Published in final edited form as:

Compr Physiol. 2011 January ; 1(1): 123–139. doi:10.1002/cphy.c090004.

Pulmonary Vascular Disease Related to Hemodynamic Stress in the Pulmonary Circulation

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I. Introduction

Hemodynamic stress in the pulmonary vasculature, manifesting as chronic pressure and/or volume overload, is a recognized causative factor in the development of pathophysiologic vessel wall remodeling and dysfunction. Typically, such vessel wall dysfunction clinically leads to pulmonary hypertension (PH), an overarching term referring to a set of clinical conditions defined generally by increased pulmonary vascular pressures. If untreated, PH leads to right ventricular failure, volume overload, and death (1). The most recent clinical classification of PH (Venice, 2003) proposes a classification system based on distinct etiologies that involve both hemodynamic and non-hemodynamic triggers. Such disease entities that increase hemodynamic stress in the pulmonary vasculature include left-heart disease, which can lead to chronic volume and pressure overload in the pulmonary vascular bed, and chronic thromboembolic disease, which may, perhaps, induce more localized hemodynamic stress in the vascular bed upstream of thrombotic lesions (Table 1). Furthermore, this classification system includes a subdivision for pulmonary arterial hypertension (PAH), a clinical entity more specifically defined by elevated pulmonary *arterial* pressures in the absence of elevated left heart pressures. Like PH, PAH can be caused either by primary (“idiopathic”) or a variety of secondary causes. One such significant secondary cause includes congenital heart diseases associated with systemic-to-pulmonary shunts, which increase both pulmonary blood flow and hemodynamic stress ultimately leading to pulmonary vascular remodeling. The extreme manifestation of PAH in this setting is known as Eisenmenger syndrome, as described by Victor Eisenmenger, an Austrian physician, in 1897. Morbidity and mortality due to PAH associated with such shunts continue to increase as the population of adults with congenital heart disease expands at an estimated rate of 5% per year (2).

While the physiological determinants of pulmonary vascular changes in response to hemodynamic stressors are reasonably well understood, relatively little is known regarding the exact molecular responses to such chronic pressure and volume overload in the pulmonary circulation and their correlation to left sided heart disease, congenital heart disease, and other pathological states. In part, this stems from the fact that control of pulmonary vascular function at the cellular and molecular levels is extraordinarily complex. Much of our knowledge of these processes is derived from various studies of peripheral vessels with extrapolation to the pulmonary circulation. Some of these data have been subsequently verified in the context of the pulmonary vasculature; however, other

mechanisms and responses have been based on incomplete, circumstantial evidence. Currently, it appears that a number of distinct but complex stimuli are induced by pressure and volume overload and can alter pulmonary vascular function via control of multiple intersecting signaling pathways, ultimately leading to the release of a common set of vasoactive effectors and stereotyped end-stage dysfunction and disease (Figure 1). This chapter will describe our current understanding of the clinical and physiological changes associated with pressure/volume overload in the context of the complex and incompletely characterized interplay of exogenous upstream stimuli and downstream vasoactive effectors that ultimately regulate these pulmonary vascular responses.

II. Physiological and histological changes associated with increased pressure and volume overload

Pulmonary hypertension (PH) develops in 68% to 78% of patients with chronic severe left ventricular systolic dysfunction (LVSD), and is commonly associated with right ventricular (RV) dysfunction (3). In the setting of left heart failure and elevated retrograde pressure in the pulmonary circulation, vascular changes typically begin in the pulmonary veins, due first to passive effects of increased congestion and later active vessel remodeling with increased pulmonary vascular resistance, increased vasomotor tone, and vascular cell proliferation. If allowed to progress, vascular changes steadily evolve to include pulmonary capillaries and ultimately larger pulmonary arterioles. Clinical manifestations are dominated by hypoxic exposure, pulmonary hemorrhage, thrombotic complications, ischemic complications, risk of infection, right-sided heart failure, and sudden cardiac death (4).

In the case of pulmonary vascular dysfunction due to left heart failure, certain etiologies may be associated with a predominance of either pressure or volume overload in the pulmonary circulation. Most common causes that initiate predominantly chronic pulmonary venous pressure overload include chronic systemic hypertension, anatomic fixed obstructions including aortic stenosis, or anatomic anomalies such as coarctation of the aorta. Additionally, development of cardiac fibrosis due to chronic ventricular remodeling after ischemic disease and infarction can reduce systolic function as well as induce a “stiffening” of the left ventricle. In these cases, from a physiological perspective, pulmonary venous pressures are elevated throughout the cardiac cycle, both in systole and diastole. In contrast, development of cardiac hypertrophy, either from genetic predisposition or environmental/toxic factors, primarily leads to increases in diastolic rather than systolic pressure. The association of diastolic dysfunction with pulmonary hypertension has recently been better appreciated. However, it is unclear if the development or severity of pulmonary hypertension is controlled by these physiological differences in the response to pressure overload.

Additionally, etiologies that predominantly induce volume overload can also lead to PH. Most commonly, these include valvular dysfunctions such as mitral and aortic regurgitation. From a physiological perspective, such cardiac diseases lead to cardiac remodeling, left ventricular systolic dysfunction, and increased volume and secondarily increased pressure in the pulmonary venous circulation. Again, significant physiological differences exist among causes of volume overload and pressure overload, but, currently, their specific impact on the development of PH has not been fully developed.

In contrast to pulmonary vascular disease in the setting of left-sided heart failure, congenital heart diseases associated with left-to-right shunts induce flow-mediated vascular changes typically occurring at the level of the small peripheral pulmonary arteries, while sparing other anatomic locations, leading to pulmonary arterial hypertension (PAH). From a physiological perspective, this distinction is due to the shunt increasing pulmonary arterial

pressure primarily via increased blood flow through the pulmonary vasculature. In response, increased pulmonary blood flow likely represents the primary stimulus to trigger pulmonary vascular remodeling and PAH. Furthermore, in the setting of congenital heart disease, critical differences in disease manifestation can develop based on anatomic location, direction, and magnitude of an anatomic shunt (Table 2). In general, certain types of congenital heart disease with functional systemic-to-pulmonary shunts, such as unrestricted ventricular septal defects (VSD), large patent ductus arteriosus (PDA), or other more complex congenital lesions (i.e., truncus arteriosus, transposition of the great arteries, and single ventricle with unrestricted pulmonary blood flow), invariably lead to pulmonary vascular remodeling and Eisenmenger's syndrome during childhood. Presence of atrial septal defects (ASD) with systemic-to pulmonary shunts may also lead to PAH over time (5). Yet, in contrast to the cases of unrestricted pulmonary blood flow, only 10–20% of all persons with atrial septal defects progress to PAH (6). This observation may reflect differences in the response of the pulmonary vasculature to pressure overload (as seen in unrestricted VSDs) as compared to volume overload (as seen in shunts with ASD); however, the underlying mechanisms are unclear. Furthermore, a widely held hypothesis suggests that patients with ASD may harbor a specific, unidentified genetic predisposition to the development of PAH that may work in concert with or independently of the increased volume load to the pulmonary circulation (7).

