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Chapter

Endothelial Dysfunction and Disruption in Pulmonary Hypertension

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Abstract

A number of systemic diseases lead to pulmonary hypertension (PH), a serious disorder with a high morbidity and mortality rate. Irrespective of the underlying disease, endothelial dysfunction or disruption plays a key role in the initiation and progression of PH. Endothelial dysfunction and disruption result in impaired vascular relaxation response, activation of proliferative pathways leading to medial hypertrophy and PH. Endothelial cells (EC) play a crucial role in regulating vascular tone and maintaining homeostasis. Caveolin-1, a 21-22 kD membrane protein, interacts with a number of transducing factors and maintains them in a negative conformation. Disruption of EC results in endothelial caveolin-1 loss and reciprocal activation of proliferative pathways leading to PH, and the accompanying loss of PECAM1 and vascular endothelial cadherin results in barrier dysfunction. These changes lead to the irreversibility of PH. Hypoxia-induced PH is not accompanied by endothelial disruption or caveolin-1 loss but is associated with caveolin-1 dysfunction and the activation of proliferative pathways. Removal of hypoxic exposure results in the reversal of the disease. Thus, EC integrity is an important factor that determines irreversibility vs. reversibility of PH. This chapter will discuss normal EC function and the differences encountered in PH following EC disruption and EC dysfunction.

Keywords: caveolin-1, endothelial cells, membrane integrity, smooth muscle cells, pulmonary hypertension

1. Pulmonary hypertension

A number of systemic diseases such as cardiopulmonary, infectious, inflammatory and autoimmune diseases, hematological disorders, drug toxicity and several genetic mutations lead to pulmonary hypertension (PH), a devastating disease with a high morbidity and mortality rate. Based on clinical diagnosis, PH has been classified into five major groups that were updated in 2013 [1]. Group 1, labeled as pulmonary arterial hypertension (PAH), includes idiopathic and heritable PAH, PAH associated with congenital heart defect (CHD), connective tissue diseases, portal hypertension, HIV, schistosomiasis and drug-/toxin-induced PAH. In addition, mutation of several genes such as *BMPRII* (bone morphogenetic protein receptor type 2), *CAV1* (caveolin-1), *ENG* (endoglin), *SMAD9* (SMAD family member 9), *ACVRL1* (activin A receptor like type 1) and *KCNK3* (potassium two

pore domain channel subfamily K member 3) are among the well-documented causes of PAH. Pulmonary veno-occlusive disease (PVOD)/pulmonary capillary hemangioma and persistent pulmonary hypertension of the newborn (PPHN) are included in Group 1 as subcategories 1′ and 1″, respectively. Recently, mutation of *EIF2AK4* (eukaryotic translation initiation factor 2a kinase 4) has been shown to be associated with PVOD and capillary hemangioma [2]. Included in Group 2 are PH associated with congenital and acquired left heart diseases; Group 3 comprises PH due to lung diseases and/or hypoxia. Group 4 includes chronic thromboembolic pulmonary hypertension (CTEPH). PH associated with hematological disorders, myeloproliferative diseases, splenectomy and a number of miscellaneous systemic and metabolic disorders is included in Group 5. Up until recently, the diagnosis of PAH was considered when the mean pulmonary artery pressure (PAP) of ≥25 mmHg, pulmonary capillary wedge pressure of ≤ 15 mmHg, and a pulmonary vascular resistance (PVR) of > 3 Wood units were observed at rest. During the 6th World Symposium on PH, the mean PAP of threshold was lowered to >20 mmHg and PVR was maintained as >3 Wood units [3]. These changes are based on the evaluation of 47 studies from 13 countries, which showed that independent of age the normal mean PAP rarely exceeded 20 mmHg [4]. The worldwide prevalence of PH is estimated to be 1%, increasing to 10% in patients older than 65 years of age. Globally, left heart diseases (Gr 2) and lung diseases (Gr 3) are considered the most common causes of PH. About 80% of patients are from developing countries; the common causes of PH in these patients are CHD, rheumatic heart disease and infection such as HIV and schistosomiasis. These patients tend to be younger than 65 years [5]. With modern therapy, the survival in patients with idiopathic and heritable PAH and PAH associated with anorexigen drugs has improved to 92%, 75% and 66% at 1, 3 and 5 years, respectively [6]. However, the underlying vascular changes remain progressive [7]. There is still a significant delay between the onset of symptoms and the final diagnosis. A recent retrospective study revealed a delay of 3.9 years between the onset of symptoms and the diagnosis of idiopathic PAH [8]. Thus, by the time the diagnosis is made, patients often have significant pulmonary vascular disease, which is a serious challenge to therapy. Interestingly, in an animal model of PH, significant disruption of endothelial cells and the activation of pro-proliferative pathways have been shown to occur before the onset of PH, indicating that the vascular pathology is already present by the time the symptoms appear [9].

