstrated to exhibit augmented responses to stimulation of the protein kinase C (PKC) pathway.17,52 Hypoxia-induced activation of GPCRs, with subsequent signaling through Gαi- and Gαs-mediated activation of extracellular signal-regulated kinase 1/2 (ERK1/2) has also been shown to be necessary for hypoxia-induced proliferation.53 Differences in cAMP response-element binding protein (CREB) expression have also been shown to function as molecular determinants of SMC proliferative capability under hypoxic conditions.54 Whether hypoxia inducible factor (HIF)-1α, the prototypic hypoxia-inducible transcription factor, is directly involved in
hypoxia-induced proliferation of specific medial SMC populations remains unclear at present. Collectively, these observations support the existence of distinct regional cell populations with membrane-bound receptors sensitive to hypoxic activation that engage specific intracellular signaling pathways conferring unique hypoxic proliferative responses.

As mentioned above, marked increases in collagen and elastin accumulation are observed in proximal vessels in response to hypoxic exposure and contribute to medial and adventitial thickening. Less is known of the pathways stimulating matrix protein production under hypoxic conditions than of those responsible for cell proliferation. There is reason to believe the former are distinctly different and perhaps even more complex than those regulating proliferation. For instance, hypoxia decreases elastin production in cultured bovine main PA SMCs, whereas in vivo, in chronically hypoxic animals of all examined species, marked increases in elastin accumulation in the PA media are observed. On the other hand, hypoxia appears to directly increase collagen production and expression of transforming growth factor (TGF)-β, an important regulator of collagen synthesis in fibroblasts, both in vivo and in vitro. The importance of TGF-β in hypoxia-induced collagen production and vascular remodeling is demonstrated in recent studies in which a dominant negative mutation in the TGF-β receptor blocks chronic hypoxia-induced pulmonary hypertension in rats. In addition, administration of rh relaxin, a hormone with direct effects on collagen and fibronectin production by fibroblasts, and other antifibrotic agents reduce hypoxia-induced pulmonary hypertension. Findings that again emphasize the importance of matrix accumulation even in large vessels in hypoxia-induced pulmonary hypertension.

More work is needed to determine the mechanisms regulating proliferation and matrix protein production by the various cells comprising the vascular wall of the proximal large PAs under hypoxic conditions, as these changes obviously have significant effects on vascular wall compliance and thus on pulmonary blood flow and right heart function. Many studies now support the concept that the changes observed in large blood vessels in response to chronic hypoxia and other stimuli are important and have significant effects on right heart function. Increases in wall stiffness in large vessels contribute to the overall increases in impedance and contribute as much as 30% to 40% of the increase in load to which the right ventricle is exposed. In addition, this increase in stiffness, associated with vascular remodeling in proximal vessels, leads to an inability of conductance vessels to store and deliver entire stroke volume of the right ventricle and ultimately results in a loss of pulmonary flow during diastole. This disturbance of flow not only increases the load on the right ventricle but also may contribute to decrease in NO production in distal PA endothelial cells, which require steady pulsatile flow for optimal NO production.

Vascular Remodeling in Distal Muscular Pulmonary Arteries

The distal pulmonary circulation, which is the primary site of hypoxic pulmonary vasoconstriction, also undergoes significant structural change in response to chronic hypoxic exposure. Therefore, an important question is whether the cellular mechanisms that act to cause structural changes in the distal vessels are the same as those used in the proximal arteries. Studies in various species raise the possibility that they are different. Significant differences in the electrophysiologic properties between proximal and distal SMCs exist and contribute to the unique nature of the distal circulation. Distal PA SMCs cultured from normal calves, demonstrate a resistance to traditional growth-promoting stimuli, including platelet-derived growth factor (PDGF)-BB, angiotensin II, and TGF-β1, as well as to serotonin and endothelin. Importantly, hypoxia also consistently inhibited growth of distal PA cells in the presence or absence of serum. These responses to growth factors and cytokines differ significantly from those described in the far more commonly used bovine vascular SMCs (VSMCs) derived from the media of the main PA, where PDGF, serotonin, endothelin, and angiotensin stimulate cell proliferation rather than cell hypertrophy. The responses are also different from those observed in SMCs derived from the rat or mouse PA, where, again, proliferative responses to these factors as well as to hypoxia have been reported. Thus, it appears that the medial SMCs in the distal pulmonary circulation, at least in some species, comprise a uniform population of well-differentiated and relatively growth-resistant cells. Studies in the human pulmonary circulation also suggest differences in proliferative capacity between SMCs derived from proximal and distal vessels. In some reports, cells described as “SM-like” derived from the distal vessels exhibit greater proliferative responses to mitogens than cells from the proximal vessels. However, it is unclear whether the cells obtained were only from normotensive patients or whether pulmonary hypertensive patients were also included. Furthermore, cells were characterized as SMCs based only on expression of α-SM-actin (but no other SM-specific marker), raising the possibility that nonmedial cells were cultured and studied, as might occur with myofibroblasts (also α-SM-actin+ cells).

Observations of a uniform population of growth-resistant SMCs in the distal circulation raise a question as to what, besides hyperplasia of the resident medial SMCs, could explain the marked medial thickening in response to chronic hypoxic exposure. Importantly, in every study assessing cell proliferation in animals exposed to hypoxic conditions, early and significant increases in fibroblast proliferation were observed. In addition, hypoxia has been demonstrated to induce differentiation of a fibroblast into a myofibroblast phenotype and to increase myofibroblast numbers in the PA adventitia. In the systemic circulation, early activation, differentiation, and subsequent migration of fibroblasts/myofibroblasts into the media have been suggested as a mechanism used to contribute to thickening. The possibility that this or a similar mechanism is operating to affect medial thickening in small PAs was suggested more than 20 years ago by Sobin et al and, more recently, by Jones and colleagues. Both investigators have presented evidence in support of the idea that either an adventitial fibroblast or an interstitial lung fibroblast is activated by hypoxia, recruited,
and differentiates into a SM-like cell in the "newly muscularized" vessel.

It seems logical that cells with a larger repertoire of functional capabilities, such as adventitial fibroblasts or even recently described adventitial stem-like cells, may be more capable of proliferating, migrating, and secreting matrix proteins than highly differentiated contractile medial SMCs, the function of which is to control vascular tone and blood flow within the distal circulation. Recent in vivo and in vitro studies evaluated the cellular composition of the distal PA media of normoxic and chronically hypoxic neonatal calves. The media of control vessels was comprised of a phenotypically uniform population of SMCs, whereas at least 2 distinct cell populations were detected in chronically hypoxic vessels. One was comprised of highly differentiated and significantly hypertrophied SMCs, and another was an undifferentiated cell population with high proliferative and migratory capabilities. Experimental data suggested that this latter cell population was of a nonresident, nonmedial origin (be it adventitial, interstitial, or circulating) and that these cells have migrated into the media. This possibility was supported by recent studies showing that hypoxia induces recruitment of mesenchymal precursors, including fibrocytes, into the vessel wall. Fibrocytes comprise a subpopulation of circulating leukocytes (CD45⁺, CD11b⁺) that, at the site of tissue injury, can assume a mesenchymal phenotype (α-SM-actin⁺, type-I collagen⁺) and the functions of resident fibroblasts or myofibroblasts. Fibrocytes have been described as contributing to fibrosis in a number of other fibroproliferative conditions in the lung.

