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Pathogenic Mechanisms of Pulmonary Arterial Hypertension

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Abstract

Pulmonary arterial hypertension (PAH)¹ is a complex disease that causes significant morbidity and mortality and is clinically characterized by an increase in pulmonary vascular resistance. The histopathology is marked by vascular proliferation/fibrosis, remodeling, and vessel obstruction. Development of PAH involves the complex interaction of multiple vascular effectors at all anatomic levels of the arterial wall. Subsequent vasoconstriction, thrombosis, and inflammation ensue, leading to vessel wall remodeling and cellular hyperproliferation as the hallmarks of severe disease. These processes are influenced by genetic predisposition as well as diverse endogenous and exogenous stimuli. Recent studies have provided a glimpse at certain molecular pathways that contribute to pathogenesis; these have led to the identification of attractive targets for therapeutic intervention. We will review our current understanding of the mechanistic underpinnings of the genetic and exogenous/acquired triggers of PAH. The resulting imbalance of vascular effectors provoking pathogenic vascular changes will also be discussed, with an emphasis on common and overarching regulatory pathways that may relate to the primary triggers of disease. The current conceptual framework should allow for future studies to refine our understanding of the molecular pathogenesis of PAH and improve the therapeutic regimen for this disease.

Keywords

pulmonary arterial hypertension; molecular mechanism; vasoconstriction; thrombosis; proliferation; genetic association; acquired risk; vascular effector

¹Abbreviations

Atrial septal defect (ASD); B isoform of vascular endothelial growth factor (VEGF-B); bone morphogenetic protein receptor type 2 (BMP2 gene which encodes for BMP2 protein); carbon monoxide (CO); caveolin-1 (CAV-1); cyclic adenosine monophosphate (cAMP); endothelin-1 (ET-1); endothelial isoform of nitric oxide synthase (eNOS); 5-lipoxygenase (5-LO); guanosine triphosphate (GTP); heme oxygenase-1 (HO-1); human herpes virus 8 (HHV-8); human immunodeficiency virus (HIV); hydrogen sulfide (H₂S); Kv1.5 channel gene (KCNK5 gene); matrix metalloproteinase (MMP); nitric oxide (NO); nitric oxide synthase enzymes (NOS); patent ductus arteriosus (PDA); peroxisome proliferator-activated receptor gamma (PPARγ); pulmonary arterial hypertension (PAH); serotonin (5-HT); selective serotonin re-uptake inhibitor (SSRI); serotonin transporter (5-HTT); tissue inhibitor of MMP (TIMP); transforming growth factor-β (TGF-β); vascular endothelial growth factor (VEGF); vascular endothelial growth factor receptor-1 (VEGFR-1/KDR); vascular endothelial growth factor receptor-2 (VEGFR-2/Flt); vasoactive intestinal peptide (VIP); ventricular septal defect (VSD); voltage-gated potassium channel (Kv).

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Introduction

Pulmonary arterial hypertension (PAH) is clinically characterized by increasing pulmonary arterial pressure in the absence of elevated left heart pressure. If untreated, PAH leads to right ventricular failure, volume overload, and death [1]. No cure is available for PAH, but recent advances in our understanding of the molecular mechanisms of disease progression have led to therapeutic approaches that improve survival and quality of life [2].

Previously, PAH had been classified as a primary form (“idiopathic”) or secondary forms, associated with a variety of diverse clinical entities. Recently, investigators have increasingly recognized that secondary disease states may precipitate pulmonary vascular disease resembling idiopathic PAH in histopathologic changes. This suggests a degree of commonality in mechanisms of disease progression. Currently, the term PAH comprises the idiopathic forms (formerly, primary PAH) as well as manifestations resulting from other disease, such as collagen vascular disease, hemoglobinopathies, congenital cardiovascular disease with systemic-to-pulmonary shunts, human immunodeficiency virus infection, portal hypertension, drugs and toxins, persistent pulmonary hypertension of the newborn, and myeloproliferative disorders, among others [3]. While right ventricular dysfunction certainly contributes to clinical decompensation, the inciting pathogenic processes appear localized to the pulmonary vasculature itself. Common histologic features in nearly all types of PAH occur at the level of the small peripheral pulmonary arteries; these include intimal fibrosis, distal localization and proliferation of vascular smooth muscle, and pulmonary arterial occlusion [4]. Furthermore, a hallmark of severe, end-stage disease is the formation of a vessel “neointima,” characterized by increased deposition of extracellular matrix and myofibroblasts [5]. Plexiform lesions can predominate, characterized by over-proliferation of endothelial-like cells encroaching upon the vessel lumen [6].

