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Pharmacotherapy of Pulmonary Hypertension

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Pharmacotherapy of Pulmonary Hypertension

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Preface

Over the past decade, we have witnessed major advancements in the understanding of the molecular mechanisms and pathophysiology of pulmonary hypertension (PH) that occurred in parallel with the discovery and development of new therapies. Pharmacological agents that modulate the main pathophysiological pathways of pulmonary arterial hypertension, including endothelin-1, nitric oxide, and prostacyclin, have changed the course of this devastating disease by relieving symptoms and improving and prolonging patients' lives. Moreover, molecular biological tools have supplied a wealth of knowledge about the roles of established and emerging signaling pathways in PH. This book is an example of how pharmacotherapy can further expand our understanding of such a heterogeneous group of diseases as PH.

Written by leading experts in the field, this volume focuses on current, evidence-based pharmacological treatments of various forms of PH and also provides a comprehensive review of most recent developments in this area. The first part of the book covers definition, classification, pathophysiology, pathology, biomarkers, and animal models of the disease, thus laying the conceptual basis for what follows. The middle section provides an overview of the established therapies such as calcium channel blockers, prostanoids, endothelin receptor antagonists, phosphodiesterase-5 inhibitors, and inhaled nitric oxide. The last section explores novel pathways and emerging therapeutic approaches including soluble guanylate cyclase stimulators, Rho-kinase inhibitors, inhibitors of serotonin receptors and transporters, peptide growth factors, vasoactive peptides, modulators of redox equilibrium and cyclic nucleotides homeostasis, as well as immunosuppressive and anti-proliferative agents. Particular attention is given to clinical applications of these experimental therapies, which are on the horizon, thus spanning the continuum from basic science to clinical applications. The catalogue of signaling cascades in PH is rapidly growing and with it the wish to modulate these pathways for therapeutic reasons. We can expect to see further progress and achievements in developing novel therapeutics over the next decade, with new improved agents in established drug classes, new drug classes in established pathways, and novel drugs aimed at new molecular targets.

We hope that this book will help the reader appreciate the importance of elucidating the pathophysiology of PH and the research on new signaling pathways and their biochemical and genetic background. It also showcases the commitment of academia and industry to drug discovery and development focusing on this devastating condition. Unfortunately, despite recent advances in the understanding of the molecular mechanisms and pathology of PH, this disease remains incurable. There is still a significant need for identifying new therapeutic targets and developing new treatment options. Undoubtedly, exciting times lie ahead of us, for both the science and the patients. If this book helps to stimulate further research and improve patient care, the wishes of the authors, the editors, and the editorial manager, Susanne Dathe, will be fulfilled.

Paris, France
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Part I
Pulmonary Hypertension: Conceptual
Bases of the Disease

Definition and Classification of Pulmonary Hypertension

Marc Humbert, David Montani, Oleg V. Evgenov, and Gérald Simonneau

Abstract Pulmonary hypertension is defined as an increase of mean pulmonary arterial pressure ≥ 25 mmHg at rest as assessed by right heart catheterization. According to different combinations of values of pulmonary wedge pressure, pulmonary vascular resistance and cardiac output, a hemodynamic classification of pulmonary hypertension has been proposed. Of major importance is the pulmonary wedge pressure which allows to distinguish pre-capillary (pulmonary wedge pressure ≤ 15 mmHg) and post-capillary (pulmonary wedge pressure > 15 mmHg) pulmonary hypertension. Pre-capillary pulmonary hypertension includes the clinical groups 1 (pulmonary arterial hypertension), 3 (pulmonary hypertension due to lung diseases and/or hypoxia), 4 (chronic thrombo-embolic pulmonary hypertension) and 5 (pulmonary hypertension with unclear and/or multifactorial mechanisms). Post-capillary pulmonary hypertension corresponds to the clinical group 2 (pulmonary hypertension due to left heart diseases).

Keywords Pulmonary arterial hypertension • Pulmonary artery pressure • Pulmonary hypertension • Right heart catheterization

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1 Introduction

Pulmonary hypertension (PH) has previously been called an orphan disease, that is, a condition which affects a few individuals and is overlooked by the medical profession and pharmaceutical industry (Humbert et al. 2007). Although undoubtedly rare, the concept that PH is overlooked cannot be considered to be the case today. Indeed, there have been a number of important discoveries in recent years that have significantly improved our understanding of the disease, helped guide patient management, and laid foundations for future research (Simonneau et al. 2009; Galiè et al. 2009). The fifth world symposium on PH has taken place in 2013 in Nice, France, 40 years after the World Health Organization sponsored the first international conference in Geneva, Switzerland, on a mysterious condition named primary pulmonary hypertension (PPH), spurred by the interest created by the sudden increase in the disease in patients who had used an anorectic pill (aminorex fumarate) (Humbert 2012). Significant achievements have been made in the field since then, as reflected by the results presented at the second world symposium on PPH (Evian, France, 1998), the third world symposium on pulmonary arterial hypertension (PAH) (Venice, Italy, 2003), and the fourth world symposium on PH (Dana Point, US, 2008) (Humbert 2012).

Table 1 Hemodynamic definitions of pulmonary hypertension. Adapted from Galiè et al. (2009)

Definition	Characteristics	Clinical groups(s) ^a
PH	$\bar{P}_{pa} \geq 25$ mmHg	All
Pre-capillary PH	$\bar{P}_{pa} \geq 25$ mmHg $P_{pcw} \leq 15$ mmHg CO normal or reduced ^b	1. Pulmonary arterial hypertension 3. PH due to lung diseases 4. Chronic thromboembolic PH 5. PH with unclear and/or multifactorial mechanisms
Post-capillary PH	$\bar{P}_{pa} \geq 25$ mmHg $P_{pcw} > 15$ mmHg CO normal or reduced ^b	2. PH due to left heart disease
Passive	TPG ≤ 12 mmHg	
Reactive (out of proportion)	TPG > 12 mmHg	

\bar{P}_{pa} mean pulmonary arterial pressure; P_{pcw} pulmonary capillary wedge pressure; CO cardiac output; TPG transpulmonary pressure gradient ($\bar{P}_{pa} - \bar{P}_{pcw}$)

^aAccording to Table 2

^bHigh CO can be present in cases of hyperkinetic conditions such as systemic-to-pulmonary shunts (only in the pulmonary circulation), anemia, hyperthyroidism, etc.

All values measured at rest

2 Definition

Pulmonary hypertension is defined as an increase of mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest as assessed by right heart catheterization (RHC) (Galiè et al. 2009). This value has been utilized for selecting patients in all randomized controlled trials on PAH and in PH registries. The normal mPAP at rest is 14 ± 3.3 mmHg and the upper limit of normal, according to these data, is 20.6 mmHg (Kovacs et al. 2009). It is currently undefined how to consider the values of mPAP between 21 and 24 mmHg (Galiè et al. 2009). In addition, the definition of PH during exercise as a mPAP > 30 mmHg as assessed by RHC is not supported by convincing published data, and healthy individuals can reach higher values (Galiè et al. 2009; Kovacs et al. 2009; Whyte et al. 2012). Therefore, no definition for PH during exercise is currently accepted (Galiè et al. 2009). According to different combinations of values of pulmonary wedge pressure (PWP), pulmonary vascular resistance (PVR), and cardiac output (CO), a hemodynamic classification of PH has been proposed (Table 1) (Galiè et al. 2009). Of major importance is the PWP, which allows to distinguish pre-capillary PH (PWP ≤ 15 mmHg) and post-capillary PH (PWP > 15 mmHg). Pre-capillary PH includes the following clinical groups: 1 (PAH), 3 (PH due to lung diseases and/or hypoxia), 4 (chronic thromboembolic PH, CTEPH), and 5 (PH with unclear and/or multifactorial mechanisms) (Simonneau et al. 2009; Galiè et al. 2009). Post-capillary PH includes the clinical group 2 (PH due to left heart diseases) (Simonneau et al. 2009; Galiè et al. 2009). In group 2, the transpulmonary gradient (TPG = mPAP – PWP) allows the distinction of so-called passive “proportionate” post-capillary PH (TPG ≤ 12 mmHg) and reactive “out of proportion” post-capillary PH (TPG > 12 mmHg) (Galiè et al. 2009). However, the concept of “proportionate” post-capillary PH is currently debated and the inclusion of other parameters such as the difference between

mPAP and diastolic PAP may allow a more accurate analysis of the pulmonary hemodynamics. In this chapter, the features of each clinical PH group are discussed in specific sections with particular attention to PAH.

3 Classification

The PH classification has gone through a series of changes since the first classification proposed in 1973, which identified only two main categories, namely “primary” or “secondary” PH, depending on the presence or absence of identifiable causes or risk factors (Simonneau et al. 2009; Galiè et al. 2009; Hatano and Strasser 1975). During the second world symposium on PPH (Evian, France, 1998), a new classification was proposed and attempted to create categories of PH that shared similar pathogenesis, clinical features, and therapeutic options (Simonneau et al. 2009). This classification defined homogenous groups of patients to help in conducting clinical trials and obtaining approval for specific therapies. In 2003, the third PH conference (Venice, Italy) proposed only minor changes, with the exception of the introduction of the terms of idiopathic PAH (IPAH), familial PAH, or associated PAH defining three PAH subgroups sharing broadly similar physiopathology and response to therapy (Simonneau et al. 2009). The other prominent change was to move pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) from separate categories into a single subcategory of PH associated with substantial venous or capillary involvement (Simonneau et al. 2009). Indeed PAH and PVOD share similarities with idiopathic PAH, including clinical presentation, hemodynamic characteristics, and risk factors, that justified placing them together in group 1. However, PVOD and PCH differ from PAH in terms of pathological changes and worst responses to vasodilator therapies (Simonneau et al. 2009).

In 2008, the fourth world PH conference held in Dana Point, California, proposed to revise previous classifications in order to accurately reflect information published over the past 5 years, as well as to clarify some areas that were ambiguous (Simonneau et al. 2009). The current Dana Point classification is presented in Table 2.

4 Group 1: Pulmonary Arterial Hypertension

The nomenclature of the PAH subgroups and associated conditions has evolved with time, and additional modifications were included in the 2008 revision (Simonneau et al. 2009; Galiè et al. 2009).

Table 2 Updated clinical classification of pulmonary hypertension. Adapted from Simonneau et al. (2009)

Updated clinical classification of pulmonary hypertension (Dana Point, 2008)

1. Pulmonary arterial hypertension (PAH)
 - 1.1. Idiopathic PAH
 - 1.2. Heritable
 - 1.2.1. *BMPR2*
 - 1.2.2. *ALK1*, endoglin (with or without hereditary hemorrhagic telangiectasia)
 - 1.2.3. Unknown
 - 1.3. Drug- and toxin-induced
 - 1.4. Associated with
 - 1.4.1. Connective tissue diseases
 - 1.4.2. HIV infection
 - 1.4.3. Portal hypertension
 - 1.4.4. Congenital heart diseases
 - 1.4.5. Schistosomiasis
 - 1.4.6. Chronic hemolytic anemia
 - 1.5. Persistent pulmonary hypertension of the newborn
 - 1'. Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)
2. Pulmonary hypertension owing to left heart diseases
 - 2.1. Systolic dysfunction
 - 2.2. Diastolic dysfunction
 - 2.3. Valvular disease
3. Pulmonary hypertension owing to lung diseases and/or hypoxia
 - 3.1. Chronic obstructive pulmonary disease
 - 3.2. Interstitial lung disease
 - 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
 - 3.4. Sleep-disordered breathing
 - 3.5. Alveolar hypoventilation disorders
 - 3.6. Chronic exposure to high altitude
 - 3.7. Developmental abnormalities
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms
 - 5.1. Hematologic disorders: myeloproliferative disorders, splenectomy
 - 5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans' cell histiocytosis; Lymphangioleiomyomatosis, neurofibromatosis, vasculitis
 - 5.3. Metabolic disorders: glycogen storage disease, Gaucher's disease, thyroid disorders
 - 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

ALK1 activin receptor-like kinase type 1; *BMPR2* bone morphogenetic protein receptor type 2; *HIV* human immunodeficiency virus

4.1 Group 1.1/1.2: Idiopathic and Heritable PAH

IPAH corresponds to sporadic disease in which there is neither a familial history of PAH nor identified risk factors (Simonneau et al. 2009; Galiè et al. 2009). When PAH occurs in a familial context, germline mutations in the gene coding for the

bone morphogenetic protein receptor 2 (*BMPR2*), a member of the transforming growth factor beta (TGF- β) signaling family, can be detected in more than 70 % of cases (Simonneau et al. 2009; Sztrymf et al. 2008). *BMPR2* mutations have also been detected in 11–40 % of apparently idiopathic cases with no familial history (Sztrymf et al. 2008; Girerd et al. 2010). Indeed, the distinction between idiopathic and familial PAH with *BMPR2* mutations is artificial, as all patients with a *BMPR2* mutation have heritable disease. Thus, it was decided to abandon the term “familial PAH” in favor of the term “heritable PAH” (Simonneau et al. 2009). Heritable forms of PAH include IPAH with germline mutations (mainly *BMPR2* but also *ACVRL1* or *endoglin*) and familial cases with or without identified mutations (Simonneau et al. 2009; Sztrymf et al. 2008; Girerd et al. 2010). Idiopathic and heritable PAH is more common in women than in men with a gender ratio around 2:1 (Sztrymf et al. 2008; Girerd et al. 2010).

Recent studies have shown that heritable PAH patients carrying a *BMPR2* mutation (irrespective of the familial history of other PAH cases) were younger at diagnosis, had more severe disease, and were less likely to demonstrate vasoreactivity than IPAH patients without a *BMPR2* mutation (Sztrymf et al. 2008; Girerd et al. 2010; Elliott et al. 2006). It is now commonly accepted that genetic testing should be performed as part of a comprehensive program that includes genetic counseling and discussion of the risks, benefits, and limitations of such testing (Girerd et al. 2010).

4.2 Group 1.3: Drug- and Toxin-Induced PAH

In the recent revision of the PAH classification aminorex, fenfluramine, dexfenfluramine, and toxic rapeseed oil represent the only identified “definite” risk factors for PAH (Simonneau et al. 2009; Galiè et al. 2009) (Table 3).

Aminorex fumarate structurally resembles adrenaline and ephedrine and is a potent appetite suppressant and central stimulant. Its use in the 1960s led to an outbreak of rapidly progressive PAH (then termed PPH) in Switzerland, Austria, and Germany, with a median exposure-to-onset time, when known, of 8 months (ranging from 3 weeks to over 1 year), as first described in a Swiss medical clinic (Follath et al. 1971; Gurtner 1979). The incidence of PAH in patients who had used aminorex was shown to be about 0.2 % overall, and proportional to the amount of drug taken. Furthermore, if discontinued early enough, a regression of PPH could be seen. Subsequently, aminorex was withdrawn from the market in 1968.

Both fenfluramine and dexfenfluramine have been marketed as appetite suppressants. An outbreak of drug-induced PAH has been identified with these two agents in the 1980s–1990s (Brenot et al. 1993; Abenheim et al. 1996; Simonneau et al. 1998; Souza et al. 2008). PAH cases in patients exposed to fenfluramine derivatives share clinical, functional, hemodynamic, and genetic features with IPAH. This suggests that fenfluramine exposure represents a potential trigger for PAH without influencing its clinical course (Souza et al. 2008).

Table 3 Updated risk factors for pulmonary arterial hypertension. Adapted from Simonneau et al. (2009)

Definite	Possible
Aminorex	Cocaine
Fenfluramine	Phenylpropanolamine
Dexfenfluramine	St. John's Wort
Benfluorex	Chemotherapeutic agents
Toxic rapeseed oil	SSRI
Likely	Unlikely
Amphetamines	Oral contraceptives
L-tryptophan	Estrogen
Methamphetamines	Cigarette smoking

PAH pulmonary arterial hypertension; *SSRI* selective serotonin reuptake inhibitor

The association of fenfluramine and dexfenfluramine intake with the development of PAH was confirmed by the Surveillance of Pulmonary Hypertension in America (SOPHIA), which enrolled 1,335 subjects at tertiary PH centers in the USA between 1998 and 2001 (Walker et al. 2006). Of note, these agents have also been associated with an increased risk of valvular heart diseases, presumably because of their serotonergic properties (Connolly et al. 1997). As a result, fenfluramine and dexfenfluramine were withdrawn from the market in the late 1990s (Souza et al. 2008; Humbert et al. 2006).

Benfluorex is a benzoate ester that shares similar structural and pharmacologic characteristics with dexfenfluramine and fenfluramine (Boutet et al. 2009; Savale et al. 2012; Frachon et al. 2010). The active and common metabolite of each of these molecules is norfenfluramine, which itself has a chemical structure similar to that of the amphetamines. Given its pharmacological properties, benfluorex would be expected to have toxic effects similar to the fenfluramine derivatives. An outbreak of valvular heart diseases and/or PAH induced by benfluorex use has been evidenced in France in the 2000s (Boutet et al. 2009; Savale et al. 2012; Frachon et al. 2010). Eighty-five cases of PH associated with benfluorex exposure were identified by the French PH network from June 1999 to March 2011 (Savale et al. 2012). Of these 85 cases, 70 patients had confirmed pre-capillary PH. Interestingly, 33 % of all patients also had prior exposure to fenfluramine or dexfenfluramine, and an additional risk factor for PH was identified in 30 % of the patients with pre-capillary PH (Savale et al. 2012). A quarter of patients in this series showed coexisting PH and mild-to-moderate cardiac valve involvement. These results, together with the accumulated data regarding the known toxic effects of fenfluramine and dexfenfluramine, strongly suggest that benfluorex exposure is a potent trigger for PAH. Benfluorex was withdrawn from the French market in 2009.

St John's Wort and over-the-counter antiobesity agents containing phenylpropanolamine also increase the risk of developing PAH (Walker et al. 2006). The SOPHIA study examined intake of a variety of nonselective monoamine reuptake inhibitors, selective serotonin reuptake inhibitors, antidepressants, and anxiolytics

and found no increased risk for developing PAH (Walker et al. 2006). However, case–control studies of selective serotonin reuptake inhibitors used during pregnancy showed an increased risk of developing persistent PH of the newborn in the offspring (Chambers et al. 2006). Based on this study, selective serotonin reuptake inhibitors have been reclassified to the “possible” category.

Amphetamines use represents a “likely” risk factor, although they are frequently used in combination with fenfluramine. A recent comprehensive, retrospective study suggested a strong correlation between the use of methamphetamine (inhaled, smoked, oral, or intravenous) and the occurrence of PAH (Chin et al. 2006). Based primarily on the results of this study, methamphetamine use is now considered a “very likely” risk factor for the development of PAH.

Cases of pre-capillary PH fulfilling the criteria of drug-induced PAH have been reported in chronic myelogenous leukemia patients treated with the tyrosine kinase inhibitor dasatinib (Montani et al. 2012). At diagnosis, patients had moderate-to-severe pre-capillary PH with functional and hemodynamic impairment. Clinical, functional, and hemodynamic improvements were observed within a few months of dasatinib discontinuation in most patients (Montani et al. 2012). However, after a median follow-up of 9 months (min–max 3–36), the majority of patients failed to demonstrate complete clinical and hemodynamic recovery and no patients reached a normal value of mPAH (≤ 20 mmHg). The lowest estimate of incident PH occurring in patients exposed to dasatinib in France was 0.45 % (Montani et al. 2012). Thus, dasatinib may induce severe pre-capillary PH, fulfilling the criteria of PAH, suggesting a direct and specific effect of dasatinib on pulmonary vessels (Montani et al. 2012).

4.3 Group 1.4.1: PAH Associated with Connective Tissue Diseases

Pulmonary arterial hypertension associated with connective tissue diseases represents an important clinical subgroup (Humbert et al. 2006; Hachulla et al. 2005). The high prevalence of PAH in patients with systemic sclerosis has been well studied. Two prospective studies, using echocardiography as a screening method and RHC for diagnosis and confirmation, found a prevalence of PAH ranging from 8 % to 12 % (Hachulla et al. 2005; Mukerjee et al. 2003). Several long-term studies have showed that the outcomes of patients with PAH associated with systemic sclerosis are worse than those of IPAH patients. However, PAH does not represent the only cause of PH in systemic sclerosis. PH due to chronic lung disease such as pulmonary fibrosis and PH due to heart failure such as diastolic dysfunction are also frequent, emphasizing the importance of a complete evaluation to accurately classify and manage PH (Hachulla et al. 2009).

In systemic lupus erythematosus (Tanaka et al. 2002; Asherson et al. 1990) and mixed connective tissue disease (Burdet et al. 1999), the prevalence of PAH remains unknown, but likely occurs less frequently than in systemic sclerosis. Importantly,

corticosteroids and immunosuppressants may improve outcomes in a subset of patients with PAH complicating the course of systemic lupus erythematosus or mixed connective tissue disease, while this is not the case in IPAH or PAH associated with systemic sclerosis (Jais et al. 2008; Sanchez et al. 2006). In the absence of chronic pulmonary parenchymal involvement, PAH has been reported infrequently in other connective tissue diseases such as Sjögren syndrome (Launay et al. 2007), polymyositis (Bunch et al. 1981), or rheumatoid arthritis (Dawson et al. 2000).

4.4 Group 1.4.2: Human Immunodeficiency Virus Infection

Pulmonary arterial hypertension is a rare complication of human immunodeficiency virus (HIV) infection (Speich et al. 1991; Opravil et al. 1997; Mehta et al. 2000; Nunes et al. 2003; Sitbon et al. 2008; Zuber et al. 2004; Opravil and Sereni 2008; Degano et al. 2010). This PAH subcategory is the strongest evidence of virus-induced PAH. The HIV-associated PAH has clinical, hemodynamic, and histologic characteristics similar to those of IPAH. Epidemiologic data in the early 1990s, a time when highly active antiretroviral therapy was not yet available, showed a PAH prevalence of 0.5 % and a dramatic early mortality (Speich et al. 1991; Opravil et al. 1997). Since the wide use of highly antiretroviral therapies, a stable prevalence of 0.46 % was confirmed, while the incidence of PAH has declined (due to the longer survival of the HIV-associated PAH patients) (Sitbon et al. 2008; Opravil and Sereni 2008). Uncontrolled studies show that patients with severe HIV-associated PAH benefit from specific PAH therapies (Degano et al. 2010). Interestingly, normalization of hemodynamics has been regularly reported in a substantial number of the HIV-associated PAH patients treated with specific PAH drugs and highly active antiretroviral therapy (Degano et al. 2010).

4.5 Group 1.4.3: Portopulmonary Hypertension

Portopulmonary hypertension (PoPH) is defined by the development of PAH associated with increased pressure in the portal circulation (Hadengue et al. 1991; Rodriguez-Roisin et al. 2004; Kawut et al. 2008; Le Pavec et al. 2008). Prospective hemodynamic studies have shown that 2–6 % of patients with portal hypertension have PH (Hadengue et al. 1991; Rodriguez-Roisin et al. 2004). As always, RHC is mandatory for the accurate diagnosis of PoPH, because several mechanisms may increase mPAP in the setting of advanced liver disease: hyperdynamic circulatory state with high CO, fluid overload, and diastolic dysfunction; in such circumstances, PVR is within the normal range. Pathologic changes in the small arteries appear identical to those seen in IPAH. A recent multicenter case–control study conducted in the USA identified that female gender and autoimmune hepatitis were independent risk factors for the development of PoPH and that hepatitis C infection was associated

with a decreased risk (Kawut et al. 2008). Long-term prognosis of PoPH was related to the presence and severity of cirrhosis as well as to right heart function in a recently reported large French cohort study (Le Pavec et al. 2008).

4.6 Group 1.4.4: Congenital Heart Diseases

A significant proportion of patients with congenital heart disease, in particular those with systemic-to-pulmonary shunts, will develop PAH if left untreated (Simonneau et al. 2009; Galiè et al. 2009; Wood 1958; Hoffman and Rudolph 1965). Eisenmenger's syndrome is defined as congenital heart disease with an initial large systemic-to-pulmonary shunt that induces progressive pulmonary vascular disease and PAH, with resultant reversal of the shunt, hypoxemia, polycythemia, and cyanosis (Wood 1958; Hoffman and Rudolph 1965). It represents the most advanced form of PAH associated with congenital heart disease. The prevalence of PAH associated with congenital systemic-to-pulmonary shunts in Europe and North America has been estimated to be between 1.6 and 12.5 cases per million adults, with 25–50 % of this population affected by Eisenmenger's syndrome. These numbers are much higher in developing countries. The histological and pathophysiological changes seen in patients with PAH associated with congenital systemic-to-pulmonary shunts, in particular endothelial dysfunction, are similar to those observed in idiopathic or other associated forms of PAH. Following the fourth world symposium on PH, it was decided to update the pathologic and pathophysiological classification of congenital heart disease with systemic-to-pulmonary shunts (Table 4). In order to provide a more detailed description of each condition, four distinct phenotypes have been individualized (Table 5) (Simonneau et al. 2009; Galiè et al. 2009).

4.7 Group 1.4.5: Schistosomiasis

Embolic obstruction of pulmonary arteries by *Schistosoma* eggs was initially thought to be the main mechanism responsible for the development of PH in schistosomiasis (Simonneau et al. 2009; Chaves 1966; Lapa et al. 2009). However, it has been demonstrated that PH associated with schistosomiasis may have a similar clinical presentation and histologic findings than IPAH (Lapa et al. 2009). The mechanisms of PH in patients with schistosomiasis are probably multifactorial including PoPH and local vascular inflammation, whereas mechanical obstruction by *Schistosoma* eggs seems to play a minor role. Therefore, PH associated with schistosomiasis has been included in Group 1 (PAH) in the Dana Point updated classification (Simonneau et al. 2009; Galiè et al. 2009). More than 200 million people are infected by *Schistosoma* worldwide and 4–8 % of the infected individuals subsequently develop hepatosplenic disease. Therefore, PAH associated with schistosomiasis represents a frequent form of the disease,

Table 4 Anatomic-pathophysiological classification of congenital systemic-to-pulmonary shunts associated with PAH. Adapted from Simonneau et al. (2009)

1. Type
1.1. Single pre-tricuspid shunts
1.1.1. Atrial septal defect (ASD)
1.1.1.1. Ostium secundum
1.1.1.2. Sinus venosus
1.1.1.3. Ostium primum
1.1.2. Total or partial unobstructed anomalous pulmonary venous return
1.2. Simple post-tricuspid shunts
1.2.1. Ventricular septal defect (VSD)
1.2.2. Patent ductus arteriosus
1.3. Combined shunts (describe combination and define predominant defect)
1.4. Complex congenital heart disease
1.4.1. Complete atrioventricular septal defect
1.4.2. Truncus arteriosus
1.4.3. Single ventricle physiology with unobstructed pulmonary blood flow
1.4.4. Transposition of the great arteries with VSD (without pulmonary stenosis) and/or patent ductus arteriosus
1.4.5. Other
2. Dimension (specify for each defect if >1 congenital heart defect)
2.1. Hemodynamic (specify Qp/Qs) ^a
2.1.1. Restrictive (pressure gradient across the defect)
2.1.2. Nonrestrictive
2.2. Anatomic
2.2.1. Small to moderate (ASD ≤2.0 cm and VSD ≤1.0 cm)
2.2.2. Large (ASD >2.0 cm and VSD >1.0 cm)
3. Direction of shunt
3.1 Predominantly systemic-to-pulmonary
3.2 Predominantly pulmonary-to-systemic
3.3 Bidirectional
4. Associated cardiac and extracardiac abnormalities
5. Repair status
5.1. Unoperated
5.2. Palliated (specify type of operation(s), age at surgery)
5.3. Repaired (specify type of operation(s), age at surgery)

^aRatio of pulmonary (Op)-to-systemic (Qs) blood flow

especially in countries where the infection is endemic. Data from Brazil based on invasive hemodynamic measurements indicated the prevalence of PAH in patients with hepatosplenic disease of 4.6 % (Lapa et al. 2009) Of note, the prevalence of post-capillary PH was also important (3.0 %) reinforcing the need of RHC to appropriately characterize PH (Lapa et al. 2009).

Table 5 Clinical classification of congenital systemic-to-pulmonary shunts associated with PAH. Adapted from Simonneau et al. (2009)

A. Eisenmenger's syndrome	Includes all systemic-to-pulmonary shunts resulting from large defects and leading to a severe increase in PVR and a reversed (pulmonary-to-systemic) or bidirectional shunt; cyanosis, erythrocytosis, and multiple organ involvement are present
B. PAH associated with systemic-to-pulmonary shunts	Includes moderate to large defects; PVR is mildly to moderately increased, systemic-to-pulmonary shunt is still prevalent, and no cyanosis is present at rest
C. PAH with small defects	Small defects (usually ventricular septal defects <1 cm and atrial septal defects <2 cm of effective diameter assessed by echocardiography); clinical picture is very similar to idiopathic PAH
D. PAH after corrective cardiac surgery	Congenital heart disease has been corrected, but PAH is still present immediately after surgery or recurs several months or years after surgery in the absence of significant postoperative residual lesions

PAH pulmonary arterial hypertension; PVR pulmonary vascular resistance

4.8 Group 1.4.6: Chronic Hemolytic Anemia

There has been increasing evidence that PH is a complication of chronic hereditary and acquired hemolytic anemias, including sickle cell disease (Castro et al. 2003; Gladwin et al. 2004; Parent et al. 2011; Fonseca et al. 2012) and thalassemia (Aessopos et al. 1995). Pulmonary hypertension has been reported most frequently in patients with sickle cell disease; however, the prevalence of PAH is not clearly established. A large US study of patients with sickle cell disease, which screened PH by means of Doppler echocardiography by the presence of tricuspid regurgitation jet velocity (TRV) ≥ 2.5 m/s, found that 32 % of patients met this criteria (Gladwin et al. 2004). However, TRV ≥ 2.5 m/s is low threshold and leads to a substantial number of false-positive cases of PH if not confirmed by RHC (Parent et al. 2011; Fonseca et al. 2012). In this study, RHC was carried out in only 18 of 63 patients with TRV ≥ 2.5 m/s and PH defined by a mPAP ≥ 25 mmHg was confirmed in 17 patients (Gladwin et al. 2004). Nevertheless, PWP was elevated in a number of patients, emphasizing the complex mechanisms of PH in sickle cell patients. Indeed, a substantial proportion of sickle cell disease patients develop post-capillary PH (Parent et al. 2011). In addition, some patients present with a hyperkinetic state with moderate elevation in mPAP and normal PVR. Thus, the prevalence of pre-capillary PH in sickle cell disease is undoubtedly much lower than 32 %.

Two recent studies (Parent et al. 2011; Fonseca et al. 2012) have addressed the precise prevalence of pre- and post-capillary PH in sickle cell disease. In these two studies, patients with sickle cell disease underwent Doppler echocardiography with measurement of TRV. RHC had to be performed in all patients in whom PH was suspected on the basis of TRV ≥ 2.5 m/s. Pulmonary hypertension was defined as a

mPAP \geq 25 mmHg. The results of these two studies performed in France (Gladwin et al. 2004) and in Brazil (Parent et al. 2011) are remarkably similar showing a prevalence of PH of 6.2 % and 10 %, respectively, which was lower than previously reported. Interestingly, post-capillary PH was the most frequent cause with a prevalence of 3.3 % and 6.2 %, respectively, whereas the prevalence of pre-capillary PH was only 2.9 % and 3.8 %, respectively (Parent et al. 2011; Fonseca et al. 2012). In addition, these two studies clearly demonstrated that when a threshold TRV of \geq 2.5 m/s was used to define PH, the positive predictive value of echocardiography for the detection of pulmonary hypertension was only 25 % and 32 %, respectively (Parent et al. 2011; Fonseca et al. 2012). Lastly, pre-capillary PH associated with sickle cell disease appears substantially different from other forms of PAH, in terms of both hemodynamic profile and response to specific PAH therapies. These observations call into question the rationale of keeping sickle cell disease in group 1 (PAH) of the clinical PH classification (Parent et al. 2011).

4.9 Lessons from the French PAH Registry in the Modern Management era

Pulmonary arterial hypertension is a rare and severe vascular disease for which the trend is to manage the patients in designated centers with multidisciplinary teams working in a shared-care approach (Humbert et al. 2006; Humbert et al. 2010a; Humbert et al. 2010b). The French registry was initiated in 17 university hospitals following at least 5 newly diagnosed patients per year. All consecutive adult (\geq 18 years) patients seen between October 2002 and October 2003 were to be included and followed up for at least 3 years: 674 patients (age of 50 ± 15 years (mean \pm SD); range 18–85) were entered in the registry. Idiopathic, familial, anorexigen, connective tissue diseases, congenital heart diseases, portal hypertension, and HIV-associated PAH accounted for 39.2 %, 3.9 %, 9.5 %, 15.3 %, 11.3 %, 10.4 %, and 6.2 % of the population, respectively (Humbert et al. 2006). At the time of diagnosis, 75 % of patients were in the New York Heart Association (NYHA) functional class III or IV (Humbert et al. 2006). Six-minute walk distance was 329 ± 109 m. mPAP, cardiac index, and PVR index were 55 ± 15 mmHg, 2.5 ± 0.8 L/min/m², and 20.5 ± 10.2 mmHg/L/min/m², respectively (Humbert et al. 2006). Delay between symptom onset (mainly dyspnea on exercise) and PAH diagnosis was still \geq 2 years (27 months), similar to that observed in the National Institutes of Health Registry in the 1980s, emphasizing the need for better PAH awareness and diagnostic strategy (Humbert et al. 2006). The low estimates of prevalence and incidence of PAH in France were 15.0 cases/million of adult population and 2.4 cases/million of adults per year, respectively (Humbert et al. 2006). Among the 354 consecutive adult patients with idiopathic, familial, or anorexigen-induced PAH who were prospectively enrolled, 56 had incident disease (PAH diagnosis was made at the time of recruitment in the registry) and

298 were prevalent cases (PAH diagnosis was made months or years before recruitment in the registry) (Humbert et al. 2010a; Humbert et al. 2010b). Patients were followed for 3 years and survival rates were analyzed. For incident idiopathic, familial, and anorexigen-induced PAH, estimated survival (95 % confidence intervals) at 1, 2, and 3 years was 85.7 % (76.5–94.9 %), 69.6 % (57.6–81.6 %), and 54.9 % (41.8–68.0 %), respectively (Humbert et al. 2010a). In a combined analysis population (incident patients and prevalent patients diagnosed within 3 years prior to study entry; $n = 190$), 1-, 2-, and 3-year survival estimates were 82.9 % (72.4 %–95.0 %), 67.1 % (57.1 %–78.8 %), and 58.2 % (49.0 %–69.3 %), respectively (Humbert et al. 2010a). Individual survival analysis identified the following factors as significantly and positively associated with survival: being female, NYHA functional class I/II, a greater six-minute walk distance, a lower right atrial pressure, and a higher cardiac output (Humbert et al. 2010a). Multivariable analysis showed that being female and having a greater six-minute walk distance and a higher cardiac output were jointly significantly associated with improved survival (Humbert et al. 2010a). This contemporary registry highlights current practice and shows that PAH is still detected late in the course of the disease with a majority of patients displaying severe functional and hemodynamic compromise. In the modern management era, idiopathic, familial, and anorexigen-induced PAH remains a progressive, fatal disease. Mortality is most closely associated with male gender, right ventricular hemodynamic function, and exercise limitation (Humbert et al. 2010a; Humbert et al. 2010b).

5 Group 1': Pulmonary Veno-Occlusive Disease and Pulmonary Capillary Hemangiomas

Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomas (PCH) are uncommon conditions, but they are increasingly recognized as causes of PH (Montani et al. 2008). Similarities in pathological features and clinical presentation suggest that PVOD and PCH may in fact overlap, and it has been shown that PCH may be an angioproliferative process frequently associated with PVOD (Lantuejoul et al. 2006). Both conditions are difficult to categorize, as they share characteristics with PAH but also have a number of distinct differences. Given the current evidence, it has been decided at the Dana Point world symposium that PVOD/PCH should be a distinct category but not completely separated from PAH, and they are currently designated as a category 1' in the updated classification (Simonneau et al. 2009; Galiè et al. 2009).

Pulmonary veno-occlusive disease and pulmonary capillary hemangiomas share a number of characteristics with PAH. First, some histologic changes in the small pulmonary arteries (intimal fibrosis and medial hypertrophy) observed in PAH are also found in PVOD/PCH. Second, clinical presentation and hemodynamic characteristics of PVOD/PCH and PAH are often indistinguishable (Montani et al. 2008; Montani et al. 2009). Third, PVOD/PCH and PAH share similar risk

factors, including connective tissue diseases such as systemic sclerosis (Dorfmüller et al. 2007; Günther et al. 2012), HIV infection (Escamilla et al. 1995), and anorexigen intake (Montani et al. 2009). Last, familial occurrence has been reported in both PVOD/PCH, and mutations in the *BMPR2* gene have been documented in patients with PVOD (Montani et al. 2008; Montani et al. 2009). These findings suggest that PVOD, PCH, and PAH may represent different components of a single spectrum of disease.

Although PVOD/PCH may present similarly to PAH, there are also a number of important differences. These include the presence of crackles on clinical examination, radiologic abnormalities best seen on high-resolution computed tomography of the chest (nodular ground glass opacities, septal thickening, mediastinal lymph node enlargement) (Montani et al. 2008; Montani et al. 2009), hemosiderin-laden macrophages on bronchoalveolar lavage (Rabiller et al. 2006), a more pronounced hypoxemia, and a lower diffusing capacity for carbon monoxide (DLCO) in patients with PVOD/PCH (Montani et al. 2008; Montani et al. 2009). In addition, the response to PAH therapy and prognosis of PVOD/PCH are worse than in PAH, with an increased risk of pulmonary edema with continuous intravenous epoprostenol, calcium-channel blockers, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors (Montani et al. 2008; Montani et al. 2009; Humbert et al. 1998).

6 Group 2: Pulmonary Hypertension due to Left Heart Disease

Left-sided ventricular or valvular diseases may produce an increase in left atrial pressure, leading to a backward transmission of the pressure and a passive increase of pulmonary pressures. Left heart disease probably represents the most frequent cause of PH (Simonneau et al. 2009; Galiè et al. 2009; Oudiz 2007). In this situation, PVR is normal or near normal (< 3 mmHg/L/min) and there is no gradient between mPAP and PWP ($TPG \leq 12$ mmHg) (Galiè et al. 2009). The increasing recognition of the left-sided heart dysfunction with a preserved ejection fraction has led to changes in the subcategories of Group 2, which now includes left heart systolic dysfunction, left heart diastolic dysfunction, and valvular diseases (Simonneau et al. 2009; Galiè et al. 2009). In some patients with left heart disease, the elevation of mPAP is out of proportion to that expected from the elevation of left arterial pressure ($TPG > 12$ mmHg) and PVR is higher than 3 mmHg/L/min (Galiè et al. 2009). Some patients with valvular disease or left heart dysfunction can develop severe PH of the same magnitude as that seen in PAH (Zener et al. 1972). The elevation of mPAP and PVR may be due to either the increase of pulmonary artery vasomotor tone and/or pulmonary vascular remodeling (Delgado et al. 2005; Moraes et al. 2000). No large, randomized controlled trial using medications approved for PAH have been performed in this patient population, and the efficacy and safety of PAH medications are currently unknown.

7 Group 3: Pulmonary Hypertension due to Lung Diseases and/or Hypoxia

In this group, the predominant cause of PH is alveolar hypoxia as a result of either chronic lung disease, impaired control of breathing, or living at high altitudes (Simonneau et al. 2009; Galiè et al. 2009; Weitzenblum et al. 1981; Thabut et al. 2005; Chaouat et al. 2005; Chaouat et al. 2008; Cottin et al. 2005). However, the precise prevalence of PH in all these conditions remains largely unknown. In the updated PH classification, a category of lung disease characterized by a mixed obstructive and restrictive pattern was added, including combined pulmonary fibrosis and emphysema (Simonneau et al. 2009; Cottin et al. 2005). In PH associated with parenchymal lung disease, the increase of mPAP is usually modest (mPAP < 35 mmHg) (Simonneau et al. 2009; Weitzenblum et al. 1981; Chaouat et al. 2008). In a retrospective study of 998 patients with chronic obstructive pulmonary disease who underwent RHC, only 1 % had severe PH defined as mPAP above 40 mmHg (Chaouat et al. 2005). These patients with severe PH had a mild-to-moderate airway obstruction, severe hypoxemia, hypocapnia, and a very low DLCO (Thabut et al. 2005; Chaouat et al. 2005). Large, randomized controlled trials of PAH therapies in patients with parenchymal lung disease and severe PH have not been performed, and there is no current recommendation supporting the use of PAH drugs in this group of patients (Humbert and Simonneau 2010).

8 Group 4: Chronic Thromboembolic Pulmonary Hypertension

Even if the exact incidence and prevalence of chronic thromboembolic pulmonary hypertension (CTEPH) are uncertain, it represents a frequent cause of PH and occurs in up to 4 % of patients after an acute pulmonary embolism (Tapson and Humbert 2006; Pengo et al. 2004). In the previous classification, CTEPH was divided into two subgroups: proximal CTEPH and distal CTEPH, depending on the technical possibility to manage PH by means of surgical pulmonary endarterectomy (Simonneau et al. 2009). However, there is no clear consensus about the exact definitions of proximal and distal CTEPH, and the decision regarding surgical treatment may vary depending on individual centers (Simonneau et al. 2009). Thus, in the new classification, it was decided to keep in Group 4 only a single category of CTEPH without distinction between proximal and distal forms (Simonneau et al. 2009; Galiè et al. 2009). Patients with suspected or confirmed CTEPH need to be referred to a center with expertise in the medical and surgical management of this condition, in order to consider the feasibility of performing surgery. The latter depends on the location of the obstruction, the correlation between hemodynamic findings, and the degree of mechanical obstruction assessed by pulmonary angiography, comorbidities, the willingness of the patient, and the experience of the medical and surgical teams

(Simonneau et al. 2009; Galiè et al. 2009; Kim 2006; Darteville et al. 2004; Jamieson et al. 2003). Patients who are not candidates for surgery may benefit from off-label use of PAH medical therapy (Suntharalingam et al. 2008; Rubin et al. 2006). However, further evaluation of these therapies in randomized control trials is needed (Rubin et al. 2006).

The clinical characteristics and management of patients enrolled in an international CTEPH registry have been recently reported (Pepke-Zaba et al. 2011). The international registry included 679 newly diagnosed (≤ 6 months) consecutive patients with CTEPH (February 2007 to January 2009). Diagnosis was confirmed by RHC, ventilation–perfusion lung scintigraphy, computerized tomography, and/or pulmonary angiography. At diagnosis, a median of 14.1 months had passed since first symptoms; 427 patients (62.9 %) were considered operable, 247 (36.4 %) non-operable, and 5 (0.7 %) had no operability data; 386 patients (56.8 %, ranging 12.0–60.9 % across countries) underwent surgery (Pepke-Zaba et al. 2011). Operable patients did not differ from non-operable patients relative to symptoms, NYHA functional class, and hemodynamics. A history of acute pulmonary embolism was reported for 74.8 % of patients (77.5 % operable, 70 % non-operable) (Pepke-Zaba et al. 2011). Associated conditions included thrombophilic disorder in 31.9 % (37.1 % operable, 23.5 % non-operable) and splenectomy in 3.4 % of patients (1.9 % operable, 5.7 % non-operable) (Pepke-Zaba et al. 2011). At the time of CTEPH diagnosis, 37.7 % of patients initiated at least one PAH therapy (28.3 % operable, 53.8 % non-operable) (Pepke-Zaba et al. 2011). Pulmonary endarterectomy was performed with a 4.7 % documented mortality rate (Pepke-Zaba et al. 2011). Long-term follow-up of this CTEPH cohort is currently being studied with a goal to analyze outcomes according to clinical presentation and treatment strategy.

9 Group 5: Pulmonary Hypertension with Unclear or Multifactorial Etiologies

9.1 Group 5.1: Hematologic Disorders

Pulmonary hypertension has been reported in chronic myeloproliferative disorders including polycythemia vera, essential thrombocythemia, and chronic myelogenous leukemia (Guilpain et al. 2008; Adir and Humbert 2010). Several mechanisms may be implicated in PH associated with chronic myeloproliferative disorders including high cardiac output, spleen involvement (asplenia or spleen enlargement), direct obstruction of pulmonary arteries by circulating megakaryocytes, CTEPH, portopulmonary PH, drug-induced PAH (dasatinib), and congestive heart failure (Montani et al. 2012; Adir and Humbert 2010). Splenectomy, as a result of trauma or as a treatment for hematologic disorders, may increase the risk of developing PH (Adir and Humbert 2010). CTEPH and several cases of PAH with medial hypertrophy, intimal fibrosis, and plexiform lesions in the pulmonary vasculature have been reported in association with splenectomy (Hoepfer et al. 1999; Jais et al. 2005).

9.2 Group 5.2: Systemic Disorders

The second subgroup includes systemic disorders, including sarcoidosis, pulmonary Langerhans' cell histiocytosis, lymphangioleiomyomatosis, neurofibromatosis, or vasculitis (Simonneau et al. 2009; Galiè et al. 2009).

Sarcoidosis is a common systemic granulomatous disease of an unknown etiology. PH is an increasingly recognized complication of sarcoidosis, with a reported prevalence of 1–28 % (Shorr et al. 2005; Nunes et al. 2006). PH is often attributed to the destruction of the capillary bed by the pulmonary fibrotic process and/or results from chronic hypoxia (Shorr et al. 2005; Nunes et al. 2006). However, the severity of PH does not occasionally correlate to the severity of parenchymal lung disease and blood gas abnormalities, suggesting that other mechanisms may contribute to the development of PH (Nunes et al. 2006). Among these mechanisms, one can consider extrinsic compression of large pulmonary vessels by lymph node enlargement or mediastinal fibrosis, granulomatous infiltration of the pulmonary vasculature, affecting especially the pulmonary veins (which sometimes mimics PVOD), cardiac sarcoidosis which may cause heart failure and post-capillary PH, and hepatic sarcoidosis which may cause portopulmonary PH (Shorr et al. 2005; Nunes et al. 2006).

Pulmonary Langerhans' cell histiocytosis is an uncommon lung disease that predominantly affects young adults and develops almost exclusively in those with a history of current or prior cigarette smoking. In pulmonary Langerhans' cell histiocytosis, pre-capillary PH is frequently observed in patients with advanced lung destruction, though no clear relationship exists between PH and the extent of parenchymal lung disease and/or hypoxia (Fartoukh et al. 2000). This observation suggests that alternative or additional mechanisms contribute to an intrinsic pulmonary vasculopathy that involves both the pre-capillary arterioles and post-capillary venous compartment, in addition to possible PVOD-like lesions (Fartoukh et al. 2000). Patients with pulmonary Langerhans' cell histiocytosis that develop PH have a particularly poor prognosis and an early referral for lung transplantation assessment is recommended (Fartoukh et al. 2000; Le Pavec et al. 2012). Recent encouraging data suggest that agents licensed for use in PAH confer improvements in pulmonary hemodynamics and are generally well tolerated. Further investigations into the use of PAH medical therapy in this population are warranted (Le Pavec et al. 2012).

Lymphangioleiomyomatosis is a rare multisystem disorder predominantly affecting women, characterized by cystic lung destruction, lymphatic abnormalities, and abdominal tumors. PH is relatively uncommon in patients with lymphangioleiomyomatosis (Cottin et al. 2012). A retrospective, multicenter study evaluated 20 patients with lymphangioleiomyomatosis and pre-capillary PH (Cottin et al. 2012). This study confirmed that PH of mild hemodynamic severity may occur in patients with lymphangioleiomyomatosis, even with mild pulmonary function impairment (Cottin et al. 2012). Off-label use of PAH therapies might improve hemodynamic parameters but requires further investigations (Cottin et al. 2012).

Neurofibromatosis type 1, also known as von Recklinghausen's disease, is an autosomal dominant disease that can be recognized by characteristic "café au lait" skin lesions and by cutaneous fibromas (Montani et al. 2011; Stewart et al. 2007). Neurofibromatosis type 1 is occasionally complicated by systemic vasculopathy. Several cases of PH have recently been reported in patients with von Recklinghausen's disease (Montani et al. 2011; Stewart et al. 2007). The mechanism of PH is unclear, and lung fibrosis and CTEPH may play a role in the development of PH. In rare cases, histological examination found both arteries and veins narrowed by medial and/or intimal hypertrophy and fibrosis (Montani et al. 2011; Stewart et al. 2007).

Last, some rare cases of PH have been observed in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis with clinical presentation similar to PAH; however, histological data are not available (Launay et al. 2006).

9.3 Group 5.3: Metabolic Disorders

PH has been reported in a few cases of type Ia glycogen storage disease, a rare autosomal recessive disorder caused by a deficiency of glucose-6-phosphatase (Hamaoka et al. 1990; Humbert et al. 2002; Pizzo 1980). The mechanisms of PH are uncertain, but portocaval shunts, atrial septal defects, severe restrictive pulmonary disease, or thromboembolic disease are thought to play a role. In one case, postmortem examination revealed the presence of plexiform lesions (Pizzo 1980).

Gaucher's disease is a rare disorder characterized by a deficiency of lysosomal B glucosidase, which results in an accumulation of glucocerebroside in reticuloendothelial cells. PH has been reported in Gaucher's disease with several potential mechanisms, including interstitial lung disease, chronic hypoxia, capillary plugging by Gaucher's cells, and splenectomy (Elstein et al. 1998; Theise and Ursell 1990).

The association between thyroid diseases and PH has been reported in a number of studies (Badesch et al. 1993; Chu et al. 2002; Li et al. 2007). High prevalence of autoimmune hypo- and hyperthyroidism suggests that these conditions may share a common (auto)immune susceptibility (Badesch et al. 1993; Chu et al. 2002; Li et al. 2007).

9.4 Group 5.4: Miscellaneous Conditions

The last subgroup includes a number of miscellaneous conditions, including tumoral extrinsic or intrinsic obstruction, fibrosing mediastinitis, and chronic renal failure with dialysis.

A progressive obstruction of proximal pulmonary arteries leading to PH may be observed when a tumor grows into the central pulmonary arteries with additional thrombosis. Such cases are mainly associated with pulmonary artery sarcomas (Anderson et al. 1995; Mayer et al. 2001). The differential diagnosis with CTEPH can be difficult and findings on angiography by computed tomography or

magnetic resonance imaging, as well as 18 F-fluorodeoxyglucose positron emission tomography, may be useful to differentiate an obstruction by tumor or thrombotic material. Occlusion of the microvasculature by metastatic tumor emboli represents another rare cause of rapidly progressive PH (Roberts et al. 2003; Dot et al. 2007). The initial laboratory evaluation often shows severe hypoxemia. CT scanning does not reveal proximal thrombi but often shows thickening of septa. In contrast, the ventilation/perfusion lung scan is generally abnormal with multiple subsegmental perfusion defects. Pulmonary microvascular cytology sampling through a pulmonary artery catheter in the wedge position is an important diagnostic tool (Dot et al. 2007). The majority of reported cases occur in association with breast, lung, or gastric carcinoma (Roberts et al. 2003; Dot et al. 2007).

Fibrosing mediastinitis may be associated with severe PH due to compression of both pulmonary arteries and veins (Davis et al. 2001; Loyd et al. 1988; Goodwin et al. 1972). Ventilation/perfusion lung scan, computerized tomography of the chest, and pulmonary angiography are useful for accurate diagnosis; however, findings can mimic proximal thrombotic obstruction (Seferian et al. 2012). The predominant etiologies are histoplasmosis (Loyd et al. 1988; Goodwin et al. 1972), tuberculosis (Goodwin et al. 1972; Seferian et al. 2012), and sarcoidosis (Nunes et al. 2006).

Lastly, PH has been reported in patients with end-stage renal disease maintained on long-term hemodialysis (Yigla et al. 2003). Based on echocardiographic studies, elevated estimated PAP may be high in this patient population (Yigla et al. 2003). There are several potential explanations for the development of PH in these patients: mPAP may be increased by high CO (resulting from the arteriovenous access and anemia), as well as fluid overload. In addition, diastolic and systolic left heart dysfunctions are also frequent in this setting, leading to a significant proportion of post-capillary PH (Yigla et al. 2003; Nakhoul et al. 2005). Furthermore, hormonal and metabolic derangement associated with end-stage renal disease might promote dysfunction of pulmonary vascular tone.

10 Conclusion

Since the mid-twentieth century, a significant progress has been made in the PH field starting from the development of RHC techniques and the first description of PPH. The National Institutes of Health Registry and the world symposiums on PH have played an important role in this process. The most common causes of PH remain post-capillary PH due to left heart diseases (group 2) and pre-capillary PH due to chronic lung diseases and/or hypoxia (group 3). Besides these two common causes, CTEPH is of a major importance because it can be surgically managed. Lastly, PAH patients should be identified and classified early, as several targeted therapies have been approved in this group of diseases. The proceedings of the fifth world PH symposium will highlight current knowledge of PH that should help the international community to better manage PH patients (Humbert 2012).

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Pulmonary Hypertension: Pathophysiology and Signaling Pathways

Bradley A. Maron and Joseph Loscalzo

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 ₄	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 _A	Endothelin-type A receptor
ET _B	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 [•]	Nitric oxide
NOX	NADPH oxidase

$\cdot\text{O}_2^-$	Superoxide
O_2NOO^-	Peroxynitrate
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- γ	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
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 (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

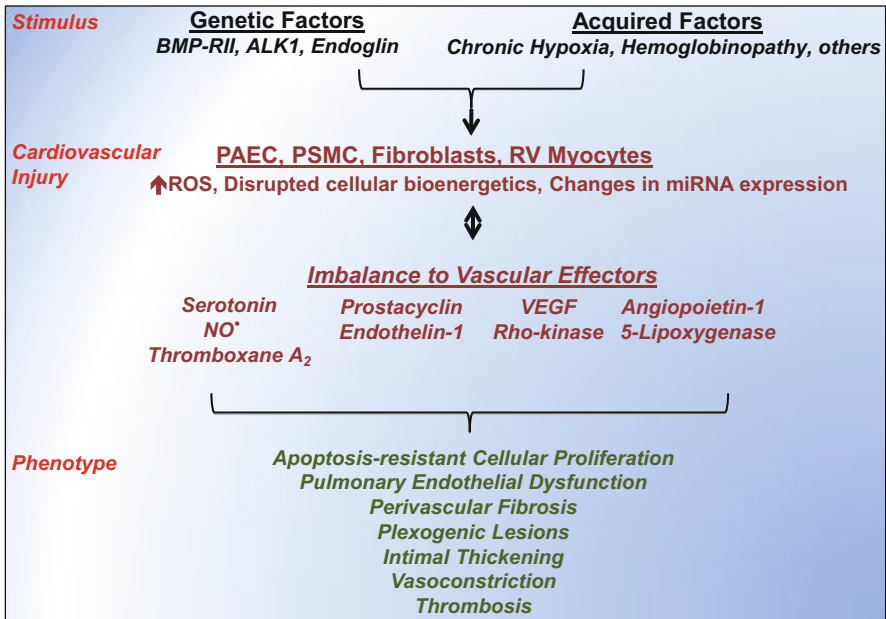


Fig. 1 Pathobiology of pulmonary arterial hypertension. The “two-hit” hypothesis of PAH contends that in the presence of certain genetic and/or molecular risk factors, exposure to environmental and/or biological stimulators of pulmonary/cardiovascular injury increases oxidant stress levels, promotes 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

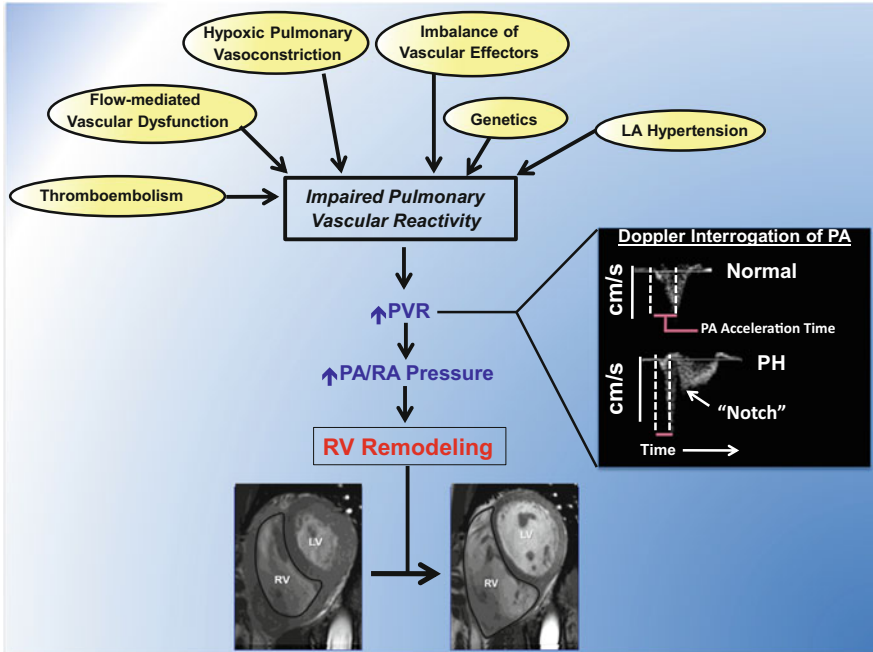


Fig. 2 Pathophysiology of pulmonary hypertension. Pulmonary hypertension (PH) is characterized by impaired pulmonary vascular reactivity due to a heterogeneous range of pathological cellular/molecular processes that mediate pulmonary vascular injury. Pulmonary vascular dysfunction in PH is detected clinically 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^{*}) 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^{*} 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^{*} 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 Ca^{2+} levels and, subsequently, levels of 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 (Stuedel et al. 1998; Gangopahyay et al. 2011). Specifically, loss of 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 α (HIF-1 α), 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 [BH_4]) necessary for normal eNOS function. Under these conditions, eNOS is ‘uncoupled,’ resulting in the preferential generation of superoxide (O_2^-) over 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 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 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 PaO₂-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[•]-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., BH₄), or inactivate NO[•] such as in the case of [•]O₂⁻ which reacts with NO[•] to generate peroxynitrite (ONOO⁻). Additionally, the interaction of [•]O₂⁻ with the stable NO[•] by-product nitrite (NO₂⁻) forms peroxynitrate (O₂NOO⁻) and, thus, decreases levels of NO₂⁻, which is a key substrate for NOS-independent synthesis of NO[•] (2HNO₂→N₂O₃ + H₂O; N₂O₃→NO[•] + NO₂[•]) (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*^{+/-}) develop PH spontaneously in vivo due, in part, to increased pulmonary vascular ROS generation, eNOS uncoupling, and decreased NOS-inhibitable NO[•] 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- β , 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^{*} (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_A) and -type B (ET_B) receptors in PSMCs and vasodilatory ET_B receptors in PAECs, which do not constitutively express ET_A. Endothelin-type A and ET_B 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_A and ET_B 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 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_B 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 (α_1/β_1 or α_1/β_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 (Fe^{2+}) prosthetic group of sGC results in the formation of a hexa-coordinated histidine–heme–NO[•] intermediate. Subsequent cleavage of the heme–histidine bond leads to a dramatic upregulation of enzyme activity: nanomolar concentrations of NO[•] 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 (Fe^{2+}) to ferric (Fe^{3+}) state that converts sGC to an NO[•]-insensitive state, presumably owing to decreased affinity of NO[•] 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 H₂O₂ induce the formation of higher cysteinyl thiol oxidative states of Cys-122 on the β_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[•]-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 α_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 μM 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[•] vasodilatory signaling and NO[•]-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 (RiociguatTM) 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⁻¹) over 12 weeks decreased pulmonary vascular resistance by 215 dyne s cm⁻⁵, 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 GAF¹ 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 μM (pH = 5.2) to 0.15 μM (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).

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

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

Isoenzyme	K_m cGMP (μM)	V_{\max} cGMP (μM)	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^{*}, 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 lipooxygenase (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

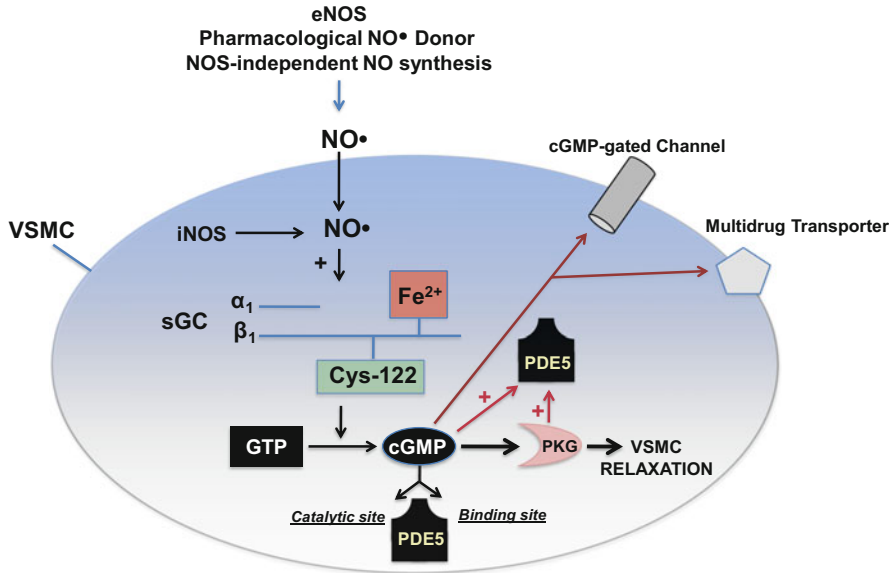


Fig. 3 Influence of phosphodiesterase type-5 on the nitric oxide signaling axis. Nitric oxide (NO[•]) derived from endothelial nitric oxide synthase (eNOS), pharmacological NO[•] donors, or via induction of inducible NOS in vascular smooth muscle cells (VSMCs) activates soluble guanylyl cyclase (sGC) 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 β₁-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 A₂ (TXA₂), and prostaglandin I₂ (prostacyclin), which exerts opposing effects to TXA₂, including vasodilation and inhibition of platelet activation. Prostacyclin is synthesized from PGH₂ 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 TXA₂/PGI₂ 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 PGI₂ synthase mRNA and protein expression levels (Tuder et al. 1999). In contrast, elevated levels of

TXA₂ and increased pulmonary vascular *sensitivity* to TXA₂ 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 B₂, a stable metabolite of TXA₂, and decreased levels of the stable prostacyclin metabolite, 2,3-dinor-6-keto-prostaglandin F1 α (Christman et al. 1992).

Shear stress, ET-1, hypoxia, and BMP-RII dysfunction are each associated with overactivation of the TXA₂ synthesis pathway relative to PGI₂ 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_A 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 B₄, for example, exerts both chemotactic and chemokinetic activity on polymorphonuclear leukocytes and eosinophils (Ford-Hutchinson et al. 1980), and leukotrienes C₄, D₄, and E₄ (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 (O₂) via the tricarboxylic acid (TCA) cycle and β -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 H_2O (Wallace 2005). Protons are pumped across the inner mitochondrial membrane to establish the significantly negative electrochemical gradient ($\Delta\psi_m$: ~ -200 mV) across that membrane, which provides the electromotive force necessary for ATP synthesis.

Changes to mitochondrial membrane permeability, and, hence, the normal $\Delta\psi_m$, 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., NADH/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 α (Archer et al. 2008). In turn, HIF-1 α 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 NO^* (Fijalkowska et al. 2010). Interestingly, in these experiments, increased HIF-1 α expression correlated inversely with low numbers of mitochondria, indicating that negative control of mitochondrial biogenesis by HIF-1 α 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 $\cdot\text{O}_2^-$ that is dismutated to form H_2O_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 H_2O_2 generation, opening of O_2 -sensitive Kv1.5 channels, and subsequent activation of L-type 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 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 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- γ

Peroxisome proliferator-activated receptor (PPAR- γ) 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- γ , 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

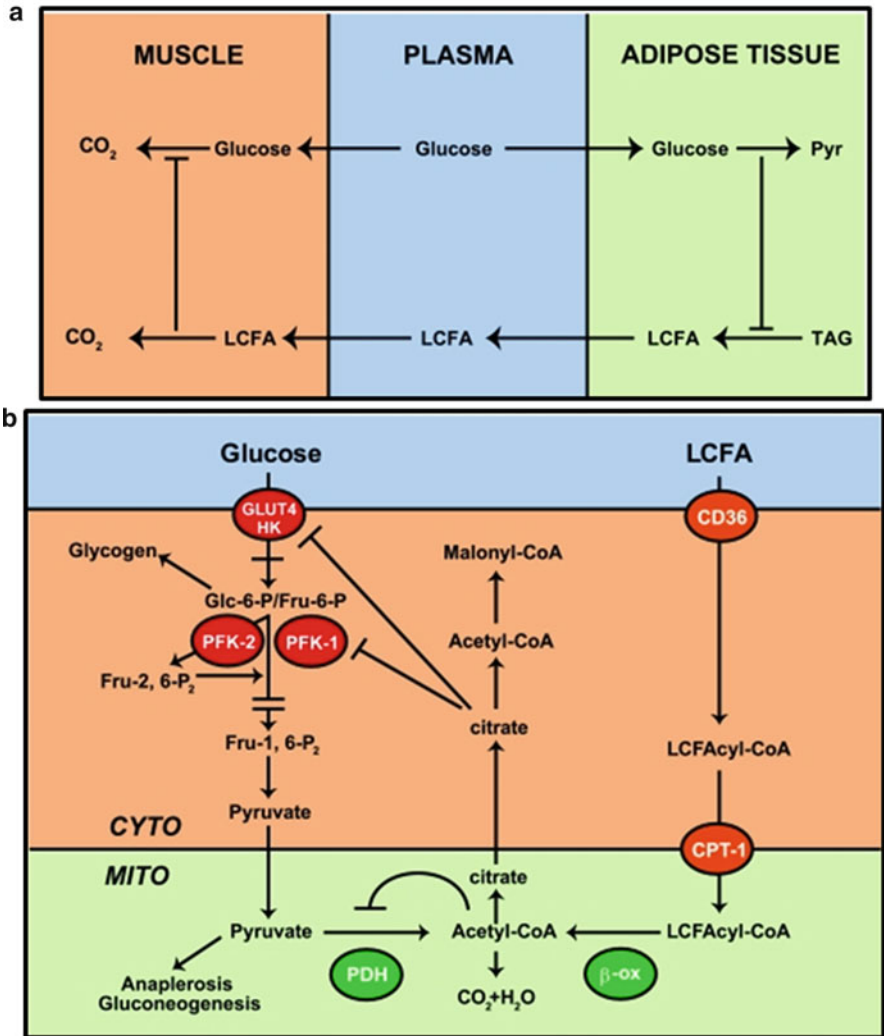


Fig. 4 Mitochondrial bioenergetics. (a) The “glucose–fatty acid cycle” or *Randle Cycle* describes a mechanism to maintain homeostatic control of circulating levels of glucose and fatty acids. (b) Inhibition of glucose utilization by fatty acid oxidation is regulated most strongly by pyruvate dehydrogenase (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 1* carnitine palmitoyltransferase I, *β-ox* β-oxidation. Reproduced with permission from Hue et al. (2009) *Am J Physiol Endocrinol Metab* 297:E578–E591

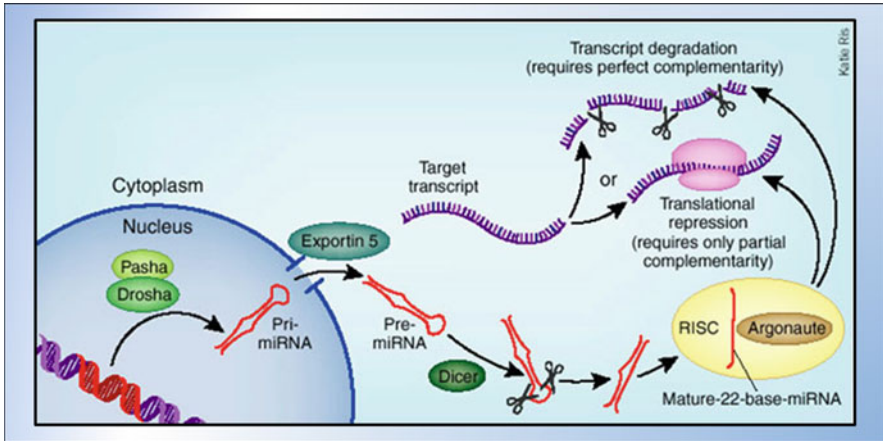


Fig. 5 MicroRNA processing. MicroRNA (miRNA) transcription in the nucleus is facilitated by the RNase III enzymes, drosha and pasha, to generate hairpin-looped molecules known as primary miRNAs (pri-miRNA), which are processed further to form miRNA precursors (pre-miRNA). Once exported from the nucleus 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- γ binding to DNA, which, in turn, stimulates apoE synthesis and secretion (Hansmann et al. 2007). Transgenic ApoE knockout mice (ApoE^{-/-}) fed a high-fat diet demonstrate spontaneous development of PH, which is reversible through pharmacological stimulation of PPAR- γ with pioglitazone (Hansmann et al. 2007). In human PAECs, BMP-RII signaling appears to induce a PPAR- γ / β -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 α -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 α -dependent disruptions to electron transport chain function (Chan et al. 2009). Importantly, HIF-1 α 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 α (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- α 1C expression through a mechanism involving the interaction of miR-328 with the 3' untranslated region of the L-type calcium channel- α 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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Pulmonary Hypertension: Pathology

Peter Dorfmueller

Abstract Pulmonary hypertension (PH) is a life-threatening and often fatal disease, characterized by elevated pulmonary vascular resistance and secondary right ventricular failure. Since etiologies of PH are multiple and its pathogenesis is complex, histology from lungs of patients with PH may help us to determine different etiological factors of the disease. The degree of involvement of various cell types and structures within the lung tissue represents an important indicator of the pathophysiological process. So even if the role for pathologists in routine management of PH is limited, lessons can be learned from morphology. The present chapter outlines the current understanding of this disease from the pathologist's point of view.

Keywords Pulmonary hypertension • Histology • Intimal fibrosis • Plexiform lesion • Medial hypertrophy • Pulmonary veno-occlusive disease

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1 Introduction

The morphologic study of diseased lungs from patients with PH represents an important landmark on the chart of pathophysiologic concepts: a real status and probably the histopathologic correlate to increased pulmonary vascular resistance. The knowledge of pathological anatomy in PH must be of importance to the researcher, as well as to the clinical physician, since observation and interpretation always precede deduction and effective action. Therefore, the histologic inventory of PH vasculopathy has been addressed in the consensus papers of the past World Symposia (Simonneau et al. 2004, 2009). Despite these publications being enriched with up-to-date scientific knowledge from the scientific community, the foundations of descriptive histopathology reach back to the 1960s and 1970s, when such historical figures as Heath, Edwards, or Wagenvoort performed their meticulous studies on large case numbers (Heath and Edwards 1958; Wagenvoort and Wagenvoort 1970). Nonetheless, recent histology-oriented papers have helped to differentiate morphological phenotypes and peculiarities in PH subgroups, stimulating the ongoing discussion within the PH community. The following account of histological observations is paralleled with historical and actual concepts of PH pathophysiology.

2 Arterial Lesions

The pulmonary arterial tree comprises at least three levels with different anatomical/histological architectural pattern. Arteries from the main arterial trunk (27 mm in diameter) to the segmentary level (0.5 mm in diameter) belong to the elastic type, characterized by an important proportion of elastic fibers within the medial layer of the vessel wall. Since their architecture is similar to large systemic arteries, chronic vessel wall stress through increased pressure will manifest in atheromatous lesions as found, e.g., in the aorta of patients with systemic hypertension. In patients displaying PH all stages from subendothelial foamy macrophages to large atheroma plaques can be encountered, even if such lesions will never reach the significance and severity of aortic plaques. Also, occlusive or near-occlusive lesions as they can be seen in carotid arteries are usually not found in PH. The increase in pulmonary vascular resistances is most probably due to the distal arterial level, comprising arteries of the muscular type:

the proportion of elastic fibers within the medial layer decreases and the share of smooth muscle cells will increase in vessels beneath 0.5 mm in diameter. From here on, peculiar lesions of the vessel wall can be found in patients with PH, lesions with different microscopic appearance, owing to the difference of the prevailing cell type. Those different cell types are physiologically present and some of them even constitute the three arterial wall compartments. In consequence, they also determine the pathological anatomy of the artery. The principal cells involved in pulmonary arterial lesions of patients suffering from PH are smooth muscle cells, fibroblasts/myofibroblasts, endothelial cells, and mononuclear cells. Figure 1 shows all different arterial lesions and the cell type prevailing (Dorfmüller and Humbert 2012).

