

# Pulmonary Hypertension: Pathophysiology and Signaling Pathways

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**Abstract** Pulmonary hypertension (PH) is characterized by pathological changes to cell signaling pathways within the alveolar-pulmonary arteriole–right ventricular axis that results in increases in pulmonary vascular resistance and, ultimately, the development of right ventricular (RV) dysfunction. Cornerstone histopathological features of the PH vasculopathy include intimal thickening, concentric hypertrophy, and perivascular fibrosis of distal pulmonary arterioles. The presence of plexogenic lesions is pathognomonic of pulmonary arterial hypertension (PAH); when present, this severe form of remodeling is associated with subtotal obliteration of the blood vessel lumen. The extent of RV remodeling in PH correlates with clinical symptom severity and portends a poor outcome. Currently available PH-specific pharmacotherapies that aim to improve symptom burden by targeting pulmonary vasodilatory/vasoconstrictor cell signaling pathways do not fully reverse pulmonary vascular remodeling and, thus, are largely unsuccessful at maintaining normal cardiopulmonary hemodynamics long term. Thus, determining the molecular mechanisms that are responsible for pulmonary vascular remodeling in PH is of great potential therapeutic value, particularly pathways that promote apoptosis-resistant cellular proliferation, disrupt normal cellular bioenergetics to alter cell function, and/or modulate severely abnormal responses to pulmonary vascular injury. This chapter reviews current insights into PH pathophysiology and disease mechanisms, and discusses novel cell signaling pathways that implicate microRNAs and mitochondrial dysfunction in the development of the PH phenotype.

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## Abbreviations

|                 |                                        |
|-----------------|----------------------------------------|
| BH <sub>4</sub> | Tetrahydrobiopterin                    |
| BMP-RII         | Bone morphogenetic protein receptor II |
| cAMP            | Cyclic adenosine monophosphate         |
| cGMP            | Cyclic guanosine monophosphate         |
| COX             | Cyclooxygenase                         |
| EGFR            | Epidermal growth factor receptor       |
| eNOS            | Endothelial nitric oxide synthase      |
| ET-1            | Endothelin-1                           |
| ET <sub>A</sub> | Endothelin-type A receptor             |
| ET <sub>B</sub> | Endothelin-type B receptor             |
| FeNO            | Iron-nitrosyl                          |
| GTP             | Guanosine triphosphate                 |
| HAPE            | High altitude pulmonary edema syndrome |
| HHT             | Hereditary hemorrhagic telangiectasia  |
| HIF             | Hypoxia-inducible factor               |
| HIV             | Human immunodeficiency virus           |
| IL              | Interleukin                            |
| ISCU1/2         | Iron-sulfur cluster assembly proteins  |
| KL              | Kruppel-like factor                    |
| LO              | Lipoxygenases                          |
| LV              | Left ventricle                         |
| MAPK            | Mitogen-activated protein kinase       |
| miR             | MicroRNA                               |
| NO <sup>•</sup> | Nitric oxide                           |
| NOX             | NADPH oxidase                          |

|                          |                                            |
|--------------------------|--------------------------------------------|
| $\cdot\text{O}_2^-$      | Superoxide                                 |
| $\text{O}_2\text{NOO}^-$ | Peroxynitrate                              |
| $\text{ONOO}^-$          | Peroxynitrite                              |
| PAEC                     | Pulmonary artery endothelial cells         |
| PAH                      | Pulmonary arterial hypertension            |
| PDE                      | Phosphodiesterase inhibitor                |
| PDGF                     | Platelet-derived growth factor             |
| PDK                      | Pyruvate dehydrogenase kinase              |
| PG                       | Prostaglandin                              |
| PH                       | Pulmonary hypertension                     |
| PKG                      | Protein kinase G                           |
| PPAR- $\gamma$           | Peroxisome proliferator-activated receptor |
| PSMC                     | Pulmonary artery smooth muscle cells       |
| PTPC                     | Permeability transition pore complex       |
| ROS                      | Reactive oxygen species                    |
| RV                       | Right ventricle                            |
| sGC                      | Soluble guanylyl cyclase                   |
| SOD                      | Superoxide dismutase                       |
| TAPSE                    | Tricuspid annular plane systolic excursion |
| TGF                      | Transforming growth factor                 |
| $\text{TXA}_2$           | Thromboxane                                |
| VEGF                     | Vascular endothelial growth factor         |

## 1 Introduction

Maladaptive changes to the phenotype of pulmonary arterioles resulting in pulmonary vascular dysfunction, right ventricular (RV) pressure loading, and, ultimately, right heart failure are a central pathophysiological mechanism leading to the development of clinically evident pulmonary hypertension (PH). The “two-hit” hypothesis of PH proposes that in the presence of a predisposing genetic and/or molecular substrate, exposure to certain environmental or biological mediators of vascular injury initiates a cascade of adverse cell signaling events culminating in gross structural malformation and functional deterioration to pulmonary arterioles. Although no single inciting event is known to trigger universally the development of PH, pulmonary endothelial dysfunction and decreased levels of bioavailable nitric oxide ( $\text{NO}^*$ ) are observed in early stages of many PH disease forms. Importantly, the pulmonary vascular bed hosts the greatest density of vascular tissue within the human circulatory system (Barst and Rubin 2011); thus, even subtle perturbations to signaling pathways that regulate structure and function of cells within the alveolar-pulmonary circulation interface may translate into meaningful changes to cardiopulmonary performance.

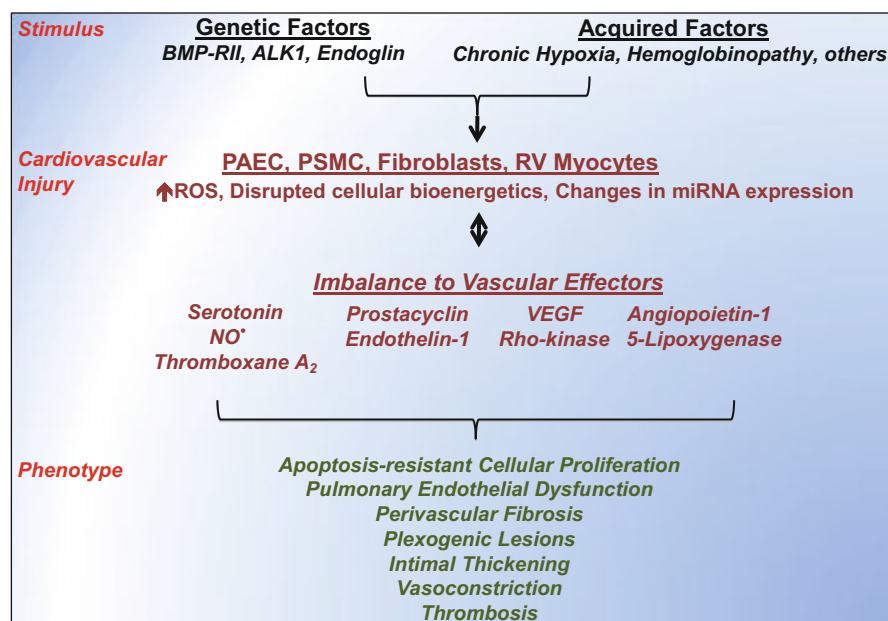
The cornerstone histopathological feature of PH is adverse remodeling of distal pulmonary arterioles that is characterized by intimal thickening (Farber and

Loscalzo 2004), dysregulated proliferation of apoptosis-resistant pulmonary artery endothelial cells (PAECs) and pulmonary vascular smooth muscle cells (PSMCs) (Abe et al. 2010), increased perivascular fibrosis, and, in certain forms of PH, the genesis of plexogenic lesions (Archer et al. 2010). Subtotal luminal obliteration of small- and medium-sized pulmonary arterioles, abnormal pulmonary vascular reactivity, and increased pulmonary blood vessel tone contribute to elevations in pulmonary vascular resistance and uncoupling of RV-pulmonary circulatory function (Rondelet et al. 2010). Enhanced understanding of cross talk between signaling pathways in PAECs, PSMCs, lung fibroblasts, and RV myocytes that occurs in response to injury has led to the development of PH-specific pharmacotherapies. These treatments aim to improve pulmonary vascular tone by restoring nitric oxide (NO)- or prostacyclin-mediated signaling pathways, or through inhibition of endothelin-1 (ET-1)-dependent and -independent activation of vascular calcium channels that promotes vascular mitogenesis and vasoconstriction (Schneider et al. 2007; McLaughlin et al. 2009). Despite this progress, however, clinical outcome in PH remains poor, particularly among patients afflicted with pulmonary arterial hypertension (PAH), in which mortality rates approach 10 % within 1 year of diagnosis (Benza et al. 2010). This observation has stimulated novel dimensions of investigation that emphasize abnormalities in mitochondrial function, cellular metabolism, and microRNA (miR)-dependent responses to hypoxia as potentially under-recognized mechanisms involved in the pathogenesis of PH.