The major causes of PH in children are CHD, PPHN, PH associated with disruption of normal pulmonary vascular and alveolar development in preterm infants, and congenital defects associated with hypoplasia of the lungs [10, 11]. A significant number of pediatric patients (>80%) have transient PH. These include resolution of PPHN and the majority of CHD cases that become free of PAH after the surgical correction of the defect [12]. However, preterm birth itself has an increased risk of developing PH even after adjusting for known factors such as heart and lung diseases, congenital diaphragmatic hernia and chromosomal abnormalities [13]. Furthermore, poor outcome has been reported in children with *BMPRII* mutation associated with idiopathic or heritable PAH [14]. Mutation of *TBX4* (T-box transcription factor 4 gene) is associated with skeletal, cardiac and neurologic defects. It also leads to a form of developmental lung disease that has been shown to be associated with severe PH during infancy and childhood [15]. It is worth noting that infants over the age of 2 years who had CHD exhibited increased pulmonary artery pressure and PVR even after surgical correction of the defect [16]. Pulmonary vascular lesions found in PAH associated with CHD are reported to be similar to what is found in idiopathic PAH [17]. However, the plexiform lesions in idiopathic PAH have monoclonal cell population, whereas Eisenmenger disease (PAH associated

with CHD) displays polyclonal cells [18], indicating a distinct difference between two forms of PAH.

Endothelial cells (EC) play a key role in maintaining vascular homeostasis in response to various stimuli and regulate vascular tone, permeability, coagulation, inflammation through mediators such as nitric oxide (NO), endothelium-derived hyperpolarization factor (EDHF), endothelin-1 (ET1), cell adhesion molecules, cytokines and chemokines. Regardless of the underlying disease, endothelial dysfunction/disruption plays a key role in the pathogenesis of PH. The genetic and environmental factors act as an initial trigger leading to endothelial cell injury and impaired regeneration resulting in vascular remodeling and loss of small pulmonary arteries [19]. Endothelial dysfunction, impaired vascular dilatation, alterations in the expression of NO, ET1 and serotonin, increased expression of inflammatory cytokines and chemokines, loss of endothelial caveolin-1 and disordered proteolysis of extracellular matrix contribute to the pathogenesis of PAH [20, 21]. Increased expression of chemokines such as CX(3)C (fractalkine) and RANTES (CCL5) has been reported in PAH; importantly, both these chemokines are produced in EC [22, 23]. In sugen + hypoxia model (mice), the deletion of CCL5 resulted in reduction in PH via caveolin-1–dependent amplification of BMPR2 signaling. It stabilized surface caveolin-1, restored BMPR2 signaling and enhanced BMPR2 and caveolin-1 interaction [24]. This observation further supports the role for inflammation in PH. In addition, perivascular infiltration with inflammatory cells (T and B cells) is present in plexiform lesions [25, 26]. Increased expression of interleukin-1 (IL-1) and IL-6 occurs in human PAH and monocrotaline (MCT)-induced PH, and inhibition of IL-6 expression and bioactivity as a preventive measure results in the abrogation of MCT-induced PH [27, 28].

In addition to the imbalance of vasoactive mediators and vascular remodeling, abnormality in ion channels (Ca^{2+}, K^{+}) and growth factors such as VEGF, EGF, TGF beta, MMPs, BMPR2 and Notch1 has been implicated in pathophysiology of PAH, leading to vasoconstriction, abnormal remodeling and plexiform lesions [29]. Proliferative EC reveals increased expression of angiogenesis and survival-related molecules such as VEGF, VEGFR2, Hif-1 α, and 1β and reduced expression of p27/kip1. Signal transducer and activator of transcription (STAT3) is essential for cell proliferation and survival, and antiapoptotic function [30]. In the MCT model of PH, the loss of endothelial caveolin-1 was shown to be associated with reciprocal activation of STAT3 (PY-STAT3) and increased proliferating cell nuclear antigen (PCNA) [31]. Furthermore, EC in plexiform and concentric lesions exhibits increased expression of PY-STAT3 [32]. Importantly, the inhibition of STAT3 prevents neointima formation by inhibiting cell proliferation and promoting the apoptosis of neointimal SMC [33]. *BMPRII* mutations linked to PAH are associated with the activation of STAT3. Furthermore, BMPR2 deficiency promotes inflammatory response resulting in increased IL-6 levels and PY-STAT3 activation [34]. BMPR2, a cell surface receptor, is essential for differentiation and proliferation of EC and SMC. Without altering the *BMPRII* mRNA levels, miR-17/92 modulates BMPR2 protein levels. Importantly, IL-6 regulates the expression of miR-17/92 in human pulmonary arterial EC via STAT3 signaling. Persistent activation of STAT3 results in the upregulation of miR-20, which leads to the reduction in the expression of BMPR2 protein [35]. BMPR2 expression is decreased also in patients with heritable and idiopathic PAH, without associated mutation [36]. Importantly, levels of SMAD-specific E3 ubiquitin protein ligase 1 (Smurf1), a key negative regulator of BMPPR2, has been shown to be increased in hypoxia and MCT models of PH in rats [37]. Increased Smurf1 immunoreactivity has also been reported in EC and SMC in the explanted lungs from patients with PAH. Furthermore, Smurf1 deletion protects mice from sugen + hypoxia-induced PH [38]. Interestingly, elafin reverses obliterative changes in pulmonary arteries via elastase inhibition

and caveolin-1–dependent amplification of BMPR2. In addition, elafin promotes angiogenesis via increasing interaction of BMPR2 and caveolin-1 via mediating stabilization of endothelial surface caveolin-1 [39].