Despite the differences in the development of PH due to retrograde pressure and volume overload in left heart failure and the development of PAH due to anterograde pressure overload with increased pulmonary blood flow in congenital heart disease, common end-stage histological features are similarly recognized. These involve intimal fibrosis, distal localization and proliferation of vascular smooth muscle, and pulmonary vessel occlusion (8). End-stage PAH may also hold some unique histopathological features distinct from PH. For example, a hallmark of severe, end-stage PH and PAH is the formation of a vessel “neointima,” characterized by increased deposition of extracellular matrix and myofibroblasts (9). However, plexiform lesions can predominate in PAH, characterized by over-proliferation of endothelial-like cells encroaching upon the vessel lumen (10).

Multiple cell types in the pulmonary arterial wall and pulmonary arterial circulation contribute to these specific responses to injury and the development of vessel remodeling (Figure 1) (9). First, the endothelium has long been appreciated as a central sensor of injurious stimuli, especially in the setting of cyclic stretch or shear stress induced by various types of pressure/volume overload and/or increased pulmonary blood flow. Dysregulation of downstream vascular effectors may be the result of initial endothelial cell injury or dysfunction. However, more recent studies suggest that dysfunction of alternative vascular components in response to these mechanical stimuli, such as adventitial fibroblasts or components of the extracellular matrix, may also initiate pulmonary vascular disease (11). Downstream of these initiating processes, an imbalance of secreted vasoactive factors ensues and directs vascular remodeling via pathological cellular processes. These include excessive cell proliferation, vasoconstriction, and thrombosis, which are associated with more complex patterns of inflammation and angiogenesis. Transdifferentiation of endothelial cells to vascular smooth muscle cells may also contribute (12,13). Again, amplification in plexiform populations has been identified, both in idiopathic PAH and in the setting of secondary causes, such as flow-dependent PAH (14), which has led to speculation of a model of end-stage PAH similar to that of progression to cancer, with dysregulation of the cell-cycle and apoptosis as predominant features (15). Furthermore, inflammatory cells and activated platelets appear to predominate in later stages of PAH (16); yet, our understanding is limited regarding the mechanistic role of these cellular populations in disease progression (17). Finally, circulating or resident progenitor cells have been proposed to factor significantly in vessel wall repair after injury; dysregulation of these

reparative functions may also contribute to PH (18). Beyond these common features, more definitive understanding of pulmonary vascular disease in the setting of hemodynamic stress can be difficult due to the dynamic nature of changes as well as the marked heterogeneity of disease manifestation even under conditions of seemingly similar underlying etiologies (4).

III. Pressure/Volume Overload Models of Pulmonary Vascular Disease

The difficulty of *in vivo* study of pulmonary vascular disease secondary to pressure/volume overload and altered hemodynamic flow has limited our understanding of the pathogenic mechanisms of PH due to hemodynamic stress. Notably, these studies have been hampered by the lack of a tractable small animal model that can be followed from initiation through progression of disease. Because human tissue specimens in patients with PH due to left-sided heart failure or with PAH due to congenital heart disease are only available late in the clinical course, study of the relevant molecular pathways has been observational and retrospective (16,19–21). A number of vascular changes seen in PH with left-sided heart disease can be observed in murine models of fixed aortic obstruction (i.e., aortic banding) or genetic murine models of hypertrophic or dilated cardiomyopathy. However, these models have typically been used to interrogate mechanisms of ventricular function rather than pulmonary vascular pathophysiology. Moreover, invasive and non-invasive studies of pulmonary blood flow have been difficult owing to the small body size and inability to achieve high pulmonary arterial pressures [(22–27), to cite a few studies].

By contrast, larger animal models offer advantages in characterizing the effect of pressure/volume overload or increased shunt flow on the pulmonary vasculature. For example, such mammalian models have been established by surgical placement of systemic-to-pulmonary shunts in adult animals (28–35) and *in utero* (36). However, identification of contributors in the molecular process of pulmonary remodeling has been difficult, owing to incomplete genetic data and tools specific for these larger animals (37,38).

A few successful studies increased *in vivo* pulmonary flow in the rat via pneumonectomy (39,40), or placement of systemic-to-venous shunts (aortocaval shunt) (41–44) or systemic-to-pulmonary shunts (left subclavian artery-to-pulmonary artery) (45). In the case of systemic-to-pulmonary shunts, development of elevated to near-“systemic” pulmonary pressures was observed (45). With regard to pulmonary hemodynamic effects, placement of a systemic-to-pulmonary shunt alone leads to a moderate degree of vascular remodeling as manifested by medial hypertrophy after prolonged hemodynamic exposure (42,43). In contrast, placement of a shunt in combination with monocrotaline injury causes additional neointimal proliferation and plexigenic lesions (39,45). It has, therefore, been hypothesized that vascular injury, possibly to the endothelium specifically, in combination with alteration in hemodynamic flow is instrumental in full histological progression to end-stage PAH (38). The completion of the sequencing of the genome of the rat and perhaps larger mammals and the growing availability of molecular and genetic reagents for non-murine species offer the opportunity for more specific molecular characterization of *in vivo* pulmonary vascular change. As a result, genetically tractable non-murine mammals may represent potentially powerful models for studying both moderate and severe histological disease seen in the pulmonary vasculature over time.

IV. Mechanotransduction in the Pulmonary Vasculature

Upstream of the histopathological processes that predominate later stages of this type of pulmonary vascular disease, it is assumed that the initiating triggers of vascular remodeling in the setting of pressure or volume overload are modulated by vascular “mechanosensors” recognizing specific hemodynamic stress. It is known that vascular endothelial cells act as a central sensor for a variety of physiological stimuli and injurious stimuli (such as hypoxia,

shear stress, inflammation, and toxins) to modulate a variety of vascular responses. Correspondingly, stemming from results of classic *ex vivo* studies of arterial explants, active modulation of vascular tone and other functions in both peripheral and pulmonary arteries relies predominantly on the function of an intact endothelial monolayer. This function primarily involves the tightly-regulated production and secretion of specific vasoactive effectors from the endothelium. These factors tend to act either in an autocrine fashion to autoregulate endothelial function or in a paracrine fashion to influence neighboring vascular smooth muscle cells (VSMCs) or other cell types in the vascular compartment (Figure 2).