## 2 PH Pathophysiology

In PH, pulmonary circulatory performance is impaired as a consequence of adverse changes to the compliance of medium- and small-sized pulmonary arterioles that occur in response to chronic pulmonary vascular injury. In the majority of patients, these changes occur owing to hypoxic pulmonary vasoconstriction; vascular congestion in the setting of left atrial hypertension (i.e., impaired left ventricular [LV] function, mitral valve disease); or impedance to pulmonary blood flow as a consequence of primary lung, cardiac, pulmonary, or vascular thromboembolic disease (Maron and Loscalzo 2013). In PAH, the interplay between specific molecular and genetic factors induces the effacement of pulmonary arterioles and disrupts homeostatic mechanisms that control normal blood vessel tone and platelet function. This results in the classic PAH phenotypic triad of microvascular thrombosis, increased pulmonary vascular reactivity, and plexiform lesions (Fig. 1).

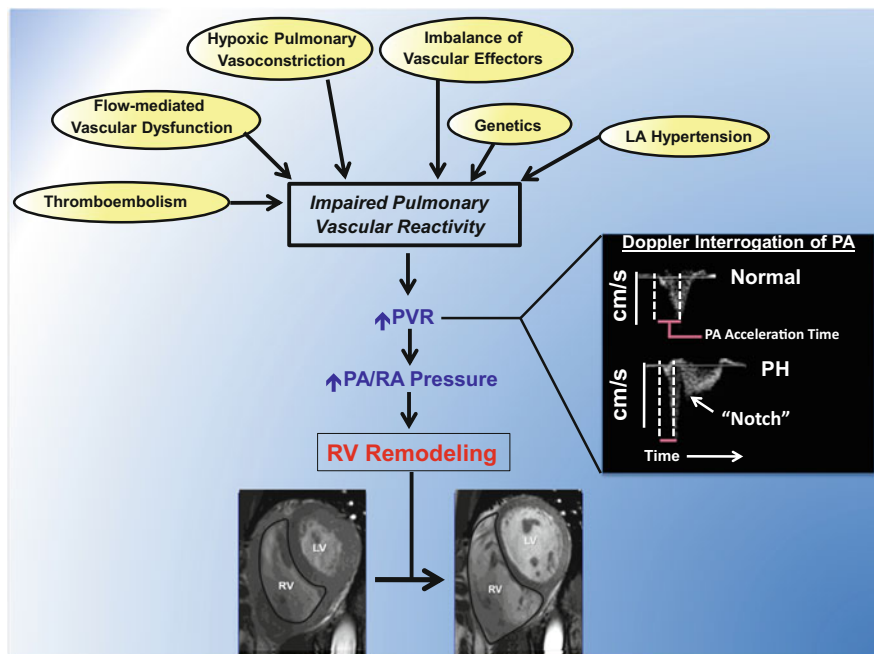
The contemporary definition of PH stipulates that the following hemodynamic criteria be met: a sustained elevation in mean pulmonary artery pressure ( $>25$  mmHg) and pulmonary vascular resistance ( $>3$  Wood units) in the setting of a normal pulmonary capillary wedge pressure. These measures emphasize *pulmonary vascular dysfunction* as the central determinate mitigating the diagnosis of PH. This distinction departs from previous iterations of this definition by identifying the pulmonary circulatory system as a specific entity within the larger



**Fig. 1** Pathobiology of pulmonary arterial hypertension. The “two-hit” hypothesis of PAH contends that in the presence of certain genetic and/or molecular risk factors, exposure to environmental and/or biological stimulators of pulmonary/cardiovascular injury increases oxidant stress levels, promotes mitochondrial dysfunction, and upregulates specific microRNAs, which, in turn, dysregulate cell signaling pathways responsible for maintaining normal pulmonary vascular structure and function. Ultimately, these changes are associated with the classical PAH vasculopathy that is characterized by pulmonary endothelial dysfunction, plexogenic lesions, vasoconstriction, and microvascular thrombosis. *BMP-RII* bone morphogenetic protein receptor II, *PAEC* pulmonary artery endothelial cell, *PSMC* pulmonary smooth muscle cell, *RV* right ventricle, *ROS* reactive oxygen species, *NO* nitric oxide, *VEGF* vascular endothelial growth factor, *miRNA* microRNA

cardiopulmonary apparatus. This approach furthermore reflects the fact that traditional PH treatment strategies, which emphasize PH-associated comorbidities (i.e., hypoxic lung disease, impaired left ventricular diastolic function) to alleviate symptoms, are often unsuccessful at providing patients with sufficient and sustained improvements to cardiopulmonary hemodynamics. Analyzing PH pathophysiology and, thus, the pursuit of novel therapies in the modern era must be predicated upon an understanding of biological/molecular factors that drive disease progression.

Increases in pulmonary vascular resistance are tolerated poorly by the RV, which, compared to the LV, is a thin-walled and non-compacted structure. Chronic changes to RV volume- and/or pressure-loading conditions result in adverse remodeling of the RV that is characterized by increased end-diastolic volume, geometric conformational changes from a normal tetrahedron to a crescentic trapezoid, and RV free wall hypertrophy (Voelkel et al. 2006) (Fig. 2). Eventual RV systolic dysfunction may be accelerated or compounded in severity by



**Fig. 2** Pathophysiology of pulmonary hypertension. Pulmonary hypertension (PH) is characterized by impaired pulmonary vascular reactivity due to a heterogeneous range of pathological cellular/molecular processes that mediate pulmonary vascular injury. Pulmonary vascular dysfunction in PH is detected clinically by elevations in pulmonary vascular reactivity, which may be assessed invasively by cardiac catheterization or noninvasively by transthoracic echocardiography (*inset*). Under normal conditions, Doppler interrogation of the pulmonary artery outflow tract results in a broad-based triangular signal envelope. In the presence of decreased vascular compliance, as is the case in moderate to severe forms of PH, the time to peak blood flow acceleration is decreased (PA acceleration time) and a reflected (i.e., retrograde) Doppler signal is detected as a mid- or late-systolic 'notch.' Increased right ventricular (RV) pressure loading results in geometric conformational changes to the RV cavity (outlined in black) as well as RV hypertrophy and systolic dysfunction. LA left atrial, PA pulmonary artery, RA right atrial, *cm/s* centimeters/second. Cardiac magnetic resonance images are reproduced with permission from Fernandez-Friera L, Alvarez-Garcia A, Guzman G et al. (2011) Apical right ventricular dysfunction in patients with pulmonary hypertension demonstrated with magnetic resonance. *Heart* 97:1250–1256

progressive tricuspid valve regurgitation that increases RV end-diastolic volume and enhances cavitory dilation. The pathobiological mechanisms involved in the development of frank, irreversible RV failure (i.e., cor pulmonale) are unresolved, but likely involve RV (subendocardial) ischemia (Gautier et al. 2007), strain/stress-induced intramural replacement fibrosis (Umar et al. 2012), and torsional effects on RV myocytes that are mediated by global changes to RV shape (Puwanant et al. 2010).

Pulmonary artery pressure is dependent partly upon RV systolic function; thus, in the setting of diminished RV contractility, pulmonary artery pressure may be normal despite severe pulmonary vascular disease. Along these lines, decremental changes in RV systolic function in patients with PH are associated with worsening symptomatology (e.g., dyspnea, fatigue, abdominal/peripheral edema), decreased functional capacity, and increased mortality. This is the case in patients with even *mild* heart failure (New York Heart Association Class II) and left atrial hypertension-associated PH due to LV systolic dysfunction, in which an RV ejection fraction  $\leq 39\%$  is an independent predictor of early mortality (de Groote et al. 1998). Similarly, in patients with PAH, decreases in tricuspid annular plane systolic excursion (TAPSE), an echocardiographic measurement of RV systolic function, correlate inversely with 1-year mortality rates (Forfia et al. 2006). In turn, clinical benefits afforded to PAH patients by endothelin receptor antagonists and prostacyclin replacement therapy (see Part II, Olschewski 2013; Clozel et al. 2013) occur by virtue of their favorable effect on RV loading conditions, which promotes reverse RV remodeling and restores RV-pulmonary vascular coupling (Oikawa et al. 2005; Chin et al. 2008).