In summary, there is experimental data to suggest that the cellular mechanisms contributing to remodeling of the distal vasculature (as schematically depicted in Figure 2) are different from those of the proximal circulation (Figure 1), especially in larger mammalian species, in which proximal vessels are comprised of heterogeneous cell populations with distinct growth and differentiation properties. In vessels in which the media is comprised of a uniform population of differentiated SMCs, adventitial cells as well as circulating progenitors may provide important cellular sources of the media thickening (Figure 2), which has traditionally been ascribed to resident SMC proliferation. More studies are needed in this area to determine the mechanisms controlling medial thickening of the distal circulation, because it constitutes a critical part of the hypoxic pulmonary vascular remodeling response.

**Structural Changes in Nonmuscular Alveolar Wall Vessels**

Perhaps the most characteristic, yet least understood, change in the pulmonary vasculature that occurs in response to chronic hypoxic exposure is the "muscularization" of the vascular segments that normally lack a muscular coat converting the precapillary segment into a resistance structure. It is the first cellular event to occur in response to chronic hypoxic exposure, as well as the first to disappear on withdrawal of the hypoxic stimulus. Mural cells are reported to increase in size and number, and a media and adventitia are formed to produce a new structural vessel profile. Several mechanisms have been invoked to explain the distal muscularization process. Certain vessels, termed "partially muscular," contain pericytes and/or "intermediate cells." These cells exhibit SM-like characteristics, although they can be distinguished morphologically and biochemically from a differentiated SMC. In vessels where either pericytes or intermediate cells are present, it has been suggested that hypoxia induces differentiation and proliferation of these cells such that they may directly contribute to the observed muscularization. Other mechanisms, including those discussed below may also operate to contribute to "distal muscularization."

In the smallest alveolar wall vessels, which normally lack any elastic lamina and possess a capillary-like wall structure,
edge in hypoxic pulmonary disease, is that lung epithelial cells may transdifferentiate into mesenchymal cells. Epithelial/mesenchymal transition (EMT) is critical during normal embryonic development and is increasingly appreciated to contribute to fibrosis and cancer development and progression. It is also possible that in these vessels, especially perivascular, TGF-β and Wnt signaling are important transducers of the EMT process. These systems are known to be regulated by hypoxia, thus supporting the idea that EMT could also contribute to the distal PA muscularization process. A better understanding of distal muscularization could provide new opportunities for therapy.

**Cell-Specific Changes Induced by Hypoxia**

**Changes in Endothelial Cell Phenotype in Chronic Hypoxic Pulmonary Hypertension**

As stated above, chronic hypoxic exposure increases intimal thickness in the pulmonary circulation by causing hypertrophy and hyperplasia of the endothelial cells and thickening of the subendothelial space. There can be focal disruption of the pulmonary endothelial cell basement membrane, creating a patchy appearance of microfibrillar material and a thickened subendothelial space, much like that reported in the aorta of hypertensive rats, observations consistent with experiments showing that hypoxic exposure increases lung vascular permeability measured by direct protein extravasation. Influx of plasma proteins into the vessel wall may activate vascular wall proteases, initiating a series of remodeling events in a series of events described by Rabinovitch. Endothelial cells, in response to hypoxia, have been shown to produce more laminin, fibronectin, and elastin, all of which affect endothelial as well as SMC function. On the other hand, chronically hypoxic endothelial cells produce and secrete less heparan sulfate proteoglycans than do normoxic cells, suggesting that chronic hypoxia impairs the ability of the endothelium to produce growth-restrictive molecules for the SMCs. Importantly, the endothelial changes observed differ at various sites along the longitudinal axis of the PA. It is also interesting that significant differences are noted between PA endothelial cells of the yak, a species resistant to the development of pulmonary hypertension, and the closely related bovine species, the most susceptible to hypoxia-induced pulmonary hypertension of all species examined, observations also supporting the idea that the endothelium plays a critical role in mediating many of the changes observed in the hypoxia-induced hypertensive process.

These changes in pulmonary endothelial cell structure and local environmental milieu are accompanied by alterations in their physiological and metabolic functions. Hypoxic exposure decreases antithrombotic potential, increases permeability and expression of inflammatory cell markers, and interferes with a variety of cell plasma membrane-dependent receptors, metabolic and transport functions of the endothelial cell. Hypoxia exerts profound effects on the physical state and composition of endothelial cell plasma membrane lipids, membrane fluidity, and plasma membrane phospholipid and fatty acid composition. The mechanisms through which hypoxia alters plasma membranes in the pulmonary circulation are not clear. Increased contents of

**Figure 3.** Schematic representation of the potential cellular mechanisms involved in hypoxia-induced remodeling of a non-muscular pulmonary artery. At least 2 possibilities have been implicated in this process. Hypoxia can induce phenotypic modulation of a resident interstitial lung fibroblast into a myofibroblast, and its recruitment to the pulmonary vessel wall, where it ultimately assumes a SMC-like phenotype. Another possibility is that a subset of circulating leukocytes with the potential to express mesenchymal characteristics (termed fibrocytes) is exuded from the vessel, incorporate themselves into the vascular wall, and assume mesenchymal characteristics.

The mechanisms of muscularization may be different and are probably more complex than simply migration of pericytes down the vessel. One suggested mechanism is the recruitment of interstitial fibroblasts through a process of migration, alignment, and incorporation into the vessel wall (Figure 3). It is also possible that in these vessels, especially so-called “corner vessels” in the alveolus, there is extravasation of inflammatory and mesenchymal precursor cells. Similar to their role in muscularized vessels discussed above, these cells could differentiate into SM-like cells and contribute to hypoxia-induced distal muscularization (Figure 3). The possibility that endothelial cells transdifferentiate into a SM-like cell and contribute to distal muscularization also needs to be considered. Endothelial/mesenchymal transdifferentiation plays an important role in vascular development of the pulmonary and systemic circulations. Microvascular endothelial cells have been reported to have the capability to transdifferentiate into mesenchymal-like cells. Another intriguing possibility, although never explored to our knowl-
malondialdehyde and conjugated dienes suggest that lipid peroxidation occurs and plays a role. Another potential mechanism is activation of membrane phospholipases, which would account for the reduction in the content of plasma membrane phospholipids and the concomitant increase in the release of fatty acids. Increases in phospholipase A and C and diacyl glycerol (DAG) lipase activity have been demonstrated. Membrane changes observed in hypoxic endothelial cells contribute directly to the alterations in growth factor receptor and function, as well as in the transport and processing of biogenic amines, extracellular nucleotides, and adenosine, and thus could have significant effects on pulmonary vascular tone and structure.