Owing to this critical role of the endothelium in the pulmonary vascular response to pressure and volume overload, there have been attempts at *ex vivo* modeling of pulmonary vascular endothelium, specifically in the setting of shear stress. At the molecular level, the physiological flow patterns of laminar shear stress, turbulent flow, and cyclic strain are all recognized by endothelial cells, leading to transduction of intracellular signals and modulation of a wide variety of phenotypic changes [as reviewed in (46)]. Significant prior work has focused mainly on the endothelium of the peripheral vasculature, suggesting that laminar flow induces a vasoprotective, quiescent vascular state, while turbulent flow leads to a pro-inflammatory and thrombogenic state. It is unclear if these flow-dependent phenotypes are recapitulated in the pulmonary vasculature, given the difficulty of studying directly the *in vivo* flow patterns at the anatomic level of the pulmonary arteriole. Attempts at computer modeling have shown promise in deciphering the complex nature of these flow patterns in health and disease (47,48). In addition, recent *ex vivo* modeling of pulsatile flow with high levels of shear stress and chronic VEGF inhibition has demonstrated apoptosis of pulmonary artery endothelial cells followed by outgrowth and selection for proliferating apoptosis-resistant cells (49). Therefore, chronically elevated flow may allow for selection of cells with dysregulated endothelial cell growth and resulting clonal or polyclonal expansion to plexiform lesions. These *ex vivo* models may play important roles in the future for better delineating the molecular effects of increased flow in the pulmonary vasculature.

The exact molecular identity of the primary mechanosensor(s) in the pulmonary vascular endothelium, however, remains elusive. Proposed molecular mechanisms of endothelial mechanosensing in the peripheral vasculature include stretch-sensitive ion channels, protein kinases associated with the cytoskeleton, integrin-cytoskeletal interactions, cytoskeletal-nuclear interactions, and oxidase systems capable of generating reactive oxygen species [as reviewed in (50)]. Notably, the functions of integrins have been particularly interrogated in this regard, as they serve as a class of receptors for extracellular matrix proteins that can mediate force transmission, by virtue of their connections with the cell matrix and cytoskeleton [as reviewed in (51)]. More recently, calcium ion influx through endothelial TRPV4 channels has been identified as a molecular mechanism contributing significantly to carotid endothelial mechanosensing in TRPV4 genetically deficient mice (52). Nonetheless, it is unclear whether multiple sensing mechanisms exist, how those mechanisms distinguish differing modes of mechanical deformation (i.e., shear stress versus cyclic strain), and if other contributory pathways of mechanotransduction are present in alternative compartments of the vessel wall (i.e., VSMCs or fibroblasts). Furthermore, very little investigation in this regard has focused specifically on the pulmonary vasculature. Therefore, our understanding of the transduction of hemodynamic forces in the vasculature is incomplete and remains an active field of ongoing study.

V. Genetics associated with pulmonary vascular remodeling in pressure/volume overload

In the setting of this complex process leading to vascular remodeling with hemodynamic stress, combinations of hemodynamics and genetic predisposition may initiate dysregulation

of signaling molecules that regulate pulmonary vascular tone, and may progress and ultimately develop into end-stage disease (1,38,53). Recently, advances in our understanding of the genetic features in the development PAH have fueled speculation that these or related genetic predispositions may also contributed to pulmonary vascular diseases initiated by hemodynamic stressors.

Mutations in the transforming growth factor- β receptor (TGF- β receptor) superfamily have been genetically linked to PAH and likely play a causative role in the development of disease. A rare group of patients with hereditary hemorrhagic telangiectasia and idiopathic PAH harbor specific mutations in *ALK1* or *endoglin*, genes encoding for two such members of the TGF- β receptor superfamily (54,55). However, a more prevalent cohort of patients carries mutations in another superfamily member, the bone morphogenetic protein receptor type 2 (*BMPR2* gene which encodes for BMPR-II) (56,57). Over 140 mutations in *BMPR2* have been reported in patients with familial PAH (58), located throughout the gene, in the extracellular ligand-binding domain, in the cytoplasmic serine/threonine kinase domain, and in the long carboxyterminal domain (59). The mechanism of action of BMPR-II is complex, and its role in PAH progression is still incompletely understood. It functions as a receptor with serine/threonine kinase activity [as reviewed in (60)]. In the adult, BMPR-II is expressed predominantly in the pulmonary endothelium, medial smooth muscle cells, and macrophages (61). Under normal conditions, BMP ligands bind BMPR-II to suppress the growth of vascular smooth muscle cells (62,63). In contrast, binding of BMP2 and BMP7 to BMPR-II in pulmonary endothelium leads to protection from apoptosis (64). Patients harboring *BMPR2* mutations generally suffer from haploinsufficiency with decreased expression of BMPR-II in pulmonary tissue (58,61,65,66). As a result, BMP signaling appears altered but not completely abolished (67,68). A widely held hypothesis contends that failure of the suppressive effects of BMP ligands on vascular smooth muscle (69) and failure of the protective effects of BMP ligands on endothelium may trigger vascular proliferation and remodeling common to multiple types of pulmonary hypertension, including pressure/volume overload. Some data exist to support a predisposition to developing PAH in patients with congenital heart disease (70,71). It remains unclear if this increased susceptibility to developing PH also applies to individuals with pre-existing left heart failure.