### 3 Cell Signaling Mechanisms in the Pathobiology of PH

#### 3.1 Endothelial Nitric Oxide Synthase in PH

Nitric oxide (NO<sup>•</sup>) is a 30 Da lipophilic gaseous molecule, which may diffuse through PAEC/PSMC membranes to participate in intercellular signaling. Nitric oxide is synthesized in mammalian tissues via activation of three nitric oxide synthase (NOS) isoforms, each of which are homodimeric enzymes containing a calmodulin-binding domain that separates an N-terminal heme-binding domain and a C-terminal reductase domain (Porter et al. 1990). Nitric oxide synthases catalyze the formation of NO<sup>•</sup> from L-arginine in a reaction that consists of two distinct monooxygenation steps. In the first monooxygenation step, two moles of electrons are donated by one mole of NADPH to a heme-bound oxygen via flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). This allows for the two-electron oxidation of a guanidine nitrogen of L-arginine to form one mole each of omega-N-hydroxy-L-arginine and water (Delker et al. 2010). In the second monooxygenation step, one-half mole of NADPH transfers one electron to a second heme-bound oxygen, and omega-N-hydroxy-L-arginine undergoes a three-electron oxidation to form one mole each of NO<sup>•</sup> and L-citrulline (Griffith and Stuehr 1995).

Activation of endothelial NOS (eNOS), such as in response to vascular endothelial shear stress, is modulated by various intracellular posttranslational modifications, including S-nitrosylation (e.g., Cys94, Cys99), phosphorylation (e.g., Ser1177, Ser65, Thr495), and palmitoylation, among others (Dudzinski et al. 2006). The classical extracellular signaling pathways involved in eNOS

activation include G-protein-coupled receptor signal transduction, which increases intracellular  $\text{Ca}^{2+}$  levels and, subsequently, levels of  $\text{Ca}^{2+}$ -calmodulin; Akt signaling via sphingosine 1-phosphate; vascular endothelial growth factor (VEGF) via phosphatase calcineurin; and hormonal stimuli (e.g., estrogen and insulin) (Murata et al. 2002; Egom et al. 2011; Cerqueira et al. 2012). Decreased pulmonary vascular eNOS activity is observed in numerous animal models of PH in vivo and in humans with this disease (Steudel et al. 1998; Gangopahyay et al. 2011). Specifically, loss of  $\text{NO}^*$  bioavailability is linked to impaired endothelium-dependent and -independent vasodilation, increased PSMC mitogenesis, and platelet aggregation. Proposed mechanisms to account for diminished levels of functional eNOS in PH are provided below.

### 3.1.1 Hypoxia and eNOS in PH

The mechanism(s) by which hypoxia influences eNOS gene expression is (are) controversial, as PAEC exposure to  $\text{PaO}_2 < 70$  mmHg has been associated with both increased *and* decreased eNOS protein expression levels. Fish and colleagues demonstrated that hypoxia induces a decrease in acetylation and lysine 4 methylation of eNOS proximal promoter histones to decrease eNOS gene transcription (Fish et al. 2010). In contrast, others have suggested that hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), a master transcription factor that modulates a wide range of cellular processes in response to hypoxia, binds to a HIF response element near the promoter region of eNOS to increase eNOS gene expression (Coulet et al. 2003). However, in this scenario, hypoxia-mediated upregulation of eNOS expression does not necessarily imply increased eNOS *activity*. To the contrary, tonic stimulation of eNOS is associated with a paradoxical *decrease* in eNOS activity, likely owing to the consumption and subsequent depletion of key cofactors (i.e., 5,6,7,8-tetrahydrobiopterin [ $\text{BH}_4$ ]) necessary for normal eNOS function. Under these conditions, eNOS is ‘uncoupled,’ resulting in the preferential generation of superoxide ( $\text{O}_2^-$ ) over  $\text{NO}^*$  (see Sect. 3.3). Data from PH experiments in vivo support this claim: eNOS deficiency and/or impaired eNOS function is a key factor in disease pathogenesis. For example, eNOS knockout mice (eNOS $^{-/-}$ ) exposed to *mild* hypoxia demonstrate significantly increased RV systolic pressure and diminished markers of eNOS bioactivity as compared to wild-type controls (Fagan et al. 1999). Diminished eNOS activity is also implicated in inflammatory (monocrotaline), genetic (bone morphogenetic protein receptor II [BMP-RII] deficient), and angioproliferative (VEGF inhibition with SU-5416) experimental models of PAH in vivo (Tang et al. 2004).

Hypoxia may also decrease eNOS activity by inducing posttranslational modification(s) of eNOS and/or caveolin-1, which decreases  $\text{Ca}^{2+}$  sensing by eNOS and results in dissociation of eNOS from its regulatory proteins, heat shock protein 90 and calmodulin (Murata et al. 2002). Alternatively, hypoxia may decrease levels of bioavailable  $\text{NO}^*$  through eNOS-independent mechanisms. In red blood cells, for example, hypoxia promotes increased levels of heme iron-nitrosyl (FeNO) that

limits hemoglobin *S*-nitrosylation, which, in turn, is a key PaO<sub>2</sub>-sensitive mechanism implicated in the regulation of pulmonary vascular tone (McMahon et al. 2005).

### 3.1.2 Oxidant Stress and eNOS

Perturbations to the redox status of PAECs, PSMCs, RV myocytes, and lung fibroblasts due to activation of reactive oxygen species-generating (ROS) enzymes, such as NADPH oxidase (NOX), xanthine oxidase, and uncoupled eNOS, or via disrupted electron transport chain function in mitochondria promote pulmonary vasculopathy characterized by impaired NO<sup>•</sup>-dependent vasodilation, intimal thickening, and perivascular fibrosis (Mittal et al. 2012). In humans, increases in pulmonary vascular ROS generation may occur as a pathological response to chronic hypoxia, or increased pulmonary vascular blood flow (e.g., secondary to intracardiac shunt); or due to impaired antioxidant enzyme function, as is the case in sickle cell anemia-associated PH in which glutathione peroxidase deficiency is observed (Gizi et al. 2011). ROS may impair eNOS activity through the oxidation of enzyme cofactors (i.e., BH<sub>4</sub>), or inactivate NO<sup>•</sup> such as in the case of <sup>•</sup>O<sub>2</sub><sup>-</sup> which reacts with NO<sup>•</sup> to generate peroxynitrite (ONOO<sup>-</sup>). Additionally, the interaction of <sup>•</sup>O<sub>2</sub><sup>-</sup> with the stable NO<sup>•</sup> by-product nitrite (NO<sub>2</sub><sup>-</sup>) forms peroxynitrate (O<sub>2</sub>NOO<sup>-</sup>) and, thus, decreases levels of NO<sub>2</sub><sup>-</sup>, which is a key substrate for NOS-independent synthesis of NO<sup>•</sup> (2HNO<sub>2</sub>→N<sub>2</sub>O<sub>3</sub> + H<sub>2</sub>O; N<sub>2</sub>O<sub>3</sub>→NO<sup>•</sup> + NO<sub>2</sub><sup>•</sup>) (Lundberg et al. 2011); (Spiegelhalder et al. 1976).

### 3.1.3 Genetic Mediators of eNOS in PH

BMP-RII is a serine–threonine kinase and member of the transforming growth factor-β (TGF-β) superfamily of receptors (Rosenzweig et al. 1995). Approximately 70 % of familial PAH cases involve mutations in BMP-RII, and receptor dysfunction is increasingly recognized as a contributor to non-PAH forms of PH (Machado et al. 2006). Although BMP-RII is believed to contribute to remodeling of pulmonary blood vessels through a wide range of signaling pathways, including SMAD-dependent PSMC migration (Long et al. 2009), it was recently demonstrated that two BMP-RII ligands, BMP2 and BMP4, are involved in BMP-RII-dependent phosphorylation of eNOS at Ser-1177 to upregulate eNOS activity (Gangopahyay et al. 2011). Similarly, abnormalities in the function of endoglin, a key BMP receptor accessory protein in human PAECs, are linked to the development of PAH when present in the setting of the clinical syndromes hereditary hemorrhagic telangiectasia (HHT) type 1 and type 2. Mice heterozygous for vascular endothelial endoglin expression (*Eng*<sup>+/-</sup>) develop PH spontaneously in vivo due, in part, to increased pulmonary vascular ROS generation, eNOS uncoupling, and decreased NOS-inhibitable NO<sup>•</sup> production (Toporsian et al. 2010).