Hypoxia alters surface coagulant properties of endothelial cells (Figure 4), suppressing normal thrombomodulin production and inducing procoagulant activity, largely through the increased expression of tissue factor. Hypoxia also increases interleukin (IL)-1 and IL-6 mRNA levels and IL-1α production in cultured endothelial cells, probably through nuclear factor κB (NF-κB) or NF-IL-6. Hypoxia (and/or IL-1) can also increase expression of endothelial leukocyte molecule (ELAM)-1, intracellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 on endothelial cells. These observations suggest that hypoxia induces changes in the endothelial cell, leading to prothrombotic and proinflammatory interactions with circulating cells that collectively participate in the pulmonary hypertensive response. Indeed, increased adherence of both leukocytes and platelets have been observed in chronically hypoxic rats. This proinflammatory response could also facilitate recruitment and migration of progenitor cells into the vessel wall.

Chronic hypoxic exposure is associated with a number of changes in the production and release of potent vasoactive substances by the endothelium which can exert significant effects not only on the contractile state of SMCs but on their proliferative and synthetic state as well. Decreased production and/or activity of prostacyclin and NO have been reported. Increases in the production of platelet-activating factor (PAF), leukotrienes, HETEs, endothelin, serotonin, and PDGF have been observed. In addition, recent reports have suggested that there is a release of extracellular ATP by PA endothelial cells under hypoxic conditions. This is interesting because ATP is also released under conditions such as osmotic swelling, which, as discussed above, has been described in the setting of hypoxic exposure. Extracellular ATP has direct effects not only on the endothelial cell, where, acting through purinergic receptors and AMP-activated protein kinase (AMPK), it participates in controlling NO release, but also on other endothelial cell functions. Released nucleotides also directly activate SMCs and fibroblast proliferation and migration.

The direct relevance of the release (or lack thereof) of many of these molecules to the hypoxia-induced hypertensive process has been confirmed in whole animal studies. Rats treated with PAF inhibitors and 5-lipoxygenase inhibitors, as well as mice lacking 5-lipoxygenase, demonstrate less pulmonary hypertension and vascular remodeling in response to hypoxia. Endothelin receptor antagonists inhibit hypoxia-induced pulmonary hypertension. Germline deletions in NO synthase 3 (NOS3) are associated with increased right ventricular pressure and distal muscularization in both normoxia and hypoxia. However, 1 group of investigators found a decrease in pulmonary arterial muscularization early in the course of hypoxic exposure. Increased production of the NOS3 cofactor tetrahydrobiopterin is associated with attenuated development of hypoxic pulmonary hypertension and vascular remodeling, whereas NOS3 deficiency results in enhanced pulmonary hypertension and remodeling. Deficiency in other NOS isoforms has not been clearly associated with increased pulmonary vascular responses to hypoxia. Decreases in other vasodilator genes, such as atrial natriuretic peptide adenomedullin, and heme oxygenase-1, and prostacyclin, are all also associated with enhanced hypoxia-induced pulmonary hypertension and/or remodeling. Conversely, increased expression of the serotonin (5-hydroxytryptamine [5-HT]) transporter (5-HTT) gene is associated with enhanced hypoxia pulmonary hypertension. Decreased expression of the 5-HT-1B receptor is associated with decreased hypoxia responses. Deficiency in the endothelin-1B receptor in rats is associated with increased hypoxia pulmonary hypertension and remodeling. Interestingly, germline overexpression of endothelin (ET)-1 in a mouse did not result in significant pulmonary hypertension.

### Table: Chronic Hypoxia Influences Release of Vasoconstrictors, Growth Factors, Matrix Proteins, and Adhesion Molecules in Endothelial Cells

<table>
<thead>
<tr>
<th>Vasoconstrictors / Vasodilators</th>
<th>Adhesion Molecules / Cytokins / Procoagulants</th>
<th>Matrix Molecules</th>
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<tbody>
<tr>
<td>PDGF-B</td>
<td>P-selectin</td>
<td>Laminin</td>
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<tr>
<td>IGF</td>
<td>ICAM</td>
<td>Fibronectin</td>
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<tr>
<td>VEGF</td>
<td>VCAM</td>
<td>Thrombospondin-1</td>
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<tr>
<td>bFGF</td>
<td>VEGF-1</td>
<td>(Heparan Sulfate Proteoglycans)</td>
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<tr>
<td>Serotonin</td>
<td>PAI-1</td>
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</tr>
<tr>
<td>Endothelin</td>
<td>IL-1</td>
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<tr>
<td>LTC4 / D4</td>
<td>IL-8</td>
<td></td>
</tr>
<tr>
<td>(Nitric Oxide)</td>
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<td>(PGI2)</td>
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but was associated with pulmonary inflammation and fibrosis. The collective body of evidence described above demonstrates that the endothelial cell is a direct target of hypoxia. Marked changes in its permeability, coagulant, inflammatory, and protein synthetic capabilities are observed in response to hypoxic exposure. These changes can affect directly or indirectly underlying SMCs and adventitial fibroblasts, thus contributing to chronic abnormalities in vascular tone and structure.

**Changes in SMC Phenotype in Chronic Hypoxic Pulmonary Hypertension: Specific Contribution to Vascular Tone and Structure**

The severity of chronic hypoxic pulmonary hypertension is determined, at least in part, by the extent of structural changes in the medial compartment of the pulmonary arterial wall. These changes include SMC proliferation, hypertrophy, matrix protein production, and recruitment of adventitial or circulating cells. Hypoxia, blood-borne and locally produced cytokines, and mechanical stress collectively act to drive the cellular responses within the media. These stimuli activate a cascade of intracellular signaling mechanisms, including tyrosine kinases, mitogen-activated protein kinases (MAPKs), PKC, phosphoinositidyl 3-kinase (PI3K), SMAD phosphorylation, calcium (Ca\(^{2+}\)) entry and Rho kinases, which collectively act to control SMC contractility, growth, differentiation, and matrix protein synthesis (Figure 5). Synergy between different stimuli and the resulting “cross-talk” between signal transduction pathways augment the extent of vascular changes within the media. Susceptibility to these stimuli is enhanced when inhibiting mechanisms, such as endothelial barrier function, local production of heparan sulfates and prostaglandin I\(_2\) (PGI\(_2\)) and NO-induced increases in cyclic nucleotides, are impaired. Intrinsic (genetic, developmental, acquired, or epigenetic) differences in growth and matrix synthetic capacity, as well as local and regional phenotypic heterogeneity of pulmonary SMCs also regulate the pattern of remodeling of the tunica media in response to chronic hypoxia. Changes in receptor expression or function...
as well as in Ca^{2+} handling have been observed in SMCs from hypertensive pulmonary arteries. Examples of these changes are discussed.