Multiple cell types in the pulmonary arterial wall and pulmonary arterial circulation contribute to the specific response to injury and the development of vessel remodeling (Figure 1) [5]. The endothelium serves as a central sensor of injurious stimuli, such as hypoxia, shear stress, inflammation, and toxins. 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, such as adventitial fibroblasts or components of the extracellular matrix, may also initiate pulmonary vascular disease [7]. Regardless of the initiating process, an imbalance of secreted vasoactive factors ensues and directs vascular remodeling via pathologic 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 [8,9]. Furthermore, the identification of monoclonal amplification in plexiform populations [10] 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 [11]. Inflammatory cells and activated platelets appear to predominate in later stages of PAH [12]; yet, our understanding is limited regarding the mechanistic role of these cellular populations in disease progression [13]. Finally, circulating or resident progenitor cells have been proposed to factor significantly in vessel wall injury and repair; dysregulation of these functions may also contribute to PAH [14].

Based on observation of these histologic changes in humans and animal models, a number of recent advances have allowed for the development of a conceptual framework to better delineate the molecular pathways of PAH. In this review, we will highlight our current understanding of the complex and incompletely characterized interplay of genetic and exogenous upstream stimuli with downstream vascular effectors that ultimately lead to clinical PAH.

Genetic Association

An understanding of the mechanism of genetic predisposition to PAH is of paramount importance for identification of the root pathogenesis. The familial variety of idiopathic PAH accounts for at least 6 percent of all cases of PAH [15-17]. Pedigree analysis has demonstrated an autosomal dominant inheritance but with variable penetrance, as only 10-20% of putative genetic carriers develop clinical PAH. Genetic anticipation is present, as each successive generation of affected families is afflicted at a younger age and greater severity compared with the preceding generation.

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 [18,19]. However, a more prevalent cohort of patients carries mutations in another member, the bone morphogenetic protein receptor type 2 (*BMPR2* gene which encodes for BMPR-II) [20,21]. Over 140 mutations in *BMPR2* have been reported in patients with familial PAH [22], mainly located in the extracellular ligand-binding domain, in the cytoplasmic serine/threonine kinase domain, or in the long carboxyterminal domain [23]. These account for 70% of all familial pedigrees of PAH and 10-30% of idiopathic PAH cases [16, 20,23-26]. *BMPR2* loss-of-function mutations have only been found in the heterozygous state. This fact likely reflects the critical role of the BMP pathway in vascular development, as demonstrated by the embryonic lethality of the *BMPR2*-homozygous null mouse [27]. The absence of *BMPR2* mutations in some familial cohorts and in most of the sporadic cases indicates that additional, unidentified genetic mutations can also predispose to development of PAH. Furthermore, the presence of incomplete penetrance and genetic anticipation suggests that *BMPR2* mutations are necessary but insufficient alone to result in clinically significant disease.

The mechanism of action of BMPR-II is complex, and its role in PAH progression is still unclear. It functions as a receptor with serine/threonine kinase activity, and it activates a broad and complex range of intracellular signaling pathways (as reviewed in [28]). Upon binding one of many possible BMP ligands, BMPR-II forms a heterodimer with one of three type-I receptors. BMPR-II phosphorylates the bound type-I receptor, which, in turn, phosphorylates one of the Smad family of proteins to allow for nuclear translocation, binding to DNA, and regulation of gene transcription. Alternatively, BMPR-II activation can also lead to signaling via the LIM kinase pathway, the p38/MAP kinase/ERK/JNK pathways, or the c-Src pathway [29], independent of Smad activation [30,31].

Correlating with these complexities of signaling, the cellular effects of BMPR-II activation are multiform. In the adult, BMPR-II is expressed predominantly in the pulmonary endothelium, medial smooth muscle cells, and macrophages [32]. The mutations in *BMPR2* that lead to PAH likely exert their pathogenic action predominantly by modulating function in endothelium and/or smooth muscle; however, it is possible that specific effects in other tissues or at other times in development may, in part, explain the mechanism of genetic predisposition. Under normal conditions, BMP ligands bind BMPR-II to suppress the growth of vascular smooth muscle cells [33,34]. In contrast, binding of BMP2 and BMP7 to BMPR-II in pulmonary endothelium leads to protection from apoptosis [35]. While certain *BMPR2* mutations may affect receptor function differently [36-39], altered function *in vivo* does not stem from somatic mutations leading to homozygous loss-of-function mutations in both *BMPR2* alleles [40]. Rather, patients harboring such mutations generally suffer from haploinsufficiency with decreased expression of BMPR-II in pulmonary tissue [22,26,32,41]. As a result, BMP signaling appears altered but not completely abolished [42,43].