Several eNOS polymorphisms are implicated in the development of PH and other vascular diseases. For example, a single nucleotide polymorphism leading to a substitution of aspartic acid for glutamic acid at position 298 (Glu298Asp) of eNOS and an increased NOS4a allelic frequency of 27-bp variable number of repeats increase susceptibility to developing the high altitude pulmonary edema (HAPE) syndrome, including elevations in pulmonary artery pressure and pulmonary vascular resistance. These changes may occur owing to function-limiting changes in the conformation of eNOS, although the precise mechanism by which to account for the phenomenon is unknown (Miyamoto et al. 1998; Droma et al. 2002; McDonald et al. 2004).

### 3.2 Endothelin-1 System

Endothelin-1 (ET-1) is a 21-amino acid vasoactive peptide that contains two disulfide bridges between Cys1-Cys15 and Cys3-Cys11 (Yeager et al. 2012), which are necessary for endothelin converting enzyme-mediated proteolytic cleavage of ET-1 from its precursor, 'Big ET-1.' Endothelin-1 is constitutively expressed in a wide range of mammalian cell types, including hepatic sinusoidal cells, renal epithelial cells, and PAECs (Huggins et al. 1993). Endothelin-1 gene expression levels are upregulated significantly in RV myocytes, PAECs, PSMCs, and lung fibroblasts in the presence of stimuli associated with pulmonary vascular injury in PH, including cytokines that mediate vascular inflammation (i.e., TGF- $\beta$ , IL-6) (Olave et al. 2012), increased levels of pulmonary vascular ROS (An et al. 2007), hypoxia (Yamashita et al. 2001), and decreased levels of bioavailable NO<sup>\*</sup> (Kourembanas et al. 1993). In fact, plasma ET-1 levels may be increased fourfold in patients with PAH or PH due to left atrial hypertension, and anti-ET-1 immunohistochemical analysis demonstrates significantly increased immunoreactivity in PAECs and PSMCs of plexiform lesions compared to blood vessels harvested from normal controls (Giaid et al. 1993). Endothelin-1 is also released from sickled red blood cells and interacts with the blood vessel wall to promote vasoconstriction in a process that contributes to the systemic and pulmonary vasculopathy of sickle cell anemia (Gladwin and Vichinsky 2008).

Endothelin-1 regulates pulmonary vascular tone through its interaction with the vasoconstrictor endothelin-type A (ET<sub>A</sub>) and -type B (ET<sub>B</sub>) receptors in PSMCs and vasodilatory ET<sub>B</sub> receptors in PAECs, which do not constitutively express ET<sub>A</sub>. Endothelin-type A and ET<sub>B</sub> receptors are members of the superfamily of G-protein-coupled receptors and are overall highly homologous (55 %), with the exception of the cysteine-rich 35-amino acid sequence distal to the seventh transmembrane domain, in which homology between receptors is only 75 % (Doi et al. 1999). Since cysteine(s) in this region are believed to regulate G-protein coupling to both ET<sub>A</sub> and ET<sub>B</sub> receptors, and, thus, are integral to receptor signal transduction, it has been postulated that differences in this region between receptor subtypes may account, in part, for their differential functions (Okamoto et al. 1997).

In PSMCs, stimulation of  $ET_{A/B}$  receptors by ET-1 induces  $G_i$  and  $G_q$  coupling to modulate phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate ( $IP_3$ ). In turn, opening of  $IP_3$ -sensitive calcium ( $Ca^{2+}$ ) channels as well as ET-1-mediated opening of the store-operated and nonselective  $Ca^{2+}$  channels induces an increase in intracellular  $Ca^{2+}$  flux ( $[Ca^{2+}]_i$ ),  $Ca^{2+}$  waves, and  $Ca^{2+}$  oscillations that promotes vasoconstriction (Liu et al. 2012). Importantly, ET-1-induced vasoconstriction persists following ET-1 dissociation from the  $ET_A$  receptor, indicating that the  $[Ca^{2+}]_i$  flux response mediated by ET-1 is robust,  $Ca^{2+}$ -dependent hyperpolarization is delayed during ET-1 signaling, or both (Zhang et al. 2003; Liu et al. 2012). The functional consequence of  $ET_A$  receptor signaling on vascular tone is noteworthy: relative to norepinephrine, the concentration of ET-1 required to induce 50 % blood vessel contraction (i.e.,  $EC_{50}$ ) in pig coronary arteries, rat aorta, and rat pulmonary artery is 0.52, 1.4, and 0.68, respectively (Huggins et al. 1993). ET-1 binding to  $ET_A$  receptors ( $K_i = 0.6 \text{ nmol l}^{-1}$ ) also promotes vascular smooth muscle cell mitogenesis by activating various signaling intermediaries that regulate protein synthesis, including protein kinase C; mitogen-activated protein kinase (MAPK); p70S6K, which targets the ribosomal protein S6K to increase cellular protein synthesis; and epidermal growth factor receptor (EGFR) via tyrosine phosphorylation (Iwasaki et al. 1999; Kapakos et al. 2010). Interestingly, upregulation of the proto-oncogene transcription factor *c-fos* by ET-1 (or hypoxia) is linked to cellular proliferation and fibrosis of PSMCs, lung fibroblasts, and myocytes in experimental animal models of PH (Rothman et al. 1994; Nishimura et al. 2003; Recchia et al. 2009), providing molecular evidence to account for the proliferative phenotypic overlap between plexogenic lesions of PAH and various solid tumors.

In contrast to PSMCs, ET-1 binding to the  $ET_B$  receptor ( $K_i$  of  $0.12 \text{ nmol l}^{-1}$ ) in PAECs results in the activation of eNOS and synthesis of  $NO^*$ , which is required to maintain normal pulmonary vascular tone and prevent vascular remodeling. Endothelin-type B receptor-dependent activation of eNOS is believed to occur via G-protein coupling to the  $ET_B$  receptor that stimulates  $[Ca^{2+}]_i$  flux and subsequent elevations in  $Ca^{2+}$  binding to calmodulin, which is a key allosteric modulator of eNOS activity. Recent work from our laboratory has demonstrated that pathophysiological levels of the mineralocorticoid hormone aldosterone akin to those observed in humans with PH increase NOX4-dependent ROS generation in PAECs in vitro, which is associated with  $ET_B$  receptor dysfunction, impaired  $ET_B$  receptor-dependent activation of eNOS, and oxidation of  $NO^*$  to  $ONOO^-$  (Maron et al. 2012).

Endothelin-type B receptor signal transduction also results in the synthesis of vasodilatory prostaglandins (PG). In isolated guinea pig lungs exposed to ET-1, a ~50-fold increase in  $ET_B$  receptor-dependent  $PGI_2$  synthesis is observed (D'Orleans-Juste et al. 1991), although the mechanism by which  $ET_B$  receptor activation stimulates  $PGI_2$  synthesis is not well characterized. Internalization of the  $ET_B$  receptor/ET-1 complex and subsequent proteasomal degradation is the chief mechanism by which ET-1 elimination occurs. This conclusion is supported

in vivo by experiments involving the transgenic spotting lethal rat (*sl/sl*), which lacks constitutively expressed vascular ET<sub>B</sub> receptors. Compared to wild-type rats, these rats demonstrate significantly higher circulating levels of ET-1 and more severe PH following monocrotaline injection to induce pulmonary vascular injury (Nishida et al. 2004).