2.1 Medial Hypertrophy

This abnormality of the vessel wall can be observed in all subgroups of pulmonary arterial hypertension (PAH, Group 1, Dana Point) and may be encountered in other forms of PH, e.g., Group 2 or 3 (PH owing to left heart disease and PH owing to lung diseases and/or hypoxia). The lesion corresponds to a proliferation of smooth muscle cells within the tunica media (Fig. 2a). The histological criterion of medial hypertrophy (which corresponds more precisely to hypertrophy and hyperplasia, i.e., increase in volume and number of smooth muscle cells, respectively) is fulfilled when the diameter of a single medial layer, delineated by its internal and external elastic lamina, exceeds 10 % of the artery's cross-sectional diameter. In the PH lung, hyperplasia of smooth muscle cells is virtually always observed at the level of pre-capillary arterioles; it should be avoided to use the term "medial hypertrophy" in this context, since human arterioles of the lung are physiologically quasi non-muscularized and do not show the classic arterial layers.

Isolated hypertrophy of the medial layer may be considered as an early and even reversible event, as has been shown for pulmonary hypertension due to hypoxia at high altitude (Heath and Williams 1989). Also, as recently stated by Sakao et al. (2010), new therapeutic concepts that appear to work in experimental PH, e.g., the tyrosine kinase receptor inhibitor imatinib, solely reduce the pulmonary arterial/arteriolar muscularization, an observation which underlines the reversibility of muscular remodeling. In a recent study and review of the literature, Penalzo and Arias-Stella (2007) noted that healthy natives of high altitude regions over 3,500 m above sea level have pulmonary hypertension, right ventricular hypertrophy and increased amount of smooth muscle cells in the distal pulmonary arterial branches. Clinical and histological findings from 30 healthy high altitude natives and 30 sea level natives revealed that the main factor responsible for pulmonary hypertension in healthy highlanders is the increased amount of smooth muscle cells in the distal pulmonary arteries and arterioles. Vasoconstriction is a secondary factor in this setting, because the administration of oxygen decreases the pulmonary arterial pressure only by 15–20 %. The adaptive increase in cardiac and smooth muscle

Diversity of Lesions in PAH - Variation of the Cell Type Prevailing

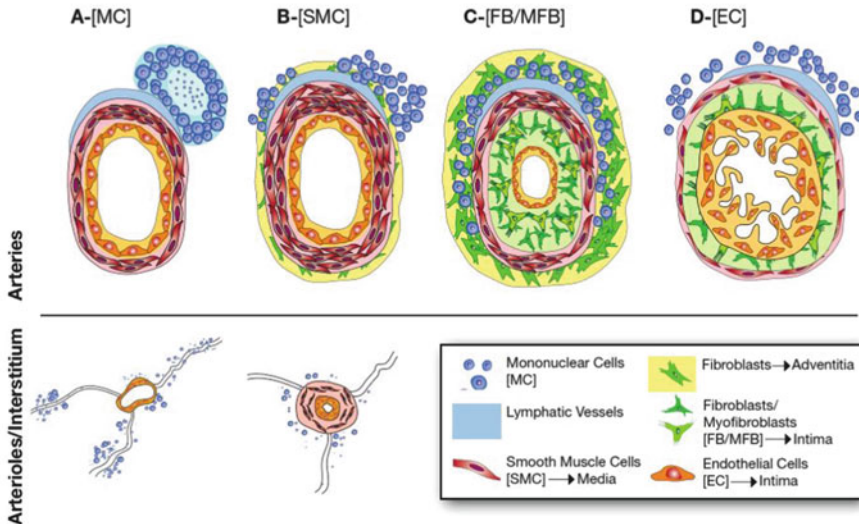


Fig. 1 Different lesions or different snap shots of one process in time? Typical histological lesions encountered in lungs from patients with PAH. (a) Tertiary lymphoid tissue (lymphoid follicles) can be observed in the vicinity of inconspicuous and remodeled arteries of patients with PAH, frequently associated with dilated lymphatic vessels (*top*); interstitial inflammatory infiltrates, mostly mononuclear cells, are frequently seen (*bottom*). (b) Hypertrophy/hyperplasia of the media is encountered in different forms of PH and corresponds to a proliferation of smooth muscle cells (SMC) (*top*). SMC are also the prevailing cell type involved in the muscularization of normally non-muscularized arterioles (*bottom*); perivascular lymphocytic infiltrates are regularly observed; most animal models of PH display this reversible lesion type with prevailing SMC. Fibrosis of the adventitia may be present. (c) Concentric intimal fibrosis (non-laminar or laminar) is constantly found in PAH; fibroblasts and myofibroblasts prevail and occlude the lumen through deposition of collagen. The lesion is often combined with hypertrophy of the media and fibrous broadening of the adventitia; perivascular inflammation is frequently observed. (d) Plexiform lesions are considered as a hallmark of PAH histology and correspond to an exuberant proliferation of endothelial cells within a dilated, thin-walled arterial lumen. Inflammation is classically described in the range of plexiform lesions. Figure reprinted with permission of the American Thoracic Society. Copyright © 2012 American Thoracic Society. Dorfmueller P, Humbert M (2012) Progress in pulmonary arterial hypertension pathology: relighting a torch inside the tunnel. *Am J Respir Crit Care Med* 186(3):210–212. Official journal of the American Thoracic Society

cell mass reverses after a prolonged residence at sea level. In PAH, medial hypertrophy is commonly associated to the remodeling of other vascular compartments, which are discussed below.

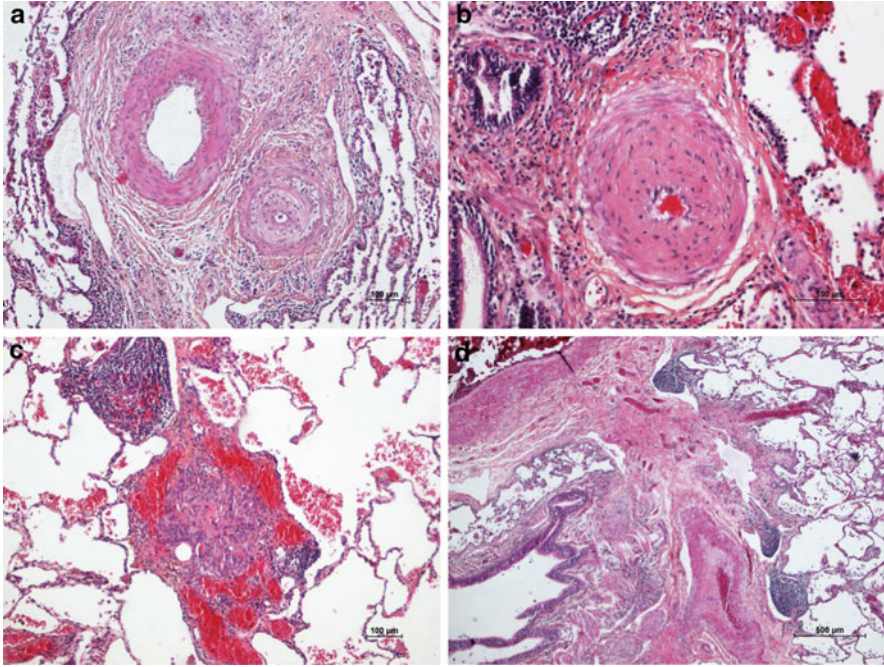


Fig. 2 Pulmonary arterial lesions from patients with PAH. Hematoxylin–eosin–safran staining. **(a)** Two arterial walls displaying different histological patterns; the left artery shows important medial hypertrophy, while the right artery predominantly presents with intimal fibrosis (concentric, partially laminar). **(b)** Small pulmonary artery with important concentric, non-laminar thickening of the intima. Note the discrete perivascular lymphocytic infiltrate. **(c)** Complex lesion with central plexiform pattern and peripheral dilation lesions. Perivascular inflammatory infiltrate and a lymphoid follicle are present in the range of the diseased vessel. **(d)** Remodeled artery with adjacent bronchus. Note the tertiary lymphoid tissue in the vicinity of the vessel

2.2 *Intimal Fibrosis*

Fibrotic lesions of the intimal layer are frequent in PH lungs. The intima may be thickened by proliferation and recruitment of fibroblasts, myofibroblasts, and other connective tissue cells, and consequently by the interstitial deposition of collagen (Fig. 2a, b). In a purely descriptive approach, this thickening may be uniform and concentric, or focally predominating and eccentric. Both forms can lead to a complete occlusion of the artery. The gain in intimal cellularity is generally understood as a reaction of the inner arterial layer to a luminal stimulus, e.g., chronic pressure and shear stress or as a scarring process after primary endothelial damage (Voelkel and Tuder 1995). In many cases, adventitial fibrosis is associated with intimal changes but remains difficult to evaluate, due to the lack of a clear anatomical delimitation.

Eccentric intimal thickening is frequently observed in cases with thrombotic events and probably represents residues of wall-adherent, organized thrombi. Thrombotic lesions, or so-called in situ thrombosis, are a characteristic pattern of chronic thromboembolic pulmonary hypertension (CTEPH) but also regularly encountered in different PAH subgroups and in PVOD; organization and recanalization of totally occluding thrombotic material may lead to bizarre, fibrotic multi-channel lesions (so-called colander-like lesions) which can easily be confounded with proliferative complex lesions (see below).

A conspicuous phenotype of fibrosis with laminar arrangement of collagen layers within the intima of the diseased vessel may be observed in PAH (Fig. 2a). This lesion is also known as “onion-skin” or “onion-bulb” lesion. The scar-like, collagen-rich arterial lesions may be found in lungs of patients suffering from different forms of PAH, including PAH associated with connective tissue disease (Cool et al. 1997). Nevertheless, the observation that intimal thickening proximal to plexiform lesions in supernumerary arteries usually displays a concentric laminar phenotype seems to closely associate these two lesions. Immunohistochemical analysis reveals fibroblasts, myofibroblasts, and smooth muscle cells.

2.3 *Complex Lesions*

Complex pulmonary arterial lesions comprise different patterns, which are commonly observed in close topographic association. However, the pathophysiologic significance of this typical PAH lesion has yet to be elucidated and its peculiar structure hitherto remains a mystery. It is still unclear if this arterial anomaly represents a cause for increased vascular resistances or if it is simply a secondary phenomenon resulting from chronic pulmonary hypertension. In this context, it should be mentioned that Stacher et al. (2012) have recently published an exhaustive histological analysis of 62 cases of PAH, taking into account associated PAH-specific treatment. Interestingly, the authors found a significant positive correlation between the quantity of plexiform lesions and case history of prostacyclin treatment, a finding which puts too mechanistic conclusions of primary luminal obstruction through endothelial proliferation into question.

The plexiform pattern is the main criterion and at the core of complex lesions (Fig. 2c). It has been first described in congenital heart disease-associated PAH as well as in idiopathic PAH (formerly called primary pulmonary hypertension) (Heath and Edwards 1958; Wagenvoort and Wagenvoort 1970). Eventually, plexiform lesions have been shown to occur in other PAH subgroups such as anorexigen intake associated PAH or portopulmonary hypertension (Widgren 1986; Krowka and Edwards 2000). The peculiar lesion concerns various vascular compartments. A focal intimal thickening of small pulmonary arteries, preferably beyond branching points, is followed by an aneurysm-like widening of the vessel: the lumen is obstructed by an exuberant proliferation of endothelial cells, leading to the formation of capillary-like, sinusoidal channels on a fibroblast-rich background (Tuder

et al. 2009). This intra-arterial capillary convolute, resembling sometimes to the glomerulum of a kidney, appears to feed into dilated, vein-like congestive vessels, which are perceivable at low magnification and may be helpful as sentinel lesions when tracing plexiform lesions. The latter anomaly is called dilation lesion and is virtually always associated with plexiform lesions (Fig. 2c). Arteritis with transmural inflammation and fibrinoid necrosis, as firstly described by Heath and Edwards (1958), maybe observed, but has become a rather infrequent phenomenon in PAH, possibly due to recent advances in medical therapy and/or associated prolonged survival of patients. However, perivascular inflammatory infiltrates in the vicinity of complex lesions and other diseased pulmonary arteries in PAH patients are commonly found (Stacher et al. 2012). The infiltrates mainly consist of T and B lymphocytes, to a lesser extent macrophages, as well as scattered mast cells. This inflammatory phenotype, in most cases, is evaluated as “mild” to “moderate”.

From all phenotypes of arterial remodeling encountered in pulmonary hypertension, complex lesions appear to be found, if not specifically, than at least typically in PAH (Group 1 of the Dana Point Classification) (Tuder et al. 2009). There have been controversial publications in the past, questioning this specificity: in a publication from 1993 (Moser and Bloor 1993), plexiform lesions were detected in 20 out of 31 patients with confirmed chronic major vessel thromboembolic pulmonary hypertension, suggesting a possible relation between organizing thromboembolic and complex lesions, which mirrors an old theory first inspired by Harrison in 1958 (Harrison 1958). Recently, this finding has been confirmed in a review by Piazza and Goldhaber (2011). Another historic hypothesis explaining the pathogenesis of the glomerulum-like exuberant endothelial cell proliferation within the diseased pulmonary artery is the formation of arteriovenous shunts (Kucsko 1953). Wagenvoort explained the generation of plexiform lesions in cardiac left-to-right shunting by an increased blood-flow eliciting reflective vasoconstriction with subsequent development of endothelial alteration (Wagenvoort and Wagenvoort 1970). He speculated that vascular necrosis with arteritis might result from intense vasospasm, as seen in the systemic circulation. The pathogenetic plot, here, would be that plexiform lesions develop in the focal areas of fibrinoid necrosis by active cellular reorganization and recanalization of the thrombus composed of fibrin and platelets, usually present in this setting and observed within plexiform lesions. In fact, the only animal model leading to obvious plexiform lesions was achieved by producing necrosis of pulmonary arterioles in dogs after 2 weeks of severe pulmonary hypertension due to the creation of a shunt between pulmonary and systemic circulations (Saldaña et al. 1968). This explanation would take into consideration that elements of inflammation are consistently present in the range of plexiform and other vascular lesions in PAH (Tuder et al. 1994; Dorfmueller et al. 2003). On the other hand, Lee et al. (1998) have proposed a neoplastic approach to the hardly understandable proliferation of intraluminal neovessels at the core of plexiform lesions: they found that a large proportion of the endothelial cells in this area show monoclonality, raising the question of possible tumor-like growth. These different observations of arterial wall alteration within plexiform lesions are probably connected in a temporary line. However, they could fit into a broader concept of pathogenesis, first

mentioned by Wagenvoort, but interestingly developed by Yaginuma et al. (1990). They submitted histological data from 11 patients with pulmonary hypertension due to congenital heart disease to a computer-based three-dimensional image reconstruction and found that plexiform lesions mostly occurred in supernumerary arteries branching apart from larger pulmonary arteries, proximal-to-arterial lesions with intimal fibrosis and medial hypertrophy. They also gathered evidence for generation of indirect anastomoses between the post-plexiform arterial segment to bronchial arteries running along the close bronchiole, passing via arterioles and the capillary bed and thereby creating the thin-walled congestive dilation lesions. In this view, the generation of proximal complex lesions might be the mere attempt of the pulmonary vasculature to bypass the primary downstream obstruction and to ensure capillary oxygenation through overt contact with arterial blood from proximal pulmonary and bronchial arteries. On the other hand, plexiform lesions are not restricted to supernumerary branching and can be observed after distal dichotomous branching of pulmonary arteries. Cool and coworkers have come to different conclusions after a computerized three-dimensional study on five patients with severe pulmonary hypertension of different cause. In their view, plexiform lesions are functionally important because blood flow is severely obstructed along the entire length of a vessel affected by a single lesion (Cool et al. 1999). This, in fact, puts at least a working shunt concept into question. They hypothesize that the plexiform lesion could be an early vascular alteration in severe pulmonary hypertension, independent of a component of medial smooth muscle cell hypertrophy. At a later time point in disease evolution, the plexiform lesion could transform into an intraluminal concentric obstruction composed of endothelial cells and recruited myofibroblasts, following the path of the plexiform lesion and thus representing a fibrous scar of the latter. The close association that can be observed between concentric laminar intimal fibrosis and plexiform lesions in lungs of patients suffering from PAH, indeed, makes this thought a tempting assumption. Nevertheless, a shunt hypothesis would not be contradictory if seen in the light of a failed attempt to shortcut other correlates of obstruction such as medial hypertrophy, intimal non-laminar fibrosis, and thrombotic lesions.

2.4 Perivascular Inflammation and Tertiary Lymphoid Tissue

It has not yet been elucidated whether the inflammatory pattern seen in plexiform lesions and other intimal lesions is of pathogenetic importance, or if it represents a pure epiphenomenon within disease evolution. The reported evidence of proinflammatory mediators, so-called chemokines, released by altered endothelial cells of PAH lungs strongly indicates a self-supporting and self-amplifying process (Balabanian et al. 2002; Dorfmueller et al. 2002; Sanchez et al. 2007). Although elements of inflammation seem to be present in diseased arteries of patients displaying PAH in various associated conditions, as well as in idiopathic PAH,

the specific role of immune cells within installation and/or maintenance of obstructive lesions remains unclear. It seems unlikely that intimal proliferation and medial hypertrophy of pulmonary arteries could be the sole result of “scarring” vasculitis-like lesions, because lymphocytic and macrophagic infiltrates observed in the setting of PAH remain perivascular and are less abundant than in pulmonary forms of vasculitis, as seen e.g., in Wegener’s granulomatosis (Dorfmueller et al. 2003). Nonetheless, Wagenvoort and coworkers had discussed such a possibility 40 years ago, and it cannot be excluded that a phase of intense inflammatory activity precedes a clinically symptomatic arterial remodeling with proliferation and recruitment of smooth muscle cells, endothelial cells, and fibroblasts/myofibroblasts. In fact, recent investigations provide evidence that chemokines with specific chemotactic activity for T lymphocytes and macrophages can increase smooth muscle proliferation (Perros et al. 2007).

A recent study from Perros et al. (2012) provides evidence for lymphoid neogenesis in lungs from patients with idiopathic PAH (IPAH). The authors analyzed lung histology of 21 patients with IPAH, of 5 patients with Eisenmenger syndrome associated PAH (ESPAH), and of 21 control patients (healthy lung areas from lobectomies of patients with adenocarcinoma or squamous carcinoma). They found pulmonary tLTs ranging from small lymphoid aggregates to large accumulations of lymphocytes resembling highly organized lymphoid follicles (Fig. 2d). tLTs were distributed throughout the pulmonary vasculature, from small distal remodeled arteries to larger pulmonary arteries; a 20-fold increase in pulmonary tLTs was measured by morphometrical means in patients with IPAH as compared with control subjects. Patients with ESPAH were not different from controls regarding lung tLT density. Lymphoid neogenesis in the target organ is considered to be a hallmark of autoimmune diseases. As such, tertiary lymphoid tissues tLTs or lymphoid follicles are commonly found in salivary glands of patients suffering from Sjögren syndrome, in thyroid gland of patients with Hashimoto thyroiditis, in joints and lungs of patients with rheumatoid arthritis, and around airways of patients with chronic obstructive pulmonary disease (Hogg et al. 2004; Aloisi and Pujol-Borrell 2006; Carragher et al. 2008). Several studies suggest that lymphoid neogenesis correlates with local autoantibody production (Carragher et al. 2008). Interestingly, Perros and coworkers also showed that lymphoid follicles in IPAH patients display canonical cellularity and structure of bona fide tLTs and lymphorganogenic chemokines such as CXCL13 and CCL19/CCL21 are overexpressed. Also, the study revealed that an IL-17-mediated response may promote the formation of tLTs in IPAH. Indeed, Rangel-Moreno et al. (2011) provide evidences that this signaling is essential for lymphoid neogenesis in lung. The presence of pulmonary tLTs in IPAH lungs could provide a structural basis for a local autoimmune response occurring in this disease. In the initially cited study, Perros and colleagues found accumulation of long-lived antibody-secreting CD¹³⁸⁺ plasma cells together with immunoglobulin deposits around pulmonary vascular lesions, which appears to be consistent with locally produced autoantibodies, a prominent feature of IPAH (Tamby et al. 2005; Terrier et al. 2008; Dib et al. 2012).

3 Venous and Venular Lesions

During the Dana Point meeting in 2008, a consensus was reached, formally separating Group 1 (pulmonary arterial hypertension) of the classification of pulmonary hypertension from the rare entities of PVOD and PCH, now categorized as the new Group 1' (one prime): pulmonary venous hypertension. This differentiation seemed necessary because of particularities regarding therapeutic strategies and the cautious, if not restrictive use of potent vasodilating drugs, such as intravenous epoprostenol, in the case of pulmonary venous involvement. From the pathologist's standpoint of view, this separation is comprehensible, but not ideal; in fact some cases of PAH (Group 1) show PVOD-like pattern (see beneath), and the vast majority of PVOD and PCH cases (Group 1') present at least mild arterial alteration. The frequent occurrence of mixed vascular involvement seems to demand a less absolutistic evaluation into pulmonary vascular lesions either predominating the arterial or the venous system.

3.1 *Pulmonary Veno-occlusive Disease/Pulmonary Capillary Hemangiomatosis*

PVOD is a rare pulmonary vascular disease causing pulmonary hypertension and has been considered together with pulmonary capillary hemangiomatosis (PCH) a subgroup of pulmonary arterial hypertension (PAH) until recently, according to the Venice classification of 2003. It shows an estimated prevalence of 0.1–0.2 per million persons and per year (Mandel et al. 2000). Historical reports and large case studies have extrapolated a proportion of PVOD in PAH ranging from 5 to 25 % (Rich et al. 1987; Mandel et al. 2000). In PVOD as in PCH, vascular lesions predominate on the post-capillary level of pulmonary vasculature. However, lesions frequently involve both veins *and* arteries in lungs of patients with PVOD. Interestingly, a recent report indicates that certain subgroups of PAH regarded as pre-capillary forms simultaneously display a PVOD-like pattern (see beneath). In PVOD, the observed post-capillary lesions involves septal veins and pre-septal venules and frequently consist of loose, fibrous remodeling of the intima, which may totally occlude the lumen (Fig. 3a, b). The involvement of pre-septal venules should be considered as necessary for the histological diagnosis of PVOD; fibrous occlusion of large septal veins may be seen in many forms of pulmonary venous hypertension, including a frequently reported obstruction of large pulmonary veins following catheter ablation for cardiac atrial fibrillation (Di Biase et al. 2006). While septal veins usually display a paucicellular, cushion-like fibrous obstruction, intimal thickening of pre-septal venules can present with a dense pattern and increased cellularity. Anti- α -actin staining may reveal involvement of smooth muscle cells and/or myofibroblasts within such venular lesions. Also, thrombotic occlusion of small post-capillary microvessels has been observed, corresponding to

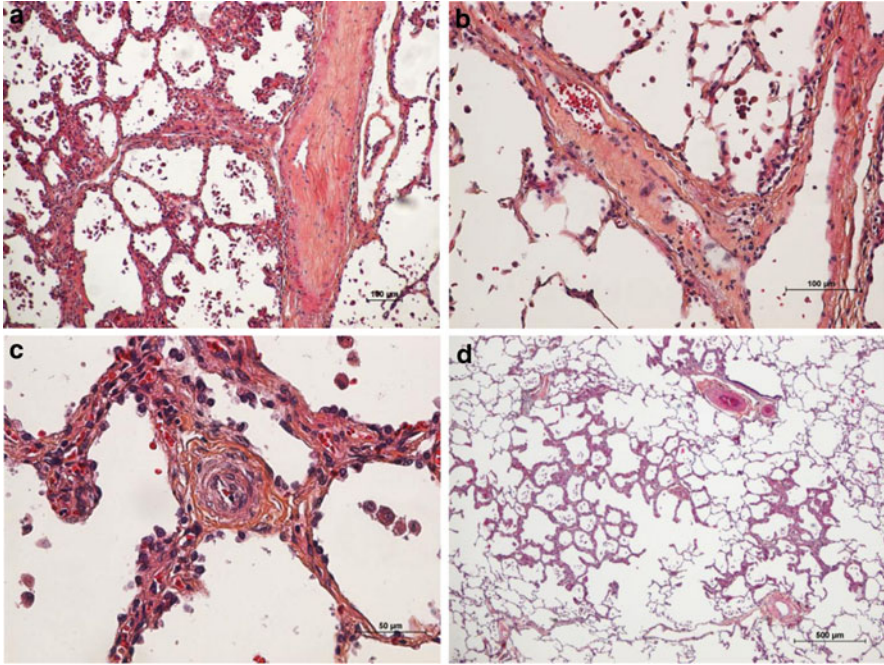


Fig. 3 Histological pattern in lungs from patients with PVOD. Hematoxylin–eosin–safran staining. (a) Septal vein with fibrous, collagen-rich occlusion. The adjacent alveolar septa show thickening with discrete interstitial fibrosis and multiplication of capillaries. Note numerous intra-alveolar macrophages. (b) Fibrous remodeling of the intima in a smaller, pre-septal venule. (c) Microvessels (either arterioles or venules) frequently display muscularization. (d) Typical patchy pattern of capillary hemangiomatosis-like foci. Alveolar septa are thickened by capillary proliferation. Note a remodeled pulmonary artery (*top right*) and a pulmonary vein with fibrous intimal thickening (*bottom right*)

“colander-like” lesions, which can be seen otherwise in small pulmonary arteries. The tunica media may be muscularized in both septal veins and pre-septal venules. The latter can be difficult to distinguish from muscularized arterioles of less than 80 μm in diameter, usually present in lungs from patients with PVOD (Fig. 3c). Pleural and pulmonary lymphatic vessels are usually dilated (Pietra 1997). The presence of calcium-encrusting elastic fibers in the vessel wall or the perivascular space, and inflammatory activation through a foreign body giant cell response is considered as an argument in favor of PVOD as compared to secondary venous hypertension (Pietra et al. 2004). Importantly, occult pulmonary hemorrhage regularly occurs in patients displaying PVOD. This particularity, which is certainly due to the post-capillary block, is of diagnostic interest, as bronchoalveolar lavage can reveal occult hemorrhage. The degree of hemorrhage can be evaluated semiquantitatively and qualitatively using the Golde score, which assesses intra-alveolar siderin-laden macrophages by Perls Prussian blue staining (Golde et al. 1975; Capron 1999). In addition to an increased number of siderophages, large amounts

of hemosiderin can be found in type II pneumocytes, and within the interstitial space. Moreover, post-capillary obstruction may frequently lead to capillary angiectasia and even capillary angioproliferation; in PVOD cases, doubling and tripling of the alveolar septal capillary layers may be focally present (Fig. 3a, d). Lately, this histological peculiarity has raised questions concerning a possible overlap between PVOD and cases of PCH, a disease classically characterized by an aggressive patch-like angioproliferation of capillaries; indeed, Lantuéjoul et al. (2006) recently reported 35 cases of PVOD and PCH with more or less similar pattern and evoke the possibility of a same disease entity. Historically, PCH was described by Wagenvoort and other authors as an aggressive capillary proliferation with patchy to nodular distribution; several rows of capillaries along alveolar walls progressed to nodules and sheets of back-to-back capillaries in advanced lesions (Wagenvoort et al. 1978). A malignant disorder which had been evoked with the term of “angiomatous growth” however seems unlikely, because cytological atypia and mitoses are usually absent. An infiltration of bronchiolar structures had also been described. A possible explanation to this angiomatoid expansion might be a hemodynamically relevant post-capillary block. Occult hemorrhage or hemosiderosis, therefore, is frequently found. As in PVOD, these characteristics lead to compensatory muscularization of arterioles and medial hypertrophy in pulmonary arteries (Fig. 3c). As already mentioned, the similarities in clinical and histological presentation indeed suggest that PVOD and PCH are a same disease entity with either a vein-predominating or a capillary-predominating phenotype (Lantuéjoul et al. 2006).

4 Forms of Pulmonary Hypertension with Significant Arterial and Venous Remodeling

4.1 PAH Associated with Connective Tissue Disease

Until recently, lesions of the pulmonary arterial system, more or less similar to those occurring in idiopathic PAH, have been held responsible for pulmonary hypertension in patients with connective tissue-associated PAH. In a published analysis of eight patients suffering from CTD-associated PAH, we observed that six out of eight patients (75 %) exhibited occlusive lesions of pulmonary veins and venules, as it can be typically seen in PVOD. In contrast, only 5 out of 29 non-CTD control patients with the primary diagnosis PAH displayed venous involvement (Dorf Müller et al. 2007). Though all investigated CTD-PAH patients exhibited pulmonary arterial changes, venous and venular fibrous remodeling, when present, was more pronounced than arterial changes, concerning the quantity of diseased vessels. All cases of CTD-associated PAH revealed pulmonary arterial lesions, involving small muscular vessels on the pre- and intra-acinar level. The vessel wall remodeling corresponded to arterial changes found in PAH, ranging from intimal

constrictive and non-constrictive lesions to associated medial hypertrophy and adventitial thickening. In another report on this subject, Overbeek et al. (2009) analyzed lung tissue of eight PAH patients with limited cutaneous SSc and compared it to samples of 11 idiopathic PAH patients. They found that all SSc-PAH patients displayed arterial and venous remodeling, while venous lesions were present in only 3 of the 11 IPAH patients. It is notable that four SSc-PAH cases displayed a PVOD-like pattern with patchy capillary congestion and signs of occult alveolar hemorrhage.

Systemic sclerosis represents one of the leading pathological conditions associated with pulmonary hypertension and CTD-associated pulmonary hypertension belongs to Group 1 of the Dana Point Classification. Prevalence of PAH in certain forms of CTD has been estimated to up to 50 % of cases (Ungerer et al. 1983). In a recent cross-sectional national screening, at least 8 % of scleroderma patients displayed moderate-to-severe PAH (Hachulla et al. 2005). In patients with CTD, PAH is the leading cause of mortality and necessitates intensive medical treatment, which frequently proves difficult and with mixed results (Sanchez et al. 1999). Treatment with vasodilators like continuous intravenous epoprostenol has shown improvement of exercise capacity and cardiopulmonary hemodynamics, but the response is less effective than in IPAH and survival remains poor among patients with associated CTD (Badesch et al. 2000; Ramirez and Varga 2004). Equally, endothelin receptor antagonists seem to show less impressive effects in systemic sclerosis patients than in other forms of PAH (Humbert and Simonneau 2005). In addition, adverse effects of vasoactive treatment in PAH associated with CTD can occur and may lead to severe pulmonary edema (Humbert et al. 1999). Considering this background, the described findings of a PVOD-like setting in CTD-associated PAH highlight the important hemodynamic effect of post-capillary occlusion on the pulmonary vasculature. Resistance of CTD-associated PAH to common vasodilator therapies and complications, such as pulmonary edema, could be the mere consequence of a higher prevalence of veno-occlusive remodeling in these patients, as compared to other forms of PAH.

5 Conclusion

Characteristic vascular lesions in pulmonary hypertension may concern the lung vasculature and microvasculature from the pre- to the post-capillary level. Differentiation of histological phenotypes has helped to categorize pulmonary hypertension into different groups and subgroups, now summed up under the actual Dana Point Classification (which will be shortly revisited in the upcoming Fifth World Symposium on PH in 2013). It is important to stress that the differentiation of lesions and their topography within the vascular bed of the lung can be of significant importance considering outcome and specific therapy. However, a clear-cut separation of different forms of pulmonary hypertension through recognition of a histological phenotype will always be difficult or even impossible; different

etiologic factors (e.g., hypoxia versus anorexic intake) may trigger a final common step in the cascade of pathologic events (e.g., oxidative stress and consecutive growth factor expression) and finally lead to the same morphologic pattern (e.g., medial hypertrophy). Nonetheless, the correct interpretation of the pathological vascular anatomy remains an indispensable roadmap to the successful coordination of clinical, biological, and pharmaceutical research dedicated to pulmonary hypertension.

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Pulmonary Hypertension: Biomarkers

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Abstract Physicians look to biomarkers to inform the management of pulmonary hypertension (PH) at all stages, from assessing susceptibility through screening, diagnosis, and risk stratification to drug selection and monitoring. PH is a heterogeneous disorder and currently there are no accepted blood biomarkers specific to any manifestation of the condition. Brain natriuretic peptide and its N-terminal peptide have been most widely studied. Other candidate prognostic biomarkers in patients with pulmonary arterial hypertension (PAH) include growth and differentiation factor-15, red cell distribution width, uric acid, creatinine, inflammatory markers such as interleukin-6, angiotensins, and microRNAs. Combining the measurement of biomarkers reflecting different components of the pathology with other modalities may enable better molecular characterisation of PH subtypes and permit improved targeting of therapeutic strategies and disease monitoring.

Keywords Pulmonary hypertension • Biomarkers • Pulmonary arterial hypertension • Brain natriuretic peptide • Cytokines • Inflammation • Iron • microRNA

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1 Biomarkers

The US National Institutes of Health Biomarkers Definitions Working Group have defined a biomarker as “any characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson et al. 2001). That is to say, a biomarker is any objective measure that informs on the state of health of an individual.

To be useful it has to satisfy a number of criteria. The strength and consistency of the relationship between the biomarker and the disease parameter it is reporting are an important consideration; associations must be convincing (statistically significant) and reproducible across different cohorts before a relationship can be considered true. A biomarker’s *specificity* is the proportion of true negatives over the true negatives and false positives—and so a measure of the ability of a biomarker to accurately rule out disease. *Sensitivity*, conversely, is the proportion of true positives over the true positives and false negatives—and so the ability of a biomarker to predict disease. The *temporality* of a biomarker is also important; some biomarkers respond rapidly to events, while others may accumulate and integrate information. The *biological gradient* refers to the dynamic range of the measurement and together with the quality of the assay used must be sufficient to differentiate accurately between groups.

Biomarkers may help in the management of PH at several stages, from susceptibility, screening, diagnosis, and risk stratification to therapeutic selection and monitoring.

1.1 Biomarkers of Susceptibility to PH

Genomic information holds the greatest potential for identifying susceptible individuals, i.e. individuals at risk but with no manifestations of the disease. Family members of patients with PAH can be tested for mutations in known risk genes,

namely *BMPR2*, *ALK1*, or *ENG* (see Sect. 2.1). Following appropriate counselling, those harbouring mutations can be seen at regular intervals to detect and manage the disease at its earliest occurrence.

Other scenarios in which biomarkers for susceptibility to PH may be identified include identifying those individuals most likely to develop PH following pulmonary embolism/thromboendarterectomy, or in other at-risk populations including but not limited to those where PAH is associated with connective tissue disorders, HIV, or schistosomiasis.

1.2 Biomarkers for Screening for PH

A considerable proportion of the pulmonary vascular bed needs to be ‘lost’ before pulmonary vascular disease becomes symptomatic (Lau et al. 2011), and even in patients with mildly symptomatic PAH, there is already a marked increase in pulmonary vascular resistance (Galië et al. 2008). Screening individuals for sub-clinical disease requires a non-invasive test and the most practical tool for this purpose is the echocardiogram. No blood biomarker currently identified is sensitive enough to detect subclinical disease.

1.3 Biomarkers for Making the Diagnosis of PH

The insidious onset and non-specific nature of presenting symptoms in PH means that the diagnosis is delayed while other pathologies are excluded. The definitive diagnosis of PH is dependent on demonstrating a raised resting mean pulmonary artery pressure at cardiac catheterization. While a normal level of the cardiac stress-marker brain natriuretic peptide (BNP) or its N-terminal peptide (NT-proBNP) may help rule out PH, there is no blood biomarker that is diagnostic of the condition. The cost and invasive nature of cardiac catheterization drive the search for alternative non-invasive tests. Further developments in other imaging technologies such as cardiomagnetic resonance (CMR) imaging or 3D echocardiography may provide a solution but remain research tools at present. Cardiac output is a prognostic marker in PH patients and inert-gas rebreathing may represent a non-invasive means of measuring this important parameter (Clinical Trials.gov Identifier NCT01606839), detecting for example treatment responses in patients with PAH (Lee et al. 2011).

1.4 Biomarkers for Risk Stratification in PH

Risk stratification can inform not just prognosis but may help optimization of treatment and the achievement of specific goals aimed at improving longer-term survival. Multicentre patient registries—National Institutes of Health (NIH), French PAH registry, and US-based Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management (REVEAL)—have been

very useful in identifying independent factors that are associated with prognosis of PAH, including age, sex, disease aetiology, World Health Organization (WHO) functional class, 6 min walk distance (6MWD), and haemodynamic parameters (D'Alonzo et al. 1991; Rich et al. 2000; Humbert et al. 2010; Benza et al. 2010). But these are blunt instruments and we remain poorly equipped to make judgements at the individual patient level (Thenappan et al. 2012). That is, to predict exactly which individuals will survive for years with their condition versus those who will succumb within months or weeks of their original diagnosis and may have benefitted from earlier intervention with combination therapy or more aggressive treatment (Hoepfer et al. 2005; Nickel et al. 2011a).

Measurements of circulating BNP and NT-proBNP levels remain the most commonly used blood biomarkers for stratifying PH patients and are recommended in the European guidelines for the diagnosis and treatment of PH (Galie et al. 2009). It is recognised, however, that some patients with advanced disease have circulating levels within the normal range and the levels for an individual patient have to be interpreted in the context of all the clinical information available. Repetitive measurements may add information and improve their utility in disease management (Nickel et al. 2011a).

Novel candidates for prognostic biomarkers in PH, which will be described in more detail below, include growth and differentiation factor-15 (Nickel et al. 2008; Rhodes et al. 2011a), red cell distribution width (Hampole et al. 2009; Rhodes et al. 2011a), inflammatory markers such as interleukin-6 (Humbert et al. 1995; Soon et al. 2010), creatinine (Leuchte et al. 2007; Shah et al. 2008), uric acid (Nagaya et al. 1999), angiopoietins (Kumpers et al. 2010), and microRNAs (Caruso et al. 2010; Courboulin et al. 2011).

1.5 Biomarkers for Therapeutic Selection and Monitoring

There is interest in the PH community in goal-orientated treatment strategies, whereby treatments are titrated according to response. The biomarkers used to assess response are 6 min walk distance, cardiopulmonary exercise testing, and BNP levels (Hoepfer et al. 2005).

With the increase in number of drugs available to treat PH, the possibilities for individualising treatment have increased. Biomarkers that can identify which patients will respond best to particular therapies enable expensive treatments to be used more cost effectively and reduce unnecessary drug exposures. This point is well illustrated by the use of acute vasodilator testing at cardiac catheterization to identify patients suitable for treatment with calcium antagonists. A small number (<10 %) of patients with PAH who show an acute reduction in pulmonary artery pressures, when exposed to nitric oxide or adenosine, respond well to calcium channel blockers with excellent haemodynamic recovery and long-term survival (Sitbon et al. 2005; Tonelli et al. 2010). The recent finding that over 60 % of patients with PAH are iron deficient (in the absence of anaemia) may identify a

subgroup of patients who will benefit from iron, but this awaits formal testing (Rhodes et al. 2011b).

While there is most interest in biomarkers of efficacy it is important not to forget toxicity and the role of safety biomarkers. For PH patients on warfarin, the International Normalised Ratio (INR) is used to monitor the clotting tendency of blood and thereby ensure the correct dosage. Patients on endothelin antagonists also have their liver enzymes monitored for early signs of liver toxicity, which is generally reversible upon discontinuation of the drug. A new protein kinase inhibitor-induced toxicity has been identified, pre-capillary PH having been associated with dasatinib therapy (Montani et al. 2012). Cardiac biomarkers such as Troponin I and BNP may represent an early means of detecting cardiac toxicity in patients exposed to protein kinase inhibitors (ClinicalTrials.gov Identifier NCT00532064).

1.6 Statistical Evaluation of Novel Biomarkers

Key to the discovery and validation of biomarkers is access to well-phenotyped patient populations from cohort studies and clinical trials. The data and/or samples are mined based on plausible candidates or using unbiased screens. These datasets can be very large, particularly if fed from platform technologies, such as proteomics or metabolomics.

Before an analysis can be started, general requirements for statistical testing must be met. Whether the biomarker of interest is normally distributed (i.e. fits to a “normal” bell-shaped curve) will determine whether parametric (for normal or Gaussian data) or non-parametric tests can be deployed. Normality can be assessed informally by box and whisker plots/histograms and formally by the Kolmogorov–Smirnov test. If required, distribution of a marker can be transformed by logging, square-rooting, or inverting all numbers to achieve normality.

Tests for two groups (e.g. control versus patient) of continuous data (i.e. not categories) include the Student’s *t*-test and Mann–Whitney *U*-test. Multiple groups require analysis of variance (ANOVA or Kruskal–Wallis) followed by post hoc testing. Correlations can be assessed by Pearson’s test or Spearman’s rank test. Chi-squared analysis is required when assessing relationships between categorical variables, for example, levels of a marker above or below a cut-off level against WHO functional class, or diagnosis (see Fig. 1).

The first step in evaluating a novel biomarker is to understand its association with the disease of interest. This usually involves comparing levels measured in a patient population with appropriate control subjects. In PH, appropriate controls may simply be healthy subjects, but could include non-PH patients with a chronic disease (e.g. asthma, COPD without PH) or different diagnostic classes of PH to that being investigated (e.g. chronic thromboembolic PH versus idiopathic PAH).

Once an association with a disease state has been demonstrated, the meaning and importance of this association can be further investigated by assessing correlations

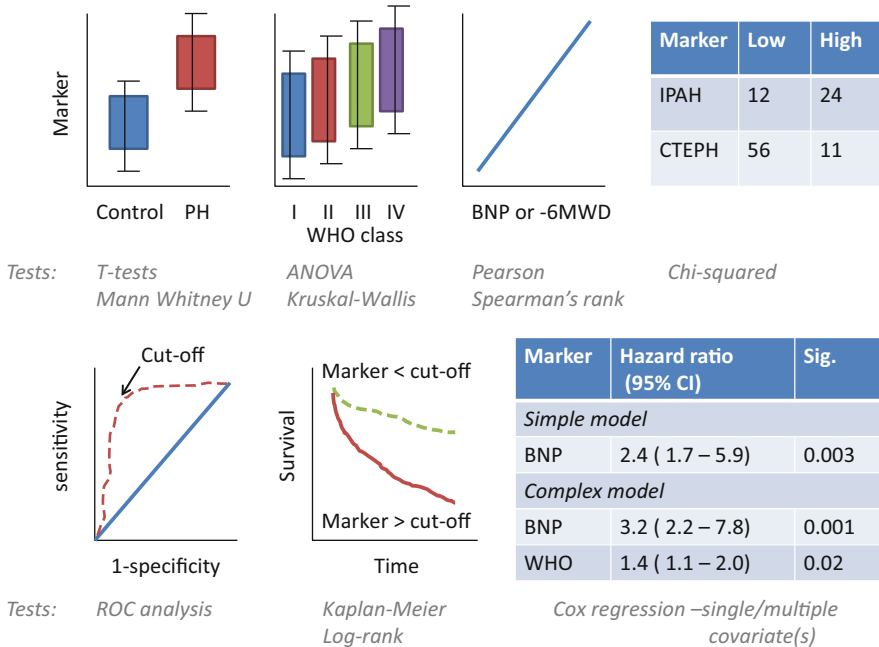


Fig. 1 Common data types and statistical analyses employed in biomarker assessment

between markers of disease severity (e.g. 6MWD, WHO functional class, pulmonary haemodynamic measurements) and circulating levels of markers such as BNP. Demonstrating the relevance of a marker to disease severity can also be hypothesis-generating; for example a marker that strongly correlates to BNP may be more likely to reflect myocardial stress than one that did not. Unless these investigations are hypothesis-driven, considerations for multiple testing may be necessary (see below).

Ultimately, if a biomarker truly reflects the severity and progression of disease, then its levels should reflect the prognosis of an individual patient. For this it is necessary to censor the survival/mortality of patients in a cohort at one given time point, and to calculate the time between biomarker measurement and the censor date. Receiver operating characteristic (ROC) analysis can then be used to assess the relationship between potential cut-off levels of a biomarker and survival over a set period, for example 1-, 2-, or 3-year survival. It plots the sensitivity versus 1-specificity for each possible cut-off level of a marker, and the area under the curve represents the power of the marker to predict survival over this time period. ROC analysis also allows for direct comparisons of performance between candidates, as those with larger area-under-the-curve (AUC) values (also known as *c*-statistic) will best discriminate survivors and non-survivors. The cut-off furthest deviating from the line $x=y$ will represent an optimum cut-off for this purpose (Fig. 1) and may be determined by plotting biomarker concentrations against their respective

sensitivity and specificity data (Schumann et al. 2010). Cut-offs can also be derived from distributions in the general/healthy populous or simply by taking median/quartile values.

The relationships of biomarker cut-offs to survival are frequently illustrated by Kaplan–Meier survival estimates and tested statistically by the log-rank test. The predictive value of stepwise changes in biomarker levels (i.e. 4–5, or 5–6, or 7.4–8.4) to the hazard rate (relative risk of mortality in a set period of time, i.e. year 1–2, year 2–3, year 3.4–4.4) can be assessed by Cox regression analysis. If a biomarker performs well by ROC, Kaplan–Meier, and Cox analysis then it can be said to have prognostic power in the population studied.

To determine whether a prognostic biomarker might add to current clinical practice, it must be compared to established gold standards, and multiple variable Cox regression analysis is the test of choice for this purpose. If a biomarker is still significantly related to survival in a Cox model including the best current prognostic measures (e.g. haemodynamic values, exercise capacity, BNP levels), then it can be said to be an “independent” prognostic marker which adds to the information currently available.

Important assumptions must be met for simple Cox analysis including that the hazard rate stays constant over time (so a high BNP would imply the same increased risk from year 1 to 2 as from year 3 to 4) and the same amplitude of change has the same hazard whatever the starting level of biomarker is (so an increase in BNP from 300 to 400 is as bad as an increase from 700 to 800). Normalisation of biomarkers prior to Cox analysis is standard and most likely aids the meeting of the latter criteria.

If a biomarker appears useful following the above tests, then the reproducibility of any findings must then be validated in further independent cohorts (see below), and eventually, its use in a clinical setting tested in specifically designed prospective clinical trials.

1.6.1 Cautionary Points

Over-fitting. An important consideration in any regression model, including Cox analysis, is not to over-fit the model. A general rule of thumb in regression is to only consider 1 covariate for each 10 data points and in Cox analysis this applies to the events (e.g. death, transplantation, hospitalisation) being considered. So a study with 100 patients with only 10 deaths would not be sufficient to assess the predictive value of a novel biomarker in comparison to BNP on death by Cox regression analysis.

Multiple Testing. This is a particular issue with the advent of high-throughput methodologies, such as genomics, proteomics, and metabolomics. If corrections are severe (such as the simplistic Bonferroni method) they may mask true differences but if ignored then false positives will arise. Estimations of the false discovery rate of a methodology may help gauge confidence in hits, but there remains no substitute

for validation of hypotheses in independent cohorts. Intrinsic to a discovery study is that identified candidates will be those that best perform, leading to over-estimation of their value compared to established markers previously identified. Hence discovery and validation cohorts are required to prove the value of a novel biomarker, and as the validation cohort will be used to assess an a priori defined hypothesis, then corrections for multiple testing will no longer be required.

Missing Data. Another problem commonly faced in biomarker discovery and development is that of missing data. This may reflect the retrospective nature of many studies which are considered secondary in importance to more orthodox clinical studies of novel therapeutics (Jenkins et al. 2011).

Incident and Prevalent Disease. It is notable that the recent REVEAL and French registry studies included prevalent as well as treatment naïve patients with incident disease (Benza et al. 2010; Humbert et al. 2010). Survival modelling with both groups of patients has its limitations and statistical techniques were used to take account of potential survivor bias arising from the inclusion of patients with prevalent disease. These methodological issues may still have resulted in overoptimistic survival rates due to an underestimation of the proportion of patients with severe disease who died soon after diagnosis (McLaughlin and Suissa 2010). Nonetheless, it is the response to treatment, captured through clinical observations and biochemical measurements, rather than the presence of treatment itself that is important in determining patient prognosis. For biomarker studies, careful analysis of survival from the date of blood sample collection is required in order to stratify risk at clinical follow-up appointments (Rhodes et al. 2011a).

1.6.2 Additional Criteria

Several groups have proposed criteria for evaluating new biomarkers and emphasised the need for prospective validation (Hlatky et al. 2009; Pletcher and Pignone 2011; Wang 2011). The lack of prospective clinical studies directly designed to assess the efficacy of adding in biomarker measurements to treatment decisions demonstrates that we are still some distance from incorporating any of the novel biomarkers proposed into standard guidelines. An appropriate trial design could be the randomization of front-line therapeutic strategies following grouping of subjects by biomarker measurement, or the randomization of patients to either normal treatment algorithms or algorithms that incorporate biomarker measurements into treatment decisions (Fig. 2).

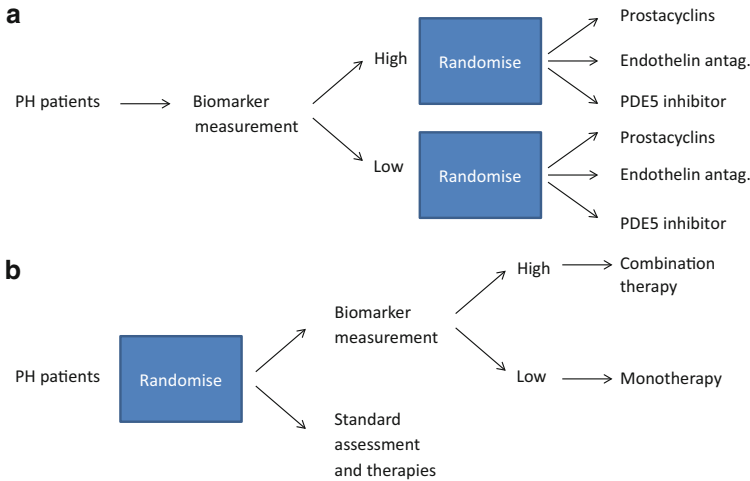


Fig. 2 Potential trial designs to test the value of biomarkers in treatment decisions in PH. **(a)** Comparison of treatment efficacies in “high” and “low” biomarker groups. **(b)** Comparison of example treatment algorithms with and without biomarker measurements. *Antag.* antagonists, *PDE5* phosphodiesterase type 5. Modified from Sargent et al. (2005)

2 Individual Biomarkers for PH

2.1 Genetic Biomarkers

Mutations in the bone morphogenetic receptor type 2 gene (*BMPR2*) are found in 50–80% of hereditary PAH (Deng et al. 2000; Lane et al. 2000; Machado et al. 2001; Morisaki et al. 2004), 10–20 % of idiopathic PAH patients (Newman et al. 2008; Girerd et al. 2010), and rarely in secondary PAH associated with anorexic drugs or congenital heart disease (Humbert et al. 2002; Roberts et al. 2004). Most predict loss of function (Machado et al. 2006). Patients with *BMPR2* mutations present approximately 10 years earlier than PAH patients without a mutation and have more severe haemodynamic disturbance leading to death at a younger age (Sztrymf et al. 2008). These patients are also less likely to respond to vasodilator therapy (Rosenzweig et al. 2008).

Two other genes in the tumour growth factor (TGF)- β receptor superfamily, activin receptor-like kinase 1 (*ALK1*) and endoglin (*ENG*), have also been causally linked to the development of PAH in hereditary haemorrhagic telangiectasia (Harrison et al. 2003). *ALK1* mutations are also found in hereditary PAH patients, leading to earlier presentation and rapid clinical decline even in comparison to *BMPR2* mutation carriers (Girerd et al. 2010). In addition, gene mutations in downstream intermediaries of the TGF- β /BMP-signalling pathway, namely Smads 1, 4, 8, and 9, have been identified as an infrequent cause of PAH (Nasim et al. 2011; Shintani et al. 2009).

Penetrance of PAH in *BMPR2* mutation carriers is generally around 20% and can vary depending on TGF- β polymorphisms (Phillips et al. 2008). Reduced penetrance suggests the presence of modifying factors that influence disease risk and the need for a so-called second hit to make the disease clinically expressed. Several genetic variants have been investigated, including polymorphisms of serotonin transporter, prostacyclin synthase, endothelial nitric oxide synthase, angiotensin-converting enzyme type 1, angiotensin II receptor type 1, carbamoyl phosphate synthetase 1, smooth muscle potassium channels (KCNQ, KCNA5), and cytochrome P450 1B1 (Fessel et al. 2011). With the cost of genome sequencing falling, the opportunities to investigate the genomic influence on PAH will increase. The sizable datasets that accompany these studies will however require careful analysis.

2.2 *Clinical and Haemodynamic Indices*

Before a novel marker can be considered useful it must be compared against the current gold standard. In PH, definitive diagnosis is dependent on invasive haemodynamic measurements by right heart catheterization—mean pulmonary artery pressure, mean right atrial pressure, pulmonary vascular resistance, and cardiac index all reflect disease severity and have been shown to hold prognostic information (D’Alonzo et al. 1991; McLaughlin et al. 2002; Sitbon et al. 2002). The current therapies for PAH have been approved on the basis of improvements in 6MWD as a surrogate for survival (Galie et al. 2005, 2008; Rubin et al. 2002). Indeed this measure has been shown to predict survival in PH (Miyamoto et al. 2000; Sitbon et al. 2002), although its variability between centres limits its use. WHO functional class is a simple clinical assessment of the symptoms of right heart failure that has been consistently demonstrated to predict survival in PH (McLaughlin et al. 2002; Sitbon et al. 2002; Humbert et al. 2010). All these measures should be considered, together with parameters such as age, diagnosis, sex, disease duration, and therapies, when assessing the potential value of a putative new biomarker in PH.

An empirically derived equation, based on baseline haemodynamic measurements, was developed to estimate survival of patients with primary PAH in the NIH registry (D’Alonzo et al. 1991). Clinical trials have used this equation to compare observed and predicted survival, but the management and treatment of PAH have changed dramatically over the last decade and contemporary registries show that the NIH equation significantly underestimates current survival (Benza et al. 2010; Humbert et al. 2010; Thenappan et al. 2010). New risk prediction equations have been produced in order to better model survival in patients with category 1.1–1.3 PAH (Humbert et al. 2010; Thenappan et al. 2010) or all groups of PAH (Benza et al. 2010). These equations have since been applied to prospectively collected data from PAH cohorts and a simplified risk score calculator developed

that may be more applicable to general clinical practice (Benza et al. 2012; Thenappan et al. 2012).

2.3 *Natriuretic Peptides*

The natriuretic peptides—atrial and brain, ANP and BNP—are synthesised by myocardium and secreted into the circulation, regulating natriuresis, diuresis, and vasomotor tone (Yoshimura et al. 1991; Nakao et al. 1992). Pressure and volume overload increases secretion and circulating levels of ANP and BNP and acts to reduce the workload of the heart, albeit ineffectively in PH.

BNP is produced as a 108 amino acid prohormone, which is cleaved and secreted as a biologically active sequence (BNP-32) and inactive N-terminal proBNP (NT-proBNP) peptide. The former has a short half-life of around 20 min and NT-proBNP a longer half-life of several hours, but both fragments are cleared by the kidney and influenced by renal function (Holmes et al. 1993; Yandle et al. 1993). Other non-cardiac factors (e.g. age, obesity, diabetes mellitus) are also known to influence NT-proBNP/BNP plasma levels.

Circulating levels of ANP, BNP-32, and NT-proBNP correlate with haemodynamic measures in PAH (Nagaya et al. 1998; Leuchte et al. 2005; Souza et al. 2005) and reduced BNP levels following long-term vasodilator therapy also correlate strongly ($p < 0.001$) with falls in PVR, suggesting that BNP measurements may reflect treatment efficacy (Nagaya et al. 1998).

Natriuretic peptide levels are elevated by left as well as right ventricular dysfunction and therefore not specific to PH. Nonetheless, the right ventricle is a major determinant of prognosis in PH (van de Veerdonk et al. 2011) and circulating BNP/NT-proBNP levels predict survival and can be used to risk stratify patients (Nagaya et al. 2000; Fijalkowska et al. 2006; Nickel et al. 2008; Hampole et al. 2009; Rhodes et al. 2011a). In a study of 55 PAH patients with a mean follow-up time of 36 months, a cut-off NT-proBNP level of 1,400 pg/ml predicted mortality with 88 % sensitivity and 53 % specificity (log rank, $p < 0.01$) (Fijalkowska et al. 2006). The influences of renal insufficiency were also examined in a later study of 118 patients, which showed in a multiple covariate analysis that NT-proBNP levels and creatinine clearance outperformed BNP, and were independent predictors of mortality (Leuchte et al. 2007).

2.4 *Growth and Differentiation Factor-15*

Growth and differentiation factor (GDF)-15 is a member of the TGF- β superfamily (Bootcov et al. 1997) that protects mice from developing ventricular hypertrophy following pressure overload (Xu et al. 2006). It is ubiquitously expressed at low levels in most tissues and is induced in response to tissue injury and inflammation.

Mechanical stretch, as well as other stimuli, triggers its release from cardiomyocytes and it is thought that GDF-15 has a role in protecting myocytes against apoptosis and induction of hypertrophy (Heger et al. 2010).

Circulating GDF-15 levels have prognostic power in several cardiovascular diseases (Kempf and Wollert 2009; Khan et al. 2009) and hence this biomarker is not specific to PH. GDF-15 expression has also been localised to plexigenic lesions in PAH patients (Nickel et al. 2011b) but, as it expressed elsewhere, the source and major stimulus for GDF-15 production in PAH are unclear. This may in part explain why GDF-15 levels added prognostic information to BNP in one study (Nickel et al. 2008), but appeared redundant in another where other markers of inflammation and general cardiovascular health were also measured (Rhodes et al. 2011a).

2.5 Red Cell Distribution Width and Other Iron Parameters

Anaemia is a recognised predictor of survival in both chronic heart failure and PAH (Rhodes et al. 2011b). Iron deficiency, measured using a number of indicators—red cell distribution width (RDW), serum ferritin, and soluble transferrin receptor—predicts a poor survival in PAH even in the absence of anaemia (Rhodes et al. 2011c). Inappropriately raised hepcidin levels suggest impaired absorption of iron from the gut. Interestingly, hepcidin is regulated by BMP6 and the possibility that dysregulated hepcidin production and PAH may be linked through a perturbation of BMP signalling merits further investigation. Nevertheless, hepcidin is an acute phase protein and hepatic expression is induced by circulating pro-inflammatory cytokines such as interleukin-6 (IL-6, an inflammatory cytokine linked to the development of PH, see below).

Inflammation can induce iron deficiency, but the relationship of iron deficiency to survival in PAH seems independent of measures of inflammation. In 139 IPAH patients (Rhodes et al. 2011a), RDW was assessed as a prognostic biomarker alongside several other previously identified plasma biomarkers: NT-proBNP, GDF-15, creatinine (a marker of renal dysfunction, see below), and IL-6, as well as baseline haemodynamics and coincident clinical measurements. All 5 plasma markers predicted survival, though RDW was the strongest performer in ROC analysis, with an area under the curve of 0.82 (GDF-15 and 6 min walk distance 0.76/0.75, NT-proBNP, and IL-6 0.67/0.66). Again, a “normal” RDW level of 15.7 % was the best cut-off, levels above identifying a group of patients with around 40 % 3-year survival compared to over 70 % in those with lower levels. Haemodynamic measurements (mPAP, mRAP, CI, and PVR) also predicted survival, as did WHO functional class, but a Cox regression model comprising just 6 min walk distance, RDW, and NT-proBNP levels could not be improved by the addition of any further measure identified as prognostic in single variable regressions.

2.6 *Inflammatory Markers*

Inflammation has long been appreciated as an element of PH pathology (Hassoun et al. 2009) and patients with PAH associated with connective tissue disorders generally exhibit a poorer prognosis and increased mortality risk when compared with other forms of the disease (Condliffe et al. 2009; Benza et al. 2010). The inflammatory marker C-reactive protein (CRP) has been shown to predict outcome, the normalisation of CRP levels being associated with improved haemodynamic and functional capacity as well as long-term survival (Quarck et al. 2009; Sztrymf et al. 2010). The levels of interleukins (ILs) such as IL-1 β and IL-6 are also raised in PAH patients (Humbert et al. 1995) and a study characterising circulating endothelial progenitor cells in PAH demonstrated elevated tumour necrosis factor (TNF)- α , as well as the levels of IL-6 (CRP) in both IPAH and Eisenmenger congenital heart disease PAH patients (Diller et al. 2008). Increased IL-6 has also been demonstrated in inoperable congenital heart disease patients (Lopes et al. 2011). Interestingly, IL-6 can be induced by loss of BMPR2 expression, interacts with BMP signalling (Hagen et al. 2007), and has been implicated in the development of PH in experimental models (Savale et al. 2009; Steiner et al. 2009). Furthermore, high IL-6 levels may contribute to the reduced BMPR2 expression seen in IPAH through activation of the microRNA (miR)-17/92 cluster (Brock et al. 2009).

A recent screen of serum TNF- α , interferon-gamma, and several interleukins (IL-1 β , -2, -4, -5, -6, -8, -10, -12p70, and -13) in 60 idiopathic and heritable PAH patients showed that all the cytokines measured (except IL-5 and IL-13) were elevated when compared with healthy volunteers (Soon et al. 2010). In addition, Kaplan–Meier analysis indicated that levels of IL-6, -8, -10, and -12p70 predicted survival. Another study of 139 IPAH patients also found that raised IL-6 was a good predictor of survival, but may not offer prognostic information above that provided by NT-proBNP, RDW, and 6MWD (Rhodes et al. 2011a). However, a direct measure of the inflammatory component in PH may yet prove useful in guiding therapeutic decisions.

2.7 *Creatinine*

Failure of the right ventricle is associated not only with reduced cardiac output but also back-pressure on the liver and kidney. In a study highlighting the dependence of NT-proBNP levels on renal function, estimates of creatinine clearance were shown to predict survival in over 100 PH patients of mixed aetiology (Leuchte et al. 2007). In one of the largest studies of biomarkers in PH to date, serum creatinine levels not only correlated with pulmonary haemodynamics, functional class, and exercise capacity but also predicted survival, particularly in a subgroup of patients with lower mRAP (<10 mmHg) measurements (Shah et al. 2008). In a more recent study of idiopathic PAH patients, creatinine levels correlated with baseline mRAP and CI, predicting survival in single variable analyses but not independently of NT-proBNP, RDW, and 6MWD (Rhodes et al. 2011a).

2.8 *Uric Acid*

Uric acid is the final degradation product of purines and an endogenous free radical scavenger that is elevated following tissue ischaemia and hypoxia. Raised levels have been described in chronic obstructive pulmonary disease (Braghiroli et al. 1993), chronic heart failure (Leyva et al. 1998), and cyanotic congenital heart disease (Hayabuchi et al. 1993; Leyva et al. 1998). Circulating uric acid levels are also widely reported to be elevated in PAH, with values around 50 % higher than seen in controls (Hoepfer et al. 1999; Nagaya et al. 1999; Bendayan et al. 2003; Voelkel et al. 2000). Correlations of uric acid serum levels with cardiac output, PVR, WHO functional class, and mortality suggested its potential usefulness as a biomarker in PH (Nagaya et al. 1999). This was also underlined by reductions of uric acid levels in response to chronic vasodilator therapy. Potential confounding factors however include renal dysfunction and diuretic use and hence uric acid levels must be interpreted in the context of these factors.

2.9 *Angiopoietins*

Angiopoietin (Ang)-1 and its antagonist Ang-2 are ligands for the Tie2 tyrosine kinase receptor on endothelial cells and, in balance with vascular endothelial growth factor (VEGF), they act to control vascular development and maturation (Augustin et al. 2009). Overexpression of Ang-1 has been proposed as a murine model of PH (Chu et al. 2004), whereas other studies have suggested that the pathway is protective (Kugathasan et al. 2009). Circulating levels of Ang-1, Ang-2, VEGF, and soluble Tie2 (which inhibits circulating Ang-1) were all shown to be elevated in a study of IPAH patients (Kumpers et al. 2010). Of these four markers only Ang-2 levels correlated with disease severity (as assessed by mRAP, PVR, CI, and WHO functional class) and also correlated with elevated NT-proBNP levels. Furthermore, Ang-2 levels predicted disease outcomes; the best cut-off for predicting death or transplantation at 3 years was 2.9 ng/ml (sensitivity 85 % and specificity 58 %), a level rarely reached in controls measured. A small second cohort also demonstrated changes in Ang-2 following the initiation of treatment that correlated with alterations in parameters such as mRAP, PVR, and 6MWD (Kumpers et al. 2010). The usefulness of this interesting new biomarker, which might potentially reflect the dysfunctional endothelium in PAH, must be validated in independent studies.

2.10 *MicroRNAs*

Recently, several non-protein coding microRNAs (miRs) have been identified that modulate gene expression at the post-transcriptional level, regulating a wide range of physiological and pathological processes in the cardiovascular system, and circulate in the bloodstream (Creemers et al. 2012). Several miRs have been implicated in the pathogenesis of PH and could have utility as biomarkers as well as therapeutic targets in PAH (McDonald et al. 2012). These include the miR-17/92 cluster—mainly miR-20a and miR-17—(Brock et al. 2009, 2012; Pullamsetti et al. 2012), miR-21 (Caruso et al. 2010), and miR-204 (Courboulin et al. 2011). Most of these studies focused on changes in miRs in hypoxia- and monocrotaline-induced experimental models of PH, but miR-21 was shown to be reduced in human lung tissue and serum from patients with IPAH (Caruso et al. 2010). Downregulation of miR-204 has also been described in lung tissue, cultured pulmonary artery smooth muscle cells, and circulating buffy coat cells from patients with PAH (Courboulin et al. 2011). Interestingly, a recent *in silico* investigation, using a network-based bioinformatics approach, identified these miRs as ones predicted to be disease modifying in PH (Parikh et al. 2012). In addition to their potential role as biomarkers, circulating miRs are now thought to have a role in mediating intercellular communication. For example, miR-150 released from monocytic cells affects endothelial cell function and miR-143/145 from endothelial cells modulates smooth muscle cell phenotypes (Hergenreider et al. 2012). Circulating miRs might provide new insights into the pathogenesis of PH, particularly as they are accessible, relatively stable, and in some cases tissue-specific. Research in circulating miRs is however still at an early stage and the methodology is challenging, requiring careful consideration of study design, standards, statistical analysis, and validation in independent cohorts (Zampetaki et al. 2012).

2.11 *Other Potential Biomarkers in PH*

While the focus of this chapter has been primarily on blood biomarkers which represent possibly the simplest objective measure of disease at relatively low cost, other methodologies relevant to PH are being improved and may in future become more commonly adopted in disease management. *Cardiopulmonary exercise testing (CPET)* is an improvement upon more standard exercise measures recommended in Europe for risk stratification, which can discern between PH of different aetiologies (Zhai et al. 2011) and has prognostic value (Ferrazza et al. 2009; Deboeck et al. 2012), but requires expertise and specialist equipment that are not available at all centres. *Radionuclide imaging*, such as positron emission tomography (PET), and the availability of novel radiotracers for studies of relevant signal transduction pathways (Jakobsen et al. 2006) offer the potential to monitor disease progression and response to therapy in PH. There has been

particular interest in the use of 18 fluorodeoxyglucose PET in PAH (Xu et al. 2007), providing a surrogate marker of changes in glycolytic activity, vascular structure, and immune/inflammatory activation in the lung (Marsboom et al. 2012) as well as a marker of right ventricular dysfunction in PAH (Bokhari et al. 2011). Imaging techniques such as *3D echocardiography* (Amaki et al. 2009; Grapsa et al. 2012a, b) and *cardiomagnetic resonance (CMR) imaging* (Vonk Noordegraaf and Galie 2011; Bradlow et al. 2012) also offer greater accuracy and reproducibility in the assessment of the right ventricle and can provide volumetric measurements that reflect its function. CMR in particular has been utilised to assess the efficacy of therapeutics in recent clinical trials (Wilkins et al. 2005, 2010) and provides prognostic information in PAH (van Wolferen et al. 2007; van de Veerdonk et al. 2011), but the expense and the expertise required to operate the machinery and interpret results may have inhibited its more widespread use.

Many other blood biomarkers have also been associated with PH and are summarised in Table 1.

2.12 Comments on Biomarkers in PH

While many different biomarkers have been proposed for use in the management of PH, most have been inadequately validated in larger, independent cohorts and few have met the criteria to support routine measurement and use in clinical practice. The evidence for the utility of BNP and NT-proBNP as indices of right ventricular stress in PH is strong and their measurement is recommended by European guidelines (Galie et al. 2009). Despite this, the lack of validated specific cut-offs and an understanding of the meaning of proportional changes in BNP levels over time limits their use in practice. GDF-15 and RDW have been particularly strong performers in recent biomarker studies (Nickel et al. 2008; Rhodes et al. 2011a), possibly because they report on multiple facets of the disease pathology. Other markers such as IL-6, creatinine, uric acid, and angiotensins may reflect more specific components, inflammation, renal dysfunction, oxidative stress, and endothelial cell dysfunction, respectively. The relatively recent discovery of microRNAs opens up a new avenue for biomarker discovery in PH. But before any of these biomarkers will be used routinely, circulating levels have to be shown to provide additional prognostic information that aids clinical decision-making and the necessary laboratory tests are readily available and reliable. The use of multiple markers, reflecting different aspects of PH pathology and incorporating imaging, may best represent the overall status of a patient and help guide future therapeutic decisions as more specific therapies become available. A critical question however is whether such a panel of biomarkers can identify not just a cohort but an individual patient with PH who is likely to derive benefit from a particular treatment?

Table 1 Other potential biomarkers in pulmonary hypertension

Biomarker	Biology	Evidence
Insulin resistance	Control of blood glucose levels	Insulin resistance twice as common in females with PAH (Zamanian et al. 2009)
Soluble TWEAK	Member of tumour necrosis factor (TNF) receptor superfamily	Predicts hemodynamic impairment and functional capacity in patients with PAH (Filusch et al. 2011)
Osteoprotegerin (OPG)	TNF/apoptotic pathway	Serum OPG elevated in IPAH patients and predicts survival (Lawrie et al. 2008; Condliffe et al. 2012)
Apelin	Vasodilator	Plasma levels decreased in IPAH, chronic lung disease, and heart failure (Goetze et al. 2006)
Endothelial progenitor cells (EPCs)	Endothelial repair/dysfunction	Heterogeneous population; altered circulating cell number and/or function of cultured cells in PAH (Diller et al. 2008, 2010; Asosingh et al. 2008; Toshner et al. 2009)
Circulating microvesicles	Endothelial function	Increased endothelial microvesicles predict haemodynamic severity and outcome in PH (Amabile et al. 2008, 2009)
Von Willebrand factor (vWf)	Endothelial function	Elevated levels prognostic in a small study of PAH associated with congenital heart disease (Lopes et al. 2011)
Cyclic GMP	Nitric oxide (NO) pathway	cGMP levels increased in PH (Bogdan et al. 1998), decreased by iloprost (Wiedemann et al. 2001), and correlate with PVR (Ghofrani et al. 2002)
Asymmetric dimethyl arginine (ADMA)	Inhibitor of NO synthase	Upregulated in PAH associated with congenital heart disease (Gorenflo et al. 2001). Levels correlate with haemodynamics and mortality in IPAH (Kielstein et al. 2005) and CTEPH (Skoro-Sajer et al. 2007)
Osteopontin	Inflammatory cytokine	Raised levels in IPAH correlate with 6MWD, mPAP, and functional class and predict survival (Lorenzen et al. 2011)
Isoprostanes	Oxidative stress	Increased in PAH and urinary levels associated with survival (Cracowski et al. 2001, 2012)
Nitric oxide (NO)	Vasodilator	Exhaled NO and urine NO metabolites reduced in IPAH and reversed by bosentan treatment (Girgis et al. 2005)
Serotonin	Vasoconstrictor	Raised levels in PAH correlate with PVR, but unresponsive to vasodilator treatment (Kereveur et al. 2000)
Endothelin-1	Vasoconstrictor	Raised plasma levels correlate with RAP, PVR, and disease severity in PAH (Stewart et al. 1991; Cacoub et al. 1993; Nootens et al. 1995; Rubens et al. 2001)
Cardiac troponins T & I	Myocardial injury	Troponin T & I levels predict disease severity and survival in PAH (Torbicki et al. 2003; Heresi et al. 2012)

(continued)

Table 1 (continued)

Biomarker	Biology	Evidence
Fatty acid binding protein	Myocardial injury	Negative prognostic marker in CTEPH (Lankeit et al. 2008) and pulmonary embolism (Puls et al. 2007)
Complement C4a des Arg	Complement cascade	Elevated levels of C4a des Arg (C4a activation fragment) in IPAH (Abdul-Salam et al. 2006)
Matrix metallo-proteinases (MMPs)	Tissue remodelling	Elevated plasma levels of MMP-2, MMP-9, and tissue inhibitor of MMP (TIMP)-4 in PH (Elias et al. 2008; Hiremath et al. 2010; Schumann et al. 2010)

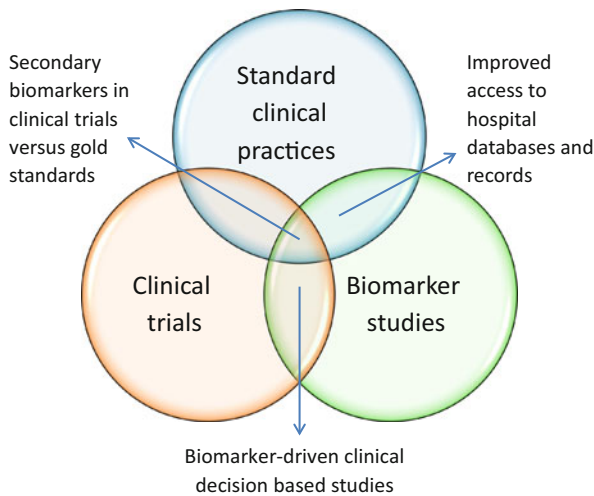


Fig. 3 Areas where improvements are required to take biomarker science forward in PH

3 Future Directions and Conclusions

The advent of “unbiased” genomic, metabolomic, and proteomic screening methodologies has not yet provided a major improvement in our understanding of disease biology or identified many new biomarkers. As the technologies are refined and improved upon, however, they will inevitably generate novel targets for therapy or biomarker use. MicroRNA screens in particular may generate novel hits, with panels of miRNAs being used to assess disease progression and response to therapy. The replication of findings using independent cohorts from other centres is vital in order to validate a potential biomarker and comparisons against current gold standards may be accelerated using hospital databases and registries in research protocols.