Similar to the BMP pathway, the serotonin (5-hydroxytryptamine or 5-HT) signaling pathway has also been implicated as a potential causative factor in PAH through observation of genetic and physiological influences (72), and in PH through a number of observations in idiopathic and familial disease and congenital heart disease [as reviewed by (73)]. Serotonin is both a vasoconstrictor and mitogen that promotes smooth muscle hyperplasia and hypertrophy (74). Primarily stored in platelet granules, secreted serotonin binds G-protein-coupled serotonin receptors present on pulmonary artery smooth muscle cells. Activation of these receptors leads to a decrease in adenylyl cyclase and cyclic AMP, resulting in an increase in contraction. Furthermore, the cell-surface serotonin transporter (5-HTT) allows for transport of extracellular serotonin into the cytoplasm of smooth muscle cells, thereby activating cellular proliferation directly through the action of serotonin or indirectly via potential pleiotropic mechanisms (75). Serotonin can influence other regulatory pathways involved in PAH progression; notably, serotonin can inhibit BMP signaling via modulation of downstream Smad proteins. In correlation, exposure to increased serotonin has led to worsened pulmonary hypertension in a *BMPR2* +/- heterozygote murine model (25). Serotonin may also regulate the angiotensin-1/Tie2 signaling pathway, another potential pathogenic contributor, which, in turn, can modulate signaling of BMPR-II via inhibition of endothelial expression of one of its heterodimeric partners, BMPR-IA (26). Notably, animal models of pulmonary hypertension have also implicated the activated serotonin pathway in this way in disease progression; however, this effect has been observed mostly in the setting

of hypoxic triggers rather than hemodynamic stress. Nonetheless, taken together, these data not only further implicate the serotonergic and BMP pathways as pathogenic factors, but also represent useful evidence of potential common regulatory pathways in this complex disease (Figure 3).

Despite these advances, a clear mechanistic explanation of the impact of *BMPR2* and the serotonin pathway on the pathogenesis of pulmonary vascular disease due to hemodynamic stress is still lacking, owing to a number of factors. First, the cellular effects of the BMP pathway, in particular, vary in the setting of diverse combinations of extracellular ligands, receptors, and intracellular signaling cascades (68). This combinatorial signaling complexity is further complicated when coupled with the multitude of signaling pathways that are activated under various types of hemodynamic stress. Furthermore, responses in cell culture may not accurately reflect *in vivo* events, considering the relative lack of sophistication of *ex vivo* modeling of pulmonary vascular flow. A genetically tractable animal model of pulmonary vascular disease would seem ideal to delineate better the precise pathogenic contribution of the BMP and serotonin pathways to vascular phenotypes in pressure/volume overload.

VI. Vasoactive Effectors of Pulmonary Vascular Remodeling in the Setting of Hemodynamic Stress

Operating downstream of the initiating hemodynamic stressors and underlying genetic predisposition, a variety of molecular effectors have been identified that contribute to events of pulmonary vascular remodeling in the setting of pressure or volume overload [as reviewed by (1,53)]. Exposure to only one or a subset of these factors may lead to varying manifestations of disease in response to pressure or volume overload and may contribute to the differences in some histopathological features of PH due to left-sided heart disease and PAH due to increased pulmonary blood flow in congenital heart diseases. Nonetheless, the induction of vasoactive effectors is common to later stages of both PH and PAH arising from both hemodynamic and non-hemodynamic triggers, leading to vasoconstriction, cellular proliferation, and thrombosis. These processes are influenced by a complex and dysregulated balance of vascular effectors controlling vascular tone, proliferation, and thrombogenesis. Most of these effectors have been described in previous comprehensive reviews. Our discussion here will be adapted from such a prior review (73) to specifically describe the known roles of these effectors in the pathogenesis of PH common to multiple etiologies, including pressure/volume overload (Table 3).

Gaseous vasoactive molecules regulate pulmonary vascular homeostasis, and alterations in their endogenous production have been linked to the progression of PH induced by hemodynamic stimuli. Nitric oxide (NO) is the best described of these factors. It is a potent pulmonary arterial vasodilator as well as a direct inhibitor of platelet activation and vascular smooth muscle cell proliferation. The synthesis of NO is mediated by a family of NO synthase enzymes (NOS). The endothelial isoform (eNOS) is regulated by a multitude of vasoactive factors and physiological stimuli, including hypoxia, inflammation, and oxidative stress. Reduced levels of eNOS have been demonstrated in the pulmonary vasculature of patients with PH and PAH, suggesting a mechanism of dysregulated vasoconstriction (76,77). Correspondingly, a murine model that genetically lacks eNOS is more susceptible to developing pulmonary hypertension in response to other endogenous stimuli as compared with the wildtype control (78,79). Furthermore, the impact of NO has been reflected in its therapeutic role in hemodynamically associated pulmonary vascular disease, as inhaled NO [as reviewed in (80)] and the NO-dependent phosphodiesterase type-5 inhibitor sildenafil (81) [and as reviewed in (82)]. Nonetheless, the exact mechanisms of eNOS and NO dysregulation in pulmonary hypertension have not been fully elucidated. First, increased

levels of eNOS have been described specifically in plexiform lesions (83) and may provoke endothelial cell proliferation in these lesions; however, this link has not been verified. Second, dysregulation of NO depends not only upon NOS activity but also upon still incompletely characterized processes of NO transport in blood (84). Third, NO concentrations and bioactivity are intricately associated with oxidative stress, another likely but incompletely described regulator of pulmonary hypertension (85). Finally, specific polymorphisms of NOS have been associated with pulmonary hypertension (86,87); however, mechanistic details are lacking. Again, further characterization of the dysregulated action of NO and potential interactions with other pathogenic factors may depend upon the development of a genetically tractable animal model of severe PAH.

Similar to NO, carbon monoxide (CO) is an endogenously produced gaseous vasodilator, a deficiency of which may promote development of PAH. Mice that are null in heme oxygenase-1 (HO-1), a key enzyme that produces CO in the pulmonary vasculature, exhibit less tolerance for hypoxia with resulting right ventricular dysfunction (88). In contrast, overexpression of HO-1 in the lung prevents the development of pulmonary hypertension in murine models of chronic hypoxia (89) and in rat models of monocrotaline-induced PAH (90). However, studies elucidating the precise regulation of CO production during hemodynamic stress are currently lacking.