### ***3.3 Soluble Guanylyl Cyclase and Phosphodiesterase Inhibition in PH***

Nitric oxide is the primary biological activator of the heterodimeric ( $\alpha_1/\beta_1$  or  $\alpha_1/\beta_2$ ) enzyme soluble guanylyl cyclase (sGC) that catalyzes the conversion of cytosolic GTP to cGMP, which is a critical secondary signaling molecule necessary for activation of cGMP-dependent protein kinase (i.e., protein kinase G [PKG]) to promote PSMC relaxation and inhibit platelet aggregation and thrombosis. Nitric oxide binding to the heme ( $\text{Fe}^{2+}$ ) prosthetic group of sGC results in the formation of a hexa-coordinated histidine–heme–NO<sup>•</sup> intermediate. Subsequent cleavage of the heme–histidine bond leads to a dramatic upregulation of enzyme activity: nanomolar concentrations of NO<sup>•</sup> may induce an appropriate 100-fold increase in sGC activation (Evgenov et al. 2006). In PH, elevated levels of ROS accumulation may impair sGC activity through the oxidation of heme from the ferrous ( $\text{Fe}^{2+}$ ) to ferric ( $\text{Fe}^{3+}$ ) state that converts sGC to an NO<sup>•</sup>-insensitive state, presumably owing to decreased affinity of NO<sup>•</sup> for oxidized heme. Alternatively, others have demonstrated that sGC activity is influenced by the redox status of functional sGC cysteinyl thiol(s) in a manner that is independent of the heme redox state (Fernhoff et al. 2009; Yoo et al. 2012). Work from our laboratory has demonstrated that pathophysiological concentrations of H<sub>2</sub>O<sub>2</sub> induce the formation of higher cysteinyl thiol oxidative states of Cys-122 on the  $\beta_1$  subunit of sGC in vascular smooth muscle cells, including sulfenic acid, sulfinic acid, and the disulfide form. Posttranslational oxidative modification of Cys122, in turn, functions as a redox ‘switch’ that regulates enzyme function, resulting in decreased NO<sup>•</sup>-sensing by sGC and impaired enzyme activity (Maron et al. 2009). The importance of abnormal sGC function in the pathogenesis of PH is well established. Transgenic mice deficient in the sGC  $\alpha_1$ -subunit develop exaggerated elevations in RV systolic pressure and muscularization of intraacinar pulmonary arterioles following exposure to chronic hypoxia compared to wild-type mice (Vermeersch et al. 2007). Moreover, hypoxia alone is associated with decreased mRNA and protein levels of sGC as well as sGC-dependent cGMP formation (Hassoun et al. 2004).

Collectively, these observations implicate the pharmacotherapeutic potential of heme-independent sGC activators in PH. Work from Ko and colleagues and drug discovery experiments performed at Bayer HealthCare in the early 1990s identified YC-1 [3-5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] and 5-substituted-2-furaldehyde-hydrazone derivative compounds (i.e., BAY compounds), respectively,

as synthetic heme-(in)dependent activators of sGC (Ko et al. 1994; Stasch et al. 2006). BAY 58-2667, perhaps the best studied among these compounds, activates sGC with a  $K_m$  of 74  $\mu\text{M}$  and a  $V_{\max}$  of 0.134  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  (Schmidt et al. 2003), and although the precise mechanism by which this (and other) BAY compounds activates heme-oxidized sGC is not fully resolved, one leading hypothesis contends that BAY 58-2667 competes with the oxidized heme moiety for binding to the sGC-activating motif to induce enzyme activation (Pellicena et al. 2004) (see Part III, Sect. 1). The effect of these compounds on sGC-NO<sup>•</sup> vasodilatory signaling and NO<sup>•</sup>-dependent vascular remodeling has been assessed in PH in vivo. In one study, the administration of YC-1 to hypoxic mice decreased PSMC proliferation and pulmonary artery pressure (Huh et al. 2011). The effect of BAY 63-2521 (Riociguat<sup>TM</sup>) on cardiopulmonary hemodynamics was also assessed in a small cohort of patients with PAH, chronic thromboembolic PH, or PH from interstitial lung disease. Drug therapy (3.0–7.5 mg day<sup>-1</sup>) over 12 weeks decreased pulmonary vascular resistance by 215 dyne s cm<sup>-5</sup>, which was associated with an increase in the median 6-min walk distance by 55.0 m from baseline (Ghofrani et al. 2010).

### 3.4 Phosphodiesterase Inhibition in PH

In 1962, Butcher and Sutherland implicated phosphodiesterase enzymatic activity in endogenous degradation of adenosine 3', 5' phosphate (cAMP) (Butcher and Sutherland 1962). Eleven PDE isoforms have since been detected in mammalian tissue (Table 1). The fields of PDE biochemistry and PH intersected following the identification of cGMP-specific PDE type-5, at elevated concentrations in PSMCs, platelets, and myocytes. Phosphodiesterase type-5 regulates cGMP bioactivity via (1) the hydrolysis of cGMP to 5'-GMP, and (2) allosteric binding of cGMP to PDE-5 GAF<sup>1</sup> domains, which induces a conformational change to the structure of PDE-5 and positively feeds back to promote cGMP metabolism (Fig. 3). PDE-5-cGMP binding ranges from  $K_d$  of 2.4  $\mu\text{M}$  (pH = 5.2) to 0.15  $\mu\text{M}$  (pH = 9.5) (Turko et al. 1999). The pH-dependent manner by which this occurs is in concert with reports demonstrating a regulatory role for the cyclic nucleotide ionizing residues (i.e., pH-sensitive) Asp-289 and Asp-478 in modulating PDE-5 function (McAllister-Lucas et al. 1995). The intracellular concentration of cGMP may also be influenced by flux through membrane-bound cGMP-gated channels and the multidrug transporter, although the contribution of these to total levels of bioactive cGMP is negligible in pulmonary vascular tissue (Serre et al. 1995).

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<sup>1</sup>GAF is an acronym of the various tissues in which these domains were originally described: cGMP-dependent phosphodiesterases (PDEs), *nabaena* adenyl cyclases, and *E. coli* FhlA (Francis et al. 2010).

**Table 1** cGMP-specific kinetic properties of phosphodiesterases and their tissue distribution

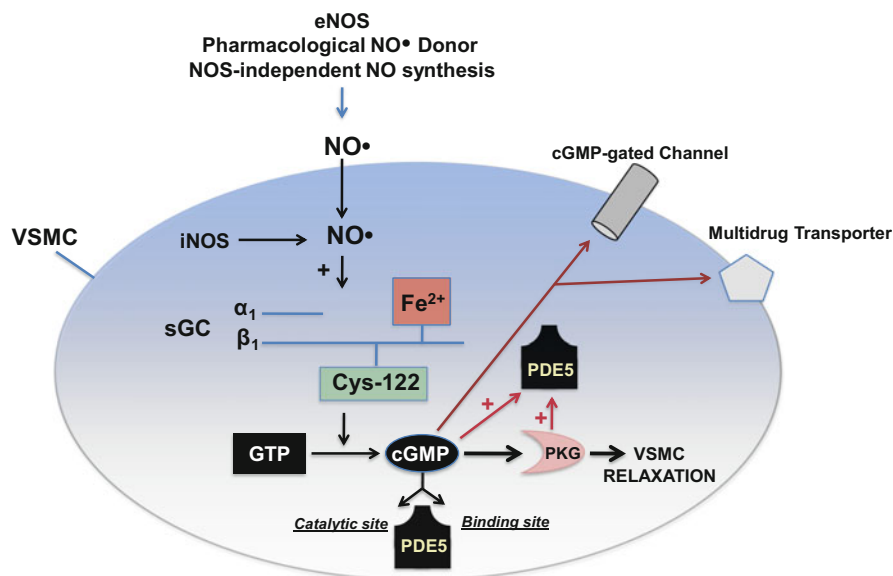
| Isoenzyme | $K_m$ cGMP<br>( $\mu$ M) | $V_{max}$ cGMP<br>( $\mu$ M) | Tissue distribution                                                 |
|-----------|--------------------------|------------------------------|---------------------------------------------------------------------|
| PDE1A     | 3–4                      | 50–300                       | VSMC, cardiomyocyte, brain                                          |
| PDE1B     | 1–6                      | 30                           | VSMC, cardiomyocyte, brain                                          |
| PDE1C     | 1–2                      | Not determined               | VSMC, cardiomyocyte, brain                                          |
| PDE2A     | 10                       | 123                          | VSMC, cardiomyocyte, brain, corpus cavernosum                       |
| PDE3A     | 0.02–0.2                 | 0.3                          | VSMC, cardiomyocyte, brain, corpus cavernosum, platelets            |
| PDE3B     | 0.3                      | 2                            | VSMC, cardiomyocytes                                                |
| PDE5A     | 1–6                      | 1–3                          | Corpus cavernosum, PSMC, skeletal muscle, platelets, cardiomyocytes |
| PDE6A/B   | 15                       | 2,300                        | Retina                                                              |
| PDE 6C    | 17                       | 1,400                        | Retina                                                              |
| PDE9A     | 0.2–0.7                  | Not determined               | Various                                                             |
| PDE 10A   | 13                       | Not determined               | Various                                                             |
| PDE 11A   | 0.4–0.2                  | Not determined               | Skeletal muscle, heart, VSMC                                        |

*PDE* phosphodiesterase, *VSMC* vascular smooth muscle cell, *PSMC* pulmonary smooth muscle cell. Adapted from Francis SH et al. (2010) cGMP-dependent protein kinase and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharm Rev* 62:525–563 and Reffelman T, Kloner RA (2003) Therapeutic role of phosphodiesterase 5 inhibition for cardiovascular disease. *Circulation* 108:239–244

In PAH, expression of PDE-5 is increased in PSMCs and in RV myocytes (Wharton et al. 2005; Nagendran et al. 2007), which is associated with decreased levels of bioactive NO<sup>\*</sup>, pulmonary vascular dysfunction, and impaired RV lusitropy (Waxman 2011). In cultured PSMCs, PDE-5 inhibition attenuates key indices of adverse remodeling, including DNA synthesis/cell growth, cellular proliferation, and suppression of apoptosis (Wharton et al. 2005). Phosphodiesterase type-5 is also linked to decreased thrombotic burden in chronic thromboembolic PH, presumably by increasing bioactive cGMP levels in platelets to inhibit platelet aggregation (Suntharalingam et al. 2007).