**Hypoxia-Induced Changes in Serotonin Transporter and Receptor Function in SMCs**

Several studies have shown a role for 5-HT in the regulation of acute and chronic hypoxic responses, particularly as they relate to contraction and growth of SMCs.\(^{130,133-135}\) 5-HT is a vasoactive and, in some SMCs, a mitogenic molecule released from platelets, pulmonary neuroendocrine cells, and endothelial cells. Moreover, increases in plasma serotonin levels have been observed under hypoxic conditions.\(^{136}\) Exposure of PA SMCs to elevated levels of 5-HT results in increased SMC contraction and proliferation.\(^{134}\) These observations have generated great interest in elucidating the mechanisms through which serotonin exerts its effects and contributes to the remodeling process. An active transporter that internalizes serotonin (5-HTT) and multiple cell surface serotonin receptors (5-HT-2A, 5-HT-1B/1D, and 5-HT-2B) have been described on SMCs, and it appears that a balance of transporter and receptor expression and function determines the cellular responses to serotonin.\(^{135}\) Overexpression of the 5-HTT gene in rats exposed to chronic hypoxia augmented vascular remodeling, as did continuous infusion of serotonin.\(^{130}\) Administration of the 5-HTT inhibitors citalopram and fluoxetine partially reduced increased pulmonary vascular resistance in chronically hypoxic mice, a finding supported by another experiment, in which hypoxic pulmonary hypertension was attenuated in knockout mice with disruption of 5-HTT.\(^{137,138}\) It has also been demonstrated that the increased contractile response to 5-HT in PAs isolated from chronically hypoxic wild-type mice is mediated through the 5-HT-1B receptor and that 5-HT-1BR knockout mice develop less pulmonary vascular remodeling and hypertensive pulmonary hypertension despite maintaining an acute response to hypoxia.\(^{139}\) Moreover, 5-HT-1BR, 5-HT-2BR, and 5-HTT are colocalized in PA SMCs, and the 5-HT-2BR functionally interacts with the 5-HT-1BR. This cross-talk between the G<sub>i</sub>-coupled 5-HT-2BR and the G<sub>i</sub>-coupled 5-HT-1BR has been suggested to facilitate the development of pulmonary hypertension.\(^{135}\) It is possible that serotonin receptors act by producing reactive oxygen species via the NADPH oxidase-like enzyme to induce SMC proliferation.\(^{140}\)

Collectively, these observations suggest that the mechanisms of action of 5-HT, its transporter, and receptors are rather complex and may be species and cell type dependent. Future studies will be needed to delineate how hypoxia affects serotonin-induced changes in SMC phenotype and the relation of these changes to hypoxia-induced vascular remodeling.

**Chronic Hypoxia-Induced Changes in Ionic Balance and Calcium Homeostasis in SMCs: Effects on Tone and Proliferation**

Accumulating evidence indicates that both subacute and chronic hypoxia cause intrinsic changes in the ionic balance and calcium homeostasis of PA SMCs, resulting in membrane depolarization, elevation in resting intracellular calcium [Ca^{2+}], persistent vasoconstriction, and changes in electrophysiological and calcium responses to vasoconstrictors and vasodilators.\(^{141-143}\)

McMurtry et al proposed almost 2 decades ago that chronic hypoxia might have some overlap with the cellular and molecular mechanisms involved in mediating acute hypoxic pulmonary vasoconstriction.\(^{144}\) Furthermore, because chronic hypoxia is also associated with variable degrees of medial SMC hypertrophy and increases in numbers of SMCs or SM-like cells, a common hypothesis is that pulmonary vasoconstriction and PA SMC proliferation use overlapping signaling processes that contribute to vasoconstrictive and structural changes. For example, hypoxia-induced increases in [Ca^{2+}], cause pulmonary vasoconstriction and also act as an important stimulus for SMC proliferation.\(^{145}\) These observations led to the hypothesis that hypoxia-induced decreases in K<sub>i</sub> channel currents, membrane depolarization, and increased [Ca^{2+}], in SMCs may serve as a shared mechanism to cause pulmonary vasoconstriction and stimulate SMC proliferation. Indeed, chronic hypoxia reduces K channel activity and also mediates changes in the transcriptional and translational control of K channel genes in PA SMCs.\(^{141,142}\) Chronic hypoxia downregulates the mRNA and protein expression of several pore-forming α-subunits of K<sub>i</sub> channels in rat PA SMCs (K, 1.1, 1.5, 1.6, 2.1, and 4.3), whereas no appreciable expression changes are seen in mesenteric artery SMCs.\(^{146}\) In addition to effects on SMC proliferation, decreases in K<sub>i</sub> channel expression may actually protect the cell from apoptosis. Inhibition of K channel activity can attenuate proapoptotic volume decreases by maintaining sufficient potassium within the cytoplasm to inhibit apoptosis.\(^{143}\) It is not known exactly what controls chronic hypoxia-induced changes in K<sub>i</sub> expression. However, many vasoactive agonists and growth factors, which are known to be released by endothelial cells (including ET-1) in response to hypoxia as described above, can have effects on K<sub>i</sub> channel expression.\(^{143,145}\)

Concordant with the observations of decreases in K<sub>i</sub> channel expression, sustained membrane depolarization, activation of L-type calcium channels, and increases in intracellular calcium, are observations that calcium channel antagonists nifedipine and verapamil attenuate hypoxia-induced pulmonary hypertension.\(^{147}\) However, other studies showed that the effects of calcium channel blockers are often only partial or temporary and are sometimes complicated by changes in cardiac output.\(^{148}\) Interestingly, in chronically hypoxic rats, nifedipine was ineffective in reducing pulmonary hypertension, whereas another vasodilator, NIP121, caused significant reduction in PA pressure and vascular resistance.\(^{149}\) More dramatically, the elevated resting [Ca^{2+}], in PA SMCs of chronically hypoxic rats was unaffected by nifedipine but was reduced instantaneously to the level of control PA SMCs by the removal of extracellular Ca^{2+}.\(^{150}\)
These observations suggest that Ca\(^{2+}\) influx pathways, other than voltage-dependent calcium channels (VDCCs), are involved in chronic hypoxic pulmonary vasoconstriction and remodeling.

Nonselective cation channels encoded by the canonical transient receptor potential (TRPC) gene family constitute alternative pathways of Ca\(^{2+}\) entry into VSMCs. Multiple TRPC subtypes have been identified in PA SMCs, with the existence of functionally distinct receptor-operated and store-operated cation entry pathways.\(^{151-154}\) The store-operated Ca\(^{2+}\) channel TRPC-1 and the receptor-operated Ca\(^{2+}\) channel TRPC-6 are coexpressed in PA SMCs. Chronic hypoxic exposure upregulated TRPC expression and enhanced both store-operated and receptor-operated calcium entry into PA SMCs.\(^{152}\) Upregulation of TRPC by hypoxia appears subtype specific, such that TRPC-1 and TRPC-6 expression is doubled or tripled, whereas other TRPCs are unaltered. The mechanisms regulating hypoxia-induced upregulation of these Ca\(^{2+}\) channels are currently unclear. However, recent studies show that, during serum-induced proliferation, TRPC-1 mRNA is upregulated, resting Ca\(^{2+}\) is elevated, and store-operated Ca\(^{2+}\) entry is enhanced and that TRPC expression is increased by PDGF in PA SMCs.\(^{151,155}\) PA SMC proliferation can be blocked by antisense oligonucleotides against TRPC, supporting the idea that their upregulation is a required step in certain SMC growth processes. Increases in store- and receptor-operated calcium entries in chronically hypoxic PA SMCs may also contribute to alterations in pulmonary vasoreactivity. Increases in TRPC expression could contribute to the increase in vasoreactivity to vasoconstrictors such as endothelin, angiotensin II, and serotonin observed in PAs from chronically hypoxic animals. This is attributable to the fact that these vasoconstrictors activate GPCRs, which stimulate phospholipase C, to generate inositol-1,4,5-trisphosphate (IP\(_3\)) and DAG, which in turn may work synergistically to promote Ca\(^{2+}\) entry through TRPC channels. Thus, upregulation of TRPC channels, in conjunction with elevated circulating levels of vasoconstrictors and increased agonist receptors (ie, ET-A receptor and 5-HT-2BR) in PA SMCs may provide a powerful mechanism for increasing and maintaining vascular tone in chronic hypoxic pulmonary hypertension (Figure 5).