Genetic factors in PH can be useful in estimating susceptibility but currently apply only to a relatively small proportion of the overall PH population. More biomarker-focused clinical studies and better integration of pre-clinical biomarker studies into standard practices and clinical therapy trials are needed to take biomarkers in PH to the next level (Fig. 3).

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Rodent Models of Group 1 Pulmonary Hypertension

John J. Ryan, Glenn Marsboom, and Stephen L. Archer

Abstract World Health Organization category 1 pulmonary hypertension (PH) is a heterogeneous syndrome in which PH originates in the small pulmonary arteries and is therefore also referred to as pulmonary arterial hypertension (PAH). Common pathophysiologic features include endothelial dysfunction, excessive proliferation and impaired apoptosis of vascular cells, and mitochondrial fragmentation. The proliferation/apoptosis imbalance relates in part to activation of the transcription factors hypoxia-inducible factor-1 α (HIF-1 α) and nuclear factor of activated T-cells (NFAT) and apoptosis repressors, such as survivin. Perivascular inflammation, disruption of adventitial connective tissue, and a glycolytic metabolic shift in vascular cells and right ventricular myocytes also occur in PAH. There are important genetic and epigenetic predispositions to PAH. This review assesses the fidelity of existing animal models to human PAH. No single model can perfectly recapitulate the many diverse forms of PH in Category 1; however, acceptable models exist. PAH induced by monocrotaline and chronic hypoxia plus SU-5416 (CH+SU) in rats display endothelial dysfunction, proliferation/apoptosis imbalance, and develop the glycolytic metabolic profile of human PAH. Histologically, CH+SU best conforms to PAH in that it develops complex vascular lesions, including plexiform lesions. However, the monocrotaline model can be induced to manifest complex vascular lesions and does manifest the tendency of PAH patients to die of right ventricular (RV) failure. Murine models offer greater molecular certainty than

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rat models but rarely develop significant PH, have less right ventricular hypertrophy (RVH) and pulmonary artery (PA) remodeling, and are harder to image and catheterize. The use of high fidelity catheterization and advanced imaging (microPET-CT, high frequency echocardiography, high field strength MRI) and functional testing (treadmill) permit accurate phenotyping of experimental models of PAH. Preclinical trial design is an important aspect of testing experimental PAH therapies. The use of multiple complementary models with adequate sample size and trial duration and appropriate endpoints are required for preclinical assessment of experimental PAH therapies.

Keywords Hypoxia • Monocrotaline • Sugen 5416 • Right ventricular hypertrophy • Plexiform lesions

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Non-standard Abbreviations and Acronyms

5-HTT	Serotonin transporter
Ang-1	Angiopoietin-1
BMPR-2	Bone morphogenetic protein receptor-2
CH + SU	Chronic hypoxia plus Sugden-5416
CO	Cardiac output
DRP-1	Dynamin-related protein 1
ET-1	Endothelin-1
FHR	Fawn-Hooded rat
HHV	Human herpes virus
HIF-1 α	Hypoxia-inducible factor-1 α
HIV	Human immunodeficiency virus
HPV	Hypoxic pulmonary vasoconstriction
LVEDP	Left ventricular end diastolic pressure
mPAP	mean pulmonary artery pressure
MRI	Magnetic resonance imaging
NFAT	Nuclear factor of activated T cells
PA	Pulmonary artery
PAAT	Pulmonary artery acceleration time
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
RVH	Right ventricular hypertrophy
SOD2	Superoxide dismutase 2
SU-5416	Sugden-5416
TAPSE	Tricuspid annulus plane systolic excursion
VEGF	Vascular endothelial growth factor
VTI	Velocity time integral

1 Introduction

In Group 1 PH (or pulmonary arterial hypertension, PAH) not only is mPAP greater than 25 mmHg, but the designation requires the exclusion of thromboembolic disease, lung disease, and left heart disease (the left ventricular end diastolic pressure should be <15 mmHg). Group 1 PH occurs in a rare idiopathic form and an even less common familial form. Most Group 1 PH is associated with an identifiable risk factor (such as connective tissue disease, congenital heart disease, or anorexigen consumption). The female/male ratio in PAH is >4/1 (Archer and Rich 2000; Pugh and Hemnes 2010). There is considerable heterogeneity within

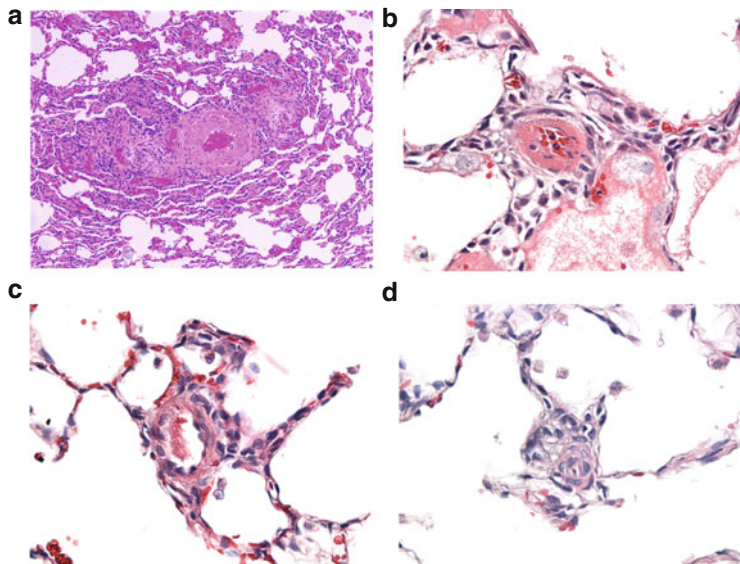


Fig. 1 Histology from human and rat lung. (a) Histology from scleroderma patient with PAH showing medial hypertrophy. (b) Vascular obliteration in monocrotaline rat. (c) Chronic hypoxia rat with mild medial hypertrophy. (d) Plexiform lesions in Sugen-Hypoxia rat

Group 1 with associated conditions as diverse as congenital heart disease, hemolytic anemia, schistosomiasis, and HIV. PAH has a significant mortality with 5-year survival of only 60 % (Thenappan et al. 2010), despite advances in therapies. The estimated incidence of PAH is 2.4–7.6 cases/million annually (Humbert et al. 2006).

Common histological features of Group 1 include medial hypertrophy and adventitial hyperplasia of small pulmonary arteries. Often there are complex vascular lesions (including intimal hyperplasia and plexiform arteriopathy) (Fig. 1a) (reviewed in Tuder et al. 2007). Plexiform lesions are reminiscent of glomeruli, containing multiple, endothelial-lined, slit-like channels interspersed with myofibroblasts and pulmonary artery smooth muscle cells (PASMCS). Clusters of small, dilated pulmonary arteries, often found distal to these plexiform lesions, are called angiomatoid lesions. Other common features of Group 1 PH are platelet deposition, thrombus in situ, and pulmonary hemorrhage (Tuder et al. 2007; Pogoriler et al. 2012). The histological features of human PAH that have been hardest to reproduce in animal models include the intimal proliferation, plexiform lesions, and pulmonary veno-occlusive lesions. These histologic factors (combined with an element of exaggerated vasoconstriction) increase right ventricular afterload (measured as increased pulmonary vascular resistance, $PVR > 3$ Wood units) and reduce vascular compliance (evident as shortened pulmonary artery acceleration time, PAAT).

Even in human PAH there is histological diversity. Plexiform lesions are uncommon in scleroderma-associated PAH (Pogoriler et al. 2012). Within the lungs of a single patient, there can be considerable regional heterogeneity in the

localization of complex vascular lesions. As in human PAH, the pathology in most animal models of Group 1 PH, is confined to the lung vasculature, although here again there is diversity among forms of Group 1 PH. Scleroderma patients more often manifest pulmonary veno-occlusive disease and may have concomitant interstitial lung fibrosis, systemic vascular disease (notably Raynaud's phenomenon,) and left ventricular disease (Sweiss et al. 2010). This is perhaps not surprising, since scleroderma is a systemic disease and circulating autoantibodies that target endothelium and smooth muscle cells (SMCs) are not specific for the lung vasculature. There are currently no good models of Category 1' PH (patients with pulmonary capillary hemangiomatosis and pulmonary veno-occlusive disease). Thus, no single animal model of PH will accurately capture all the facets of Group 1 PH, perhaps because Group 1 is itself a heterogeneous collection of PH syndromes.

The other groups classified by the WHO system are not the focus of this review, but they too are best studied using models that closely replicate their central disease process. In Group 2 PH, the PH is secondary to an increase in left atrial pressure caused by left heart disease, including systolic or diastolic left ventricular dysfunction, or valvular diseases, such as mitral stenosis (Califf et al. 1997). There are no convenient and readily reproducible small animal models for Group 2 PH, although transaortic banding can induce this syndrome (Chen et al. 2012). Group 3 PH results from chronic hypoxia exposure or chronic lung disease resulting in mild elevations in PAP (Elwing and Panos 2008). The chronic hypoxic rat model is reflective of Category 3 PH (Ryan et al. 2011). Group 4 PH is secondary to chronic thromboembolic disease and small animal models are lacking. Group 5 is a collection of PH syndromes associated with miscellaneous diseases, such as extrinsic pulmonary artery (PA) obstruction and sarcoidosis (and is thus difficult to model).

Many PAH patients have multiple, identifiable pathogenic stimuli, such as a component of hypoxia and/or increased LVEDP, that contribute to their PH syndrome. Models that offer molecular purity (e.g., knockout mice) may not recapitulate the complex clinical picture of PH. Interestingly, the models induced by multiple insults, such as monocrotaline plus pneumonectomy or chronic hypoxia plus Sugen-5416 (CH + SU), best mimic human PAH.

Many experimental therapies studied in animal models have demonstrated a benefit, which fails to translate into clinical benefit in patients (Stenmark et al. 2009). In a review of 91 studies that used the monocrotaline model, therapies were demonstrated to be beneficial in 88 of the studies (Stenmark et al. 2009). The excess optimism from these animal studies originates partially from publication bias (which favors positive findings) and partly from flaws in the models (i.e., monocrotaline imperfectly mimics human PAH). However, poor study design often contributes to the confusion. Many of these "positive" studies reflect the short duration of the trial, the reliance on surrogate end points instead of mortality, and the rather poor phenotypic characterization of hemodynamics and right ventricular function. For example, by extending study duration in a rodent trial of statins for prevention of PAH, we found the same lack of efficacy (McMurtry et al. 2007a) that was ultimately noted in patients (Kawut et al. 2011). Several positive rodent studies of statins in PAH were of shorter duration, perhaps contributing to an overly optimistic conclusion.

The ideal model would reflect the distinct clinical characteristics of Group 1 PH, namely a female predominance, isolated pulmonary vascular disease, right ventricular hypertrophy, a high mortality from right heart failure, and the classical histopathological features of human PAH. Such a model has been challenging to create. In addition, for those studying pediatric Group 1 PH, a model would need to be created in neonatal animals, since the pulmonary vascular biology of neonates and adults differs substantially.

In this chapter, we review the models of Group 1 PH, emphasizing common rodent models, summarized in Table 1. We briefly discuss large animal models, including dogs, sheep, cows, and primates. These large animals oftentimes more successfully replicate human disease than do rodent models; however, their utility is limited by their size, the expense of the models, the slower disease progression, as well as less scientific emotional/ethical caveats, relating to large animal studies.

2 Molecular Signaling

The animal models of PAH should replicate the molecular and cellular signaling disorders seen in PAH patients. Plasma serotonin is increased in PAH (Herve et al. 1995), as is the ratio of endothelial-derived vasoconstrictors/dilators (Christman et al. 1992). Increased expression and activity of serotonin transporter (5-HTT) in PASMCM contributes to the proliferative excess in PAH (Marcos et al. 2003), as does increased expression of platelet-derived growth factor receptor (Perros et al. 2008). The decreased PASMCM apoptosis and increased proliferation seen in PAH raises comparisons to cancer (Merklinger et al. 2005). Apoptosis resistance is driven by several factors, including *de novo* expression of the anti-apoptotic protein survivin (McMurtry et al. 2005). Apoptosis resistance is seen in multiple rodent models, including Fawn-Hooded rats and 5-HTT overexpressing mice (Archer et al. 2010).

Mutations in bone morphogenetic protein receptor-2 (BMPR-2) (Morrell et al. 2001), the genetic basis for familial PAH (Thomson et al. 2000), also promote proliferation and impair apoptosis. The resulting loss of BMPR-2 receptor function contributes to PASMCM proliferation (Zhang et al. 2003). BMPR-2 also affects endothelial cells (Yang et al. 2011). Although most rodent models of PAH have no genetic BMPR-2 abnormality, impaired BMP signaling and acquired downregulation of BMPR-2 expression is commonly observed (McMurtry et al. 2007b; Morrell 2006). In addition, the BMP antagonist gremlin is increased in hypoxic PH and gremlin is also upregulated in the vessels of PAH patients (Cahill et al. 2012). Increased gremlin secretion by endothelial cells blocks BMP signaling thereby contributing to PASMCM proliferation and PAH.

Normoxic activation of HIF-1 α promotes PASMCM proliferation and creates a glycolytic phenotype, which can be detected by increased pulmonary uptake of fluorodeoxyglucose (FDG) on Positron Emission Tomography (PET) scans (Marsboom et al. 2012a; Fijalkowska et al. 2010). HIF-1 α activation occurs despite

Table 1 Animal models of Group 1 PAH

Animal model	Method	Medial hypertrophy	Plexiform lesions	mPAP (mmHg)	RVH	HIF-1 α activity	Kv1.5	Spontaneity	Mortality
Human PAH	Spontaneous or hereditary	+++	+++	~30–60	Varies	+	↓	+++	30 % mortality at 3 years
Monocrotaline rat	60–100 mg/kg sc Can be combined with pneumonectomy	+++	+	40–60	Moderate	+	No change	–	100 % ~2 months
Chronic hypoxia rat	10 % O ₂ for 3–4 weeks Also studied in mice	+	–	30–40	Mild	+	↓	–	Minor
Chronic hypoxia plus SU-5416 rat	20 mg/kg of SU-5416 coupled with 3 weeks of hypoxia at 10 %	++	+	40–60	Moderate	+	↓	–	10 % at 6 weeks then stable until ~20 weeks
Fawn-Hooded rat	Hereditary	++	–	30–50	Moderate	+	↓	+++	Survives up to 1 year
BMPR-2 mutation mouse	Overexpression of mutant BMPR-2 alleles	++	–	35–45	Mild	+	↓	+++	Stable up to 3 months
Endothelin-B receptor deficiency	Monocrotaline administered to ET _B -deficient rats	+	–	40–45	Mild	–	No change	–	Stable up to 3 months
Angiotensin-1 overexpression	AAV-Ang-1 is injected into RV outflow track	+	–	30–40	Mild	–	No change	–	Stable up to 3 months
5-HTT overexpressing mouse	Transgenic mice exposed to hypoxia for 2–4 weeks	+	–	45–55	Mild	–	No change	+	Stable up to 3 months
γ -Herpesvirus reactivation mice	Virus given to 1 year old S100A4/Mts mice	–	+	20–30	None	–	?	–	Stable up to 1 year
Schistosomiasis mice	Female C57/BL6 adult mice infected sc with <i>S. mansoni</i>	+	+	20–30	None	Undefined	?	–	Stable up to 20 weeks

(continued)

Table 1 (continued)

Animal model	Method	Medial hypertrophy	Plexiform lesions	mPAP (mmHg)	RVH	HIF-1 α activity	Kv1.5	Spontaneity	Mortality
Stachybotrys chartarum mice	Intratracheal injection of fungal spores into mice over 4–12 weeks	+	–	20–30	Moderate	Undefined	?	–	Stable up to 12 weeks
High fat diet to Apo E knockout mice	Starting at 4 weeks of age, mice are fed high fat chow for 11 weeks	+	+	20–30	Mild	Undefined	?	–	Stable up to 3 months
Monocrotaline in primates	Administered interscapularly 1/month for 4 months	+	–	40–60	Moderate	+	?	–	Stable up to 6 months
HIV-associated PH in primates	SHIV- <i>nef</i> infection	+	+	Limited data	Moderate	Undefined	?	–	Stable up to 6 months
Radiation exposure to Wistar rats	Exposed to protons with shoot-through technique	+	–	25–35	Mild	Undefined	?	–	Stable up to 2 months
Pulmonary artery banding rodent	Main PA ligated with a silk suture	–	–	Normal	Severe	–	No change	–	Stable up to 12 weeks
Aorto-caval shunt rodent	Needle advanced through abdominal aorta into IVC. Can be combined with monocrotaline	+	–	25–35	Severe	–	No change	–	Stable up to 12 weeks
Brisket disease in cattle	Cattle reared at 7,000 ft	+	–	60–80	Severe	+	?	–	Stable up to 6 months

? represents the unknown effect of the animal model on Kv1.5

5-*HIT* serotonin transporter, *Ang-1* angiotensin-1, *BMPR-2* bone morphogenetic protein receptor-2, *CH + SU* chronic hypoxia plus Sugen-5416, *ET-1* endothelin-1, *FHR* Fawn-Hooded rat, *HIV* human herpes virus, *HIF-1 α* hypoxia-inducible factor-1 α , *HHV* human immunodeficiency virus, *mPAP* mean pulmonary artery pressure, *PA* pulmonary artery, *PAH* pulmonary arterial hypertension, *PH* pulmonary hypertension, *RVH* right ventricular hypertrophy, *SU-5416* Sugen-5416

normal PO_2 and (at least in some cases) is the result of epigenetic silencing of superoxide dismutase 2 (SOD2) (Archer et al. 2010). Decreased SOD2 expression impairs mitochondrial redox signaling and permits normoxic activation of HIF-1 α (Archer et al. 2010). HIF-1 α activation increases the transcription of the pyruvate dehydrogenase kinases (PDKs), through interaction of HIF-1 α with the PDK gene's hypoxia-responsive element (HRE) (Kim et al. 2006). PDKs phosphorylate and inhibit the pyruvate dehydrogenase (PDH) complex, thereby shifting PASMC metabolism to glycolysis (McMurtry et al. 2004; Michelakis et al. 2002; Guignabert et al. 2009). This abnormal mitochondrial-SOD2-HIF-1 α pathway is seen in Fawn-Hooded rats, as well as in the monocrotaline and chronic hypoxic models. The glycolytic phenotype is reminiscent of the Warburg phenomenon seen in cancer cells (Bonnet et al. 2007a). Recently it has been noted that the mitochondria in PAH PASMC are not only functionally impaired, but also they are structurally abnormal—specifically they are fragmented (Archer et al. 2008). Mitochondrial fragmentation reflects a disorder in two tightly regulated processes: mitochondrial fission (which is increased) and mitochondrial fusion (which is depressed). The excessive fission is caused by activation of dynamin-related protein 1 (DRP-1) (Marsboom et al. 2012b). HIF-1 α activation increases the activity of DRP-1 by phosphorylation of DRP-1 at serine 616. This phosphorylation is coupled to mitosis because the same regulatory kinase (cyclin B1/CDK1) phosphorylates DRP-1 and permits mitosis. For the cell to undergo mitosis, the mitochondria must divide and DRP-1 inhibition prevents both mitotic fission and cell division. For example, the DRP-1 inhibitor, mdivi-1, arrests PAH PASMCs at the G2/M interphase. Thus the mitochondria looms large in the pathogenesis of PAH, which is interesting, since mitochondria are also the redox sensor for hypoxia and are central to hypoxic pulmonary vasoconstriction (Weir et al. 2005).

Kv1.5 and other voltage-gated potassium channels are decreased in human PAH and rodent models of PAH. Loss of Kv1.5 contributes to constriction and proliferation of PASMCs (Michelakis et al. 2001). Loss of Kv channels depolarizes PASMC membrane potential, thereby increasing influx of Ca^{2+} via the activation of voltage-gated calcium channels (Fig. 2) (Archer and Rich 2000; Reeve et al. 2001; Yuan et al. 1998). This raises cytosolic calcium promoting vasoconstriction and proliferation. If the increase in cytosolic calcium (which results from loss of Kv channels and activation of TrpC6 channels (Zhang et al. 2007)) is sustained, this activates nuclear factor of activated T cells (NFAT), a Ca^{2+} -calcineurin-dependent transcription factor that increases PASMC proliferation (Bonnet et al. 2007b). Loss of Kv channels also increases cytosolic K^+ , which inhibits caspases and promotes apoptosis resistance. Downregulation of Kv1.5 seen in human PAH (Yuan et al. 1998; Weir et al. 1998; Geraci et al. 2001) is recapitulated in many rodent models including those induced by chronic hypoxia (Pozeg et al. 2003) (\pm Sugen-5416) and the PH in Fawn-Hooded rats, BMPR-2 dominant negative transgenic mice (Young et al. 2006), and serotonin transporter (5-HTT) transgenic mice (Guignabert et al. 2006).

The role of inflammation in PAH is evident from the strong association with connective tissue disease and the histological presence of perivascular mononuclear

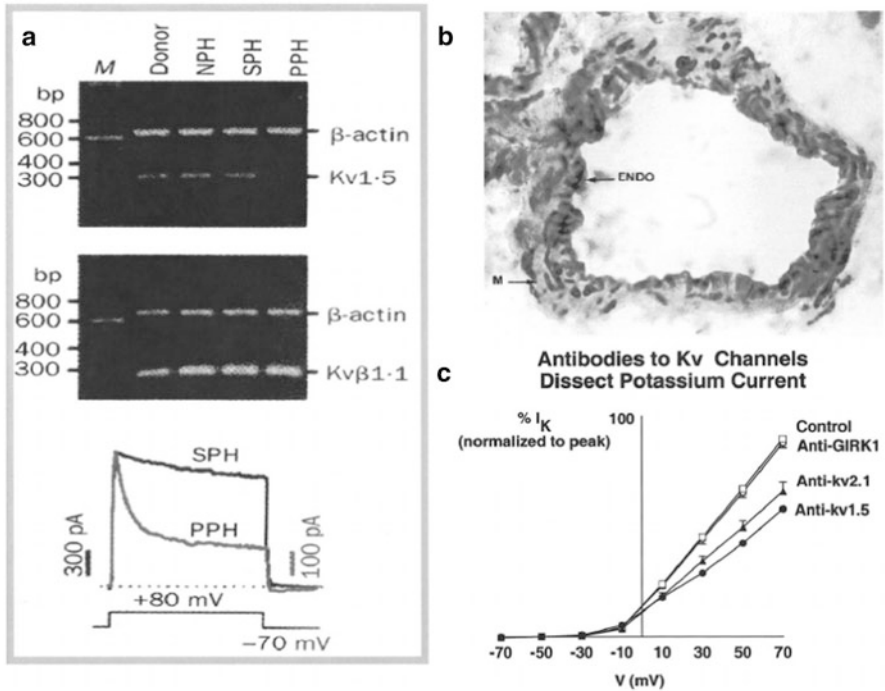


Fig. 2 Potassium channel abnormalities in PPH. **(a)** Reverse-transcriptase polymerase chain reaction shows that patients with PPH have less mRNA for Kv1.5 than donors, chronic lung disease patients with normal PVR (NPH), or secondary PHT patient (SPH). Expression of other mRNA is unaffected (such as B-actin and Kv β 1.1 subunit). **(b)** Immunohistochemistry from rat pulmonary arteries with PH shows Kv1.5 channel in PSMCs (*light gray*). Kv1.5 is also present in endothelium (ENDO, *arrow*) but is not seen because slide is double-stained with an antibody to endothelial NO synthase (*dark gray*). M indicates media. **(c)** Whole-cell potassium current (IK) is inhibited by internal application of an antibody directed against Kv1.5 and Kv2.1 on whole-cell patch clamping of normal rat PSMCs but not by an antibody against a K1 channel that is not expressed in PSMCs (GIRK-1). This demonstrates that Kv1.5 and Kv2.1 contribute to electrical activity of rat PSMCs. Reprinted with permission from Archer et al. (1998)

cell infiltrates adjacent to pulmonary arteries in PAH. Mononuclear cells have also been seen around the characteristic plexiform lesions (Cool et al. 1997). Reproducing this inflammatory infiltration is important in animal models of PAH. Inflammation is present in the monocrotaline model but is notably absent in the CH + SU model (Zhao 2010).

3 Outcome Assessments in Animal Models

A brief summary of the techniques required to comprehensively assess PAH in rodent models is provided. Exercise capacity, accurate hemodynamic measurements, and advanced imaging tools are necessary to properly categorize,

quantify, and monitor disease progression and regression of PH in animal models. Rodent imaging has been revolutionized by miniaturization of micromanometer-tipped catheters, high field-strength magnetic resonance imaging (MRI), high-frequency echocardiography, and advanced application of nuclear imaging (Urboniene et al. 2010; Thibault et al. 2010; Marsboom et al. 2012a).

4 Exercise Capacity

Exercise capacity can be assessed using treadmill testing. However, there are multiple determinants of maximal exercise capacity, including not only RV function and the status of the pulmonary circulation but also pulmonary function, left ventricular performance, skeletal muscle function, and activity of the peripheral and central nervous systems. Typical rodent treadmills are motorized and one varies the speed and slope following a standardized protocol (summarized in Fig. 3). The animal is motivated to run by a shock bar that delivers a mild noxious stimulus should they cease running (Fig. 3a). Maximal exercise capacity is defined as the distance covered before the animal fatigues and comes to rest on the shock grid (at which time they are immediately removed from the device). Concern has been raised about the reproducibility and reliability of this metric (Knab et al. 2009). Despite its limitations, assessment of exercise capacity is an important outcome. It is helpful as a surrogate for cardiac output and, when interpreted in light of findings on catheterization and echocardiography, is useful in assessing the response to therapy. Indeed, exercise capacity is the parameter used by the FDA to approve medications for patients with PAH (Miyamoto et al. 2000).

5 Catheterization and Echocardiography

Anesthesia. Both catheterization and echocardiography are performed using inhaled isoflurane mixed with medical air or 100 % O₂. To induce anesthesia in rats, the rodent is placed in an anesthesia induction chamber with gas flow at 2–3 l/min. Anesthesia is induced by administration of isoflurane at 3–4 %. After induction, anesthesia is maintained using isoflurane 1.5–2 % (for echocardiography) or 3 % (for more invasive procedures, such as cardiac catheterization). Anesthesia should be sufficient to prevent responses to noxious stimuli (like paw pinch) but sufficiently light that the heart rate is >300 bpm in rats or >500 bpm in mice.

Hemodynamics. Complete hemodynamic assessment needs to be performed to fully understand the PH models, including measurement of PVR. Without a left and right heart catheterization one cannot exclude Group 2 PH. In rodents and other animals used to study PH, the normal PAP is the same as in normal humans. However, in rats and mice the heart rate is 5–10 times faster than in humans,

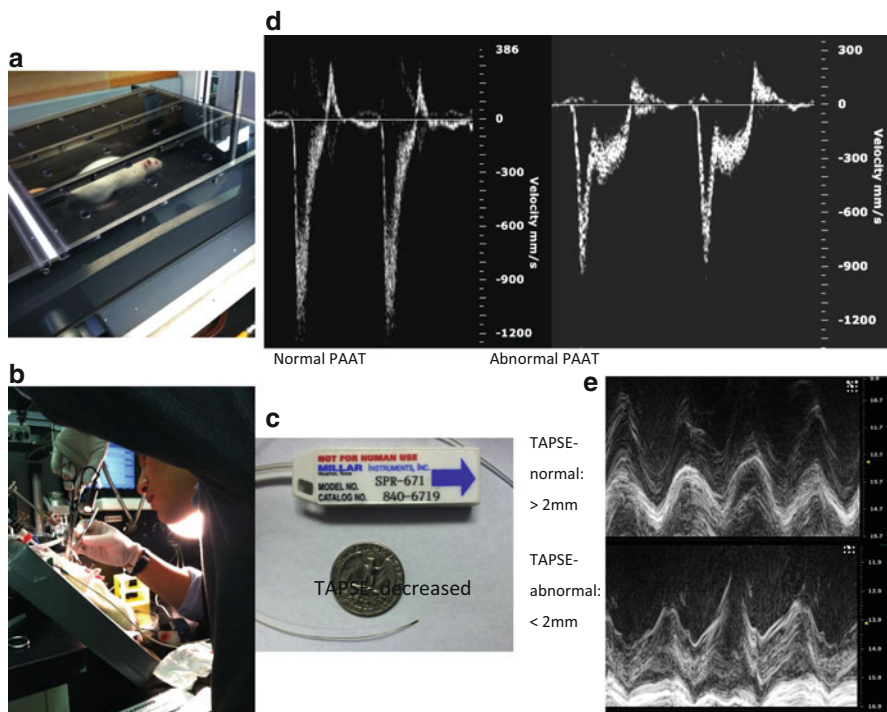


Fig. 3 (a) Rodent treadmill. Exercise capacity is tested by measuring maximal distance run on a motorized treadmill (Simplex II Instrument; Columbus Instruments, Columbus, OH). The initial treadmill speed is 5 m/min and increased 5 m/min every 5 min for 30 min or until the rat fatigued. (b) Right heart catheterization on rodent. (c) Microtipped catheter compared to 25 cents piece. (d) Pulmonary artery acceleration time as measured by Doppler echocardiogram. (e) Tricuspid annular plane systolic excursion measured on M mode echocardiogram

respectively. Hemodynamics in a group of normal and pulmonary hypertensive rats studied in our laboratory are shown in Table 2. A complete summary of the technique for rat catheterization is provided in ref (Urboniene et al. 2010).

Pulmonary hemodynamics are optimally measured in lightly anesthetized, close-chested animals. Although cannulating the PA is faster and requires less expertise in the open-chest preparation, opening the thorax reduces cardiac output, heart rate, and PA pressures (particularly end diastolic pressures). It is acceptable to use the open chest technique but this technical detail and its limitations should be noted. When performing pressure–volume assessments, we have found that, hemodynamics are more precisely obtained in prone or upright positions (rather than in the commonly used supine position) (Fig. 3b). Measurement of mPAP, systolic PAP, and LVEDP are best performed using micromanometer-tipped catheters that have sufficient frequency response to measure pressures at heart rates of greater than 300 bpm (Fig. 3c). Fluid-filled catheters produce damped (and thus inaccurate) pressure tracings at these heart rates. In addition to the right heart catheterization, it is important to measure LVEDP by retrograde catheterization of the LV (performed

Table 2 Rat hemodynamics

Rat hemodynamics	Normal Sprague-Dawley rat ($n = 8$)	Pulmonary hypertensive rat: Monocrotaline rat ($n = 6$)
HR (beats/min)	372 \pm 16	311 \pm 9
PAAT (ms)	33.4 \pm 1.1	22.1 \pm 1.4
sPAP (mmHg)	20.7 \pm 1.5	51.2 \pm 3.8
mPAP (mmHg)	14.6 \pm 1.6	32.8 \pm 2.0
PVR (dyn/s/cm ⁵)	0.15 \pm 0.02	0.52 \pm 0.06
SV (ml)	0.31 \pm 0.02	0.22 \pm 0.01
CO (ml/min)	111 \pm 8	66 \pm 6
CI (ml/min/g)	0.36 \pm 0.01	0.20 \pm 0.01
Treadmill distance (m)	405 \pm 75	125 \pm 54

BW body weight, *CI* cardiac index, *CO* cardiac output, *HR* heart rate, *PAAT* pulmonary artery acceleration time, *PACsa* pulmonary artery cross sectional area, *SV* stroke volume, *VTI* velocity time integral

via a carotid arteriotomy). For right heart measurements, the catheter is placed into the right internal jugular vein via a venotomy. A fluid-filled catheter is advanced through a curved sheath into the RV and subsequently the PA. Once PA pressures are obtained, the more fragile Millar catheter replaces the fluid filled catheter. This is challenging to perform in mice and open-chested preparations using Millar catheters may be required.

Measurement of cardiac output using thermodilution techniques is reliable and easily performed in rodents. The technique involves bolus infusion of cold saline in the internal jugular vein with temperature recording by a thermistor in the aorta (Physitemp Instruments, Clifton, NJ). Changes in temperature at the thermistor are inversely related to cardiac output. One must be mindful of the small volumes of fluid that rodents can safely tolerate to avoid perturbing hemodynamics. (we use boluses of 0.1 ml of iced saline). Normal cardiac output in adult male rats is >110 ml/min.

Echocardiography. Because catheterization is performed by the cut-down technique, (rather than percutaneously) rodents are typically euthanized at the end of the procedure. Serial studies are more easily performed using echocardiography. The challenge in measuring PAP by echocardiogram in rodent models is the absence of tricuspid regurgitation. This precludes the use of Bernoulli's equation, the technique commonly used to assess PA pressure in patients (Rich et al. 2011), to provide a noninvasive estimate of PA systolic pressure. However, in rodents the PA Doppler signal is easily obtained and this permits assessment of PA compliance and PA pressure using the PA acceleration time (PAAT) (Fig. 3d).

For echocardiography the anesthetized rat is placed in a supine position. After shaving the chest and applying gel to improve acoustic coupling, two-dimensional images are obtained (we use the Vevo 2100 (VisualSonics, Toronto, ON) equipped with a 25 MHz transducer). The two-dimensional echocardiogram allows measurement of PA diameter and with color guidance allows optimal placement of the

Table 3 Measurement of hemodynamics

Rat hemodynamic measurements	Formula
Stroke volume	$PA_{CSA} \times VTI$
Cardiac output	$SV \times HR$
Cardiac index	CO/BW
Mean PA pressure	$58.7 - (1.21 \times PAAT)$

BW body weight, *CI* cardiac index, *CO* cardiac output, *HR* heart rate, *PAAT* pulmonary artery acceleration time, *PA_{CSA}* pulmonary artery cross sectional area, *SV* stroke volume, *VTI* velocity time integral

pulsed-wave Doppler signal in the main PA. The PA pulsed Doppler envelope is analyzed to determine the PAAT (defined as the time to peak flow velocity) and flow velocity time integral (VTI). Two-dimensionally guided M-mode echocardiography is used to measure RV wall thickness

PA pressure is best measured using PAAT, which is inversely related to mean PA pressure. By performing simultaneous Doppler and right heart catheterization studies, one can create a regression equation that accurately relates PAAT to mPAP (Urboniene et al. 2010). The normal PAAT in an adult rat is 30–35 ms and a PAAT < 20 ms indicates significant PH. PAAT correlates inversely with mPAP on catheterization ($r^2 = 0.76$) and our laboratory's regression equation is $mPAP = 58.7 - (1.21 \times PAAT)$ (Urboniene et al. 2010). Ideally, each research laboratory should create its own regression equation.

In the rat monocrotaline PAH model, good correlation has been found between echocardiography and MRI assessment of PA pressure by PAAT versus the gold standard of right heart catheterization, offering the potential for noninvasive techniques to ultimately replace cardiac catheterization in rat models (Urboniene et al. 2010; Jones et al. 2002). PAAT also correlates well with mPAP in mice. A decrease in the ratio of acceleration time to ejection time (AT/ET) correlates closely with increases in murine RVSP (Thibault et al. 2010).

In PH, there is also mid-systolic notching of the PA Doppler envelope due to a retrograde cancellation wave that reflects back from the noncompliant distal vasculature (Fig. 3d). Echocardiographic estimates of CO correlate well with results of thermodilution studies. RV stroke volume is calculated through multiplying PA VTI by PA cross-sectional area (πr^2 , where r = the radius of the main PA). CO is calculated by multiplying stroke volume by heart rate (Table 3).

The RV undergoes systolic shortening due to a deep layer of vertically arrayed RV fibers and this shortening is diminished when RV function is impaired. Shortening of the RV can be measured by orienting the M-mode cursor longitudinally across the tricuspid annulus in the direction of the RV apex. The excursion of the annulus (base to apex) is measured in mm and is called the tricuspid annular plane systolic excursion (TAPSE) (Fig. 3e). TAPSE is a useful noninvasive assessment of RV function (Forfia et al. 2006). TAPSE is measured in rodents and can assess RV function. The normal value in our laboratory is >2 mm. In rats with severe PAH this value decreases to <1 mm.

We will now review the most established and reproduced models of Group 1 PH and subsequently briefly review the less frequently used animal models, including transgenic mice and surgical models of congenital heart disease.

5.1 Monocrotaline-Induced PAH

Although the monocrotaline rat model has recently been subject to considerable criticism (Gomez-Arroyo et al. 2012), it does capture many of the cardinal features of human PH, such as endothelial cell damage, medial hypertrophy of small PAs, vascular inflammation, RVH, and RV failure. Monocrotaline is derived from the seeds of *Crotalaria spectabilis* and has been shown to induce pulmonary vascular injury primarily after hepatic generation of monocrotaline pyrrole. In a 1954 study of Jamaicans who used herbal medicines derived from *Crotalaria spectabilis*, Bras et al. (1954) implicated monocrotaline in the development of pulmonary veno-occlusive disease. Pulmonary vasculitis was observed in rats fed with monocrotaline in 1961 by Lalich (Lalich and Merkow 1961). Lalich then demonstrated that these rats develop right ventricular hypertrophy. A reliable and reproducible model for monocrotaline-induced PH was developed by Heath in 1967 (Kay et al. 1967).

Method. A single administration of monocrotaline (60–100 mg/kg, subcutaneously) to rats predictably results in PH within approximately 3 weeks and mortality in most rats after 6–8 weeks (Urboniene et al. 2010). The quick advance to death, secondary to RV failure likely decreases the ability of this model to develop compensatory mechanisms. There is a dose–response to monocrotaline and lower dose of monocrotaline (20 mg/kg) result in milder PH and a nonlethal, compensated model with RV hypertrophy rather than RV failure (Buermans et al. 2005).

Features of model. Monocrotaline is converted to the active metabolite dehydromonocrotaline in the liver by cytochrome P-450 enzyme CYP3A4. It exerts multiple toxicological effects on the pulmonary endothelium including induction of DNA damage (Wagner et al. 1993), abnormalities in nitric oxide signaling, and activation of proliferation factors (Huang et al. 2010). Mitochondrial swelling and increased production of reactive oxygen species in the lung have been observed in monocrotaline rats (Rosenberg and Rabinovitch 1988; Archer et al. 1990). Monocrotaline also causes cell-cycle arrest to pulmonary artery endothelial cells, as well as to glial cells, pneumocytes, and hepatocytes. These effects on pneumocytes likely contribute to the extravascular effects of monocrotaline, namely pulmonary edema and alveolar cell septal hyperplasia (Woods et al. 1999). Some of the toxic effects of monocrotaline are also likely mediated through hematological effects. Dehydromonocrotaline accumulates in the erythrocytes and circulates systemically in red blood cells (Pan et al. 1991). Platelet-mediated thrombosis and fibrin activation are observed (Schulze and Roth 1998). The role played by platelets in monocrotaline PAH is highlighted by

the protective effect of thrombocytopenia (Schultze and Roth 1998). Inflammation plays an important role in this model, with mononuclear cells accumulating in the adventitia early in the evolution of PAH (Wilson et al. 1989). Macrophages accumulate and contribute to the proliferation of smooth muscle cells (Wilson et al. 1989). The liver toxicity observed in monocrotaline model contributes to development of portal hypertension and this may predispose to PH (Epstein et al. 1992).

The initial visible insult in monocrotaline PAH is endothelial injury. This endothelial damage is seen on electron microscopy (Fig. 4) (Rosenberg and Rabinovitch 1988). The endothelial injury can also result in edema in the adventitia (Wilson and Segall 1990). In an alternate model, rats that received monocrotaline in their drinking water had decreased angiotensin converting enzyme (ACE) activity early in the development of PH, again reflective of endothelial damage (Molteni et al. 1984). Within 8 days of administration of monocrotaline subcutaneously, extension of SMCs peripherally into the small PAs is observed with changes occurring at a later stage in the larger PAs (approximately 2 weeks after monocrotaline administration). The predominant histology on light microscopy is medial hypertrophy of small (20–100 μm) PAs (Fig. 1b). There is also obliteration of these arteries, which reduces vascular density. The first hemodynamic changes are not observed until \sim 2 weeks after administration of monocrotaline. Mean PA pressures in monocrotaline rats increase to greater than 35 mmHg by 4–6 weeks. The development of RVH follows the development of PH, being evident at 3–4 weeks (Marsboom et al. 2012a; Pan et al. 1991).

When monocrotaline is combined with pneumonectomy, mean PA pressures are increased even further (>45 mmHg) (White et al. 2007). To create this model, 12-week-old Sprague-Dawley rats are subjected to pneumonectomy and 1 week later, monocrotaline is administered (60 mg/kg subcutaneously). The histological findings are also more severe than with monocrotaline alone, with advanced medial hypertrophy and neointimal lesions observed, including plexiform-like lesions. This exacerbation is likely secondary to increased pulmonary blood flow or hypoxia in the residual lung (Okada et al. 1997). An unexplained finding is that the plexiform lesions seen in this model develop within 1 week of administration of monocrotaline—before the onset of PH. These lesions resemble human plexiform lesions and also have a similar molecular profile expressing vascular endothelial growth factor receptor-2 and contain PASM rich in tissue factor (White et al. 2007).

The right ventricle. The RV in monocrotaline PAH is hypertrophied and has a glycolytic shift in metabolism characterized by PDK activation and inhibition of PDH. This RV has a phenotype similar to hibernating myocardium (Piao et al. 2010a).

Limitations of model. The monocrotaline model has been subject to criticism, such as the development of concomitant myocarditis in some animals (Gomez-Arroyo et al. 2012), the lack of plexiform lesions, and damage to airways [pulmonary fibrosis and alveolar septal cell hyperplasia (Dumitrescu et al. 2008)].

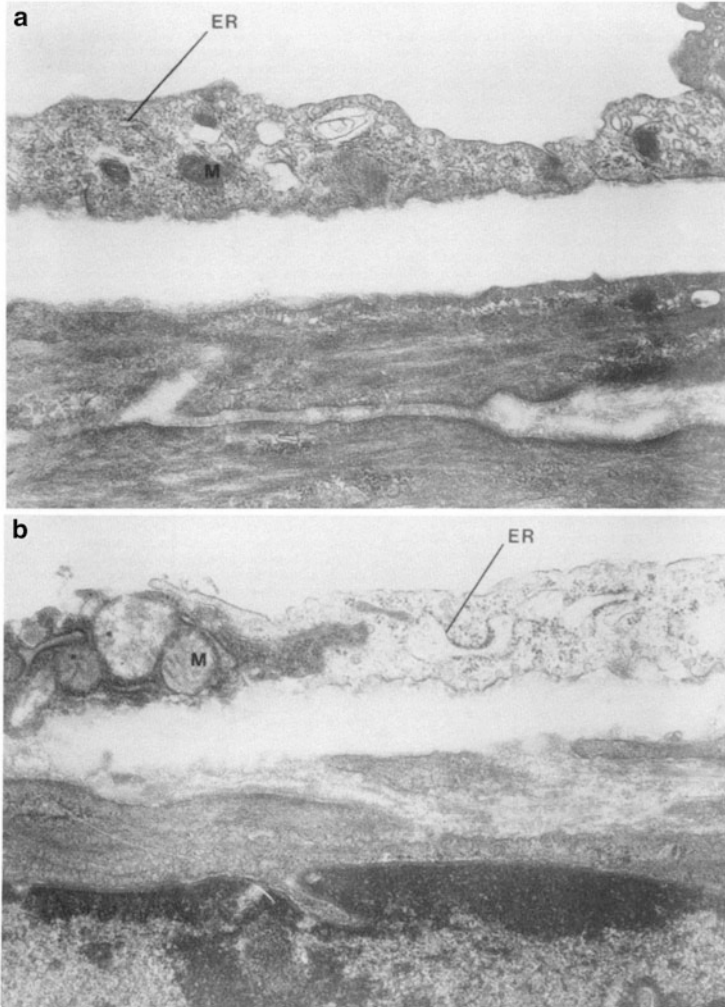


Fig. 4 Photomicrographs of small preacinar artery 4 days after injection with monocrotaline (**b**) or normal saline (**a**). Endothelium from monocrotaline-injected animals appears swollen and less dense. Note swollen mitochondria (M) and dilated endoplasmic reticulum (ER). Reproduced with permission from Rosenberg and Rabinovitch (1988)

Monocrotaline has also been found to cause obstructive pulmonary vein thrombosis (Lalich et al. 1977). However, this post-capillary obstruction is similar to the pathology observed in pulmonary veno-occlusive disease (PVOD) and thus the monocrotaline model reflects aspects of Group 1' PH.

Monocrotaline has been studied in other animals. Importantly, monocrotaline does not lead to the development of pulmonary hypertension in mice for an

unknown reason (Dumitrascu et al. 2008). PAH arteriopathy has been shown in young beagles who have been exposed to dehydromonocrotaline (Gust and Schuster 2001). Eight weeks after venous injection of dehydromonocrotaline (3 mg/kg) in 12-week-old beagles, pulmonary artery pressures increase to 30–35 mmHg. Medial hypertrophy of small PAs develops and an increase in neointimal proliferation is seen. However this model is infrequently used, primarily because this canine model offers minimal advantage over the corresponding rodent model. Likewise, monocrotaline (30 mg/kg) causes PAH in baboons and macaca monkeys leading to RV disease (Raczniak et al. 1978). Monthly administration of monocrotaline for 4 months leads to clinical manifestations and histological changes very similar to those found in humans with PAH (Chesney and Allen 1973). Although, limited hemodynamics are available in this model (Raczniak et al. 1978), these monkeys live 220 days after injection of monocrotaline on average (range 100–355 days). RV dilatation and RVH is pronounced, with wide separation of the RV myocytes and an increase in RV collagen. The authors report that these myocytes had an increase in the number of mitochondria (Raczniak et al. 1978). However, the use of monocrotaline in monkeys offers very little advantage (if any) over the rodent monocrotaline model and introduces economic and ethical challenges. It does suggest that ingestion of monocrotaline could cause PAH in humans and is a reminder about the need for caution in the ingestion of biologicals, which may occur as health supplements or herbal teas.

Despite limitations, the monocrotaline rat remains a convenient, reliable, and reproducible form of PH (Stenmark et al. 2009) that mimics many of the cellular and molecular components of PAH. The fact that RV failure seems to be important to the death of these animals is a strength of the model. There appears to be some poorly characterized individual variability in susceptibility to monocrotaline since we find that not all exposed animals develop PAH (approximately 10 % of exposed animals fail to develop PH in our protocols).

5.2 Hypoxia Models

Chronic hypoxia is a model of Group 3 PH that is often used to make inferences regarding Group 1 PH. The PH and the vascular remodeling are mild and animals usually do not die of RV failure. We discuss the chronic hypoxic model because it is relevant to the CH + SU model (injection of Sugen 5416 + 3 weeks of chronic hypoxia 10 %). Moreover, HIF-1 α activation (seen in chronic hypoxia) is also seen in Group 1 PH. Upregulation of HIF-1 α occurs in the setting of hypoxia and results in mitochondrial fragmentation and subsequent smooth muscle cell proliferation (which is more easily demonstrated in vivo than in cell culture). Mice that are haploinsufficient for HIF-1 α are relatively protected from this form of PH (Shimoda et al. 2001), implicating a central role for HIF-1 α in the pathogenesis of this form of PH.

Of note, there is remarkable interspecies variation in the response to hypoxia. Animals that are genetically acclimated to high altitude such as llamas or yaks have no demonstrable PH or vascular remodeling upon exposure to chronic hypoxia (Stenmark et al. 2009; Rabinovitch et al. 1979). Rats are intermediate responders to chronic hypoxia, however, not all breeds of rats respond equally. Fawn-Hooded rats (FHR) respond most significantly, with mPAP 45–50 mmHg (Bonnet et al. 2006). After Fawn-Hooded rats, Sprague-Dawley rats are the most susceptible to hypoxia-induced pulmonary hypertension with mPAP 30–35 mmHg (Kentera et al. 1988). Fischer rats are relatively resistant to hypoxia-induced PH and develop minimal PH (mPAP 25–30 mmHg) or vascular remodeling and thus should preferentially not be used to create this model of PH (Zhao et al. 2001). It has also been observed that younger rats are also more sensitive to hypoxia (Rabinovitch et al. 1981; Stenmark et al. 2006). Mice are very poor responders to hypoxia (Stenmark et al. 2009). The difference between mice and rats in response to hypoxia may be due to differences in the up- and downregulation of various genes. For example, chronic hypoxia in the mice results in downregulation of genes associated with vascular smooth muscle cell proliferation (Hoshikawa et al. 2003), whereas in rats hypoxia causes an increase in genes associated with proliferation of endothelial cells and a decrease in genes involved in apoptosis (Bull et al. 2007).

5.3 Chronic Hypoxia Rats

Method. In chronic hypoxic rats, most experimental protocols gradually decrease FiO_2 over 3–5 days to acclimate rodents and then maintain the animals at 10 % O_2 (approximately P_aO_2 40 mmHg, which simulates an altitude of 18,000 ft) for up to 4 weeks. This model is tolerated well with minimal mortality, provided time for acclimatization is allowed. Hypobaric and normobaric hypoxia yield similar PH; although normobaric hypoxia is often used, because it is not complicated by the noise of the vacuum pump, which is required for the hypobaric model. However, the hypobaric model is cheaper as it does not require premixed gases. Importantly, to rule out any effects of HPV on measured pressures, animals should be kept in normal oxygen tensions for at least 1 h prior to catheterization.

Features of model. There is usually little evidence of vascular obstruction or rarefaction. The hallmark is medial hypertrophy of small PAs, which develops after ~2 weeks of hypoxic exposure (Zhao 2010) (Fig. 1c). Hypoxic pulmonary vasoconstriction (HPV) plays a role early in the disease process with vascular remodeling becoming more important over time. HPV is selectively decreased in chronic hypoxia-induced pulmonary hypertension (whereas constriction to other stimuli is enhanced) (Reeve et al. 2001; McMurtry et al. 1976). Within 2 days of exposure to hypoxia, HPV is no longer marked as impaired redox signaling develops in the mitochondria and vascular remodeling plays a more critical role in elevating PA pressures (Fig. 5) (Reeve et al. 2001).

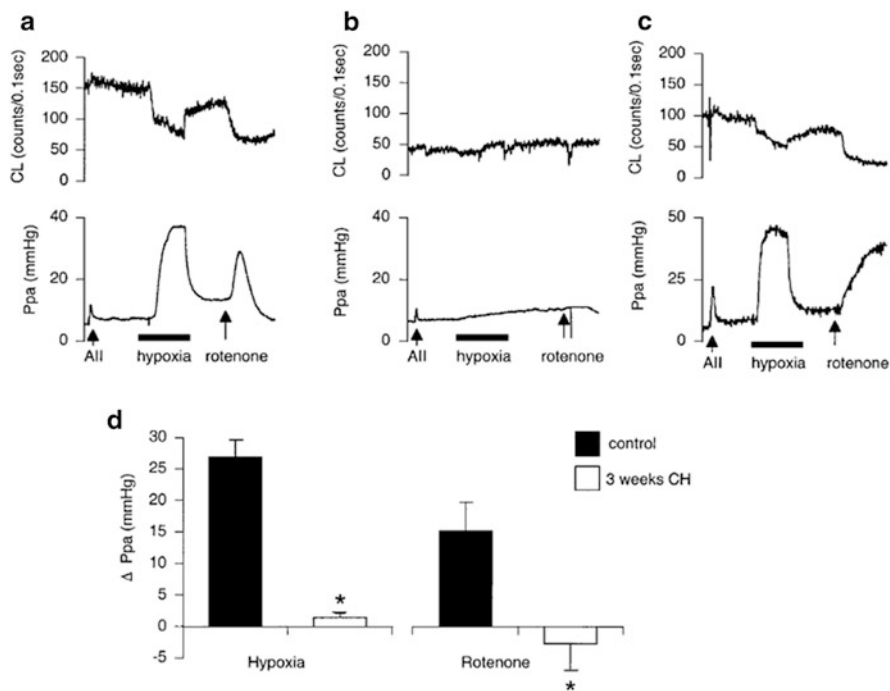


Fig. 5 Representative traces from isolated, perfused lungs showing simultaneous recording of chemiluminescence (CL) and pulmonary arterial pressure (Ppa). (a) Response in control lung to angiotension II (All), hypoxia, and rotenone (10 mM). (b) Response in 3-week chronic hypoxia (CH) lung to All, hypoxia, and rotenone. (c) Response in 3-week CH returned to normoxia for 2 days to All, hypoxia, and rotenone. Rotenone added at arrow. (d) Means \pm SE changes (Δ) in Ppa in control and CH rat lungs to acute hypoxia and rotenone (10 μ M). * $P < 0.05$, different from control. Reproduced with permission from Reeve et al. (2001)

The vascular remodeling in chronic hypoxia increases PVR. It has been observed that luminal area is preserved in the rat model of hypoxia in vessels between 30 and 200 μ m (Hyvelin et al. 2005). Potentially the medial thickening can grow in an outward direction in some vessels, thereby not affecting the luminal area (Stenmark and McMurtry 2005). Histologically, the increased medial hypertrophy is characterized by an increase in α -SM-actin. Fibroblasts and myofibroblasts increase deposition of elastin, collagen, and fibronectin thereby contributing to adventitial thickening (Davie et al. 2006). Distal extension of smooth muscle into normally nonmuscularized pulmonary blood vessels is a characteristic feature of chronic hypoxic PH (Stenmark et al. 2006). Inflammation plays an important role in the chronic hypoxia model and both an early and a late stage of inflammation is observed (Burke et al. 2009). Mesenchymal precursor cells and mononuclear cells that infiltrate the vessel wall may contribute to the vascular remodeling in chronic hypoxia (Frid et al. 2006).

The RV. The RV in this model undergoes mild hypertrophy. A local increase in ACE activity has been observed in the RV of chronic hypoxic rats, similar to the increase reported in monocrotaline rats (Molteni et al. 1984; Morrell et al. 1997).

Limitations of model. Limitations from this model include its failure to reflect the severe obstructive remodeling and complex vascular lesions in Group 1 PH. In addition, the hemodynamic abnormalities are modest and RV failure is not a significant feature of the model in rodents. Also this model is accompanied by polycythemia (which can increase PH), which is not a feature of PAH. Finally, many experimental treatments (including many of which are ineffective in more severe models) are “effective” in regressing chronic hypoxic PH, suggesting the model is insufficiently robust for preclinical assessment of PAH therapies (Girgis et al. 2003).

Brisket disease is a form of chronic hypoxic PH induced by exposure of lowland cattle to high altitude (>7,000 ft). This form of hypoxic PH is severe and does lead to right heart failure, evident by fluid accumulation in the dependent “brisket” region of the cow (Grover and Reeves 1962). This disease is extensively reviewed in ref (Rhodes 2005). We mention Brisket disease in this review to highlight the ability to study chronic hypoxia in cows, which are robust responders to hypoxia (unlike rodents). Medial hypertrophy of the small pulmonary arteries is observed in these animals and is proportional to the extent of the increase in mPAP (Alexander and Jensen 1963). Some cattle such as *Bos taurus* (Astrup et al. 1968) have a heritable disposition to develop severe PH secondary to high altitude, although the disease typically occurs in about 20 % of steers brought to high altitude (Grover 1965). Systolic PAP in the affected animals is typically 80–85 mmHg, whereas it is 30 mmHg in the resistant animals. Recently genetic studies performed on herds at high altitude have identified single nucleotide polymorphisms and candidate genes which cause and protect against high altitude pulmonary hypertension (Newman et al. 2012).

5.4 Fawn-Hooded Rats

Three major FHR strains are known: (a) PAH prone FHR/EurMcwiCrl, (b) a systemically hypertensive FHR strain (prone to glomerulonephritis,) and (c) a strain prone to depression/substance addiction (Cowley et al. 2004; Brown et al. 1998; Kuijpers and de Jong 1987; van Rodijnen et al. 2002; Kuijpers and Gruys 1984). PAH-prone FHR have normal renal function and no systemic hypertension. FHR spontaneously develop PAH, largely through an increase in HIF-1 α , and mitochondrial-metabolic abnormalities reflecting a complex, heritable etiology. The FHR was originally an outbred strain using “German brown” albino rats and Long Evan’s rats at the University of Michigan (Tschopp and Weiss 1974; Tobach et al. 1984). In FHR the PAH is heritable, occurring in 68 % of male and female FHR offspring (Kentera et al. 1988).

Method. FHR are allowed to age and monitored periodically using echocardiography for the development of PH. The time to onset of PH varies with altitude. FHR are sensitive to hypoxia and when raised at high altitude demonstrate marked vasoconstrictor response (Nagaoka et al. 2006). The development of PH in FHR is accelerated by mild hypoxia, even at levels that would not cause PH in normal rodents (Le Cras et al. 1999, 2000). In Denver at an elevation of 5,200 ft, FHR animals develop PAH within 1 month of birth (Sato et al. 1992). For FHR raised in Denver, the risk of PH can be decreased by the administration of mild, neonatal hyperbaria, which simulates sea-level PO_2 (Le Cras et al. 2000). In Edmonton, Alberta, at half the altitude, PAH develops much later in life, approximately between 20 and 40 weeks (Bonnet et al. 2006). In Chicago Illinois (sea level), PH does not develop until age 40–60 weeks (Piao et al. 2012).

Features of disease. FHR have increased expression of lung endothelin (ET-1) and decreased endothelial nitric oxide synthase, as is also seen in human PAH (Le Cras et al. 2000; Nagaoka et al. 2001). Platelet abnormalities observed in human PAH are also seen in FHR with PH, along with increased vasoconstrictor response to serotonin (Ashmore et al. 1991). FHR have a normal serotonin transporter gene, despite abnormal serotonin storage (Gonzalez et al. 1998). FHR and Tester-Moriyama rats share pigmentation and platelet disorders, but differ at 62 % of microsatellite markers, indicating their genetic differences (Hamada et al. 1997). Although Tester-Moriyama rats share a similar serotonin platelet disorder, they have no PAH, suggesting that the serotonin pathway does not cause FHR PAH (Nagaoka et al. 2001). The characteristic FHR coat color is also observed to be localized to chromosome 1, as is the defect in the platelet storage pool (Brown et al. 1996). FHR PASMCM, like PASMCM from human PAH, has an exaggerated predilection to proliferate (Morrell et al. 2009), particularly in response to growth factors, such as Epidermal Growth Factor. This may contribute to their pulmonary vascular remodeling and PAH (Janakidevi et al. 1995). In addition, FHR are hypoxia sensitive, being prone to develop PAH and alveolar simplification (Le Cras et al. 1999, 2000) when exposed to mild hypoxia [at levels that do not affect other rodents (Sato et al. 1992)]. Alveolar simplification (a phenotype common in perinatal lung injury) manifests as fewer and larger alveoli with reduced numbers of septae, loss of small PAs, and decreased capillary density. In neonatal FHR, mild hypoxia that does not adversely affect other rat strains, causes severe PAH that is associated with increased lung endothelin (ET-1) expression (Nagaoka et al. 2001). However, the ET_A receptor-antagonist, BQ-123, only inhibits excessive FHR PASMCM proliferation partially and under specific culture conditions [0.3 but not 0.1 or 10 % serum (Zamora et al. 1996)], indicating that excess ET-1 is also not the fundamental cause of FHR's PAH. Also, as in human PAH, the PASMCMs in FHR are hyperproliferative and the resulting histologic abnormality is medial hypertrophy of small PAs (Bonnet et al. 2006). FHR have not been reported to spontaneously develop complex vascular lesions.

We believe that much of the hypoxia sensitivity and hyperproliferative phenotype of FHR relates to abnormalities in mitochondrial structure and defects in oxygen sensing that they share with human PAH (Bonnet et al. 2006). Activation

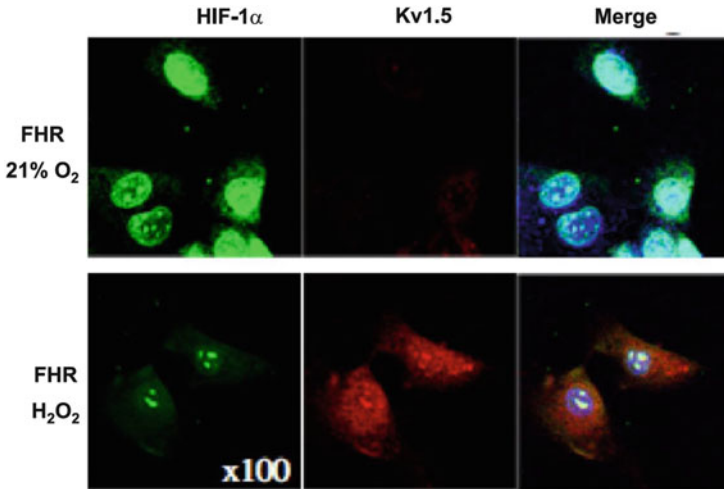


Fig. 6 Disruption in mitochondrial fusion in PAH. 48-h incubation in H₂O₂ reverses nuclear translocation of HIF-1 and restores Kv1.5 (red) in FHR PSMCs, consistent with the hypothesis that a loss of mitochondrial ROS production causes the FHR's abnormalities. *iPAH* idiopathic PAH, *BN1* consomic rat. Reproduced with permission from Archer et al. (2008)

of HIF-1 α is observed in the PSMC of FHR, even when cell cultures are maintained at 120 mmHg (Archer et al. 2010; Marsboom et al. 2012b; Bonnet et al. 2006) (Fig. 6). This normoxic activation of HIF-1 α is associated with fragmentation of mitochondria as well as a decrease in mitochondrial expression of SOD2 (Archer et al. 2010). SOD2 downregulation in FHR is the result of epigenetic silencing of the SOD2 gene. Increased activation of DNA methyltransferases 1 and 3B lead to methylation of two key CpG islands in the promoter and intron 2 (Archer et al. 2010). 5-azacytidine, a DNA methyltransferase inhibitor, restores SOD2 expression by demethylating the CpG islands. With return of SOD2 function, proliferation of FHR PSMC decreases. The mitochondrial fragmentation in FHR (and human PAH) relates in a large part to activation of dynamin-related protein (DRP-1) (Marsboom et al. 2012b). Inhibition of mitochondrial fission in PAH PSMC prevents mitotic division of mitochondria and arrests the cells in G2/M phase of the cell cycle. The resulting growth inhibition plus induction of apoptosis suggest this recently identified abnormality may contribute to PAH in the FHR model (and humans).

It is important to remember that when using the FHR model, one needs to use an appropriate control. The FHR-BN1 consomic rat can be used as this control—these rats are made through introgression of chromosome 1 from the hypoxia resistant Brown Norway rats into an isogenic FHR background (Cowley et al. 2004). Although these animals are genetically identical to FHR except for chromosome 1, there is neither activation of HIF-1 α or PDK nor any mitochondrial abnormality and they do not develop PAH. Thus the PAH-initiating gene in FHR is likely present on chromosome 1 (Bonnet et al. 2006).

The RV. The right ventricle in FHR undergoes a glycolytic shift secondary to elevated PA pressures as well as RVH (Piao et al. 2009).

Limitations. The main disadvantage of this model is that the animals develop PH slowly, although this can be expedited by exposing the animals to mild hypoxia. Also, the FHR manifest some degree of alveolar simplification, raising the issue that FHR are not a pure model of PH (Le Cras et al. 1999). Finally, the availability of consomic rats is limited.

5.5 *Chronic Hypoxia and Sugen-5416*

Sugen-5416 (SU-5416) is a small molecule inhibitor of vascular endothelial growth factor receptor 2 (VEGFR-2) that was developed as a potential anticancer drug, semaxinib. Taraseviciene-Stewart et al. (2001) developed this rat model of PH by combining SU-5416 with chronic hypoxia. The original model was created by subcutaneous injection of 200 mg/kg of SU-5416, three times a week for 3 weeks at Denver's altitude, although subsequently it was found that a lower dose of SU-5416 was sufficient and that more prolonged observation in normoxia (after the hypoxic exposure) lead to the development of complex vascular lesions (Abe et al. 2010).

Method. This model has now evolved and is typically made by one subcutaneous injection of 20 mg/kg of SU-5416 coupled with 3 weeks of hypoxia at 10 % with a return to normoxia for 2–9 weeks. The pulmonary hypertension continues to progress after removal from the hypoxic stimulus. Thus, 5–6 weeks after subcutaneous injection these animals have severe and progressive PAH, with mean PAP ~50 mmHg. By 5–6 weeks, medial hypertrophy develops in all pulmonary arteries and neointimal proliferation is seen in small pulmonary arteries (those <50 μ m). A concentric laminar neointima is seen 8 weeks after administration of SU-5416. Plexiform lesions, similar to those in human PAH, develop at approximately 10–12 weeks (Abe et al. 2010).

Features of disease. The model is well tolerated with a much lower mortality than that seen in monocrotaline rats despite the fact that the PA pressures are typically higher in SU-5416 rats (Sakao and Tatsumi 2011). This model has minimal response to vasodilators such as iloprost and thus bears a similarity to human PAH (Oka et al. 2007). The low mortality, the simplicity of creating the model plus its replication of the key features of human PH, particularly plexiform lesions, recommends the Chronic Hypoxia-SU-5416 (CH + SU) model (Fig. 1d).

SU-5416 causes death of PA endothelial cells and this apoptosis is followed by obliteration of precapillary arterial lumen by the emergence of apoptosis-resistant proliferating endothelial cells. This subset of cells with a predisposition to survival is evident by an increase in IGF-1, Bcl-2, and Bcl-X expression (Taraseviciene-Stewart et al. 2001).

The CH + SU model has recently been adapted for mice (Ciuclan et al. 2011). The murine protocol uses a weekly dose of 20 mg/kg SU-5416 subcutaneously for 3 weeks, administered while the mice are in hypoxia (FiO₂ 10 %). Although used by a limited number of investigators thus far, this model has identified abnormalities in TGF- β , BMP, and SMAD signaling as well as increases in HIF-1 α and IL-6 typical of PAH. CH + SU in mice does result in a more robust increase in RVSP (>45 mmHg) than is seen in other murine models.

Limitations. By its nature, this model depends on VEGF inhibition to achieve its features. However, the role of VEGF inhibition in human PAH is unclear. Additionally, the SU-5416 model has a notable absence of perivascular inflammation, which is observed in other models [monocrotaline (Wilson et al. 1989)] and in human PAH (Price et al. 2012). This may be an important deficiency since monocytes and macrophages and other inflammatory cells may mediate pulmonary vascular remodeling in hypoxic models of PH (Zhao 2010).

6 Transgenic Models

6.1 *BMPR-2 Mutation Models*

Familial PAH is associated with multiple mutations in the BMPR-2 gene (Lane et al. 2000), but the abnormal genotype is not sufficient to cause PAH in most cases. Carriers of BMPR-2 mutations have only a 20 % risk of developing PAH, indicating that other factors are required to cause PH (Newman et al. 2001). While a complete deletion of BMPR-2 is lethal (see, for example, Beppu et al. 2000), mice with a BMPR-2 gene haploinsufficiency do not develop PH (Liu et al. 2007). To circumvent embryonic lethality of a complete deletion, several genetically modified mice have been developed. We have previously reviewed animal models of BMPR2 extensively in Ryan et al. (2011) and this discussion is based on that review.

7 Global Knockout Mice

Beppu et al. (2000) generated mice in which the BMPR-2 exons 4 and 5 were deleted globally. Homozygous mutant mice died during gastrulation. Beppu et al. (2004) found that the heterozygous mice had mild PH and pulmonary vascular remodeling. However, other investigators did not find PH at baseline in these mice, although increased pulmonary vascular remodeling and increased tone in response to serotonin or overexpression of 5-lipoxygenase are observed (Song et al. 2005; Long et al. 2006).

8 Conditional Knockout Mice

Beppu et al. (2005) also generated mice containing loxP sequences flanking exons 4 and 5, allowing conditional deletion of the transmembrane domain and rendering the resulting protein incapable of initiating downstream signaling. It is feasible to eliminate BMPR-2 signaling in a cell type-specific manner by crossing these mice with mice expressing Cre recombinase in specific cell types. These conditional knockout mice have been used together with a Cre transgene that is selectively expressed in endothelial cells to generate another mouse model of PH (Hong et al. 2008). PH develops in a subset of mice when the BMPR-2 is deleted in mature animals. In those mice with RSVP >30 mmHg, there is medial hypertrophy of small PAs and RVH. When Beppu et al. (2009) used the TIE2-Cre transgene to knockout BMPR-2 in endothelial cells during development, the mice died in utero with extensive cardiac defects.

9 BMPR-2-Dominant Negative Transgenic Mice

These are mice in which human mutant BMPR-2 alleles are overexpressed in a tissue-specific and inducible manner. West et al. (2004) induced expression of a kinase-dead, dominant-negative BMPR-2 mutation in mice. This mutation leads to a loss of the entire intracellular domain. By using a SMC-specific promoter, transgene activity is limited to the SMCs after treatment with the activator doxycycline. Doxycycline was fed to the mothers after giving birth and was continued in the diet until mice reached adulthood. In the transgenic mice, the RVSP was increased to 55–60 mmHg. Modest increased medial thickness of the PAs is observed in this model.

West et al. (2008) then developed a second series of mice with a transgene containing a BMPR-2 with a different mutation observed in familial PAH (a carboxyl terminal stop codon at position 899). Interestingly, when expression of this transgene was induced in SMC, the mice developed PH and extensive pulmonary vascular pruning. Compared to mice with a mutation in the kinase domain, this study suggests that it is worse to have a dysfunctional BMPR-2 (only missing the regulatory carboxy terminal domain) (West et al. 2008) than have an almost complete deletion of BMPR-2 (West et al. 2004). Overexpression of this dominant-negative BMPR-2 gene is associated with downregulation of the voltage-gated potassium channel, Kv1.5, and causes PA vasoconstriction. Nifedipine, a selective L-type calcium channel blocker, reduces PH in these mice. This suggests that activation of L-type calcium channels, caused by reduced expression of Kv1.5 channels and membrane depolarization, mediates PH in these mice (Young et al. 2006). A concern with these transgenic models is that mutated

BMPR-2 isoforms are expressed at supraphysiological levels, thereby potentially disrupting the entire BMP signaling system.

10 Short Hairpin (shRNA) BMPR-2 Knockdown Mice

Liu et al. (2007) generated mice in which BMPR-2 gene expression was globally reduced, using a shRNA-based strategy. Silencing BMPR-2 expression by RNA interference does not cause PAH (although the knockdown of BMPR-2 is approximately 90 %) but instead causes gastrointestinal hyperplasia, incomplete mural cell coverage of vessel walls, and mucosal hemorrhage. It appears that BMP receptor signaling regulates angiogenesis by maintaining the expression of endothelial guidance molecules and if this is disrupted, vessels are malformed. However, there remain moderate amounts of BMPR2 in the lungs of these animals, thereby suggesting that downregulation of BMPR2 alone is not sufficient to induce PAH, in line with the aforementioned conditional knockout mice (Beppu et al. 2005).

10.1 5-HTT Overexpressing Mice

Serotonin (5-HT) increases proliferation of PASMC and migration of fibroblasts and causes pulmonary vasoconstriction. Expression of the serotonin transporter (5-HTT) is increased in Group 1 PH (Eddahibi et al. 2001). Transgenic overexpression of 5-HTT has been achieved in mice both globally (MacLean et al. 2004) and in SMC-specific (Guignabert et al. 2006) models to assess the role this transporter plays in PAH.

Method. When 5-HTT transgenic mice are exposed to hypobaric hypoxia for 14–28 days, the RVSP increases to mPAP 45–50 mmHg. When bred in normoxia, these 5-HTT transgenic mice have been found to have elevated RVSP (mPAP 30–35 mmHg) (MacLean et al. 2004). The SMC-specific, 5-HTT transgenic mouse, is created using a SM22 promoter, and RVSP increases to 30–35 mmHg in this model after 15 days of 10 % O₂ (Guignabert et al. 2006).