Recent studies have shown the involvement of Notch1 signaling in PAH. Increased expression of Notch1 has been reported in the lungs of patients with IPAH and in rats with sugen + hypoxia-induced PH. Notch1 positively regulates EC proliferation by downregulating p21 and negatively regulating apoptosis via Bcl2 and survivin. *In-vitro* studies with human pulmonary arterial EC revealed increased expression of Notch1 during hypoxia exposure, and Notch1 downregulation decreased cell proliferation [40]. Furthermore, Notch1 under hypoxia contributes to increased proliferation, migration and survival in cancer cells [41]. Notch1 is essential for VEGF-induced proliferation, migration and survival of EC [42]. Thus, Notch1 plays a significant role in the pathogenesis of PH. However, Notch1 also plays a key role in vascular morphogenesis, EC quiescence, junction stability and vascular homeostasis. Reduction in Notch1 activity destabilizes cellular junction and triggers EC proliferation and results in the loss of arterial identity and incorporation of these cells into veins. Notch1 is sensitive to shear stress and it requires VEGFA and VEGFR2 for growth [43, 44]. Interestingly, Notch-mediated inhibition of proliferation requires phosphatase-tensin homolog (PTEN), a dual lipid/protein phosphatase. PTEN localization is cell cycle dependent, negatively regulates cell cycle progression and has a restrictive role on angiogenesis [45]. Recent studies have shown significant loss of PTEN concomitant with caveolin-1 dysfunction in hypoxia-induced PH [46]. Fibroblasts from idiopathic pulmonary fibrosis lungs exhibit low membrane PTEN associated with low membrane caveolin-1 levels, and overexpression of caveolin-1 restores membrane PTEN levels. PTEN contains a caveolin-1–binding motif and, in part, colocalizes in caveolae [47]. Thus, caveolin-1 expression determines the membrane PTEN levels through its binding sequence. Furthermore, PTEN has also been shown to negatively regulate STAT3 and its activation, and importantly, membrane localization of PTEN is considered responsible for the inactivation of STAT3 [48].

2. Endothelial cell function

EC forms a monolayer in contact with blood flow and mechanical forces and underlying SMC. It is a non-thrombogenic and a selective barrier to circulating macromolecules. Juxtaposition of EC and SMC facilitates cross talk, and EC maintains SMC in quiescent state. Myoendothelial gap junction plays an important role in Ca^{2+} exchange between EC and SMC. EC is crucial for delivery of O_2 and nutrients to underlying organs. EC maintains a balance between vasodilatation and vasoconstriction, apoptosis and cell proliferation, participate in immune and metabolic function, and maintain anticoagulant state [21, 49]. In addition, EC converts mechanical information into biological responses through mechanotransduction processes. EC adapts to mechanical inputs while maintaining crucial vascular barrier function. Failure of EC to adapt to changes has effects on vascular permeability, an important cause of vascular diseases [50].

2.1 Caveolae, caveolin-1 and cavin-1

Caveolae, a subset of specialized lipid rafts (50–100 nm), first described in 1950s by Palade [51] and Yamada [52], is found on plasma membranes of a variety of cell types including EC, SMC, fibroblasts and adipocytes. Caveolae are non-clathrin–coated plasma membrane vesicles (50–100 nm) enriched in glycosphingolipids,

cholesterol, sphingomyelin and lipid-anchored membrane proteins. They form an important signaling platform that compartmentalizes and integrates a number of signaling molecules and allows cross talk between different signaling pathways, and mediates and integrates signaling events at the cell surface. EC contains 5000–10,000 caveolae per cell [53]. In addition, caveolae act as plasma membrane sensors and respond to stress. Caveolae flatten in response to membrane stretch. The flattening is a protective mechanism; it buffers the membrane and prevents its rupture [54, 55]. Caveolin-1 is a major protein (21–22 kDa) constituent of caveolae that maintains the shape of caveolae; EC has the highest levels of caveolin-1 [56]. Caveolin-1 is involved in multiple cellular processes such as molecular transport, cell proliferation, adhesion, migration and signal transduction. Caveolin-1 has an integral role in endocytosis. However, overexpression of caveolin-1 inhibits endocytosis [57, 58]. Caveolin-1 is synthesized in endoplasmic reticulum and then transported to Golgi complex. During its biosynthesis, it is associated with lipid rafts and become detergent resistant. From a structural standpoint, caveolin-1 contains a hairpin loop structure and three palmitoylation sites and a scaffolding domain that facilitates interaction with the plasma membrane [59, 60]. Caveolin-1 functions through protein-protein interaction and regulates and stabilizes several proteins including Src family of kinases, G proteins (α-subunits), G protein-coupled receptors, H-Ras, PKC, endothelial NO synthase (eNOS), integrins, and growth factor receptors such as VEGFR2, EGFR and PDGFR in an inhibitory conformation. Importantly, a 20-amino acid membrane proximal region of the cytosolic amino-terminal domain, termed caveolin-scaffolding domain (residue 82–101), is sufficient to mediate these interactions [61, 62]. Caveolin-1 also functions as a suppressor of cytokine signaling (SOCS), the family of proteins that are upregulated by cytokines and that in turn inhibit cytokine signaling via modulating JAK-STAT pathway [63]. Caveolin-2 is present associated with caveolin-1 in all cell types. It requires caveolin-1 for its transport from Golgi body to the plasma membrane. Caveolin-2 is not necessary for caveolae formation or caveolar localization of caveolin-1, but the coexpression results in a more efficient formation of caveolae [64]. In the absence of caveolin-1, caveolin-2 is degraded, and the decreased expression of caveolin-2 promotes increased cell proliferation [65, 66]. Furthermore, caveolin-2 knockout mice display increased proliferation of endothelial cells, hyper-cellular lung parenchyma and cell cycle progression [67].