Given these findings, a widely held hypothesis contends that failure of the suppressive effects of BMP ligands on vascular smooth muscle [44] and failure of the protective effects of BMP ligands on endothelium may trigger vascular proliferation and remodeling. Accordingly, in vascular smooth muscle cells derived from patients with familial PAH harboring *BMPR2* mutations, exposure of BMP ligands does not suppress proliferation [39,44,45]. Furthermore, unlike the response in wildtype endothelium, exposure of endothelial cells cultured from patients with idiopathic PAH to BMP2 does not protect against apoptosis [35]. These dysfunctional signaling pathways have been corroborated in some rodent models of PAH [46]. In correlation, pulmonary levels of *BMPR-II* are reduced both in familial cases of PAH without any *BMPR2* mutation and in cases of secondary PAH [32]. As a result, abnormal growth responses to BMP/TGF- β stimulation have been noted in pulmonary vascular smooth muscle cells derived from PAH patients [47]. Furthermore, response of pulmonary vascular smooth muscle cells to BMP signaling appears regulated by hypoxia, a known precipitant of pulmonary hypertension [48]. Thus, dysregulation of the BMP signaling pathway may be a common pathogenic finding in multiple types of PAH due to genetic or exogenous stimuli (Figure 2).

Despite these advances, a clear mechanistic explanation of the impact of *BMPR2* mutations on pathogenesis is still lacking, owing to a number of factors. First, the cellular effects of the BMP pathway vary in the setting of diverse combinations of extracellular ligands, receptors, and intracellular signaling cascades [43]. Therefore, responses in cell culture may not accurately reflect *in vivo* events that are coordinately regulated by multiple intersecting signaling pathways. Second, the definitive *in vivo* effects of these mutations have been difficult to decipher. While adenoviral overexpression of wildtype *BMPR-II* appears to ameliorate pulmonary hypertension in a hypoxic rat model [49], overexpression of *BMPR-II* fails to attenuate PAH that is chemically induced (via monocrotaline) in a rat model [50]. More definitive *in vivo* studies have been limited. Specifically, mouse models harboring specific *BMPR2* heterozygous mutations have failed to exhibit robust PAH under static conditions [38,51], again suggesting that dysfunctional *BMPR-II* is likely insufficient alone to cause disease. Provocation with hypoxia has led to minimally increased pulmonary vascular reaction, while exposure to an experimental inflammatory stimulus or additional cytokines has provoked graded vascular responses [52]. Murine models harboring different *BMPR2* mutations may show promise with a more robust phenotype [53]. Yet, until genetically tractable animal models of disease become readily available, the precise pathogenic contribution of the BMP pathway to PAH will likely remain elusive.

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 physiologic influences [54]. Serotonin is both a vasoconstrictor and mitogen that promotes smooth muscle hyperplasia and hypertrophy [55]. 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 [56].

A number of observations in idiopathic and familial disease, congenital disease, and environmental exposure have implicated the proliferative effects of serotonin in PAH. In idiopathic PAH, pulmonary expression of serotonin receptors is increased [57,58], and plasma levels of serotonin are chronically elevated [59]. A mouse model of hypoxic pulmonary hypertension parallels these changes [58]. A positive association has been noted among patients with congenital platelet defects in serotonin uptake (i.e., delta storage pool disease) and

development of PAH [60]. Chronic exposure to anorexigens, such as dexfenfluramine (an inhibitor of serotonin reuptake and stimulator of serotonin secretion), has led to increased levels of circulating, free serotonin. In mice, these changes are accompanied by increased 5-HT receptor type 2B response [58] and inhibition of 5-HTT responses. In turn, these changes correlate with an increased risk for development of PAH in humans [61]. Finally, the *L*-allelic variant of 5-HTT is associated with increased expression of the transporter and enhanced smooth muscle proliferation; in homozygous form, this variant has been reported in 65% of idiopathic PAH patients but only in 27% of controls, suggesting an increased risk of PAH in the homozygous population [62,63]. A larger study of 5-HTT polymorphisms, however, has not confirmed this association [64].

Animal models of pulmonary hypertension have also implicated the activated serotonin pathway in disease progression. Treatment with serotonin and chronic hypoxia in a rat model has led to worsened hemodynamics and increased vessel remodeling [65]. Similarly, overexpression of the *5-HTT* gene in mice has resulted in spontaneous development of PAH in the absence of hypoxia and exaggeration of pulmonary hypertension after hypoxic stimulus [66,67]. Conversely, vessel remodeling and hypoxic pulmonary hypertension are reduced in a *5-HT1B* receptor-null mouse [68] and are abrogated in a *5-HT2B* receptor-null mouse [58].