Features of model. Hypoxic pulmonary vasoconstriction is decreased in this model. In the hypoxia treated transgenic mice, the distal PAs become more muscularized and there is an increase in proliferation of PASMCs. Curiously, monocrotaline, which as mentioned earlier does not normally cause PH in mice, causes an increase in RVSP to 25–35 mmHg when administered to these transgenic mice (5 mg/kg subcutaneously).

Limitations. The main limitation of this model is that both the PH and histological changes are not severe.

10.2 Models That Require Further Study

Whereas the animal models discussed heretofore have been studied by many investigators and are quite reproducible, there are a number of intriguing, but less widely studied, models that merit mention. These models require further study to determine their relevance as PH models.

10.3 Broiler Fowl Disease

Although not a transgenic model, PAH spontaneously develops in 3 % of young domestic fowl bred for meat production (broiler chickens, broilers) when raised in conditions that promote maximal growth. In this setting the rapid growth is accompanied by increases in cardiac output that enters lungs which have not increased in size as quickly as the increase in cardiac output (Wideman 2000). Relevance of this model to 5-HTT overexpressing mice is suggested by the observed increase serotonin in these birds (Chapman et al. 2008). Although too sporadic to make a robust model of PH without some modification, broiler fowl remains the only animal “model” that spontaneously develops plexiform lesions (Wideman and Hamal 2011).

10.4 Endothelin B Receptor Deficiency in Rats

Endothelin-1 (ET-1) interacts with endothelin B (ET_B) receptors in the endothelium, which causes pulmonary vasodilatation through increased prostacyclin and nitric oxide production. ET-1 is increased in human PAH (Giaid et al. 1993) and in the chronic hypoxic PH model, ET_B receptors and endothelin-1 levels are increased in the lungs (Li et al. 1994). The ET_B receptor deficiency model is based on the molecular elimination for a compensatory vasodilator pathway (ET_B-mediated vasodilatation).

Method. Exposing ET_B-deficient rats [ET_B (sl/sl)] to monocrotaline (60 mg/kg subcutaneously at 4–6 weeks of age) results in neointimal lesions, similar to those observed in human PAH (Ivy et al. 2005).

Features of model. In ET_B-deficient rats exposed to monocrotaline, mean PAP increases to 40–45 mmHg, with a reduction in cardiac output to 30 ml/min. The total pulmonary resistance in monocrotaline-treated, ET_B-receptor deficient rats increases considerably from a baseline of 400 mmHg/l/min to 1,400 mmHg/ml/min. Thus, deficiency of the ET_B receptor accelerates progression of monocrotaline-induced PAH in rats. This suggests an antiproliferative effect of the ET_B receptor in pulmonary vascular homeostasis and is another example of how

multiple insults (removal of a compensatory mechanism) exacerbate experimental PH. Significant perivascular inflammation is observed in this model.

Limitations of model. The biggest limitation of this model is that these rats are not readily available for purchase. Also, plexiform lesions are not seen in this model (Ivy et al. 2002).

10.5 Angiotensin-1 Overexpression in Rats

Angiotensin-1 (Ang-1) plays a controversial role in PAH with two groups reporting opposite findings. Thistlewaithe et al. reported that expression of angiotensin-1 and phosphorylation of Tie2 (the endothelial-specific receptor of Ang-1) are upregulated in the lungs of patients with a variety of forms of PH (Chu et al. 2004). It is noted that Ang-1 expression correlated directly with the severity of PH (Du et al. 2003), supporting their contention that overexpression of Ang leads to PH in rodents. In contrast, Stewart et al. found that decreased activity of the Ang-Tie2 pathway worsens PAH by decreasing endothelial cell-survival signaling (Zhao et al. 2003). They note that transfer of the Ang-1 gene improves PAH (Kugathasan et al. 2005). This controversy needs to be resolved before Ang-overexpression can be used widely as a PAH model.

Method. To create a rat model of PH through overexpression of Ang-1, 2×10^{10} genomic particles of adeno-associated virus-angiotensin-1 (AAV-Ang-1) are injected into the RV outflow track of anesthetized Fischer rats at 12 weeks of age (Chu et al. 2004). Rats are subsequently sacrificed 1–2 months later. The average survival of these rats, if not sacrificed, is approximately 5–6 months after injection.

Features of model. In the animals that express high levels of Ang-1 protein, PH is observed and histological changes such as medial hypertrophy and muscular hyperplasia of the small PAs. RVSP typically reaches 50 mmHg in the Ang-1 overexpressing rats. There are no complex lesions and the endothelium does not seem to be adversely affected.

Limitations. The inflammatory changes, which are often observed in the monocrotaline model and in human PAH, are not seen in this model. The complex histologic lesions seen in the CH + SU5416 model and in human PAH are not seen. Also, the role of Ang-1 in human PAH remains undefined and the model has not been replicated, thus limiting its applicability at this time.

10.6 High Fat Diet to Apo E Knockout Mice

Metabolic syndrome in humans has been identified as a risk factor for PH (Robbins et al. 2009) and thus ApoE knockout mice have been studied to determine if the

high fat diet can also cause a PH-syndrome (Hansmann et al. 2007). In this model, starting at 4 weeks of age, ApoE knockout mice are fed high fat chow for 11 weeks (Pendse et al. 2009). RVSP increases to 30 mmHg and the pulmonary arteries are observed to develop mild medial hypertrophy, however, no plexiform lesions are observed. Another limitation of this model is that the male rats develop the bulk of the PH, which contrasts with the female preponderance observed in humans.

11 Inflammatory Models

Several lines of evidence indicate that inflammation plays an important role in the development of PH (Hassoun et al. 2009). For example, autoimmune diseases such as scleroderma are a common cause of PAH and HIV infection is also associated with PAH. In addition, schistosomiasis contains an important inflammatory component. While inflammation is induced as a component of the monocrotaline model, a number of models exist that specifically look at how inflammation influences the development of PAH.

11.1 *γ -Herpesvirus Reactivation Model*

The importance of viral infections in the development of PAH has been suggested by the identification of human herpes virus 8 (HHV-8) in the lungs of PAH patients (Cool et al. 2003), but this finding is somewhat controversial because other authors have not found signs of HHV-8 infection in PAH (Valmary et al. 2011). Nevertheless, the murine γ -Herpesvirus 68, which resembles HHV-8, leads to increased perivascular inflammation and vascular remodeling. However, this vascular remodeling only occurs in transgenic mice overexpressing the small calcium binding protein S100A4 and even in these mice no significant increase in RV pressures was observed (Greenway et al. 2004; Spiekerkoetter et al. 2008).

Method. γ -Herpesvirus 68 (γ HV68) is administered to S100A4/Mts mice at 1 year of age. The model does not result in PH if the animals are inoculated at less than 3 months of age.

Features of model. Using this late inoculation, the animals develop neointimal hyperplasia, which is maintained (Fig. 7A). However, the increase in RVSP, which is mild 6 weeks after administration of virus (RVPS ~ 30 mmHg) is no longer evident 3 months later. In order to cause persistent PH, a mutant M1- γ HV68 strain can be administered, which has a fivefold higher efficiency in reactivation and causes neointimal proliferation in all S100A4/Mts1 mice.

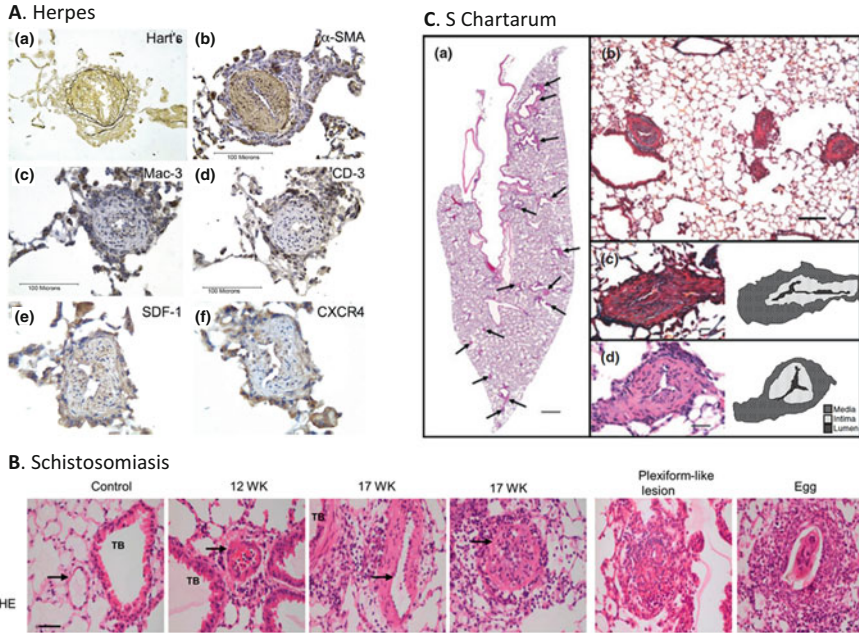


Fig. 7 (A) Herpes- γ : the PVD lesion is characterized by fragmented elastin, a neointima composed largely of α -smooth muscle actin (α -SMA)-positive cells, and a perivascular inflammatory infiltrate consisting of macrophages and T cells. Representative PVD lesions from 1.5-year-old S100A4/Mts1 mice were characterized by Hart's stain showing fragmented elastin fibers (a) and positive immunohistochemistry for α -SMA (b). In the perivascular region surrounding the PVD lesion and occasionally within the vessel wall, cells show positive immunoreactivity for the macrophage marker Mac-3 (c), the T lymphocyte marker CD-3 (d), stromal cell-derived factor-1 (SDF-1; e), and its cognate receptor, CXCR4 (f). Original magnification $\times 400$. Bars: 100 μ m. Reproduced with permission from Spiekerkoetter et al. (2008). (B) Schistosomiasis—representative photomicrographs of serial lung sections from control mice and mice chronically infected with *S. mansoni* at 12, 17, and 20 weeks. Reproduced with permission from Crosby et al. (2010). (C) Histopathological findings in the lungs of a mouse from the 12-week exposure of *Stachybotrys chartarum* at 1×10^4 spores/mouse (18 times) and were sacrificed 1 day after the last injection. (a) Pulmonary arteries with thickened walls were diffusely distributed in the lung (haematoxylin and eosin stain). Arrows indicate representative pulmonary arteries with stenotic changes. (b) Symmetrical thickening of the intima and media of the vascular wall is demonstrated at the periphery of the pulmonary arteries (elastica Masson's double stain). (c) Intima and media thickening of the vascular wall caused pulmonary artery stenosis (elastica Masson's double stain). (d) Myointimal cell proliferation in the intima caused symmetric thickening of the arterial wall and extensive stenosis (haematoxylin and eosin stain). Scale bars, 500 μ m (a), 100 μ m (b), 10 μ m (c, d). Reproduced with permission from Ochiai et al. (2008)

Limitations of model. The hemodynamic changes observed in this model remain modest and the complexity required to generate PH suggest that much is left to understand before this could be used widely as a murine PH model.

11.2 *Schistosomiasis*

Parasitic worms from the *Schistosoma* genus have a complicated life cycle which needs two different hosts to complete (one being a mammal or bird and another being a specific kind of snail). Eggs released in the urine or feces of infected humans or animals will infect snails, which in turn leads to the formation of cercariae, which are able to swim and penetrate the skin of humans. Therefore, poor sanitation is responsible for maintaining this cycle, explaining why schistosomiasis almost exclusively occurs in developing countries (Graham et al. 2010). From a global perspective, Schistosomiasis is a common cause of PH, especially in underdeveloped countries. While the mechanism of the disease process is still not fully defined, it has been suggested that egg embolism, increased pulmonary blood flow, and especially inflammation can contribute to the onset of PAH (Graham et al. 2010). Currently, there is only limited knowledge about whether therapies used in other patients with PAH will have therapeutic benefit in PAH associated with schistosomiasis.

Method. The disease process and the subsequent PH can be mimicked in an animal model (Crosby et al. 2010) by transcutaneously infecting female C57/BL6 adult mice with a suspension of approximately 30 cercariae of the Puerto Rican strain of *S. mansoni*.

Features of model. After approximately 5 weeks, the worms mature and start making eggs. Since worms preferentially inhabit the portal veins, initially eggs will accumulate in the liver. However, eggs ultimately appear in the lungs and lead to extensive inflammation with severe thickening of the media of the small PA and plexiform lesions from 12 weeks onwards (Fig. 7B).

Limitations of model. Despite the success in generating the classical histological features of human PH, these animals do not develop PH or RVH. Further study is required to understand the lack of PH in this model.

11.3 *Stachybotrys chartarum*

Although it is unclear if *Stachybotrys chartarum* causes PH in humans, repeated intratracheal injected of 10,000 spores of this fungus into mice 6–18 times over 4–12 weeks has been shown after 4 weeks to cause mild increases in PA pressures as well as medial hypertrophy and intimal thickness (Fig. 7C) (Ochiai et al. 2008). RVH is observed at 3 months. The RVSP measured in this mouse model is mild (20 mmHg compared to normal values at approximately 10 mmHg). However, the combination of chronic inflammation with histological findings of pulmonary vascular remodeling makes this potentially an attractive model, worthy of further study.

11.4 HIV-Associated Pulmonary Hypertension

HIV associated with pulmonary hypertension has been observed since the late 1980s (Kim and Factor 1987), but it remains unclear how infection leads to an increased risk for PAH. In humans with HIV the prevalence is low (0.46 %) (Sitbon et al. 2008).

Method. A nonhuman primate models of HIV-1 infection can be created with simian immunodeficiency virus (SIV) or using a HIV-1 nef gene, which is crucial for maintaining a high viral load by downregulating CD4 (Marecki et al. 2006). Alternatively, HIV-1 transgenic rats can be studied which develop increased RVSP and PA remodeling (Lund et al. 2011).

Features of model. Both SIV (Chalifoux et al. 1992) and SIV modified to overexpress nef (Marecki et al. 2006) can recapitulate many of the lung pathologies observed in patients with PH secondary to HIV, although with limited or no hemodynamics changes (Marecki et al. 2006). In this model, complex, plexiform lesions, coupled with luminal obliteration, medial hypertrophy, and thrombosis have been observed in SIV-*nef* animals, but not in SIV infected animals. There is a high prevalence of mononuclear cells in this model supportive of the inflammatory role played by HIV (Humbert 2008).

Limitations of model. Monkeys are very costly to house. Moreover, the exact hemodynamics in this model remain undefined. A fundamental limitation is likely the low prevalence of PAH even in humans with HIV (Sitbon et al. 2008).

11.5 Radiation Exposure

Recently a novel animal model of PAH was developed in the form of a lung injury from radiation (Ghobadi et al. 2012).

Method. Male adult Wister rats were exposed to 150 MeV protons from a cyclotron, using a shoot-through technique so that rats' lungs were irradiated at different doses (Fig. 8). Eight weeks after lung irradiation, the animals were sacrificed and hemodynamics were measured.

Features. Lung irradiation caused PH (systolic PAP ranged from 30 to 60 mmHg). The severity of the hemodynamic changes of PH was proportional to the irradiated lung volume. Histologically, lung irradiation caused medial hypertrophy, adventitial thickening, and obliteration of small pulmonary vessels. These features were also seen out of the field of radiation. This is an interesting model of Group 1 PH, although replication by other groups is required.

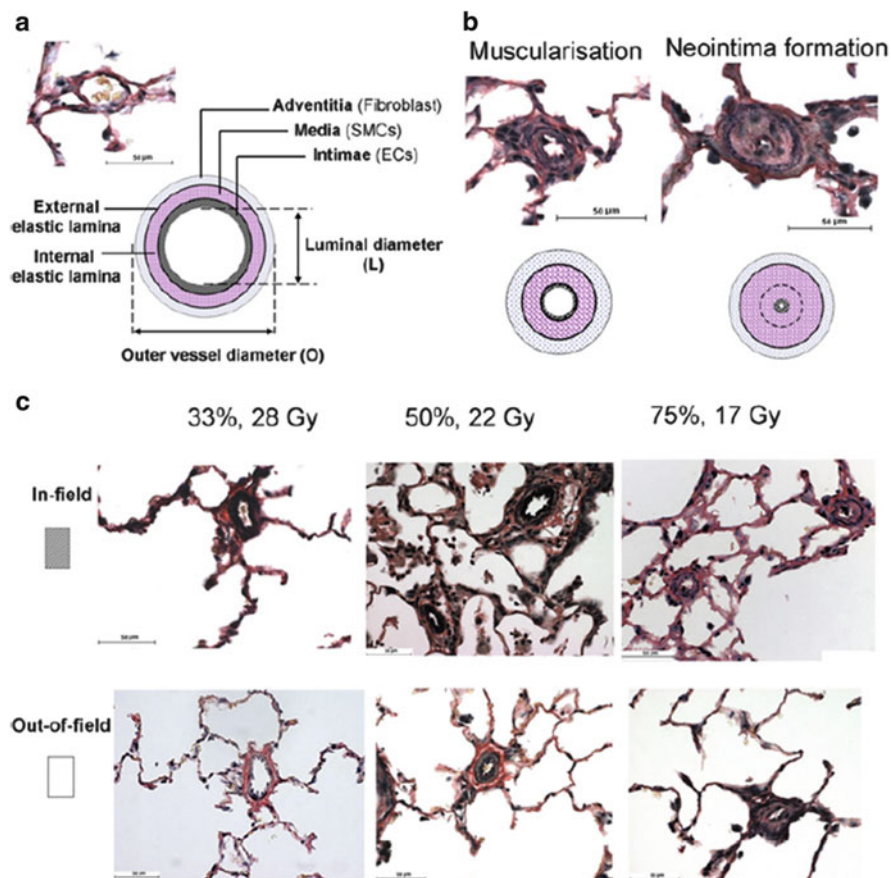


Fig. 8 Early radiation response of lung vasculature leads to global pulmonary vascular remodeling. (a) Anatomy of nonirradiated control pulmonary intra-acinar vessel (<50 μm). Pulmonary vascular remodeling was assessed by immunohistochemical staining (Verhoeff's elastica stain) of pulmonary intra-acinar vessels 8 weeks postirradiation of 33, 50, and 75 % of rats' lungs to 28, 22, and 17 Gy, respectively. (b) Lung irradiation induces vascular remodeling such as muscularization, adventitia thickening, and neointima formation. (c) Vascular remodeling was present in-field and out-of-field showing a global pulmonary vascular response in all irradiated volumes. *L* luminal diameter, *O* outer vessel diameter, *SMC* smooth muscle cell. Magnification $\times 400$. Ghobadi G, Bartelds B, van der Veen SJ et al (2011) Thorax

Limitations. Irradiation has an effect on both vasculature as well as airway tissue thus this is not a pure Group 1 PH model. The exact mechanism through which it achieves it changes remain unclear as this model has not been studied extensively, but primarily the changes appear mediated through endothelial cell damage.

12 Surgical Models

Surgical models provide insight in particular to congenital cardiac disease, which are an important cause of Group 1 PH. They provide valuable hemodynamic information as well as insight into adaptive and maladaptive response of the right ventricle (Piao et al. 2010b). Increased pulmonary blood flow leads to increased shear stress on the vascular wall and smooth muscle cell hypertrophy and proliferation (Buus et al. 2001). Since right ventricular failure is an important cause of death in PAH patients, it is noteworthy that different models have been developed to induce RVH in the absence of pulmonary disease. Both pressure overload (by pulmonary artery banding) and volume overload (by shunting blood from the aorta to the vena cava) have been used to study RV hypertrophy.

12.1 Pulmonary Artery Banding Model

Prognosis and functional status in congenital heart disease is determined to a large extent by right ventricular function. Pulmonary artery banding (PAB) replicates pulmonic stenosis (either isolated or after tetralogy of Fallot repair) and does not cause pulmonary hypertension. As such it is a useful control model to separate pulmonary disease from cardiac disease.

Methods of model. To create this model, under anesthesia, via a limited median sternotomy, the main pulmonary artery is dissected from the ascending aorta. A small needle [16G in rats (Fang et al. 2012), 23G in mice (Bartelds et al. 2011)] is then placed parallel to the main PA and ligated with a 4-0 silk suture (7-0 in mouse model). Withdrawal of the needle then creates a fixed PA stenosis.

Features of model. The PAB model demonstrates a decrease in cardiac output and exercise performance. Right ventricular hypertrophy develops 3–4 weeks after banding. This model has been useful to study right ventricular metabolism in the pressure-overloaded right ventricle (Fang et al. 2012). Using this model, it has been observed that fatty acid oxidation (FAO) is increased in the right ventricle and inhibiting FAO in PAB rats increased right ventricle ATP production and improved exercise capacity, as well as RV performance.

Limitations of model. As with all the surgical models, technical expertise is required to develop a consistent disease in animals.

12.2 Aorto-Caval Shunt

Aorto-caval shunt is a successful model of RV volume overload.

Methods of model. To create this model in mice, through a midline laparotomy, a 25G needle is advanced through the abdominal aorta toward the inferior vena cava. The distal vein is compressed throughout the procedure. Upon removal of the needle, tissue glue is used to close the aortic puncture site.

Features of model. When studied in mice, aorto-caval shunting develop a similar extent of RVH to PAB (Bartelds et al. 2011). Aorto-caval shunting can also be combined with monocrotaline to further accentuate the PA pressures in addition to PA flow. To create this model, 7 days after monocrotaline is administered (60 mg/kg), aorto-caval shunt surgery is performed (van Albada et al. 2005). Compared to aorto-caval alone or monocrotaline alone, MCT plus aorto-caval shunt develop functionally more severe PH with increased mortality over 5–6 weeks, although the pressures observed in MCT and MCT plus aorto-caval shunt are similar (systolic PAP 55–60 mmHg) as is the extent of neo-intimal proliferation and muscularization. The combined insult does result in a greater amount of RVH and RV dilatation. This further highlights the vital impact of right ventricular function on morbidity and mortality.

Limitations of model. This technique, although reproducible and rapid, has been associated with a 10 % mortality at 24 h postsurgery (Garcia and Diebold 1990).

13 Summary

When determining which animal model of Group 1 PH to study, the following characteristics are important to consider:

1. Accurate representation of human PAH
2. Hemodynamic changes
3. Pathologic changes, including inflammation
4. Ease, cost and reproducibility of the model
5. Cellular or molecular question being addressed
6. Local expertise and resources

Group 1 PH is not a uniform disease and a single model cannot successfully capture the all the features of disparate diseases within this group. Comprehensive assessment using advanced imaging, catheterization, and molecular characterization are essential. Adequate trial design and selection of appropriate endpoints are as important as the model itself in obtaining results that are relevant to human PAH.

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Part II
Pulmonary Hypertension: Established
Therapies

General Supportive Care

Ioana R. Preston

Abstract In addition to PAH-specific therapies proven to be effective in achieving a better outcome and/or symptomatic improvement, the care of patients with pulmonary arterial hypertension (PAH) requires a complex strategy that includes general pharmacological and non-pharmacological measures. This chapter will detail the general supportive measures beneficial for PAH patients as adjuvant treatment to targeted therapies.

Keywords Diuretics • Anticoagulation • Pulmonary rehabilitation • Travel • Pulmonary arterial hypertension

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1 Introduction

Pulmonary arterial hypertension (PAH) is a complex disease that requires an integrated approach between multiple disciplines. In addition to targeted therapies, general measures have been recommended to prevent complications that may lead to disease decompensation and to treat nonspecifically certain aspects of PAH. A summary of recommendations for general care is presented in Table 1. Among those, only supervised exercise therapy has been formally studied in randomized trials in PAH. Therefore, the recommendations in this chapter are mostly based on small, nonrandomized reports and clinical experience. The principles of general care described below aid in maximizing the clinical care of PAH patients. General supportive care measures can be divided into pharmacological and non-pharmacological measures.

2 General Pharmacological Measures

2.1 Diuretics

Diuretics are often used in decompensated right heart failure in clinical practice. Although there are no clinical trials testing the effectiveness of diuretics in PAH, clinical experience suggests that a tight volume control achieved with diuretics improves symptoms. There are no guidelines regarding the choice or dose of diuretics. Unlike the experience in left heart failure, where aldosterone antagonists have shown to improve survival in patients with systolic left ventricular failure (Hamaguchi et al. 2010), there are no data of their efficacy in right heart failure. Frequent monitoring of renal function and electrolytes, as well as close weight control, is advisable. In randomized trials of left heart failure, adjustment of cardiovascular medications based on regular monitoring of brain natriuretic peptide (BNP) or its precursor, N-terminal pro-BNP (NT-pro-BNP) led to better control of heart failure symptoms and fewer hospitalizations (Januzzi et al. 2011). Prospective studies on PAH patients showed that a decrease in NT-pro-BNP levels with therapy was associated with improved outcome (Mauritz et al. 2011). Although there are no studies proving the benefit of actively adjusting medical therapy (i.e., diuretics) based on BNP levels in PAH, BNP or NT-pro-BNP levels can be monitored as a measure of compensated right heart failure.

2.2 Oxygen

Most PAH patients have a minor degree of hypoxemia and many present with nocturnal desaturations, even in the absence of obstructive sleep apnea (Minai et al. 2007). There are few exceptions where a more severe degree of hypoxemia

Table 1 Summary of recommendations for general supportive care of PAH patients

Pharmacological measures	Considerations
Diuretics	The goal is to achieve a tight control of volume status. The choice of diuretic is variable
Oxygen	In hypoxemic patients, oxygen supplementation can be considered. Traveling requires assessment before the flight for possible oxygen supplementation
Anticoagulants	Only warfarin has been studied to date. Long-term anticoagulation should be considered in idiopathic PAH, heritable PAH, and PAH associated with anorexigen use
Digoxin	Goal to treat tachyarrhythmias
Non-pharmacological measures	Considerations
Physical exercise and pulmonary rehabilitation	Exercise is encouraged in patients stable on targeted therapies. Patients benefit from supervised exercise programs
Pregnancy and birth control	Pregnancy is contraindicated and birth control measures are encouraged
Screening for depression and psychosocial support	Screening for depression is important and patients may benefit from a multidisciplinary approach
Infection control	Preventive vaccinations are recommended and catheter care using sterile techniques are important in avoiding infections
Elective surgery	The increased risk of complications requires careful assessment and planning prior to surgery

is encountered: patients with congenital systemic to pulmonary shunts and right to left physiology (Eisenmenger's syndrome), those with patent foramen ovale and right to left shunts, and those with scleroderma-associated PAH who have a more severe gas exchange abnormality and a low diffusion capacity for carbon monoxide. Oxygen was shown to be an acute vasodilator (Atz et al. 1999), but long-term treatment with oxygen has not been studied in PAH patients. One exception constitutes patients with Eisenmenger syndrome, in whom long-term chronic oxygen therapy did not bring any benefit (Sandoval et al. 2001). Currently, the recommendations for oxygen therapy in PAH are extrapolated from the chronic obstructive lung disease data. Long-term oxygen can be considered in PAH when partial pressure of oxygen is <60 mmHg, or oxygen saturation <88 % (Weitzenblum et al. 1985). In addition, oxygen supplementation can be used for exertional and nocturnal hypoxemia. Most stable PAH patients can safely travel by plane, although patients who require oxygen replacement, either continuously, or with exertion will most likely require oxygen during a flight. In a retrospective study in which 159 out of 430 PAH patients traveled at least once (Thamm et al. 2011), 57 patients were on long-term ambulatory oxygen therapy, and 29 used supplemental oxygen while traveling. There were 20 adverse events, most of them mild to moderate, and only 7 required medical intervention. A more recent study investigated prospectively 34 PAH patients and found that hypoxemia is common during flight and is associated with cabin pressures equivalent to

>6,000 ft above sea level, ambulation, and flight duration (Roubinian et al. 2012). Therefore, while most stable PAH patients can safely travel by plane, we recommend evaluation for supplemental in-flight oxygen for patients taking long flights, if not already on supplemental therapy.

2.3 Anticoagulants

The role of chronic thrombosis in PAH is controversial (Wagenvoort 1980; Fuster et al. 1984). It is not clear whether the thrombotic arteriopathy described in pathological samples from PAH patients are an epiphenomenon or an integral part of the pulmonary vascular remodeling. The experience with anticoagulation is derived from one retrospective cohort and two prospective studies that showed that the use of warfarin was associated with a better outcome in PAH (Fuster et al. 1984; Rich et al. 1992; Kawut et al. 2005). These studies looked only at patients with idiopathic PAH, heritable PAH, and PAH due to anorexigen use. To date, there is no information on the effects of anticoagulation in other forms of PAH. Therefore, current guidelines for chronic anticoagulation include these three categories of PAH. The consideration for anticoagulation must be weighed against the potential risk of bleeding. In particular, patients with PAH associated with scleroderma, those with congenital heart disease, and portopulmonary hypertension patients may be at increased risk due to the presence of gastrointestinal, or pulmonary arteriovenous malformations, or an abnormal coagulation profile, respectively. When anticoagulation is considered, the goal is to maintain an international normalized ratio between 1.5 and 2.5 (as it is practiced in the USA) or between 2.0 and 3.0 (the European experience). Anticoagulation can be stopped for invasive procedures without the need to bridge with heparins, unless patients carry the diagnosis of chronic thromboembolic pulmonary hypertension, or have other reasons for anticoagulation.

2.4 Digoxin

Digoxin was shown to improve cardiac output acutely in PAH patients (Rich et al. 1998), but long-term studies are lacking. The current recommendations include its use in tachyarrhythmias. As the therapeutic window of digoxin is narrow, caution needs to be exerted in patients with prerenal azotemia, in order to avoid toxicity.

3 Non-pharmacological Measures

3.1 Physical Exercise and Pulmonary Rehabilitation

Once started on targeted therapies, PAH patients are encouraged to gradually increase their activity to a level that brings on mild to moderate dyspnea. Activities that elicit presyncope, syncope, or chest pain should be discouraged. In addition, patients who are on chronic anticoagulation should avoid contact sports. Patients with both early and advanced disease benefit from supervised rehabilitation programs. To date, four studies showed that supervised rehabilitation programs both in inpatient and outpatient settings improve exercise capacity and quality of life, as well as skeletal muscle function, when added to pharmacological therapy (Mereles et al. 2006; de Man et al. 2009; Fox et al. 2011; Grunig et al. 2012). The most recent study evaluated the effect of exercise training in an inpatient setting for 3 weeks followed by outpatient training for 15 weeks in a cohort of patients with different forms of pulmonary hypertension (Grunig et al. 2012). Compared with baseline, 3- and 15-week assessments showed improvement in 6-min walk distance (6MWD), scores of quality of life, functional class, peak oxygen consumption, oxygen pulse, heart rate and systolic pulmonary artery pressure at rest, and maximal workload. The improvement in 6MWD was similar across the different forms and functional classes of pulmonary hypertension. Adverse events such as respiratory infections, syncope, or presyncope occurred in 13 % of patients, suggesting that exercise training in pulmonary hypertension is an effective but not a completely harmless add-on therapy and patients should be closely monitored. Once patients complete the supervised program, they are encouraged to continue their maintenance exercises regularly.

3.2 Pregnancy and Birth Control

PAH is considered a contraindication to pregnancy because it has been previously associated with 30–50 % mortality in the peripartum period, although recent reports show more favorable outcomes, especially in long-term responders to calcium channel blockers (Jais et al. 2012). Barrier contraceptive methods are safe, but not very reliable. Hormonal replacement therapy is a suitable option, if high doses of estrogen are avoided due to their association with increased risk of thrombosis. Alternatively, progesterone-only preparations such as medroxyprogesterone acetate and etonogestrel are effective approaches to contraception. Intrauterine devices (IUDs) are a safe and reliable method of long-term contraception, although special care needs to be taken during the implantation. We recommend the procedure be done in the hospital setting with placement of two peripheral intravenous lines and atropine readily available in case of vasovagal reactions. PAH patients who become pregnant should be informed of the high risk of pregnancy and termination of

pregnancy discussed. Patients who choose to continue pregnancy should be closely monitored by a team involving high-risk obstetricians and PAH specialists, preferably in a PAH center. Treatment with PAH-specific therapies should be initiated or modified, taking into account the risk profile of PAH therapies, and a planned elective delivery should be scheduled (Bendayan et al. 2005; Bonnin et al. 2005).

3.3 Screening for Depression and Psychosocial Support

Similar to other chronic conditions, many patients with PAH are diagnosed and/or being treated for depression. Some patients are reluctant to discuss their psychosocial limitations with their PAH specialists and many PAH specialists do not have formal training to recognize and treat depression. The type of therapy chosen is a complex matter. Serotonin receptor antagonists (SSRIs) are commonly used for depression. Nevertheless, the role of serotonin in PAH remains controversial (see Chap. 4.4) and the decision of what pharmacological therapy, should one be indicated, needs to be weighted and discussed among the various healthcare professionals providing for the care of the PAH patient. A report that surveyed two cohorts of patients showed that the use of SSRIs was associated with decreased mortality after adjustment for age, gender, etiology of PAH, and obesity (Shah et al. 2009), while in the large Registry to Evaluate Early And Long-term PAH disease management (REVEAL) incident SSRI use was associated with increased mortality and a greater risk of clinical worsening (Sadoughi et al. 2013). Conversely, the use of SSRIs in pregnant women was associated with an increased risk for primary pulmonary hypertension of the newborn in the offspring (Chambers et al. 2006; Kieler et al. 2012). Therefore, an interdisciplinary approach involving the PAH treating physician, the primary care provider, a psychologist, social worker, and/or a psychiatrist should be considered in determining the best approach to screening and treating depression in PAH. In addition to pharmacological intervention, patient support groups may provide psychosocial and emotional support.

3.4 Infection Control

Similar to other patients with chronic cardiopulmonary conditions, vaccinations against influenza and pneumococcal pneumonia are recommended. Special catheter care, as well as maintenance of sterile techniques while mixing the drug are advised in patients on long-term intravenous infusion with prostacyclin analogues. The involvement of specialized nurses to train the patient in the technique of sterile handling of the parenteral drug and catheter site care is extremely important to ensure a good practice to avoid blood stream infections.

3.5 Elective Surgery

Patients with pulmonary hypertension, in particular those with PAH, are at high risk for complications when undergoing anesthesia and major surgery (Kaw et al. 2011). The perioperative management can be challenging as it may be complicated by a systemic inflammatory response, hypoxemia, and the development of decompensated right heart failure. The mortality rate in the perioperative period was reported between 7 and 18 % (Minai et al. 2006; Kaw et al. 2011; Meyer et al. 2013). Risk factors for complications included emergency surgery, major surgery, and a long operative time (Kaw et al. 2011; Meyer et al. 2013). Patients on oral PAH-specific therapies may require temporary conversion to parenteral therapies (inhaled, intravenous, or subcutaneous). It is not clear what type of anesthesia is better tolerated, as both epidural and general anesthesia cause peripheral vasodilation with a possible decrease in cardiac output. Careful assessment and planning prior to surgery by the treating physicians and anesthesiologist team are advised.

In conclusion, in the intricate strategy of PAH management, general supportive measures play an important role in aiding to the pharmacological targeted therapy in ensuring stability, avoiding the development of complications, and providing symptomatic improvement. These measures need to be integrated in a complex approach, best achieved with the collaboration between the PAH treating physician and multiple other specialties.

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Calcium-Channel Blockers in Pulmonary Arterial Hypertension

Marie-Camille Chaumais, Elise Artaud Macari, and Olivier Sitbon

Abstract Voltage-activated calcium channels are a family of membrane proteins that provide the major influx pathway for calcium in many different types of cells. Calcium-channel blockers inhibit the calcium influx into vascular cells leading to relaxation of smooth muscle cells and vasodilatation. Vasoconstriction of small pulmonary arteries is recognized as a component of the pathogenesis of pulmonary arterial hypertension and treatment with calcium-channel blockers appears to be rational in this setting. No randomized controlled trial has been performed to demonstrate the beneficial effects of calcium-channel blockers in the treatment of patients with pulmonary arterial hypertension. However, uncontrolled studies have suggested that long-term administration of high-dose calcium antagonists dramatically improves survival in a small subset of patients who respond acutely to those drugs, compared with unresponsive patients. The initial response to an acute vasodilator test with inhaled nitric oxide or intravenous prostacyclin or adenosine accurately identifies patients with pulmonary arterial hypertension who are likely to respond to long-term treatment with calcium-channel blockers.

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Abbreviations

Ca ²⁺	Calcium
CCBs	Calcium-channel blockers
CO	Cardiac output
DHP	Dihydropyridine
NO	Nitric oxide
NYHA	New York Heart Association
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PVR	Pulmonary vascular resistance
SMC	Smooth muscle cells

Calcium channel antagonists or calcium channels blockers (CCBs) inhibit the calcium influx into vascular cells leading to relaxation of smooth muscle cells (SMC) and vasodilatation. For more than 25 years, development of CCBs led to the management of hypertension, angina pectoris or cardiac dysrhythmia and these drugs represent nowadays a major pharmacological approach.

Beside proliferation of endothelial and SMC, vasoconstriction of small muscular pulmonary arteries is recognized as a component of the pathogenesis of pulmonary arterial hypertension (PAH) and treatment with CCBs appears to be rational in this setting. Unfortunately, CCBs can be used only in a minority of patients with PAH. This chapter focuses on pharmacology of CCBs and their use in the management of patients with PAH

1 Pharmacology of calcium channel blockers

1.1 Mechanism of Action

Voltage-activated calcium channels are a family of membrane proteins that provide the major influx pathway for calcium in many different types of cells. Long-lasting voltage-gated calcium channels (L-type Ca^{2+} channels) are a major subclass of voltage-activated calcium channels present in cardiac, skeletal, and SMC, in various types of cells in the brain, and in many nonexcitable cells. L-type Ca^{2+} channels open in response to a change in membrane potential and are regulated by a variety of receptor-activated signaling pathways. In cardiac and SMC, activation of L-type Ca^{2+} channels initiates contraction directly by increasing cytosolic calcium concentration and indirectly by activating calcium-dependent calcium release by ryanodine-sensitive calcium release channels in the sarcoplasmic reticulum (Tsien 1983; Bers 2002).

Four subunits make up the SMC and cardiac L-type Ca^{2+} channels. The α -1 subunit associated with the disulfide-linked α -2 δ subunit and the intracellular β subunit (Fig. 1). The α -1 subunit of 190–250 kDa is the largest subunit, and it incorporates the conduction pore, the voltage sensor and gating apparatus, and most of the known sites of channel regulation by second messengers, drugs, and toxins (Catterall et al. 2005). Several α -1 subunit genes that give rise to L-type currents have been identified, including α -1S which is found in skeletal muscle, α -1C which is expressed in heart, smooth muscle, and brain, as well as α -1D which is found in neuroendocrine cells (De Waard et al. 1996). Diversity in the α -1 subunits also arises owing to alternative splicing of the DNAs; for example, the α 1C-subunit gene undergoes alternative splicing to give rise to isoforms found in heart (α 1c-a), smooth muscle (α 1c-b), and brain (α 1c-c) (Hosey et al. 1996). In 2000, a rational nomenclature was adopted (Ertel et al. 2000) based on the well-defined potassium channel nomenclature (Chandy and Gutman 1993). Calcium channels were named using the chemical symbol of the principal permeating ion (Ca^{2+}) with the principal physiological regulator (voltage) indicated as a subscript (CaV). The numerical identifier corresponds to the CaV channel α -1 subunit gene subfamily (1–3 at present) and the order of discovery of the alpha-1 subunit within that subfamily (1 through n). According to this nomenclature, the CaV1 subfamily (CaV1.1–CaV1.4) includes channels containing α 1S, α 1C, α 1D, and α 1F, which mediate L-type Ca^{2+} channels and where CaV1.2 is the isoform in the heart and SMC. α -1 subunit is organized in four homologous domains (I–IV), with six transmembrane segments (S1–S6) in each (Fig. 1). The S4 segment serves as the voltage sensor (Catterall et al. 2005). The pore loop between transmembrane segments S5 and S6 in each domain determines ion conductance and selectivity, and changes of only three amino acids in the pore loops in domains I, III, and IV will convert a sodium channel to calcium selectivity. Although auxiliary subunits modulate the properties of the channel complex, the pharmacological and electrophysiological diversity of calcium channels arises primarily from the existence of multiple alpha-1

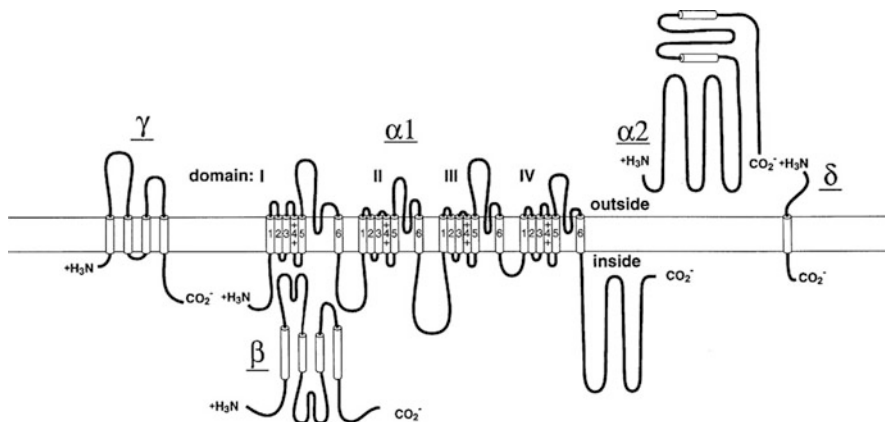


Fig. 1 L-voltage Ca^{2+} channels' structure (from Hockerman et al. 1997). *Cylinders* represent alpha helical transmembrane segments. *Bold lines* represent the polypeptide chains of each subunit

subunits (Hofmann et al. 1994). Phosphorylation of channel-forming subunits by different kinases is one of the most important ways to change the activity of L-type Ca^{2+} channels. L-type Ca^{2+} channels in cardiac cells, neurons, skeletal muscle, and chromaffin cells are regulated by a protein kinase A-dependent phosphorylation (McDonald et al. 1994). In addition, other protein kinases (for example, protein kinase C and calcium-dependent kinases) and phosphoprotein phosphatases have been shown to modify channel activity (Catterall 2011; Brette et al. 2006).

The α -1 subunit is the binding site for CCBs. By inhibition of L-type Ca^{2+} channel, CCBs decrease calcium influx into SMC of arterial wall and myocardium cells leading to vasodilatation and a decrease of systemic blood pressure.

1.2 Classification of Calcium-Channel Blockers

The three major classes of CCBs are chemically distinct and exhibit different functional effects depending on their biophysical, conformation-dependent interactions with the L-type Ca^{2+} channel. Dihydropyridines (DHP) have exclusively vascular effects, whereas non-dihydropyridines (regrouping phenylalkylamines and benzothiazepines) have both vascular and cardiac effects. Drugs in these three subclasses bind to separate sites on the L-type calcium channel: Bound phenylalkylamine (verapamil-like) makes both hydrophobic contacts with residues in transmembrane segments IIIS6 and IVS6 and ionic interactions with the glutamate residues in the pore region of the α -1 subunit. The benzothiazepine (diltiazem-like) calcium antagonists bind to the cytoplasmic bridge between motif III (IIIS) and motif IV (IVS) and more specifically the S6 segments of these domains. The benzothiazepine site is therefore located in close proximity to the phenylalkylamine receptor site (Kimball et al. 1993). The DHP (nifedipine-like) calcium antagonists bind to transmembrane segment 6 of

Table 1 Characteristics of available calcium-channel blockers on cardiac and vascular smooth muscle cells

CCB group	Agent	Vascular effects	Cardiac effects	Indications	
Dihydropyridines	Nifedipine	++++	–	Hypertension/ angina	
	Nicardipine	++++	–	Hypertension	
	Felodipine	++++	–	Hypertension	
	Nitrendipine	++++	–	Hypertension	
	Isradipine	++++	–	Hypertension	
	Nisoldipine	++++	–	Hypertension	
	Amlodipine	++++	–	Hypertension/ angina	
	Lercanidipine	++++	–	Hypertension	
Non-dihydropyridines	Benzothiazepines	Diltiazem	+++	++	Hypertension/ angina/ dysrhythmia
		Phenylalkylamines	Verapamil	++++	++++

CCBs have different effects on vascular and cardiac tissues. The non-dihydropyridine agents have inotropic and chronotropic negative effects leading to their use in angina pectoris and dysrhythmia, whereas dihydropyridine agents have only peripheral vasodilator effect and are prescribed mainly for hypertension

both motif III (IIS6) and motif IV (IVS6) and segment 5 of motif III (IIS5) (Hockerman et al. 1997). Whereas the three major classes of CCBs all interact with receptor sites formed by the same general region of the α -1 subunit, binding with specific separate sites on this α -1 subunit allows a pharmacologic rationale for their combination, especially for diltiazem and dihydropyridines (Elliott and Ram 2011).

All currently available CCBs cause vasodilation, with lowering of blood pressure. The DHPs are more potent vasodilators and generally have less cardiodepressant activity than representatives of other classes of CCBs due to their high selectivity on the vasculature. On the opposite, verapamil has a high cardiac selectivity for the L-type Ca^{2+} channel and diltiazem has an intermediary profile (Lee and Tsien 1983; Herzig et al. 1992). Associated with the decrease in blood pressure, non-DHP induces a depression of cardiac contractility and conduction. Verapamil and diltiazem slow conduction through the atrioventricular node and increase the atrioventricular nodal refractory period, which, in turn, results in the slowing of the ventricular response rate in atrial fibrillation or flutter or in the conversion of atrioventricular nodal reentry tachyarrhythmias to sinus rhythm by disruption of the timing of the reentry circuit (Abernethy and Schwartz 1999). All the available CCBs are described in Table 1.

1.3 Usual Indications

All CCBs have been approved by the Food and Drug Administration (FDA) for the treatment of hypertension, alone or in combination with other antihypertensive drugs. Specific CCBs could also be indicated in angina pectoris prevention or cardiac dysrhythmia (Table 1). CCBs may also be beneficial in patients with left ventricular diastolic dysfunction, Raynaud's phenomenon, migraine, preterm labor, esophageal spasm, and bipolar disorders (Abernethy and Schwartz 1999). Verapamil and diltiazem are the only non-dihydropyridine drugs (phenylalkylamine and benzothiazepine, respectively) currently used in clinical practice whereas several DHPs are approved. The newer DHP calcium antagonists are structurally related to nifedipine, but may provide greater vascular selectivity and wider clinical utility. CCBs are usually used long term by oral route. Most are available in long-acting formulations that permit once-daily administration. Only diltiazem, nifedipine, and verapamil are available in intravenous formulations.

In addition to their role in the acute changes in ion flux associated with changes in membrane potential, L-type calcium channels seem to have a role in cellular growth and proliferation. Long-term treatment with CCBs for hypertension has been reported to decrease arteriolar and left ventricular hypertrophy at different levels regarding models and drugs (Jesmin et al. 2002; Kyselovic et al. 2001).

1.4 Side Effects

The most common side effects with CCB treatment are related to their vasodilator properties, with headache, flushing, dizziness, and nausea. Leg edema may occur in patients on long-term CCBs, the mechanism of this side effect being however unknown. Other side effects such as constipation (principally with verapamil), rash, and drowsiness may also occur. For short-acting DHPs, a reflex increase in heart rate has been reported (Meredith and Elliott 2004). Bradycardia is usually observed with diltiazem. Gingival hyperplasia may occur in patients who are given a DHP, but it is rare in those given verapamil or diltiazem.

CCBs are generally not recommended for patients with or high risk for heart failure due to reduction of left ventricular function. Verapamil and diltiazem are contraindicated in patients with hypotension, sick sinus syndrome, and second- or third-degree atrioventricular block and in patients with atrial flutter or atrial fibrillation. In addition, verapamil is contraindicated in patients with severe left ventricular dysfunction, whereas diltiazem is contraindicated in patients with acute myocardial infarction and pulmonary congestion (Elliott and Ram 2011).

1.5 Drug Interactions

All CCBs have drug interactions due to their metabolism by the CYP 3A4. Indeed, strong enzyme inducer as rifampicin or phenytoin or enzyme inhibitor as ketoconazole or cimetidine could significantly alter CCBs concentrations. Verapamil and diltiazem inhibit also the clearance of other substrates of CYP 3A4 (e.g., cyclosporine, carbamazepine, cyclosporine, tacrolimus, simvastatin, midazolam, and HIV-protease inhibitors), whereas the dihydropyridine drugs do not. Moreover, verapamil inhibits P-glycoprotein-mediated drug transport, which may alter the intestinal absorption of several drugs and their distribution into peripheral tissues and the central nervous system (Doppenschmitt et al. 1999). Associated with inhibition of P-glycoprotein-mediated transport of digoxin into peripheral tissues, verapamil also decreases its clearance (Kuhlmann 1987). After an initiation with verapamil in a patient who is receiving digoxin, the cardiac glycoside should be measured in serum after it has reached a new steady state to determine whether the dose should be reduced. Finally, due to their antiarrhythmic properties and additive negative inotropic effects, verapamil and diltiazem have to be used with caution when combined with β -blockers.

2 Calcium-Channel Blockers in Pulmonary Arterial Hypertension

Vasoconstriction of small pulmonary arteries is recognized as a component of the pathogenesis of PAH (Rich et al. 1992). Pure vasodilators alleviate vasoconstriction with little effect on fibrotic and proliferative changes that usually predominate over vasoconstriction in PAH (Rubin 1997). The goal of vasodilator therapy is to reduce pulmonary arterial pressure (PAP), increase cardiac output (CO), and thus decrease pulmonary vascular resistance (PVR) without symptomatic systemic hypotension. Before availability of PAH-targeted therapies (prostacyclin analogues, endothelin-receptor antagonists, PDE5 inhibitors), the question of the overall efficacy of administering vasodilators was of concern, as well as the way of safely identifying the patients who may benefit from long-term vasodilator therapy. Many vasodilators have been studied in PAH with opposite effects (Rubin and Peter 1980; Packer 1985; Hughes and Rubin 1986; Packer et al. 1982; McGoon and Vlietstra 1984). Among these, only CCBs have been proved to be effective long term in rare carefully selected PAH patients (Rich et al. 1992; Packer 1985; Sitbon et al. 1998, 2005).

2.1 Acute Vasodilator Testing

Pulmonary vasodilator testing establishes the relative contribution of reversible vasoconstriction in patients with PAH. It is generally accepted that the initial response to an acute pulmonary vasodilator testing accurately identified patients

with PAH who were likely to respond to chronic vasodilator therapy (Reeves et al. 1986; Sitbon et al. 1998, 2005; Morales-Blanhir et al. 2004; Galie et al. 2009). Multiple criteria for a positive acute vasodilator test in patients with PAH have been suggested: a decrease in both mean PAP of at least 20 % and PVR of more than 20 or 30 % (Barst et al. 1999; Rich et al. 1992); a decrease in PVR of more than 30 % with a decrease in mPAP of more than 10 % (Groves et al. 1993); a decrease in PVR of 50 % with a mean PAP lower than 30 mmHg (Raffy et al. 1996). Conclusions from the third World Symposium on PAH (Barst et al. 2004) and guidelines from the European Society of Cardiology/European Respiratory Society (Galie et al. 2004) and from the American College of Chest Physicians (Badesch et al. 2004) proposed a decrease in mean PAP of at least 10 mmHg to reach an absolute value of 40 mmHg or less without decrease in CO, which is currently recommended to consider a pulmonary vasodilator test as positive (Fig. 2). The current criteria derive from a retrospective analysis of 557 idiopathic PAH patients who received long-term treatment with CCBs after a positive acute pulmonary vasodilator test by older criteria (fall in both mean PAP and PVR > 20 %). Of 12.6 % of the patients who exhibited acute pulmonary vasoreactivity, only half (6.8 %) improved long term with the use of CCBs (Sitbon et al. 2005). The mean PAP reached during acute vasodilator testing was less than 40 mmHg in the majority of patients who were considered long-term CCB responders (Sitbon et al. 2005).

2.2 Drugs Used for Acute Vasodilator Testing

Drugs used for acute vasodilator testing have been chosen based on selectively for the pulmonary circulation and short duration of action to minimize systemic adverse effects (Naeije and Vachiery 2001). The agents currently recommended to evaluate the pulmonary vasoresponsiveness include inhaled nitric oxide, intravenous adenosine, or epoprostenol (Galie et al. 2004; Badesch et al. 2009; Zuo et al. 2012; Nootens et al. 1995; Sitbon et al. 1995). In addition, inhaled iloprost has acquired substantial evidence for this use, although this agent is not currently recommended by the most updated guidelines (Jing et al. 2009; Hoepfer et al. 2000). There is a lack of consensus on the preferred agent for determining acute pulmonary vasoreactivity (Ghofrani et al. 2008). NO has the advantages to be most selective agent for pulmonary vascular bed and to have an easy route of administration, good safety, and low cost. The ACCP/AHA 2009 Expert Consensus Document on Pulmonary Hypertension recommends inhaled NO as the preferred vasodilator agent and considers IV epoprostenol and adenosine acceptable alternatives (McLaughlin et al. 2009). NO doses of 10–80 ppm for up to 15 min are usually given, even though two studies have shown that the vasodilator response to NO was not dependent upon the NO concentration (Sitbon et al. 1995; Ricciardi et al. 1998). Doses of 10–20 ppm for 5 min are usually used in clinical practice.

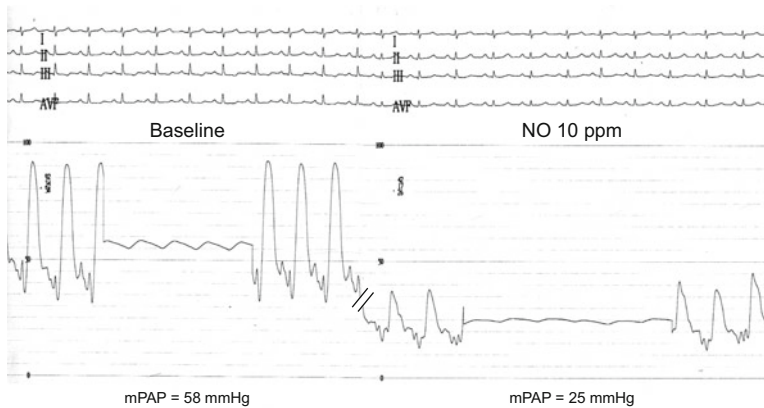


Fig. 2 Acute vasodilator testing with inhaled NO in a 45-year-old male with idiopathic PAH. Mean PAP dropped from 58 to 25 mmHg during acute vasodilator testing. Patient subsequently received long-term treatment with diltiazem (360 mg daily) leading to major and sustained clinical and hemodynamic improvement

2.3 Place of Calcium-Channel Blockers in PAH

A small uncontrolled study has suggested that long-term administration of high-dose CCBs dramatically improves survival in a small subset of patients who respond acutely to those drugs, compared with unresponsive patients (Rich et al. 1992). A large retrospective study from the French reference center for PAH has shown that less than 10 % of patients with idiopathic and anorexigen-associated PAH may respond to long-term CCB therapy (Sitbon et al. 2005). Patients with heritable PAH are less likely to exhibit a significant response to acute vasodilator testing (Elliott et al. 2006; Rosenzweig et al. 2008; Sztrymf et al. 2008; Montani et al. 2010). In forms of PAH other than idiopathic PAH and PAH associated with anorexigens (i.e., PAH associated with connective tissue diseases, HIV infection, portal hypertension, or congenital cardiac shunts), the rate of acute responders is even rare, and long-term response to CCBs is exceptional in these patients (Montani et al. 2010).

The updated evidence-based treatment algorithm for patients with PAH recommends to perform an acute vasodilator testing in all patients with PAH at the time of first evaluation by right heart catheterization, acknowledging that patients with iPAH, and anorexigen-associated PAH are more likely to respond to CCB treatment (Barst et al. 2009; Galie et al. 2009). In the ESC/ERS guidelines, the degree of recommendation to perform an acute vasodilator testing is strong (I-C) for patients with idiopathic and anorexigen-associated PAH and weak (IIb-C) for patients with other forms of PAH (Galie et al. 2009).

2.4 *Clinical Use of Calcium-Channel Blockers in PAH*

No randomized controlled trial has been performed to confirm the beneficial effects of CCBs in the treatment of PAH, predominantly because of the rarity of the condition (Badesch et al. 2004), and CCB therapy in PAH has not received regulatory approval anywhere because of this. However, open-label prospective studies have shown significant improvement outcome in selected PAH patients treated with CCBs. In 1987, Rich et al. have shown a decrease in PAP, PVR, and right ventricular hypertrophy in more than half of patients with idiopathic PAH (formerly primary pulmonary hypertension) who responded favorably to acute vasodilator testing and long-term treatment with CCBs (Rich and Brundage 1987). In this study, high doses of CCBs were required to produce marked hemodynamic response (Rich and Brundage 1987). These results were confirmed by the same group in 1992 in a larger group of patients (Rich et al. 1992). They found that 27 % of patients with idiopathic PAH responded favorably to an acute vasodilator testing with high doses of CCBs and the majority experienced clinical and hemodynamic improvement on long-term treatment with such drugs. In addition, there was a major difference in survival favoring responders versus nonresponders (Rich et al. 1992). This benefit in survival in CCB responders was confirmed in a large retrospective study performed in France with a survival rate of 97 % at 5 years in responders versus 48 % in patients who failed on long-term CCBs (Sitbon et al. 2005). In that study, a sustained benefit with CCBs was defined as the improvement or being maintained in NYHA functional class I or II with near-normalization in pulmonary haemodynamics after a year of treatment (Sitbon et al. 2005).

The current approach of treatment with CCBs in PAH is to initiate the drug if the patient displays a positive vasodilator response, an adequate systemic blood pressure (above 90 mmHg) and a stable NYHA functional class I–III prior to initiation of therapy (Barst et al. 2009; Galie et al. 2009). The agents commonly recommended are diltiazem, nifedipine, or amlodipine (Barst et al. 2009; Galie et al. 2009). The choice between nifedipine/amlodipine and diltiazem is guided by heart rate at rest: diltiazem is preferred in patients with resting HR > 80 bpm (Rich et al. 1992). Verapamil is not recommended due to its prominent negative inotropic effect (Packer et al. 1984). Moreover, CCBs are contraindicated in patients with right heart failure or hemodynamic instability (McLaughlin et al. 2009). Finally, high doses of CCBs must not be used in patients with negative pulmonary vasodilator test as these agents can lead to systemic side effects, worsening of right ventricular function, pulmonary edema, and ultimately death in those patients (Partanen et al. 1993; Farber et al. 1983).

Acute testing with CCBs as proposed by Rich (Rich et al. 1992) has been progressively abandoned for a progressive up-titration of the dose over a few weeks (Gaine 2000). Nifedipine is usually initiated at a dose of 30 mg/day and up-titrated to 60 mg/day and sometimes over up to 240 mg/day. Diltiazem is started at 180 mg/day and up-titrated to 360 mg/day, up to 720 mg/day (Rich et al. 1992).

Table 2 Use of calcium-channel blockers in PAH

CCB agent	Started dosage (mg/day)	Usual dosage (mg/day)	Most frequent side effects
Nifedipine	30	60–240	Systemic hypotension, edema, headache, nausea
Amlodipine	5	20–40	Not reported
Diltiazem	180	360–720	Systemic hypotension, bradycardia, edema, headache, nausea

Nifedipine, diltiazem, and to a less extent amlodipine are the three CCBs used in PAH patients who are responders to acute vasodilator testing. CCBs are given at high dosages with a progressive up-titration. Side effects are related to their vasodilator properties (Sitbon et al. 1998)

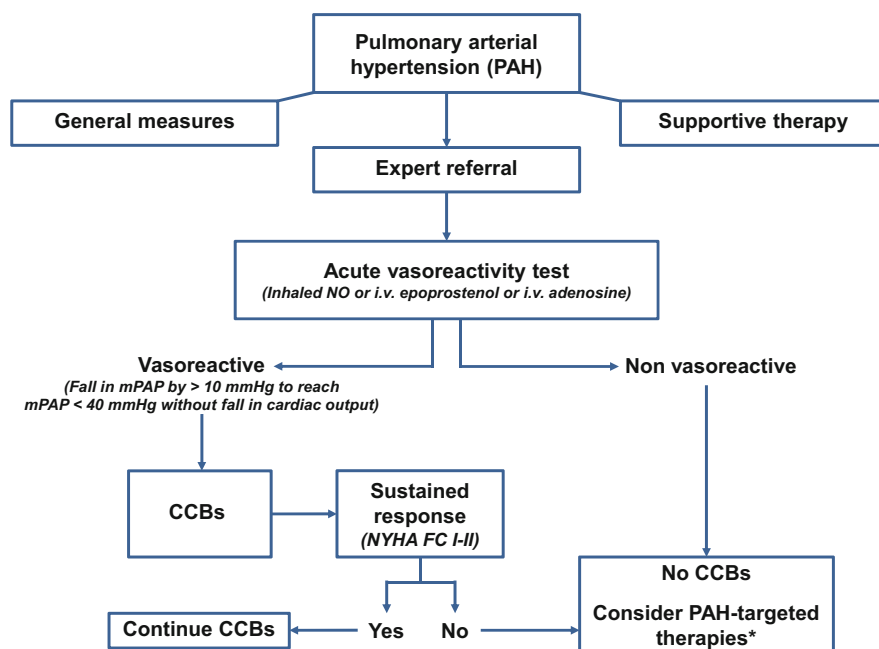


Fig. 3 Place of calcium-channel blockers in the PAH treatment algorithm (adapted from Galie et al. 2009). Abbreviations: CCBs calcium-channel blockers, *mPAP* mean pulmonary artery pressure, *NO* nitric oxide, *NYHA FC* New York Heart Association functional class. *Asterisk*: PAH-targeted therapies: prostacyclin analogues, endothelin-receptor antagonists, phosphodiesterase type-5 inhibitors

The target dose of amlodipine is 20 mg/day (Woodmansey et al. 1996; Barst et al. 2009) (Table 2). The side effects most frequently reported with CCBs in PAH are systemic hypotension and edema of the lower limbs (Sitbon et al. 1998). Association with low dose of diuretics and elastic stockings will help to reverse these side effects.

Patients with iPAH who meet the criteria for a positive vasodilator response and receive treatment with CCBs should be followed closely for both safety and efficacy with an initial reassessment after 3–4 months on therapy including clinical and hemodynamic evaluation. We recommend repeating acute vasodilator testing during the first reassessment on CCBs in order to determine the possibility for further increase in CCB daily dose in case of demonstration of an additional pulmonary vasodilator response. Finally, if NYHA functional class I or II has not been achieved, then additional PAH-targeted therapies are suggested (Fig. 3).

3 Conclusion

CCBs are the first drugs that have been shown to improve long-term survival in patients with PAH. Unfortunately, treatment with those agents should be restricted to rare patients with idiopathic or anorexigen-related PAH who demonstrate an acute pulmonary vasodilator response during first assessment with right heart catheterization. It is widely accepted that the initial response to an acute vasodilator test with inhaled nitric oxide or intravenous prostacyclin or adenosine accurately identify patients with PAH who are likely to respond to chronic CCBs therapy. Calcium antagonists must not ultimately be used in patients with PAH in the absence of positive vasodilator response.

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Prostacyclins

Horst Olschewski

Abstract Prostacyclins have a favourable pharmacological profile for treatment of pulmonary hypertension as they possess vasodilative, antiproliferative, anti-aggregatory, and anti-inflammatory properties that may compensate the main pathologic changes in the small pulmonary arteries. In severe pulmonary hypertension these vessels show a deficit in the endogenous prostacyclin secretion. The therapeutic potential of prostacyclin for pulmonary hypertension has been known since 30 years, and since nearly 20 years prostacyclin has been approved for idiopathic PAH. There are intravenous, subcutaneous, and inhaled approaches of different substances who share many but not all pharmacologic properties. However, none of these approaches are easy and free of adverse effects. Long-term experience and careful decision-making are instrumental to achieve favourable clinical long-term results.

Keywords Prostacyclin • Epoprostenol • Iloprost • Treprostinil • Beraprost • Selexipag

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1 Introduction

Prostacyclin and its stable analogues possess a chemical prostaglandin structure and preferentially bind to the prostaglandin I (IP) receptor. Prostacyclins are potent vasodilators and possess antithrombotic, antiproliferative, and anti-inflammatory properties. They also reduce matrix secretion in smooth muscle cells (SMC), endothelial cells, and fibroblasts (Fig. 1). The endothelial cells are the major source of endogenous prostacyclin. Due to the short half-life, the prostacyclin action will be directed both on the local vascular wall and on passing blood cells that get in contact with the endothelium. Pulmonary arterial hypertension (PAH) is associated with vasoconstriction, thrombosis, proliferation, inflammation, and a lack of endogenous prostacyclin secretion. This provides a strong rationale for prostacyclin use as therapy for PAH.

2 History of Prostacyclin and Analogues

Endogenous prostacyclin was discovered in 1976 (Moncada et al. 1976). In the same year, prostacyclin was chemically synthesised (epoprostenol) and investigated in animals and humans (Gryglewski 1980). Chemically stable analogues were synthesised in the 1980s. Clinical studies in PAH patients were performed with epoprostenol, iloprost, beraprost, and treprostinil while cicaprost, a highly specific IP-receptor agonist, was not clinically developed.

The first applications of prostacyclin in pulmonary hypertension were published in 1980 (O'Grady et al. 1980) and the first long-term therapy for severe idiopathic pulmonary arterial hypertension (PAH) was applied in 1984 (Higenbottam et al. 1984). Despite many new developments in PAH therapy, prostacyclins remain a mainstay in the treatment of PAH.

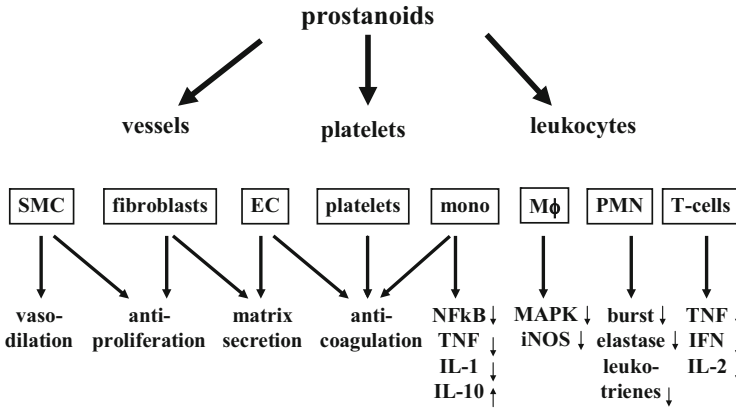


Fig. 1 Effects of prostacyclins on the vessel wall and adherent blood cells. *SMC* smooth muscle cells; *EC* endothelial cells; *mono* mononuclear cells; *Mφ* macrophages; *PMN* polymorphonuclear neutrophils; *T-cells* T-lymphocytes; *TNF* transforming nuclear factor α ; *IL* interleukin; *MAPK* mitogen-activated protein kinase; burst, generation of reactive oxygen species, elastase, elastase secretion; *IFN* interferon γ . From Olschewski et al. (2001b)

3 Pharmacologic Properties

The main target of prostacyclin and its analogues is the human IP receptor which is abundantly expressed in blood vessels, leucocytes, and thrombocytes and is rapidly activated by binding of the ligand. The IP receptor in small animals is much less sensitive to prostacyclin as the human receptor. Therefore predictions about dose-response relationships based on such animal models may not be reliable. The IP receptor is coupled with Gs proteins and activates adenylate cyclase, leading to increased cAMP levels in the target cells which explain most of the biologic effects. Principally, it also couples with Gq proteins and might activate vasoconstrictive pathways under certain instances (Chow et al. 2003; Wise 2003). In addition, prostacyclin and analogues are not highly specific to the IP receptor but may also activate EP receptors (Abramovitz et al. 2000) which are located on the cell surface as well as in the nucleus (Bhattacharya et al. 1998, 1999). They may further activate the peroxisome proliferator-activated receptor delta (PPAR δ) (Gupta et al. 2000). Both PPAR α and PPAR δ may also be activated via IP receptor-dependent PKA activation, but the intracellular prostacyclin produced by the endogenous PGI synthase seems to specifically activate the apoptosis pathway by activation of PPAR δ (Hatae et al. 2001) (Fig. 2). There is evidence that PPAR δ is also involved in the acute signalling in prostacyclin-induced vasodilatation (Li et al. 2012). Specifically, prostacyclins activate the most important potassium channels, the TWIK-related acid-sensitive potassium channel (TASK1) and the calcium-activated potassium channel (KCA) and thereby cause membrane hyperpolarization and inhibition of L-type calcium channels in SMCs. Figure 3 represents the current knowledge about the mechanisms underlying the powerful activation of potassium current

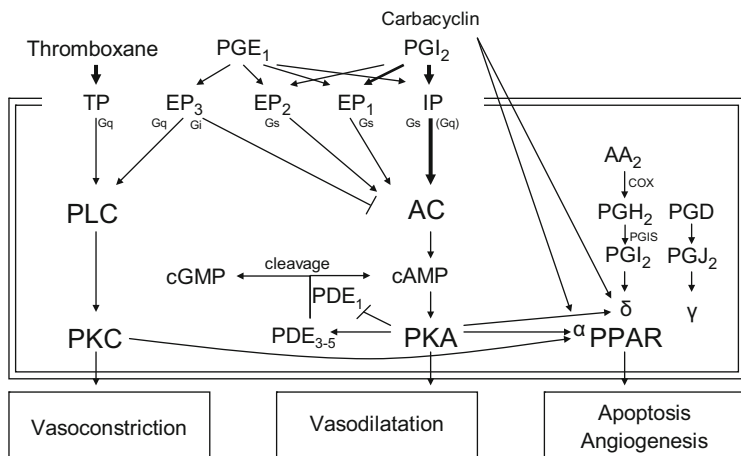


Fig. 2 Intracellular targets of prostacyclin. Extracellular PGI₂ mainly but not specifically acts on the IP receptor and the EP1 receptor while PGE₁ is more specific to EP receptors. *Gs*, *Gq*, and *Gi* G-proteins, *PLC* phospholipase C, *PKC* proteinkinase C, *AC* adenylyl-cyclase, *PKA* protein kinase A, *AA₂* arachidonic acid, *COX* cyclooxygenases 1 and 2, *PGH₂* prostaglandin H₂, *PGIS* prostacyclin synthase, *PPAR* peroxisome proliferator-activated receptors, *PGD* prostaglandin D, *PGJ₂* prostaglandin J₂, *PDE* phosphodiesterases. From Gomberg-Maitland and Olschewski (2008)

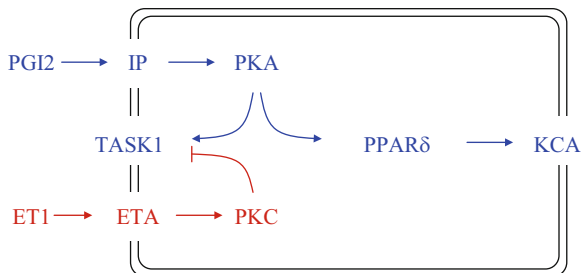


Fig. 3 Prostacyclin-induced activation of potassium channels prostacyclin (PGI₂) activates proteinkinase A (PKA) via IP receptors of the cell membrane. PKA activates both the TWIK-related acid-sensitive K channel (TASK1) and the calcium-activated potassium channel (KCA). The activation of KCA is critically dependent on activation of PPAR δ , while TASK1 activation is independent of PPAR δ . Endothelin1 (ET1) counteracts the activation of TASK1 via proteinkinase C (PKC) activation. Data from Li et al. (2012) and Tang et al. (2009)

from TASK1 and KCA channels by prostacyclin and the opposite effects of endothelin-1 (Tang et al. 2009). In human pulmonary arterial smooth muscle cells, TASK1 is the only active potassium channel at physiologic membrane potential and is therefore mainly responsible for the negative membrane potential which provides a low vascular tone (Olschewski et al. 2006). It has recently been shown that TASK1 activity depends on both oxygen and src tyrosine kinase activity (Nagaraj et al. 2012).

3.1 Differences Between Prostacyclin Analogues

Epoprostenol is provided as a dry powder with a highly basic glycine buffer as a solvent drug. After mixing drug powder with the solvent, the solution has to be used within 12–24 h due to spontaneous degradation of the compound. A new formulation that was FDA approved in 2009 (Veletri[®]) allows for 48 h use at 25 °C and 5 days of storage at 2–8 °C. All other prostanoids are chemically stable in solution and their plasma half-life is much longer. It is about 30 min with iloprost and beraprost and up to 4.5 h with treprostinil. Vein irritation is common to all prostanoids. All stable prostanoids have been provided as oral preparations; however, only beraprost has received approval for PAH in Japan and some Eastern countries (see below). Iloprost has been approved as inhalative therapy and treprostinil as subcutaneous infusion or intravenous infusion or inhalation (see below). The doses that are needed for strong vasodilative effects differ considerably among prostanoids. A continuous intravenous dose of 50 ng/kg/min of poprostenol is, on average, translated into 100 ng/kg/min of treprostinil but just 3 ng/kg/min of iloprost. The reasons for these huge differences are not known.