### 3.5 Prostacyclin Signaling in PH

Arachidonic acid, or 5,8,11,14-eicosatetraenoic acid, is released from membrane phospholipids in response to mechanical or chemical stimuli, resulting in the synthesis of two major classes of eicosanoids: prostanoids, via cyclooxygenase (COX) pathway, and leukotrienes, via the lipoxygenase (LO) pathway. Cyclooxygenase (COX) exists in at least two isoforms. The constitutively expressed form, COX-1, is present in various cell types including the vascular endothelium, gastric mucosa, and platelets. In contrast, COX-2, which is the inducible form of COX, is present in cells involved in inflammation, particularly macrophages. COX-2 is also present in normal vascular endothelial cells and appears to be upregulated in



**Fig. 3** Influence of phosphodiesterase type-5 on the nitric oxide signaling axis. Nitric oxide (NO•) derived from endothelial nitric oxide synthase (eNOS), pharmacological NO• donors, or via induction of inducible NOS in vascular smooth muscle cells (VSMCs) activates soluble guanylyl cyclase (sGC) to generate cGMP. Normal NO•–sGC signaling is regulated by the redox status of the prosthetic heme ligand and functional cysteinyl thiol(s), such as Cys122, which are present near the catalytically active region of the β<sub>1</sub>-subunit of sGC. The interaction of cGMP with its most relevant biological target, protein kinase G (PKG), results in VSMC relaxation. This vasodilatory effect of this pathway is offset through the interaction of cGMP with phosphodiesterases (PDEs), which decreased bioactive cGMP levels through hydrolysis of cGMP to form 5'GMP, or by allosteric binding of cGMP to PDE. In the case of PDE type-5 (PDE5), increased levels of cGMP and/or PKG upregulates PDE5 activity. The contribution of gated cGMP channels and the multidrug transported to the regulation of bioactive cGMP levels is negligible in pulmonary vascular tissue. Adapted from Francis SH et al. (2010) cGMP-dependent protein kinase and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharm Rev* 62:525–563, with permission

response to stimuli associated with vascular injury, such as shear stress (Topper et al. 1996). Key products of the COX pathway that are pertinent to the pathophysiology of PH include the potent vasoconstrictor and stimulus for platelet aggregation, thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and prostaglandin I<sub>2</sub> (prostacyclin), which exerts opposing effects to TXA<sub>2</sub>, including vasodilation and inhibition of platelet activation. Prostacyclin is synthesized from PGH<sub>2</sub> by prostacyclin synthase in a reaction that occurs primarily in vascular endothelial cells. Early investigators speculated that the pulmonary vasoconstriction phenotype in PAH was a consequence of imbalanced TXA<sub>2</sub>/PGI<sub>2</sub> synthesis. This hypothesis was supported by the observation that, compared to normal pulmonary blood vessels, pre-capillary pulmonary arterioles harvested from patients with PAH, hepato-pulmonary PH, and HIV-associated PH demonstrate significantly decreased PGI<sub>2</sub> synthase mRNA and protein expression levels (Tuder et al. 1999). In contrast, elevated levels of

TXA<sub>2</sub> and increased pulmonary vascular *sensitivity* to TXA<sub>2</sub> have been reported in PH, such as in the lamb model of hypoxia-induced neonatal persistent pulmonary hypertension (Hinton et al. 2007). These observations are consistent with other reports in adults demonstrating that, compared to healthy controls, patients with PAH or PH due to hypoxic lung disease demonstrate elevated levels of urinary excretion of 11-dehydro-thromboxane B<sub>2</sub>, a stable metabolite of TXA<sub>2</sub>, and decreased levels of the stable prostacyclin metabolite, 2,3-dinor-6-keto-prostaglandin F1 $\alpha$  (Christman et al. 1992).

Shear stress, ET-1, hypoxia, and BMP-II dysfunction are each associated with overactivation of the TXA<sub>2</sub> synthesis pathway relative to PGI<sub>2</sub> in vascular tissue (Zaugg et al. 1996; Song et al. 2005; Racz et al. 2010). Although the precise mechanism by which to account for this imbalance is unresolved, abnormal COX-2 function in the setting of vascular injury may play a role. It was recently demonstrated that compared to control mice, COX-2 knockdown mice administered monocrotaline to induce vascular inflammation exhibit a robust increase in NOX4 gene expression, dihydroethidium fluorescence (indicative of ROS accumulation), and ET<sub>A</sub> receptor expression in pulmonary arterioles, whereas prostacyclin levels were decreased significantly (Seta et al. 2011). These findings are consistent with reports in cultured COX-2-deficient PSMCs, in which hypoxia results in a hypertrophic remodeling response and a vasoconstrictor phenotype (Fredenburgh et al. 2008).

5-lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid ultimately into various leukotrienes that mediate cellular processes involved in vascular remodeling and cellular responses to injury. Leukotriene B<sub>4</sub>, for example, exerts both chemotactic and chemokinetic activity on polymorphonuclear leukocytes and eosinophils (Ford-Hutchinson et al. 1980), and leukotrienes C<sub>4</sub>, D<sub>4</sub>, and E<sub>4</sub> (each of which contain a cysteine) are implicated in pulmonary vasoconstriction and increased pulmonary vascular permeability. Although rats that overexpress 5-LO do not develop PAH spontaneously, pulmonary vascular dysfunction and abnormal cardiopulmonary hemodynamics are accelerated in the presence of pulmonary vascular inflammation (Jones et al. 2004). This supports other observations indicating that an inflammatory milieu is conducive to 5-LO-dependent synthesis of the vasoactive cysteinyl leukotrienes (Listi et al. 2008). Molecular inhibition of the 5-LO-activating protein (FLAP) has, in turn, been shown to prevent pulmonary hypertension in rats exposed to chronic hypoxia (Voelkel et al. 1996).

### 3.6 Mitochondrial Dysfunction

Mitochondria regulate bioenergetics, cellular respiration, and the intracellular redox status and, thus, have the potential to regulate PAEC/PSMC signaling pathways linked to cell survival, proliferation, and ROS production. Hydrogen (hydride) derived from dietary carbohydrate and fats is oxidized by molecular oxygen (O<sub>2</sub>) via the tricarboxylic acid (TCA) cycle and  $\beta$ -oxidation pathways, respectively, to

generate adenosine triphosphate (ATP). These biochemical events occur via the electron transport chain, in which two electrons donated by  $\text{NADH} + \text{H}^+$  flow sequentially from complex I to ubiquinone (coenzyme Q) to complex III (ubiquinol: cytochrome c oxidoreductase) and then to cytochrome c. Electrons are then transferred to complex IV to reduce  $\frac{1}{2}\text{O}_2$  and generate  $\text{H}_2\text{O}$  (Wallace 2005). Protons are pumped across the inner mitochondrial membrane to establish the significantly negative electrochemical gradient ( $\Delta\psi\text{m}$ :  $\sim -200$  mV) across that membrane, which provides the electromotive force necessary for ATP synthesis.

Changes to mitochondrial membrane permeability, and, hence, the normal  $\Delta\psi\text{m}$ , are antecedent to reversible structural and functional changes in mitochondria and, if unchecked, commit the cell to apoptosis (Kroemer and Reed 2000; Michelakis et al. 2008). Numerous mechanisms to account for the relationship between mitochondrial membrane permeability and changes to cell survival have been proposed and include increased permeability of the voltage-sensitive permeability transition pore complex (PTPC), alkalization of the local pH, and perturbations to the intramitochondrial redox status that results in oxidation of a key thiol involved in regulating PTPC opening and/or oxidation of pyridine nucleotides (i.e.,  $\text{NADH}/\text{NAD}^+$ ) to favor PTPC opening (Woodfield et al. 1998; Zamzami and Kroemer 2001). Collectively, these changes afford egress of apoptosis-associated proteins (e.g., Bax, Bcl-2, others) from the intramitochondrial to extramitochondrial space, thereby activating programmed cell death signaling pathways (Mossalam et al. 2012).