In addition to caveolin-1, caveolae require polymerase 1 and transcript release factor (PTRF) also known as cavin-1. It is an essential component of caveolae; it regulates membrane curvature by stabilizing caveolin-1 in caveolae. The loss of cavin-1 results in the loss of caveolae and the release of caveolin-1 into the plasma membrane. Importantly, caveolin-1 is required for cavin-1 recruitment to plasma membrane [68, 69]. Loss of caveolin-1 is accompanied by a marked loss of caveolin-2 and partial reduction in cavin-1 expression in the lungs. The re-expression of caveolin-1 rescues both caveolin-2 and cavin-1 [70]. In a carotid artery-injury model, the local loss of cavin-1 is reported to promote neointima formation. Furthermore, in cultured vascular SMC, the overexpression of cavin-1 suppresses SMC proliferation and migration, whereas its inhibition promotes cell proliferation and migration [71]. Cavin-1 knockout mice display lung pathological changes such as remodeled pulmonary vessels, PH and right ventricular hypertrophy. In addition, these mice have altered metabolic phenotype with insulin resistance [72, 73].

Recent studies have shown other accessory proteins required in caveolae biogenesis. The accessory protein pacsin2 also known as syndapin2 contains F-BAR domain associated with generation and maintenance of caveolae. It partially colocalizes with caveolin-1 at plasma membrane level. Loss of pacsin2 function results in the loss of caveolae and accumulation of caveolin-1 within the plasma membrane. Interestingly, overexpression of F-BAR domain can cause loss of caveolae. Another

protein EH 15 homology domain 2 (EHD2) is present in caveolae, and it binds to pacsin2 that partially colocalizes with caveolin-1. It is a dynamin-related ATPase that confines caveolae to cell surface. Furthermore, regulation of EHD2 oligomerization in a membrane-bound state is crucial in order to restrict caveolar dynamics in cells [74, 75]. Importantly, caveolar coat controls a large number of signaling circuits; a defect in any of these pathways can lead to several systemic diseases such as vascular dysfunction, cardiomyopathy, cancer, muscular dystrophy and lipodystrophy [76].

The role of caveolin-1 is well established in the pathogenesis of PH. Caveolin-1 knockout mice are viable but have dysregulated NO synthesis, impaired NO and Ca^{2+} signaling, cell proliferation, increased vascular permeability accompanied by cardiomyopathy and PH. Reconstituting endothelial caveolin-1 has been shown to recover dysregulated NO synthesis, cardiomyopathy and PH [77, 78]. In addition, caveolin-1 knockout mice exhibit low-grade systemic pro-inflammatory status and moderately increased IL-6 and TNFα levels [79]. EC-specific *CAV1* knockout mice and LPS-treated wild-type mice exhibit reduced BMPR2 expression and eNOS uncoupling, accompanied by increased TGF-β–promoted TGFβRI-dependent SMAD-2/3 phosphorylation. In addition, human lung sections from patients with ARDS reveal reduced endothelial caveolin-1 expression, increased TGF-β levels and severe pulmonary vascular remodeling. These results further support the view that the loss of endothelial caveolin-1 promotes pulmonary vascular remodeling in inflamed lungs via oxidative stress-mediated reduction in BMPR2 expression [80]. Furthermore, endothelial dysfunction during inflammation leads to endothelium-mesenchymal transition (End MT). These cells lose endothelial characteristics and acquire mesenchymal phenotypes and express mesenchymal specific markers such as smooth muscle α-actin, fibroblast-specific protein 1 and Notch1 [81]. In addition, caveolin-1 is a determinant of oxidative stress and is a regulator of metabolic switch and autophagy [82].

2.2 Vascular relaxation

NO, EDHF and prostacyclin (PGI2) induce endothelium-dependent vascular relaxation. NO is produced by eNOS via its action on L-arginine and oxygen. NO activates guanylate cyclase, which catalyzes the conversion of guanosine triphosphate to cyclic guanylate monophosphate. eNOS expressed in endothelial cells and cardiac myocytes is targeted to caveolae. It directly binds to caveolin-1 scaffolding domain and is held in an inhibitory state. This interaction prevents eNOS activation leading to inappropriate NO production under basal conditions. The eNOS/caveolin-1 regulatory cycle is a reversible protein-protein interaction controlled by Ca^{2+}/calmodulin and by enzyme palmitoylation. Increase in intracellular Ca^{2+} with calmodulin disrupts the caveolin-1/eNOS complex resulting in eNOS activation and NO production leading to vascular relaxation. Calmodulin is a direct allosteric competitor promoting the caveolin-1 and eNOS dissociation. Heat shock protein (HSP) 90 binds to eNOS in Ca^{2+}/calmodulin-dependent manner and it reduces the inhibitory effects of caveolin scaffolding domain on eNOS, thus promoting eNOS activation [83–85]. Furthermore, increase in vascular flow and pressure rapidly activates caveolar eNOS with its dissociation from caveolin-1 and association with calmodulin [86]. Thus, caveolin-1 and eNOS have a dynamic relationship. Importantly, caveolin-1 contained within non-caveolar lipid rafts fails to exert its inhibitory effect on eNOS [87]. The loss of endothelial caveolin-1 leads to eNOS uncoupling, oxidative stress and endothelial injury [88]. Interestingly, under conditions of stress, caveolin-1 increases eNOS trafficking in plasma membrane and primes eNOS for flow-mediated activation. Caveolin-1 plays a positive role in shear-induced

eNOS activation by targeting eNOS to plasma membrane. Importantly, the coupling of flow stimulus to activate eNOS is lost in the absence of caveolin-1 and caveolae. Thus, caveolin-1 exerts dual role of post-translational regulation of eNOS activity [89]. In addition, caveolin-1 plays a critical role in VEGFR2 stimulation and downstream mediators of angiogenesis, but higher levels of caveolin-1 repress this function. [90]. Interestingly, EC migration, tube formation and angiogenesis are impaired both in caveolin-1 and eNOS knockout mice but are fully restored by double knockout [91].