Apart from differences in pharmacokinetics and pharmacodynamics, there may be other specific differences between the prostacyclin analogues. The antiproliferative effects of treprostinil appeared more impressive than those of other compounds (Clapp et al. 2002), but due to differences in chemical stability such results must be interpreted with caution. Non-IP-receptor effects of prostacyclin analogues have attracted some interest in tumour biology (Keith and Geraci 2006) and might be interesting for pulmonary hypertension as well. Iloprost and cicaprost were found to have different effects in the murine corneal model of angiogenesis. While iloprost caused significant angiogenesis, comparable to VEGF, cicaprost had no such effects (Pola et al. 2004). The explanation may be that iloprost and carbacyclin but not cicaprost activate peroxisome proliferator-activated receptors (PPAR) (Reginato et al. 1998), which results in VEGF secretion (Forman et al. 1995; Barger 2002). VEGF increase may antagonise endothelial dysfunction and represents a potential beneficial factor (le Cras et al. 2002; Taraseviciene-Stewart et al. 2001).

Some effects have only been described in one of the compounds but may be common to all prostacyclin analogues. Treprostinil augments the positive inotropic effects of catecholamines in isolated ventricular myocytes (Fontana et al. 2007), although it has no positive inotropic effects of its own. This effect may have clinical relevance because during right heart failure there is an increased catecholamine drive. These effects may add on indirect effects that originate from systemic vasodilation and subsequent baroreflex activation (Fig. 4) and improved ventriculoarterial coupling (Kerbaul et al. 2007).

Iloprost suppresses neutrophil adhesion, respiratory burst, and elastase secretion (Rose et al. 2003). This may be important because inflammation appears to play a role among the pathologic mechanisms of pulmonary arterial hypertension (Stacher et al. 2012). In SMC, a number of beneficial changes in gene expression

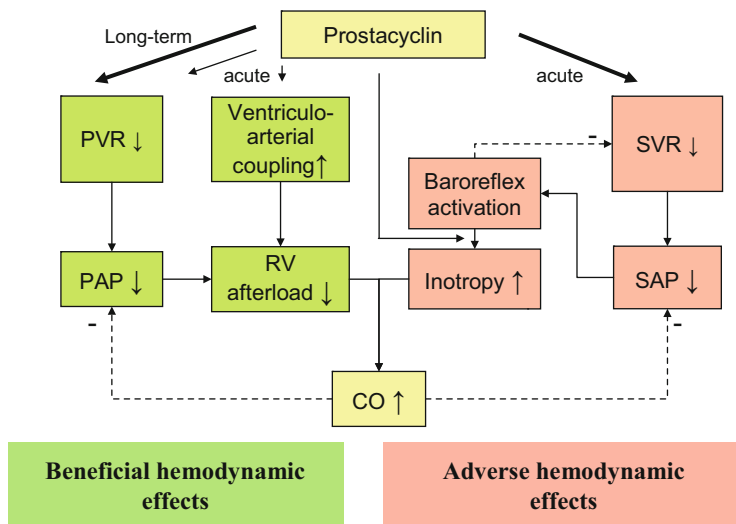


Fig. 4 Hemodynamic effects of systemically applied prostacyclins or its analogues. Within minutes, prostacyclins cause a strong vasodilatation which in most patients is more prominent on the systemic (SVR) compared to the pulmonary vascular resistance (PVR). The decrease in systemic pressure (SAP) activates the arterial baroreflex which increases SVR and has positive inotropic effects. The reduction in PVR would decrease pulmonary arterial pressure (PAP); however, in the acute phase, the increase in cardiac output counteracts this resulting in an unchanged PAP. Within weeks, PVR decreases more prominently and SVR does not further decrease which may result in a significant PAP decrease. For ventriculoarterial coupling and inotropic effects of prostacyclin, see text. Adapted from Olschewski and Olschewski (2011)

(hyaluronidonic acid, COX 2, and VEGF upregulation; monocyte chemotactic protein and plasminogen activator inhibitor-1 downregulation) were shown after incubation with iloprost (Meyer-Kirchrath et al. 2004; Sussmann et al. 2004). If we consider that endothelial dysfunction or damage may play a major role in the pathologic development of pulmonary artery remodelling, this translates into a number of beneficial effects of iloprost (Fig. 5).

3.2 Hemodynamic Effects

The hemodynamic effects and the associated side effects of all prostacyclin analogues are similar while the mode of application and the applied doses differ. In the case of continuous intravenous infusion there is a relatively fast dose increase with systemic prostacyclin analogues over the first weeks, followed by a gradual dose increase over many months. Interestingly, there is no loss of pharmacological effect if the infusion rate is adequately adapted. The doses for epoprostenol typically start at 4 ng/kg/min and increase to about 20–60 ng/kg/min after a year.

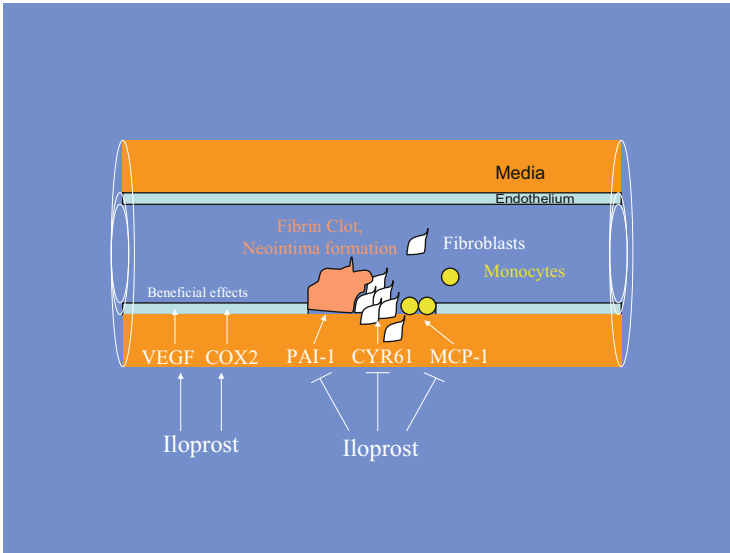


Fig. 5 Hypothesis for neointimal formation and possible role of prostacyclins. After endothelial damage, monocytes, fibroblasts, and the coagulation system will form a neointima, which leads to intima fibrosis. Iloprost has been shown to inhibit the mRNA expression of important mediators of these processes. *VEGF* vascular endothelial growth factor, *COX2* cyclooxygenase 2, *PAI-1* plasminogen activator inhibitor, *Cyr 61* cysteine-rich angiogenic protein, *MCP-1* monocyte chemotactic protein. From Gomberg-Maitland and Olschewski (2008)

In the majority of patients, the beneficial hemodynamic effects, characterized by a significant decrease in pulmonary vascular resistance, will occur after some days of therapy; however, they may be obscured by systemic side effects. It takes several weeks until the patient will fully anticipate the beneficial effect of the drug because the pulmonary effects become more and more prominent while the systemic side effects fade away.

If the prostacyclin analogues are applied by inhalation every 2–3 h, there is no need for dose increase in the majority of patients (Olschewski et al. 1996, 2000, 2003, 2010), suggesting that this mode of application may not cause receptor desensitisation.

4 Clinical Application

4.1 Epoprostenol: Intravenous

Continuous intravenous prostacyclin (epoprostenol) was the first therapy for idiopathic and heritable PAH. Case reports and small case series (Higenbottam

et al. 1984; Weir et al. 1989; Rubin et al. 1982) demonstrated improvement in symptoms and hemodynamics and were followed by a prospective, randomised open-label controlled trial (Barst et al. 1996) for 12 weeks. The 6 min walk distance (6MWD) improved by 32 m in the epoprostenol group compared with a decrease of 15 m in the control group. The mean pulmonary vascular resistance decreased by 21 % in the epoprostenol group vs. an increase of 9 % in the conventional group. This study also suggested a survival benefit as those eight patients who died during the 12-week trial were all in the conventional therapy group.

Long-term effects of epoprostenol have never been compared with placebo because this is considered unethical due to the substantial treatment benefit after 3 months of therapy. Observational cohort analyses comparing the survival of epoprostenol-treated IPAH and HPAH patients with historical controls suggested long-term benefits (McLaughlin et al. 2002; Sitbon et al. 2002).

In scleroderma-associated PAH, intravenous epoprostenol improved exercise capacity (Badesch et al. 2000). Similar improvements in functional capacity and functional class have been seen in subjects with congenital left-to-right cardiac shunts (Rosenzweig et al. 1999) and infection with the human immunodeficiency virus (HIV) (Nunes et al. 2003; Aguilar and Farber 2000) while results are ambiguous in portopulmonary hypertension (Krowka and Swanson 2006) and show unfavourable effects in pulmonary hypertension secondary to left heart failure (Califf et al. 1997).

Increasing use of epoprostenol was associated with a considerable decrease in the number of lung transplantations for pulmonary hypertension. A US survey indicated that treatment with epoprostenol allowed two-thirds of subjects to deactivate from the transplant list (Robbins et al. 1998). It is unclear how long the disease will remain stable and therefore frequent clinical follow-up with examination, exercise, and hemodynamics is recommended (Badesch et al. 2007; Galie et al. 2009).

4.1.1 Combination with Other PAH Medication

In the BREATHE 2 study, subjects with PAH were started on epoprostenol with up-titration for 16 weeks and randomised in a 2:1 ratio to bosentan or placebo (Humbert et al. 2004). Addition of bosentan caused a trend, but no significant benefit in clinical or hemodynamic measurements.

Simonneau et al. reported the results of a 16-week multinational, double-blind, placebo-controlled trial assessing the safety and efficacy of sildenafil in addition to epoprostenol (PACES) (Simonneau et al. 2008). Patients had improvements in exercise capacity (adjusted increase 26 m, $p < 0.001$), hemodynamics, and time to clinical worsening in favour of sildenafil. The long-term open-label extension at 1 year maintained this benefit.

4.1.2 Limitations of Intravenous Epoprostenol

Limitations are mainly based on the pharmacology of epoprostenol with its chemical instability and extremely short half-life after infusion. Long-term administration of epoprostenol requires a permanent central venous catheter and a portable infusion pump. Conventional medication needs to be prepared daily and to be kept cold, requiring ice packs to be worn with medication in 24 h cassettes. The new formulation provides more stability after dissolving the drug, but the plasma half-life remains unchanged.

Patients need education of sterile techniques, operation of the pump, taking care of the catheter, and a strong support system to help ensure a safe environment prior to initiation. A “caregiver,” a family member, or a friend who lives in close proximity to the patient is strongly recommended to obviate problems if the subject is too ill or unable to prepare medication on a given day. Serious complications include infection and thrombosis of the catheter and temporary interruption of the infusion because of inadvertent disconnection or pump malfunction. The incidence of catheter-related sepsis ranges from 0.04 to 0.6 cases per patient-year in experienced centres (Sitbon et al. 2002; Humbert et al. 1999; Kitterman et al. 2012) but may be much higher in less experienced centres.

Side effects are usually well tolerated, provided the dose is uptitrated at an adequate rate. The most common side effects include flushing, headache, nausea, loose stool, jaw pain, and foot pain and ascites (Barst et al. 1996; McLaughlin et al. 2002; Sitbon et al. 2002). While jaw pain is mostly transient and well tolerated, foot pain and ascites may deteriorate physical activity and quality of life.

4.2 *Iloprost: Intravenous*

Intravenous iloprost has been approved for pulmonary hypertension in New Zealand, but it has also been used in other countries like Germany, Switzerland, England, Australia, Thailand, Israel, Argentina, and Brazil. Intravenous iloprost has similar acute hemodynamic effects as epoprostenol (Higenbottam et al. 1998). It comes as concentrated solution in glass ampoules and does not require cooling. Due to its longer half-life it is less risky in the case of accidental therapy disruption. In Europe, the typical starting dose is 0.5 ng/kg/min and the long-term dose of infused iloprost is mostly 3 ng/kg/min (Ewert et al. 2007). In a few cases doses up to 10 ng/kg/min were applied. It has not been formally tested if infused iloprost has the same clinical efficacy as infused epoprostenol or treprostinil.

4.3 *Treprostinil: Intravenous*

The FDA approved the use of intravenous treprostinil for patients who do not tolerate subcutaneous treprostinil therapy. The advantage over epoprostenol is the chemical stability and the longer half-life that may also decrease the risk of rebound pulmonary hypertension in case of inadvertent cessation of the infusion. When patients transitioned from IV epoprostenol to IV treprostinil they finished on more than twice the dose of treprostinil compared with epoprostenol (Gomberg-Maitland et al. 2005a; Sitbon et al. 2007). It is unclear why a higher treprostinil dose is required compared to epoprostenol as, theoretically, its longer half-life would suggest the opposite. There were significantly more septic events with treprostinil as compared to epoprostenol in the USA; particularly the number of gram-negative sepsis episodes was increased (Kitterman et al. 2012). This might be associated with the different pump system, the slower infusion rate, or pharmacologic effects of the drug. There was no indication that the drug itself was contaminated.

4.4 *Treprostinil: Subcutaneous*

Treprostinil was originally approved as subcutaneous (sc) infusion. Subcutaneous therapy avoids a permanent central venous catheter. Medication is infused via a small self-inserted sc needle with continuous injection by syringe by a battery-driven pump. The infusion site should be changed every 3–4 days according to the package insert. In clinical practice patients often leave sites for 2–4 weeks (Lang et al. 2006). The drug comes as a ready-to-use solution and there is no need for cooling. The most common side effect occurring in up to 85 % of the patients is local infusion site pain (Simonneau et al. 2002). Patients are treated with variable response with a combination of local anaesthetic solutions, nonsteroidal anti-inflammatory agents, gabapentin, pregabalin, or low-dose narcotics. The pain does not appear to be dose related and cannot be predicted before therapy starts.

Class IV patients and patients receiving higher doses of treprostinil had a more significant improvement. There was also an improvement in hemodynamic parameters, including right atrial pressure, mean pulmonary artery pressure, pulmonary vascular resistance, and cardiac output. The drug was FDA approved for the treatment of NYHA class II–IV PAH patients in 2002 and EMA approved for PAH NYHA III in 2005.

Recent retrospective and observational studies of sc treprostinil suggest long-term clinical improvement and survival benefits. Lang and colleagues evaluated clinical outcomes in 99 PAH patients and 23 patients with inoperable chronic thromboembolic pulmonary hypertension for a mean follow-up of 26 ± 17 months in the open-label phase of a randomised clinical trial (Lang et al. 2006). The mean dose was 40 ± 3 ng/kg/min (range 16–84 ng/kg/min). Patients maintained their improved exercise tolerance (6MWD) and FC at 3 years. Event-free survival was

83 % at 1 year and 69 % at 3 years and overall survival was 89 % at 1 year and 71 % at 3 years, similar rates to those observed on iv epoprostenol.

Evaluation of all patients who had been enrolled in randomised controlled trials with open-label extension and available long-term observations revealed 860 patients who were treated for up to 4 years (Barst et al. 2006). Survival in patients on monotherapy censoring patients with the addition of targeted PAH therapy (130 patients) or premature discontinuation due to adverse events (199 patients) was 88, 79, 73, and 70 % at 1, 2, 3, and 4 years, respectively; however, only few patients remained on therapy for more than 2 years. The most frequently reported side effect was site pain.

Addition of sildenafil to sc treprostinil improved exercise capacity (6MWD) and functional class (Gomberg-Maitland et al. 2005b) and the same was found for the addition of sildenafil to inhaled treprostinil (Voswinckel et al. 2008).

4.4.1 Treprostinil Oral

The oral form of treprostinil is an extended release formulation. This therapy had a significant benefit in treatment-naïve PAH patients (FREEDOM-M) but no significant effect in patients on other targeted PAH medications (FREEDOM-C). Recently, the FDA decided not to approve oral treprostinil based on the moderate effects on 6 min walk distance and no evidence for a delay in clinical worsening achieved in the FREEDOM trials.

4.5 Beraprost: Oral

Beraprost is absorbed rapidly after oral administration and has an elimination half-life of 20–40 min. In Europe, 130 FC II and III subjects were enrolled in a 12-week randomised double-blind placebo-controlled trial of beraprost (Galie et al. 2002). Subjects had pulmonary arterial hypertension caused by IPAH, connective tissue diseases, congenital left-to-right shunts, portal hypertension, and HIV. At a median dose of 80 µg, four times daily, the 6MWD improved by 25 m, compared to placebo ($p = 0.04$). Subjects with IPAH had a mean increase of 46 m whereas PAH from other causes had no significant improvement. In the USA, PAH patients in functional class II and III were included in a 12-month study. The 6 min walk distance improved at 3 and 6 months compared with placebo, but this benefit was not sustained at 9 and 12 months (Barst et al. 2003). Beraprost has not been approved in the USA and Europe but is approved in Japan and several other Far East countries.

4.6 *Selexipag*

Selexipag is a non-prostanoid substance that specifically activates the IP receptor (Morrison et al. 2012). It has been investigated in a phase II study and had significant hemodynamic effects and side effects as expected for a prostacyclin analogue (Simonneau et al. 2012). Currently, a phase III study is performed.

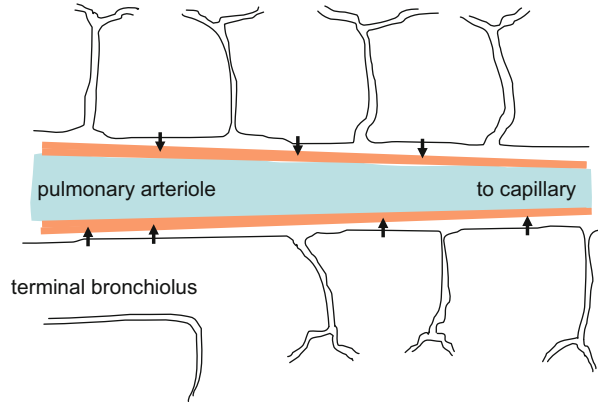
4.7 *Iloprost: Inhaled*

The lung anatomy allows direct access to the precapillary vessels of the lung via drug deposition in the alveolar space or the surface of the bronchiolus terminalis (Fig. 6). Inhalation of prostacyclin as compared to infusion was shown to provide intrapulmonary selectivity and pulmonary selectivity with less systemic side effects (Olschewski et al. 1996, 2003; Walmrath et al. 1993, 1996). The inhalative approach was used with epoprostenol from the early 1990s, with iloprost from 1994 and with treprostinil from 2003.

When iloprost is delivered by different appropriate nebulizers, the pharmacodynamic and pharmacokinetics applied by the same dose are very similar (Olschewski et al. 2003). To ensure alveolar deposition, the delivery system produces small aerosolized particles with a median diameter of 3.0–5.0 μm . Iloprost must be inhaled six to nine times a day to achieve good clinical results. The inhaled doses for significant clinical effects were much smaller with inhaled iloprost (about 0.31 ng/kg/min) (Olschewski et al. 2002), compared to the intravenous infusion of iloprost for PAH.

Inhaled iloprost was approved for PAH, NYHA III + IV in the USA; for PAH, NYHA III and IV as well as inoperable CTEPH in Australia; and for IPAH, NYHA III in Europe. In the European study patients with IPAH, HPAH, PAH associated with connective tissue diseases, or inoperable CTEPH in FC III or IV were enrolled in a 12-week multi-centre placebo-controlled trial (Olschewski et al. 2002). The primary endpoint was a combined clinical endpoint where four criteria had to be met to be counted as a responder: a 10 % increase in 6MWD, an improvement in NYHA functional class, no deterioration, and no death. Seventeen percent of patients on iloprost reached this endpoint compared with 4 % in the placebo group ($p = 0.007$). The mean increase in 6MWD was 37 m ($p = 0.004$) and 59 m amongst subjects with primary pulmonary hypertension. Subjects' well-being improved as evidenced by quality of life scores and the Mahler dyspnea index. Hemodynamic trough values (before inhalation) at 12 weeks improved slightly but significantly in the treatment group compared with placebo ($p < 0.001$). There was a major improvement when post-inhalation values were considered. Side effects consisted of symptoms related to systemic vasodilation. More syncopal episodes (8 vs. 5, NS) occurred in the iloprost group, although they were not associated with clinical deterioration.

Fig. 6 The inhaled route of application for prostacyclin or its analogues. *Black arrows* mark areas where locally deposited drug penetrates the airway wall and diffuses into the pulmonary artery wall. From Gomberg-Maitland and Olschewski (2008)



Iloprost also improved hemodynamics and physical capacity in a small series of lung fibrosis patients (Olschewski et al. 1999), decompensated right heart failure (Olschewski et al. 2000), and HIV patients (Ghofrani et al. 2004). In a long-term observational study from Germany, the survival after 1 and 2 years was 92 % and 87 % and event-free survival was 84 % and 74 % in the IPAH patients (Olschewski et al. 2010). These results are comparable with the recent long-term data with endothelin receptor antagonists.

4.7.1 Combination Studies with Inhaled Iloprost

Hoeper et al. studied 20 patients with IPAH that were on either inhaled iloprost or oral beraprost. Three months of therapy with bosentan added to the prostacyclin resulted in improvement in exercise capacity (6MWD) and maximal oxygen consumption (Hoeper et al. 2003).

The reverse order, addition of inhaled prostacyclin to oral endothelin antagonist, was used in the STEP trial (Iloprost inhalation solution safety and pilot efficacy trial in combination with bosentan for evaluation in pulmonary arterial hypertension), a double-blind placebo-controlled trial of 67 FC III and IV patients on a stable dose of bosentan for 3 months (McLaughlin et al. 2006). Subjects were randomised to either iloprost 5 µg six to nine times daily or placebo with 6MWD distance post-inhalation as the primary endpoint. The mean improvement of 26 m vs. placebo did not meet statistical significance ($p = 0.051$), but improvements in secondary endpoints including functional class and time to clinical worsening favoured inhaled iloprost.

Hoeper et al. in their 12-week study randomised stable IPAH patients FC III, currently on bosentan (125 mg twice daily) to either inhaled iloprost 5 µg six times daily or no additional therapy. According to slow recruitment and the results of the interim analysis (36 completed and 40 enrolled patients), this trial was early terminated (Hoeper et al. 2006).

Sildenafil may act synergistically with prostanoids due to its interaction with cAMP breakdown via inhibition of phosphodiesterase 3 via cGMP (Olschewski et al. 2001a). Ghofrani et al. (2002) demonstrated impressive additive effects of inhaled iloprost on sildenafil-induced pulmonary vasodilatation and beneficial clinical effects of adjunct therapy with sildenafil in patients on inhaled iloprost. Over 9–12 months of follow-up, exercise capacity and hemodynamics improved (Ghofrani et al. 2003).

4.8 Treprostinil: Inhaled

Inhaled treprostinil has effects similar to inhaled iloprost; however, there are significant differences in the pharmacodynamics (Voswinckel et al. 2006). This allows for a shorter inhalation time and less inhalations per day. It may be even possible to inhale the full dose with one single breath (Voswinckel et al. 2009). The maximally tolerated dose with a near maximal acute decrease in pulmonary vascular resistance occurred with the 30 μg dose.

In a small cohort, the addition of inhaled treprostinil to oral PAH therapy found significant improvements in 6MWD with nearly all patients improving physical capacity and functional class (Channick et al. 2006). The same was found in a large multinational study (TRIUMPH-1). Study drug or placebo was inhaled with a special ultrasonic nebulizer four times daily. Patients were on a stable treatment with bosentan or sildenafil before enrollment and this therapy was not changed during the study period. Patients on inhaled treprostinil improved significantly as compared to placebo ($p < 0.001$) both post-inhalation and pre-inhalation. Side effects were mostly mild and did not involve toxic effects (McLaughlin et al. 2010). Based on these results inhaled treprostinil was approved by the FDA, while the EMA declined approval based on formal issues.

Combination of sildenafil with inhaled treprostinil had synergistic hemodynamic effects (Voswinckel et al. 2008).

5 Management of Prostacyclin Therapy

5.1 Decision for a Prostacyclin or Its Analogues

The decision for a prostacyclin therapy is—in most of the cases—equivalent with a lifelong commitment for this kind of drug. Because all approved drugs employ demanding application modes and are prone to side effects, this decision must be based on solid ground.

5.2 *Potential Treatment Hazards*

Left ventricular dysfunction, either systolic or diastolic, represents a contraindication for prostacyclins due to the results of the FIRST study (Califf et al. 1997). In this study, 471 patients with left ventricular failure and mild pulmonary hypertension were randomised to continuous intravenous epoprostenol or placebo. After 6 months there was a significantly higher mortality in the epoprostenol group compared to control.

There was no study in patients with mild left ventricular failure and severe pulmonary hypertension and there is no consensus how to treat “out of proportion” pulmonary hypertension in left heart failure. However, there is consensus, although not formally studied, not to employ prostacyclin or its analogues in patients with a PAWP > 15 mmHg. As a consequence, all candidates for this therapy should have undergone right heart catheterization and a number of further diagnostic tests before a prostacyclin therapy is started.

There are several diseases and conditions that may amplify adverse effects of prostacyclins even if they are not clinically manifest before application. This is the case in pulmonary veno-occlusive disease (PVOD) (Gunther et al. 2012; Rabiller et al. 2006), where prostacyclins can have beneficial effects but are prone to lung edema and hypoxemia (Montani et al. 2009), and in chronic thromboembolic pulmonary hypertension (CTEPH), where any vasodilators will primarily increase the blood flow of the hyper-perfused areas, resulting in worsening of hypoxemia. Patients with obstructive lung disease or lung fibrosis have both heterogeneous ventilation and heterogeneous perfusion and are prone to ventilation/perfusion mismatch and shunt blood flow with any kind of pulmonary vasodilator.

The inhalative application as compared to the systemic application of prostacyclins may be advantageous in patients who are prone to V/Q mismatch (Olschewski et al. 1999). This also applies for liver diseases (Krowka and Swanson 2006; Reichenberger et al. 2006). However, prostacyclin or its analogues have not been approved in any of these conditions.

5.3 *Practical Issues of Prostacyclin Treatment*

As prostacyclin patients represent a small population with a life-threatening disease and a risky medication, a strong partnership between the patient and the health professionals is very important. In most specialised centres there is a long-lasting personal relationship between individual patients and nurses and/or physicians.

Most patients experience some degree of side effects. They must learn to accept this for the sake of their physical capacity and survival. They often need to talk with an experienced professional about these issues. Both water retention and overnutrition can cause significant clinical deterioration. Patients must learn to control their water, salt, and calorie intake and to adapt the diuretic dose to their needs. Infection management is very important, particularly in patients on iv or sc medication. This may be very demanding for both the patient and the caregiver (Hall et al. 2012).

Patients profit from a written plan for the correct response to a given situation including antibiotic treatment and eventually removal of the intravenous line or change of the sc line.

It seems that an oral-first treatment strategy is not associated with a disadvantage in patients with pulmonary hypertension (Cornwell et al. 2011). Actually, most of the patients who start a prostacyclin treatment have been treated with non-prostanoid medication like endothelin receptor antagonists or phosphodiesterase-5 inhibitors. If they are in WHO class III or IV, they receive a prostacyclin or analogue on top of their oral therapy. A complete switch is mostly due to severe side effects from the oral drugs.

Patients who are primarily started on prostacyclin therapy are mostly in NYHA III or IV with signs of right heart decompensation and severely reduced cardiac index and central venous oxygen saturation. Sometimes these patients need to stay in an intensive care unit. It may be advantageous to start these patients on upfront combination therapy, but the evidence for such an approach is based on small uncontrolled case series (Kemp et al. 2012).

Before starting the therapy with prostacyclin or its analogues, water retention is treated with iv diuretics. If systemic pressure is too low, patients receive low-dose catecholamines in parallel. There is some experience with dobutamine, dopamine, adrenaline, and noradrenalin in such a setting. Noradrenalin should only be used in parallel with a continuous prostacyclin because it may further constrict the pulmonary vessels and promote right heart decompensation and death. The prostacyclin dose is then uptitrated while the resting medication is downtitrated as soon as possible.

If a patient is recompensated on prostacyclin treatment, it may be possible to switch to a less demanding therapy like an endothelin receptor antagonist, a phosphodiesterase inhibitor, or even a calcium channel blocker if the patient is a responder.

5.4 Switch from iv Prostacyclins to Other Forms of Application

If a patient is on intravenous medication, it may be considered to switch to a less demanding application form. Transition from iv epoprostenol to inhaled iloprost or from iv treprostinil to inhaled treprostinil is possible. The advantage is to have less systemic side effects. There are small series of patients where this transition worked in most of the cases (Ivy et al. 2008; Perez et al. 2012).

5.5 Switch from Inhaled Prostacyclins to Continuous Infusion

There are three major reasons to consider this switch: (1) the inhalations are too time-consuming and demanding, (2) there are specific side effects (mostly

coughing), and (3) the effect is not sufficient. The first reason normally becomes evident within the first days or weeks of therapy and the second normally goes away after the first days of therapy, but the third may become evident after months or years. Although the dose of inhaled prostacyclin analogues is quite stable over long periods of time, the needed dose may gradually increase.

6 Conclusion

In pulmonary hypertension, a lack of prostacyclin is an important feature of the pathologic changes. Therapy with prostacyclin or its analogues restores physiological pathways involving the pulmonary arterial vessel wall, the platelets, and inflammatory cells and exerts beneficial clinical effects antagonising vasoconstriction, inflammation, and aggregation of circulating cells in the pulmonary vessels. Prostacyclin therapy has been shown to be efficacious, particularly in advanced disease. Combination of other targeted therapies with prostacyclin or its analogues appears to be effective and safe. Despite demanding application methods, this class of drugs remains a mainstay in the treatment of PAH.

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Endothelin Receptor Antagonists

Martine Clozel, Alessandro Maresta, and Marc Humbert

Abstract Three pathways have been identified in the pathogenesis of pulmonary arterial hypertension (PAH): the endothelin (ET), nitric oxide (NO) and prostacyclin pathways. These pathways represent the targets of approved PAH therapies and their discovery has facilitated significant progress in the understanding and treatment of PAH. The ET system is well established as a key player in the pathophysiology of PAH, with deleterious effects mediated by both the ET_A and ET_B receptors. Endothelin receptor antagonists (ERAs) are an important part of PAH therapy, with two ERAs currently approved for the treatment of PAH and a novel ERA that has recently been investigated in a Phase III clinical trial. This chapter describes the role of ET in the pathogenesis of PAH, reviews experimental data and examines the clinical status of ERAs in PAH treatment.

Keywords Endothelin • Endothelin receptor antagonist • Pulmonary arterial hypertension

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1 The ET System

1.1 Endothelins

ETs represent a group of three distinctive 21-amino acid peptides: ET-1, ET-2, and ET-3 (Inoue et al. 1989), with ET-1 being the main isoform produced in the cardiovascular system. Each ET isoform is synthesized as a large pre-pro-endothelin (pre-pro-ET) that is cleaved to pro-ET (big ET-1, -2, and -3) and then further processed by ET-converting enzymes (ECE-1 and ECE-2) to yield mature ET (Kedzierski and Yanagisawa 2001).

ET-1 is one of the most potent vasoconstrictor ever identified (Yanagisawa et al. 1988). It is probably also one of the most potent co-proliferative agents. ET-1 is released, predominantly from vascular endothelial cells, but also by every other cell type. Its secretion can be constitutive or regulated, and it contributes to the maintenance of vascular tone (Russell and Davenport 1999). In endothelial cells, ET-1 is not stored, but secreted predominantly toward the interstitium and smooth muscle (80 %) and to a lesser extent into the circulation (20 %) (Wagner et al. 1992), supporting a paracrine/autocrine role of ET-1, rather than hormonal. The paracrine activity of ET-1 is also corroborated by a strong affinity for its receptors combined with a high tissue receptor density (e.g., 10–100 nM in the vascular wall), resulting in newly secreted ET-1 being immediately trapped by receptor binding (Frelin and Guedin 1994). These unique features explain why plasma ET-1 concentrations are a poor marker of activity of the ET system.

The production of ET-1 is upregulated in response to a range of stimuli including mechanical stress (Yoshizumi et al. 1989), hypoxia (Rakugi et al. 1990), thrombin (Golden et al. 1998), angiotensin II (Imai et al. 1992), vasopressin (Imai et al. 1992), human immunodeficiency virus glycoprotein 120 (HIV gp120) (Ehrenreich et al. 1990), and growth factors (Olave et al. 2012). Conversely it is downregulated by NO (Bourque et al. 2011), prostacyclin (Prins et al. 1994), natriuretic peptides (Emori et al. 1993), and estrogens (Akishita et al. 1998). ET-1 can also be produced, although to a lower extent, by a large number of different cell types including smooth muscle cells (Markewitz et al. 2001), cardiac myocytes (Ito et al. 1993), lung fibroblasts (Davie et al. 2006), macrophages (Ehrenreich et al. 1990), and leukocytes (Sessa et al. 1991).

1.2 *ET Receptors*

ET-1 exerts its action through two pharmacologically distinct G protein-coupled receptors, namely, ET_A and ET_B receptors (Davenport 2002). The ET_A receptor is mostly expressed on vascular smooth muscle cells, fibroblasts, and cardiomyocytes and mediates the vasoconstrictor and proliferative effects of ETs (Hosoda et al. 1991; MacLean et al. 1994; Davie et al. 2002). The ET_B receptor is expressed on vascular endothelial cells where it induces endothelial-dependent vasodilatation through NO production (Hosoda et al. 1991; Hirata et al. 1993) and clearance of circulating ET-1 (Dupuis et al. 1996a). The ET_B receptor is also present on vascular smooth muscle cells, where its activation induces vasoconstriction and cell proliferation (Davie et al. 2002; Clozel et al. 1992), and on fibroblasts, where it mediates cell proliferation, matrix contraction, and collagen production (Shi-Wen et al. 2001, 2004). In the kidney, the ET_B receptor modulates sodium and water handling (Kohan 1997). The ET_B receptors on smooth muscle cells and fibroblasts are inducible (Adner et al. 1998). In a number of pathological situations, there is a shift in the expression of ET_B receptors, which experience a downregulation on endothelial cells and a marked upregulation on smooth muscle cells (Iglarz and Clozel 2010). In rats with systemic hypertension, diabetes mellitus, or hypercholesterolemia, ET_B receptor immunostaining is reduced in endothelial cells and increased in vascular smooth muscle (Kakoki et al. 1999). Upregulation of smooth muscle ET_B receptors has been observed in animal models of heart failure (Gray et al. 2000) and subarachnoid hemorrhage (Roux et al. 1995) and in human diseases including chronic thromboembolic pulmonary hypertension (PH) (Bauer et al. 2002), atherosclerosis (Dagassan et al. 1996), ischemic heart disease (Dimitrijevic et al. 2009), and scleroderma (Shi-Wen et al. 2001, 2004). In such pathological situations, both ET_A and ET_B receptors mediate the detrimental actions of ET-1. Because of a “cross talk” between ET_A and ET_B receptors (Clozel and Flores 2006; Clozel and Gray 1995), selective antagonism of only one receptor subtype may result in compensation by the other receptor subtype and may be insufficient, whereas dual antagonism may be necessary for complete inhibition.

The molecular basis of this cross talk between receptors could be the formation of ET_A–ET_B heterodimers in cells expressing both receptor subtypes, which may further modulate the functionality of the receptors (Sauvageau et al. 2007; Gregan et al. 2004).

1.3 The ET System and the Pulmonary Circulation

The pulmonary circulation is the major site of production of ET-1. In rats, the lung displays the highest immunoreactive ET concentrations (Matsumoto et al. 1989), with mRNA expression levels five times higher than in any other organ studied, including heart, kidney, and brain (Firth and Ratcliffe 1992). The human lung possesses high-affinity binding sites for ET which are variably distributed (Zhao et al. 1995). In human pulmonary arteries, the ET_A receptor is predominant in large vessels, with an increasing proportion of ET_B receptor in more distal pulmonary arteries (Davie et al. 2002).

The lung has also the capacity to clear plasma ET-1 from the circulation and removes about 50 % of circulating ET-1 through the ET_B receptors (Dupuis et al. 1996b). In the normal lung, the amount of ET-1 extracted is quantitatively similar to the amount produced, resulting in a physiological balance of ET-1 across the pulmonary circulation.

2 The Role of the ET System in the Pathogenesis of PH

2.1 Imbalance of Endothelial Mediators

The pathogenesis of PH is a multifactorial process that involves endothelial cell dysfunction and altered production of endothelial cell-derived vasoactive mediators (Tuder et al. 2001). The result is an imbalance between vasodilators/antimitotics, such as prostacyclin and NO, and vasoconstrictors/mitogens, such as thromboxane A₂, serotonin, angiotensin-2, and ET-1, in favor of vasoconstriction and proliferation (Archer and Rich 2000; Kumpers et al. 2010). ET-1 seems to play a major role in endothelial dysfunction and in the pathogenesis of PH, because of its abundance in pulmonary endothelial cells and its increased production in response to many insults. ET-1 is a major initiator of endothelial dysfunction and its production is inversely correlated with that of NO. ET-1 decreases NO bioavailability either by decreasing its production or by increasing its degradation (Iglarz and Clozel 2007). Impairment of these signaling pathways, which regulate normal vascular tone, can lead to the development of PH.

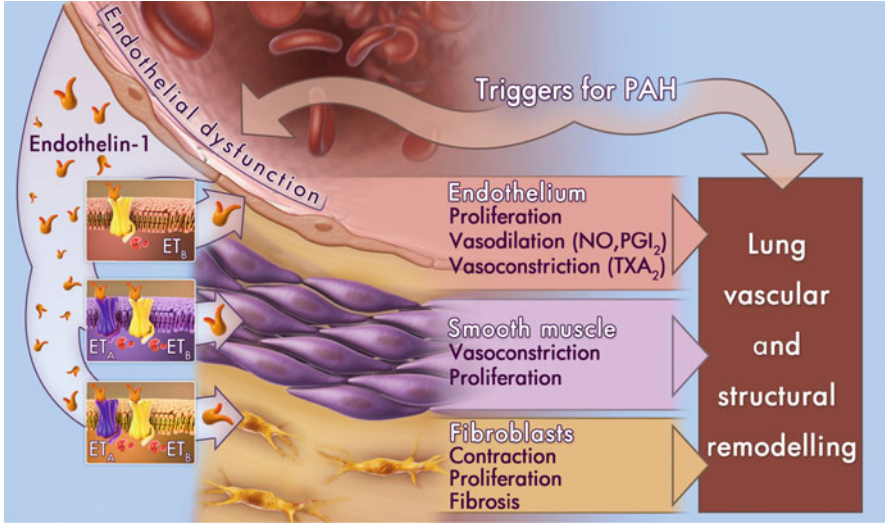


Fig. 1 The pathological roles of ET-1 in PAH

2.2 Effects of ET-1 Contributing to PAH

The effects of ET-1 are in line with the pathological changes that are typical of PAH: PAH is characterized by pulmonary arterial wall remodeling leading to formation of fibrotic neointimal and plexiform lesions, endothelial injury associated with vasoconstriction and in situ thrombosis, and infiltration of inflammatory cells (Stenmark et al. 2009). ET-1 may contribute to each mechanism involved in the pathogenesis of PAH (Fig. 1). In addition to its pressor effects, ET-1 can cause both an abnormal growth pattern in smooth muscle cells (Davie et al. 2002), endothelial cells (Morbidelli et al. 1995), and fibroblasts (Peacock et al. 1992) and an inhibition of cell apoptosis (Kulasekaran et al. 2009; Jankov et al. 2006; Shichiri et al. 1997). ET-1 significantly increases the chemotaxis of endothelial cells and fibroblasts (Morbidelli et al. 1995; Peacock et al. 1992). It also induces fibrosis by stimulating the activation and contraction of fibroblasts (Shi-Wen et al. 2001, 2004), and the synthesis of extracellular matrix compounds (Shi-Wen et al. 2001). Another effect of ET-1 is the induction of inflammation through various mechanisms, including the increase in vascular permeability (Filep et al. 1995), the stimulation of pro-inflammatory cytokine production (Browatzki et al. 2000), the activation of platelet and neutrophils, and the promotion of cellular adhesion (Helset et al. 1996). Finally, there is increasing evidence suggesting that ET-1 increases reactive oxygen species production in the vasculature (Loomis et al. 2005). This was confirmed in a transgenic mouse model where endothelium-restricted ET-1 overexpression led to endothelial dysfunction, vascular hypertrophy, and increased activity and expression of NADPH oxidase (Amiri et al. 2004).

Taken together, these enhanced biological activities shifted in favor of constriction, proliferation, fibrosis, and inflammation suggest that disturbances in the control of ET-1 production can contribute to the pathogenesis of PAH.

2.3 Upregulation of the ET System in PH

The most compelling evidence for activation of the ET system in PH comes from the observation that ET-1 is overexpressed in a number of conditions associated with PH, including idiopathic PAH (Stewart et al. 1991; Giaid et al. 1993; Rubens et al. 2001), PAH associated with connective tissue diseases (Zamora et al. 1990; Julkunen et al. 1991; Vancheeswaran et al. 1994), HIV infection (Rolinski et al. 1994, 1999), portal hypertension (Benjaminov et al. 2003), congenital heart diseases (Yoshiyoshi et al. 1991), sickle cell disease (Benza 2008), but also persistent PH of the newborn (Rosenberg et al. 1993), chronic thromboembolic PH (Bauer et al. 2002), and PH complicating chronic obstructive pulmonary disease (Carratu et al. 2008), and interstitial lung disease (Trakada et al. 2003). One can speculate that the release of ET-1 is reflecting a common endothelial injury in response to a variety of stimulants, such as HIV gp120, anti-endothelial cell antibodies, shear stress, and *BMPR2* mutation, and that the common response will be a sequence of pathological alterations leading to PAH. Furthermore, the increase in plasma ET-1 concentrations tends to correlate with disease severity. In patients with PAH, the increase in plasma ET-1 concentrations is correlated with right atrial pressure, mixed venous oxygen saturation, and pulmonary vascular resistance (Cacoub et al. 1997; Nootens et al. 1995). The elevated circulating ET-1 concentrations associated with PH are not related to a decrease in ET_B receptor-mediated clearance, as indicated by normal or near-normal clearance in patients with PAH, suggesting that increased synthesis could be a primary abnormality (Langleben et al. 2006).

Changes in the ET signaling system in PH are not only limited to an increased production of ET-1 but also affect the ET receptor gene expression. ET receptors are upregulated in PAH. PAH is associated with a significant upregulation of ET binding sites in the distal pulmonary artery circulation, and the relative proportions of both receptor subtypes (60 % ET_A; 40 % ET_B) generally remain the same in normal and remodeled vessels (Davie et al. 2002). In patients with chronic thromboembolic PH, the ET_B receptor is overexpressed in pulmonary arterial smooth muscle cells where it mediates contraction and proliferation, whereas the mRNA levels of ET_A receptor in both endothelium and media are unchanged (Bauer et al. 2002).

3 ET Receptor Antagonism: Preclinical Studies

ET receptor blockade is the most efficient way to antagonize the ET system. Inhibition of ECE is also possible but less effective (Davenport and Maguire 2006). ERAs are classified as ET_A-selective, ET_B-selective, or dual ET_A/ET_B

antagonists depending on their relative affinities for the ET_A and ET_B receptors, as determined either in in vitro studies in cell culture, and/or in vivo by measurement of ET-1 plasma concentrations since they are elevated by ET_B antagonism (Attina et al. 2005). No pharmacological definition of selectivity has been clearly established and ET_A-selective antagonists can bind to both receptors at high concentrations (Goddard and Webb 2002), whereas dual antagonists usually demonstrate a higher affinity for the ET_A receptor. The majority of preclinical studies describing ET receptor antagonism in PH have been performed using in vitro cell culture assays (animal and human), ex vivo tissue preparations (animal and human), and animal models.

3.1 ET Receptor Antagonism and Vasoconstriction/ Vasodilation

Given the role of the ET_B receptor in vasodilation (Hirata et al. 1993), the beneficial effects of combining ET_A and ET_B antagonism have been challenged (Hirata et al. 1993; Dupuis et al. 1996a; Sato et al. 1999). Whether ET_B antagonism results in arterial vasoconstriction or vasodilation appears to depend upon the model and vessels investigated. The ET_B receptor antagonist BQ-788 significantly inhibited ET-1-induced contraction in human internal mammary arteries, but not in radial arteries, whereas the ET_A receptor antagonist BQ-610 produced a potent inhibitory effect in both arteries (Liu et al. 1996). Dilatory effects have been observed with both selective ET_A receptor blockade and dual antagonism. Pretreatment with the ET_A-selective antagonists sitaxentan and BQ-123 or with the dual antagonist bosentan completely inhibited the pulmonary vasoconstrictor response to acute hypoxia in rats (Tilton et al. 2000; Oparil et al. 1995; Chen et al. 1995). Other dual ET_A/ET_B receptor antagonists (BSF 420627, SB 217242 and SB 209670) also demonstrated vasodilatory properties in hypoxia models of PH in rats and dogs (Jasmin et al. 2001; Underwood et al. 1998; Willette et al. 1997). In isolated perfused rat lungs, blockade of both ET_A and ET_B receptors with the ET_A-selective BQ-123 and ET_B-selective BQ-788 antagonists was required for maximal inhibition of ET-1-induced contraction (Sato et al. 1995). Dual blockade was also necessary in isolated human bronchi (Fukuroda et al. 1996) and in isolated small pulmonary arteries from rats with induced myocardial infarction (Sauvageau et al. 2006) to inhibit the contractive effect of ET-1. In arteries from rats with monocrotaline-induced PH, the dual antagonist bosentan and the ET_A receptor antagonist A-147627 potently reduced the contraction induced by ET-1, while bosentan also reduced vascular sensitivity to ET-1 (Sauvageau et al. 2009). These studies suggest that combined ET_A/ET_B receptor blockade may be necessary for complete inhibition of ET-1-induced vasoconstriction. This synergistic effect may be explained by a cross talk between the ET_A and ET_B receptors and the formation of functional heterodimers (Sauvageau et al. 2007).

3.2 *ET Receptor Antagonism and Vascular/Cardiac Hypertrophy*

Beyond vasodilation, the impact of ET-1 antagonists on vascular hypertrophy and right ventricle afterload is essential for pharmacological evaluation, especially in conditions where vascular remodeling is already present. Experimental animal models have been used to reproduce the pathophysiology of PH and its cardiac consequences such as right ventricular hypertrophy and failure. These models mimic some of the clinical conditions that may lead to PH, such as hypoxia, endothelial injury/inflammation, left-to-right shunt, or vascular obstruction.

In several models of acute PH, such as acute hypoxia in rats (Chen et al. 1995; Eddahibi et al. 1995), pigs (Holm et al. 1996), and piglets (Pearl et al. 1999), cardiopulmonary bypass in pigs (Carteaux et al. 1999), and endotoxic shock in pigs (Weitzberg et al. 1996), bosentan blocked the progression of PH and increased cardiac output. The ET_A antagonist, sitaxentan, demonstrated similar effects in hypoxic rats (Tilton et al. 2000).

In chronic models of hypoxia-induced PH in rats, sitaxentan (Tilton et al. 2000) and bosentan (Chen et al. 1995) prevented the development of PH. Furthermore, both drugs, when initiated after the hypoxic injury, produced a significant reversal of established hypoxia-induced PH with reduction of right ventricular hypertrophy and pulmonary vascular remodeling, despite continuous hypoxic exposure. Likewise, selective ET_A antagonists [BQ-123 (DiCarlo et al. 1995) and A-127722 (Chen et al. 1997)] have been examined in this model and found to consistently attenuate or even prevent right ventricular hypertrophy and pulmonary artery medial thickening.

Mixed results have been reported in the model of monocrotaline-induced PH in rats. Sakai et al. (2000) reported that dual antagonism with SB 209670 had the potential to worsen PH compared with selective ET_A blockade with BMS 193884, because blockade of the ET_B receptor inhibited the hypotensive effect of ET-1 on pulmonary circulation. However, the dual antagonist bosentan was found to reduce right ventricular hypertrophy and medial hypertrophy in this model (Hill et al. 1997; Clozel et al. 2006). Using different time courses of administration, Hill et al. (1997) evidenced that bosentan acts mainly during the remodeling phase rather than during the acute lung injury following monocrotaline injection. ET_A-selective antagonists, including sitaxentan (Tilton et al. 2000), BQ-123 (Miyachi et al. 1993), LU 135252 (Prie et al. 1997), ABT-627 (Nishida et al. 2004), and YM598 (Yuyama et al. 2004), have similarly demonstrated a protective effect in this model although a reversal of medial hypertrophy was not systematically observed (Prie et al. 1997). In contrast, ET_B-selective antagonism with A-192621 aggravated PH, whereas combination with the ET_A-selective antagonist ABT-627 improved PH to the same extent as ET_A antagonism alone (Nishida et al. 2004). This exaggerated response to monocrotaline during ET_B receptor blockade was attributed to ET_A receptor activation, suggesting an exclusive contribution of ET_A receptor-mediated action to the pathogenesis of monocrotaline-induced PH.

This interpretation was challenged by a comparative study in rats with monocrotaline-induced PH, where the dual ET receptor antagonist BSF 420627 doubled survival compared with the untreated animals and increased it by 10 % compared with the animals receiving the ET_A-selective antagonist LU 135252 (Jasmin et al. 2001). Reduction of right ventricular hypertrophy was only seen in the animals receiving the dual ET receptor antagonist, suggesting that blockade of both receptors was necessary to prevent the deleterious effects of ET-1 in this model.

A direct comparison between the dual ET receptor antagonist bosentan and the ET_A-selective antagonist ambrisentan in the monocrotaline-induced PH model (Schroll et al. 2008) indicated that ambrisentan significantly increased prostacyclin synthase I expression compared with bosentan. However, no benefit on pulmonary hemodynamics was observed: both antagonists had similar effects on right ventricular systolic pressure, pulmonary vascular remodeling, and right ventricular hypertrophy.

More recently, macitentan, a novel ET receptor dual antagonist, was also compared with bosentan in the monocrotaline-induced PH rat model (Iglarz et al. 2008). Macitentan prevented right ventricle hypertrophy and the development of PH at a ten times lower dose than bosentan. Prior studies have shown that dual antagonists could significantly reduce mortality in experimental PH (Jasmin et al. 2001; Clozel et al. 2006). Macitentan had a major effect of decreasing mortality in this animal model. In the Dahl-salt rat model of systemic hypertension, macitentan administered on top of the maximally effective dose of bosentan further reduced mean arterial pressure (MAP). In contrast, bosentan administered on top of the maximally effective dose of macitentan failed to elicit any further reduction in MAP. The add-on effect of macitentan on top of bosentan suggests that macitentan is able to achieve a more complete blockade of ET receptors (Iglarz et al. 2012). The potential for increased potency and efficacy of macitentan has been attributed to physicochemical and binding properties promoting tissue penetration (Iglarz et al. 2011). Overall, both selective and dual ERAs have proven to be efficacious in monocrotaline-induced PH in rats and the Dahl-salt rat model of systemic hypertension.

In models of overcirculation-induced PH, the objective is to experimentally mimic PH caused by congenital heart diseases with left-to-right shunts. This situation can be reproduced by anastomosis of the left subclavian artery to the pulmonary arterial trunk in piglets and results in pronounced chronic PH, with medial hypertrophy, and increased pulmonary vascular resistance (Morin 1989; Rondelet et al. 2003). In this model, bosentan and sitaxentan have been shown to prevent and reverse pulmonary vascular remodeling and right heart hypertrophy (Rondelet et al. 2003, 2007).

In models of chronic thromboembolic PH, experimental vascular obstruction or occlusion results in a sustained increase in pulmonary vascular resistance and remodeling of the pulmonary vascular bed. In a canine model where PH was induced by repeated embolization with ceramic beads, treatment with bosentan not only reduced medial thickening of pulmonary arteries, but also reduced

adventitial thickening and prevented intimal fibrosis and peripheral neomuscularization (Kim et al. 2000). The lack of effect on hemodynamics was attributed to the fact that hypertension was due to mechanical obstruction in this model.

The above studies document that dual and ET_A-selective ERAs prevented, attenuated, or even reversed vascular remodeling and/or hypertrophy associated with experimental PH.

3.3 ET Receptor Antagonism and Fibrosis

The evaluation of ERAs in PH also includes the benefit derived from their anti-proliferative and anti-fibrotic effects. Smooth muscle cell proliferation, adventitial thickening, altered angiogenesis, and fibrosis are generally observed in PH, especially in PH associated with connective tissue disease or PH due to chronic lung disease, which are characterized by a wide range of vascular inflammatory and fibrotic complications (Crystal et al. 1984).

At the cellular level, proliferation of distal human pulmonary artery smooth muscle cells was found to be modulated by both the ET_A and ET_B receptors and to be inhibited by the dual ET_A/ ET_B receptor antagonist PD145065 (Davie et al. 2002). In contrast, human lung fibroblast growth was stopped by the ET_A-selective receptor antagonist BQ-123 but not by the specific ET_B receptor antagonist BQ-788 (Gallelli et al. 2005). In human skin fibroblast cultures exposed to ET-1, interstitial collagenase downregulation and collagen matrix contraction were inhibited by bosentan and by the ET_A-selective receptor antagonist PD156707, but dual antagonism was necessary to prevent the production of collagen type I and III (Shi-Wen et al. 2001). Both antagonists inhibited ET-1-induced collagen matrix contraction in fibroblasts from patients with scleroderma showing an enhanced contractile phenotype (Shi-Wen et al. 2004). In human pulmonary endothelial cells exposed to cigarette smoke extract, ET_B receptor blockade, either with bosentan or with BQ-788, attenuated cell dysfunction (Milara et al. 2010). In these sensitized endothelial cells, the ET_B receptor is overexpressed and may mediate vasoconstriction via reduced NO synthesis and increased thromboxane A₂ productions, and potentiate endothelial cell dysfunction induced by ET-1.

The above *in vitro* results, suggesting a role for ET antagonism in countering the pro-fibrotic and pro-inflammatory effects of ET-1, are supported by several experimental models. Using the bleomycin-induced pulmonary fibrosis model in rats, Park et al. (1997) reported that bosentan reduced lung fibrosis, as measured by tissue morphometry. However, this finding was not confirmed by Mutsaers et al. (1998), who observed that neither dual ET receptor antagonism with bosentan nor single ET_A blockade with BQ-485 prevented the accumulation of collagen, despite the inhibition of ET-1 expression in lung tissue. A more recent study, however, confirmed the beneficial effects of bosentan in this model both on the development of PH and of pulmonary fibrosis. Bosentan-treated rats had improved exercise capacity, less right ventricular hypertrophy, and lower hydroxyproline

content in lung tissue (Schroll et al. 2010). The novel dual antagonist macitentan improved right ventricular hypertrophy and hydroxyproline content at a dose three times lower than bosentan (Iglarz et al. 2011).

In a model of cigarette smoke-induced chronic obstructive disease associated with PH in rats, BQ-123 and bosentan both suppressed the development of emphysema, and the benefit of specifically blocking the ET_B receptor suggested by *in vitro* studies (Milara et al. 2010) was not confirmed. Both ERAs similarly inhibited pulmonary apoptosis, reduced matrix metalloproteinases (MMP-2 and MMP-9) activities, decreased inflammatory cytokine levels, and increased serum antioxidant activity in the cigarette smoke extract-treated rats.

4 ET Receptor Antagonism for the Treatment of PAH: Several Molecules with Different Characteristics

Two ERAs are currently approved for the treatment of PAH: bosentan (Tracleer[®], Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) and ambrisentan (Letairis[®], Gilead Sciences, Foster City, California, USA and Volibris[®], GlaxoSmithKline, London, UK). Sitaxentan (Thelin[®], Pfizer Ltd, Kent, UK) was withdrawn from the market in December 2010 for safety concerns (Galie et al. 2011). Macitentan (Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) is a novel ERA that has completed the Phase III clinical trial in PAH [SERAPHIN: Study with an Endothelin Receptor Antagonist in Pulmonary arterial Hypertension to Improve cliNical outcome (ClinicalTrials.gov)].

4.1 Pharmacological Profile

These ERAs differ in several aspects, including chemical structure, receptor affinity, and pharmacokinetics (Table 1). Ambrisentan is ET_A selective, whereas bosentan and macitentan are dual ERAs, with a slightly higher affinity for the ET_A receptor. The specificity of macitentan combines several unique features: it has demonstrated an effect on survival in animal models of PAH; it is a slow-offset competitive antagonist with high receptor occupancy; it is optimized for tissue penetration, including an optimal shape, a high distribution coefficient for lipid versus aqueous solutions, and a minimal ionization state. *In vitro* experiments investigating the receptor affinity and receptor binding kinetics of macitentan and other ERAs, demonstrated that macitentan has a receptor occupancy half-life 15-times greater than bosentan and ambrisentan (Gatfield et al. 2012) (Fig. 2). All of these features are expected to result in improved and enhanced targeting of the autocrine–paracrine ET-1 system (Fig. 3), and especially in pathological situations associated with upregulation of ET receptors, such as PAH (Iglarz et al. 2008;

Table 1 Profiles of the endothelin receptor antagonists

	Bosentan	Ambrisentan	Macitentan
Status	Approved in 2001 in USA, 2002 in Europe	Approved in 2007 in USA, 2008 in Europe	Phase III completed
Chemical structure	Pyrimidinyl sulfonamide	Diphenylpropanoic acid	Propylsulfamide
Receptor selectivity	Dual	ETA-selective	Dual
Tissue selectivity	No	No	Yes
Log D	20:1	1:2.5	800:1
pKa	5.1	3.5	6.2
Peak plasma concentration (hours)	3–5	2	8
Bioavailability (%)	50	90	30–90 ^a
Elimination half life	5.4	9	16
Protein binding (%)	>98	99	99
<i>Metabolism</i>			
Route	Hepatic + biliary excretion	Hepatic (glucuronidation) + biliary excretion	Hepatic + biliary excretion
Enzyme involved	CYP 3A4, 2C9	CYP 3A4, 2C19, UGTs (1A9S, 2B7S, 1A3S)	CYP 3A4
Excretion in urine (%)	<3	Low	49.7 %
<i>Effect on CYP and hepatobiliary transporters</i>			
CYP	↑CYP 3A4, ↑2C9, (↑2C19)	↑CYP 3A4	↑CYP 3A4
NTCP/OATP inhibition	Yes/no	No/no	No/no
BSEP inhibition	Yes	No	No
<i>Adverse effects</i>			
Liver enzyme	Yes (11 %)	No (0 %)	Yes (3 %)
Peripheral edema	Yes (13.2 %)	Yes (17 %)	Yes (18 %)
Anemia/hemoglobin decrease	Yes (9.9 %)	Yes (7 %)	Yes (13 %)
Teratogenicity	Yes	Yes	Yes
<i>Significant drug–drug interactions</i>			
Cyclosporine A	Yes	Yes	No
Warfarin	Yes	No	No
Sildenafil	Yes	No	No
Ketoconazole	Yes	No	No
Fluconazole	Yes		
Rifampicin	Yes	No	
Glibenclamide	Yes		No
References	Actelion Pharmaceuticals Ltd. (2011)	Gilead Sciences, Inc. (2011), Raja (2010a)	Iglarz et al. (2008), Raja (2010a), Sidharta et al. (2011), Sidharta et al. (2012a), Sidharta et al. (2012b),

(continued)

Table 1 (continued)

Bosentan	Ambrisentan	Macitentan
		Bruderer et al. (2012a), Bruderer et al. (2012b), Sidharta et al. (2010), Raja (2010b)

↑ drug induces

CYP: cytochrome P450, *Log D*: distribution coefficient, *NTCP*: sodium-dependent taurocholate cotransporter, *OATP*: organic anion transporting polypeptide, *pKa*: ionization dissociation constant, *UGTs*: uridine glucuronosyltransferases

^aIn dogs and rats (dose-dependent)

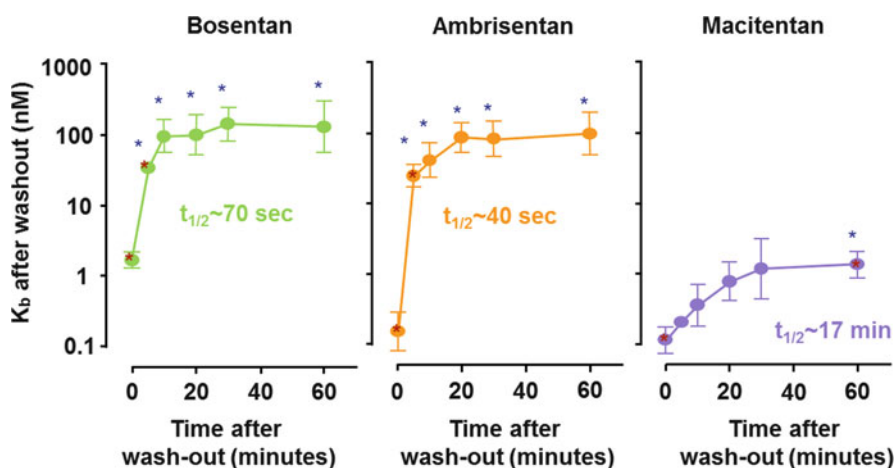


Fig. 2 Receptor binding half-lives of bosentan, ambrisentan and macitentan

Iglarz et al. 2011). Macitentan has a long elimination half-life allowing for a once-a-day dosing regimen, as ambrisentan, whereas bosentan is dosed twice daily. All three ERAs are metabolized by cytochrome P450 (CYP) enzymes in the liver, but the major hepatic metabolism pathway for ambrisentan is glucuronidation by hepatic uridine glucuronosyltransferases (UGTs). Subsequent excretion is primarily in the bile for all three agents.

4.2 Safety Profile

The main difference between ERAs appears to be in safety profiles, with higher rates of peripheral edema observed with the ET_A -selective ambrisentan and a greater frequency of serum liver function abnormalities occurring with bosentan.

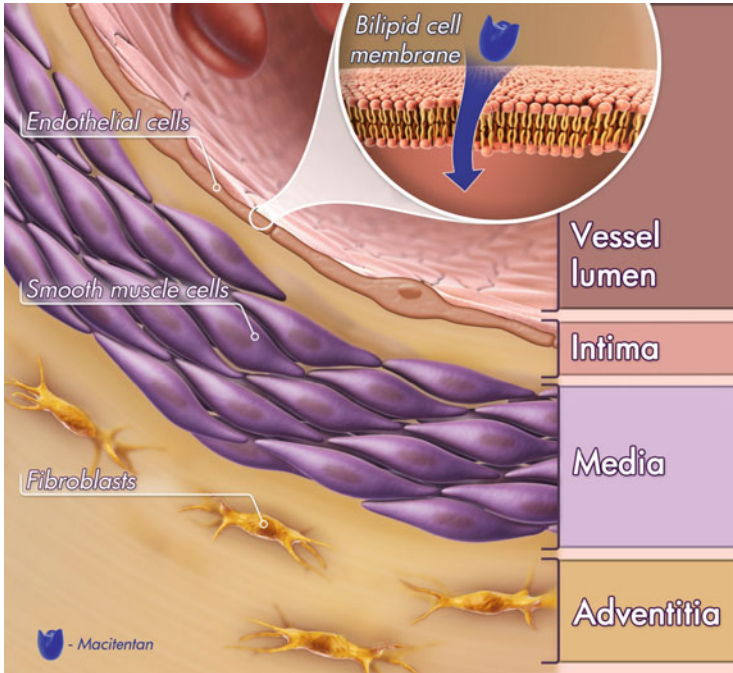


Fig. 3 Tissue-targeting potential of macitentan

Peripheral edema is a clinical consequence of worsening PH, but it is also a known class effect of ERAs (Battistini et al. 2006; Kohan et al. 2011). Even though ET_B receptors have been suggested to play a role in sodium excretion, ET_A -selective antagonists pose a greater risk of fluid overload than dual ET_A/ET_B antagonists (Barton and Kohan 2011; Trow and Taichman 2009). The mechanism of this fluid retention has been studied in rodents and the results show that ET_A -selective receptor antagonists cause water retention by stimulating, via the activation of un-antagonized ET_B receptors, the release of vasopressin and the expression of V2 receptors.

Liver abnormalities have also been associated with ERAs but may vary widely among individual agents (Battistini et al. 2006). In pivotal clinical trials, the incidence of serum aminotransferase elevations was lower with ambrisentan [0 % (Gilead Sciences, Inc. 2011)] than that with bosentan [11 % (Actelion Pharmaceuticals Ltd. 2011)]. In the long-term extension trial with ambrisentan, the incidence of serum aminotransferase elevations observed with ambrisentan was of 3.9 % (Oudiz et al. 2009). In the SERAPHIN trial, elevations of liver aminotransferases were observed in 4 % of patients on 3 mg macitentan and in 3 % of patients on 10 mg macitentan (Pulido et al. 2013).

The mechanism of hepatotoxicity of ERAs has been evaluated in sandwich-cultured human hepatocytes (Hartman et al. 2010) and in vivo (Fattinger et al. 2001; Mano et al. 2007). It has been shown that the mechanism of liver enzyme elevations

with bosentan was due—at least in part—to an inhibition of the biliary efflux of taurocholate mediated by the bile salt export pump (BSEP). The observation that in humans an elevation in bile salts preceded an elevation of aminotransferases and a potentiation by glibenclamide, also an inhibitor of BSEP, suggests that this hypothesis also stands in man. Macitentan has been designed to avoid an interaction with bile salt elimination.

Reductions in hemoglobin concentration and hematocrit have been reported with the use of ERAs (Battistini et al. 2006). The cause of this reduction is not a direct anemia but is secondary to hemodilution. As a class, ERAs have shown teratogenic effects in animals, and pregnancy should be avoided.

4.3 Drug Interaction Profile

Trough concentrations of bosentan are increased 30-fold by cyclosporine and bosentan should therefore not be co-administered with cyclosporine A. The mechanism of the interaction has been suggested to be an inhibition of OATP-mediated uptake of bosentan into hepatocytes (Treiber et al. 2007). Bosentan should also not be used concomitantly with glibenclamide, due to an increased risk of elevated liver aminotransferases. Bosentan is metabolized by CYP2C9 and CYP3A4 and inhibition of these isoenzymes may increase the plasma concentration of bosentan. Therefore administration with fluconazole, which inhibits CYP2C9 and also CYP3A4, and administration with both a potent CYP3A4 inhibitor (such as ketoconazole, itraconazole, or ritonavir) and a CYP2C9 inhibitor (such as voriconazole) are not recommended. Bosentan is also a mild inducer of CYP2C9 and CYP3A4, and possibly CYP2C19. Consequently, plasma concentrations of substances metabolized by these isoenzymes such as sildenafil, simvastatin, hormonal contraceptives (ethinyl estradiol and norethindrone), and to a lower extent warfarin, are decreased by bosentan coadministration. Conversely, bosentan concentration rises by up to 50 % with concomitant sildenafil therapy due to inhibition of OATP-mediated transport (Treiber et al. 2007). Inducers of CYP2C9 and CYP3A4 are expected to decrease the concentration of bosentan. As such, rifampicin reduces bosentan steady-state concentrations, although an initial concentration increase has been attributed to inhibition OATP-mediated uptake of bosentan into hepatocytes (Treiber et al. 2007).

Ambrisentan is sensitive to drug–drug interaction with cyclosporine, as its concentrations are increased two-fold by cyclosporine A. If coadministration is necessary, a reduction of the dose of ambrisentan should be considered.

Macitentan has a very low propensity for drug–drug interactions, as shown by clinical pharmacology studies, which showed no significant interactions between macitentan and sildenafil, warfarin, cyclosporine, and only a two-fold increase with ketoconazole (Raja 2010b). Macitentan and its metabolites do not interfere with the BSEP-mediated elimination of bile salts (Raja 2010b, Bolli et al. 2012), which may mean that there is no drug–drug interaction with glibenclamide. Therefore,

concomitant use of macitentan and glibenclamide was permitted in the completed SERAPHIN trial.

Thus, both ET_A-selective and dual ERAs have demonstrated improvements in hemodynamics, right ventricular hypertrophy, pulmonary arterial remodeling, pulmonary vessel endothelial function, and survival in experimental PH. The registration of bosentan for the treatment of PAH has constituted a first step forward in the treatment of patients with PAH and has been followed by the registration of ambrisentan. Understanding how to improve efficacy and the mechanisms of edema and liver enzyme changes have led to the development of macitentan, a dual ERA with maximal tissue-targeting properties, enhanced affinity for the ET receptors and long-lasting receptor occupancy, and minimal risk of fluid retention and interaction with bile salts. Only clinical trials can appropriately describe the efficacy and safety of each of these molecules. Together with other classes of drugs, phosphodiesterase type 5 inhibitors, and prostacyclin receptor agonists, the treatment paradigm will continue to evolve. The goal of 'standard of care treatment' will shift from improving quality of life and symptoms, to slowing the progression of this very severe disease.

5 ET Receptor Antagonism in PAH: Human Studies

5.1 Bosentan

Bosentan was the first oral therapy approved for PAH. Studies have demonstrated improvements in exercise capacity, functional class, and hemodynamic parameters and an increase in time to clinical worsening in PAH patients. The first randomized, double-blind, placebo-controlled, multicenter trial (study 351) with bosentan was designed to assess its effects on exercise capacity and cardiopulmonary hemodynamics, as well as safety and tolerability (Channick et al. 2001). Eligible patients had severe idiopathic PAH or PAH associated with scleroderma (in functional classes III or IV, according to the New York Heart Association (NYHA) classification), despite prior treatment including vasodilators, anticoagulants, diuretics, cardiac glycosides, or supplemental oxygen as clinically indicated. Thirty-two patients were randomized to receive bosentan or placebo (2:1 ratio). Patients randomized to bosentan received 62.5 mg bid for the first 4 weeks, followed by the target dose (125 mg bid) unless drug-related adverse events were observed. After 12 weeks, the 6-min walk distance (6MWD) increased by 70 m (from 360 ± 19 m [m \pm SE] at baseline to 430 ± 14 m at week 12; $P < 0.05$) in the bosentan arm, whereas no improvement was seen with placebo (355 ± 25 m at baseline and 349 ± 44 m at week 12). The median change from baseline was +51 m with bosentan versus -6 m with placebo. The difference between treatment arms in the mean change in 6MWD was 76 ± 31 m (mean \pm SEM) in favor of bosentan (95 % CI, 12–139; $P = 0.021$). Bosentan improved cardiac index, with the difference between

treatment groups in the mean change at week 12 being 1.0 ± 0.2 L/min/m² (mean \pm SEM) in favor of bosentan (95 % CI, 0.6–1.4 L/min/m², $P < 0.001$). Bosentan decreased pulmonary vascular resistance, whereas it increased with placebo ($P < 0.001$). Asymptomatic increases in hepatic aminotransferases were observed in two bosentan-treated patients, but these normalized without discontinuation or change of dose.

A larger double-blind, placebo-controlled study (BREATHE-1) evaluated bosentan in 213 patients with PAH (idiopathic or associated with connective tissue diseases) who were randomized to placebo, bosentan 125 or 250 mg bid for a minimum of 16 weeks (62.5 mg bid for 4 weeks and then target dose) (Rubin et al. 2002). The primary endpoint was the change in exercise capacity assessed by 6MWD, and secondary endpoints included changes in Borg dyspnea index, NYHA functional class, and time to clinical worsening. Patients had symptomatic, severe PAH (NYHA functional class III or IV) despite treatment with anticoagulants, vasodilators, diuretics, cardiac glycosides, or supplemental oxygen as clinically indicated. Glibenclamide was contraindicated since it was increasing the risk of liver enzyme elevations. Patients were randomized to receive either bosentan 62.5 mg bid for 4 weeks followed by the target dose (125 or 250 mg bid) or matching doses of placebo (144 patients received bosentan and 69 received placebo). After 16 weeks, bosentan improved the 6MWD by 36 m whereas a deterioration (-8 m) was seen with placebo, and the difference between treatment groups in the mean change in 6MWD was 44 m in favor of bosentan ($P = 0.0002$). A dose response for efficacy could not be ascertained. The risk of clinical worsening was reduced by bosentan compared with placebo ($P = 0.0015$). The most frequent adverse event in both the bosentan and placebo treatment groups was headache. Abnormal hepatic function [as indicated by elevated levels of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST)], syncope, and flushing occurred more frequently in the bosentan groups. Increases in hepatic enzymes over three times the upper limit of normal occurred in 12 % of the bosentan 125 mg group and in 14 % of the bosentan 250 mg group; two patients (3 %) in the 125 mg bid group and five (7 %) in the 250 mg bid group had elevations $> 8 \times$ ULN. Hepatic function abnormalities were transient except for three patients (all in the 250 mg dosage bosentan group); these patients were withdrawn prematurely from the study. All hepatic transaminases increases have been reported to be reversible with continued treatment, decrease in dose, or discontinuation. Three patients died during the course of the study: two on placebo and one on bosentan.