As a result, serotonin signaling modulates pulmonary smooth muscle function in both normal and disease states and likely contributes to disease progression of PAH. However, the exact contribution to this mechanism remains to be characterized. For example, selective serotonin re-uptake inhibitors (SSRIs), which increase serotonin levels but inhibit serotonin transport, abrogate, rather than enhance, development of PAH in the setting of hypoxia [69]. In contrast, a possible causative association between maternal SSRI use and persistent pulmonary hypertension of the newborn has been reported [70]. As a result, it is currently unclear if serotonin itself or its associated effectors primarily drive PAH development. Serotonin can also 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 [71]. Serotonin may also regulate the angiotensin-1/Tie2 signaling pathway, another potential pathogenic contributor (see discussion below). This, in turn, can modulate signaling of *BMPR-II* via inhibition of endothelial expression of one of its dimerized partners, *BMPR-IA* [72]. 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 2).

Acquired/Exogenous Factors

In addition to genetic predisposition, development of PAH likely depends on a variety of physiologic, acquired, and/or exogenous stimuli. Some of these factors have been studied to a sufficient degree to hypothesize a potential pathogenic mechanism(s) (Figure 3). These include chronic hypoxia, hemoglobinopathies, autoimmune vascular disease, viral infections, congenital heart disease with systemic-to-pulmonary shunt, and “serotonergic” anorexigen use (see discussion above). Factors associated with PAH that perhaps carry even less defined mechanisms of disease include thrombocytosis, central nervous system stimulants, portal hypertension, persistent pulmonary hypertension of the newborn, and female gender predilection.

The pulmonary vascular response to hypoxia has been well studied in cell culture and in animal models (as reviewed in [73,74]), but its impact on PAH is unclear. In general, pulmonary vascular responses in acute and chronic hypoxia likely allow for the propagation of PAH, and, therefore, may contribute to the later stages of disease. Acute hypoxia induces vasodilatation

in systemic vessels, but induces vasoconstriction in pulmonary arteries. This acute and reversible effect is mediated in part by upregulation of vasoconstrictors, such as endothelin-1 and serotonin, and, in part, by hypoxia- and redox-sensitive potassium channel activity in pulmonary vascular smooth muscle cells. Coordinately, these events lead to membrane depolarization in smooth muscle cells, increase in cytosolic calcium, and vasoconstriction [75]. In contrast, chronic hypoxia induces vascular remodeling and less reversible changes, including migration and proliferation of vascular smooth muscle cells and deposition of extracellular matrix. These cellular events in chronic hypoxia correlate with the remodeling events in end-stage PAH. However, because the histopathology of hypoxic pulmonary hypertension does not recapitulate all aspects of PAH, some mechanistic differences in pathogenesis certainly exist but have not yet been fully identified. These are important considerations, especially in the context of interpreting studies of hypoxic pulmonary hypertension and extrapolating those findings to the pathogenesis of PAH.

PAH is associated with hemoglobinopathies, especially thalassemias [76,77] and sickle cell anemia [78-80]. Hemolysis accompanying these disorders may lead to destruction of bioactive nitric oxide by free hemoglobin [81] or reactive oxygen species [82,83]. Furthermore, reactive oxygen species may lead to increased levels of oxyhemoglobin, which further impairs the delivery of nitric oxide to the vessel wall. As a result of the lack of available nitric oxide, an inflammatory and proliferative cascade may ensue with culmination in PAH. Accordingly, decreased nitric oxide bioavailability with development of pulmonary hypertension has been reported after hemolysis in a murine model of sickle cell disease [84]. *In vivo* correlation to human disease is pending.

Pulmonary arteriopathy complicates autoimmune diseases, especially in the setting of the CREST variant of limited systemic sclerosis and, to a lesser degree, in mixed connective tissue disease, systemic lupus erythematosus, and rheumatoid arthritis [85-88]. The occurrence of PAH in each disease has been associated with Raynaud's phenomenon, suggesting at least some similarities in pathogenesis [89]. The presence of interstitial lung disease and pulmonary fibrosis, seen at varying frequency in these autoimmune syndromes, may represent a common pathogenic factor in the development of PAH. Accordingly, in the setting of pulmonary fibrosis and hypoxia, significant inflammation and deposition of extracellular matrix have been observed, which may increase vasoconstriction, proliferation, and vessel remodeling. Murine models of interstitial lung disease utilizing chemical (bleomycin) exposure may prove important in further elucidating the pathogenic mechanisms [90,91].