The transient receptor potential (TRP) channels are the link between caveolae and EDHF. TRP channels facilitating the capacitive Ca^{2+} entry directly interact with caveolin-1 in EC. Ca^{2+}-activated K^{+} channels play a key role in endothelium-dependent hyperpolarization and vascular tone regulation. Absence of caveolin-1 impairs Ca^{2+} homeostasis in EC and decreases the activity of TPRV4 cation channels that participate in NO and EDHF activation. Caveolin-1 is required for EDHF-related relaxation, modulating TRPV4 and connexins. Caveolin-1 knockout arteries exhibit fewer gap junctions and altered myoendothelial communication. Furthermore, caveolin-1 deficiency is associated with impaired EDHF-mediated vascular relaxation in response to shear stress and acetylcholine [92–94]. Colocalization of PGI2 synthase and caveolin-1 regulates angiogenesis [95]. Thus, caveolin-1 interacts with relaxing factors to maintain homeostasis.

2.3 Barrier function

Endothelial barrier controls the passage of fluids, nutrients and cells through vascular wall. Glycocalyx coats the luminal surface of EC and forms an important barrier. It modulates permeability, prevents leukocyte and platelet adhesion to EC, serves as an anti-inflammatory, anti-adhesive and anti-coagulant barrier, and allows selective permeability. In addition, it mediates mechanotransduction of shear stress. Under normal conditions, the apoptosis rate in EC is very low, but the activated EC exhibits a reduction in the EC surface layer, the glycocalyx, and an increased rate of apoptosis [96, 97]. Disturbed flow has been shown to inhibit glycocalyx expression as well as to reduce caveolin-1 expression in systemic arterial EC [98]. Inflammatory mediators lead to the disruption of glycocalyx resulting in the weakening of vascular protection. Integrity of vascular glycocalyx is inversely related to the degree of inflammation. Inflammatory mediators lead to the loss of glycocalyx resulting in the weakening of vascular protection. Furthermore, destruction of glycocalyx has been reported in the MCT model of PH [99].

Ca^{2+}-dependent vascular endothelial cadherin (VE-Cad) and its associated catenins control cell-cell adhesion and paracellular barrier function and are important for the tight junction complexes. VE-Cad is tissue specific for EC and is expressed at the intercellular clefts and mediates cell-cell adhesion, maintains barrier function, and contributes to the inhibition of cell growth. Association of caveolin-1 and VE-Cad catenin complex is essential for barrier function [100–102]. Depletion of caveolin-1 reduces VE-Cad levels and facilitates endothelial cell permeability [103]. Furthermore, VE-Cad interacts with various growth receptors, regulates endothelial proliferative signaling and mediates contact inhibition of cell growth [104, 105]. In adult EC, VE-Cad and VEGFR2 are physically linked. This maintains VEGFR2 stable and prevents its endocytosis. VEGF-induced permeability is facilitated by decoupling of VE-Cad and VEGFR2 [106]. Loss of VE-Cad and PECAM1 has been shown to occur in the MCT-induced PH [31, 107]. PECAM-1 supports EC integrity and maintains barrier function [108]. Importantly, BMPR2 also plays a role in maintaining vascular integrity by dampening inflammatory signals in pulmonary vasculature [109].

3. Endothelial disruption/dysfunction

Vascular injury from different conditions such as inflammation, hypoxia, increased flow and pressure, and shear stress leads to endothelial dysfunction. Injury can lead to disruption of EC and endothelial caveolin-1 loss or endothelial dysfunction without EC disruption. Both lead to the activation of proliferative pathways, vascular remodeling and PH [96, 110]. Recent studies have shown the loss of myocyte enhancer factor 2 (MEF2) in dysfunctional EC from PAH patients. MEF2 regulates a number of transcription factors involved in pulmonary vascular homeostasis [111]. Furthermore, these dysfunctional ECs exhibit increased production of leptin, and SMCs overexpress leptin receptor contributing to SMC proliferation [112]. In addition, pulmonary arterial ECs from PAH patients have been shown to produce increased FGF2 leading to increased proliferation and survival response by constitutive activation of ERK1/2 and decreased apoptosis associated with the activation of Bcl2 and Bcl-xL. It is thought that FGF2 in PAH may contribute to abnormal EC phenotype [113]. Furthermore, there is evidence that pro-inflammatory cytokine macrophage migration inhibitory factor and its receptor CD74 are markedly increased in idiopathic PAH, which may contribute to pro-inflammatory phenotype of EC [114].