With respect to longer term data on bosentan therapy, McLaughlin et al. (2005) reported that first-line therapy with bosentan monotherapy, with the addition of or transition to other therapy as needed, resulted in Kaplan–Meier survival estimates of 96 % at 12 months and 89 % at 24 months. At 12 and 24 months, 85 % and 70 % of patients, respectively, remained alive and on bosentan monotherapy. Sitbon et al. (2005) compared survival in 139 NYHA functional class III idiopathic PAH treated with bosentan with historical data from 346 similar idiopathic PAH patients treated with continuous intravenous epoprostenol. Baseline characteristics suggested that the epoprostenol cohort had more severe disease. Kaplan–Meier

survival estimates after 1 and 2 years were 97 % and 91 %, respectively, in the bosentan-treated cohort and 91 % and 84 %, respectively, in the epoprostenol cohort.

In a retrospective study of 86 children with idiopathic PAH and PAH associated with congenital heart disease or connective tissue diseases (Rosenzweig et al. 2005), bosentan was used with or without concomitant intravenous epoprostenol or subcutaneous treprostinil. At data cutoff, 68 patients (79 %) were still treated with bosentan, 13 (15 %) were discontinued, and 5 (6 %) had died. Median exposure to bosentan was 14 months. In 90 % of the patients ($n = 78$), NYHA functional class improved (46 %) or was unchanged (44 %) with bosentan treatment. Kaplan–Meier survival estimates at 1 and 2 years were 98 % and 91 %, respectively. Galliè et al. (2006) evaluated bosentan therapy in a multicenter, double-blind, randomized, placebo-controlled study in patients with functional class III Eisenmenger syndrome (BREATHE-5). Fifty-four patients were randomized 2:1 to bosentan vs. placebo for 16 weeks. Resting systemic arterial oxygen saturation did not worsen with bosentan therapy, and, compared with placebo, bosentan decreased pulmonary vascular resistance ($P < 0.05$) and mean pulmonary arterial pressure ($P < 0.05$), and increased exercise capacity ($P < 0.01$). Four patients discontinued due to adverse events: 2 (5 %) in the bosentan arm and 2 (12 %) in the placebo arm. An open-label multicenter study with bosentan therapy in 16 patients with HIV-associated PAH similarly demonstrated favorable clinical, functional, and hemodynamic effects (Sitbon et al. 2004).

While bosentan is used relatively widely in patients with PAH, close follow-up over time of both efficacy and safety is encouraged. After approval of bosentan for treatment of PAH in the European Union, European authorities required the introduction of a post-marketing surveillance system to obtain further data on its safety profile (Humbert et al. 2007). For that purpose, a prospective, Internet-based post-marketing surveillance system was developed which solicited reports on elevated aminotransferases, medical reasons for bosentan discontinuation, and other serious adverse events requiring hospitalization. Captured data included demographics, PAH etiology, baseline functional status, and concomitant PAH-specific medications. Safety signals captured included death, hospitalization, serious adverse events, unexpected adverse events, and elevated aminotransferases. Within 30 months, 4,994 patients were included, representing 79 % of patients receiving bosentan in Europe. 4,623 patients were naïve to treatment; of these 352 had elevated aminotransferases, corresponding to a crude incidence of 8 % and an annualized rate of 10 %. Bosentan was discontinued due to elevated aminotransferases in 150 bosentan-naïve patients (3 %) with increased hepatic transaminases reported to normalize in all patients after stopping bosentan. Safety results were consistent across subgroups and etiologies. Therefore, liver function tests should be checked monthly. In addition to hepatotoxicity, other side effects include decrease in hemoglobin (hematocrit may be checked every 3 months) and peripheral edema.

Women treated with bosentan should use effective contraception because pregnancy and labor increase the demand on the heart-pulmonary system and is contraindicated in patients with PH; in addition bosentan is also potentially teratogenic. Of note, bosentan may reduce the effects of hormonal contraception and it is recommended that women who are taking bosentan use a second method of contraception. In males, there is concern that the ERAs, as a class may be capable of causing testicular tubular atrophy and male infertility. Younger males who may consider conceiving in the future should be informed regarding this possibility prior to taking these drugs.

5.2 *Sitaxentan*

Sitaxentan, a selective ETA receptor antagonist, when given at a dose of 100 mg/day orally, was approved in the European Union and other countries, but not in the USA, for the treatment of PAH in adult patients. Indeed, multicenter, randomized placebo-controlled clinical trials demonstrated that sitaxentan has beneficial effects on exercise capacity (i.e., 6MWD), NYHA functional class, and hemodynamic parameters in PAH patients (Barst et al. 2004, 2006). However, sitaxentan had to be removed from the world market because of the occurrence of acute severe idiosyncratic hepatotoxicity (Barst et al. 2006).

5.3 *Ambrisentan*

Ambrisentan is another oral, once-daily, ETA-selective ERA approved for the treatment of PAH. In Phase III clinical trials in patients with PAH, ambrisentan (2.5–10 mg orally once daily) improved exercise capacity, Borg dyspnea index, time to clinical worsening, NYHA functional class, and quality of life compared with placebo (Galiè et al. 2008). Ambrisentan in Pulmonary Arterial Hypertension, Randomized, Double-Blind, Placebo-Controlled, Multicenter, Efficacy Study 1 and 2 (ARIES-1 and ARIES-2) were concurrent, double-blind, placebo-controlled studies that randomized 202 and 192 patients with PAH, respectively, to placebo or ambrisentan (ARIES-1, 5 or 10 mg; ARIES-2, 2.5 or 5 mg) orally once daily for 12 weeks (Galiè et al. 2008). The primary endpoint for each study was change in 6MWD from baseline to week 12. Clinical worsening, NYHA functional class, Short Form-36 Health Survey score, Borg dyspnea score, and B-type natriuretic peptide plasma concentrations were also assessed. In addition, a long-term extension study was performed. The 6MWD increased in all ambrisentan groups; mean placebo-corrected treatment effects were 31 m ($P = 0.008$) and 51 m ($P < 0.001$) in ARIES-1 for 5 and 10 mg ambrisentan, respectively, and 32 m ($P = 0.022$) and 59 m ($P < 0.001$) in ARIES-2 for 2.5 and 5 mg ambrisentan, respectively. Improvements in time to clinical worsening (ARIES-2), NYHA functional class

(ARIES-1), Short Form-36 score (ARIES-2), Borg dyspnea score (both studies), and B-type natriuretic peptide (both studies) were observed. Elevation of liver aminotransferases have been reported with ambrisentan use. In most cases alternative causes of liver injury could be identified. Cases consistent with autoimmune hepatitis, including possible exacerbation of underlying autoimmune hepatitis, hepatic injury, and hepatic enzyme elevations, which could be potentially related to therapy, have been mentioned in the Summary of Product Information labeling text of Volibris/Letairis). In 280 patients completing 48 weeks of treatment with ambrisentan monotherapy, the improvement from baseline in 6MWD at 48 weeks was 39 m.

In light of marked aggravation in patients with pulmonary hypertension associated with idiopathic pulmonary fibrosis and pulmonary fibrosis without pulmonary hypertension, ambrisentan is now contraindicated in EU for these patients.

5.4 Macitentan

The dual ERA macitentan has been tested in a large pivotal, long-term, event-driven randomized, placebo-controlled study (SERAPHIN, Study with an Endothelin Receptor Antagonist in Pulmonary arterial Hypertension to Improve cliNical outcome) (Pulido et al. 2013). This study was designed to evaluate the efficacy and safety of macitentan through the primary endpoint of time to first morbidity and all-cause mortality event in 742 patients with symptomatic PAH with a mean duration of treatment of 85, 100 and 104 weeks for placebo, macitentan 3 mg and macitentan 10 mg respectively. Macitentan, at both the 3 and 10 mg dose, has met its primary endpoint, decreasing the risk of a morbidity and mortality event over the treatment period versus placebo (Pulido et al. 2013). This risk was reduced by 45 % in the 10 mg dose group ($P < 0.001$). At 3 mg, the observed risk reduction was 30 % ($P = 0.01$). The statistically significant effect of macitentan 10 mg on this endpoint was observed irrespective of background PAH therapy. Treatment with macitentan in the SERAPHIN study was safe and well tolerated. Secondary efficacy endpoints, including change from baseline to month 6 in 6MWD and change from baseline to month 6 in NYHA functional class (FC) and time—over the whole treatment period—to either death due to PAH or hospitalization due to PAH, also showed a dose-dependent effect ($P < 0.05$ for either dose). Macitentan had a significant effect on the baseline-adjusted mean change in 6MWD ($P = 0.04$ for macitentan 3 mg and $P = 0.007$ for macitentan 10 mg). The effect of macitentan on change from baseline to month 6 in 6MWD was also examined by baseline FC. For patients in FC I/II, the baseline-adjusted mean change in 6-MWD was 6.3 m (97.5 % CL, -19.0 to 31.6 m) for macitentan 3 mg and 12.3 m (97.5 % CL, -8.1 to 32.7 m) for macitentan 10 mg. For patients in FC III/IV the baseline-adjusted mean change in 6-MWD was 32.8 m (97.5 % CL, 2.4 to 63.1 m) for macitentan 3 mg and 37.0 m (97.5 % CL, 5.4 to 68.6 m) for macitentan 10 mg.

The safety set comprised 741 patients (randomized 1:1:1), who received at least one dose of study treatment. Macitentan in this patient population was safe and well tolerated. The number of adverse events reported and patients discontinuing treatment due to adverse events was similar across all groups. Elevations of liver aminotransferases greater than three times the upper limit of normal were observed in 4 % of patients receiving placebo, in 4 % of patients on 3 mg of macitentan, and in 3 % of patients on 10 mg of macitentan. In addition, no difference was observed between macitentan and placebo on fluid retention (edema). A decrease in hemoglobin—reported as an adverse event—was observed more frequently on macitentan than placebo, with no difference in treatment discontinuation between groups.

In conclusion, several ERAs have been successfully evaluated in randomized controlled clinical trials in patients with symptomatic PAH. Compounds have been shown to be different from each other and the differentiation is not only related to whether the compound belongs to the class of selective ET_A receptor antagonists or dual ET_A/ET_B receptor antagonists. Progress in diagnostic and treatment in PAH has been markedly influenced by the ERAs; however, many questions remain. Whether various PAH subgroups such as PAH associated with connective tissue diseases or associated with congenital heart diseases may respond more or less favorably to one or another molecule is currently unknown. Also, whether an ERA should be first-line monotherapy, i.e., as opposed to a phosphodiesterase type 5 inhibitor or a prostacyclin analogue, is also unclear. Whether there is an additive or synergistic effect of an ERA with a prostanoid (e.g., epoprostenol, iloprost, or treprostinil) and/or a phosphodiesterase type 5 inhibitor (e.g., sildenafil or tadalafil) is yet to be determined. As observed in many chronic diseases, combination therapy (an area of active investigation) may further improve the overall efficacy for treating patients with PAH.

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Phosphodiesterase-5 Inhibitors

Barbara A. Cockrill and Aaron B. Waxman

Abstract Nitric oxide (NO) signaling plays a key role in modulating vascular tone and remodeling in the pulmonary circulation. The guanylate cyclase/cyclic guanylate monophosphate-signaling pathway primarily mediates nitric oxide signaling. This pathway is critical in normal regulation of the pulmonary vasculature, and is an important target for therapy in patients with pulmonary hypertension. In the pulmonary vasculature, degradation of cGMP is primarily regulated by PDE-5, and inhibition of this enzyme has important effects on pulmonary vasculature smooth muscle tone. Large randomized placebo-controlled trials of PDE-5 inhibitors demonstrated improved exercise capacity, hemodynamics and quality of life in adult patients with PAH. This chapter will discuss the mechanisms of NO signaling in the vasculature, characteristics of the PDE5-inhibitors approved for treatment of PH, and review available data on the use of phosphodiesterase inhibitors in PH.

Keywords cGMP • Phosphodiesterase 5 • Sildenafil • Tadalafil • Guanyl cyclase

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List of Abbreviations

6MWD	6-min walk distance
ANP	Atrial natriuretic peptide
BNP	Brain-type natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cGK	cGMP-dependent protein kinases
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CrCL	Creatinine clearance
EMA	European Medicines Agency
eNOS	Endothelial nitric oxide synthase
FDA	United States Food and Drug Administration
GTP	Guanosine triphosphate
HFpEF	Heart failure preserved ejection fraction
HFrEF	Heart failure reduced ejection fraction
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressure
PDE	Phosphodiesterase
PH	Pulmonary hypertension
PKG	Protein kinase-G
QOL	Quality of life
RHC	Right heart catheterization
RV	Right ventricle
sGC	Soluble guanylate cyclase

1 Introduction

Abnormalities in nitric oxide (NO) pathway signaling pathways are present in patients with pulmonary hypertension (PH) (Giaid and Saleh 1995; Ghofrani et al. 2004a) leading to defective vasorelaxation and abnormal vascular remodeling. The recognition that inhaled NO caused pulmonary vasodilation via increased cyclic guanosine monophosphate (cGMP) led to investigation of phosphodiesterase inhibitors, which block the breakdown of cGMP, as treatment for PH. This chapter discusses the mechanisms of NO signaling in the vasculature and compound characteristics of sildenafil, vardenafil, and tadalafil and reviews available data on the use of phosphodiesterase inhibitors in PH.

2 Signaling Pathways

2.1 Nitric Oxide

Nitric Oxide (NO) is an important player in mechanisms of cardiovascular homeostasis and is involved in vasorelaxation and platelet aggregation. NO is produced and released from endothelial cells and acts in a paracrine fashion. Defects of NO production and/or bioavailability have been implicated in varied conditions including hypertension, hypercholesterolemia, aging, diabetes, erectile dysfunction, heart failure, and pulmonary vascular disease. Endothelial cells are the major source of plasma NO that is synthesized via endothelial nitric oxide synthase (eNOS). NO synthesis and release from endothelial cells are increased in response to the mechanical shear stress of blood flow. NO crosses the plasma membrane of smooth muscle cells and acts as a signaling molecule and induces changes in target protein function. NO binds to a prosthetic heme on the β -subunit of soluble guanylate cyclase (sGC), activating the enzyme (Derbyshire and Marletta 2009). Activation of sGC drives the conversion of guanosine triphosphate (GTP) to cyclic-guanosine monophosphate (cGMP), resulting in increased concentration of cGMP (Waldman and Murad 1988). cGMP can also be synthesized by membrane-bound guanylyl cyclases that are activated by specific peptides including atrial natriuretic peptide (ANP) (Chinkers 1994), brain-type natriuretic peptide (BNP) (Schulz et al. 1989), and c-type natriuretic peptide (CNP) (Koller et al. 1991). However, NO-induced increase in cGMP is crucial in a number of physiologic processes including relaxation of vascular smooth muscle, inhibition of platelet aggregation, blunting of cardiac hypertrophy, and protection against ischemia reperfusion injury of the heart. NO-induced increase in cGMP and the downstream effects on contraction in smooth muscle appear to be mediated specifically by protein kinase-G (PKG) (Hofmann et al. 2000).

2.2 cGMP-Dependent Protein Kinases (PKG)

PKG represents the principal intracellular mediator of cGMP signals. There are two PKG families, or cGMP-dependent protein kinases (cGK). They are both serine/threonine kinases that are widely distributed in eukaryotes and are encoded by two genes, *prkg1* and *prkg2* that code for PKG1 and PKG2, respectively. Two isozymes, PKG1 α and PKG1 β , are generated from the *prkg1* gene and differ only in the N-terminal 100-amino acids (Hofmann et al. 2009). The PKG1 family is more commonly involved when NO mediates cGMP signaling. cGMP binds to allosteric sites in the PKG1 regulatory domain and increases phosphotransferase activity three- to tenfold. The N-terminal binding site in PKG1 isozymes has higher affinity for cGMP than does the more C-terminal site and is found in particular subcellular membrane fractions, in complex with certain cytosolic proteins, and as free cytosolic proteins (Klein et al. 2007; Schlossmann and Desch 2009).

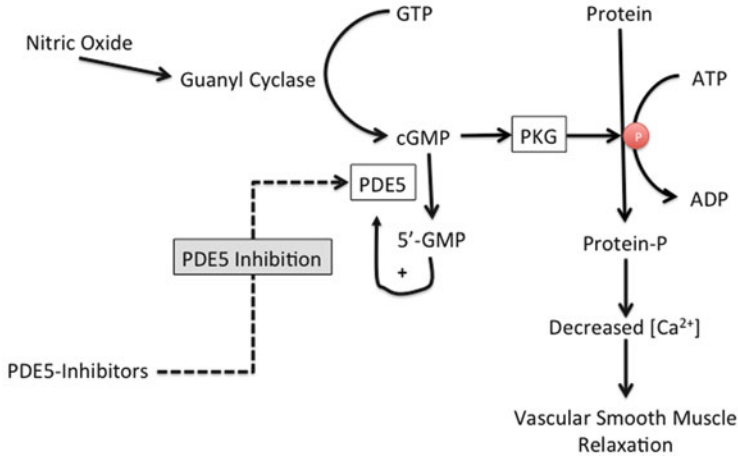


Fig. 1 Schematic diagram of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/cyclic nucleotide phosphodiesterase-5 (PDE-5) pathway and the site of action of PDE-5 inhibitors

Ligand-induced elevation of cGMP triggers activation of PKG1 leading to the catalytic transfer of the γ -phosphate from adenosine triphosphate (ATP) to a serine or threonine residue on the target protein. This phosphorylated protein then mediates the translation of the extracellular stimulus (NO) into a specific biological function. There are specific physiological substrates for PKG1 in smooth muscle (Fig. 1).

Smooth muscle tone is regulated by the rise and fall of the intracellular Ca^{2+} concentration. In most cells, contraction is initiated by receptor-mediated generation of inositol 1,4,5-triphosphate (IP_3). IP_3 induces Ca^{2+} release from intracellular stores followed by an influx of extracellular Ca^{2+} through voltage-dependent and independent Ca^{2+} channels. The contractile state of smooth muscle is largely determined by the phosphorylation state and activity of the myosin light chain. The rise in intracellular calcium concentration initiates contraction by activation of the Ca^{2+} /calmodulin-dependent myosin light chain kinase, which phosphorylates myosin light chain and, subsequently, activates myosin ATPase. Smooth muscle contraction can be modulated when the intracellular Ca^{2+} concentration is held constant by changing the Ca^{2+} sensitivity of the contractile apparatus (Ogut and Brozovich 2003).

Myosin light-chain phosphatase reverses smooth muscle contraction by catalyzing the dephosphorylation of the myosin light chain. NO-induced activation of cGMP-dependent PKG1 increases the open probability of large conductance Ca^{2+} -activated K^+ channels (Fukao et al. 1999) and negatively regulates Ca^{2+} release from IP_3 sensitive stores (Schlossmann et al. 2000), resulting in a marked lowering of intracellular free calcium, desensitization of contractile proteins to Ca^{2+} , and smooth muscle relaxation. NO/cGMP/PKG1 signaling and phosphorylation of multiple intracellular proteins contributes to a reduction of intracellular Ca^{2+} concentration through decreased mobilization from intracellular stores and decreased entry from the extracellular space, or reduction in sensitivity to Ca^{2+} leading to decreased smooth muscle tone (Schlossmann et al. 2003; Vanderheyden et al. 2009).

2.3 *Phosphodiesterases and Phosphodiesterase-5*

The level of intracellular cGMP is regulated by both synthesis and degradation. Decreased synthesis of cGMP occurs as the NO signal declines and NO dissociates from NO-sGC, which rapidly returns sGC to the inactive state. Degradation of cGMP is regulated by cyclic nucleotide phosphodiesterases (PDE), a superfamily of metallophosphohydrolases, which are involved with catabolism of cAMP and GMP signaling (Rybalkin et al. 2003a). PDEs fall into 11 families according to their substrate specificity, mechanism of action, and subcellular location; PDE-5 is specific for cGMP and abundantly present in the lung vascular smooth muscle (Soderling and Beavo 2000). PDE-5, as a major cGMP-hydrolyzing PDE expressed in smooth muscle cells, can effectively control the NO/cGMP/PKG1 signaling pathway, especially under conditions of low calcium. In adult rat lungs, PDE-5 is highly expressed in smooth muscle cells in the medial layer of pulmonary arteries and veins (Sebkhi et al. 2003) and increased in hypoxia-induced pulmonary hypertension (Maclean et al. 1997). PDE-5 plays a key role in vascular smooth muscle tone particularly in the venous system of the corpus cavernosum and the pulmonary vascular bed. PDE-5 has been found in all types of vascular and visceral smooth muscle cells as well as cardiac myocytes.

Phosphodiesterase-5 contains two major functional domains: the catalytic domain (C domain) located in the more C-terminal portion of the protein and a regulatory domain (R domain) located in the more N-terminal portion (Corbin and Francis 1999; Francis and Corbin 1999). Via the catalytic C-domain, intracellular cGMP is rapidly degraded by PDE-5. PDE-5 limits the activity of cGMP on its substrate PKG1 by specifically cleaving the 3', 5'-cyclic phosphate moiety of cGMP to produce 5'-GMP, an inactive molecule that has no second messenger activity.

The R domain contains specific allosteric cGMP-binding sites that regulate enzyme function. Regulatory allosteric cGMP-binding sites are located in a GAF subdomain in the N-terminal portion of the respective proteins (Francis et al. 2006). The allosteric cGMP-binding sites and phosphorylation of a single serine near the N-terminus by PKG activate the PDE-5 catalytic activity and provide negative feedback regulation of cGMP (Francis et al. 2001; Mullershausen et al. 2003; Rybalkin et al. 2003b). When cGMP binds to the allosteric site a conformational change occurs that exposes the serine allowing for phosphorylation and activation of the PDE-5 catalytic activity (Francis et al. 2001). When cGMP occupies the allosteric site, the affinity of the catalytic site for cGMP is enhanced, which further enhances the PDE-5 catalytic site function (Mullershausen et al. 2003).

In addition to the upregulation caused by cGMP feedback, a second feedback mechanism upregulates PDE-5 activity in the NO/cGMP/PKG1 signaling pathway. Once activated, PKG1 phosphorylates and activates the cGMP-specific PDE-5 in smooth muscle cells causing increased activity (Corbin et al. 2000; Wyatt et al. 1998). In humans, phosphorylation of PDE-5 occurs at serine (Ser-102) in

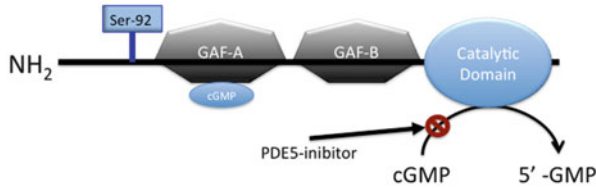


Fig. 2 Domain structure of PDE-5. Binding of cGMP to GAF-A via a high-affinity binding site results in activation of the catalytic domain. Within the catalytic domain, cGMP can interact with PDE-5 to undergo hydrolysis to 5'-GMP. GAF-A binding also enhances phosphorylation of serine-92, inducing conformational changes and increasing the affinity for cGMP

the regulatory domain and increases both the affinity of the enzyme for the allosteric sites of cGMP substrate, and the V_{\max} of the enzyme (Corbin et al. 2000; Rybalkin et al. 2002; Thomas et al. 1992), thus increasing cGMP hydrolysis. The high affinity cGMP binding sites on PDE-5 are on the N-terminal regulatory GAF-A domain of the enzyme (Zoraghi et al. 2005) (Fig. 2). The principal result of PDE-5 activity is to decrease cGMP levels leading to decreased stimulation of PKG1. Binding of cGMP to a GAF domain induces a conformational change in the PDE to activate its catabolic activity for cGMP. GAF binding enhances phosphorylation at Ser-92 of PKG1 (Turko et al. 1998). PKG1 phosphorylation induces conformational changes increasing cGMP-binding affinity in the regulatory GAF domain and enhancing cGMP catalytic activity (Corbin et al. 2000). Allosteric binding of cGMP to PDE-5 could sequester the nucleotide as an intracellular storage site, providing protection from catalysis and possibly releasing the pool when cGMP levels decline as a result of other signaling events (Kotera et al. 2004). Both the binding of cGMP to allosteric sites in PDE-5 and its phosphorylation by PKG1 provide a potent negative feedback on NO/cGMP/PKG1 signaling.

PDEs can be differentially regulated in chronic conditions and may contribute to pathophysiology of specific diseases. PDE-5 upregulation in both expression and activity levels has been observed in disease conditions such as pulmonary hypertension (Corbin et al. 2005), congestive heart failure (Forfia et al. 2007), and right ventricular hypertrophy (Nagendran et al. 2007). The upregulation of PDE-5 underlies reduced responsiveness to NO as well as an enhanced responsiveness to PDE-5 inhibitors; a potential mechanism is the feedback activation of the PDE-5 promoter by increased levels of cGMP (Lin et al. 2001). The physiological importance of PDE-5 in regulation of smooth muscle tone has been most effectively demonstrated by the successful clinical use of its specific inhibitors, sildenafil, vardenafil, and tadalafil in the treatment of erectile dysfunction (Ravipati et al. 2007) and pulmonary hypertension (Galie et al. 2005). By inhibiting PDE-5 hydrolytic activity, sildenafil causes a higher rate of accumulation of cGMP in response to NO, thus enhancing NO's vasodilatory effect.

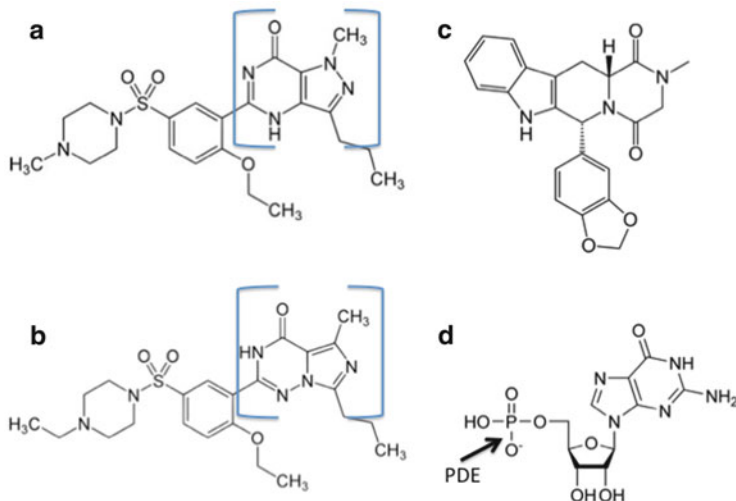


Fig. 3 Comparison of the molecular structures of (a) sildenafil, (b) vardenafil, (c) tadalafil, and (d) cyclic guanosine monophosphate (cGMP). The *arrow* indicates the site of the phosphodiester bond that undergoes PDE hydrolysis. The area between the brackets is the ringed structure that mimics the purine moiety of cGMP

2.4 PDE-5 Inhibitors

A number of studies confirm that basal activity of sGC, driven by NO, generates cGMP concentrations sufficient to induce strong vasorelaxation when PDE-5-dependent cGMP breakdown is reduced in rat aorta (Teixeira et al. 2006), intrapulmonary arteries (Andersen et al. 2005), and guinea pig basilar artery (Kruuse et al. 2001). The selective PDE-5 inhibitors sildenafil, vardenafil, and tadalafil evoked sustained relaxation of endothelium-intact aortic rings through mechanisms involving both NO-dependent and -independent pathways. The relaxation effect evoked by PDE-5 inhibitors was markedly reduced after endothelial cell removal, following inhibition of NO synthesis, inhibition of sGC, or trapping of endothelium-derived NO (Teixeira et al. 2006).

Sildenafil, tadalafil, and vardenafil are competitive and reversible inhibitors of cGMP hydrolysis by the catalytic site of PDE-5. The structures of sildenafil and vardenafil are similar in that both contain a structural mimic of the purine ring of cGMP (Fig. 3) that contributes to their capacities to act as competitive inhibitors of PDE-5. However, there are differences in the molecular structure of sildenafil and vardenafil that are understood to convey greater potency (Teixeira et al. 2006). The distinguishing components of the molecular structures are what provide for interactions with the catalytic site of PDE-5 and dramatically improves the affinity of these compounds for PDE-5 compared with cGMP, and the selectivity of these compounds for PDE-5 compared with other PDEs (Gupta et al. 2005).

2.5 Clinical Pharmacology

In the USA and Europe, there are currently three PDE-5 inhibitors approved for the treatment of PAH, sildenafil, vardenafil, and tadalafil. All three drugs were initially approved for treating erectile dysfunction. There are clear differences between the compounds as to their selectivity and specificity for PDE inhibition. Sildenafil and vardenafil are very similar in chemical structure, while tadalafil is quite different based on a methylidione structure (Fig. 3). These differences are reflected in the different pharmacokinetics.

Sildenafil citrate was the first selective PDE-5 inhibitor approved. It is a potent inhibitor of PDE-5 (IC_{50} of 3.9 nM), with a high selectivity (>1,000-fold) for human PDE-5, moderate selectivity (>80-fold) over PDE-1, and approximately tenfold selectivity over PDE-6. The overlap with PDE-6, which is found in the photoreceptors of the retina, is thought to account for color vision abnormalities observed with high plasma levels of sildenafil (Center for Drug Evaluation and Research 2000). Vardenafil has a high potency in vitro ($IC_{50} = 0.1\text{--}0.7$ nm) and a high selectivity for PDE-5 compared with the other known phosphodiesterase inhibitors. Tadalafil is a selective and potent inhibitor of PDE-5 with an IC_{50} of 0.94 nm. It exhibits high selectivity for PDE-5 compared to other PDEs (Rosen and Kostis 2003). Tadalafil is structurally different from both sildenafil and vardenafil and has distinct differences in the clinical pharmacology.

Sildenafil is relatively lipophilic with a weakly basic center in the piperazine tertiary amine ($pK_a = 6.5$), resulting in only partial ionization at physiological pH (Walker et al. 1999). Following oral administration, sildenafil is rapidly absorbed and reaches peak plasma concentrations within 1 h (range, 0.5–2 h). The mean absolute oral bioavailability of sildenafil is approximately 38–41 %. However, 92 % of the administered dose is absorbed, and the relatively low oral bioavailability of sildenafil is a result of extensive gut wall and hepatic first-pass metabolism (Walker et al. 1999). Bioavailability of sildenafil is further reduced by 11 % after a high-fat meal (Nichols et al. 2002).

Both sildenafil and its major active metabolite (*N*-desmethyl sildenafil) are highly bound to plasma proteins (≈ 96 %). Sildenafil is extensively metabolized, with no unchanged sildenafil being detected in either urine or feces. Metabolites are predominantly excreted into the feces (73–88 %) and to a lesser extent into the urine (6–15 %) (Muirhead et al. 2002a). Plasma concentrations of sildenafil and its *N*-desmethyl metabolite were reported to decline biexponentially, with a mean terminal half-life of 3–5 h for both of them, independent of the route of administration (Nichols et al. 2002). Vardenafil is a class 2 low-solubility/high-permeability drug that is rapidly absorbed, with plasma concentrations being detected in all subjects within 8–15 min after oral administration. Peak plasma concentrations were observed 0.25–3 h after administration, with a median of 0.7 h for the 20- and 40-mg dose level, and slightly later, with 0.9 h for the 10-mg dose level (Center for Drug Evaluation and Research 2003a).

Sildenafil is primarily metabolized by the cytochrome P-450 (CYP) isoenzyme CYP3A4 and to a lesser extent CYP2C9. Since CYP3A4 is the major metabolic pathway for sildenafil, all inhibitors of CYP3A4 have the potential to interfere with sildenafil elimination (Fukao et al. 1999). The principal routes of metabolism are *N*-demethylation, oxidation, and aliphatic hydroxylation (Schlossmann et al. 2003). Grapefruit juice, an inhibitor of intestinal CYP3A4, alters the pharmacokinetics of sildenafil and its metabolite, *N*-desmethyl sildenafil. Grapefruit juice increases sildenafil bioavailability, tends to delay sildenafil absorption (by 0.25 h), and increases sildenafil AUC (1.23-fold) and the AUC of *N*-desmethyl sildenafil (1.24-fold), which, based on the average change, may be judged not clinically significant.

The absolute bioavailability of vardenafil was described as approximately 15 % (range, 8–25 %; 95 % confidence interval, 12–18 %). Based on in vitro investigations in human plasma, approximately 93–95 % of the drug is bound to plasma proteins, approximately 80 % to albumin, and 11 % to α_1 -acid glycoprotein (Center for Drug Evaluation and Research 2003a).

Vardenafil is extensively metabolized and predominantly excreted as metabolites in the feces (\approx 91–95 % of administered oral dose) and to a small extent in urine (\approx 2–6 % of administered oral dose). Only 1 % of the administered dose is excreted into urine in unchanged form (Corbin et al. 2005). Metabolism is predominantly mediated by CYP3A4 and to a smaller extent by CYP3A5 and CYP2C isoforms. The systemic exposure of vardenafil was shown to be largely increased when coadministered with potent CYP3A4 inhibitors such as erythromycin, ketoconazole, indinavir, and ritonavir. Grapefruit juice as a weak inhibitor of CYP3A gut wall metabolism may give rise to modest increases in vardenafil systemic exposure when coadministered (Center for Drug Evaluation and Research 2003a).

Severe renal impairment can lead to an increase in systemic exposure for all three drugs that merits dose reductions for sildenafil and tadalafil in affected patients. Patients with severe renal impairment (CrCL <30 mL/min) have a reduced oral clearance of sildenafil compared to healthy subjects, resulting in an approximately twofold higher AUC and C_{\max} of the drug and its metabolite. There was no significant effect on sildenafil systemic exposure in patients with mild (CrCL 50–80 mL/min) or moderate (CrCL 30–49 mL/min) renal dysfunction (Muirhead et al. 2002b). In subjects with hepatic dysfunction (Child-Pugh A and B), sildenafil oral clearance was reduced by 46 %, resulting in an 84 % increase in AUC, a 47 % increase in C_{\max} , and a 34 % prolongation of $t_{1/2}$. *N*-desmethyl sildenafil AUC and C_{\max} increased by 87 % and 154 %, respectively. The larger effect of hepatic impairment on *N*-desmethyl sildenafil compared to sildenafil increased the AUC ratio from metabolite to parent drug from 52 to 71 %, suggesting that hepatic dysfunction affects elimination of the metabolite more than the parent drug (Muirhead et al. 2002b).

In the case of vardenafil, patients with moderate (CrCL 30–50 mL/min) and severe renal impairment (CrCL < 30 mL/min) had a slightly higher vardenafil exposure as compared to healthy subjects, with increases of 31 % and 21 %, respectively.

respectively. Plasma concentrations were similar, however, between healthy subjects and patients with mild renal impairment (Center for Drug Evaluation and Research 2003a).

Tadalafil is a class 2 low-solubility/high-permeability drug that is rapidly absorbed after oral administration with a median time to reach peak plasma concentration of 2 h (range, 0.5–6 h) (Center for Drug Evaluation and Research 2003b). While the absolute bioavailability of tadalafil following oral dosing has not been reported, at least 36 % of the dose is absorbed from an oral solution. The absorption and pharmacodynamic properties of tadalafil are not affected by either food or alcohol, and thus the drug can be administered without regard for food or alcohol consumption (Abdel-Aziz et al. 2011).

Tadalafil is excreted primarily as inactive metabolites, largely in the feces (≈ 61 % of the administered oral dose) and to a lesser extent in urine (≈ 36 % of the administered oral dose). The mean elimination half-life for tadalafil was 17.5 h, and the mean apparent oral clearance was 2.5 L/h in healthy subjects. Tadalafil is primarily metabolized by CYP3A4 to a catechol metabolite, which further undergoes extensive methylation and glucuronidation to form methylcatechol and methylcatechol glucuronide metabolites (Center for Drug Evaluation and Research 2003b). Renal impairment increased the systemic exposure to tadalafil but had a greater effect on the levels of its renally cleared methylcatechol metabolite and its conjugated form than the parent compound. In mild (CrCL 51–80 mL/min) to moderate (31–50 mL/min) renal insufficiency, tadalafil systemic exposure was twice as high compared to that of healthy subjects, and in end-stage renal disease (ESRD) patients, it was 3.7- to 4.1-fold higher (Gupta et al. 2005).

3 Clinical Efficacy

Although treatment of pulmonary hypertension (PH) has progressed dramatically in the last two decades, definitive randomized clinical trials are particularly difficult to perform in this population. Because PH is a relatively rare condition and large numbers of patients are not available for clinical trials, most clinical studies are small. The diagnosis of PH requires a right heart catheterization (RHC) (Galie et al. 2009a). However, because this is an invasive and expensive procedure, both clinicians and investigators have attempted to use alternative means to identify patients with PH. Cardiac echocardiography, while a good screening test, commonly misclassifies patients (Taleb et al. 2013). Unfortunately, making the diagnosis of PH by means other than hemodynamic data measured invasively is fraught with error and may misidentify patients and invalidate conclusions of therapeutic investigations. The remainder of this chapter reviews existing data on the use of PDE-5 inhibitors, and when available will note the criteria used to identify the diagnosis of PH.

3.1 Group-1 PAH

In 2000, the first case report describing the use of sildenafil in a single patient with PAH was published (Prasad et al. 2000). The first randomized trial of sildenafil treatment in PAH was a single center placebo-controlled double-blind cross-over study that investigated the use of sildenafil (dose range 25–100 three times per day) vs. placebo for 6 weeks. The diagnosis of PAH was based on echocardiography. Twenty-two patients completed the trial. After 6 weeks, improvements in exercise time (475 ± 168 s prior to treatment vs. 686 ± 224 s after sildenafil, $p < 0.0001$), cardiac index as measured by echocardiography (2.80 ± 0.9 to 3.45 ± 1.1 L/m², $p < 0.0001$) and quality of life (QOL) were noted (25 % improvement in dyspnea, $p = 0.009$; 8 % improvement in fatigue, $p = 0.04$) (Sastry et al. 2004).

The first multicenter double-blind placebo-controlled trial to demonstrate efficacy of a PDE-5 inhibitor to treat PH (SUPER-1) randomized 278 patients to receive placebo or sildenafil (20, 40 or 80 mg) three times daily for 12 weeks (Galie et al. 2005). Patients with PAH, PH related to connective tissue diseases, or repaired congenital systemic to pulmonary shunts as determined by RHC were eligible. The primary outcome measurement was 6-min walk test distance (6MWD). Secondary endpoints included hemodynamic measurements, symptoms, and time to clinical worsening (defined as death, transplantation, hospitalization, or the need for additional PAH therapy). Idiopathic PAH was the diagnosis in 2/3 of patients and 96 % were WHO functional class II and III. Baseline characteristics were well matched between study groups. In this study, sildenafil treatment was associated with improvement in exercise capacity at 4, 8, and 12 weeks in all study groups compared with placebo, with a trend toward greater improvement at 12 weeks in the group receiving 80 mg three times per day. At 12 weeks, the mean placebo-corrected treatment effects among 266 patients were 45 m among those receiving 20 mg of sildenafil (99 % confidence interval, 21–70; $p < 0.001$), 46 m for those receiving 40 mg (99 % confidence interval, 20–72; $p < 0.001$), and 50 m for those receiving 80 mg (99 % confidence interval, 23–77; $p < 0.001$). At 12 weeks compared with placebo, all treatment groups demonstrated an improvement in mean pulmonary artery pressure (PAP) (mean change PAP vs. placebo in mmHg with confidence intervals from baseline to week 12: 20 mg -2.1 (-4.3 to 0.0), $p = 0.04$; 40 mg -2.6 (-4.4 to -0.9), $p = 0.01$; 80 mg -4.7 (-6.7 to -2.8) $p < 0.001$), cardiac index, pulmonary vascular resistance and WHO functional class. No differences were found in clinical worsening or survival.

Patients who completed the first 12 weeks of the SUPER-1 study were eligible to enter a long-term open-label observational extension study of sildenafil treatment (SUPER-2) (Rubin et al. 2011). Primary endpoints were 6MWD and WHO functional class. The dose was titrated to 80 mg three times per day as tolerated. Two hundred fifty-nine of the 277 patients initially randomized in SUPER-1 entered the extension study; the study was extended until the last patient received 3 years of sildenafil treatment. At 3 years, 183 patients remained on sildenafil treatment and 87 % were receiving 80 mg three times per day. For patients who completed the

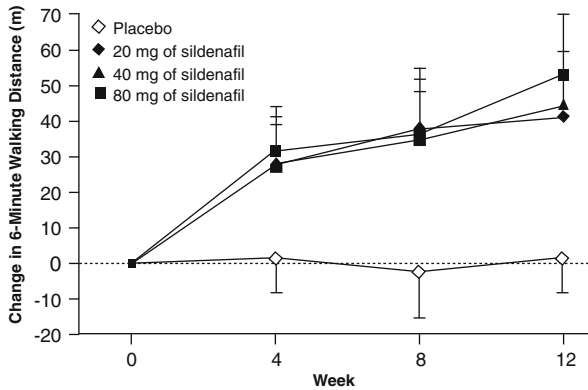


Fig. 4 Comparison of changes in 6-min walk distance with sildenafil treatment compared with placebo. The figure shows mean changes from baseline, with 95 % confidence intervals, in the 6-min walking distance at week 12 in the placebo and sildenafil groups. With the use of a two-sample *t*-test stratified according to baseline walking distance and cause of pulmonary arterial hypertension, $p < 0.001$ for the comparison of sildenafil in doses of 20, 40, and 80 mg with placebo. In this intention-to-treat analysis, 266 patients for whom outcome data were available were included. The dosing schedule for all study medication was three times daily (Galie et al. 2005)

study, compared to the baseline at the time of randomization in SUPER-1, 127 (69 %) increased 6MWD (81 patients with >60 m improvement, 22 patients with >30 – 60 m improvement, 24 patients with 0 – 30 m improvement) (Fig. 4), 49 (22 %) decreased 6MWD (18 patients with ≥ 0 – 30 m worsening, 12 patients with >30 – 60 m worsening, 19 patients with >60 m worsening), and WHO functional class was improved in 81 (44 %) (71 patients improved one class, 10 patients improved two classes, 86 patients had no change, and 15 patients worsened by one class), maintained in 86 (47 %), and declined in 15 (8 %). At 3 years from post-SUPER-1 baseline, 187 patients were alive, 53 had died, and 37 did not have data available. Of patients with known outcomes the survival rate was 79 %: a conservative estimate assuming all censored patients had died was 68 %.

Unfortunately, the SUPER-2 study design was based on dosing strategies from SUPER-1, and 87 % of patients who completed the extension study received sildenafil 80 mg three times per day. While SUPER-2 was ongoing, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved a maximum dose of sildenafil 20 mg three times per day and thus the findings of SUPER-2 may not be applicable.

Tadalafil, a longer acting PDE-5 inhibitor that can be taken once per day, became available shortly after sildenafil. A small case series investigated the transition from sildenafil to tadalafil in 12 patients with PAH (4 iPAH, 4 congenital heart disease, and 2 connective tissue disease) (Tay et al. 2008). Sildenafil had resulted in significant improvement in 6MWD, New York Heart Association score (NYHA), and dyspnea; improvements were maintained after transition to tadalafil. Compared with baseline, the mean increase in 6MWD after sildenafil was 56.45 m

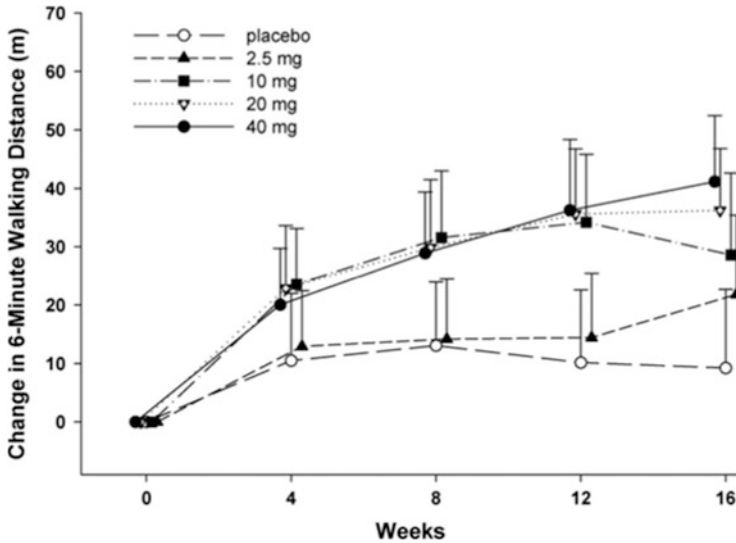


Fig. 5 Tadalafil mean changes from baseline, with 95 % CIs, in the 6-min walking distance at week 16 in the tadalafil and placebo groups. With the use of a permutation test on rank stratified according to baseline walking distance, cause of PAH, and bosentan use, $p = 0.402$, $p = 0.047$, $p = 0.028$, and $p < 0.001$ for the comparison of tadalafil in doses of 2.5, 10, 20, and 40 mg, respectively, with placebo. In this intention-to-treat analysis, 392 patients for whom outcome data were available were included in descriptive statistics. The dosing schedule for all study medications was once daily (Galie et al. 2009b)

and the improvement was unchanged after transition to tadalafil (56.73 m, $p = 0.99$.) Compared with baseline, 4/12 patients improved NYHA score after sildenafil and 5/12 were improved after tadalafil. The mean improvement in Borg Dyspnea Index after sildenafil was 2.25 and after tadalafil was 3.00 ($p = 0.486$).

Tadalafil was studied in the double-blind placebo-controlled, multicenter Pulmonary Arterial Hypertension and Response to Tadalafil (PHIRST-1) study (Galie et al. 2009b). Four hundred five patients with iPAH or associated PH were randomized to receive placebo or tadalafil at doses of 2.5, 10, 20, or 40 mg once daily. Patients who had PH confirmed by RHC and were treatment naïve or on background therapy were eligible: 53 % of subjects were already taking bosentan at the time of study entry. The primary endpoint was 6MWD. Similar to the SUPER-1 trial of sildenafil, most patients had iPAH (61 %) and were WHO functional class II (32 %) or III (65 %). At doses of 10 mg and above, tadalafil treatment was associated with a dose-dependent increase in 6MWD; however only the 40 mg dose met predefined criteria for a significant change ($p < 0.01$) (Fig. 5). The mean placebo-corrected improvements in 6MWD were 20 m for patients receiving tadalafil 10 mg ($n = 78$; 95 % CI 1–39); 27 m for patients receiving 20 mg ($n = 82$; 95 % CI 11–44); and 33 m for patients receiving 40 mg ($n = 70$, 95 % CI, 15–50). Tadalafil 40 mg/day was also associated with an improvement in time to clinical worsening ($p = 0.041$) and QOL. Two measures of QOL were used and

both showed improvement in the tadalafil 40 group; at week 16 compared with baseline, statistically significant improvements were found in 6/8 domains of the Medical Outcomes Study 36-item short form version 2 health survey (all $p < 0.01$) and for all sections of the EuroQol-5D questionnaire (all $p < 0.02$). Unlike the SUPER-1 study, there was no change in WHO functional class.

The PHIRST-1 study also had an extension protocol (PHIRST-2) (Oudiz et al. 2012). Patients who completed PHIRST-1 were eligible to continue treatment with tadalafil at either 20 or 40 mg. Because the results of PHIRST-1 were not known at the beginning of the extension study, the 20 mg dose was included. Subjects were discontinued from the study if they initiated prostacyclin analogs, PDE-5 inhibitors, and/or endothelin receptor antagonists.

As discussed previously, vardenafil appears to be a more potent inhibitor of PDE-5 than either sildenafil or tadalafil (Rosen and Kostis 2003; Corbin and Francis 2002). As with sildenafil and tadalafil, early reports indicated favorable treatment effects in patients with PH (Jing et al. 2009; Ghofrani et al. 2004b). Vardenafil was recently studied in a randomized placebo-controlled trial in China (Jing et al. 2011). Sixty-six patients with Group-1 PAH on no other specific PAH treatment were randomized 2:1 to receive vardenafil or placebo. Subjects received 5 mg once daily for 4 weeks, increasing to 5 mg twice daily for a total of 12 weeks. The primary endpoint was 6MWD; secondary endpoints were cardiopulmonary hemodynamics, the Borg dyspnea index, WHO functional class and time to clinical worsening. After 12 weeks, compared with placebo, there was significant improvement in the treatment group in all of the endpoints including the occurrence of clinical worsening. With vardenafil treatment 6MWD increased by 59 m compared with a decrease of 10 m in the placebo group yielding a mean placebo-corrected treatment effect of 59 m (95 % CI 41–98 m, $p = 0.001$). Hemodynamic variables were also significantly improved with treatment: placebo-corrected mean PAP was 5.3 mmHg lower (95 % CI -10.6 to -0.1 , $p = 0.047$), cardiac index was 0.4 L/min/m² higher (95 % CI 0.1–0.7, $p = 0.005$) and pulmonary vascular resistance (PVR) was 4.7 Wood Units lower (95 % CI -7.8 to -1.7 , $p = 0.003$). More patients in the treatment group improved WHO function class compared with placebo. After 12 weeks of vardenafil treatment, 10 (22.7 %) of 44 patients improved at least one WHO functional class, 32 (72.8 %) remained stable, and two (4.5 %) deteriorated to class III and IV ($p < 0.032$). In the placebo group, only 1 (5 %) of 20 patients improved from class III to class II, 15 (75 %) remained stable, two (10 %) deteriorated from class II to class III, and two deteriorated from class III to class IV (the two patients who died were classified as class IV) ($p \leq 0.0585$). Notably, vardenafil is the only PDE-5 inhibitor to demonstrate a delay in clinical worsening as a monotherapy at approved dosing. During the 12-week study period, five patients had clinical worsening events: four patients in the placebo group (20 %) vs. one in the vardenafil group (2.3 %, $p = 0.044$). Patients who completed 12 weeks of treatment were eligible for an open-label study of vardenafil 5 mg twice daily for 12 more weeks. Improvements in 6MWD were maintained in the treatment group, and subjects initially on placebo showed significant improvement during the extension phase.

Currently, there are no long-term investigations to guide the decision regarding which PDE-5 inhibitor is the best choice for patients. The acute effects of sildenafil, tadalafil, and vardenafil on hemodynamics and oxygenation were studied in 60 patients undergoing RHC (Ghofrani et al. 2004b). Patients received short-term NO inhalation at 20–40 ppm. After the hemodynamic status had returned to baseline, patients received one oral dose of a PDE-5 inhibitor. All three drugs caused significant pulmonary vasodilation. Compared with baseline sildenafil 50 mg caused a decrease of mPAP by -16.2% (95 % CI -11.6 to -21.5 , $p < 0.001$). Tadalafil, given at doses of 20 and 40 mg caused a significant decrease of mPAP of -12.6% (CI -3.6 to -24.4 , $p = 0.01$), -18.3% (CI -13.9 to -21.8 , $p < 0.001$). The decrease in mPAP with tadalafil 60 mg did not reach statistical significance (-10.0% (CI -22.2 to 1.1)). Oral vardenafil at doses of 10 and 20 mg caused a significant decrease of mPAP of -14.3% (CI -5.6 to -23.1 , $p < 0.001$) and -12.1% (CI -7.3 to -15.8 , $p = 0.001$), respectively. A reduction in pulmonary to systemic vascular resistance ratio was seen with both sildenafil 50 mg (15.5 % decrease) and tadalafil only at 40 mg (16.0 % decrease), but not vardenafil at either 10 or 20 mg dose. Only sildenafil was associated with an improvement in arterial oxygenation that was equivalent to that of inhaled NO.

Survival benefit with PDE-5 inhibitor treatment has not been confirmed in a randomized trial. However, given the proven benefit on other clinical parameters, it is unlikely that further placebo-controlled trials will be done. Recently, a small retrospective observational study from Japan indicated that treatment with sildenafil was associated with improved survival (Zeng et al. 2012). Patients were not randomized, and the criteria for the treatment decision cannot be determined from the report. However, survival rates in PAH patients treated with sildenafil after 1, 2, and 3 years were 88 %, 72 %, and 68 % compared with 61 %, 36 %, and 27 % for patients treated with previously conventional therapy.

3.2 Pediatric Pulmonary Arterial Hypertension

Currently, no specific PAH drugs are approved for treatment of PAH in children. However, because of similar clinical characteristics and histopathology, the data for use of treatment in adults has been extrapolated to the pediatric population. Evaluating the efficacy of PAH treatment in children is fraught with difficulty. Most of the standard trial endpoints—6MWD, functional class, and QOL surveys—are not applicable or validated in children. In addition, lung vasculature continues to develop after birth and epigenetic influences may be more prominent. These issues are reinforced by the recent Pulmonary Vascular Research Institute Taskforce recommendation to develop a classification of pulmonary vascular hypertensive disease that is specific to children (Cerro et al. 2011). Because sildenafil is available in an oral liquid form, it is especially suited for treatment of children. However, as discussed below, recent data raise serious questions regarding the safety of PDE-5 use in the pediatric population.

Sildenafil has been studied in a randomized, double-blind, placebo-controlled trial (Sildenafil in Treatment-Naïve Children, Aged 1–17 Years with Pulmonary Arterial Hypertension—STARTS-1.) (Barst et al. 2012). In this multicenter international trial, 235 treatment-naïve children were randomized to receive sildenafil at low-, medium-, or high dose or placebo for 16 weeks. The primary endpoint, peak oxygen consumption as measured by cycle ergometry, was determined only for the 115 patients who could exercise reliably. Secondary endpoints, cardiopulmonary hemodynamics and functional class, were measured in all patients. After 16 weeks, the primary endpoint showed a placebo-corrected increase from baseline of 7.7 % in treated subjects, which was not statistically significant ($p = 0.056$). However, in post hoc analysis, the low-dose treatment was not different from placebo: when only medium- and high-dose groups were included, peak oxygen consumption did show a significant increase from placebo-controlled baseline (9.7 %, $p = 0.023$). Compared with placebo, high-dose sildenafil treatment was associated with improvement in hemodynamic parameters: mPAP decreased by 7.3 mmHg (CI -12.4 to -2.1 , $p = 0.006$) and PVRI ratio decreased to 0.727 (CI 0.612–0.863, $p < 0.001$); medium dose was also associated with a decrease in PVRI ratio (0.819, CI 0.684–0.981, $p = 0.031$), but the decrease in mPAP (-3.9 mmHg) was not statistically significant. Low-dose sildenafil subjects did not show hemodynamic improvement. Major safety concerns surfaced regarding the use of sildenafil in children after an interim analysis of the extension trial, STARTS-2, prompting the FDA to issue a recommendation that sildenafil not be used in children (Administration. USFDA 2012). All patients entered into the STARTS-1 trial were eligible for entry into an extension study of low, medium and high dose. There were 35 deaths during the extension phase: 26 patients died while still on therapy, and 9 after completing treatment. The interim analysis of patients treated for ≥ 3 years revealed an apparent dose-related higher mortality rate in patients started on treatment with high-dose sildenafil compared with medium- or low-dose sildenafil (20 % vs. 14 % vs. 9 %, respectively). Patients who died had more severe disease at baseline: Of patients who died, 74 %, 69 %, and 71 % had baseline values above median values for PVRI (15.1 Wood units m^2), mPAP (62 mmHg), and right atrial pressure (7 mmHg), respectively; 40 % were classified as FC III or IV at baseline (versus 15 % of the overall study population). In contrast to the FDA warning, the authors concluded that the overall efficacy and safety profile favors the medium-dose regimen. Currently, the EMA endorses the use of sildenafil in pediatric pulmonary hypertension but recommends against high-dose treatment. All agree that in patients currently treated with sildenafil, the drug should be discontinued only under careful medical supervision.

The safety and efficacy of tadalafil in pediatric pulmonary hypertension was investigated in a retrospective study of 33 children (Takatsuki et al. 2012). Four patients were started on tadalafil as initial therapy and 29 were switched from sildenafil to tadalafil primarily due to the ease of once/day dosing. Fourteen of the 29 patients who switched medication underwent RHC while on sildenafil and again after tadalafil treatment: in this small group statistically significant improvements were found in mean PA (53.2 ± 18.3 mmHg vs. 47.4 ± 13.7 ; $p < 0.005$) and PVR

index (12.2 ± 7.0 vs. 10.6 ± 7.2 Units/m²; $p < 0.05$.) Clinical improvement was also noted in the treatment-naïve patients after starting tadalafil. The authors speculated that the improvement in hemodynamics after transition from sildenafil to the longer-acting tadalafil may have been due to improved compliance with once/day dosing and/or more consistent serum levels.

3.3 Pulmonary Hypertension and Left Ventricular Dysfunction

Patients with longstanding left ventricular dysfunction and pulmonary venous hypertension frequently develop an increase in pulmonary vascular resistance and secondary pulmonary hypertension. In fact, Group-2 patients account for the largest number of patients with pulmonary hypertension (Oudiz 2007). Mechanistically, pulmonary vasodilation without a concomitant decrease in left heart pressures would lead to increased pulmonary capillary pressures and pulmonary edema. Importantly, targeting the PH in patients with heart failure due to reduced ejection fraction (HFrEF) with epoprostenol and endothelin antagonists has been shown to be ineffective or harmful (Califf et al. 1997; Anand et al. 2004). Conversely, initial reports on the use of PDE-5 inhibitors to treat patients with Group-2 pulmonary hypertension are promising. In small studies of short duration, PDE-5 inhibitors have been shown to have a number of beneficial effects in patients with Group-2 PH including increased oxygen uptake, improved pulmonary vascular resistance, and cardiac output (Behling et al. 2008; Melenovsky et al. 2009; Lewis et al. 2007). There are a number of reasons that PDE-5 inhibitors may be effective in heart failure. PDE-5 expression is increased in hypertrophied myocardium and may be a marker of oxidative stress (Lu et al. 2010). Cyclic-GMP modulates contractility in the hypertrophied RV and inhibition of breakdown of cGMP acutely increases RV contractility (Nagendran et al. 2007).

3.3.1 PDE-5 Inhibitors in HFrEF

In short-term studies, sildenafil has been shown to increase exercise capacity and quality of life in patients with HFrEF. In a randomized trial of 34 patients with PH and systolic dysfunction, treatment with sildenafil for 12 weeks resulted in a 20 % reduction in resting and peak exercise PVR, a 38 % increase in peak exercise cardiac output, a 15 % increase in $VO_{2\text{ max}}$ and improved QOL (Lewis et al. 2007). Another study treated patients with PH and HFrEF for 24 weeks and found a 25 % reduction in systolic PA, a 25 % increase in $VO_{2\text{ max}}$ and improved ventilator efficiency (Guazzi et al. 2010).

In a recent 1-year study, 45 patients with HFrEF and PH were randomized to receive placebo or sildenafil 50 mg three times daily (Guazzi et al. 2011a). Primary

endpoints were left ventricular diastolic function, chamber dimensions, and mass; secondary endpoints were functional capacity and QOL. At 6 months, sildenafil increased LVEF (6.8 %), improved echocardiographic indices of diastolic function, reduced PASP (13 mmHg), and improved QOL ($p < 0.01$ for all measures). Improvements were maintained at 1 year.

The favorable early studies of sildenafil on hemodynamics, cardiac function, and quality of life suggest an important role for PDE-5 inhibitors in treatment of PH-HFrEF and prevention of adverse cardiac and vascular remodeling. The available data has led to extensive off-label use of PDE-5 inhibitors in HFrEF. Recently, a large multinational randomized trial, Sildenafil in Heart Failure (SiHF) was initiated by investigators to confirm earlier findings of safety and efficacy (Cooper et al. 2013).

3.3.2 PDE-5 Inhibitors in HFpEF

In a placebo-controlled trial, Guazzi et al. (2011b) randomized 44 patients with HFPEF and PH to receive placebo or sildenafil 50 mg three times per day for 1 year. The primary endpoints were improvement in pulmonary hemodynamics and right ventricular function. The patient groups were well matched at baseline. After 6 months, in comparison with baseline and the placebo group, the treatment group showed significant improvements in hemodynamic variables and right ventricular function. The pulmonary vascular resistance decreased from 3.88 ± 1.38 Wood units to 1.18 ± 2.1 Wood units ($p < 0.01$) and was associated with a small but statistically significant decrease in pulmonary wedge pressure. Pulmonary function as measured by spirometry, lung volumes and diffusing capacity show significant improvement and likely indicates a decrease in pulmonary congestion. Secondary endpoints of breathlessness, fatigue emotional function also improved with treatment and were unchanged with placebo. Importantly, the positive changes associated with sildenafil treatment were maintained at 1 year. Although not specified as entry criteria, patients in this study had markedly elevated right atrial pressures at baseline, and thus represent a particularly ill subset of HFPEF. This study also found improvements in measurements of left ventricular function. The mechanism of the decrease in improved LV function may be related to interventricular interaction or direct effects of PDE-5 inhibition on LV mechanics.

However, in a randomized controlled trial of sildenafil (20 mg three times daily) in 113 patients with HFpEF (Phosphodiesterase-5 Inhibition to Improve Clinical Status and Exercise Capacity in Diastolic Heart Failure—the RELAX trial) there were no significant differences in the primary endpoint of change in peak oxygen consumption at 24 weeks, cardiovascular or renal hospitalization, echocardiographic cardiac magnetic resonance indices of ventricular remodeling and diastolic function, pulmonary artery pressures, or QOL (Redfield et al. 2012).

3.4 Group-3 Pulmonary Hypertension

Pulmonary hypertension is a common complication of severe pulmonary parenchymal lung disease. Currently, there are no adequate trials to determine whether specific treatment of PH in this setting is beneficial. Advanced therapy is not approved by the FDA in this setting, and according to published guidelines its use is recommended only in the context of a clinical trial. The most important consideration is to treat the underlying lung disease and provide supplemental oxygen in patients who are hypoxemic. Furthermore, the use of pulmonary vasodilators in patients with parenchymal lung disease carries the added risk of worsening oxygenation by inhibiting hypoxic pulmonary vasoconstriction and increasing blood flow to poorly ventilated lung units. However, there are data to support the concept that pulmonary hypertension contributes to symptoms and prognosis in patients with parenchymal lung disease: the presence of PH is clearly associated with a worse prognosis and the degree of PH is not clearly correlated with the severity of lung function abnormalities. Thus, the use of advanced PH therapy is an area of great interest for patients with Group-3 PH.

There are a few small studies that have investigated the use of PDE-5 inhibitors in patients with parenchymal lung disease. Importantly, rather than specifically targeting patients with PH associated with parenchymal lung disease, many studies include a mix of subjects with both normal and elevated pulmonary artery pressure. In patients with moderate-to-severe COPD, the acute effects of sildenafil were investigated in 18 patients: only 5 had mPA > 25 mmHg at rest, but all developed increased PA pressure with exertion (Holverda et al. 2008). A single dose of sildenafil attenuated the rise in PA pressure with exertion and was not associated with changes in stroke volume or cardiac output. This same group studied 15 COPD patients after 3 months of sildenafil therapy and found no change in exercise capacity or stroke volume as assessed by magnetic resonance imaging (MRI) (Rietema et al. 2008). A recent study of sildenafil treatment in COPD patients *without* PH showed worsened gas exchange and QOL with no benefit in exercise capacity (Lederer et al. 2012).

A small study, which specifically enrolled COPD patients with documented PH, randomized 20 to sildenafil at low dose (20 mg three times per day) or high dose (40 mg three times per day) (Blanco et al. 2010). Baseline hemodynamic and gas exchange data were collected, and patients were treated for 3 months. Sildenafil treatment was associated with a decrease in mean PAP both at rest and during exercise (21 ± 9 % at rest, 19 ± 10 % with exercise, $p < 0.001$ for both). A decrease in PaO₂ at rest was found with treatment thought due to the inhibition of hypoxic vasoconstriction. The authors caution that treatment of patients with COPD-PH with sildenafil should be closely monitored to assess for changes in gas exchange.

Patients with diffuse parenchymal lung disease (DPLD), such as idiopathic pulmonary fibrosis (IPF) or pulmonary fibrosis associated with collagen vascular diseases, may also develop severe PH, which contributes to morbidity and mortality

(Corte et al. 2009; Helman et al. 2007). Unfortunately, as with COPD, convincing data regarding the efficacy of advanced treatment of the PH are lacking. In a randomized controlled open-label trial of 16 patients with documented PH and pulmonary fibrosis, a single dose of sildenafil acutely caused preferential pulmonary vasodilation and, in contrast to IV epoprostenol, actually improved PaO₂. This study found an improvement in ventilation–perfusion matching with sildenafil administration (Ghofrani et al. 2002).

Small-randomized studies of PDE-5 inhibition in DPLD have had variable results. Of 14 patients with PH-ILD treated for 3 months with sildenafil, 57 % had an improvement in 6MWD (Collard et al. 2007). Another study of 29 IPF patients, which targeted patients with PH, did not document any improvement in dyspnea or 6MWD. This study, however, used only echocardiography to define PH; thus, the number of subjects who actually had PH is uncertain. A third study retrospectively identified 15 patients with PH-ILD who were treated with sildenafil (Corte et al. 2010). Patients (11/15) had PH documented by right heart catheterization (mean PA = 41.3 ± 11.0 mmHg) or had markedly elevated right ventricular systolic pressure on echocardiography (RVSP = 73.8 ± 17 mmHg). After 6 months, there was an improvement in 6MWD and a decreased in BNP levels.

A prospective observational study of a mixed group of patients with nongroup-1 PAH (chronic inoperable thromboembolic disease $n = 11$, COPD $n = 6$, DPLD $n = 5$, and valvular heart disease $n = 3$) followed patients who were treated with sildenafil (Chapman et al. 2009). All patients had PH diagnosed by right heart catheterization (mean PA > 25 mmHg at rest) at baseline and had follow-up catheterization after treatment. Although the numbers were small, after 12 months of treatment with sildenafil 50 mg three times per day, there was a significant improvement in hemodynamics for patients with CTEPH, DPLD, and valvular heart disease. However, patients with COPD did not show improvement in any parameter.

The largest published investigation of PDE-5 inhibitors to treat advanced IPF enrolled 180 patients in a double-blind, randomized placebo-controlled trial (Zisman et al. 2010). Importantly, enrollment criteria were based on the degree of lung function abnormality; measurement or estimation of pulmonary artery pressure was not reported. Subjects received sildenafil (20 mg three times per day) or placebo for 12 weeks. After the initial study period, all patients received sildenafil in an open-label 12-week period. An improvement in 6MWD ≥ 20 % was the primary outcome; changes in oxygenation, dyspnea, and QOL were secondary outcomes. After sildenafil treatment, there was no difference in primary outcome. There were small, but statistically significant differences in arterial oxygenation, dyspnea, carbon monoxide diffusing capacity, and QOL all favoring sildenafil treatment. Because confirmed PH was *not* required for study entry, the number of patients who actually had PH is not known, and greatly confounds the interpretation of the results.

From the currently available data, it is not known whether treatment of Group-3 PH with PDE-5 inhibitors or any advanced PH therapy is beneficial. It is clear that treatment of “all comers” with COPD or IPF in the absence of documented PH is

not useful. It is the opinion of the authors that some early results are promising in patients with known PH and further study is needed. Critically important to the design of randomized, placebo-controlled trials is to study the subjects most likely to benefit: only patients who have verified PH should be enrolled.

3.5 Adverse Effects of PDE-5 Inhibitors

The two largest studies of PDE-5 inhibitors in patients with PAH confirmed that these agents are generally well tolerated in patients with PH. The SUPER-1 trial found the most common adverse effects attributable to the highest dose of sildenafil (80 mg three times daily) to be headache (49 % vs. 39 % with placebo), flushing (15 % vs. 4 %), dyspepsia (13 % vs. 7 %), myalgia (14 % vs. 3 %), and pyrexia (10 % vs. 2 %) (Galie et al. 2005). The PHIRST trial had similar results with tadalafil 40 mg once per day: headache (42 % vs. 15 % with placebo), myalgia (14 % vs. 3 %), and flushing (13 % vs. 2 %) being most common (Galie et al. 2009b).

Serious side effects, although not seen in the trials, are also recognized. Systemic hypotension may occur with PDE-5 inhibitor treatment, especially in patients taking nitrates. Therefore, patients should not take nitrates with PDE-5 inhibitors and should be clearly warned about this interaction. Post-marketing studies have reported sudden vision loss due to nonarteritic anterior ischemic optic neuropathy in patients using sildenafil for erectile dysfunction. However, it appears this correlation was due to patients with diabetes were at increased risk for this condition and were also more likely to use medication for erectile dysfunction. Recently, a large, multicenter trial and an in-depth case review found no increase in ocular risk in patients taking sildenafil (Wiroszko et al. 2012; Azzouni and Abu samra 2011). Sensorineural hearing loss, however, does appear to be a true risk associated with the use of PDE-5 inhibitors; it has been reported with all three available drugs and may be related to elevated cGMP level effects on cochlear hair cells (Snodgrass et al. 2010; Khan et al. 2011; Maddox et al. 2009).

4 Conclusion

The NO/cGMP/PKG1 signaling pathway is critical in normal regulation of the pulmonary vasculature, and patients with PH have documented abnormalities in this system. In the pulmonary vasculature, degradation of cGMP is primarily regulated by PDE-5, and inhibition of this enzyme has salutatory effects on pulmonary vasculature smooth muscle tone. Large randomized placebo-controlled trials of PDE-5 inhibitors demonstrated improved exercise capacity, hemodynamics, and quality of life in adult patients with PAH. Studies of these agents in patients with other forms of PH are promising but inconclusive. Currently, there is no definitive evidence that PDE-5 treatment prolongs survival.

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Inhaled Nitric Oxide for the Treatment of Pulmonary Arterial Hypertension

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Abstract Following the recognition of nitric oxide (NO) as the “endothelium-derived relaxing factor,” an explosion of laboratory and clinical research led to the development of inhaled NO as a potential therapy for patients with pulmonary arterial hypertension (PAH). Despite clear demonstration of its selective and potent pulmonary vasodilator properties, inhaled NO therapy has only been formally approved by the US Food and Drug Administration and European Medicine Evaluation Agency for clinical use in the treatment of term and near-term infants with severe persistent pulmonary hypertension of the newborn (PPHN) with acute hypoxemic respiratory failure. Over the past decades, inhaled NO remains the central therapy for PPHN and is commonly used for acute pulmonary vasoreactivity testing during right heart catheterization and for treating pediatric and adult patients with PAH associated with postoperative cardiac surgery, severe respiratory failure, pulmonary hypertension crises, and other disorders. This review will describe the current use of inhaled NO in clinical practice and briefly discuss its potential role for the treatment of chronic PAH.

Keywords Persistent pulmonary hypertension of the newborn • Acute respiratory distress syndrome • Cyclic GMP • Soluble guanylate cyclase • Phosphodiesterase inhibitors • Pediatric pulmonary hypertension

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1 Introduction

Nearly all pharmacologic therapies that are currently available for the treatment of pulmonary arterial hypertension (PAH) are based on studies of adult patients and are exclusively approved by the Food and Drug Administration (FDA) for use in adults. Children with PAH are generally treated with the same drug therapies as adults, representing off-label use of these medications. In striking contrast, inhaled NO has been most extensively studied and is FDA approved exclusively for the treatment of term and near-term neonates with pulmonary hypertension and acute hypoxemic respiratory failure. Interestingly, this represents only 25 % of the current use of inhaled NO in most centers, including hospitals in the USA and in Europe. That is, most of the clinical use of inhaled NO is off-label, including its use for testing acute pulmonary vasoreactivity during right heart catheterization, postoperative cardiac patients, acute respiratory failure, and other settings. This chapter provides a brief overview of the physiologic basis and roles for inhaled NO therapy in the treatment of sick newborns, including term and preterm infants. Additional studies regarding its potential application in the care of pediatric and adult patients with PAH in settings such as cardiac catheterization, postoperative care, and acute and chronic lung disease will be reviewed. Finally, the potential role for inhaled NO for the chronic treatment of PAH is also discussed.

2 Physiologic Basis for Inhaled NO Therapy

2.1 Mechanisms of NO–cGMP Signaling

The Nobel Prize-winning work of Furchgott, Murad, and Ignarro led to the initial recognition and identification of the “endothelium-derived relaxing factor” as NO and set the stage for extensive work regarding its complex biology. NO, a highly diffusible gas with a very short half-life (seconds), is synthesized from the terminal nitrogen of L-arginine by the enzyme NO synthase (NOS). These enzymes are complex oxidoreductases that function as dimers and exist as three distinct isoforms, including the endothelial (or type III) NOS (eNOS). NO production is partly dependent upon substrate availability (L-arginine), as well as the presence of essential cofactors, including heat shock protein 90 (Hsp 90), an intracellular chaperone, and tetrahydrobiopterin (BH₄), a bioactive form of folic acid. Depletion of these cofactors or inhibition of Hsp90–eNOS interactions leads to “uncoupled” eNOS activity, which promotes generation of the toxic reactive oxygen species, superoxide (O₂[−]), rather than NO. Multiple factors contribute to the regulation of NO production and activity, including oxygen tension, shear stress, diverse hormones and growth factors, and endogenous inhibitors, such as asymmetric dimethyl-arginine. NO also interacts with O₂[−], thereby limiting NO bioavailability and resulting in the formation of the potent oxidant peroxynitrite (ONOO[−]).