An additional, putative vasoactive factor that may play an important and novel role in flow-dependent pulmonary vascular disease is hydrogen sulfide (H₂S). H₂S has recently been proposed as an endogenous signaling molecule -- a third such significant gaseous *in vivo* transmitter in addition to NO and CO. Its role in a variety of physiologic and pathologic processes has been reported, including memory, neuro-modulation, sepsis, and inflammation [as reviewed by (91,92)]. It modulates a growing number of pleiotropic, tissue-specific, and cell-specific effects, including cell membrane hyperpolarization, smooth muscle relaxation, regulation of cell growth, and regulation of neuronal excitability. Importantly, H₂S appears to regulate vascular activity and vascular tone [as first described in (93)], and understanding of its physiological role is only just emerging. Hydrogen sulfide is produced endogenously from L-cysteine by cystathionine beta-synthase (CBS) or by cystathionine gamma-lyase (CSE) (94,95). CSE, expressed in vascular smooth muscle cells, appears to be the dominant enzymatic regulator of H₂S production in the pulmonary and peripheral vasculature (96). Using an *ex vivo* apparatus of rat aortic rings, past studies demonstrated that H₂S can induce vasorelaxation (97,98) in an endothelium-dependent, yet cyclic-GMP independent, mechanism (97). These results suggest that this mechanism of action is at least partially independent of the vasodilatory mechanisms initiated by NO (97). The mechanism may be mediated by regulation of K(ATP) channels to allow for hyperpolarization of smooth muscle cells (96). Furthermore, it was recently reported that, using similar *ex vivo* assays, in contrast to exposure to higher concentrations of H₂S (200–1600 micromolar) that induce vasorelaxation, lower concentrations (10–100 micromolar) of H₂S can, surprisingly, induce vasoconstriction of aortic rings (98). Regulation of these vasoconstrictor effects by H₂S may depend partially upon the action of NO (98) as well as the anatomic location of the arterial bed (99). The *in vivo* importance of H₂S on vascular activity has been demonstrated in mutant mice lacking CSE which suffer from pronounced systemic hypertension and diminished endothelium-dependent vasodilation (100). In correlation, regulatory effects of H₂S on vascular tone and remodeling have been suggested in models of systemic hypertension (101) as well as pulmonary hypertension (62,92,102–104).

These data suggest that endogenous H₂S may affect not only the homeostatic regulation of pulmonary vascular activity in general but also the pathogenesis of flow-dependent pulmonary hypertension in particular. Further understanding of the effect of H₂S on the pulmonary vasculature is limited. It is unclear if pulmonary vascular tone is modulated by

H₂S in a concentration-specific manner, as shown in peripheral aortic tissue (98). The molecular signaling pathways induced by H₂S in the pulmonary vasculature have not yet been identified. Most importantly, the *in vivo* role of H₂S in pulmonary vascular homeostasis has yet to be explored in the setting of normal hemodynamics compared with the setting of PAH secondary to increased pulmonary flow.

The arachidonic acid metabolites prostacyclin and thromboxane A₂ also play crucial roles in vasoconstriction, thrombosis, and, to a certain degree, vessel wall proliferation. Prostacyclin (prostaglandin I₂) activates cyclic adenosine monophosphate (cAMP)-dependent pathways and serves as a vasodilator, an antiproliferative agent for vascular smooth muscle, and an inhibitor of platelet activation and aggregation. In contrast, thromboxane A₂ increases vasoconstriction and activates platelets (105). Protein levels of prostacyclin synthetase are decreased in small and medium-sized pulmonary arteries in patients with PAH, particularly with the idiopathic form (106). Biochemical analysis of urine in patients with PAH has shown decreased levels of a stable metabolic of prostacyclin (6-keto-prostacyclin F₂alpha), accompanied by increased levels of a stable metabolite of thromboxane A₂ (thromboxane B₂) (107). Therefore, it appears that production of these effectors is coordinately regulated with the imbalance toward thromboxane A₂ favored in the development of PAH. The mode of this regulation remains to be characterized. Nonetheless, recognition of this imbalance has led to the success of prostacyclin therapy and improvement of hemodynamics, clinical status, and survival in patients with severe PAH due to congenital heart disease [as reviewed in (108)].

Endothelin-1 (ET-1) is expressed by pulmonary endothelial cells and has been identified as a significant vascular mediator in PAH. It acts as both a potent pulmonary arterial vasoconstrictor and a mitogen of pulmonary smooth muscle cells (109,110). The vasoconstrictor response relies upon binding to the endothelin receptor A (ET_A receptor) on vascular smooth muscle cells, which leads to an increase in intracellular calcium, along with activation of protein kinase C, mitogen-activated protein kinase, and the early growth response genes *c-fos* and *c-jun* (9). The mitogenic action of ET-1 on pulmonary vascular smooth muscle cells can occur through either the ET_A receptor and/or the ET_B receptor subtype, depending on the anatomic location of cells. The ET_A receptor predominantly mediates mitogenesis in the main pulmonary artery, whereas mitogenesis in resistance arteries relies upon contributions from both subtypes. The resulting vasoconstriction, mitogenesis, and vascular remodeling are thought to lead to significant hemodynamic changes in the pulmonary vasculature and to PAH. Plasma levels of endothelin-1 are increased in animal and human subjects suffering from PAH of a variety of etiologies (77,111). Again, improvement in hemodynamics, clinical status, and survival of PAH patients and more recently PH in the context of left-heart failure treated with chronic ET receptor antagonists highlights the significance of these effects [as reviewed in (112)]. However, the complete upstream pathogenic mechanisms that result in elevated levels of endothelin-1 and dysregulated signaling have not been fully clarified.

Downregulation of vasoactive intestinal peptide (VIP) may also play a pathogenic role. VIP is a pulmonary vasodilator, an inhibitor of proliferation of vascular smooth muscle cells, and an inhibitor of platelet aggregation (113,114). Decreased concentrations of VIP have been reported in serum and lung tissue of patients with PAH (115). *VIP*-null mice suffer from moderate pulmonary hypertension (116). Furthermore, both pulmonary arterial pressure and pulmonary vascular resistance decrease after treatment with VIP (117–119). Improvement of hemodynamics and clinical course has also been observed with inhaled VIP in a small number of PAH patients (115). Key questions regarding the mode(s) of regulation of VIP expression and its putative causative or contributory role in PH due to hemodynamic stress remain unanswered.

Vascular endothelial growth factor (VEGF) is a well-studied endothelial cell mitogen and angiogenic factor. In the pulmonary circulation, it binds endothelial cells via tyrosine kinase receptors (two subtypes: VEGFR-1/KDR and VEGFR-2/Flt). Production of VEGF and its receptors is upregulated in human pulmonary tissue in both acute and chronic hypoxia (120). Increased VEGF expression has also been observed in PAH, accompanied by elevated levels of VEGFR-1 in the affected pulmonary endothelium and specifically elevated levels of VEGFR-2 in plexiform lesions (10,121). However, other signaling molecules that are typically involved in the VEGF angiogenic signaling cascade appear to decrease: phosphoinositide-3-kinase, Akt, and Src. As a result, a dysregulated response to VEGF has been proposed to influence critically endothelial cell survival, proliferation, and apoptosis. Correspondingly, mice with homozygous deletions in the B isoform of VEGF (*VEGF-B*) demonstrate less vascular remodeling compared to wildtype controls when exposed to hypoxia (122), indicating that VEGF-B can exacerbate vascular proliferative changes. In rat models, a combination of chronic blockade of VEGFR-2 and hypoxia leads to pulmonary endothelial cell apoptosis and to the outgrowth of selected apoptotic-resistant, proliferating endothelial cells with severe PAH (123). Apoptosis followed by selection of apoptosis-resistant cells may be a crucial event in PAH, triggered by VEGFR-2 blockade. Correlation of these findings to *in vivo* human disease is as yet uncertain. However, in aggregate, these observations may offer a partial mechanistic explanation of disordered angiogenic response, clonal expansion of endothelial cells, and resulting plexiform lesions in PAH of Eisenmenger's syndrome.