An association between the human immunodeficiency virus (HIV) infection and pulmonary hypertension has been noted in approximately 0.5% of all patients with HIV infection, a rate 6 to 12 times higher than the general population [92,93]. Notably, HIV does not infect pulmonary arterial endothelium [94]; however, mechanisms of disease have been proposed that directly stem from effects of HIV infection (as reviewed by [95]). These include infection of smooth muscle cells with subsequent dysregulation of proliferation, imbalance of vascular mitogens in response to systemic HIV infection, and endothelial injury precipitated by HIV-infected T cells [96]. Recently, the direct actions of HIV-encoded proteins have been implicated in PAH development. The HIV gp120 protein may induce pulmonary endothelial dysfunction and apoptosis [97]. In a macaque model of simian immunodeficiency virus infection, a pathogenic interaction of the viral Nef protein with the pulmonary vessel wall has been reported, leading to pulmonary arteriopathy [98]. Cell culture studies have also demonstrated a role for the HIV Tat protein in repression of BMPR-II transcription, potentially provoking a proliferative response in the vessel wall [99]. Finally, it has been proposed that human herpes virus 8 (HHV-8), the causative agent of Kaposi's sarcoma and an opportunistic pathogen highly associated with HIV infection, may play a role in PAH development with progression to plexiform lesions. Although it was initially reported that HHV-8 infection is associated with

idiopathic PAH [100], that link has not been consistently validated after study of additional populations [101-106]. Nonetheless, PAH in the setting of HIV infection likely results from multifactorial effects, and the underlying pathogenesis may involve both direct results of viral infection and indirect consequences of associated pathogens.

Increased flow through the pulmonary circulation has long been associated with development of PAH. Certain types of congenital heart disease with functional systemic-to-pulmonary shunts, such as unrestricted ventricular septal defects (VSD) and large patent ductus arteriosus (PDA), invariably lead to pulmonary vascular remodeling and the clinical syndrome of PAH during childhood (Eisenmenger's syndrome). Presence of atrial septal defects (ASD) with systemic-to-pulmonary shunts may also lead to PAH over time [107]. Yet, in contrast to the cases of unrestricted VSD and PDA, only 10-20% of all persons with atrial septal defects progress to PAH [108]. This observation may reflect differences in the response of the pulmonary vasculature to pressure overload (as seen in shunts with VSD and PDA) 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. This putative predisposition does not appear to rely upon *BMPR2* function, as *BMPR2* mutations do not specifically cluster with the presence of ASD [109]. At the molecular level, the physiologic 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 [110]). 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. In part, this stems from the difficulty of directly studying the *in vivo* flow patterns at the anatomic level of the pulmonary arteriole. Recently, *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 [111]. Therefore, chronically elevated flow may allow for selection of cells with dysregulated endothelial cell growth and resulting clonal or polyclonal expansion to plexiform lesions. A number of animal models have been developed to study "flow-dependent" PAH, notably involving surgical creation of a shunt in rodents and larger mammalian species (as reviewed in [112]). In addition, attempts at computer modeling have shown promise [113,114]. These may play important roles in the future for better delineating the molecular effects of increased flow in the pulmonary vasculature.

PAH has been reported in patients suffering from other clinical syndromes such as chronic myelodysplastic syndromes with thrombocytosis [115,116] and idiopathic thrombocythemia [117,118]; as well as in persons exposed to stimulants of the central nervous system such as methamphetamines and cocaine [119-121]. Dysregulation of serotonergic signaling may contribute but does not explain these associations entirely. PAH can also develop in patients suffering from portal hypertension [122-124], and in neonates with failure of the normal fetal-to-neonatal circulatory transition (persistent pulmonary hypertension of the newborn). Finally, idiopathic PAH demonstrates a gender predilection, with a high predominance of affected females [125]. It remains unknown if these associated states alone act as primary triggers of PAH or if confounding factors otherwise predominate pathogenesis.

Vascular Effectors

Downstream of the genetic and acquired triggers of PAH, the histopathologic processes that predominate later stages of disease include vasoconstriction, cellular proliferation, and

thrombosis. These processes are influenced by a complex and dysregulated balance of vascular effectors controlling vasodilatation and vasoconstriction, growth suppressors and growth factors, and pro- versus anti-thrombotic mediators. Most of these effectors have been described in previous comprehensive reviews and will be described here specifically in regard to their known roles in the pathogenesis of PAH (Table 1).