As a gas, NO diffuses freely from the endothelium to subjacent vascular smooth muscle cells (SMC). The biologic effects of NO in vascular SMC are largely dependent upon direct activation of soluble guanylate cyclase (sGC), which converts GTP to cGMP and relaxes SMC through stimulation of cGMP-gated ion channels and activation of cGMP-dependent protein kinase type I (Francis et al. 2010; Gao 2010; Hofmann et al. 2009; Somlyo and Solyo 2003). The actions of cGMP are limited through catabolism by phosphodiesterases (PDE), especially by type 5 cGMP-dependent PDE (PDE5), which are key determinants of the magnitude and duration of the vasodilator response to endogenous and exogenous NO.

NO has multiple effects on cell signaling, inflammation, growth and differentiation, and metabolism. NO based signaling via soluble GC explains much of its actions; however, exciting new studies suggest that the effects of NO on vasodilation are far more complex than provided by this simple paradigm. NO also elicits physiologic effects via cGMP-independent mechanisms, which include direct activation of K⁺ channels and through interactions with other heme-containing molecules and proteins containing reactive thiol groups (reviewed in Hare and Stamler 2005). Alternative NO signaling pathways likely exist through the reaction of NO with protein thiols to form S-nitrosothiols (SNO), which may induce vasodilation or protein modification. That is, NO regulates protein activities through S-nitrosylation, in which addition or removal of NO modulates protein function. SNO homeostasis is a balance between S-nitrosylation and de-nitrosylation, which can either up- or downregulate protein functions (Torko et al. 2012). Furthermore, the view that NO gas directly diffuses to airway and vascular cells to mediate

its effects has been challenged. Extracellular NO may increase intracellular SNO accumulation in vascular endothelium and SMC through nitrosation of extracellular L-cysteine to form SNO-L-cysteine, which is then imported via type L amino acid transporters.

2.2 Physiologic Effects of Inhaled NO

Inhaled NO is a selective pulmonary vasodilator that can acutely lower pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) without altering systemic arterial pressure (Frostell et al. 1991; Pepke-Zaba et al. 1991). The local effects of NO inhalation on the lung are primarily due to the rapid absorption and scavenging through avid binding to hemoglobin, followed by rapid oxidation to form nitrite, which interacts with oxyhemoglobin, leading to the formation of nitrate and met-hemoglobin (metHgb). In the presence of oxygenated hemoglobin (Hgb), NO is rapidly metabolized to nitrate with formation of met-Hgb, which is rapidly reduced to ferrous-Hgb by met-Hb reductase in red blood cells. In contrast with inhaled NO, systemically administered vasodilators, including prostacyclin, sildenafil, and pharmacologic NO donors, can dilate the pulmonary vasculature, but their efficacy is often limited by systemic hypotension. Additionally, in the setting of acute lung injury or with chronic lung disease, systemic vasodilator drugs can impair matching of ventilation with perfusion leading to systemic arterial hypoxemia.

In addition to its lung-selective effects, inhaled NO has the unique ability to regulate the distribution of pulmonary blood flow in the setting of parenchymal lung disease, including acute respiratory distress syndrome (ARDS) (Rossaint et al. 1993). This “micro-selective” effect of inhaled NO accounts for its ability to increase pulmonary blood flow to well-aerated and ventilated lung regions in preference to poorly inflated lung regions. NO-induced improvement in oxygenation appears largely dose dependent; although higher doses of inhaled NO (20–80 ppm) can cause progressive pulmonary vasodilation in many settings, low doses of inhaled NO (1–10 ppm) achieve better improvements in oxygenation than that observed with higher doses. In fact, higher doses of inhaled NO may paradoxically worsen oxygenation in some patients with acute respiratory failure due to the loss of this “micro-selective” effect (Gerlach et al. 1993).

Since the effects of NO are short-lived, inhaled NO is generally administered continuously or with a pulsing device that is rapidly triggered with the onset of inspiration. Inhaled NO has been administered via pulsed nasal delivery, which may provide an effective mode of delivery for ambulatory therapy in the setting of chronic cardiopulmonary disease (as discussed below). NO is a colorless and odorless gas that readily reacts with oxygen to form nitrogen dioxide (NO₂), which can cause lung injury. As a result, NO gas must be stored in nitrogen or another inert gas and administered to the patient in a manner designed to minimize the duration of exposure to oxygen. Because the pulmonary vasodilator effects of

NO are transient when the gas is discontinued, NO must be administered continuously with careful monitoring of NO and NO₂ concentrations.

Routinely available commercial equipment permits the safe delivery of NO gas in intubated and non-intubated patients. Met-hemoglobin is a source of potential toxicity with exposures to high doses of NO (≥ 80 ppm) especially for prolonged periods. Met-hemoglobinemia typically responds to a reduction of the inhaled concentration or to discontinuation of NO therapy. Abrupt discontinuation of NO inhalation can result in “rebound” pulmonary hypertension leading to a decreased cardiac output and systemic hypotension. Rebound pulmonary hypertension after NO withdrawal may be due to suppression of endogenous NOS activity by exogenous NO (Assruey et al. 1993). The risk of rebound pulmonary hypertension can be minimized by gradually weaning the concentration of inhaled NO gas delivered to the patient. In the presence of severe left ventricular (LV) failure, administration of NO to relieve pulmonary vasoconstriction may augment LV filling and increase left atrial and pulmonary capillary wedge pressure, causing pulmonary edema. Thus, caution should be exercised when patients with LV failure or diastolic dysfunction are treated with inhaled NO. Similarly, inhaled NO in the setting of pulmonary veno-occlusive disease can induce “flash pulmonary edema,” causing marked hypoxemia and acute respiratory distress, which has been observed during acute vasoreactivity testing during evaluation of patients with PAH.

3 Inhaled NO and Neonatal Pulmonary Hypertension

3.1 NO and the Developing Lung

PVR is high throughout fetal life, especially in comparison with the low resistance of the systemic circulation. As a result, the fetal lung receives less than 8 % of combined ventricular output, with most of the right ventricular output crossing the ductus arteriosus (DA) to the aorta. Mechanisms that contribute to high basal PVR in the fetus include low oxygen tension, relatively low basal production of vasodilator products, such as NO and prostacyclin (PgI₂), increased production of vasoconstrictors, including endothelin-1 (ET-1) or leukotrienes, and altered smooth muscle cell reactivity including high myogenic tone (Gao and Raj 2010a; Rudolph et al. 1977). The fetal pulmonary circulation is characterized by a progressive increase in responsiveness to vasoactive stimuli during development. In the ovine fetus, the pulmonary circulation is largely unresponsive to increased oxygen tension during the early canalicular period, but the vasodilator response to high and low fetal PO₂ increases with gestation (Morin et al. 1988; Rasanen et al. 1998). These observations parallel clinical findings that maternal hyperoxia does not increase pulmonary blood flow between 20 and 26 weeks gestation, but increased PO₂ causes pulmonary vasodilation in the 31–36-week fetus (Rasanen et al. 1998). Thus, in addition to structural maturation and growth of the developing lung

circulation, the vessel wall also undergoes functional maturation, leading to enhanced vasoreactivity during fetal life.

Several mechanisms contribute to progressive changes in pulmonary vasoreactivity during development, including maturational changes in endothelial cell function, especially with regard to NO production (Abman et al. 1990; 1991; North et al. 1994; Halbower et al. 1994; Parker et al. 2000). Lung eNOS mRNA and protein are present in the early fetus and increase with advancing gestation in utero and during the early postnatal period in rats and sheep (Parker et al. 2000). Endothelial NOS expression and activity are regulated by several factors, including oxygen tension, hemodynamic forces, hormonal stimuli (e.g., estradiol), paracrine factors (including vascular endothelial growth factor, VEGF), substrate and cofactor availability, superoxide production (which inactivates NO), and others. NO causes vasodilation by stimulating sGC in vascular SMC, increasing SMC cGMP content. The ability to respond to endogenous or exogenous NO is operative very early during fetal life, suggesting critical roles for NO–cGMP throughout lung development (Kinsella et al. 1994a). Fetal lung PDE5 expression and activity are high in comparison with the normal postnatal lung, contribute significantly to the regulation of pulmonary vascular tone and reactivity in utero as well as after birth, and are further increased in PPHN (Hanson et al. 1998; Farrow et al. 2008a). The role of NO–cGMP signaling in fetal pulmonary vasoregulation has been recently reviewed (Gao and Raj 2010b).

At birth, a rapid and dramatic decrease in PVR causes an eight- to tenfold increase in pulmonary blood flow. Systemic vascular resistance increases at birth, partly due to removal of the low-resistance vascular bed of the placenta. The largest drop in PVR occurs immediately after birth, but PVR continues to progressively fall throughout infancy. As pulmonary artery pressure falls below systemic levels, blood flow through the ductus arteriosus (DA) and foramen ovale becomes left to right prior to functional closure, thereby establishing the normal postnatal circulatory pattern. Mechanisms contributing to the fall in PVR at birth include establishment of an air–liquid interface, rhythmic lung distension, increased oxygen tension, and altered production of vasoactive substances. Inhibition of NOS activity attenuates the decline in PVR after delivery of fetal lambs, suggesting that about 50 % of the rise in pulmonary blood flow at birth may be directly related to the acute release of NO (Abman et al. 1990). Failure to achieve or sustain this drop in PVR at birth leads to profound hypoxemia and constitutes the syndrome known as *persistent pulmonary hypertension of the newborn (PPHN)*.

3.2 Inhaled NO in Experimental PPHN

PPHN is associated with diverse cardiorespiratory diseases in newborns or can be idiopathic, often accompanies hypoxemic respiratory failure, and contributes to significant mortality and morbidity. Newborns with PPHN are at risk for severe asphyxia and its complications, including death, chronic lung disease,

neuro-developmental sequelae, and other problems. Successful transition of the pulmonary circulation at birth requires the precise orchestration of multiple growth factors and signaling pathways to ensure normal functional and structural maturation of the lung circulation.

Mechanisms that lead to the failure of PVR to fall at birth have been pursued in diverse animal models, including exposure to acute or chronic hypoxia after birth, chronic hypoxia in utero, placement of meconium into the airways of neonatal animals, sepsis, and others. Each model demonstrates important physiologic changes that may be especially relevant to particular clinical settings. However, most studies examined only brief changes in the pulmonary circulation, while the mechanisms underlying altered lung vascular structure, growth, and function in PPHN remain poorly understood. Clinical observations that neonates with severe PPHN who die during the first days after birth already have pathologic signs of chronic pulmonary vascular disease suggest that intrauterine events may play an important role in this syndrome (Geggel and Reid 1984; Murphy et al. 1981). Adverse intrauterine stimuli during late gestation, such as abnormal hemodynamics, changes in substrate or hormone delivery to the lung, hypoxia, inflammation, or others, may potentially alter lung vascular function and structure, thereby contributing to abnormalities of postnatal adaptation.

Pulmonary hypertension induced by early closure of the DA in fetal lambs alters lung vascular reactivity and structure, causing the failure of postnatal adaptation at delivery and providing an experimental model of PPHN (Abman et al. 1989; Morin 1989). Over days, pulmonary artery pressure and PVR progressively increased in this model, but flow remained low and PaO₂ unchanged (Abman et al. 1989). Marked right ventricular hypertrophy and structural remodeling of small pulmonary arteries develop after 8 days of hypertension. After delivery, these lambs have persistent elevation of PVR despite mechanical ventilation with high oxygen concentrations. Studies with this model show that chronic hypertension without high flow can alter fetal lung vascular structure and function. This model is further characterized by endothelial cell dysfunction and altered smooth muscle cell reactivity and growth. Other hallmarks include findings of impaired NO production and activity and downregulation of lung endothelial NO synthase mRNA and protein expression (Villamor et al. 1997; Shaul et al. 1997). Fetal pulmonary hypertension is also associated with decreased cGMP concentrations, which are also partly due to decreased sGC and increased PDE5 activities, suggesting further impairments in downstream signaling (Steinhorn et al. 1995). Thus, multiple alterations in the NO–cGMP cascade appear to play an essential role in the pathogenesis and pathophysiology of experimental PPHN by contributing to altered structure and function of the developing lung circulation, and leading to failure of postnatal cardiorespiratory adaptation. High production of reactive oxygen species, such as superoxide in the pulmonary vasculature, may further contribute to the disruption in NO–cGMP signaling in this model (Farrow et al. 2008b).

3.3 *Inhaled NO for Clinical PPHN*

Based on strong preclinical data, early pilot studies demonstrated that inhaled NO can increase oxygenation with brief exposure (Roberts et al. 1992) and cause sustained improvements with prolonged therapy, obviating the need for ECMO therapy in severe PPHN (Kinsella et al. 1992). The primary goal of PPHN therapy is selective pulmonary vasodilation. Inhaled NO therapy (5–20 ppm) improves oxygenation and decreases the need for ECMO therapy in patients with diverse causes of PPHN (Roberts et al. 1997; No Authors 1997; Clark et al. 2000; Davidson et al. 1998). Inhaled NO is well suited for the treatment of PPHN: It is a rapid and potent vasodilator, and because nitric oxide is a small gas molecule, it can be delivered as inhalation therapy to airspaces approximating the pulmonary vascular bed. In contrast, intravenous dilators such as prostacyclin, tolazoline, and sodium nitroprusside may produce nonselective effects on the systemic circulation leading to hypotension as well as increased right-to-left shunting and impaired oxygenation. Large placebo-controlled trials that enrolled infants with an oxygenation index (OI) over 25 provided clear evidence that inhaled NO significantly decreases the need for extracorporeal life support in newborns with diverse causes of hypoxemic respiratory failure and PPHN. These studies also showed that doses of 5–20 ppm were effective and that increasing the dose beyond 20 ppm in nonresponders did not improve outcomes (No Authors 1997). Further, sustained treatment with 80 ppm NO increased the risk of methemoglobinemia (Davidson et al. 1998). Weaning can generally be accomplished in 4–5 days; any prolonged need for inhaled NO therapy without resolution of disease should lead to a more extensive evaluation to determine whether other underlying anatomic lung or cardiovascular disease is present, such as pulmonary venous stenosis, alveolar capillary dysplasia, severe lung hypoplasia, and others.

These clinical trials suggest that up to 40 % of infants will not respond or sustain a response to inhaled NO (Roberts et al. 1997; No Authors 1997; Clark et al. 2000). The reasons for an inadequate response are diverse and require the clinician to carefully analyze the relative roles of parenchymal lung disease, pulmonary vascular disease, and cardiac dysfunction for each infant. For instance, if severe airspace disease is associated with PPHN, strategies such as high-frequency ventilation or more aggressive lung recruitment strategies that optimize lung expansion are likely to be effective. The two therapies used together are more effective than either used individually (Kinsella et al. 1997a). Cardiac function should also be carefully assessed longitudinally. In particular, infants with severe left ventricular dysfunction are likely to have pulmonary venous hypertension and are unlikely to respond to pulmonary vasodilation unless there is also optimization of cardiac performance.

Recent studies suggest that infants with PPHN may respond well to the administration of sildenafil, a selective PDE5 inhibitor, which has been approved by the FDA for the treatment of adult pulmonary hypertension (Ichinose et al. 2001; Weimann et al. 2000; Shekerdemian et al. 2002, 2004). Sildenafil is a potent and highly specific PDE5 inhibitor that has been used in several preclinical studies on

animal models of pulmonary hypertension. One study of a small cohort of human infants with PPHN demonstrated that enteral sildenafil improved oxygenation and survival compared to placebo in centers where inhaled NO was not available (Baquero et al. 2006). Animal studies suggest that sildenafil augments pulmonary vasodilator response to inhaled NO in experimental PPHN (Deruelle et al. 2005). A recent study of intravenous sildenafil given to infants with severe pulmonary hypertension indicates that the drug was generally well tolerated and notably improved oxygenation (Steinhorn et al. 2009). While systemic administration of sildenafil improved oxygenation, sildenafil had little effect on systemic blood pressure except with high-dose bolus infusions. Sildenafil has the potential to independently decrease PVR and improve oxygenation in human infants with PPHN; ongoing studies are exploring the relative roles for sildenafil in infants with severe PPHN who have poor or partial responses to inhaled NO.

In addition to high PDE5 activity, recent studies suggest that oxidized sGC may contribute to high PVR and the failure to respond to inhaled NO in experimental models of PPHN (Deruelle et al. 2006; Chester et al. 2009). In these studies, sGC activators and stimulators induced greater pulmonary vasodilation than inhaled NO after delivery of lambs with severe intrauterine pulmonary hypertension (Deruelle et al. 2006; Chester et al. 2009). These findings support the need for clinical studies to explore the potential role for sGC activators as a novel intervention in infants with severe PPHN who remain hypoxic despite inhaled NO therapy.

3.4 Inhaled NO and Preterm Infants

Surfactant therapy, the use of prenatal steroids, and safer and more effective modes and strategies of mechanical ventilation and airway care have markedly improved outcomes for extremely low birth weight, preterm newborns. However, some preterm infants present with severe respiratory failure associated with severe PAH. As described above, inhaled NO therapy has been proven as safe and effective for near-term and term newborns with PPHN, but its role in the treatment of the premature newborn has been far more controversial. Many early concerns included uncertainty regarding the role of PAH in preterm infants with or without respiratory distress syndrome (RDS), as well as potential risks for lung injury, inducing or extending problems with intracranial hemorrhage (ICH) and related issues associated with extreme immaturity.

The rationale and potential role for inhaled NO treatment of PAH in preterm infants has been well established through laboratory studies. Preclinical studies suggested that inhaled NO causes potent and selective pulmonary vasodilation in very immature animals and that inhaled NO lowers PVR and improves oxygenation in premature lambs with severe RDS (Kinsella et al. 1994b). Importantly, these studies and others demonstrated improved pulmonary hemodynamics without the development of pulmonary edema or hemorrhage despite the presence of a patent ductus arteriosus. Early clinical reports further supported a potential role for inhaled

NO therapy in premature newborns, as reflected by marked improvement in oxygenation caused by effective treatment of severe pulmonary hypertension and resolution of extra-pulmonary right-to-left shunting, as well as other preterm infants with severe respiratory failure.

Multiple clinical reports subsequently demonstrated that inhaled NO can acutely improve oxygenation in preterm infants with moderate or severe respiratory failure, especially in the settings of sepsis or prolonged premature rupture of membranes with oligohydramnios (Abman et al. 1993; Peliowski et al. 1995). Initial concerns regarding high risks for adverse effects, including ICH due to potential impairment of platelet function and prolonged bleeding time, were not confirmed by subsequent trials of low doses of inhaled NO in preterm infants (Kinsella et al. 1999). In fact, early studies suggested that low-dose inhaled NO therapy may actually attenuate the severity of ICH in preterm infants with high mortality (Kinsella et al. 1999).

Thus, strong evidence suggests that inhaled NO can effectively lower PVR and improve oxygenation in preterm infants with severe hypoxemia and PPHN-type physiology. A recent NIH workshop suggested that further placebo-controlled studies would be helpful to better ascertain the role of inhaled NO in these profoundly ill infants (Cole et al. 2011a). Currently, however, this subpopulation of preterm newborns with marked respiratory failure and PAH is relatively small and due to the risk for high mortality in this group, most centers routinely employ inhaled NO therapy in this setting, limiting the likelihood of successfully performing a randomized controlled trial.

In addition to its potential role in the treatment of PAH, marked interest has grown regarding the potential role for inhaled NO therapy in preterm infants for the prevention of bronchopulmonary dysplasia (BPD). BPD is the chronic lung disease that results from the inhibition or disruption of normal pulmonary alveolar and vascular development due to premature birth and oxygen- and ventilator-induced lung injury (Kinsella et al. 2006a). Inhaled NO has been shown to lower PAP and PVR and improve gas exchange in preterm infants with established BPD (Mourani et al. 2004; Banks et al. 1999), but whether early treatment with inhaled NO could enhance long-term cardiopulmonary outcomes is uncertain. Laboratory studies have demonstrated that in addition to its effects on pulmonary vasodilation, inhaled NO also reduces lung inflammation in preterm lambs with RDS and attenuates acute lung injury in other animal models (Kinsella et al. 1997b). Inhaled NO has been shown to improve lung vascular and alveolar growth after hyperoxia in neonatal rats and improves lung structure in preterm lambs or baboons after prolonged mechanical ventilation.

Whether inhaled NO can prevent BPD in human preterm infants remains unclear. Several multicenter randomized trials have been performed to directly test this hypothesis, but the results have been variable and conclusions regarding its potential roles remain incomplete (Steinhorn and Porta 2007). Inhaled NO was shown to decrease the incidence of BPD and death in prematurely born infants with moderate RDS at a single-center study (Schreiber et al. 2003). This observation was partly supported by data from a subsequent multicenter trial in which inhaled NO decreased the rate of BPD and death in premature infants with a birth weight >1,000 g, but not for the entire study population (Kinsella et al. 2006b). Another

multicenter study, in which inhaled NO was provided at 7–21 days after birth, reported that NO inhalation improved survival without BPD (Ballard et al. 2006). However, a more recent large-scale MRCT failed to demonstrate any difference in the incidence of BPD with early therapy (Mercier et al. 2010).

Although these results are encouraging when considered together, additional studies need to be performed before the precise role of inhaled NO for the prevention of BPD can be defined. The study designs for the large MRCTs involved different doses and duration of treatment with inhaled NO, and there were striking differences in patients regarding postnatal age and respiratory status at enrollment. In fact, a recent NIH workshop concluded that based on extensive meta-analyses, there are currently insufficient data to recommend the routine use of inhaled NO therapy for the prevention of BPD in preterm infants (Cole et al. 2011b). Mechanisms that contribute to the failure of inhaled NO to more consistently improve long-term outcomes of preterm infants are uncertain, but may be due to impaired sGC expression or activation by exogenous NO, as suggested in an experimental model of BPD in sheep (Bland et al. 2003).

4 Inhaled NO and Acute Vasoreactivity Testing in PAH

Inhaled NO has been used extensively to perform acute vascular reactivity testing (AVT) in the cardiac catheterization laboratory in patients with PAH. In patients with PAH, demonstration of a positive response to vasodilator agents correlates with a favorable long-term clinical outcome (Rich et al. 1992). Inhaled NO has largely replaced other agents, such as prostacyclin or adenosine, in most centers due to its ease of administration, selectivity for the pulmonary vascular bed, and rapid “on and off” nature of its effects. While a number of vasodilators including intravenous prostacyclin and calcium channel blockers have been utilized for diagnostic testing in PH patients, systemic administration of these agents can produce severe hypotension, increased intrapulmonary right-to-left shunting, and death. In contrast, a number of studies have indicated that inhaled NO can be safely and effectively used to assess the capacity for pulmonary vasodilation in pediatric (Atz et al. 1999) and adult patients (Ricciardi et al. 1998) with PH, without causing systemic hypotension. The ability of inhaled NO to decrease PAP or PVR can be used to predict the subsequent response to therapy with oral vasodilators, such as nifedipine (Ricciardi et al. 1998), and a better midterm survival in adult patients with PH due to congenital heart disease (Post et al. 2004). Risks of inhaled NO or other vasodilator use during short exposures include the rapid development of pulmonary edema in patients with pulmonary veno-occlusive disease (PVOD), which is often considered diagnostic of PVOD in unsuspected cases. AVT with or without inhaled NO must also be performed cautiously in patients with left ventricular dysfunction. A positive pulmonary vasodilator response to NO inhalation has been used as a criterion to select patients for cardiac transplantation (Fojon et al. 2005).

5 Inhaled NO and Postoperative Cardiac Patients

Pulmonary hypertension leading to acute right heart failure can complicate the management of patients during and after cardiac surgery employing cardiopulmonary bypass (CPB). A number of uncontrolled studies have demonstrated that inhalation of inhaled NO (20–40 ppm) effectively decreases PAP in cases of coronary artery bypass grafting or surgery for valvular heart disease that are complicated by perioperative PAH (Fullerton et al. 1996). Pulmonary hypertension in cardiac transplant recipients is a major cause of right heart failure and early death. Inhaled NO has been reported to selectively reduce right ventricular (RV) afterload and enhance RV stroke work after cardiac transplantation (Ardehali et al. 2001). Although there is physiologic data and clinical experience, suggesting that NO inhalation may be beneficial in patients during or after cardiac surgery complicated by PAH and RV dysfunction, whether or not inhaled NO can improve clinically important outcomes remains to be determined by randomized controlled trials.

PAH contributes to significant morbidity and mortality in cardiac surgery patients with congenital heart disease (CHD) (Hopkins et al. 1991; Hoskote et al. 2010). PAH associated with CHD may be due to increased pulmonary blood flow, high PVR, or both. In some settings (Fontan patients), even modest elevation of PVR can markedly reduce cardiac output. In many children with CHD, elevated PAP is due to high PBF (due to left-to-right shunts) and PAH returns to normal after surgical correction of the anatomic lesion. In some cases, however, patients develop high PVR due to significant vascular injury, which may not resolve after surgical correction, which can be exacerbated by endothelial injury after cardiopulmonary bypass (CPB). In addition, PAH contributes to poor outcomes following cardiac transplantation (Costard-Jackle and Fowler 1992; Giglia and Humpl 2010). In these instances, PAH may be a significant clinical problem and requires treatment. The exact incidence of PAH in children undergoing surgical interventions remains unclear and has changed due to earlier surgical repairs, but it has been estimated between 10 and 30 % (Hopkins et al. 1991). PAH also affects morbidity as well as mortality (Hoskote et al. 2010). Among infants undergoing CPB, postoperative PAH is strongly associated with increased time on mechanical ventilation and longer durations of ICU admission (Brown et al. 2003; Schulze-Neick et al. 2001).

Studies of inhaled NO have been consistently shown to lower PAP and PVR without affecting systemic arterial pressure in postoperative PAH associated with CHD (Day et al. 2000; Journois et al. 1994), Fontan procedures (Miller et al. 2000), and orthotopic heart transplantation. Inhaled NO has also been shown to be effective in preventing transient episodes of postoperative PH crisis and to stabilize patients during PH crisis (Goldman et al. 1996). A retrospective study suggested significant decreases in mortality in a patient group receiving inhaled NO compared with a control group receiving conventional care (24 % vs. 56 %) (Journois et al. 2005). In a randomized study, inhaled NO use was associated with fewer pulmonary hypertensive crises and shorter time to extubation (Miller et al. 2000). A postoperative approach that includes inhaled NO may allow for shorter stays in the pediatric intensive care unit after CPB surgery (Gothberg and Edberg 2000). These

studies show that in pediatric patients with PH after surgery for CHD, inhaled NO effectively improved hemodynamics and postoperative outcomes.

Abrupt withdrawal from inhaled NO may lead to rebound PH, which may be decreased by gradually reducing inhaled NO dose to 1 ppm before withdrawal, or by the addition of a second PAH drug treatment, such as sildenafil, dipyridamole, or prostacyclin (Vonbank et al. 2003; Ivy et al. 1998a). In pediatric patients who previously failed to wean from inhaled NO after surgery for CHD, sildenafil has been shown to facilitate weaning (Lee et al. 2008).

6 Inhaled NO in Acute Respiratory Distress Syndrome

Inhaled NO is a potent vasodilator that is delivered directly to areas of the ventilated lung to improve ventilation–perfusion mismatch, resulting in improved oxygenation and lowering PAH. Early observational studies of patients with severe acute respiratory distress syndrome (ARDS) consistently demonstrated that inhaled NO can cause selective pulmonary vasodilation and improve systemic oxygenation in adults (Rossaint et al. 1993). Similar studies in children further demonstrated that inhaled NO lowers PAP and PVR, increases cardiac index, and improves oxygenation in children with ARDS (Abman et al. 1994). Despite acute improvement in pulmonary hemodynamics and gas exchange, however, subsequent randomized trials have failed to demonstrate benefits with regard to mortality, duration of mechanical ventilation, or the number of days alive and off mechanical ventilation (Dobyns et al. 1999; Dellinger et al. 1998; Michael et al. 1998; Lundin et al. 1999). Whether or not inhaled NO can improve clinical outcome in a subgroup of severely hypoxemic patients with respiratory failure and how to better identify candidates remain unknown. For example, greater benefits were observed with inhaled NO in children with ARDS who were treated with combined high-frequency oscillatory ventilation than conventional ventilation (Dobyns et al. 1999).

Past studies used various doses and duration of inhaled NO therapy, which could affect their outcomes based on the dynamic dose responsiveness over time. Therefore, the current evidence suggests that inhaled NO should not be routinely used in patients with ARDS, but may be considered as adjunctive therapy in selected patients (e.g., those with coexisting PAH) to transiently improve oxygenation in patients with severe ARDS while other therapies are considered.

7 Potential Role for Inhaled NO for Chronic Treatment of PAH

Although traditionally administered through an endotracheal tube during mechanical ventilation, inhaled NO can also be safely and effectively delivered noninvasively by face mask or nasal cannula delivery devices. Ivy et al. first described the

use of noninvasive NO delivery in a young infant with severe idiopathic PAH (IPAH), who responded well to therapy until transitioned to chronic intravenous prostacyclin therapy (Ivy et al. 1994). Nasal inhaled NO provided prolonged and effective vasodilator therapy in infants with severe CDH with persistent PAH after extubation (Kinsella et al. 2003). Several reports have described the successful ambulatory use of inhaled NO. Channick et al. reported that ambulatory NO by nasal cannula provided sustained reductions in PAP and PVR in eight adult patients with IPAH (Channick et al. 1996). Importantly, no adverse signs, such as syncope, cyanosis, or respiratory distress, were noted with brief discontinuation of therapy, although the potential for “rebound PH” with sudden withdrawal remains a concern. Additionally, Ivy et al. reported successful management in 26 children and young adults with PAH through pulsed NO delivery (Ivy et al. 2003). Another study demonstrated improvements in WHO functional class, 6 min walk distance, and brain natriuretic peptide (BNP) levels after 1 month of ambulatory inhaled NO treatment (Ivy et al. 1998b). Patients demonstrated improved pulmonary hemodynamics and cardiac index after 1 year, although additional PAH-specific therapies were often required for ongoing care. The combination of inhaled NO with PDE5 inhibition may provide an additional strategy for long-term therapy of PAH (Perez-Penate et al. 2008).

The dose of inhaled NO actually delivered to the distal lung is uncertain, but NO from nasopharyngeal aspirates was measured at roughly half of the administered dose (Kinsella et al. 2003; Ivy et al. 1998b, 2003). A potential advantage of inhaled NO, in contrast with intravenous vasodilators, is its pulmonary selectivity and the ability to avoid the need for a central line and such associated risks as line infections or thrombosis. Potential advantages of using a pulsed delivery device include greater efficiency of delivery during early inspiration, thereby allowing for more consistent alveolar delivery of gas, efficacy at lower concentrations of gas, and with less wasted delivery to the environment. Other potential candidates for prolonged ambulatory therapy could potentially include patients with COPD or other chronic lung diseases, PAH associated with sickle cell disease, as a “bridge to transplantation,” or for temporary use with disease exacerbations. Thus, despite availability of numerous other PAH-specific therapies, inhaled NO may provide an additional strategy for chronic management of severe PAH. Much still needs to be achieved to improve the applicability of ambulatory NO therapy, including the need for high concentration tanks for greater portability, studies that determine whether continuous 24/7 therapy is necessary or if shorter durations of treatment are sufficient, and other questions. Further studies are needed to determine the effect of long-term ambulatory breathing of pulsed NO and oxygen on the quality of life and mortality rate of patients with severe COPD.

8 Conclusions

Over the past 25 years, extensive basic and clinical research has increased our understanding of the pathobiology of NO–cGMP signaling in pulmonary hypertension and has led to the safe and rapid translation of inhaled NO therapy for patients with severe PAH. Based on the strength of extensive multicenter randomized controlled trials (MRCTs) in term and near-term newborns with PPHN, inhaled NO continues to provide an effective and safe therapy in this critical care setting. Although clinical observations have supported the use of inhaled NO in preterm infants with PPHN-type physiology and profound hypoxemia, MRCTs are lacking in this population to provide more clear evidence for use in this population. Although low doses of inhaled NO are safe in preterm infants, whether inhaled NO therapy prevents the development of BPD or brain injury in preterm newborns remains highly controversial, and current recommendations do not support its routine use for the prevention of chronic lung disease. Inhaled NO is commonly used for acute vasoreactivity testing of PAH in the cardiac catheterization lab, in stabilization of postoperative cardiac patients, and in selective cases of severe ARDS with PAH, but further work is needed in these settings to better prove efficacy. Finally, chronic ambulatory therapy for the long-term treatment of PAH has been suggested from case studies, and with continued improvements in technology, inhaled NO may provide an alternate strategy for chronic PAH as well as in the acute setting.

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Part III
Pulmonary Hypertension: Novel Pathways
and Emerging Therapies

Soluble Guanylate Cyclase Stimulators in Pulmonary Hypertension

Johannes-Peter Stasch and Oleg V. Evgenov

Abstract Soluble guanylate cyclase (sGC) is a key enzyme in the nitric oxide (NO) signalling pathway. On binding of NO to its prosthetic haem group, sGC catalyses the synthesis of the second messenger cyclic guanosine monophosphate (cGMP), which promotes vasodilation and inhibits smooth muscle proliferation, leukocyte recruitment, platelet aggregation and vascular remodelling through a number of downstream mechanisms. The central role of the NO–sGC–cGMP pathway in regulating pulmonary vascular tone is demonstrated by the dysregulation of NO production, sGC activity and cGMP degradation in pulmonary hypertension (PH). The sGC stimulators are novel pharmacological agents that directly stimulate sGC, both independently of NO and in synergy with NO. Optimisation of the first sGC stimulator, YC-1, led to the development of the more potent and more specific sGC stimulators, BAY 41-2272, BAY 41-8543 and riociguat (BAY 63-2521). Other sGC stimulators include CFM-1571, BAY 60-4552, vericiguat (BAY 1021189), the acrylamide analogue A-350619 and the aminopyrimidine analogues. BAY 41-2272, BAY 41-8543 and riociguat induced marked dose-dependent reductions in mean pulmonary arterial pressure and vascular resistance with a concomitant increase in cardiac output, and they also reversed vascular remodelling and right heart hypertrophy in several experimental models of PH. Riociguat is the first sGC stimulator that has entered clinical development.

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Clinical trials have shown that it significantly improves pulmonary vascular haemodynamics and increases exercise ability in patients with pulmonary arterial hypertension (PAH), chronic thromboembolic PH and PH associated with interstitial lung disease. Furthermore, riociguat reduces mean pulmonary arterial pressure in patients with PH associated with chronic obstructive pulmonary disease and improves cardiac index and pulmonary vascular resistance in patients with PH associated with left ventricular systolic dysfunction. These promising results suggest that sGC stimulators may constitute a valuable new therapy for PH. Other trials of riociguat are in progress, including a study of the haemodynamic effects and safety of riociguat in patients with PH associated with left ventricular diastolic dysfunction, and long-term extensions of the phase 3 trials investigating the efficacy and safety of riociguat in patients with PAH and chronic thromboembolic PH. Finally, sGC stimulators may also have potential therapeutic applications in other diseases, including heart failure, lung fibrosis, scleroderma and sickle cell disease.

Keywords Pulmonary hypertension • Nitric oxide • Soluble guanylate cyclase • sGC stimulator • Riociguat

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1 Soluble Guanylate Cyclase Signalling in Pulmonary Endothelium and Vascular Smooth Muscle

Nitric oxide (NO), a diffusible, short-lived, highly reactive gaseous molecule produced from L-arginine by NO synthases, plays a major role in controlling nearly every cellular and organ function in the body and performs diverse functions in human physiology and disease (Coggins and Bloch 2007; Derbyshire and Marletta 2012; Hirst and Robson 2011; Tonelli et al. 2013). The haemoprotein soluble guanylate cyclase (sGC), a key enzyme in the cardiopulmonary system, is the physiological receptor for endogenous NO. It catalyses the generation of the signalling molecule cyclic guanosine monophosphate (cGMP). This cyclic nucleotide is an important second messenger that is involved in the regulation of many vascular processes. It promotes vasodilation (i.e. vascular smooth muscle relaxation) via cGMP-dependent protein kinase G (PKG), inhibits proliferation of vascular smooth muscle cells in cooperation with bone morphogenetic protein (BMP) and Smad signalling, inhibits fibrosis and platelet aggregation, and also plays a role in protecting against vascular inflammation (Garg and Hassid 1989; Mellion et al. 1981; Pfeifer et al. 1998; Rizzo et al. 2010; Sawada et al. 2009; Schlossmann and Schinner 2012; Schwappacher et al. 2013).

The central role of sGC in the regulation of vascular tone is demonstrated by the phenotype of sGC-knockout mice, in which NO-dependent aortic relaxation is lost (Bryan et al. 2009; Friebe et al. 2007). In addition, smooth muscle relaxation and conventional haemodynamic response following NO administration are attenuated in mice that express a mutant form of sGC with only basal activity (Thoonen et al. 2013a). Further studies using these mouse models promise to help elucidate how sGC contributes to cardiovascular homeostasis (Thoonen et al. 2013b).

2 Dysregulation of the NO–sGC–cGMP Pathway in Pulmonary Hypertension

The pathophysiology of pulmonary hypertension (PH) includes endothelial dysfunction that results in insufficient stimulation of the NO–sGC–cGMP pathway. This pathway may be affected at a number of different steps in many types of PH (Fig. 1).

2.1 Nitric Oxide Production

Endogenous NO levels are reduced in patients with pulmonary arterial hypertension (PAH), PH associated with connective tissue disease, chronic obstructive pulmonary disease (COPD) and interstitial lung disease (Clini et al. 2000; Cremona et al. 1994; Girgis et al. 2005; Kaneko et al. 1998; Kawaguchi et al. 2006; Malerba et al. 2007; Riley et al. 1997). Moreover, impaired vasodilatory responsiveness to NO has been found in the pulmonary arteries of rats with experimentally induced

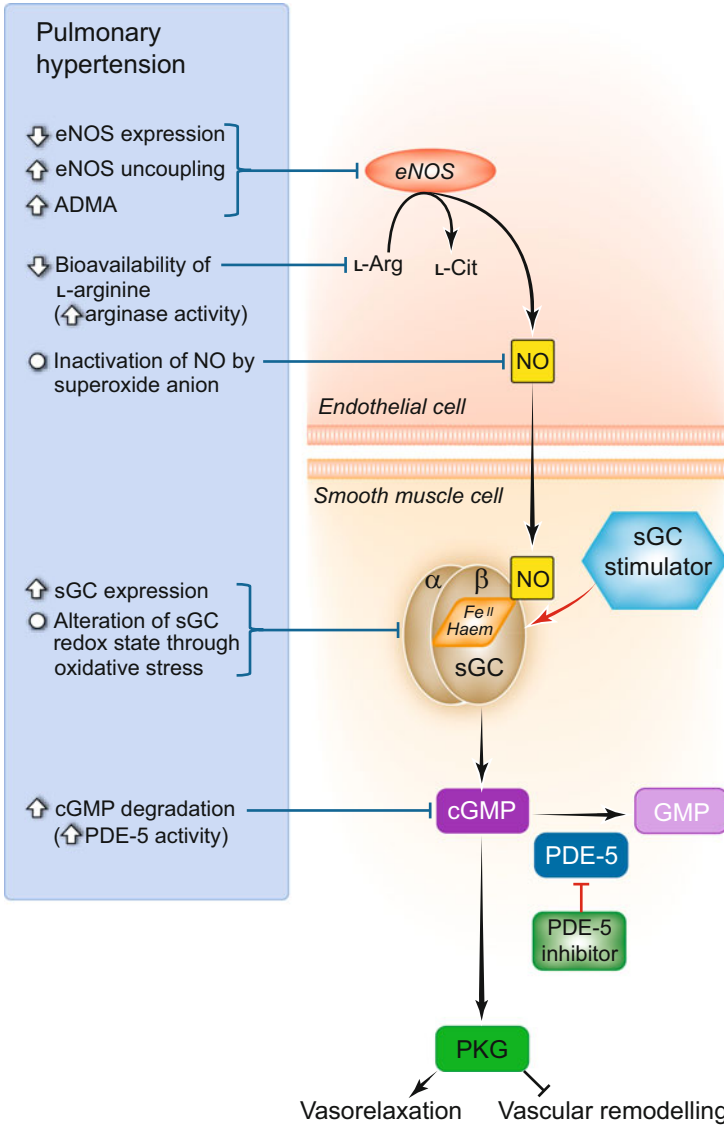


Fig. 1 Steps in the nitric oxide-soluble guanylate cyclase–cyclic guanosine monophosphate signalling pathway that may be disrupted in pulmonary hypertension. *ADMA* asymmetrical dimethylarginine, *cGMP* cyclic guanosine monophosphate, *eNOS* endothelial nitric oxide synthase, *Fe^{II}* ferrous iron, *GMP* guanosine monophosphate, *L-arg* L-arginine, *L-cit* L-citrulline, *NO* nitric oxide, *PDE-5* phosphodiesterase-5, *PKG* protein kinase G, *sGC* soluble guanylate cyclase

PH (Mam et al. 2010). The mechanisms leading to low bioavailability of NO in PH include changes in the expression and activity of endothelial nitric oxide synthase (eNOS), changes in the bioavailability of L-arginine, and oxidative stress.

Expression of eNOS is downregulated in the vascular endothelium of the pulmonary arteries in several forms of PH (i.e. PAH, PH associated with COPD, interstitial lung disease and bronchiectasis) (Giaid and Saleh 1995). Interestingly, in patients with plexiform lesions associated with PAH, eNOS expression correlates inversely with pulmonary vascular resistance (Giaid and Saleh 1995). The expression of eNOS is also reduced in platelets from patients with idiopathic PAH, which exhibit defective activation and aggregation (Aytekin et al. 2012).

In addition to the downregulation of eNOS expression, eNOS activity may be suppressed by endogenous inhibitors such as asymmetrical dimethylarginine (ADMA). Plasma levels of ADMA are elevated in patients with idiopathic PAH and chronic thromboembolic pulmonary hypertension (CTEPH) compared with healthy individuals (Kielstein et al. 2005; Skoro-Sajer et al. 2007). Plasma ADMA levels have also been found to be higher in patients with congenital heart disease accompanied by PAH than in those who do not have PAH (Sanli et al. 2012).

NO levels may also be reduced due to decreased bioavailability of its precursor L-arginine. Increased serum arginase activity and consequently reduced circulating levels of L-arginine have been observed in patients with PAH compared with healthy individuals (Xu et al. 2004). In addition, dysregulated arginine metabolism, characterised by both diminished systemic arginine bioavailability and raised ADMA levels, is directly associated with elevated pulmonary artery pressure in patients with advanced decompensated heart failure (Shao et al. 2012).

Furthermore, NO levels are reduced in conditions of oxidative stress, and biomarkers of oxidative stress are increased in the lungs of patients with idiopathic PAH and PH associated with connective tissue disease, left heart disease or hypoxia (Bowers et al. 2004; Cracowski et al. 2001; Hoshikawa et al. 2001). Under conditions of oxidative stress, NO levels are depleted by a reaction with superoxide (Laursen et al. 2001). This reaction generates the strong oxidant peroxynitrite, which, in concert with other oxidants, induces cell damage via lipid peroxidation, inactivation of enzymes and other proteins by oxidation and nitration, and activation of matrix metalloproteinases. Importantly, it also oxidises eNOS, resulting in its uncoupling. The uncoupling of eNOS ultimately abrogates NO synthesis and enhances the production of superoxide, thus amplifying the oxidative stress, which leads to further cellular dysfunction and finally cell death.

2.2 Activity of Soluble Guanylate Cyclase

Oxidative stress also impairs sGC activity. A study by Perez et al. demonstrated a link between oxidative stress and the regulation of sGC activity in newborn lambs with persistent PH. The increased levels of reactive oxygen species and decreased sGC activity that resulted from ventilating these lambs with oxygen were both normalised by a high-dose hydrocortisone treatment (Perez et al. 2012). Oxidation of the haem group on sGC causes it to dissociate from the enzyme and thus decreases the latter's activity and NO responsiveness (Roy et al. 2008; Thoonen et al. 2013a).

Although total sGC expression is increased in the vascular smooth muscle cells of small pulmonary arteries of patients with idiopathic PAH, this increase may be attributable to the increase in haem-free, NO-insensitive sGC that has been described in a range of animal models involving oxidative stress and in human cardiovascular diseases (Ahrens et al. 2011; Schermuly et al. 2008; Stasch et al. 2006).

2.3 *Cyclic Guanosine Monophosphate Degradation*

Increased cGMP degradation may also affect sGC signalling. Elevated expression and activity of phosphodiesterase (PDE)-5, resulting in reduced cGMP levels, have been found in two animal models of PH: sheep with PH associated with left heart disease and rats with hypoxia-induced PH (Black et al. 2001; Maclean et al. 1997). Furthermore, PDE-5 activity increased in response to oxidative stress in the pulmonary vascular smooth muscle cells of mice exposed to short-term hyperoxia (Farrow et al. 2012).

2.4 *Genetic Factors*

Advances in our understanding of the genetic factors associated with PH are important in further elucidating its underlying pathological mechanisms. Variants in many genes are known to be associated with systemic hypertension, indicating that they may play a central role in the control of blood pressure (Ehret et al. 2011). These include variants in three genes encoding components of pathways that regulate cGMP levels (i.e. the α and β subunits of sGC, and the natriuretic peptide clearance receptor, which sequesters the ligands of particulate guanylate cyclase) (Ehret et al. 2011). In addition, a hypertension sensitivity locus has been identified in the promoter region of the gene encoding eNOS (Salvi et al. 2012). The impact of these genetic variations on PH is yet to be determined.

3 Mechanisms of sGC Stimulation

3.1 *Activation of sGC by NO*

The heterodimeric α/β haemoprotein sGC is the physiological receptor for the endogenous gaseous second messenger NO. The prosthetic haem group of sGC is noncovalently bound to its β subunit via a haem-binding motif and the axial haem ligand histidine-105. Binding of NO to the reduced central iron atom of the haem moiety results in an up to 200-fold increase in the conversion rate of guanosine triphosphate (GTP) into cGMP (Fig. 2) (Arnold et al. 1977; Evgenov et al. 2006; Humbert et al. 1990; Lee et al. 2000; Murad 2006; Thoonen et al. 2013a; Wolin et al. 1982). However, this simple model of sGC activation is not consistent with

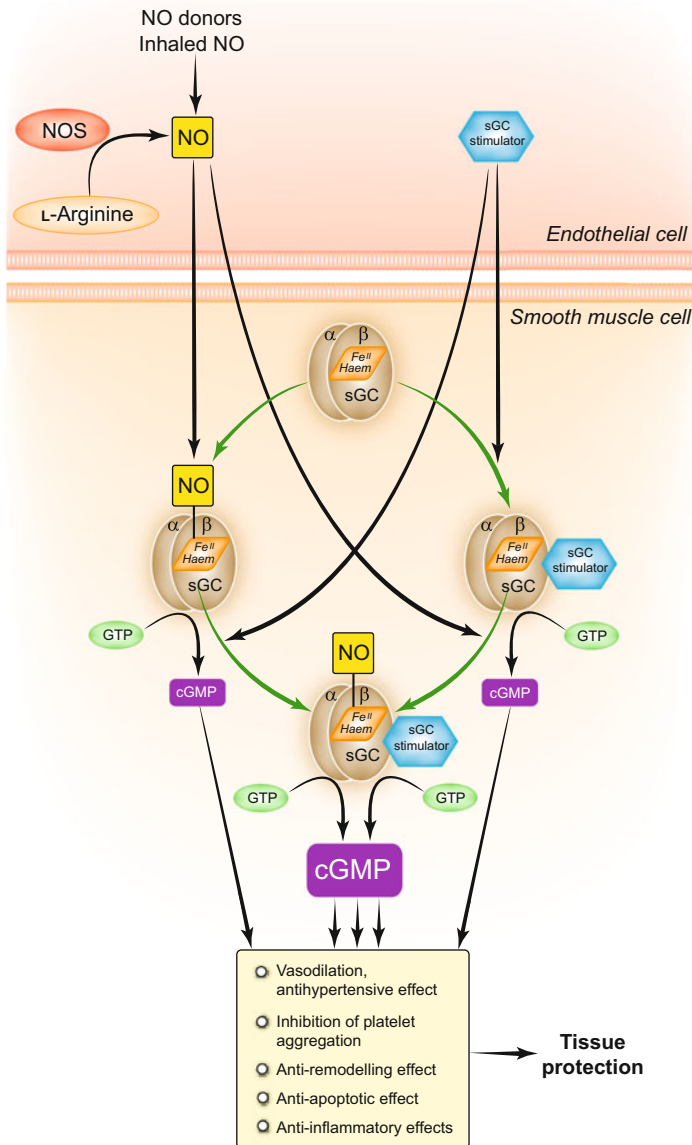


Fig. 2 Dual mode of action of soluble guanylate cyclase (sGC) stimulators. sGC is a key enzyme in the cardiopulmonary system and is the receptor for nitric oxide (NO). It catalyses the generation of the signalling molecule cyclic guanosine monophosphate (cGMP), which plays a pivotal role in regulating cellular and organ functions, such as vascular tone, proliferation, fibrosis, platelet aggregation and inflammation. Stimulators of sGC increase cGMP production by directly targeting sGC to induce its full activation via a dual mode of action. They stimulate sGC independently of NO, due to their distinct binding site on sGC, and they are also able to sensitise sGC to low levels of NO by stabilising NO–sGC binding. *cGMP* cyclic guanosine monophosphate, *Fe^{II}* ferrous iron, *GTP* guanosine triphosphate, *NO* nitric oxide, *NOS* nitric oxide synthase, *sGC* soluble guanylate cyclase

some subsequently observed activation characteristics of the enzyme. For example, some research groups have demonstrated that the conversion of the hexa-coordinated intermediate state into the penta-coordinated active nitrosyl–haem complex depends on the concentration of free NO, suggesting that there is a second binding site for NO (Cary et al. 2005; Derbyshire and Marletta 2012; Friebe and Koesling 1998).

3.2 *Pharmacological Stimulation of sGC*

Stimulators of sGC are a novel class of compounds that directly stimulate sGC with a dual mode of action (Fig. 2) (Stasch et al. 2001, 2002a; Stasch and Hobbs 2009). First, they bind at a different site than NO and can increase sGC activity in the absence of NO (Friebe et al. 1996; Mülsch et al. 1997; Stasch et al. 2001). Secondly, they can act synergistically with NO by stabilising the nitrosyl–haem complex to maintain sGC in its active conformation (Russwurm et al. 2002; Schmidt et al. 2003). Therefore, the combined effect of an sGC stimulator and NO on the catalysis of cGMP synthesis is more pronounced than the effects of each compound alone. The signalling molecule cGMP plays an important role in regulating cellular and organ functions such as vascular tone, proliferation, fibrosis and inflammation (Schlossmann and Schinner 2012; Stasch et al. 2011).

The non-haem-binding site of sGC is yet to be identified, but it has been postulated that sGC stimulators bind at the amino-terminus of the $\alpha 1$ subunit of sGC or the pseudosymmetric substrate site of its catalytic domain (Friebe et al. 1999; Lamothe et al. 2004; Stasch et al. 2001; Yazawa et al. 2006). The elucidation of the full-length structure of sGC and co-crystallisation studies will provide further insight into the mechanism of sGC activation and its potentiation by sGC stimulators.

Stimulation of sGC has several potential advantages compared with other therapies targeting the NO–sGC–cGMP pathway. The disadvantages of inhaled NO and NO donor treatment include the resistance of a significant proportion of patients with PH to NO, the development of tolerance to NO, the potential cytotoxic effects of NO and rebound PH after discontinuation of inhaled NO administration (Burney et al. 1999; Erusalimsky and Moncada 2007; Krasuski et al. 2000; Münzel et al. 2005; Radi 2004).

Stimulation of sGC may also have significant advantages over PDE-5 inhibition. Owing to their NO-independent mode of action, sGC stimulators could be effective even when NO production is severely compromised or absent; by contrast, PDE-5 inhibitors merely prevent the degradation of cGMP. When NO signalling is disrupted in PH, the production of cGMP may be severely limited, and the preservation of low cGMP levels by PDE-5 inhibitors may be ineffective. Moreover, when PDE-5 is inhibited, the activity of other PDEs may compensate for it (Stasch et al. 2011).

Additionally, sGC expression is upregulated in vascular smooth muscle cells of small pulmonary arteries in patients with PH compared with those of healthy lung donors, presenting an opportunity for sGC stimulation (Schermuly et al. 2008). This contrasts with the upregulation of PDE-5 in pulmonary arteries (Black et al. 2001; Maclean et al. 1997).

Another potential advantage of sGC stimulators is their synergistic action with the low levels of endogenous NO produced in the lungs of patients with PH. This synergistic activity could ensure optimal ventilation–perfusion matching to maintain or even enhance arterial haemoglobin oxygen saturation (Becker et al. 2011).

4 Discovery of sGC Stimulators

The search for drugs targeting sGC began at Bayer in 1994 with the testing of nearly 20,000 compounds for sGC-inducing activity in primary cultures of endothelial cells. This led to the discovery of a class of compounds, the 5-substituted-2-furaldehyde-hydrazone derivatives, that are direct NO-independent sGC stimulators (Evgenov et al. 2006). However, it was found that exposure to light increased the potency of these substances in stimulating sGC and relaxing isolated blood vessels, which made them unsuitable for further drug development (Evgenov et al. 2006). It was serendipitous that, in 1994, the synthetic bezy lindazole YC-1 was discovered to be an inhibitor of platelet aggregation mediated by intracellular cGMP increase. It had obvious structural similarity to the 5-substituted-2-furaldehyde-hydrazone derivatives and it was found to directly stimulate isolated sGC in the same NO-independent, but haem-dependent, manner (Friebe et al. 1996; Ko et al. 1994; Mülsch et al. 1997). This was the starting point for a chemical optimisation programme and the development of sGC stimulators (Stasch et al. 2001, 2002a).

4.1 YC-1

YC-1 demonstrates both NO-independent and NO-dependent sGC stimulation (Ko et al. 1994; Mülsch et al. 1997). YC-1 increases the activity of purified haem-bound sGC up to 12-fold, and synergy with NO increases this effect by an order of magnitude compared with YC-1 alone (Friebe et al. 1996). YC-1 has weak vasodilatory properties, and inhibits vascular smooth muscle remodelling and platelet aggregation (Cetin et al. 2004; Ko et al. 1994; Mülsch et al. 1997; O'Reilly et al. 2001; Pan et al. 2004; Tulis et al. 2002). YC-1 attenuates hypoxia-induced PAH, right ventricular (RV) hypertrophy and pulmonary vascular remodelling in mice (Huh et al. 2011). However, the clinical utility of YC-1 is limited by its lack of specificity: it inhibits PDEs and has many cGMP-independent effects (Friebe et al. 1998; Galle et al. 1999; Hwang et al. 2003a, b).

4.2 BAY 41-2272

BAY 41-2272 was developed during an optimisation programme that generated more than 2,000 new compounds using YC-1 as a lead structure (Stasch et al. 2001). BAY 41-2272 has a greater specificity than YC-1; up to a concentration of 10 μM it has no

PDE-5 inhibitory activity, and it does not inhibit other cGMP-metabolising PDEs such as PDE-1, PDE-2 or PDE-9 (Bischoff and Stasch 2004; Stasch et al. 2001).

BAY 41-2272 has greater potency than YC-1; in the absence of NO, it stimulates sGC approximately 20-fold above baseline, and in synergy with the NO donor diethylamine NONOate (DEA/NO), it stimulates sGC activity up to 400-fold above baseline (Stasch et al. 2001). BAY 41-2272 is also over 30 times more potent than YC-1 in causing rabbit aortic ring relaxation (Straub et al. 2001).

4.2.1 In Vitro Results

BAY 41-2272 inhibits the conversion of lung fibroblasts to myofibroblasts, which is a feature of pulmonary fibrosis (Dunkern et al. 2007). This suggests that sGC stimulation may be useful in mitigating the progression of pulmonary fibrosis.

BAY 41-2272 also has beneficial effects on other pathological processes. The compound elicits cardioprotective effects in vitro, inhibiting hypertrophy of cultured neonatal rat cardiomyocytes (Irvine et al. 2012). In addition, BAY 41-2272 has been found to protect against ischaemia–reperfusion-induced lung injury in isolated, intact rabbit lungs (Egemnazarov et al. 2009). Moreover, its administration at high doses inhibits platelet aggregation ex vivo (Roger et al. 2010; Stasch et al. 2001).

4.2.2 Studies in Experimental Models of Pulmonary Hypertension

An overview of preclinical studies of sGC stimulators is given in Table 1. The potent vasodilatory activity of BAY 41-2272 has been demonstrated in many animal models of PH. Intravenous infusion of BAY 41-2272 in sheep with acute PH induced by a thromboxane A₂ analogue caused dose-dependent pulmonary and systemic vasodilation, and enhanced and prolonged the pulmonary vasodilatory response to inhaled NO (Evgenov et al. 2004). Inhibition of nitric oxide synthase (NOS) by *N*-nitro-L-arginine methyl ester (L-NAME) in these sheep abolished the systemic but not the pulmonary vasodilatory effects, suggesting that, in the pulmonary vasculature, BAY 41-2272 acts independently of endogenous NO production (Evgenov et al. 2004). In the same model, inhalation of BAY 41-2272 produced selective pulmonary vasodilation (Evgenov et al. 2007). This was also observed following BAY 41-2272 infusion combined with NO inhalation in rabbits with acute lung injury (induced by oleic acid injection) and PH. In addition, the combination of BAY 41-2272 and inhaled NO improved arterial oxygenation and reduced intrapulmonary shunting in these rabbits (Weidenbach et al. 2005).

Intravenous infusion of BAY 41-2272 markedly attenuated heparin–protamine-induced PH in dogs. BAY 41-2272 administration also led to both pulmonary and systemic vasodilation in these dogs and improved arterial oxygen saturation compared with vehicle infusion alone (Freitas et al. 2007). In addition, intravenous infusion of low doses of BAY 41-2272 reduced pulmonary arterial pressure and pulmonary vascular resistance in a canine model of acute pulmonary thromboembolism (Cau et al. 2008).

Table 1 Preclinical studies of soluble guanylate cyclase (sGC) stimulators in pulmonary hypertension

Study design	Treatment	Effects of sGC stimulators	References
Ovine model of acute PH – Pharmacologically induced by the thromboxane A ₂ analogue U-46619	Intravenous infusion of BAY 41-2272	<ul style="list-style-type: none"> • Dose-dependent pulmonary and systemic vasodilation • Augmentation and prolongation of the pulmonary vasodilatory response to inhaled NO • Pharmacological inhibition of eNOS abolished systemic but not pulmonary vasodilator effects 	Evgenov et al. (2004)
Ovine model of severe persistent PH of the newborn – Induced by partial ligation of the ductus arteriosus	BAY 41-2272 infusion into the pulmonary artery	<ul style="list-style-type: none"> • Potent pulmonary vasodilation 	Deruelle et al. (2005a)
Ovine fetal model	Infusion of BAY 41-2272 into the left pulmonary artery	<ul style="list-style-type: none"> • Sustained pulmonary vasodilation was not attenuated by an eNOS inhibitor • More prolonged pulmonary vasodilatory response than following sildenafil administration 	Deruelle et al. (2005b)
Rabbit model of acute lung injury and PH – Pharmacologically induced by oleic acid injection	Combined administration of inhaled NO and intravenously infused BAY 41-2272	<ul style="list-style-type: none"> • Selective pulmonary vasodilation • Improved arterial oxygenation • Reduced intrapulmonary shunting 	Weidenbach et al. (2005)
Rat model of PH – Pharmacologically induced by monocrotaline	Daily oral administration of BAY 41-2272 after fully established PH	<ul style="list-style-type: none"> • Reduced RV systolic pressure 	Dumitrascu et al. (2006)
Mouse model of PH – Induced by chronic hypoxia		<ul style="list-style-type: none"> • Reversal of RV hypertrophy and pulmonary vascular remodelling 	
Neonatal rat model of PH – Induced by hypoxia	Daily intramuscular treatment with BAY 41-2272	<ul style="list-style-type: none"> • Reduced RV hypertrophy • Attenuation of pulmonary arterial wall thickening 	Deruelle et al. (2006)

(continued)

Table 1 (continued)

Study design	Treatment	Effects of sGC stimulators	References
Canine model of PH – Induced by heparin-protamine reaction	Intravenous infusion of BAY 41-2272	<ul style="list-style-type: none"> • Pulmonary and systemic vasodilation • Improved arterial oxygen saturation 	Freitas et al. (2007)
Ovine model of acute PH – Pharmacologically induced by the thromboxane A ₂ analogue U-46619	Inhalation of BAY 41-2272 or BAY 41-8543 BAY 41-8543 inhalation	<ul style="list-style-type: none"> • Selective pulmonary vasodilation • Improved systemic arterial oxygenation • Augmentation of the magnitude and duration of the pulmonary vasodilatory response to inhaled NO 	Evgenov et al. (2007)
	Concurrent administration of the phosphodiesterase inhibitor zaprinast and BAY 41-8543	<ul style="list-style-type: none"> • Augmentation and prolongation of the pulmonary vasodilator effect of BAY 41-8543 	
Canine model of acute pulmonary embolism – Induced by microsphere injection	Intravenous infusion of BAY 41-2272	<ul style="list-style-type: none"> • Pulmonary vasodilation • Systemic vasodilation with higher doses • No adverse effects on arterial oxygen saturation 	Cau et al. (2008)
Rat model of PH – Pharmacologically induced by monocrotaline	Daily oral administration of riociguat after fully established PH	<ul style="list-style-type: none"> • Partial reversal of PH • Partial reversal of RV hypertrophy and pulmonary vascular remodelling 	Schermuly et al. (2008)
Mouse model of PH – Induced by chronic hypoxia		<ul style="list-style-type: none"> • No significant effect on systemic arterial pressure 	
Neonatal rat model of PH – Induced by hypoxia	Daily intraperitoneal administration of BAY 41-2272	<ul style="list-style-type: none"> • Prevention of hypoxia-induced increase in RV systolic pressure and RV hypertrophy comparable to sildenafil treatment • Acute pulmonary and systemic vasodilation 	Thorsen et al. (2010)
Rat model of PH – Pharmacologically induced by monocrotaline	Inhaled and oral BAY 41-8543 Inhaled BAY 41-8543	<ul style="list-style-type: none"> • Decreased pulmonary vascular remodelling • Improved cardiac function • Selective pulmonary vasodilation 	Egemenzarov et al. (2010)

(continued)

Table 1 (continued)

Study design	Treatment	Effects of sGC stimulators	References
Rat model of acute PH – Pharmacologically induced by the thromboxane A ₂ analogue U-46619	Intravenous injection of BAY 41-8543	<ul style="list-style-type: none"> • Pulmonary and systemic vasodilation that was blunted by pharmacological inhibition of eNOS 	Badejo et al. (2010)
Pig model of PH – Induced by hypoxia	Right atrial infusion of BAY 41-8543	<ul style="list-style-type: none"> • Reversal of pulmonary vasoconstriction • Systemic vasodilation 	Lundgren et al. (2012)
Rat model of moderate systemic hypertension – Renin transgenic rats treated with the eNOS inhibitor L-NAME	Daily oral administration of riociguat	<ul style="list-style-type: none"> • Normalisation of blood pressure • Reduced heart and kidney damage • Improved survival 	Sharkovska et al. (2010)
Rat model of malignant systemic hypertension – Induced by 5/6 nephrectomy			
Rat carotid artery balloon injury	Perivascular administration of BAY 41-2272	<ul style="list-style-type: none"> • Reduction in neointimal growth after arterial balloon injury 	Joshi et al. (2011)
Rat model of acute pulmonary embolism – Induced by microsphere injection	BAY 41-8543 infusion	<ul style="list-style-type: none"> • Normalisation of pulmonary haemodynamics • Improved arterial oxygenation 	Watts et al. (2011)
Pig model of septic shock – Induced by endotoxin infusion	Inhalation or intravenous administration of BAY 41-8543	<ul style="list-style-type: none"> • Reduced pulmonary vascular resistance • Increase in cardiac output 	Kronas et al. (2011)
Mouse model of pulmonary fibrosis and PH – Pharmacologically induced by bleomycin	Daily oral administration of riociguat	<ul style="list-style-type: none"> • Attenuation of PH and RV hypertrophy to a greater extent than by sildenafil treatment • Amelioration of pulmonary inflammation and fibrosis • Improved survival 	Evgenov et al. (2011)
Rat model of malignant systemic hypertension – Dahl salt-sensitive rats fed a high salt diet	Daily oral administration of riociguat	<ul style="list-style-type: none"> • Attenuation of systemic hypertension and systolic dysfunction • Attenuation of fibrotic tissue remodelling and degenerative changes in the myocardium and renal cortex • Improved survival 	Geschka et al. (2011)

(continued)

Table 1 (continued)

Study design	Treatment	Effects of sGC stimulators	References
Pig model of PH – Induced by hypoxia	Right atrial infusion of BAY 41-8543	<ul style="list-style-type: none"> • Augmentation of the pulmonary vasodilatory effect of the endothelin receptor blocker tezosentan 	Lundgren et al. (2012)
Rat model of acute pulmonary embolism – Induced by microsphere injection	BAY 41-8543 infusion	<ul style="list-style-type: none"> • Amelioration of RV dysfunction • Reduction of pulmonary vascular resistance 	Watts et al. (2013)
Rat model of PH – Pharmacologically induced by monocrotaline	Intravenous administration of BAY 41-8543	<ul style="list-style-type: none"> • Decreased pulmonary and systemic arterial pressures • Slightly greater decrease in pulmonary arterial pressure in rats that also received chronic sodium nitrite therapy than in those that received BAY 41-8543 alone 	Pankey et al. (2012)
Rat model of angioproliferative PAH – Induced by hypoxia and the vascular endothelial growth factor receptor antagonist SU5416	Oral administration of riociguat	<ul style="list-style-type: none"> • Increased the open-to-occluded artery ratio and decreased the neointima-to-media ratio • Decrease in RV systolic pressure • Decrease in pulmonary vascular resistance • Increase in cardiac output • Greater decrease in RV hypertrophy and improvement in RV function than following sildenafil administration 	Lang et al. (2012)
Mouse model of COPD – Induced by tobacco smoke exposure	Daily oral administration of riociguat	<ul style="list-style-type: none"> • Prevention of PH development • Prevention of RV hypertrophy • Prevention of vascular remodelling 	Seimetz et al. (2012)

(continued)

Table 1 (continued)

Study design	Treatment	Effects of sGC stimulators	References
Diabetic eNOS-knockout mice	Co-administration of riociguat and angiotensin II receptor blocker	<ul style="list-style-type: none"> • Reduced blood pressure • Attenuation of nephropathy 	Ott et al. (2012)
Porcine model of CTEPH – Induced by left pulmonary artery ligation and weekly histocryl pulmonary artery embolisation	Administration of BAY 41-8543 or sildenafil	<ul style="list-style-type: none"> • BAY 41-8543 led to a marked decrease in pulmonary vascular resistance and concomitant increase in cardiac output to a greater extent than sildenafil 	Mercier et al. (2012)
Guinea pigs chronically exposed to cigarette smoke	Daily administration of BAY 41-2272	<ul style="list-style-type: none"> • BAY 41-2272 prevented tobacco smoke-induced pulmonary vascular remodelling and emphysema 	Lobo et al. (2013)
Rat model of angioproliferative PAH – Induced by hypoxia and the vascular endothelial growth factor receptor antagonist SU5416	Oral administration of riociguat	<ul style="list-style-type: none"> • Increased Smad 1/5 phosphorylation in PAH vascular lesions • Decreased medial thickness of PAH vascular lesions 	Schwappacher et al. (2013)

COPD chronic obstructive pulmonary disease, *CTEPH* chronic thromboembolic pulmonary hypertension, *eNOS* endothelial nitric oxide synthase, *L-NAME* *N*-nitro-*L*-arginine methyl ester, *NO* nitric oxide, *PAH* pulmonary arterial hypertension, *PH* pulmonary hypertension, *RV* right ventricular, *sGC* soluble guanylate cyclase

In newborn sheep with persistent PH (induced by partial ligation of the ductus arteriosus), a pulmonary artery infusion of BAY 41-2272 resulted in marked pulmonary vasodilation (Deruelle et al. 2005a). BAY 41-2272 infusion in sheep fetuses also induced a more prolonged pulmonary vasodilator response than sildenafil administration, and this effect was not attenuated by administration of a NOS inhibitor (Deruelle et al. 2005b).

BAY 41-2272 has been shown to have beneficial effects on other cardiopulmonary parameters in several animal models. Intramuscular injections of BAY 41-2272 into newborn rats with hypoxic PH reduced RV hypertrophy and attenuated pulmonary arterial wall thickening (Deruelle et al. 2006). Intraperitoneal administration of BAY 41-2272 in the same rat model prevented a hypoxia-induced increase in RV systolic pressure and RV hypertrophy, and resulted in acute pulmonary and systemic vasodilatation compared with vehicle administration alone (Thorsen et al. 2010).

Oral administration of BAY 41-2272 in rats with monocrotaline-induced PH and in wild-type mice with hypoxia-induced PH reduced RV systolic pressure, and reversed

RV hypertrophy and pulmonary vascular remodelling (Dumitrascu et al. 2006). BAY 41-2272 reduced vascular smooth muscle growth by PKG-specific antimigratory and protein kinase A-specific antiproliferative mechanisms (Joshi et al. 2011; Wharton et al. 2005). Its anti-remodelling activity was demonstrated by a marked reduction in neointimal growth after arterial balloon injury in animals that received perivascular BAY 41-2272 compared with vehicle-treated controls (Joshi et al. 2011).

The clinical use of BAY 41-2272 was, however, not possible because of its strong inhibition and induction of cytochrome P450 isoenzymes (Mittendorf et al. 2009).

4.3 BAY 41-8543

BAY 41-8543 was the sGC stimulator with the most promising potency, specificity and oral activity of the 2,000 compounds derived from the optimisation of YC-1 (Mittendorf et al. 2009; Stasch et al. 2002a, b). BAY 41-8543 has a greater potency than both YC-1 and BAY 41-2272; it stimulates sGC activity up to 92-fold over baseline in the absence of NO and 362-fold over baseline in synergy with the NO donor sodium nitroprusside (Stasch et al. 2002a). Its vasodilatory potency is 500-fold higher than that of YC-1 and three times higher than that of BAY 41-2272 following intravenous administration in normotensive rats (Stasch et al. 2002a; Straub et al. 2001). BAY 41-8543 also has greater specificity than YC-1; it does not inhibit PDE-1, PDE-2, PDE-5 or PDE-9 (Stasch et al. 2002a).

4.3.1 Studies in Experimental Models of Pulmonary Hypertension

BAY 41-8543 has demonstrated beneficial effects in animal models of PH, most notably potent pulmonary vasodilation (Table 1). In sheep with acute PH induced by a thromboxane A₂ analogue, BAY 41-8543 inhalation caused selective dose-dependent reductions in pulmonary artery pressure and pulmonary vascular resistance, improved systemic arterial oxygenation and augmented the magnitude and duration of the pulmonary vasodilatory response to NO inhalation (Evgenov et al. 2007). In a rat model of acute PH induced in the same way, intravenous injection of BAY 41-8543 also resulted in dose-dependent pulmonary and systemic vasodilation, which was blunted by NOS inhibition (Badejo et al. 2010).

Intravenous injection of BAY 41-8543 in rats with monocrotaline-induced PH decreased pulmonary and systemic arterial pressures. Interestingly, the decrease in pulmonary arterial pressure induced by BAY 41-8543 was slightly greater in rats that also received chronic sodium nitrite therapy than in those that received BAY 41-8543 alone (Pankey et al. 2012). Right atrial infusion of BAY 41-8543 in pigs with hypoxic PH reversed pulmonary vasoconstriction and produced systemic vasodilation (Lundgren et al. 2012). It also reversed hypoxia-induced pulmonary vasoconstriction when administered alone or in combination with the endothelin receptor blocker tezosentan in a porcine model (Lundgren et al. 2012).

Moreover, BAY 41-8543 attenuates vascular remodelling. In rats with monocrotaline-induced PH, intratracheal administration of BAY 41-8543 decreased pulmonary vascular remodelling, improved cardiac function and induced selective pulmonary vasodilation (Egemnazarov et al. 2010).