3.1 EC disruption and pulmonary hypertension

Endothelial disruption accompanied by the loss of endothelial caveolin-1 has been reported in several forms of experimental models of PH such as MCT, myocardial infarction and sugen + hypoxia [31, 115, 116]. In the MCT model, progressive loss of endothelial caveolin-1 and reciprocal activation of proliferative and anti-apoptotic pathways such as PY-STAT3 and Bcl-xL occur before the onset of PH. Loss of other membrane proteins such as PECAM-1, Tie2 and soluble guanylate cyclase (α and β) occurs in tandem with caveolin-1 loss indicating extensive disruption of endothelial cell membrane. At 2 weeks, a further loss of endothelial caveolin-1 is accompanied by the loss of cytosolic proteins such as HSP90 and IκB-α and increased pulmonary artery pressure. However, at this stage, eNOS expression is relatively well preserved. In the presence of significant loss of endothelial caveolin-1 and HSP90, eNOS gets uncoupled resulting in an increased production of reactive oxygen species (ROS). By 3 and 4 weeks, there is a significant reduction in eNOS levels, leading to normalization of ROS levels [9, 31, 117]. At 4 weeks post-MCT, extensive endothelial caveolin-1 loss is accompanied by the loss of von Willebrand factor (vWF) in 29% of the arteries; and 23% of arteries exhibit enhanced expression of caveolin-1 in SMC. Enhanced expression of caveolin-1 in SMC occurs only in the arteries with extensive endothelial caveolin-1 and vWF loss. At this stage, the expression of total caveolin-1 in the lungs remains low. In addition, there is a progressive increase in MMP2 expression and activation [117]. The rescue of endothelial caveolin-1 as a preventive measure abrogates MCT-induced PH, but once the PH is established, the treatment does not alter the progression of the disease [118–120]. Exposing MCT-treated rats to hypoxia accelerates the disease process, and by 4 weeks, extensive endothelial disruption and endothelial caveolin-1 loss are accompanied by enhanced expression of caveolin-1 in SMC in 61% of the arteries, near normalization of lung caveolin-1 expression, and neointima formation. Importantly, neointimal cells exhibit low to no caveolin-1 expression [121, 122]. Extensive loss of endothelial caveolin-1, enhanced expression of caveolin-1 in SMC and neointima formation are also observed in idiopathic and hereditary PAH, PAH associated with CHD and drug toxicity [122–125]. In *in-vitro* studies, pulmonary arterial SMCs from idiopathic PAH exhibit increased caveolin-1 expression accompanied by increased capacitive Ca^{2+} entry and DNA synthesis, which could be abrogated by silencing caveolin-1 [125]. Loss of EC exposes SMC to direct pressure and shear stress,

which is likely to result in flattening of caveolae leading to displacement of caveolin-1 to non-caveolar site on the plasma membrane. Recently, it has been shown that in the MCT + hypoxia model, at 4 weeks, the extensive loss of endothelial caveolin-1 as well as VE-Cad loss and enhanced expression of caveolin-1 in SMC are accompanied by cavin-1 loss, tyrosine phosphorylation of caveolin-1 and neointima formation. Loss of VE-Cad is indicative of loss of EC attachment to the junction [126]. Interestingly, p-caveolin-1 in cancer has been shown to make cells pro-migratory [127, 128]. As PH progresses, SMC phenotype changes from contractile to synthetic, facilitating cell migration, and neointima formation resulting in arterial occlusion. Neointima formation leads to the irreversibility of the disease [129]. In addition, increasing pulmonary blood flow either by pneumonectomy or by a shunt procedure (left subclavian and pulmonary artery) in rats treated with MCT leads to the development of neointimal lesions. Pneumonectomy or shunt alone does not lead to neointima formation [130, 131]. Furthermore, in children with significant left to right cardiac shunt, reversal of pulmonary vascular changes were seen after they underwent pulmonary artery banding to restrict the pulmonary flow. Medial hypertrophy and early intimal changes seem reversible, but not during the later stages [132, 133]. These studies demonstrate that EC injury and disruption associated with increased flow or pressure play an important role in determining the pattern of pulmonary vascular remodeling.

Apoptosis of EC in PAH is followed by proliferation of antiapoptotic EC. This concept has been confirmed in *in-vitro* studies. Sugen (VEGFR antagonist) causes initial apoptosis, and the surviving cells become hyperproliferative [134]. Importantly, increased levels of circulating EC (CEC) have been reported in PAH, and 50% of these cells expressed CD36, a marker of microvascular origin, and 25% exhibited E selectin, a marker of EC activation [135]. In children with CHD and PAH, the increased levels of CEC are reported to be associated with worse prognosis. Pulmonary ECs exhibited high expression of antiapoptotic protein Bcl-2 in cases of irreversible PAH but not in cases of reversible PAH, or in controls. Interestingly, intimal proliferation was observed only in irreversible PAH cases, but not in the reversible PAH [136, 137]. In addition, increased vWF levels in patients with PAH were reported to be associated with worse survival [138]. Interestingly, increased CEC levels were observed in PAH, but not in CTEPH [139]. These studies strongly support the view that the disruption and loss of EC are associated with severe PAH and poor prognosis.