Gaseous vasoactive molecules regulate pulmonary vascular homeostasis, and alterations in their endogenous production have been linked to the progression of PAH. 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 physiologic stimuli including hypoxia, inflammation, and oxidative stress. Reduced levels of eNOS have been demonstrated in the pulmonary vasculature of patients with PAH, suggesting a mechanism of dysregulated vasoconstriction [126,127]. 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 [128,129]. Furthermore, the impact of NO has been reflected in its therapeutic role in PAH, as inhaled NO (as reviewed in [130]) and the NO-dependent phosphodiesterase type-5 inhibitor sildenafil [131] (and as reviewed in [132]). Nonetheless, the exact mechanisms of eNOS and NO dysregulation in PAH have not been fully elucidated. First, increased levels of eNOS have been described specifically in plexiform lesions in idiopathic PAH [133]. Increased NO levels 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 [134]. Third, NO levels are intricately associated with oxidative stress, another likely but incompletely described regulator of pulmonary hypertension [135]. Finally, specific polymorphisms of NOS have been associated with pulmonary hypertension [136,137]; 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) and hydrogen sulfide (H₂S) are endogenously produced gaseous vasodilators, deficiencies of which may promote development of PAH. Mice that are null in heme oxygenase-1 (HO-1), a primary enzyme that produces CO in the pulmonary vasculature, exhibit less tolerance for hypoxia with resulting right ventricular dysfunction [138]. In contrast, overexpression of HO-1 in the lung prevents development of pulmonary hypertension in murine models of chronic hypoxia [139] and in rat models of monocrotaline-induced PAH [140]. H₂S also functions as a vasodilator and inhibitor of vessel wall proliferation, which can protect against the development of PAH in rat models [141,142]. However, studies elucidating the precise regulation of CO and H₂S production during disease progression are currently lacking.

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 [143]. Protein levels of prostacyclin synthase are decreased in small and medium-sized pulmonary arteries in patients with PAH, particularly with the idiopathic form [144]. Biochemical analysis of urine in patients with PAH has shown decreased levels of a breakdown product of prostacyclin (6-keto-prostacyclin F₂α), accompanied by increased levels of a metabolite of thromboxane A₂ (thromboxane B₂) [145]. 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 (as reviewed in [146]).

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 mitogen of pulmonary smooth muscle cells [147,148]. The vasoconstrictor response relies upon binding to the endothelin receptor A (ET_A receptor) on vascular smooth muscle cells. This 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* [5]. 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 due to a variety of etiologies [149,150]. Again, improvement in hemodynamics, clinical status, and survival of PAH patients treated with chronic ET receptor antagonists highlights the significance of these effects (as reviewed in [151]). 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 [152,153]. Decreased concentrations of VIP have been reported in serum and lung tissue of patients with PAH [154]. *VIP*-null mice suffer from moderate pulmonary hypertension [155]. Furthermore, both pulmonary arterial pressure and pulmonary vascular resistance decrease after treatment with VIP [156-158]. Improvement of hemodynamics and clinical course has also been observed with inhaled VIP in a small number of PAH patients [154]. Key questions regarding the mode(s) of regulation of VIP expression and its putative causative role in PAH 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 [159]. 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 [6,160]; 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 critically influence 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 [161], 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 [162]. 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 pending. In aggregate, however, these observations may offer a partial mechanistic

explanation of disordered angiogenic response, clonal expansion of endothelial cells, and resulting plexiform lesions in PAH.

The above vascular effectors all appear to be active in the progression of different forms of PAH. Interestingly, it has been recently reported that peroxisome proliferator-activated receptor gamma (PPAR γ) can ameliorate PAH in insulin-resistant, apolipoprotein E-deficient mice [163]. This suggests not only a link between insulin resistance/obesity and PAH but also highlights a novel protective role for PPAR γ in pulmonary vasculopathy. Other potential contributing factors to PAH progression include phosphodiesterase I [164], survivin [165], the calcium binding protein S100A4/Mts1 [166,167], the transient receptor potential channels [168], and adrenomedullin [169-171]. These may represent important but 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 [172]. 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, voltage activated potassium channels, angiopoietin-1, caveolae, 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 [173]). 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 [174,175], serotonin [176,177], and eNOS [178], among others. Recently, elevated rho-kinase activity has been demonstrated in animal models of PAH [179,180]. Furthermore, intravenous fasudil, a selective rho-kinase inhibitor, has induced pulmonary vasodilatation and regression of PAH in various animal models [179-186] as well as in patients with severe PAH who were otherwise refractory to conventional therapies [187,188]. 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 [189,190]. 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.