The above vascular effectors all appear to be active in the progression of different forms of PAH. Other potential contributing factors include peroxisome proliferator-activated receptor gamma (124), phosphodiesterase I (125), survivin (126), the calcium binding protein S100A4/Mts1 (127,128), the transient receptor potential channels (129), and adrenomedullin (130–132). These may represent important, as yet incompletely described, pathogenic contributors. Furthermore, other vascular growth factors such platelet derived growth factor, basic fibroblast growth factor, insulin-like growth factor-1, and epidermal growth factor all may play downstream roles in later stages of PAH. Yet, while our understanding of the specific action of each individual effector has improved, the *in vivo* vascular responses to their coordinate action remain unclear. In fact, none of these factors has yet been definitively linked to the root pathogenesis of disease. Insight into this topic is offered by the fact that the above effectors are likely subject to upstream, over-arching regulatory pathways that affect the action of multiple vasoactive molecules (133). Characterization of these regulatory mechanisms may eventually allow for identification of primary molecular triggers of disease and offer novel therapeutic targets for drug development. Some examples of potential overarching and overlapping regulatory pathways may include those that function through rho-kinase, angiotensin-1, caveolin-1, 5-lipoxygenase (5-LO), and vascular elastase.

Multiple cell types in the vascular wall rely upon the rho-kinase signaling pathway for homeostatic function and response to injury [as reviewed in (134)]. These cell types include endothelial and vascular smooth muscle cells, inflammatory cells, and fibroblasts. Rho is a guanosine triphosphate (GTP) binding protein that activates its downstream target, rho-kinase, in response to activation of a variety of G-protein coupled receptors. When activated, rho-kinase inhibits myosin phosphatase and conversely upregulates the ERM family of kinases. *In vitro* activation of these signaling cascades results in modulation of multiple cellular processes, including enhanced vasoconstriction, proliferation, impaired endothelial response to vasodilators, chronic pulmonary remodeling, and upregulation of vasoactive cytokines via the NF- κ B transcription pathway. Rho-kinase activity has also been linked specifically to a number of known effectors of PAH, including endothelin-1 (135,136), serotonin (137,138), and eNOS (139), among others. Recently, elevated rho-kinase activity has been demonstrated in animal models of PAH (140,141). Furthermore, intravenous

fasudil, a selective rho-kinase inhibitor, has induced pulmonary vasodilation and regression of PAH in various animal models (138,140–146) as well as in patients with severe PAH who were otherwise refractory to conventional therapies (147,148). Interestingly, in a mouse model of chronic hypoxia, pulmonary hypertension improved after therapy with simvastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor known also to inhibit rho-kinase activity (149,150). Taken together, these data suggest that rho-kinase may control a master molecular “switch” in the pulmonary artery, initiating an activated state in disease from a quiescent state in health. As a result, rho-kinase represents an attractive and novel “upstream” therapeutic target for treatment of PAH and perhaps PH.

Angiopoietin-1 is an angiogenic factor that has been linked to the regulation of multiple vascular effectors of PAH [as reviewed in (151)]. Angiopoietin-1 is produced by smooth muscle cells and pericytes during vascular development; it binds to the Tie2 receptor expressed on endothelium, which then activates smooth muscle proliferation and migration. After development, pulmonary expression is dramatically reduced; however, most non-familial forms of PAH are characterized by up-regulation of Tie2, and these levels correlate with severity of histological disease (152). The level of angiopoietin-1 itself may also be upregulated in some cases (153,154). In this context, angiopoietin-1 can stimulate pulmonary arterial endothelial cells to secrete mitogenic factors such as serotonin (155) and endothelin-1 (154). As discussed previously, angiopoietin-1 also represses endothelial expression of BMPR-1A, a receptor that dimerizes with BMPR-2 (152). It is conceivable that angiopoietin-1 may serve as a crucial link between the serotonergic and BMP pathways with resulting smooth muscle cell hyperplasia (156). In correlation, rodents expressing transgenic angiopoietin-1 in pulmonary tissue develop pulmonary vascular remodeling with diffuse smooth muscle cell hyperplasia in small pulmonary vessels (26). Furthermore, gene transfer of a Tie2 receptor antagonist ameliorates monocrotaline-induced PAH in rats (155). Paradoxically, angiopoietin-1 has also demonstrated a protective role in some forms of PAH, based on separate rodent studies (157). As a result, while angiopoietin-1 likely influences the function of multiple vascular mediators, it is unclear if it primarily acts as a direct pathogenic factor or as a homeostatic response mediator in the human manifestation of PAH or PH due to hemodynamic triggers.

The actions of caveolae and its main coat protein caveolin-1 (CAV-1) may also represent a possible upstream regulatory pathway of the development of PAH. Caveolae are flask-shaped invaginations found on the surface of the plasma membrane in a variety of vascular cell types, including pulmonary endothelium, vascular smooth muscle cells, and fibroblasts (as reviewed in (158)). Caveolae are potential regulators of signaling function that spatially organize and concentrate signaling molecules. CAV-1 is depleted in plexiform lesions and muscularized pulmonary arterioles from patients with PAH (159). Furthermore, *CAV-1*-null mice develop a dilated cardiomyopathy and pulmonary hypertension (97) with impaired NO and calcium signaling (160). A number of possible pathogenic mechanisms can be envisioned. Both eNOS (161) and endothelin-1 (162) are targeted to caveolae; disruption of these or other trafficking pathways could lead to increased inflammation and proliferation in the vessel wall (163). BMPR-2 may traffic through cholesterol-rich membrane rafts and caveolae, suggesting a possible regulatory role of caveolae for the BMP pathway and potential PAH development (164). Moreover, *CAV-1* itself can function as a tumor suppressor gene, which, if depleted in the vasculature, may directly lead to increased proliferation (165). Definitive proof, however, of the specific pathogenic actions of caveolae and CAV-1 in pulmonary vascular diseases due to hemodynamic stress is lacking.