Intracellular signaling, via GTP-RhoA and its downstream effector Rho kinase (Rho/Rho kinase signaling), is also increasingly appreciated as an important signaling pathway in the pathogenesis of chronic hypoxic pulmonary hypertension because of its potential to cause sustained vasoconstriction and its effects on the proliferative and differentiation state of vascular wall cells.\(^{156,157}\) The contractile state of VSMCs is regulated by phosphorylation (causing contraction) and dephosphorylation (causing relaxation) of the 20-kDa regulatory myosin light chain (MLC). Phosphorylation of MLC is catalyzed by Ca\(^{2+}\)/calmodulin-dependent MLC kinase (MLCK) and dephosphorylation by calcium-independent MLC phosphatase (MLCP), which is targeted to myosin by its regulatory myosin binding subunit (MBS). Thus, the balance and activity of MLCK and MLCP regulate contraction. At a given level of cytosolic Ca\(^{2+}\), the activity of both enzymes can be modulated by second messenger-mediated pathways to change MLC phosphorylation and thus force of contraction; in other words, the activity of these pathways will change the calcium sensitivity of contraction.\(^{156,157}\) There is indirect evidence that hypoxia activates RhoA in VSMCs.\(^{157,158}\) Furthermore, several vasoconstrictors speculated to participate in hypoxia-induced sustained vasoconstriction (including ET-1, 5-HT, and thromboxane) activate RhoA in VSMCs through GPCR-mediated signaling pathways.\(^{156,157}\) Interestingly, GPCR-independent signaling to RhoA also occurs and can be mediated by receptor and nonreceptor tyrosine kinases and by recruitment of other GTPases.\(^{156,157}\)

The importance of Rho kinase signaling in chronic hypoxic pulmonary hypertension is highlighted by the experiments of McMurtry et al, in which the Rho kinase inhibitor Y27632, but not nifedipine, caused marked vasodilation in the hypertensive animals.\(^{159}\) The severity of chronic hypoxic pulmonary hypertension has also been shown to be reduced by chronic blockade of the Rho/Rho kinase signaling.\(^{29,159}\)

Collectively, these observations suggest that chronic hypoxia induces changes in SMC Ca\(^{2+}\) handling and intracellular Ca\(^{2+}\) concentrations through numerous mechanisms, as well as changes in Rho/Rho kinase signaling, which act in concert to have profound effects on the contractile and proliferative state of SMCs (Figure 5).

**Changes in Adventitial Fibroblast Phenotype and Function in Chronic Hypoxic Pulmonary Hypertension**

A rapidly emerging concept is that the vascular adventitia acts as a biological processing center for the retrieval, integration, storage, and release of key regulators of vessel wall function. In fact, under certain conditions, the adventitial compartment may be considered the principle injury-sensing tissue of the vessel wall.\(^{20}\) In all species studied, hypoxic exposure induces early and often dramatic adventitial remodeling.\(^{15,20,70}\) Accumulating experimental data suggest that, in response to vascular stresses including hypoxia and overdistention, the adventitial fibroblast is the first cell to be activated, to proliferate, to upregulate contractile and extracellular matrix 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.\(^{15,20,33,70,72,77}\) Each of these hypoxia-induced changes in fibroblast phenotype modulates either directly or indirectly overall vascular function and structure.

In vitro experimental studies support the notion that adventitial fibroblasts have greater propensity to proliferate under hypoxic conditions than do resident PA SMCs.\(^{160-162}\) It has even been suggested that hypoxia-induced proliferation is a unique property of the PA fibroblast.\(^{161}\) Current evidence implies that activation of G\(_{q}\) and G\(_{i}\) family members, perhaps in a ligand-independent fashion, with subsequent stimulation of PKC and mitogen-activated PKC family members, are important regulators of hypoxia-induced fibroblast proliferation.\(^{114,118,160}\) Activation of PI3K and synergistic interaction with Akt, mammalian target of rapamycin (mTOR), and p70 ribosomal protein S6 kinase have also recently been demonstrated to be essential for the proliferative response of PA
adventitial fibroblast to hypoxia.\textsuperscript{114,118} Transcriptional targets of hypoxia and/or the aforementioned signaling pathways include Egr-1, HIF-1\(\alpha\), and HIF-2\(\alpha\). Knockdown experiments have shown that both Egr-1 and HIF-2\(\alpha\) participate in the proliferative response.\textsuperscript{163,164} It has also been shown that downstream targets of HIF-1\(\alpha\), angiotensin converting enzyme (ACE), and the angiotensin II receptor type 1 (AT\(1\)) are upregulated by hypoxia and that inhibitors of ACE and AT\(1\) can block hypoxia-induced, HIF-dependent, adventitial fibroblast proliferation.\textsuperscript{165,166} In addition, mice partially deficient in either HIF-1\(\alpha\) or HIF-2\(\alpha\) have attenuated hypoxia-induced pulmonary hypertension and remodeling.\textsuperscript{168,169}

It should be stressed that the arterial adventitia, like the lung interstitium, is composed of heterogeneous fibroblast populations and that only specific subsets of fibroblasts appear capable of proliferating in response to hypoxia.\textsuperscript{20,170} Experimental evidence suggests that a selective expansion of these “hypoxia-sensitive” fibroblast subpopulations accounts for the marked accumulation of fibroblasts in the adventitia of chronically hypoxic animals, also consistent with observations that fibrogenic foci in the lung are comprised of selective fibroblast populations.\textsuperscript{20,170}

PA adventitial fibroblasts derived from chronically hypoxic animals exhibit heightened growth responses to a number of growth promoting stimuli including hypoxia as well as alterations of proliferation associated signaling pathways, compared with fibroblasts derived from normoxic animals.\textsuperscript{161,162,171,172} For instance, the atypical PKC\(\\gamma\) isozyme acts as a proproliferative kinase in fibroblasts from chronically hypoxic animals, whereas it exhibits antiproliferative actions in fibroblasts from normoxic animals.\textsuperscript{172} These observations raise the possibility that chronic hypoxia leads to the emergence of fibroblasts in the adventitia that have lost their ability to limit stimulus-induced proliferation.