Pathological disruptions to mitochondria-dependent regulation of cell survival are a central mechanism in the pathobiology of various angioproliferative diseases, including solid tumor cancers. Apoptosis-resistant proliferation of PAECs/PSMCs is likewise a prominent pathophenotypic feature of PH, particularly with respect to plexigenic lesions in PAH. This observation has raised attention to the possibility that mitochondrial dysfunction is an under-recognized pathobiological factor by which to account for the phenotypic overlap between these two broad categories of disease. Evidence in support of this concept is derived partly from observations made in the fawn-hooded rat, a unique animal strain that develops PAH spontaneously. Pulmonary vascular smooth muscle cells harvested from these animals demonstrate mitochondria that are decreased in size and fragmented prior to the development of pulmonary vascular remodeling (Bonnet et al. 2006). The functional effects of these changes are linked to a shift in mitochondrial metabolism from oxidative phosphorylation toward glycolysis, impairment to electron flux, and subsequent activation of hypoxia-inducible factor (HIF)-1 $\alpha$  (Archer et al. 2008). In turn, HIF-1 $\alpha$  has been shown in endothelial cells cultured from patients with idiopathic PAH to target carbonic anhydrase IX, which decreases levels of the antioxidant enzyme manganese superoxide dismutase (SOD2) to increase vascular ROS generation and decrease levels of  $\text{NO}^*$  (Fijalkowska et al. 2010). Interestingly, in these experiments, increased HIF-1 $\alpha$  expression correlated inversely with low numbers of mitochondria, indicating that negative control of mitochondrial biogenesis by HIF-1 $\alpha$  may be one mechanism by which to account for abnormal cellular respiration patterns observed in in vivo models of PAH. Conventional

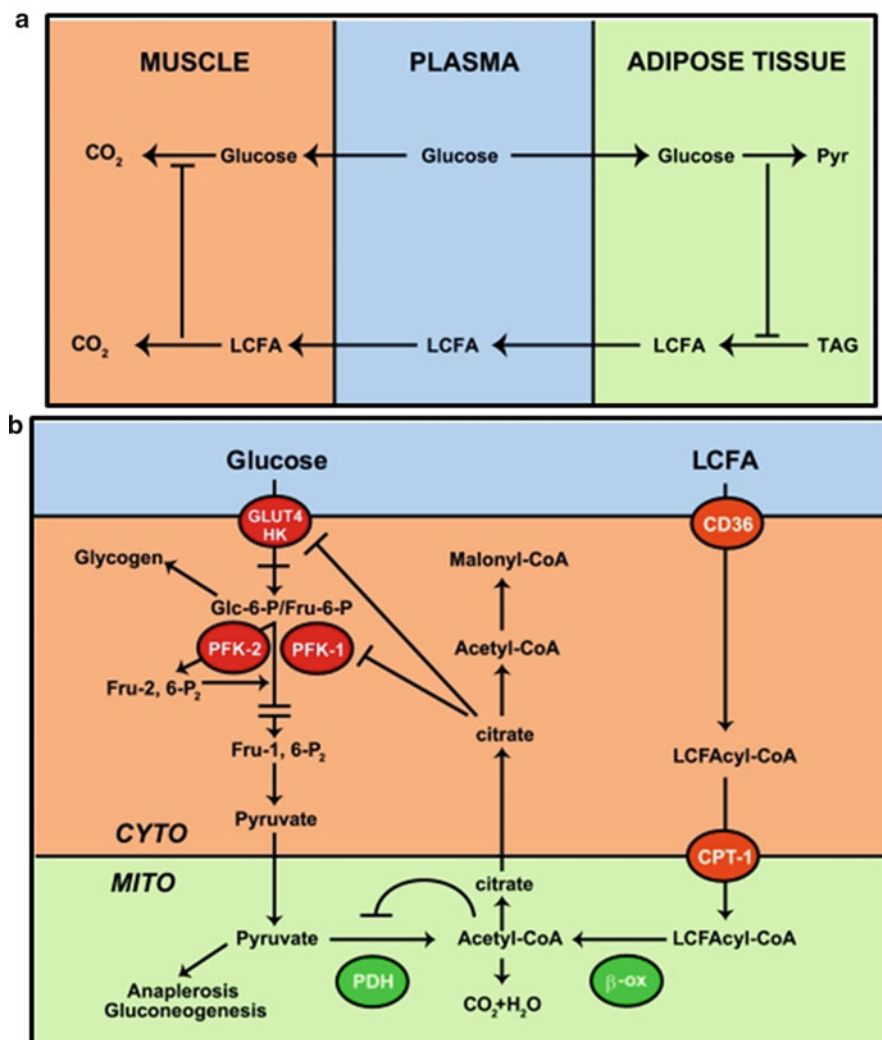
factors associated with PAH may also influence mitochondrial dysfunction directly. For example, compared to healthy controls, stimulation of PSMCs with ET-1, platelet-derived growth factor (PDGF), or IL-6 harvested from PAH patients results in Kruppel-like factor 5 (KL-5)-mediated activation of cyclin B1 that hyperpolarizes the mitochondrial inner membrane to inhibit apoptosis (Courboulin et al. 2011b).

Under normoxic conditions, electron transport chain complexes I or II generate  $\text{O}_2^-$  that is dismutated to form  $\text{H}_2\text{O}_2$ , which is a key signaling molecule required for activation of Kv channels necessary to maintain the negative electrochemical gradient of the mitochondria (Bonnet et al. 2006). At  $\text{PaO}_2 < 70$  mmHg, there is decreased intramitochondrial  $\text{H}_2\text{O}_2$  generation, opening of  $\text{O}_2$ -sensitive Kv1.5 channels, and subsequent activation of L-type  $\text{Ca}^{2+}$  channels that promotes pulmonary vasoconstriction (Archer et al. 2004). Human PSMCs in PAH, however, are deficient in Kv1.5 channels, and data from experimental animal models of PAH suggest that the effect of this deficiency is mitochondrial hyperpolarization, and, consequently, tonic activation of L-type  $\text{Ca}^{2+}$  channels associated with vasoconstriction and proliferation of PSMCs (Reeve et al. 2001).

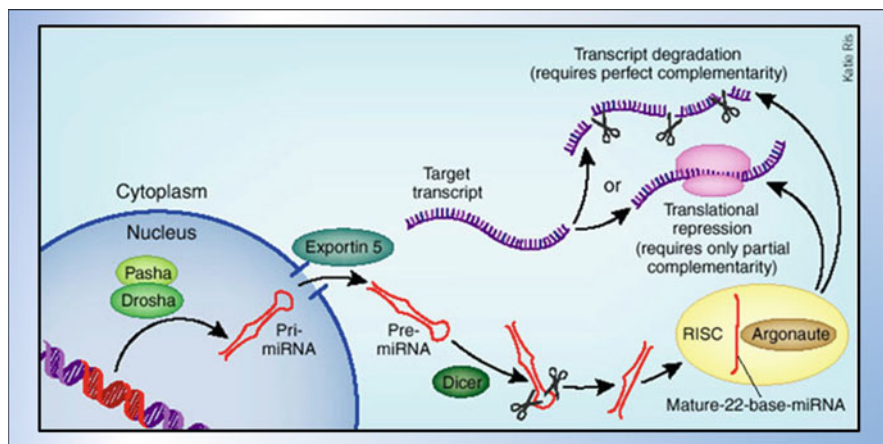
Less well established is the role of abnormal mitochondrial bioenergetics in the development of pulmonary vascular dysfunction and/or RV hypertrophy in PH. There is increasing evidence suggesting that in cardiomyocytes, an abnormal shift in cellular fuel utilization vis-à-vis the glucose–fatty acid cycle (i.e., *Randle's* cycle) (Randle et al. 1963) accounts for changes to myocardial structure (i.e., hypertrophy) and function (i.e., impaired contractility) (Fig. 4). In the monocrotaline and pulmonary artery banding rat models of PAH, for example, decreased RV  $\text{O}_2$  consumption is observed and modulates a shift from oxidative phosphorylation to glycolysis by a mechanism involving increased Glut-1 expression and upregulation of pyruvate dehydrogenase kinase (PDK) expression with consequent increased phosphorylation of pyruvate dehydrogenase leading to its inhibition (Piao et al. 2010). The functional effects of this process include impaired RV systolic function and prolongation of the QT interval, which can be reversed by PDK inhibition or through inhibition of fatty acid oxidation to induce an indirect reciprocal shift in the mitochondrial fuel source back to glucose (oxidation) (Fang et al. 2012).