Modulation of voltage-gated potassium channels (Kv) may also represent an overarching pathogenic mechanism of PAH. Kv channels are inhibited in the smooth muscle cells of resistance vessels in the pulmonary arterial tree in response to hypoxia, and they regulate hypoxic pulmonary vasoconstriction (as reviewed in [191]). Subsequent depolarization leads to the opening of voltage-gated calcium channels, an increase in intracellular calcium, and the

initiation of a number of intracellular signaling cascades promoting vasoconstriction and proliferation and inhibiting apoptosis. Expression array analysis has demonstrated a depletion of Kv1.5 channels in pulmonary tissue derived from PAH patients [192]. It is currently unknown if these Kv channel abnormalities are congenital or acquired; however, a number of polymorphisms in the Kv1.5 channel gene (*KCNA5*) have been described, which may suggest a genetic predisposition to channel depletion [193]. Appetite suppressants, such as dexfenfluramine and aminorex that are risk factors for development of PAH, can also directly inhibit Kv1.5 and Kv2.1 [194]. Inhibition of Kv currents in pulmonary smooth muscle cells may be regulated by serotonin [195], thromboxane A2 [196], and, perhaps, nitric oxide [197]. Furthermore, BMP signaling can regulate Kv receptor expression [198,199]. Taken together, the Kv pathway may represent a common point of regulation in pathogenesis. Accordingly, augmentation of Kv activation would be predicted to induce vasodilatation and, perhaps, allow for regression of vessel remodeling. *In vivo* gene transfer of Kv channels in chronically hypoxic rats has led to improvement of pulmonary hypertension and suggests its therapeutic potential [200].

Angiopoietin-1 is an angiogenic factor that has been linked to the regulation of multiple vascular effectors of PAH (as reviewed in [201]). Angiopoietin-1 is produced by smooth muscle cells and pericytes during vascular development; it binds 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 histologic disease [45]. The level of angiopoietin-1 itself may also be upregulated in some cases [202,203]. In this context, angiopoietin-1 can stimulate pulmonary arterial endothelial cells to secrete mitogenic factors such as serotonin [204] and endothelin-1 [203]. As discussed previously, angiopoietin-1 also represses endothelial expression of BMPR-1A, a receptor that dimerizes with BMPR-II [45]. It is conceivable that angiopoietin-1 may serve as a crucial link between the serotonergic and BMP pathways with resulting smooth muscle cell hyperplasia [205]. In correlation, rodents expressing transgenic angiopoietin-1 in pulmonary tissue develop pulmonary vascular remodeling with diffuse smooth muscle cell hyperplasia in small pulmonary vessels [72]. Furthermore, gene transfer of a Tie2 receptor antagonist ameliorates monocrotaline-induced PAH in rats [204]. Paradoxically, angiopoietin-1 has also demonstrated a protective role in some forms of PAH, based on separate rodent studies [206]. 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.

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 [207]). 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 [208]. Furthermore, *CAV-1*-null mice develop a dilated cardiomyopathy and pulmonary hypertension [209] with impaired NO and calcium signaling [210]. A number of possible pathogenic mechanisms can be envisioned. Both eNOS [211] and endothelin-1 [212] are targeted to caveolae; disruption of these or other trafficking pathways could lead to increased inflammation and proliferation in the vessel wall [213]. BMPR-II may traffic through cholesterol-rich membrane rafts and caveolae, suggesting a possible regulatory role of caveolae for the BMP pathway and potential PAH development [214]. Serotonin signaling may depend upon caveolae to downregulate Kv channels [195]. Moreover, *CAV-1* itself can function as a tumor suppressor gene, which, if depleted in the

vasculature, may directly lead to increased proliferation [215]. Definitive proof, however, of the specific pathogenic actions of caveolae and CAV-1 in PAH 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 PAH. These include interleukin-1 β [216], transforming growth factor- β 1 [217], bradykinin [218], monocyte chemoattractant protein-1 [219], fractalkine [220], RANTES [221], and leukotrienes [222], 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 [223]. 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 [224]. In both a monocrotaline-treated rat model [225] and a genetically susceptible *BMPR2* +/- heterozygote mouse model [52] of PAH, overexpression of 5-LO via adenoviral delivery has worsened pulmonary hypertension and vascular remodeling. Furthermore, 5-LO inhibitors have attenuated 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 [226]). 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 (i.e., epidermal growth factor) to enhance smooth muscle proliferation. Increased degradation of elastin [227] 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 [228] and tenascin C [229]. Tenascin C also is induced in pulmonary tissue of patients harboring *BMPR2* mutations and suffering from familial PAH [230]. This upregulation of elastase function may be induced by a number of vascular effectors implicated in PAH, including NO [231], serotonin [58], and theoretically, the BMP pathway [4]. Elastase inhibitors can induce apoptosis of smooth muscle cells in cell culture and can improve PAH in animal models [232-234]. While elastase function may not represent a primary trigger of PAH, 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.