Hypoxia-activated PA adventitial fibroblasts may exert significant effects on other cell types within the vessel wall through the production of paracrine factors (by HIF-1\(\alpha\)-dependent mechanisms), which have potent stimulatory effects on SMC proliferation, or production of reactive oxygen species (ROS), generated in large part through a distinct NADPH oxidase system.\textsuperscript{173,174} Fibroblast-produced ROS can exert potent effects on vascular tone either directly by stimulating contraction of SMCs and/or by acting as a sink for endothelial derived NO before its optimal response to vasoactive stimuli reported.\textsuperscript{176} Hypoxia can induce significant changes in the production of chemokines and cytokines by the adventitial fibroblast including MCP-1, SDF-1, thrombin, and VEGF.\textsuperscript{20,76} These chemokines can serve to recruit inflammatory cells and circulating progenitor cells (discussed below). In addition, activated adventitial fibroblasts are known to secrete a variety of proangiogenic factors.\textsuperscript{20,72,76} In this context, it has been reported that hypoxia induces a rapid expansion of the PA adventitial vasa vasorum.\textsuperscript{76,77} Expansion of the vasa vasorum appears important in contributing to the progression of many vascular diseases, possibly by providing a conduit for delivery of inflammatory and progenitor cells to the vessel wall.\textsuperscript{20,180}

Transcriptional Mechanisms Contributing to Phenotypic Changes in Pulmonary Vascular Wall Cells Under Hypoxic Conditions

See the online data supplement, under Section 1, available at http://circres.ahajournals.org.

Role of Inflammation, Progenitor Cells, and Vasa Vasorum in Hypoxia-Induced Vascular Remodeling

Relatively little attention has been given to the possibility that pulmonary inflammation and/or a noninflammatory accumulation of circulating monocytes/macrophages might contribute to the abnormalities of structure and function seen in the PAs following chronic hypoxic exposure. However, there is accumulating evidence to support the idea that both acute and chronic exposure of animals to even moderate hypoxia results in increased expression of lung inflammatory cytokines, chemokines, and adhesion molecules and in the accumulation of leukocytes within the lungs and in and around lung blood vessels. It is increasingly appreciated that factors produced by leukocytes have significant effects on the phenotype of local vascular wall cells, including increases in proliferation and matrix protein production and changes in the responses to vasocostricting or vasodilating substances. Additionally, these inflammatory cells induce recruitment of circulating mesenchymal precursors that could directly contribute to the remodeling process (Figure 6).

Several recent animal studies document the enhanced expression of inflammatory mediators (monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-2, IL-1\(\beta\), IL-6) and increased numbers of macrophages and neutrophils in the lungs of mice and rats exposed to acute hypoxia.\textsuperscript{128,181,182} At early time points, the increase in macrophage and neutrophil numbers was associated with an increase in albumin extravasation consistent with the development of mild vascular leak. This finding is similar to acute hypoxia-induced changes in lung vascular permeability reported by others and is also observed in response to acute hypoxia in the mesenteric circulation.\textsuperscript{183,184} Human studies also suggest that acute hypoxic exposure is associated with leukocyte recruitment and cytokine production in the lung. In
patients with high-altitude pulmonary edema (HAPE), increased numbers of neutrophils and macrophages, in bronchoalveolar lavage fluid, as compared with healthy individuals, have been observed. In addition, increased levels of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 have been noted, levels that quickly reversed to normal following return to normoxic conditions.

Chronic hypoxic exposure results in the robust and persistent appearance of mononuclear cells in the PA adventitia and media of both weanling rats and neonatal calves. Interestingly, at least in these 2 animal models, hypoxia-induced accumulation of mononuclear cells appeared specific to the pulmonary circulation because no macrophage recruitment was noted in systemic vessels (aorta, femoral, carotid). Further, no neutrophils were identified in the pulmonary perivascular areas at any of the time points (≥24 hours) evaluated. A substantial proportion of the mononuclear cells that accumulated around PAs was comprised by fibrocytes (cells characterized by dual expression of leukocytic and mesenchymal markers). As mentioned earlier, fibrocytes may play a particularly important role in the hypoxia- or injury-induced vascular remodeling processes (Figure 6). They have been described as important contributors to the fibrosis observed in lung injury, wound healing, and asthma. The recruited monocytes and fibrocytes release a variety of factors, which can act to increase the proliferation and/or differentiation of resident fibroblasts and SMCs. In addition, fibrocytes are potent producers of extracellular matrix proteins, especially collagen, raising the possibility that nonresident, recruited mesenchymal cells contribute to vascular fibrosis.

Fibrocytes also produce angiogenic factors and could contribute to the neovascularization of the vasa vasorum described above. Other investigators have also suggested a role for circulating bone marrow–derived cells in chronic hypoxia–induced remodeling.

The contribution of circulating mononuclear cells/fibrocytes to the hypoxia-induced vascular remodeling process was confirmed by depletion studies, in which the number of these cells was reduced in the circulation of hypoxic rats using intravenous injections of liposome-encapsulated clodronate or gadolinium chloride. A striking reduction in adventitial thickening as well as a near complete inhibition of the accumulation of collagen, fibronectin, and tenascin-C were documented.

Questions arise as to how leukocytes and precursor cells traffic into the vascular wall, particularly the adventitia, under conditions of chronic hypoxia. In the systemic circulation, the adventitial vasa vasorum microcirculation undergoes marked neovascularization in a number of vasculopathies and is thought to serve as a conduit for continued delivery of inflammatory and progenitor cells to the vessel wall. Similar changes have been documented in the pulmonary arteries of chronically hypoxic calves. Furthermore, experiments have shown that inhibition of plaque neovascularization reduced macrophage accumulation and progression of advanced atherosclerosis. Other experiments have shown that hypoxic fibroblasts support the growth of vasa endothelial cells. Thus, local fibroblasts and recruited inflammatory progenitor cells play a role in driving the neovascularization of the vasa and contribute to a feed-forward loop of vascular remodeling. Future experiments need to be directed at determining the factors that recruit and then retain inflammatory and progenitor cells in the vessel wall so as to ultimately design specific therapies to turn the process off.

**Chronic Intermittent Hypoxia and Pulmonary Vascular Remodeling**

See the online data supplement, under Section 2.

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None.
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Transcriptional Mechanisms Contributing to Phenotypic Changes in Pulmonary Vascular Wall Cells Under Hypoxic Conditions

As noted above, prolonged exposure to decreased oxygen concentration results in marked phenotypic changes in all the resident cells that comprise the pulmonary artery. Collectively, these changes result in significant alterations in the structure and the function of the pulmonary vasculature. Indeed, many studies in various organs and cell types have demonstrated that hypoxia has a direct and profound effect on the cellular transcriptome, an effect which is now known to be both cell type- and probably even cell state-specific1-7. The mechanisms, by which cells sense hypoxia and activate transcriptional regulators, are areas of intense investigation. Current theories suggest that the oxygen sensor(s) should be flexible and highly adaptive, allowing for graded cellular responses. This feature is particularly important because it allows cells to both detect and respond to deviations in PaO2. Because there is a wide range of PaO2 distribution in tissues, the threshold of activation varies from organ to organ and from cell to cell. Oxygen sensing heme proteins, like complex III or IV of the respiratory chain or isoforms of NADPH oxidase, and non-heme oxygen sensing proteins, like prolyl hydroxylases (PHDs) or hemexygenase-2 (HO-2), have been proposed as candidate sensor systems for the regulation of hypoxic-inducible responses. However, this topic has been the subject of several recent reviews and will not be covered here1, 8-11. Instead, we will examine the roles of various transcription factors that contribute to the complex transcriptional profile activated by hypoxia in vascular wall cells. We will focus on hypoxia inducible factor, HIF, as a master regulator of hypoxic responses, but will also consider the role that other transcription factors, such as Egr-1, NF-κB, CREB and AP-1, play in the hypoxia-induced remodeling process.
Hypoxia Inducible Factors (HIFs)