### 3.7 Peroxisome Proliferator-Activated Receptor- $\gamma$

Peroxisome proliferator-activated receptor (PPAR- $\gamma$ ) is a transcription factor most commonly associated with its regulatory effect on genes involved in fatty acid storage and glucose metabolism in adipocytes (Kilroy et al. 2012); PPAR- $\gamma$ , and its transcription target apoE, are also key downstream targets of BMP-RII signal transduction. In turn, loss of function to BMP-RII via somatic mutation or dissociation of BMP-RII-interacting proteins is associated with PSMC proliferation in vitro and the development of PAH in vivo (Merklinger et al. 2005; Chan et al. 2007; Song et al. 2008). In PSMCs, the antiproliferative effect of BMP-RII



**Fig. 4** Mitochondrial bioenergetics. (a) The “glucose–fatty acid cycle” or *Randle Cycle* describes a mechanism to maintain homeostatic control of circulating levels of glucose and fatty acids. (b) Inhibition of glucose utilization by fatty acid oxidation is regulated most strongly by pyruvate dehydrogenase (PDH) and, to a lesser extent, by 6-phosphofructo-1-kinase (PFK) and glucose uptake. Phosphofructokinase inhibition owing to citrate accumulation or via pharmacological/molecular strategies shifts glucose toward glycogen synthesis and pyruvate to gluconeogenesis or the synthesis of TCA intermediates (i.e., anaplerosis). Overactivation of PDK in right ventricular myocytes in an in vivo model of pulmonary hypertension has been associated with a shift from glucose oxidation to glycolysis and subsequent myocardial dysfunction (Piao et al. 2010). *LCFA* long-chain fatty acids, *TAG* triacylglycerol, *Pyr* pyruvate, *Cyto* cytosol, *MITO* mitochondria, *GLUT4* glucose transporter 4, *HK* hexokinase, *Glc-6-P* glucose-6-phosphate, *Fru-6-P* fructose 6-phosphate, *CPT I* carnitine palmitoyltransferase I,  $\beta$ -ox  $\beta$ -oxidation. Reproduced with permission from Hue et al. (2009) *Am J Physiol Endocrinol Metab* 297:E578–E591



**Fig. 5** MicroRNA processing. MicroRNA (miRNA) transcription in the nucleus is facilitated by the RNase III enzymes, drosha and pasha, to generate hairpin-looped molecules known as primary miRNAs (pri-miRNA), which are processed further to form miRNA precursors (pre-miRNA). Once exported from the nucleus to the cytoplasm, the RNA endonuclease, dicer, facilitates the synthesis of the mature miRNA duplex by removing the hairpin loop. The catalytic component to the RNA-induced silencing complex (RISC) is referred to as argonaute and facilitates RISC-mediated miRNA incorporation. miRNA then interacts with 3' untranslated regions of specific mRNA targets to negatively regulate gene expression. Reproduced with permission from Mack GS (2007) MicroRNA gets down to business. *Nat Biotechnol* 25:631–638

is modulated by phospho-ERK and PPAR- $\gamma$  binding to DNA, which, in turn, stimulates apoE synthesis and secretion (Hansmann et al. 2007). Transgenic ApoE knockout mice (ApoE<sup>-/-</sup>) fed a high-fat diet demonstrate spontaneous development of PH, which is reversible through pharmacological stimulation of PPAR- $\gamma$  with pioglitazone (Hansmann et al. 2007). In human PAECs, BMP-RII signaling appears to induce a PPAR- $\gamma$ / $\beta$ -catenin complex that targets the gene encoding apelin to modulate normal cellular responses to injury and, in PSMCs, suppresses cellular proliferation (Falcetti et al. 2010; Alastalo et al. 2011).

### 3.8 MicroRNA-Mediated Regulation of Cellular Responses to Hypoxia

MicroRNA (miRNA) are non-canonical and highly conserved noncoding ribonucleic acid molecules (~20 nucleotides) that participate in a heterogeneous range of cellular processes and are believed to regulate over 30 % of all mRNA transcripts (Berezikov et al. 2005). MicroRNA transcription generates hairpin-looped molecules known as primary miRNAs (pri-miRNA), which are processed in the cell nucleus to form miRNA precursors (pre-miRNA). Once exported from

the nucleus to the cytoplasm, the RNA endonuclease, dicer, facilitates the synthesis of the mature double-stranded miRNA by removing the hairpin loop (Fig. 5). miRNAs interact with 3' untranslated regions of specific mRNA targets to regulate negatively gene expression (Chan and Loscalzo 2010). More than 90 miRNAs have been identified to be upregulated in response to hypoxia, although only a select few (miR-210, miR-424, miR-17, miR-328) have been studied in detail with respect to PH disease pathophysiology (Fasanaro et al. 2008).

Hypoxia-inducible factor-1 $\alpha$ -dependent upregulation of miR-210 targets iron-sulfur cluster assembly proteins (ISCU1/2) to repress mitochondrial respiration. Under hypoxic conditions, miR-210 levels are increased in PAECs *in vitro*, which results in miR-210-dependent downregulation of ISCU1/2 that inhibits mitochondrial electron transport (i.e., Complex I) and the tricarboxylic acid cycle. In this way, miR-210 is a critical molecular intermediate that accounts, in part, for the effect of hypoxia on HIF-1 $\alpha$ -dependent disruptions to electron transport chain function (Chan et al. 2009). Importantly, HIF-1 $\alpha$  itself is likely to be under miRNA-dependent regulation. In human vascular endothelial cells, hypoxia-induced upregulation of miR-424 and subsequent targeting of the scaffolding protein, cullin 2, by miR-424 appears to be an important regulatory mechanism stabilizing HIF-1 $\alpha$  (Ghosh et al. 2010). Moreover, the observation that miR-424 promotes angiogenesis in peripheral blood vessels following ischemia (i.e., locally hypoxic environment) in mice *in vivo* raises speculation that this particular miRNA may be relevant in the angioproliferative pattern observed in pulmonary arterioles under hypoxic conditions in PH.

Along these lines, miR-17 is also implicated in hypoxia-mediated vascular endothelial cell proliferation through the negative regulation of the cell cycle inhibitor p21. In one study, overexpression of miR-17 increased PDGF-stimulated cellular proliferation in cultured PSMCs. Administration of a miR-17 antagomir to mice exposed to chronic hypoxia, however, was shown to protect against increases in pulmonary artery pressure and pulmonary arterial muscularization (Courboulin et al. 2011a, b; Pullamsetti et al. 2011).

Recently, downregulation of miR-328 by hypoxia was linked to hypoxic pulmonary vasoconstriction and negative pulmonary vascular remodeling in rats with moderate PH (Guo et al. 2012). In these experiments, hypoxia-induced suppression of miR-328-dependent inhibition of L-type calcium channel- $\alpha$ 1C expression through a mechanism involving the interaction of miR-328 with the 3' untranslated region of the L-type calcium channel- $\alpha$ 1C was associated with increased RV systolic pressure. Furthermore, miR-328 signaling suppressed insulin-like growth factor 1, and was proposed by the authors of that study as a potential mechanism by which to account for the relationship between hypoxia, miR-328, and decreased PSMC apoptosis.

Parikh and colleagues performed a network bioinformatics analysis, which predicted miR-21 to participate in PH pathobiology by regulating BMP-, BMP-RII-, inflammation-, and hypoxia-associated signaling pathways (Parikh et al. 2012). This analysis was consistent with previous observations *in vitro* implicating miR-21 in negative vascular remodeling (Ji et al. 2007). Moreover,

hypoxia-mediated miR-21 upregulation in PAECs appears to contribute to the PH vascular phenotype by decreasing BMP-RII, RhoB, and Rho kinase, which, under normal conditions, are involved in pulmonary vasodilatory signaling. miR-21 was likewise linked to disease expression in various PH animal models *in vivo*, and was observed to be highly expressed in pulmonary vascular tissue in humans with PH.

## 4 Conclusions

Pulmonary hypertension describes a complex disorder characterized by dysregulation of cell signaling pathways that maintain normal structure and function to distal pulmonary blood vessels. In severe forms of PH, this may result in an obliterative vasculopathy, severely elevated pulmonary artery pressure and pulmonary vascular resistance, and adverse RV remodeling. The development of successful PH pharmacotherapies in the future that aim to modify disease progression will likely hinge on the identification of novel molecular mechanisms that modulate pulmonary vascular remodeling. This pursuit is expected to require enhanced understanding of the processes by which miRNAs, mitochondria, and other molecular factors regulate cellular bioenergetics, survival, and proliferation to contribute to PH disease expression.

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