A severe inflammatory state predominates end-stage PAH and may play a significant role in pathogenesis. A number of soluble chemoattractants and pro-inflammatory cytokines from the pulmonary artery are upregulated in human and animal models of severe PH. These

include interleukin-1 β (166), transforming growth factor- β 1 (167), bradykinin (168), monocyte chemotactic protein-1 (169), fractalkine (170), RANTES(171), and leukotrienes (172), among others. 5-lipoxygenase (5-LO) regulates the synthesis of leukotrienes, which in turn can promote cytokine release. 5-LO may represent a possible upstream factor involved in inciting this pro-inflammatory state (173). 5-LO promotes endothelial cell proliferation in cell culture, and elevated levels of 5-LO have been detected in macrophages and pulmonary endothelium derived from patients suffering from idiopathic PAH (174). In both a monocrotaline-treated rat model (175) and a genetically susceptible *BMPR2* +/- heterozygote mouse model (27) of PAH, overexpression of 5-LO via adenoviral delivery worsened pulmonary hypertension and vascular remodeling. Furthermore, 5-LO inhibitors attenuate pulmonary hypertension in both of these models. It is tempting to speculate that 5-LO may possess an upstream regulatory role in PAH progression. Yet, it is also possible that 5-LO and its enzymatic products and derivatives may represent only the severity of the end-stage inflammatory state.

Finally, vascular-specific serine elastase activity has been implicated in the pathogenesis of PAH via regulation of the remodeling response in the extracellular matrix [as reviewed in (176)]. In pulmonary arterioles, serine elastases are secreted into the extracellular space to activate matrix metalloproteinases (MMP) and to inhibit tissue inhibitors of MMP (TIMP). Both MMP and elastases degrade most components of the extracellular matrix leading to an upregulation of fibronectin and subsequent enhancement of cellular migration. Matrix degradation also leads to increased integrin signaling with resulting expression of the glycoprotein tenascin C. Tenascin C acts cooperatively with other growth factors (e.g., epidermal growth factor) to enhance smooth muscle proliferation. Increased degradation of elastin (177) has been observed in pulmonary arteries from patients suffering from congenital heart disease and resultant pulmonary hypertension. In addition, rat models of pulmonary hypertension have demonstrated increased production and activity of vascular elastases (178) and tenascin C (179). Tenascin C is also induced in pulmonary tissue of patients harboring *BMPR2* mutations and suffering from familial PAH (180). This upregulation of elastase function may be induced by a number of vascular effectors implicated in PAH, including NO (181), serotonin (182), and theoretically, the activated BMP pathway (8). Elastase inhibitors can induce apoptosis of smooth muscle cells in cell culture and can improve PAH in animal models (183–185). While elastase function may not represent a primary trigger of PH, its function in the extracellular matrix likely coordinates multiple downstream signaling pathways involved in disease propagation and, for this reason, may also represent a novel therapeutic target.

VII. Conclusions and Future Directions

Pulmonary hypertension can develop as a result of hemodynamic stressors, including either retrograde or anterograde pressure/volume overload. The consequent vascular disease is marked by a complex but stereotyped pattern of histopathology and involves dysregulation of multiple cell types. It has become increasingly clear that such pulmonary vascular remodeling entails a multifactorial pathobiology. Certainly, if sufficiently chronic and/or severe, hemodynamic stressors lead to pulmonary hypertension of some extent in all individuals. However genetic mutations, exogenous exposures, and acquired disease states likely predispose to adverse vascular remodeling. Similar to carcinogenesis in which a susceptible person requires additional injuries to manifest graded levels of disease, a “multiple-hit” hypothesis has emerged to explain the progression to clinical PH or PAH due to pressure or volume overload (Figure 4).

More delineated mechanisms of disease have focused on the end-stage condition common to multiple etiologies, including hemodynamic triggers and the effects of the imbalance of

multiple vascular effectors. Some unifying mechanism(s) of disease have become more apparent, linking the regulation of these effectors into a more cohesive model; however, at this time, a complete understanding of these complex cellular processes at the molecular level is incomplete. In part, this fact stems from the lack of a small animal model that can be followed and genetically manipulated from initiation through progression to severe disease. There has been some success in physiological study of PH in larger mammals; however, identification of contributors in the molecular process of pulmonary remodeling has been difficult, largely owing to incomplete genetic data and tools specific for these models (37,38). Conversely, while genetic and molecular studies in the mouse are more tractable, induction of significant PH in murine models has been challenging, especially in the setting of appropriate modeling of hemodynamic stress in the pulmonary vasculature. Nonetheless, recent advances have hinted at the possibility of more tractable animal models of PAH, which would be useful for combining genetic study and hemodynamic exposures.

Our continued lack of understanding of PH may also stem from the limitations of utilizing a reductionist approach to define a complex clinical syndrome based on end-stage pathological observations. In the post-genomic era, it is conceivable that complex clinical syndromes such as PH will become redefined, based on growing genomic, transcriptional, and proteomic data. A “systems-based” network analysis can be envisioned where specific genetic determinants of disease (such as *BMPR2* mutations) may interact with environmental or acquired perturbations (in this case, pressure or volume overload), “disease” modifying genes (such as genes modifying serotonin production), and “stress response” modifying genes (such as those that modulate angiogenesis or inflammation) to lead to one of several pathophenotypes (such as PH, right ventricular failure, or thromboembolic disease) (133). With the aid of the genomic database, this resulting human disease network would offer several unique opportunities: to provide a mechanistic rather than an observational description to pulmonary vascular remodeling, to predict causative risk factors for PH, and to identify and quantify regulatory determinants that otherwise would be unrecognized. By combining a networks-based approach with more tractable animal models of PH, the results would certainly improve our ability to identify common, overarching pathways of pathogenesis and aid in our understanding of the mechanistic links among hemodynamic stress and end-stage disease. Such information would be valuable not only for prevention of disease for at-risk individuals but also, perhaps, for identification of therapeutic targets useful for regression of the pathogenic process itself.

Acknowledgments

This work was supported by the NIH-T32 Cardiovascular Training Grant 5T32HL007208-30 (Massachusetts General Hospital) and AHA Founders Affiliate Postdoctoral Grant 0825906D (S.Y.C.) and the NIH grants HL 61795, NO1 HV 28178, HL 58976, PO1 81587 (J.L.). We thank Stephanie Tribuna for expert administrative assistance.