The identification of HIF as a transcription factor was a milestone in the field of oxygen physiology. Since that landmark study it has become clear that HIF, which is now recognized as a family of proteins (HIF-1,2,3α and HIF-1β), is a global regulator of oxygen homeostasis. Both the protein half-life and the specific activity of the HIF-α subunit are precisely O2-regulated via hydroxylation events, which provide a direct mechanism for the transduction of changes in the O2 concentration to changes in gene expression. HIF is a heterodimer, consisting of an O2-regulated HIF-α subunit and a constitutively expressed HIF-β subunit. This heterodimer protein binds to consensus DNA binding motifs within the regulatory regions (hypoxia-responsive elements) of hypoxia-responsive genes. HIF-1α is the most ubiquitously expressed and best characterized of the α-family members and is recognized as a master regulator of hypoxic signaling. HIF-2α shows patterns of regulation similar to HIF-1α but its expression is restricted to select cell types. HIF-3α is less well characterized but may act as an internal repressor of the HIF system given that a HIF-3α splice variant encodes IPAS (inhibitor PAS domain protein).

Hypoxic regulation of HIF has been repeatedly demonstrated in the lung. HIF-1α is expressed in most cell types within the hypoxic lung, including bronchial and alveolar epithelium, airway and vascular smooth muscle and vascular endothelium. Additionally, exposure of cultured vascular cells to hypoxia results in induction of HIF-1α expression and HIF1 DNA binding activity. Importantly, a number of studies suggest that HIF expression can be induced in cells under normoxic conditions by a wide variety of growth factors and cytokines, including epidermal growth factor, fibroblast growth factor-II, hepatocyte growth factor, insulin-like growth factor I and II, endothelin-1, interleukin-1β, insulin, prostaglandin-E2, transforming growth factor α and β, thrombin, and tumor necrosis factor α. In contrast to the hypoxia-induced HIF-1α response, the growth factor/cytokine-induced HIF responses appear to be due to the stimulation and increased synthesis of proteins mediated by phosphatidylinositol-3-kinase and mitogen activated protein kinase pathways. Therefore, HIF may regulate cell responses even under conditions where hypoxia alone is not enough to stabilize its expression and nuclear translocation.
A variety of studies using mice that are partially deficient for HIF-1α have provided great insight into the role that this transcription factor plays in the regulation of pulmonary vascular responses to chronic hypoxic exposure. Complete HIF-1α deficiency results in embryonic lethality at mid-gestation due to cardiac and vascular defects. However, mice heterozygous for a null allele at the HIF-1α locus (HIF-1α +/-) develop normally but exhibit markedly attenuated vascular remodeling responses to chronic hypoxic exposure. Electrophysiologic studies have demonstrated that the normal increase in capacitance (a measure of cell volume) observed in wild-type mice subjected to chronic hypoxia was completely abrogated in HIF-1α +/- mice. In addition, the hypoxia-induced depolarization of myocytes, described in SMC from chronically hypoxic animals, was significantly reduced in myocytes derived from HIF-1α +/- mice. This relationship between HIF-1α and K+ channel regulation in pulmonary arterial SMC has been confirmed in additional studies, which again showed an inverse relationship between nuclear HIF-1α translocation and Kv1.5 expression. Interestingly, this study also demonstrated that fawn hooded rats, an animal with a disrupted SMC mitochondrial network, have HIF-1α activation under normoxic conditions and develop severe pulmonary hypertension associated with downregulation of the voltage gated K+ channels. This suggests a previously unsuspected link between oxygen sensing and pulmonary arterial hypertension, even in the absence of hypoxia. Another recent study has demonstrated that hypoxia-induced increases in basal intracellular [Ca2+]i and TRPC expression, as discussed above, were absent in mice partially deficient for HIF1α. Changes in TRPC expression, which lead to enhanced capacitive calcium entry through store-operated calcium channels, may contribute to hypoxic hypertension by facilitating calcium influx and increasing basal intracellular [Ca2+]i in SMC. Thus, like Kv1.5, TRPC expression in the pulmonary vasculature can be modified by HIF-1α.

Recent loss and gain of function approaches have also demonstrated that HIF-1α activity is necessary and sufficient for basal and hypoxia-induced VEGFR-1 expression in bone marrow-derived mesenchymal stromal cells (MSC). MSC, in which HIF-1 activity was inhibited, failed to migrate or activate AKT in response to VEGF or placental growth factor (PLGF). Thus, HIF-1 could contribute to the recruitment (and possibly retention) of progenitor cells under hypoxic conditions. Additionally, studies in human renal clear cell carcinoma cells have shown
that expression of E-cadherin and HIF-1α are mutually exclusive. The expression of repressors of the E-cadherin gene transcription appear to be regulated in a HIF-1 dependent manner. Thus, it is possible that HIF-1 contributes to epithelial-mesenchymal transition through direct repression of E-cadherin. It would be interesting if HIF-1α contributed to the endothelial-mesenchymal transitions, described above, and thus playing a role in the hypoxia-induced rapid “muscularization” of vessels.

HIF-2α also appears to be important in fetal lung and lung vascular development. HIF-2α null mice have impaired fetal lung maturation and die at birth but can be rescued by VEGF treatment. Interestingly, HIF-2α heterozygotes are also protected from vascular remodeling associated with chronic hypoxic exposure.

These observations in HIF-1 and 2α heterozygous mice raise important questions. Some have suggested that hypoxia induces an angiogenic response in the lung and that this is a protective or adaptive response to lessen pulmonary vascular resistance. Since many HIF-1α and -2α target genes are considered angiogenic, questions remain as to why the HIF-1α or HIF-2α heterozygotes have reduced pulmonary vascular pressures. For instance, are there mechanisms that operate in the absence or decreased production of HIF that allow angiogenic responses to proceed under hypoxic conditions? Interestingly, recent investigations have suggested that increasing the stabilization of HIF-α may increase vascularization and thus be protective in the injured, premature lung. Thus, more studies regarding the role of HIF1α in hypoxia-induced vascular remodeling are needed to unravel the mysteries of its precise role in the adaptation of the lung to chronically hypoxic conditions.