Conclusions and Future Directions

Pulmonary arterial hypertension refers to a clinical syndrome of vascular disease with a stereotyped pattern of histopathology and is related to a variety of secondary disease states. It has become increasingly clear that development of PAH entails a complex, multifactorial pathophysiology. Although genetic mutations, exogenous exposures, and acquired disease states can predispose to PAH, no one factor identified thus far is sufficient alone to drive fully the pathogenic process. Similar to carcinogenesis in which a susceptible person with a specific genetic mutation requires additional injuries to manifest disease, a “multiple-hit” hypothesis has emerged to explain the progression to clinical PAH (Figure 4).

More delineated mechanisms of disease have focused on the end-stage condition 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, 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 physiologic study of PAH in larger mammals; however, identification of contributors in the molecular process of pulmonary remodeling has been difficult, owing to incomplete genetic data and tools specific for these models [112,235]. Conversely, while genetic and molecular studies in the mouse are more tractable, induction of significant PAH in murine models has been challenging. Nonetheless, recent advances have hinted at the possibility of more tractable animal models of PAH, which would be useful for combining genetic study and pathophysiologic exposures.

Our continued lack of understanding of PAH may also stem from the limitations of utilizing a reductionist approach to define a complex clinical syndrome based on end-stage pathologic observations. In the post-genomic era, it is conceivable that complex clinical syndromes such as PAH 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 (such as hypoxia or anorexigen use), “disease” modifying genes (such as *5-HTT*), and “stress response” modifying genes (such as those that modulate angiogenesis or inflammation) to lead to one of several pathophenotypes (such as PAH, right ventricular failure, or thromboembolic disease) [172]. 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 risk factors for PAH, and to identify and quantitate regulatory determinants that otherwise would be ignored. By combining a networks-based approach with more tractable animal models of PAH, the results would certainly improve our ability to identify common, overarching pathways of pathogenesis and aid in our understanding of the mechanistic links among primary disease triggers 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.

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Figure 1. Pulmonary Arterial Pathobiology Involves the Coordinate Action of Multiple Cell Types

The histologic progression of the pulmonary vasculature from quiescence to pathogenic activation in PAH involves numerous vascular cell types and phenotypic responses. Initial injury to the endothelium and/or adventitial fibroblasts may activate pathogenic signaling pathways. These result in an imbalance of secreted vascular effectors that drive the vascular responses of vasoconstriction, proliferation, and dysregulation of apoptosis, leading to the formation of a layer of “neointima” (red arrow) and, in some cases, plexiform lesions. Activation of platelets (blue thrombus) and extravasation/migration of blood-borne inflammatory cells (green arrow) likely play prominent roles in these processes, but their exact mechanistic actions are unclear. Engraftment and differentiation of vascular progenitor cells may influence disease progression (purple arrow). Transdifferentiation of endothelial cells to vascular smooth muscle cells may contribute as well (blue arrow). 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. Common Mechanisms Promoting PAH May Rely upon the Intersecting BMPR-II and Serotonergic Pathways

In the serotonergic pathway, both genetic predisposition (i.e., platelet storage pool disease, perhaps *5-HT2B* mutations, or perhaps the *L*-allele variant of *5-HTT*) and specific exogenous stimuli (i.e., hypoxia, anorexigens) lead to vessel remodeling via increased serotonin levels, increased 5-HTT activity, and/or increased serotonin receptor signaling. 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-1A 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.

Figure 3. Physiologic/Exogenous Triggers of Pulmonary Arterial Hypertension Are Poorly Understood at the Molecular Level

While most of these clinical risk factors have been known for years, the mechanisms that lead to the imbalance of vascular mediators and dysregulated cellular phenotypes are unclear.

Figure 4. Paradigm for the “Multiple-Hit” Hypothesis Promoting Pulmonary Arterial Hypertension

Susceptible persons with genetic or acquired traits do not progress to PAH without suffering from additional insults that are synergistic in the pathogenesis of disease.

Table 1**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 (?).

Vascular Effector	Change in Activity in PAH	Effect on Vasoconstriction	Effect on Cell Proliferation	Effect on Thrombosis
Serotonin	Increased	Increased	Increased	Increased
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
Kv Channel	Decreased	Increased	Increased	(N/A)
Angiopoietin-1	Increased	(N/A)	Increased	(N/A)
Caveolae	Decreased	(?)	(?)	(?)
5-lipoxygenase (5-LO)	Increased	Increased	Increased	Increased
Vascular Elastase	Increased	Increased	Increased	Increased