Endothelial mesenchymal transition (EndMT) is a process by which ECs exhibit phenotype alteration. These cells lose endothelial characteristics and acquire the properties of myofibroblasts or mesenchymal cells. They exhibit loss of PECAM-1 and VE-Cad, in addition to caveolin-1, and express smooth muscle α-actin, fibroblast-specific protein 1 and Notch1. PECAM-1 and VE-Cad support EC integrity and junctional stability and maintain barrier function. Thus, their loss leads to the loss of barrier function and junction stability. These transformed ECs also acquire pro-inflammatory phenotype and are primed for proliferation, migration and tissue generation [81, 140]. Neointimal cells exhibit low levels of caveolin-1, but normal eNOS expression in the experimental model of PH and also in human PAH [96, 122], and sustained NO production has been shown to degrade caveolin-1 [141]. Importantly, caveolin-1 deficiency has been shown to induce spontaneous EndMT in pulmonary EC [142]. EndMT plays an important role in vascular remodeling, and it is also linked to the loss of BMPR2 in PH [143–145]. Furthermore, TGFβ1 plays a significant role in EndMT [146]. In addition, endothelial caveolin-1 depletion leads to eNOS uncoupling and oxidative stress that switches from BMPR2 signaling to TGFβR1 and thus may promote EndMT [80]. Plexogenic lesions contain increased VEGFA and VEGFR expression indicating misguided angiogenesis involving cells of EC origin. EC dysfunction in PAH model is shown to act through DNA methylation, histone protein modification and non-coding RNA [19]. Thus,

the initial apoptosis followed by the proliferation of dysfunctional and antiapoptotic EC leads to deregulation of a number of pathways resulting in neointima and plexiform lesion formation and irreversible PAH.

3.2 EC dysfunction without EC disruption and pulmonary hypertension

Exposure to acute hypoxia results in pulmonary arterial contraction and elevated pulmonary artery pressure, while sustained hypoxia leads to pulmonary vascular remodeling [147]. Hypoxia impairs endothelium-dependent relaxation response [148, 149]. In the MCT model of PH, the progressive loss of endothelial caveolin-1 is accompanied by a significant reduction in the expression of HSP90 (2 weeks post-MCT) and eNOS (3 weeks post-MCT) [9]. However, hypoxia does not alter the protein expression of caveolin-1, eNOS or HSP90 in the lungs. During hypoxia, caveolin-1 and eNOS have been shown to form a tight complex *in vivo* and *in vitro*, resulting in their dysfunction [110, 150]. Normally, in response to Ca^{2+} agonists, eNOS dissociates from caveolin-1 and binds to HSP90. Ca^{2+} activated calmodulin further aids in recruitment of HSP90, thus facilitating the release of eNOS from caveolin-1 [151]. However, hypoxia disrupts eNOS/HSP90 binding [152]. Furthermore, normally functioning caveolin-1 is required for the plasma membrane localization of TRPC4 and endothelial Ca^{2+} entry [153], and introduction of caveolin-1 scaffolding domain restores Ca^{2+} entry during chronic hypoxia [154]. Thus, the hypoxia-induced caveolin-1 and eNOS complex formation may in part be responsible for the deregulation of Ca^{2+} entry and disruption of HSP90/eNOS binding leading to impaired vascular relaxation. Statins have been shown to disrupt hypoxia-induced abnormal coupling of eNOS and caveolin-1, thus restoring eNOS function and attenuating hypoxia-induced PH [155]. Recent studies of hypoxia-induced PH in rats and cows showed no disruption of EC or any alterations in the expression of caveolin-1, VE-Cad or vWF [46, 126]. Not surprisingly, there was no enhanced expression of caveolin-1 in SMC as seen in the MCT model. However, there was evidence of caveolin-1 dysfunction, such as the activation of proliferative pathways such as PY-STAT3, β-catenin and pERK1/2, and a loss of PTEN. PTEN contains a Cav-1–binding motif and, in part, colocalizes in caveolae. Caveolin-1 determines the membrane PTEN levels through its binding sequence. The loss of PTEN during hypoxia further confirms caveolin-1 dysfunction [46].

People living at high altitude develop PH and right ventricular hypertrophy as an adaptive mechanism. Upon return to sea level, PH reverts to normal slowly [156]. These observations suggest that the absence of physical disruption of EC observed in the hypoxia model may be the reason why hypoxia-induced PH is reversible. Although hypoxia plays a role, inflammation and endothelial dysfunction are important factors that determine the development of PH in chronic obstructive pulmonary disease (COPD). The outflow obstruction in COPD results from inflammatory processes affecting airways, lung parenchyma and pulmonary vasculature. PH in COPD can develop independently of underlying parenchymal destruction and loss of lung vessels [157, 158]. Endothelial dysfunction has been observed in mild cases of COPD, and the loss of endothelium-dependent relaxation in the pulmonary vasculature correlates with the severity of the disease [159]. Importantly, the loss of endothelial caveolin-1 accompanied by enhanced expression of caveolin-1 in SMC is reported in COPD associated with PH. COPD without PH had preserved endothelial caveolin-1 [160]. In addition, severe pulmonary arterial lesions such as plexiform and angiomatoid lesions have been documented in explanted lungs after transplantation in COPD associated with severe PH. These lesions were similar to what are seen in IPAH [161]. In infants with respiratory distress syndrome, despite significantly elevated pulmonary artery pressure and significant medial thickening, pulmonary arteries

exhibit well-preserved endothelial caveolin-1, without any evidence of EC disruption or enhanced expression of Cav-1 in SMC. In contrast, loss of endothelial Cav-1 and disruption/loss of EC coupled with enhanced expression of Cav-1 in SMC were observed in infants with bronchopulmonary dysplasia and associated inflammation [123]. These results indicate that irrespective of the underlying disease, EC disruption leads to the loss of endothelial caveolin-1 and subsequent enhanced expression of Cav-1 in SMC, followed by neointima formation and irreversible PH. Thus, the EC disruption puts the patients at a higher risk of developing irreversible PH.