Hypoxia Induces Upregulation of Other Transcription Factors (Egr-1, NF-κB, CREB, AP-1, SP-1, P53, NF-IL-6)

Early growth response-1 (Egr-1) is a zinc finger transcription factor involved in a number of early responses to a variety of pathophysiologic stimuli including growth factors, hormones, neural transmitters, and hypoxia. It too has been suggested to be a master regulator of gene responses in a variety of pathologic cardiovascular processes and a wide variety of genes have
been shown to be altered in cells over-expressing Egr-1\textsuperscript{35,36}. Egr-1 binds with high affinity to a consensus DNA element to modulate the expression of genes involved in cell growth and survival, extracellular matrix remodeling and thrombosis\textsuperscript{37}. Egr-1 nuclear localization is enhanced under conditions of hypoxia and this nuclear binding is necessary for hypoxic induction of the pro-coagulant, tissue factor. Although Egr-1 up-regulation is dependent on the degree and severity of hypoxia, it’s activation is independent of HIF\textsuperscript{38,39}. In fact, up-regulation of Egr-1 has been demonstrated at oxygen concentrations, which are less than those needed to activate HIF-1\textalpha{} by hypoxia. Hypoxia has been demonstrated to induce significant increases in the expression and activity of Egr-1 in pulmonary artery adventitial fibroblasts\textsuperscript{40,41}. Attenuation of Egr-1 protein with antisense oligonucleotides reduced hypoxia-induced proliferation of pulmonary arterial fibroblasts, at least in part, by reducing expression of cyclin-D and epidermal growth factor receptor\textsuperscript{40}. The role of Egr-1 as an important contributor to the pathogenesis of hypoxia-induced pulmonary vascular remodeling is demonstrated by attenuated responses in Egr-1 knockout mice\textsuperscript{39}.

Nuclear factor κB (NF-κB) is thought to be a central transcriptional mediator of the inflammatory response\textsuperscript{42}. NF-κB responsive genes include those responsible for producing inflammatory cytokines, chemokines and cell surface adhesion molecules. As described in the body of the manuscript, there is good evidence for pro-inflammatory cytokine up-regulation in the chronically hypoxic lung vasculature. Interestingly, a number of studies have demonstrated that NF-κB is also activated by hypoxia\textsuperscript{43-45}. The signaling mechanism(s) leading to this activation remain unclear, although it has been suggested that multiple mechanisms may be capable of inducing NF-κB expression under hypoxic conditions\textsuperscript{13,43-45}. Because both inflammation and the recruitment of progenitor cells play a vital role in hypoxia-induced vascular remodeling, future emphasis should be applied to this transcription factor.

A number of other transcription factors are also known to be regulated by hypoxia. These include: CREB, AP-1, P53, SP-1 and SP-3 and NF-IL-6/C/EBP\textbeta{}\textsuperscript{10,13}. Although none of these transcription factors have been thoroughly studied in the chronically hypoxic lung vasculature, each is capable of regulating genes involved in cell proliferation, matrix protein synthesis and cell phenotype. The potential relevance of these transcription factors to hypoxia-induced vascular
remodeling is emphasized by the recent studies of Kwapiszewska et al., who utilized gene expression profiling of laser-microdissected intrapulmonary arteries to evaluate the pattern of genes induced by chronic hypoxia, specifically in the small pulmonary artery. They found, perhaps not surprisingly, that most of the genes regulated in the array experiments were involved in regulation of cell metabolism. Hypoxia regulated genes involved in glycolysis, lipid pathways, and protein synthesis and degradation. At early time points, expression of metabolic genes was more pronounced, perhaps consistent with attempts at an adaptive response. As the duration of hypoxia increased, subsequent genes related to angiogenesis were up-regulated, consistent with the vascular remodeling process. Not surprisingly, many of the genes identified at both early and late time points were documented to have hypoxic response elements (i.e. HIF-binding sites). However, it was evident that many of the genes found to be up-regulated under hypoxic conditions did not contain a well-recognized hypoxic response element. These observations are consistent with the idea that hypoxia may exert its effects through the activation of numerous transcription factors, all of which regulate a complex array of gene sets that collectively act to drive the vascular remodeling process.
Chronic Intermittent Hypoxia and Pulmonary Vascular Remodeling

Much less is known regarding the effects of intermittent exposure to hypoxia on the pulmonary circulation than that of chronic or continuous hypoxia. Intermittent hypoxia, as seen in patients with sleep disordered breathing, is associated with the development of pulmonary hypertension and is included in the 2003 Venice Classification of pulmonary hypertension. However, the incidence of pulmonary hypertension in patients with sleep apnea without significant daytime gas exchange abnormalities is not known. One recent report suggested that as many as 40% of patients with sleep apnea -- but without evidence of underlying lung disease and with normal daytime arterial blood gasses -- may develop mild sleep apnea-related pulmonary hypertension, which may be associated with enhanced acute hypoxic pulmonary vasoconstriction\(^{47}\). Patients with underlying chronic lung disease, such as COPD, but without daytime hypoxemia, have worsening hypoxemia, albeit usually mild, during sleep. This eventually results in episodically increased pulmonary arterial pressures\(^{48}\). However, the development of sustained increases in pulmonary pressures in these nocturnally hypoxic COPD patients is less clear. There have been reports of decreased or unchanged pulmonary pressures when the nocturnal intermittent hypoxia is corrected with supplemental oxygen\(^{48}\).

Studies on the vascular effects of intermittent hypoxia have, until recently, focused on the systemic circulation and the development of systemic hypertension\(^{49}\). Increased adrenergic tone is thought to play a significant role in the development of systemic hypertension, and recent studies also suggested a role for altered endothelium-dependent vasodilators (i.e. NO) and ROS\(^{50, 51}\).

In previous studies, focused on the systemic circulation, increases in right ventricular mass were variably reported and frequently not elevated compared to normoxic controls. More recently, intermittent hypoxia studies have consistently suggested increased RV mass, likely in response to increased pulmonary vascular resistance\(^{48}\). One group suggested that the “threshold” for developing increased RV mass in intermittent hypoxia was a period of sustained hypoxia for at
least 2 continuous hours of every 24 hour period. A significant problem with these previous reports has been the highly variable definition of intermittent hypoxia. Said definition ranges from as few as 15 seconds of repetitive exposure to hypoxia to hours and even days of hypoxic exposure followed by a return to normoxia, often without regard for the sleep-wake cycle of the organism.

More recently, mice exposed to brief (15 seconds to 2 minutes) cyclic hypoxia for 8 hours per day, corresponding to the sleep hours of the rodents, have shown increased right ventricular pressure and hypertrophy. In rats, similar increases in right ventricular hypertrophy have been reported. In addition, our group found an increase in muscularization of the distal pulmonary circulation in mice exposed to intermittent hypoxia, but to a lesser extent than was seen in continuous hypoxia. The mechanisms underlying the vascular and ventricular remodeling are currently being investigated and may involve, at least in part, mechanisms different from those seen in continuous hypoxia.

Several hypotheses regarding the cellular and physiological differences between intermittent and chronic hypoxia exposures have been proposed. One interesting hypothesis is that intermittent hypoxia is associated with oxidative stress. In studies on the effect of intermittent hypoxia on alterations in breathing control and long-term facilitation of carotid body responses, neuronal levels of API, HIF1-α, and NF-κB are increased. Activation of these oxidant-sensitive transcription factors leads to differential gene expression in the neurons and alterations in control of breathing. Recently, activation of HIF-1α has also been linked to some of the metabolic changes associated with intermittent hypoxia, namely increased serum triglycerides. Preliminary evidence suggests that oxidant-sensitive transcription factors are also increased in the rat lung following intermittent hypoxia (compared to both normoxia and chronic hypoxia). Further studies are needed to determine if these transcription factors are important in the pulmonary vascular and right ventricular remodeling that occur in the face of intermittent hypoxia.
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