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Figure 1. Pulmonary Hypertension Involves the Coordinated Action of Multiple Endothelial-Derived Vascular Effectors

The histological progression of the pulmonary vasculature from quiescence to pathogenic activation and vasoconstriction in PH in large part involves the dysregulation of endothelial function. Initial injury to the endothelium may initiate pathogenic signaling pathways. These pathways activate an imbalance of secreted vascular mediators that drive the vascular responses of vasoconstriction, proliferation, thrombosis, and dysregulation of apoptosis, leading to the formation of a layer of “neointima.” Pathological phenotypes that may influence disease progression include vessel inflammation, transdifferentiation of endothelial cells to vascular smooth muscle cells and transdifferentiation of fibroblasts and vascular smooth muscle cells to myofibroblasts. Engraftment and differentiation of vascular progenitor cells may contribute, as well. This figure is reproduced with modifications (73). Micrographs of pulmonary arteries are courtesy of www.scleroderma.org and Humbert *et al.*, Treatment of Pulmonary Arterial Hypertension, *New England Journal of Medicine*, 2004, 351(14):1425–1436; Copyright 2004, Massachusetts Medical Society. All rights reserved.

Figure 2. The Pulmonary Vascular Endothelium is Integral for Sensing and Responding to Hemodynamic Stress

The pulmonary vascular endothelial cell acts as a sensor of physiological and pathophysiological conditions, transmitted primarily in the forms of mechanical or hemodynamic stimuli as well as humoral, neural, and hypoxic triggers. In response, endothelial cells produce and secrete a tightly regulated balance of vasoactive effectors in the vascular milieu. These effectors can then act in an autocrine fashion to autoregulate endothelial function or, in a paracrine fashion, to mediate contractile, secretory, or proliferative function of neighboring vascular smooth muscle cells (VSMCs).

Figure 3. Common Mechanisms Promoting PH Due to Hemodynamic Stress May Rely upon the Intersecting BMPR-II and Serotonergic Pathways

In the serotonergic pathway, both genetic predisposition and specific exogenous stimuli lead to vessel remodeling via increased serotonin levels and signaling activity. In the BMPR-II pathway, genetic predisposition (such as heterozygous loss-of-function +/- *BMPR2* mutations) and, perhaps, exogenous stimuli lead to decreased receptor expression, alteration of downstream signaling, and resulting vessel remodeling. Additional pathogenic mechanisms may upregulate the angiotensin-1/Tie2 receptor pathway with resulting repression of BMPR-IA function and alteration of BMPR-II signaling. Interestingly, serotonin can directly upregulate angiotensin-1 and can modulate downstream Smad function, thereby influencing the BMP signaling cascade. This functional intersection has yet to be fully described, but may represent a common, unifying mechanism of pathogenesis. The precise details of how hemodynamic stressors regulate these pathways are under investigation. This figure is reproduced with modifications (73).

Figure 4. Paradigm for the “Multiple-Hit” Hypothesis Promoting Pulmonary Hypertension Secondary to Pressure/Volume Overload

Hemodynamic stress induces pulmonary hypertension with histopathologic complications in most, if not all, persons when subjected to pressure or volume overload chronically.

However, susceptible persons with genetic or acquired traits may progress to PH more rapidly or to a heightened extent. These factors appear synergistic in the pathogenesis of disease, leading to a common set of end-stage pathologies.

Table 1
Clinical Classification of Pulmonary Hypertension (Venice, 2003)

Adapted from reference (4).

Pulmonary Hypertension (PH)
<i>Associated with left-heart disease</i>
<i>Associated with lung disease/hypoxia</i>
<i>Associated with chronic thromboembolism</i>
<i>Miscellaneous</i>
Pulmonary Arterial Hypertension (PAH)
<i>Associated with congenital systemic-to-pulmonary shunts</i>
<i>Associated with drugs/toxins</i>
<i>Associated with portal hypertension</i>
<i>Associated with connective tissue disease</i>
<i>Associated with human immunodeficiency virus infection</i>
<i>Persistent pulmonary hypertension of the newborn</i>
<i>Associated with significant venous or capillary involvement</i>
<i>Idiopathic/Familial</i>
<i>Miscellaneous</i>

Table 2
Classification of PAH Associated with Congenital Heart Disease

Adapted from reference (4).

Type of Lesion	
Simple or Combinations	<i>Atrial septal defect (ASD)</i>
	<i>Sinus venosus defect</i>
	<i>Ventricular septal defect (VSD)</i>
	<i>Aortopulmonary window</i>
	<i>Patent ductus arteriosus (PDA)</i>
	<i>Total or partial unobstructed anomalous pulmonary venous return</i>
Complex congenital heart disease	<i>Atrioventricular septal defects</i>
	<i>Truncus arteriosus</i>
	<i>Single-ventricle physiology with unobstructed pulmonary blood flow</i>
	<i>Transposition of the great arteries with ventricular or arterial shunt</i>
Lesion dimensions	<i>Small to moderate (ASD<2.0 cm, VSD/PDA<1.0 cm)</i>
	<i>Large (ASD>2.0 cm, VSD/PDA>1.0 cm)</i>
Associated extracardiac abnormalities	
Repair status	<i>Unoperated</i>
	<i>Palliated</i>
	<i>Repaired</i>

Table 3
Dysregulation of Vascular Effectors in Pulmonary Arterial Hypertension

An imbalance of mediators of pulmonary vascular response has been observed in PAH. The predominant pathogenic consequences of these imbalances are tabulated; these influence vasoconstriction, cellular proliferation, and thrombosis. Less significant effects are denoted by (N/A); unclear effects are indicated by (?). This table is reproduced with modifications (73).

<u>Vascular Effector</u>	<u>Change in Activity in PAH</u>	<u>Effect on Vasoconstriction</u>	<u>Effect on Cell Proliferation</u>	<u>Effect on Thrombosis</u>
Nitric Oxide	Decreased (Increased in plexiform lesions)	Increased	(?) Increased in plexiform lesions	Increased
Thromboxane A ₂	Increased	Increased	Increased	Increased
Prostacyclin	Decreased	Increased	Increased	Increased
Endothelin-1	Increased	Increased	Increased	(N/A)
Vasoactive Intestinal Peptide (VIP)	Decreased	Increased	Increased	Increased
Vascular Endothelial Growth Factor (VEGF)	Increased	(N/A)	Increased	(N/A)
Rho-Kinase	Increased	Increased	Increased	Increased
Angiopoietin-1	Increased	(N/A)	Increased	(N/A)
Caveolae	Decreased	(?)	(?)	(?)
5-lipoxygenase (5-LO)	Increased	Increased	Increased	Increased
Vascular Elastase	Increased	Increased	Increased	Increased