4. Conclusions

Under normal conditions, ECs play a key role in maintaining SMCs in quiescent state and vascular homeostasis. Caveolin-1, a major protein constituent of caveolae on the cell membrane, regulates multiple cellular processes including inflammation, molecular transport, cell proliferation, adhesion, migration and signal transduction. Caveolin-1 interacts with protein molecules that are in or are recruited to caveolae and maintains them in inhibitory confirmation. Endothelial caveolin-1 loss and caveolin-1 dysfunction lead to PH.

4.1 EC disruption and caveolin-1 loss

Injury such as inflammation, increased pulmonary blood flow associated with increased pressure, drugs and toxins can cause endothelial disruption, which is usually progressive. Endothelial disruption leads to the progressive loss of endothelial membrane proteins including caveolin-1, PECAM-1 and VE-Cad. These alterations lead to deregulation of multiple pathways. As depicted in **Figure 1**, (a) the loss of caveolin-1 is accompanied by reciprocal activation of proliferative and antiapoptotic pathways leading to SMC hypertrophy and proliferation. (b) Further loss of EC exposes SMCs to direct pressure resulting in enhanced expression of caveolin-1 in SMCs. Tyrosine phosphorylated caveolin-1 could alter the phenotype and facilitate cell migration leading to neointima formation. (c) Loss of PECAM-1 and VE-Cad results in the loss of barrier function and junction stability. These alterations lead to EndMT. These cells lose endothelial properties and acquire pro-inflammatory phenotype and are primed for proliferation, migration and tissue generation and participate in neointima formation, thus leading to irreversible PH.

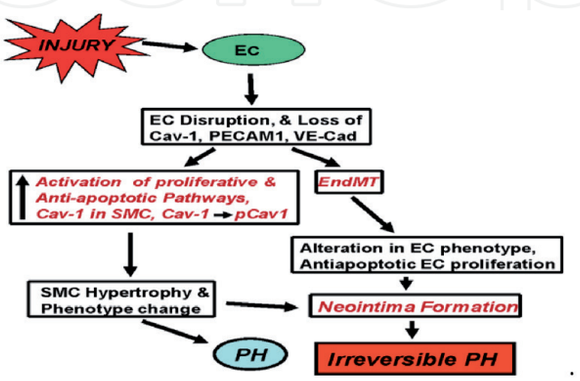

Figure 1.
This figure depicts the pathway leading from endothelial cell disruption to irreversible PH. Cav-1 = caveolin-1, EC = endothelial cells, EndMT = endothelial mesenchymal transformation, PECAM-1 = platelet endothelial cell adhesion molecule 1, pCav-1 = tyrosine phosphorylated caveolin-1, PH = pulmonary hypertension, SMC = smooth muscle cells.

4.2 EC and caveolin-1 dysfunction

Hypoxia exposure to EC leads to a tight complex formation between caveolin-1 and eNOS, resulting in the dysfunction of both factors (**Figure 2**). Importantly, there is no EC disruption or the loss of caveolin-1 or any other membrane proteins. Since there is no loss of EC, medial layer is not exposed to shear stress and pressure. Not surprisingly, there is no enhanced expression of caveolin-1 in SMCs. However, caveolin-1 and eNOS dysfunction lead to SMC proliferation, medial hypertrophy and loss of endothelial-dependent vascular relaxation. Removal of hypoxia results in the disruption of caveolin-1/eNOS tight complex leading to reversal of PH. Slowly, the pulmonary artery pressure and medial hypertrophy return to normal as seen in experimental animals and in people returning to sea level from high altitude. Hypoxia-induced PH is reversible. However, associated inflammation/shear stress in hypoxia-induced PH, resulting in EC disruption, would lead to irreversible PH.

In conclusion, EC integrity and caveolin-1 function are important factors that determine reversible vs. irreversible PH.

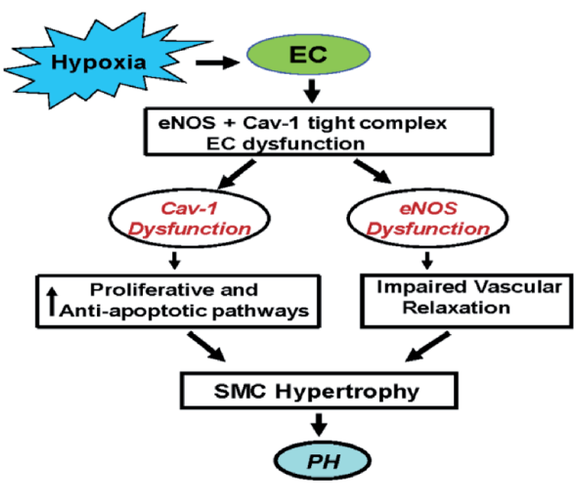

Figure 2.
This figure shows the effect of hypoxia on EC leading to eNOS/caveolin-1 complex formation, endothelial dysfunction and subsequent medial hypertrophy and PH. EC = endothelial cells, Cav-1 = caveolin-1, eNOS = endothelial nitric oxide synthase, SMC = smooth muscle cells, PH = pulmonary hypertension.

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