BAY 41-8543 administration has been shown to produce other favourable effects in animal models, for example inhibition of thrombosis (Stasch et al. 2002a, b). Infusion of BAY 41-8543 also normalised pulmonary haemodynamics, improved arterial oxygenation and ameliorated RV dysfunction after experimental pulmonary embolism in a rat model (Watts et al. 2011, 2013). Inhalation or intravenous administration of BAY 41-8543 in pigs with endotoxin-induced septic shock reduced pulmonary vascular resistance and increased cardiac output (Kronas et al. 2011). In addition, BAY 41-8543 and, to a lesser extent, sildenafil produced a marked decrease in pulmonary vascular resistance with a concurrent increase in cardiac output in a CTEPH model in piglets (Mercier et al. 2012).

Despite promising results in animal models, the clinical application of BAY 41-8543 is precluded by unfavourable drug metabolism and pharmacokinetic properties, including high clearance and dose non-linearity of plasma concentrations (Mittendorf et al. 2009).

4.4 Other sGC Stimulators

CFM-1571 is another sGC stimulator that was developed using YC-1 as a lead structure (Selwood et al. 2001). CFM-1571 synergises with NO to stimulate sGC and has no PDE inhibitory activity. However, it has low oral bioavailability and low potency (Selwood et al. 2001; Stasch and Hobbs 2009).

Acrylamide analogues such as A-350619 are direct haem-dependent stimulators of sGC with a similar mode of action to YC-1; they act independently of and in synergy with NO, although they have no structural similarity with YC-1 (Miller et al. 2003; Zhang et al. 2003). Aminopyrimidine analogues are another class of sGC stimulators currently in clinical development (Brockunier et al. 2009).

BAY 60-4552, a close chemical analogue of riociguat (see Sect. 5), has been shown to attenuate cardiac hypertrophy and improve survival in spontaneously hypertensive stroke-prone rats (SHR-SP) maintained on a high salt/fat diet (Costell et al. 2012). In patients with biventricular heart failure and PH, BAY 60-4552 induces potent vasodilation and a significant increase in cardiac index (Gheorghiade et al. 2013; Mitrovic et al. 2009). Another sGC stimulator, vericiguat (BAY 1021189), is currently in clinical phase 1 trials for heart failure (Bayer 2013).

5 Riociguat

5.1 *Discovery*

Extended exploration of the substituent-active region of the pyrazolopyridines (the compound class to which BAY 41-8543 and BAY 41-2272 belong) was performed to address their specificity and pharmacokinetic limitations (Mittendorf et al. 2009). Pharmacokinetic optimisation of more than 800 pyrimidine derivatives led to the identification of the potent sGC stimulator riociguat (BAY 63-2521), which exhibits an improved drug metabolism/pharmacokinetic profile compared with its predecessors, and favourable effects on pulmonary haemodynamics and exercise capacity in patients with PH (Mittendorf et al. 2009; Stasch et al. 2011).

5.2 *Preclinical Evidence*

5.2.1 *In Vitro Results*

Riociguat stimulates recombinant sGC in a dose-dependent manner, up to 73-fold above baseline at 100 μM (Schermulý et al. 2008). Riociguat synergises with the NO donor DEA/NO to increase sGC activity up to 112-fold above baseline (Schermulý et al. 2008). Riociguat is highly specific and does not inhibit PDEs, including PDE-1, PDE-2, PDE-5 and PDE-9 (Schermulý et al. 2008).

The vasodilatory properties of riociguat have been demonstrated in a number of *ex vivo* experiments. Riociguat reduced coronary perfusion pressure in rat heart preparations without affecting left ventricular pressure or heart rate, inhibited contraction of rabbit aortic rings and porcine coronary artery rings and promoted vasorelaxation in arteries isolated from nitrate-tolerant rabbits (Stasch and Hobbs 2009). Riociguat also decreased the acute pulmonary vasoconstriction induced by hypoxia in isolated mouse lungs (Schermulý et al. 2008).

5.2.2 *Studies in Experimental Models of Cardiopulmonary Diseases*

The vasodilatory potency of riociguat has also been demonstrated in animal models of PH (Table 1). Daily oral administration of riociguat in rats with monocrotaline-induced PH and mice with hypoxia-induced PH attenuated PH, RV hypertrophy and pulmonary vascular remodelling without a significant effect on systemic arterial pressure (Schermulý et al. 2008). In addition, riociguat effectively suppressed pulmonary vascular remodelling in other animal models; in rats with severe PH, it increased the open-to-occluded artery ratio and decreased the neointima-to-media ratio, and RV systolic pressure was reduced compared with placebo (Lang et al. 2012). A potential mechanism for the suppression of pulmonary vascular remodelling by riociguat is via its activation of PKG isotype I, which significantly

increased Smad1/5 phosphorylation and decreased the medial thickness of pulmonary vascular lesions in a rat model of PAH (Schwappacher et al. 2013). Riociguat decreased RV hypertrophy and improved RV function to a greater extent than sildenafil in rats with severe PH, and decreased pulmonary vascular resistance and increased cardiac output compared with placebo (Lang et al. 2012). Riociguat was also more effective than sildenafil in attenuating PH and RV hypertrophy in mice with bleomycin-induced PH, and reduced their mortality compared with vehicle-treated mice (Evgenov et al. 2011). Moreover, riociguat treatment prevented tobacco smoke-induced PH, RV hypertrophy and vascular remodelling in a mouse model of COPD (Seimetz et al. 2012).

Riociguat has beneficial effects on other pathological processes. For example, a daily oral administration of riociguat improved survival, normalised blood pressure and reduced heart and kidney damage in rat models of moderate and malignant hypertension (Sharkovska et al. 2010). Riociguat also improved survival and attenuated systemic hypertension, systolic dysfunction and fibrotic tissue remodelling and degeneration in the myocardium and renal cortex in another rat model of malignant systemic hypertension (Geschka et al. 2011). Administration of riociguat in combination with an angiotensin II receptor blocker reduced blood pressure in diabetic eNOS-knockout mice and attenuated nephropathy, as evidenced by reduced urinary albumin excretion (Ott et al. 2012). Finally, riociguat treatment ameliorated pulmonary inflammation and fibrosis in bleomycin-exposed mice (Evgenov et al. 2011).

5.3 *Clinical Trials*

Riociguat is the first sGC stimulator that has entered clinical development. Its dual mode of action (i.e. synergy with endogenous NO and NO-independent stimulation of sGC) restores NO–sGC–cGMP signalling, resulting in significant improvements in pulmonary vascular haemodynamics and increased exercise ability in patients with PH.

A summary of clinical studies of riociguat is given in Table 2. In a phase 1 clinical trial, a single dose of riociguat decreased mean arterial and diastolic pressures and increased heart rate compared with placebo in healthy male volunteers (Frey et al. 2008). Systolic pressure was not affected and no serious adverse events were reported. Riociguat was readily absorbed and demonstrated dose-proportional pharmacokinetics in this study. In another phase 1 study, the drug showed complete oral absorption in healthy men and a dose-proportional increase in systemic exposure (Becker et al. 2012). Riociguat was well tolerated and exhibited no clinically relevant food effects, or pharmacodynamic or pharmacokinetic interactions with aspirin or warfarin in healthy volunteers (Becker et al. 2012; Frey et al. 2011, 2013b). In addition, two exploratory studies have concluded that the safety profile of riociguat is similar in individuals with and without hepatic impairment. These studies also showed that riociguat exposure was significantly increased in patients with moderate, but not mild, hepatic impairment, compared with healthy individuals (Frey et al. 2013a).

Table 2 Clinical studies of riociguat in pulmonary hypertension

Study design	Treatment	Effects of sGC stimulators	References
Randomised, placebo-controlled, single-blinded, parallel-group, single-dose trial – 58 healthy male volunteers	Single dose of riociguat 0.25–5 mg	<ul style="list-style-type: none"> • Well tolerated with a favourable safety profile • Slight but statistically significant decreases in MAP and DBP, but not SBP 	Frey et al. (2008)
Uncontrolled, open-label, single-dose trial – 12 patients with PAH – 6 patients with CTEPH – 1 patient with PH-ILD	Single dose of riociguat, 1 or 2.5 mg	<ul style="list-style-type: none"> • Favourable safety profile • Improvements in all major pulmonary haemodynamic parameters • Mean SBP remained above 110 mmHg 	Grimminger et al. (2009)
Uncontrolled, open-label, 12-week trial – 42 patients with CTEPH – 33 patients with PAH	Dose titration of riociguat from 1 mg t.i.d. to a maximum of 2.5 mg t.i.d. according to SBP and tolerability	<ul style="list-style-type: none"> • Improved pulmonary haemodynamics • Improved exercise capacity 	Ghofrani et al. (2010b)
Uncontrolled, open-label, long-term extension (≤ 24 months) of 12-week trial – 41 patients with CTEPH – 27 patients with PAH	Riociguat 0.5–2.5 mg t.i.d. for at least 15 months	<ul style="list-style-type: none"> • Sustained improvements in exercise capacity and functional class for at least 15 months (interim analysis) 	Ghofrani et al. (2010a)
Single-centre, randomised, double-blind, placebo-controlled, crossover warfarin interaction study – 22 healthy male volunteers	The addition of a single dose of warfarin sodium 25 mg to riociguat 2.5 mg t.i.d. (10 days)	<ul style="list-style-type: none"> • Favourable safety profile • No pharmacodynamic interactions and no clinically relevant pharmacokinetic interactions with warfarin 	Frey et al. (2011)
Three open-label, randomised, crossover studies – Healthy male volunteers (a) 22 for bioavailability study (b) 24 for food effect and dose-proportionality studies	Riociguat 0.5–2.5 mg	<ul style="list-style-type: none"> • Complete oral absorption • Dose-proportional increase in systemic exposure • No clinically relevant food effect • Well tolerated 	Becker et al. (2012)

(continued)

Table 2 (continued)

Study design	Treatment	Effects of sGC stimulators	References
Randomised, open-label, crossover aspirin interaction study – 15 healthy male volunteers	Single dose of riociguat 2.5 mg and two doses of aspirin 500 mg/day	<ul style="list-style-type: none"> • No clinically relevant pharmacodynamic or pharmacokinetic interactions with aspirin 	Frey et al. (2013b)
Two non-randomised, non-blinded exploratory studies with group stratification – 32 patients with liver cirrhosis (16, Child-Pugh A and 16, Child-Pugh B)	Single oral dose of 1 mg riociguat	<ul style="list-style-type: none"> • Safety profile of riociguat similar in individuals with and without hepatic impairment • Exposure to riociguat unchanged in patients with Child-Pugh A (mild) hepatic impairment • Exposure to riociguat significantly increased in patients with Child-Pugh B (moderate) hepatic impairment 	Frey et al. (2013a)
Uncontrolled, open-label, 12-week trial – 22 patients with PH-ILD	Dose titration of riociguat 1.0–2.5 mg t.i.d. continued for 12 weeks	<ul style="list-style-type: none"> • Substantial reduction in pulmonary vascular resistance • Increases in cardiac output and 6-min walk distance 	Hoeper et al. (2013)
Uncontrolled, open-label, two-dose trial – 22 patients with PH-COPD	Single dose of riociguat, 1 or 2.5 mg	<ul style="list-style-type: none"> • Decreased mean pulmonary arterial pressure • Decreased pulmonary vascular resistance 	Ghofrani et al. (2013c)
PATENT-1 trial Phase 3, randomised, placebo-controlled, double-blind 12-week trial – 443 patients with PAH	Dose titration of riociguat 0.5–2.5 mg t.i.d., continued for 12 weeks	<ul style="list-style-type: none"> • Increased 6-min walk distance • Improved pulmonary vascular resistance, functional class, time to clinical worsening and Borg dyspnoea score • Well tolerated 	Ghofrani et al. (2013a)
PATENT-2 Phase 3, open-label, long-term extension study – 363 patients with PAH who had completed PATENT-1	Dose titration of riociguat 0.5–2.5 mg t.i.d., continued for 1 year	<ul style="list-style-type: none"> • Improved 6-min walk distance and functional class • Well tolerated 	Rubin et al. (2013)

(continued)

Table 2 (continued)

Study design	Treatment	Effects of sGC stimulators	References
PATENT PLUS Phase 2, placebo-controlled, double-blind interaction 12-week and long-term extension study – 18 patients with symptomatic PAH receiving stable sildenafil (i.e. ≥ 90 days)	Dose titration of riociguat 0.5–2.5 mg t.i.d., continued for 12 weeks and long-term extension	<ul style="list-style-type: none"> • Similar changes in blood pressure after 12 weeks of riociguat or placebo • Combined riociguat and sildenafil therapy associated with high rate of discontinuation 	Galiè et al. (2013)
CHEST-1 trial Phase 3, randomised, placebo-controlled, double-blind 16-week trial – 261 patients with inoperable or persistent CTEPH	Dose titration of riociguat 0.5–2.5 mg t.i.d., continued for 16 weeks	<ul style="list-style-type: none"> • Increased 6-min walk distance and cardiac index • Improved pulmonary vascular resistance and functional class • Reduced right atrial pressure and mean pulmonary arterial pressure • Positive trend in time to clinical worsening, Borg dyspnoea score and quality of life • Well tolerated 	Ghofrani et al. (2013b), Tse (2012), Bayer (2012), Kim et al. (2013)
LEPHT trial Randomised, placebo-controlled, double-blind dose-finding 16-week trial – 210 patients with PH associated with left ventricular systolic dysfunction	Dose titration of riociguat 0.5–2 mg t.i.d., continued for 16 weeks	<ul style="list-style-type: none"> • Improved cardiac index, pulmonary and systemic vascular resistance and quality of life • No significant changes in mean pulmonary arterial pressure and systemic blood pressure • Well tolerated 	Bonderman et al. (2013)

COPD chronic obstructive pulmonary disease, *CTEPH* chronic thromboembolic pulmonary hypertension, *DBP* diastolic blood pressure, *MAP* mean arterial pressure, *PAH* pulmonary arterial hypertension, *PH-COPD* pulmonary hypertension due to chronic obstructive pulmonary disease, *PH-ILD* pulmonary hypertension due to interstitial lung disease, *SBP* systolic blood pressure, *sGC* soluble guanylate cyclase, *t.i.d.* three times daily

Several phase 2 clinical trials assessing the safety and efficacy of riociguat in the treatment of different types of PH have been completed. The results of two large phase 3 placebo-controlled trials, PATENT-1 and CHEST-1, investigating the efficacy and safety of oral riociguat in patients with PAH and CTEPH, respectively, have also been presented (Ghofrani et al. 2013a, b).

5.3.1 Pulmonary Arterial Hypertension

In a first study of 19 patients with moderate-to-severe PH, including 12 patients with PAH, single doses of riociguat (2.5 or 1 mg) had a favourable safety profile (Grimminger et al. 2009). Riociguat significantly reduced mean pulmonary arterial pressure and pulmonary vascular resistance and increased cardiac index in a dose-dependent manner compared with baseline, and to a greater extent than inhaled NO. The drug had no effect on gas exchange or ventilation–perfusion matching. Riociguat also exerted dose-dependent systemic effects in these patients, but mean systolic blood pressure remained above 110 mmHg.

In a follow-up study of 33 patients with PAH, treatment with riociguat for 12 weeks had a favourable safety profile and improved their symptoms, pulmonary haemodynamics (e.g. reduced pulmonary vascular resistance) and exercise capacity [as demonstrated by an increased 6-min walk distance (6MWD)], compared with baseline (Ghofrani et al. 2010b). The improvements from baseline in 6MWD and functional class in patients with PAH after 12 weeks' treatment with riociguat were sustained for at least a further 15 months. Long-term riociguat treatment was also well tolerated by these patients (Ghofrani et al. 2010a).

In the phase 3 PATENT-1 trial, riociguat significantly improved pulmonary haemodynamics, dyspnoea score and functional class, and increased exercise ability in patients with PAH (Ghofrani et al. 2013a). In this trial, 443 patients with symptomatic PAH were randomised to receive placebo or riociguat for 12 weeks. Riociguat significantly increased 6MWD from baseline compared with placebo both in patients receiving endothelin receptor antagonists (ERAs) or inhaled prostanoids and in treatment-naïve patients (Ghofrani et al. 2013a). A number of other clinically relevant measures of efficacy were also consistently improved by riociguat compared with placebo, including pulmonary vascular resistance, N-terminal prohormone of brain natriuretic peptide (NT-proBNP) levels, functional class, time to clinical worsening and Borg dyspnoea score. Riociguat was well tolerated and had a favourable safety profile in this study, both as a monotherapy and in combination with ERAs or inhaled prostanoids (Ghofrani et al. 2013a). In the PATENT-2 long-term extension study, riociguat treatment for an additional year was also well tolerated and improved 6MWD and functional class compared with baseline (Rubin et al. 2013).

In the PATENT PLUS interaction study, administration of riociguat to patients with symptomatic PAH receiving stable sildenafil therapy resulted in similar changes in blood pressure as placebo after 12 weeks. However, in a long-term extension of this study, the combined administration of riociguat and sildenafil was associated with a high rate of discontinuation due to hypotension and there was no evidence that the combination exerted any favourable clinical effect (Galiè et al. 2013).

5.3.2 Chronic Thromboembolic Pulmonary Hypertension

The first study of riociguat in patients with PH included six patients with CTEPH (Grimminger et al. 2009). Results from the overall study population are described in the previous section. A sub-analysis of the patients with CTEPH showed a significant improvement in cardiac index following administration of riociguat compared with NO inhalation.

The outcome of 12 weeks' treatment with riociguat in a group of 42 patients with CTEPH was similar to that observed in patients with PAH; their symptoms and pulmonary haemodynamics (including pulmonary vascular resistance) improved and their exercise capacity increased compared with baseline (Ghofrani et al. 2010b). These beneficial effects persisted for at least 15 months and were associated with a favourable safety profile (Ghofrani et al. 2010a).

Preliminary results of the phase 3 CHEST-1 trial have demonstrated that riociguat benefits patients with CTEPH by improving pulmonary haemodynamics, dyspnoea score and functional class and increasing 6MWD and cardiac index (Bayer 2012; Ghofrani et al. 2013b; Kim et al. 2013). A total of 261 patients with inoperable, persistent or recurrent CTEPH were randomised to receive placebo or riociguat for 16 weeks (Ghofrani et al. 2013b). Riociguat treatment resulted in a significant increase in mean 6MWD of 46 m from baseline compared with placebo (Ghofrani et al. 2013b; Tse 2012).

5.3.3 Pulmonary Hypertension Associated with Interstitial Lung Disease

After 12 weeks' treatment with riociguat, a slight improvement in 6MWD, a reduction in pulmonary vascular resistance and an increase in cardiac output were observed in patients with pulmonary hypertension associated with interstitial lung disease (PH-ILD) compared with baseline. Riociguat was well tolerated by most of these patients (Hoepfer et al. 2013).

5.3.4 Pulmonary Hypertension Due to Chronic Obstructive Pulmonary Disease

Riociguat administered to patients with PH due to COPD decreased mean pulmonary arterial pressure and pulmonary vascular resistance compared with baseline. Selected parameters of lung function were slightly improved compared with baseline, whereas gas exchange was not significantly affected in these patients (Ghofrani et al. 2013c). The maintenance of gas exchange suggests that the vasodilation induced by riociguat did not cause ventilation–perfusion mismatch in these patients.

5.3.5 Pulmonary Hypertension Associated with Left Ventricular Systolic Dysfunction

Riociguat has also been shown to provide significant benefits to patients with PH associated with left ventricular systolic dysfunction already receiving optimised therapy for heart failure (Semigran et al. 2012). In the LEPHT trial, 210 patients with chronic systolic heart failure and PH underwent right heart catheterisation before and after receiving placebo or riociguat for 16 weeks (Bonderman et al. 2013). Riociguat treatment was well tolerated and improved cardiac index, pulmonary and systemic vascular resistance and quality of life in these patients, without significantly changing mean pulmonary arterial pressure or systemic blood pressure compared with placebo (Ghio et al. 2012; Semigran et al. 2012).

5.4 Ongoing Clinical Trials

The results of several clinical trials of riociguat are awaited. CHEST-2 (NCT00910429) is a long-term, open-label extension of the phase 3 trial CHEST-1 trial. Other trials include a phase 2 study of riociguat in patients with PH-ILD (NCT00694850) and a phase 2 DILATE trial (NCT01172756) assessing the haemodynamic effects and safety of riociguat in patients with PH associated with left ventricular diastolic dysfunction.

5.5 Future Directions

Stimulators of sGC may have therapeutic applications in diseases other than PH. For example the restoration of NO-sGC-cGMP signalling and the favourable haemodynamic effects and cardiac protective properties of sGC stimulators may be beneficial for patients with heart failure (Gheorghiadu et al. 2013; Mitrovic et al. 2009; Stasch and Hobbs 2009). In particular, the antifibrotic properties of sGC stimulators may benefit these patients. This possibility is supported by the results of experiments in a mouse model of chronic RV pressure overload in which riociguat treatment reduced the collagen content of the right ventricle and improved the RV ejection fraction (Schymura et al. 2012).

New evidence indicates that sGC stimulators may constitute a potential new therapy for lung fibrosis. This includes the attenuation of pulmonary fibrosis by orally administered riociguat in a mouse model, and the inhibition of the conversion of lung fibroblasts to myofibroblasts by BAY 41-2272 in combination with sildenafil (Dunkern et al. 2007; Evgenov et al. 2011). In addition, the antifibrotic activity and vasoactive properties of sGC stimulators make them promising candidates for the treatment of scleroderma. It has recently been discovered that sGC stimulators exert potent antifibrotic effects in experimental models of systemic sclerosis (Beyer et al. 2012).

NO production in the airways is reduced in patients with COPD (Clini et al. 2000). The use of sGC stimulators may therefore have beneficial effects beyond the alleviation of PH in these patients. Support for this hypothesis is provided by experiments in mice that showed that riociguat prevented the development of tobacco smoke-induced emphysema (Seimetz et al. 2011; Stasch et al. 2011). In similar experiments in guinea pigs, BAY 41-2272 prevented both tobacco smoke-induced emphysema and pulmonary vascular remodelling (Lobo et al. 2013).

Sickle cell disease is another condition that may be amenable to sGC stimulator therapy. NO bioavailability is reduced in this disease due to oxidative stress and the sequestration of NO by free haemoglobin released into the plasma following erythrocyte haemolysis. It has been suggested that sGC stimulators may be able to bypass NO scavenging by free haemoglobin in sickle cell disease (as well as in other haemolytic diseases and during the transfusion of aged blood or haemoglobin-based oxygen carriers) to reverse haemoglobin-mediated vasoconstriction (Raaf et al. 2013). It has also been shown that the increased adhesion of neutrophils, which contributes to veno-occlusion in sickle cell disease, is reduced by BAY 41-2272 (Canalli et al. 2008; Miguel et al. 2011). Furthermore, in a murine model of sickle cell disease, priapism associated with dysregulation of NO-sGC-cGMP signalling may be ameliorated by treatment with sGC stimulators (Claudino et al. 2009).

Disrupted NO-sGC-cGMP signalling is a common pathogenic feature of many cardiovascular diseases. Although clinical studies of sGC stimulators are currently focusing on the treatment of PH, their therapeutic potential has been, and continues to be, explored in a wide range of experimental disease models. In addition, research is in progress to identify and optimise new compounds in this drug class for the treatment of a number of specific diseases.

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Therapeutics Targeting of Dysregulated Redox Equilibrium and Endothelial Dysfunction

Michael G. Risbano and Mark T. Gladwin

Abstract All forms of WHO Group 1 PAH share a progressive and complex vasculopathy. At the center of this derangement lies the pulmonary vascular endothelium, which plays a crucial role in maintaining a delicate and precise balance of opposing vasoconstricting and vasodilating forces. In PAH, endothelial cell damage and dysfunction alter vascular homeostasis in favor of vasoconstriction. There is evidence of increased expression and activity in the vasoconstrictor and mitogen endothelin-1 signaling system and a decreased production of the potent vasodilator prostacyclin. These pathways have been a major focus of FDA approved PAH-specific therapies. Beyond these pathways, there is the dysfunction within the endothelial nitric oxide (NO) synthase signaling pathway and dysregulation of reactive oxygen and nitrogen species (ROS) that contribute to the pathogenesis of PAH. The dysregulation of vasodilator systems in PAH in large part involves the NO pathway, with almost every step subject to impairments. This includes a reduction in endothelial NO synthase function (eNOS), the enzymatic “uncoupling” of eNOS, increased scavenging of NO by superoxide and cell-free hemoglobin, the elaboration of endogenous competitive inhibitors of eNOS (ADMA), and the oxidation of the NO target, soluble guanylyl cyclase. The dysregulation of NO signaling pathways occurs in the setting of parallel upregulation of vascular oxidases that generate ROS. Enzymatic sources of ROS in PH that have been identified include the NAPDPH oxidases 1, 2, and 4, xanthine oxidase, uncoupled eNOS, and complex III of the mitochondrial electron transport chain. Superoxide produced from these sources reacts with NO to form the reactive nitrogen species peroxynitrate, further diverting bioavailable NO to more injurious species. In PAH, this upstream dysregulation of ROS/NO redox homeostasis severely impairs vascular tone and contributes to the pathological activation of

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mitogenic pathways, leading to cellular proliferation and obliteration of the pulmonary vasculature. Therapeutic strategies are being evaluated that target the associated dysregulated redox equilibrium and endothelial dysfunction in PAH. Therapeutic interventions reviewed in this chapter include NO donor or NO generating drugs, therapies that recouple eNOS or directly increase cGMP levels via inhibition of phosphodiesterase 5 or stimulation of soluble guanylyl cyclase, and therapies that inhibit vascular oxidases or scavenge ROS.

Keywords Endothelial dysfunction • Nitric oxide • Redox equilibrium

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List of Abbreviations

eNOS	Endothelial nitric oxide synthase
PAH	Pulmonary arterial hypertension
PH	Pulmonary hypertension
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

1 Introduction

1.1 Pulmonary Arterial Hypertension, Defined

Pulmonary arterial hypertension (PAH) is characterized by obstructive cellular proliferation and vasoconstriction of the pulmonary arterial vasculature (Tuder et al. 1994). A decrease in pulmonary artery lumen is a consequence of a

derangement in multiple, complex pathways and includes (a) endothelial cell dysfunction and increased contractility of small pulmonary arteries, (b) proliferation and remodeling of endothelial and smooth muscle cells, and (c) in situ thrombosis. This pathologic remodeling ultimately leads to an increase in pulmonary vascular resistance with an increased afterload, causing a reduction in cardiac output as the right heart fails. The diagnosis of pulmonary arterial hypertension must be demonstrated hemodynamically by right heart catheterization with a resting mean pulmonary artery pressure (mPAP) ≥ 25 mmHg without an elevation in pulmonary capillary wedge pressure (<15 mmHg) (McLaughlin et al. 2009a, b).

PAH is a rare disease with an estimated prevalence of 15 cases per million persons in France (Humbert et al. 2006) with a historical survival of a median of 2.8 years after diagnosis (McLaughlin et al. 2009a, b). Even in the current era of PAH-specific therapy prognosis remains poor with a 1-, 2-, and 3-year survival of 87 %, 76 %, and 67 % respectively (Humbert et al. 2010).

1.2 Pulmonary Arterial Hypertension: A Disorder of Vasoconstriction and Vasodilation

All forms of WHO Group 1 PAH share a progressive and complex vasculopathy. At the center of this derangement lies the pulmonary vascular endothelium, which plays a crucial role in maintaining a delicate and precise balance of opposing vasoconstricting and vasodilating forces. In PAH, endothelial cell damage and dysfunction alter vascular homeostasis in favor of vasoconstriction. There is evidence of increased expression and activity in the vasoconstrictor and mitogen endothelin-1 signaling system (Stewart et al. 1991; Giaid et al. 1993), a decreased production of the potent vasodilator prostacyclin with increased release of the vasoconstrictor thromboxane A₂ (Christman et al. 1992). These pathways have been a major focus of FDA approved PAH-specific therapies. Beyond these pathways, there is the dysfunction within the endothelial nitric oxide synthase signaling pathway and dysregulation of reactive oxygen and nitrogen species that contribute to the pathogenesis of pulmonary arterial hypertension. Investigation into these alternative pathways has produced potential PAH-specific treatments aimed at increasing nitric oxide production or generation, increasing cGMP and GSNO or reducing ROS/RNS. These therapeutic interventions may serve to compliment current existing treatment modalities.

2 Mechanisms for Decreased NO Bioavailability in PAH

2.1 NO Signaling and Endothelial Dysfunction

Nitric oxide is a diatomic gas molecule that is produced by endothelial NO synthase enzyme via the 5-electron oxidation of L-arginine to form L-citrulline and NO. NO then diffuses as a paracrine messenger from endothelium to smooth muscle, where it

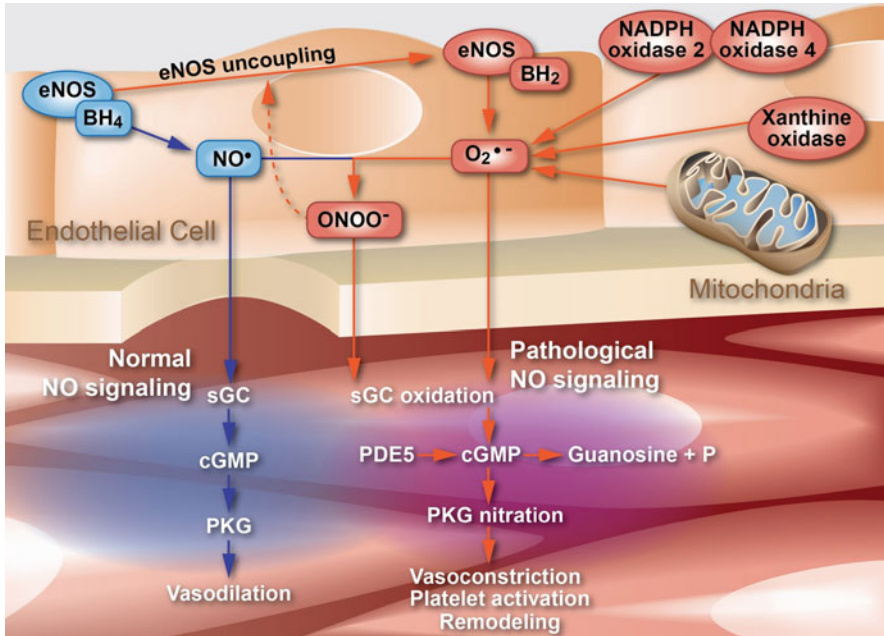


Fig. 1 Schematic representation of the various forms of free radical production from eNOS uncoupling, xanthine oxidase, NADPH, and downstream effects within the pulmonary endothelial cell. Reproduced with permission from Tabima et al. (2012)

binds to the heme group of soluble guanylyl cyclase (sGC) (see Fig. 1). This activates sGC, which converts guanosine triphosphate (GTP) to the cyclic guanosine nucleotide monophosphate (cGMP). cGMP in turn activates protein kinases to produce smooth muscle relaxation, vasodilation and increases in blood flow (Arnold et al. 1977; Ignarro et al. 1982, 1987; Palmer et al. 1987, 1988). As an endothelium-derived relaxing factor, nitric oxide (NO) is an important signaling molecule that regulates basal vasomotor tone, blood pressure, and inhibition of vascular cell growth. NO also regulates vascular smooth muscle proliferation and migration (Kibbe et al. 2000; Zuckerbraun et al. 2007). The normal production of NO by the endothelium is impaired in patients with cardiovascular disease, associated risk factors of increasing age, smoking, diabetes, obesity, hypercholesterolemia, and in PAH.

This NO–sGC–cGMP pathway regulates approximately 25 % of resting blood flow and blood pressure (Quyyumi et al. 1995; Cannon et al. 2001). With endothelial dysfunction, the NO synthase enzyme fails to produce NO. This can be measured in humans (and animals) by infusing acetylcholine into the brachial artery. Acetylcholine activates muscarinic receptors to increase intracellular levels of calcium and activate eNOS, which increases blood flow. Alternatively infusions of N-monomethyl-L-arginine (L-NMMA) - a nonselective competitive inhibitor of eNOS, will decrease blood flow. In patients with endothelial dysfunction the normal vasoconstriction to L-NMMA and vasodilation to acetylcholine are blunted, indicating a failure of endothelial eNOS to produce NO under basal and stimulated

conditions, respectively. In studies of endothelial dysfunction, the direct NO donor sodium nitroprusside is infused to test eNOS and endothelium-independent NO signaling, since NO produced by nitroprusside will directly activate sGC.

The dysregulation of vasodilator systems in PAH in large part involves the NO pathway, with almost every step subject to impairments. This includes a reduction in endothelial NO synthase function (eNOS), the enzymatic “uncoupling” of eNOS (see Fig. 1), increased arginase activity that metabolizes L-arginine (Zuckerbraun et al. 2011), increased scavenging of NO by superoxide and cell-free hemoglobin, the elaboration of endogenous competitive inhibitors of eNOS (ADMA), and the oxidation of the NO target, soluble guanylyl cyclase. The endothelial dysfunction in PAH is characterized by early impaired endothelium-dependent vasodilation, as evidenced by a lack of vasodilator response to acetylcholine, bradykinin, or calcium ionophore and an increased superoxide production in the vessel wall with subsequent decreased NO bioavailability (Rabinovitch 2008).

In the following section, we summarize key aspects of the current status of NO signaling in PAH related to (a) reduction, dysfunction, or uncoupling of eNOS, (b) downstream effects on NO–cGMP signaling, (c) Arginase I and II, and (d) catabolism of NO by ROS or hemoglobin.

2.2 *eNOS Uncoupling*

Despite reports of varying levels of eNOS measured in the lung it is the functional reduction in NO signaling that is consistently reported in the animal models and in patients with PAH. The reduction in endogenous NO formation, as measured by reduced basal or stimulated NO enzymatic synthesis, has been demonstrated in patients with primary PAH (Demoncheaux et al. 2005) and in the hypoxic rat model of PH. A decrease in NO production results in a reduction of vasodilation regardless of normal or increased eNOS protein levels (Weerackody et al. 2009). The paradox of a normal or increased eNOS expression and reduced NO signaling is most likely explained by a pathologic mechanism called “eNOS uncoupling” (see Fig. 1).

Under normal circumstances eNOS forms a homodimer, and each monomer contains an oxygenase domain at the N-terminus. This oxygenase domain contains binding sites for tetrahydrobiopterin (BH₄), the heme iron, and L-arginine (Gielis et al. 2011), while there is a reductase domain at the C-terminus (List et al. 1997; Venema et al. 1997; Forstermann and Munzel 2006). In the dysfunctional state of the uncoupled eNOS enzyme, electrons that normally transfer from the NOS reductase domain to the oxygenase domain are diverted to molecular oxygen (O₂) thus forming superoxide (O₂^{•-}) rather than transferring electrons to L-arginine (Beckman and Koppenol 1996) to form NO. eNOS therefore exhibits oxidase activity rather than NO synthase activity. NO levels are reduced by primary diversion of electrons to oxygen and via secondary NO scavenging reaction by NOS-generated superoxide, which can react to form peroxynitrite (ONOO⁻). Triggers for eNOS uncoupling include the depletion of necessary cofactors L-arginine and BH₄ (Kunuthur et al. 2011). The oxidation of BH₄ to dihydrobiopterin (BH₂) is thought to be central

to enzyme coupling (Cunnington and Channon 2010), with a number of models suggesting that the eNOS-bound BH2 may be more important than low levels of BH4. This is measured as either a decrease in the BH4:BH2 ratio or increases in BH2. A key observation is that eNOS generates superoxide when either BH4 levels decline or oxidized BH2 increases (Khoo et al. 2005; Crabtree et al. 2009). Consistent with this pathobiology, animal models of PH have low BH4:BH2 ratios. (Khoo et al. 2005; Grobe et al. 2006).

2.3 Arginase (I and II)

NO production is the result of the oxidation of L-arginine to citrulline. As expected, a decrease in the substrate L-arginine leads to a decrease in NO bioavailability. In chronic hemolytic diseases, such as sickle cell anemia, a chronic elevation in circulating arginase I has been noted, which decreases NO bioavailability (Morris et al. 2005, 2008). Arginases are increased in sickle cell disease as a result of liver dysfunction, inflammation, and most importantly the release from red blood cells during hemolysis. The levels of arginase II are also elevated in idiopathic PAH (Xu et al. 2004). Arginase catalyzes arginine to ornithine and urea, and its activity can be measured in patients as a decreasing ratio of arginine/ornithine (Morris et al. 2003, 2005; Xu et al. 2004). This reaction occurs with arginase I (the final step of the urea cycle, which occurs mainly in the cytoplasm of the liver, and also in human red blood cells) and arginase II (which occurs within mitochondrion intracellularly). Overexpression of arginases affects NO synthase activity and decreases the NO-dependent smooth muscle relaxation by depleting the L-arginine substrate that would otherwise be available to eNOS. In fact, Langle et al. (1995) has demonstrated that an acute release of hepatic arginase into the pulmonary circulation in the setting of liver transplant and reperfusion resulted in acute pulmonary vasoconstriction.

Sickle cell patients with increased plasma arginase activity have reduced L-arginine:ornithine ratios and in turn have more severe PAH and higher mortality (Morris et al. 2005). In IPAH patients plasma levels of L-arginine correlate with right atrial pressure, cardiac index and 6-minute walk distance (Beyer et al. 2008). Additionally arginase activity alone may promote the development and vasculopathy of PAH (Sharma et al. 2009) through NO-independent pathways, such as production of ornithine which may promote vascular remodeling and proliferation (Xu et al. 2004; Chen et al. 2009).

2.4 Hemolysis as a Mechanism of Endothelial Dysfunction and Vasculopathy

A number of disease states culminate in the hemolysis of erythrocytes; most notably sickle cell anemia, thalassemia, hemodialysis-associated hemolysis, malaria, and paroxysmal nocturnal hemoglobinuria (PNH). Hemolysis causes a

decompartmentalization of cellular contents into the intravascular space. The release of materials including hemoglobin, arginase I, and asymmetric dimethylarginine (ADMA) produces a net effect of resistance to NO signaling and NO donor infusions (Reiter et al. 2002; Gladwin et al. 2004; Morris et al. 2005; Schnog et al. 2005; Landburg et al. 2008; Kato et al. 2009; D'Alecy and Billecke 2010; Landburg et al. 2010).

During steady-state sickle cell disease, there is a high rate of intravascular hemolysis. This is reflected by a depletion of haptoglobin in steady state in patients with homozygous HbSS disease and measurable cell-free hemoglobin in the plasma (Reiter et al. 2002; Rother et al. 2005). Hemoglobin, when decompartmentalized from the red cell, will react with NO at the near diffusion limit, to oxidize the NO to nitrate (Doherty et al. 1998; Dou et al. 2002). This reaction is so fast and irreversible that even levels of cell-free plasma hemoglobin of only 6–10 μM are sufficient to inhibit all NO signaling and produce vasoconstriction (Reiter et al. 2002). In addition to cell-free hemoglobin, hemolysis releases other red cell enzymes that have the potential to inhibit NO signaling. Arginase I is present in abundance in red cells and metabolizes arginine to ornithine, reducing arginine availability for NO synthesis (Morris et al. 2005). Hemolysis is also associated with ADMA accumulation, an endogenous competitive NO synthase inhibitor (Schnog et al. 2005; Kato et al. 2009). Hemoglobin also drives oxidative reactions via fenton and peroxidase chemistry and possibly via the induction of vascular oxidases, such as xanthine oxidase and NADPH oxidase, which are both upregulated in mouse models of SCD (Aslan et al. 2001; Wood et al. 2005, 2008). The net effect of hemolysis is a resistance to NO signaling and NO donor infusions (Kaul et al. 2000; Reiter et al. 2002; Kaul et al. 2004). For example, in humans and in transgenic mouse models that express human hemoglobin S, there is a reduction in the vasodilator effect of infusions of NO donors. In both humans with SCD and in mouse models of SCD, the magnitude of the blunted responses to NO donors is proportional to the plasma hemoglobin level (Dasgupta et al. 2010; Reiter et al. 2002; Kaul et al. 2004). In addition to direct inhibition of NO by reaction with hemoglobin, NO resistance can also occur secondary to inactivation reactions with superoxide, as well as direct oxidation and inactivation of soluble guanylate cyclase, the target for NO (Gladwin 2006).

Most studies have supported this hypothesis over the last decade using animal models, human vascular studies, and large epidemiological cohort studies. For example, humans with SCD and transgenic mice with SCD have impaired vasodilator responses to NO that are proportional to the levels of plasma hemoglobin (Reiter et al. 2002; Kaul et al. 2004; Hsu et al. 2007). Multiple mouse models of hemolysis develop spontaneous PH and right heart failure including the Berkeley sickle cell mouse (Hsu et al. 2007), the spherocytosis mouse (Frei et al. 2008), and the alloimmune hemolysis mouse (Hsu et al. 2007). In transgenic sickle mice, the blunted vasodilator response to NO directly relates to plasma hemoglobin levels (Kaul et al. 2004) and NO metabolite levels are inversely correlated with plasma hemoglobin levels (Dasgupta et al. 2010). NO resistance, PH, and systemic hypertension can be produced experimentally in animals by induced intravascular hemolysis or by infusions of hemoglobin or hemolysate (Minnecci et al. 2005; Tofovic et al. 2009).

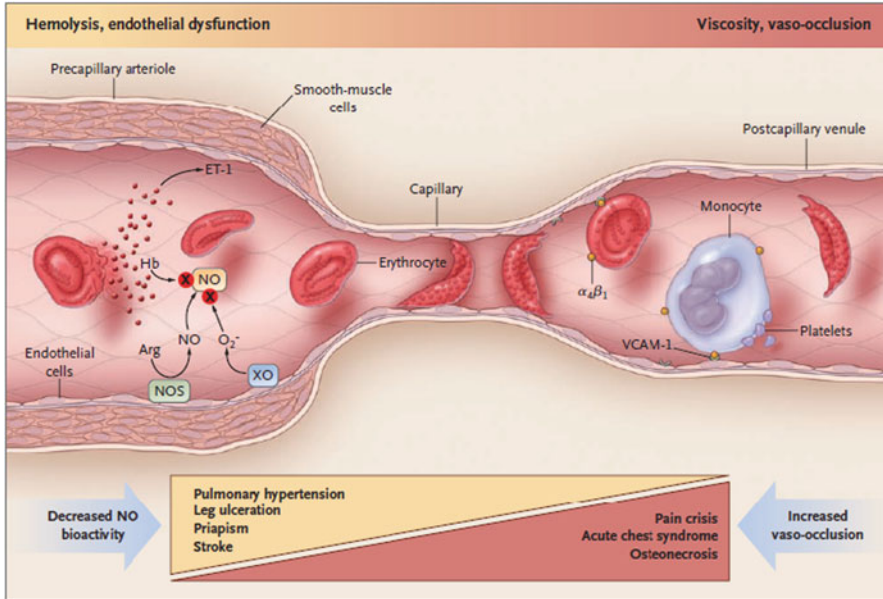


Fig. 2 Mechanisms for hypothetical vascular involvement in sickle cell anemia. There are two overlapping mechanisms proposed for vascular related disease in sickle cell anemia, (a) on the *left*, a low steady-state hemoglobin levels and high rates of intravascular hemolysis are a result of endothelial dysfunction, mediated by the inactivation of NO by plasma-free hemoglobin, increased ROS production and arginase activity; (b) on the *right*, increased steady-state leukocyte counts and increased hemoglobin levels may cause hemolysis-associated endothelial dysfunction through hemostatic activation and intimal/smooth muscle proliferation and therefore obstruction of capillaries and post-capillary venules. The relative frequency of associated clinical phenotypes with the degree of hemolysis/endothelial dysfunction and increased viscosity/vaso-occlusion are depicted by the two complementary triangles. *Arg* arginine, *ET-1* endothelin-1, *NO* nitric oxide, *NOS* nitric oxide synthase, O_2^- superoxide, *VCAM-1* vascular cell adhesion molecule-1, *XO* xanthine oxidase. Reproduced with permission Gladwin and Vichinsky (2008)

In humans, impairment in NO signaling, directly measured via venous occlusion strain-gauge plethysmography, correlates with high levels of plasma hemoglobin or its surrogate lactate dehydrogenase (Reiter et al. 2002). The relationship in humans between increasing plasma hemoglobin levels and direct measures of decreasing NO-dependent blood flow or low NO bioavailability have now been reproduced in numerous studies in disparate diseases, such as hemodialysis-associated hemolysis (Meyer et al. 2010), malaria (Yeo et al. 2007, 2009), and PNH (Hill et al. 2010).

From an epidemiological standpoint, in patients with sickle cell disease their clinical vasculopathic complications are significantly associated with markers of hemolytic anemia, including PH, priapism, and leg ulceration (Gladwin et al. 2004; Nolan et al. 2005; Kato et al. 2006; Nolan et al. 2006) (see Fig. 2). Many cohort studies have consistently associated the severity of hemolytic anemia with increasing Doppler-estimated pulmonary artery systolic hypertension and high risk of death, including the NIH-PH cohort (Gladwin et al. 2004), the Duke cohort

(De Castro et al. 2008), the UNC cohort (Ataga et al. 2006), the Muti-Centers Study of Hydroxyurea cohort (Machado et al. 2006), the Pediatric Hypoxic Response (PUSH) cohort (Onyekwere et al. 2008; Minniti et al. 2009; Naoman et al. 2010), and a cohort study from Greece (Voskaridou et al. 2010). An analysis of banked plasma samples from the classic cooperative study of sickle cell disease (CSSCD) cohort revealed that an abnormally high NT-proBNP level ≥ 160 pg/ml, a biomarker for pulmonary hypertension in patients with sickle cell disease (Machado et al. 2006), was present in 27.6 % of adult SCD patients, and high levels were associated with markers of hemolytic anemia such as a low hemoglobin level ($P < 0.001$), high lactate dehydrogenase ($P < 0.001$), and high total bilirubin levels ($P < 0.007$) (Machado et al. 2009). Most importantly, an NT-proBNP level ≥ 160 pg/ml was a major and independent predictor of mortality (RR 6.24, 95 % CI 2.9–13.3, $P < 0.0001$). The recently published analysis of >600 screening patients in the Walk-PHASST cohort confirms similar strong associations between indices of hemolytic anemia, high NT-proBNP, low walk distance and increased Doppler-echocardiographic estimates of pulmonary artery systolic pressures (Sachdev et al. 2011).

Three additional recently published studies in patients with sickle cell disease confirmed the associations between the severity of hemolytic anemia and pulmonary hypertension defined by right heart catheterization (Parent et al. 2011; Fonseca et al. 2012; Mehari et al. 2012).

2.5 Oxidative Stress, ROS, and RNS

Free radicals are oxygen-derived reactive oxygen species (ROS) that have one or more unpaired electrons, superoxide ($O_2^{\bullet-}$), hydroperoxyl (HO_2^{\bullet}), peroxy (RO_2^{\bullet}), and hydroxyl (OH^{\bullet}) that along with non-radical peroxides (ROOH) like hydrogen peroxide (H_2O_2) are regarded as ROS. These ROS oxidize various targets. Both human subjects (Joppa et al. 2007) with PAH and animal models of PH have demonstrated increased levels of oxidative stress (DeMarco et al. 2008; Jankov et al. 2008).

Reactive nitrogen species or RNS (NO^{\bullet} , NO_2^{\bullet} , nitrogen dioxide; N_2O_3 , nitrogen trioxide; $ONOO^-$, peroxynitrite) are chemically derived from NO and result from the oxidation of the terminal nitrogen of L-arginine. RNS are produced during the urea cycle in the presence of O_2 and NOS (Brown and Borutaite 2006). RNS reacts with reactive sulfur species creating sulfenic acid and other thiol or thyl radicals ($RSOH$; RS^- and RS^{\bullet}). N_2O_3 acts as a nitrosating agent to add a NO^+ group to secondary amines or thiols to produce *N*-nitrosamines and *S*-nitrosothiols (RSNO), respectively. Such *S*-nitrosation of the cysteine thiol of glutathione by NO^+ or by transnitrosation reactions with other *S*-nitrosothiols results in the formation of *S*-nitrosoglutathione (GSNO). The production of RSNO affects the downstream signaling of the NO pathway both physiologically and pathologically (Moya et al. 2002; Foster et al. 2003; Gaston et al. 2006).

It is increasingly apparent that enhanced production and dysregulation of ROS/RNS signaling contributes to the pathogenesis of PAH (DeMarco et al. 2008). The generation of ROS/RNS has been described in antimicrobial defense as well as physiological responses in redox signaling ranging from cellular differentiation and antioxidant gene expression to proliferation and apoptosis (Fialkow et al. 2007). Oxidative stress is the result of the imbalance of excessive formation of ROS and RNS and/or a decrease in antioxidant defenses. Cellular homeostasis is affected through oxidative damage and posttranslational redox modifications of proteins, lipids, and nucleic acids with resultant defective cellular function, apoptosis, and vasculopathy.

The role of reactive oxygen and nitrogen species has been recently reviewed in the pathogenesis of pulmonary hypertension (Tabima et al. 2012). For example, peroxynitrite (ONOO^-) can have variable effects that have been proposed to contribute to the pathogenesis of pulmonary hypertension. Not only can it cause toxicity in smooth muscle and endothelial cells to cause cell death but also cellular proliferation through ERK and protein kinase C activation (Agbani et al. 2011). Peroxynitrite is derived from NO and superoxide and can form from uncoupled eNOS or other oxidases that produce superoxide in the presence of NO. As an oxidant ONOO^- in turn modifies eNOS activity, reduces NO production and enhanced superoxide production, which reacts with remaining NO to produce additional ONOO^- . Peroxynitrite at low concentrations can inactivate prostacyclin synthase, reducing the vasodilatory effects of prostacyclin and increasing vasoconstrictive mediators thromboxane A2 and prostaglandin H2 (Agbani et al. 2011).

2.5.1 ROS/RNS and eNOS Uncoupling

As previously described eNOS is essential in vascular homeostasis through the production of NO. Uncoupled eNOS shifts away from the production NO to the generation of $\text{O}_2^{\bullet-}$ with subsequent reductions in vascular dilation. There is evidence that eNOS uncoupling is involved in the pathogenesis of PH which comes from studies using the Caveolin-1 (Cav-1) knockout mice. Cav-1 regulates eNOS signaling as caveolin binds to eNOS (Garcia-Cardena et al. 1997; Bernatchez et al. 2005) as a negative regulator of its activity. Released activated uncoupled eNOS produces oxidative stress and contributes to the development of cardiopulmonary disease (Wunderlich et al. 2008; Karuppiah et al. 2011). Although not all mice with uncoupled eNOS develop PH (Ozaki et al. 2002), Cav-1-deficient mice develop spontaneous PH which is reversed with treatment with L-NAME, which inhibits eNOS and reduces superoxide production (Wunderlich et al. 2008). This further supports that eNOS uncoupling can produce a PH phenotype. Additionally eNOS-dependent peroxynitrite formation from the reaction of NO and superoxide contributes to pulmonary vasculopathy (Zhao et al. 2009).

2.5.2 NOXs

Recent research shows that the oxidase systems nicotinamide adenine dinucleotide dehydrogenase (NADH) oxidase (Nox) and xanthine oxidoreductase (XOR) are involved in the development of pulmonary vascular disease and PH. Members of the Nox family that are found in the human vasculature include Nox1, Nox4, Nox5, and, the phagocyte NADPH oxidase gp91^{phox}, known as Nox2. It is notable that Nox5 is not expressed in rodents.

The primary role of Nox is ROS generation, in particular superoxide production (Takeya and Sumimoto 2003). All Nox have transmembrane helices that regulate electron transport across the membranes to reduce O_2 to $O_2^{\bullet-}$. Within the six membrane domains are two highly conserved heme binding histidine sites (heme domains) and a binding site in the cytoplasmic tail for NADPH and flavin adenine dinucleotide (FAD) (Vignais 2002; Bedard and Krause 2007). The FAD is constitutively bound to the flavin domain and the NADPH acts a substrate. The NADPH oxidases use NADPH as an electron donor to reduce molecular oxygen to $O_2^{\bullet-}$ and/or H_2O_2 while maintaining their own discrete biochemical function for activity, regulation, and expression. Two electrons are transferred from NADPH to FAD, which is reduced to $FADH_2$ on the inner C-terminal tail (Nisimoto et al. 1999). The electrons transfer from the inner heme to an outer heme and then bind to an O_2 molecule to produce $O_2^{\bullet-}$ on the external membrane (Cross and Segal 2004). Different Nox families produce different ROS, as Nox1–3 and 5 produce $O_2^{\bullet-}$ and Nox4, Duox 1, 2 produce H_2O_2 . The production of H_2O_2 by Nox is controversial and may result from the rapid dismutation by the Nox catalytic site.

Noxs have been shown to be a major source for the generation of ROS in vascular smooth muscle cells (Wedgwood et al. 2001), endothelial cells (Chatterjee et al. 2008), and fibroblasts (Pagano et al. 1997). The ROS derived from the NADPH oxidases have been implicated in the pathogenesis of pulmonary hypertension (Brennan et al. 2003; Sanders and Hoidal 2007) and in particular the development of PH related to chronic hypoxia (Mittal et al. 2007; Sanders and Hoidal 2007). Within the pulmonary vasculature, Nox1, Nox2, and Nox4 produce ROS, which blunts vascular relaxation to exogenous NO resulting in impaired tone (Brennan et al. 2003) and contractile response in hypoxic PH models (Fresquet et al. 2006; Liu et al. 2006; Mittal et al. 2007; Fike et al. 2008). Elevated superoxide levels produced within the pulmonary arterioles in the persistent PH of the newborn model (Deruelle et al. 2005; Farrow et al. 2008b) have been defined as the primary oxidant causing oxidative stress mainly derived from Nox and also secondarily from uncoupled eNOS (Wedgwood and Black 2003; Deruelle et al. 2005; Wedgwood et al. 2005; Farrow et al. 2008a, b).

Chronic hypoxia models of PH result in clear changes in NO and ROS production. Hypoxia results in increased HIF-1 gene expression, which increases TGF- β 1 levels (Sturrock et al. 2006) and increases Nox-4 expression and ROS production in PASM (Sanders and Hoidal 2007; Nisbet et al. 2010). Studies have shown that inhibition or knock-down silencing of Nox4 reduces ROS formation and reverses

PH in cultured human pulmonary artery smooth muscle cells through a reduction of smooth muscle proliferation (Sturrock et al. 2006; Mittal et al. 2007). The Nox2 isoform plays a role in the NO-dependent endothelial dysfunction in pulmonary arteries by inhibiting NO-mediated activation of sGC and cGMP production during chronic hypoxia (Li et al. 1999; Li and Forstermann 2000). Accordingly, the Nox2 knockout mouse is protected from hypoxic PH, with decreases in right ventricular pressures and hypertrophy, pulmonary artery wall thickening, and superoxide production (Liu et al. 2006). Increased ROS from Nox4 can alter the balance of $\text{NADP}^+:\text{NADPH}$ and tetrahydrobiopterin to dihydrobiopterin ($\text{BH}_4:\text{BH}_2$) and contribute to an uncoupled eNOS state. Not only are the NADPH oxidase enzymes responsible for superoxide production but also they serve as regulators of xanthine oxidase and eNOS (Wolin et al. 2005; Jankov et al. 2008).

2.5.3 Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) primarily exists in two forms, in cells as the dehydrogenase form (XDH), and in the circulation as the oxidase form (XO). Xanthine oxidoreductase catalyzes the final two steps in purine degradation hypoxanthine \rightarrow xanthine \rightarrow uric acid. Substrate-derived electrons from the oxidation of hypoxanthine to xanthine reduce NAD^+ to NADH. This process has been well characterized in gout; however, XOR has also been examined as a contributor of intracellular ROS in the setting of inflammatory conditions and vascular injury. Inflammation has been recognized as a significant contribution to vascular remodeling in human and experimental models of PH (Hassoun et al. 2009). During inflammatory conditions, the cysteine residue of XOR is oxidized and converts XDH to XO (Amaya et al. 1990), which transfers substrate-derived electrons to O_2 to generate intracellular H_2O_2 and $\text{O}_2^{\bullet-}$. Both XO and XDH are responsible for the generation of ROS (Harris and Massey 1997) and moderate hypoxia is a potent stimulus for XOR activity in arterial endothelial cells (Kelley et al. 2006).

It is important to note three crucial distinctions in the activity of XOR (1) the conversion to XO is not essential for ROS production, as XDH has partial oxidase activity during conditions where NADH/NAD^+ ratios are increased (i.e., hypoxemia) (Williamson et al. 1993; Harris and Massey 1997); (2) about 80–95 % of the ROS produced by XOR under normal and pathophysiological conditions is H_2O_2 (Kelley et al. 2010); and (3) hypoxia induces XOR expression and activity (Kelley et al. 2006). XOR can be either produced within the vasculature or distributed to critical sites in the vasculature. When this occurs XOR serves as a significant source of ROS thereby modulating vascular function. Studies have shown enhanced xanthine oxidoreductase activity within the arteries of PAH patients (Spiekermann et al. 2009), in cultured endothelial cells (Cote et al. 1996; Houston et al. 1999), and in a hypoxia exposed rat model of PH (Hoshikawa et al. 2001). It is therefore postulated that xanthine oxidoreductase can serve as a critical regulator of ROS formation within the vasculature and drive pathological processes involved in the pathogenesis of PAH.

In chronic hypoxic models of PH, an increase in vascular $O_2^{\bullet-}$ production results in impaired NO signaling and endothelial dysfunction (Houston et al. 1999; Zulueta et al. 2002). This directly contributes to vascular remodeling and has been partly attributed to enhanced xanthine oxidoreductase activity (Hoshikawa et al. 2001). Treatment of chronic hypoxia-induced PAH rats with XOR-specific inhibitors, such as allopurinol attenuates hypoxia, normalizes blood pressure, reduces RV hypertrophy as well as pulmonary vascular thickening (Hoshikawa et al. 2001; Jankov et al. 2008). It is unclear, however, if XOR's role in PAH is either contributory or as a nitrite reductase that prevents/reverses experimental PH (Zuckerbraun et al. 2010). One potential explanation for this unresolved paradox and discordant role of XOR may be explained by the presence of nitrite. Electrons may be diverted away from oxygen and pathological superoxide formation to nitrite and protective NO.

2.5.4 Mitochondria as a Source of ROS

Under normal aerobic conditions, mitochondrion in the endothelial and smooth muscle cells of pulmonary arteries converts a small amount (1–2 %) of O_2 into $O_2^{\bullet-}$ via the electron transport chain (Boveris et al. 1972; Wolin 2009). The production of superoxide can be affected by the modulation of mitochondrial redox status, biogenesis, fusion and fission processes, as well as changes in mitochondrial bioenergetics (Erusalimsky and Moncada 2007). Superoxide dismutase 2 (SOD2) converts mitochondrial superoxide to H_2O_2 , which play a variable role in regulation of vascular tone and cellular proliferation and apoptosis. There are beneficial effects when AMPK, JNK, and nuclear NF- κ B are activated and result in the upregulation of cytoprotective genes involved in glucose transportation, antiapoptosis, glycolysis, antioxidant defenses, and repair mechanisms (Biswas et al. 2003). Additionally, H_2O_2 can have a variable effect on the pulmonary artery depending on the anatomical aspect of the vessel, where H_2O_2 will either act to increase contractility or vasodilation. In the absence of PAH, under normal physiological conditions, the contribution of mitochondrial $O_2^{\bullet-}$ to $ONOO^-$ is not clear (Erusalimsky and Moncada 2007). In the setting of PAH, the ability to dismutase superoxide are either saturated or impaired (Farrow et al. 2008b; Nozik-Grayck et al. 2008) and the electron transport chain production of $ONOO^-$ (from the reaction of NO and superoxide) and $O_2^{\bullet-}$ is enhanced (Burwell et al. 2006; Dahm et al. 2006).

The reason for mitochondrial dysfunction in PAH may be explained by two distinct theories. The first suggests that the mitochondrial electron transport chain serves as a source of ROS promoting senescence, necrosis, or apoptosis (Wolin et al. 2005) thus resulting in pulmonary arterial vasculopathy. The second possibility considers that mitochondrial dysfunction and a reduction in physiologic ROS levels (H_2O_2) drive pulmonary hypertension through a net decrease in cellular redox state, which inhibits redox-sensitive voltage-gated potassium channels Kv1.512 activity, causing cellular depolarization, increased intracellular Ca^{2+} ,

and then vasoconstriction of pulmonary artery smooth muscle cells (Archer et al. 2010). A possible reconciliation of these two theories can be postulated by considering the enzymatic source and the identities and quantities of the measured ROS. In the setting of hypertension and inflammation, high levels of NADPH and XOR-derived superoxide and ONOO⁻ are likely pathologic, while the potassium-gated channels Kv1.5/12 may be tonically activated by the physiologic flux of H₂O₂ thus limiting intracellular calcium levels.

3 Therapies

The therapeutic strategies discussed target the dysregulated redox equilibrium and endothelial dysfunction that occurs in PAH. Many of the therapies discussed are experimental, and they are being tested in preclinical animal models, with some currently being tested in human clinical trials in human PAH (BH₄ and sodium nitrite).

3.1 Therapies Using NO or NO-Generating Drugs

3.1.1 Inhaled Nitric Oxide Gas

In both animal and human studies, inhaled NO (5–80 ppm) is a potent, short-acting and selective pulmonary vasodilator that reduces pulmonary vascular resistance and right ventricular afterload (Rossaint et al. 1993; Atz and Wessel 1997; Roberts et al. 1997; Markewitz and Michael 2000). iNO is delivered directly to the pulmonary vascular resistance vessels where it is immediately inactivated by hemoglobin (Hb), limiting the systemic vasodilatory effects of the drug. This selectivity of NO to Hb exemplifies the beneficial attributes of iNO as a pulmonary-specific vasodilator compared to other intravenous vasodilators, which result in a decrease in systemic vascular resistance. In patients with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), iNO is delivered to well-ventilated lung units and can improve V/Q mismatching in injured lung to improve oxygenation; however, despite significant improvements in hypoxemia, therapeutic trials with iNO have not shown a mortality benefit or shorter duration of mechanical ventilation. As a therapeutic agent in PH patients, iNO is mostly utilized as adjuvant therapy to improve gas exchange in newborns with severe PH (Ivy et al. 1997). However, its usefulness as a therapeutic treatment for other forms of PH is currently limited (Coggins and Bloch 2007). It remains a commonly used agent in the cardiac catheterization laboratory to test pulmonary vasodilator responsiveness.

iNOs therapeutic value is limited by the dose and duration of exposure, with doses higher of 80 ppm leading to methemoglobin levels above 5 % (Weinberger et al. 2001). Other safety concerns regard the withdrawal of iNO and rebound

pulmonary hypertension. Several studies have noted a potentially life-threatening increase in pulmonary vascular resistance upon acute withdrawal of iNO (Miller et al. 1995; Atz et al. 1996; Lavoie et al. 1996; Cueto et al. 1997). This form of “rebound pulmonary hypertension” is manifested by an acute increase in pulmonary vascular resistance and compromised cardiac output associated with severe hypoxemia. The mechanism behind this rebound phenomena may be in part explained by a finding that exogenous NO exposure inhibits endogenous eNOS activity (Black et al. 1999). This suggests that a transient decrease in endogenous eNOS activity (rather than endothelial NOS) likely results in a decrease in of plasma cGMP and nitrate concentrations during inhaled NO therapy resulting in the rebound pressure increase. The coadministration of a phosphodiesterase-5 inhibitor (PDE5i) (sildenafil) in children has been shown to ameliorate this rebound effect (Namachivayam et al. 2006), suggesting that inhaled NO may work synergistically with current treatment options such as phosphodiesterase-5 inhibitors (see below). New delivery systems that allow for pulsed NO delivery combined with pulsed oxygen from liquid oxygen sources may allow for small portable devices of inhaled NO. Large clinical trials of inhaled NO in patients with PAH on background phosphodiesterase-5 inhibitor therapies are required to address these questions.

In the adult PAH population, however, inhaled NO (iNO) has been primarily utilized as a diagnostic agent to assess acute pulmonary vasoreactivity to guide therapeutic options, i.e., calcium channel blockers versus PAH-specific agents (Sitbon et al. 2005). The ACCF/AHA 2009 Expert Consensus Document on Pulmonary Hypertension (McLaughlin et al. 2009a, b) favored the use of iNO over other vasodilators largely due to ease of administration compared to other agents and limited side effects when used correctly (Sitbon et al. 1998). Typical dose range is 20–80 ppm for 5–10 min, with repeated hemodynamic measurements while on iNO to measure the degree of response (Barst et al. 2004; Sitbon et al. 2005). Response to iNO may also portend improved long-term outcomes independent of treatment with calcium channel blockers (Krasuski et al. 2011; Malhotra et al. 2011).

3.1.2 NO Donors

Organic nitrates such as nitroglycerin are commonly utilized agents that relax capacitance vessels and decrease pulmonary and systemic vascular resistance at higher doses. The underlying mechanisms of action are unclear and are likely multifactorial (Elkayam 1991) and may include the metabolism to NO and nitrite with secondary activation of sGC (Artz et al. 2002; Ignarro et al. 2002). The development of nitrate tolerance with treatment can limit the class utility of these drugs.

Nitrate tolerance appears to be related to both increases in ROS formation, which inactivates the NO formed by organic nitrate metabolism, and by the oxidative inhibition of aldehyde dehydrogenase, the enzyme that converts the organic nitrates to NO. Munzel et al. (1995b) demonstrated an increase in vascular superoxide

production after nitroglycerin treatment. Tolerance was prevented by the coadministration of superoxide dismutase (SOD), thus highlighting the role of ROS in this inhibition (Munzel et al. 1995b). A later study by the same group identified a membrane-bound Nox1 as a likely source of superoxide production (Munzel et al. 1996), and more recent studies demonstrated a role for endothelin-1 in the pathway (Kurz et al. 1999). It is likely that the increase in superoxide inactivates the NO released from nitroglycerin, increasing the formation of peroxynitrite. This may in turn promotes a supersensitivity to vasoconstrictors due to a tonic activation of protein kinase C, leading to nitrate tolerance (Munzel et al. 1995a). Proposed superoxide sources include NADPH oxidase, uncoupled eNOS, and the mitochondria. Superoxide and NO rapidly combine to form peroxynitrite. This aggravates tolerance by promoting the uncoupling of NO synthase and the inhibition of sGC and prostacyclin synthase (Elkayam 1991). More recent studies have defined a new tolerance mechanism through the inhibition of mitochondrial aldehyde dehydrogenase, an enzyme that metabolizes nitroglycerin to form NO and nitrite (Sydow et al. 2004; Zhang et al. 2004).

3.1.3 Nitrite

Nitrite (NO_2^-), an endogenous anion salt, has historically been considered an inert oxidation product of NO and oxygen (see Fig. 3). There is increasing evidence over the past 10 years that has supported the use of nitrite as a potent physiological vasodilator (Gladwin et al. 2000; Modin et al. 2001; Cosby et al. 2003; Dejam et al. 2004; Lundberg et al. 2008) and reservoir for NO (Lundberg et al. 2008). Nitrite is reduced to NO by a variety of enzyme systems as oxygen tension drops along the physiological and pathological hypoxic gradient. Enzymes that have been identified with nitrite reductase activity include hemoglobin, myoglobin, neuroglobin, eNOS, xanthine oxidoreductase, and acidic disproportionation (Flogel et al. 2004; Huang et al. 2005; Rassaf et al. 2007; Shiva et al. 2007; Hendgen-Cotta et al. 2008; Shiva and Gladwin 2009; Tota et al. 2010; Tiso et al. 2011). Nitrite has been shown to mediate hypoxic pulmonary vasodilation in a deoxyhemoglobin and pH-dependent fashion (Hunter et al. 2004).

Nitrite has been proposed as a therapy for PH based on inhaled studies in newborn sheep with hypoxia- and thromboxane-induced PH (Hunter et al. 2004). Recent studies have shown that inhaled, low-dose nebulized sodium nitrite can prevent and reverse experimental PAH and heart failure in both the chronic hypoxia mouse model and monocrotaline rat model (Zuckerbraun et al. 2010). Zuckerbraun et al. (2010) showed that a low dose of sodium nitrite has a potent antiproliferative effect on smooth muscle that is dependent on nitrite reduction by the enzyme xanthine oxidoreductase. Nitrite in this context signals through cGMP to increase the levels of the cell cycle checkpoint inhibitor P21.

Recently, a study demonstrated that a hypoxia-induced PH mouse model treated with oral dietary nitrate had a reduction in right ventricular pressures and hypertrophy and with decreased pulmonary vascular remodeling (Baliga et al. 2012). Plasma and

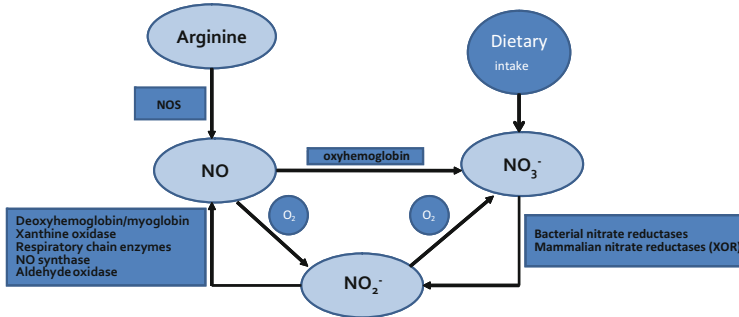


Fig. 3 The production of nitrite from nitric oxide and dietary nitrate. Within cells nitric oxide synthase (NOS) produces nitric oxide (NO) from L-arginine. The enterosalivary circulation produces NO through dietary intake of nitrate rich foods (spinach, lettuce, or beetroot juice). Ingested nitrate is taken from the circulation into the salivary glands and concentrated in the saliva. Salivary nitrate is reduced to nitrite by oral cavity bacteria, which when swallowed generates nitrogen oxide in the acidic stomach. Adapted from Lundberg et al. (2009)

lung tissue levels of nitrite and cGMP were found to be elevated in mice supplemented with nitrate. In comparison, mice that either lacked eNOS or were treated with allopurinol, a xanthine oxidoreductase inhibitor, reduced the beneficial effects of oral nitrite/nitrate supplementation.

Animal toxicology studies and phase Ia and Ib human studies have been completed with plans for a proof of concept phase II study over the next 2 years.

3.1.4 Recoupling of eNOS

Tetrahydrobiopterin

As a critical cofactor for endothelial NO synthase, suboptimal concentrations of tetrahydrobiopterin (BH₄) result in decreased synthesis of NO, increased superoxide production, which contributes to eNOS uncoupling and endothelial dysfunction. BH₄ is a potential therapeutic target for patients with pulmonary hypertension. Mice with congenital low tissue levels of BH₄ tend to develop PH with RVH due to distal vessel muscularization (Khoo et al. 2005; Nandi et al. 2005). After hypoxic challenge, treatment with BH₄ reduced muscularization of the distal pulmonary arteries (Francis et al. 2010). A BH₄ analogue, 6-acetyl-7,7-dimethyl-7,8-dihydropterin (ADDP), studied in a hypoxia-induced rat model of PH demonstrated enhanced eNOS expression in the pulmonary vascular endothelium with improved NO-mediated pulmonary artery dilation (Kunuthur et al. 2011).

Caveolin-1 (Cav1)-deficient mice, prone to developing PAH and a hypertrophied right ventricle, have enhanced eNOS activity but do not increase BH₄ levels. This causes a discordance between eNOS activity and BH₄ levels, resulting in dysfunctional eNOS signaling. Chronic supplementation with BH₄ in these mice results in a normalized BH₄/BH₂ ratio, decreased superoxide production

and a reduction in pulmonary artery pressures and right ventricular congestion manifest by a lower central venous pressure. These findings were not reproduced with a similar antioxidant tetrahydrobiopterin (H4N), which has no NOS activity (Wunderlich et al. 2008).

Supplementation with sepiapterin, an oxidized BH4 analogue, increased cellular BH4 content and NO production, recoupled eNOS, and decreased superoxide formation in the pulmonary artery endothelial cells isolated from fetal lambs with PPHN (Teng et al. 2011).

Exogenous treatment with BH4 or therapies to increase endogenous BH4 to modulate eNOS activity in humans are of great clinical interest and are currently under investigation. An open-label dose escalation safety study of the optically active form of BH4, sapropterin (6r-BH4) in 18 Group 1 PAH or inoperable CTEPH patients on concurrent oral PH therapy with either ERAs or PDE5i demonstrated tolerability without significant systemic hypotension (Robbins et al. 2011). Additionally, 6-min walk distance improved in the treatment group as well as a small but significant decrease in the inflammatory marker MCP-1. There were no significant changes in measures of NO synthesis or oxidative stress.

Other therapies that increase BH4 levels include statins (Hattori et al. 2003), certain angiotensin converting enzyme inhibitors (Oak and Cai 2007; Imanishi et al. 2008), telmisartan (Wenzel et al. 2008), and insulin (Ishii et al. 2001). Larger, placebo-controlled human studies in the use of oral BH4 or increases endogenous BH4 in the setting of PH are needed.

L-Arginine

L-arginine is the nitrogen donor to nitric oxide production. As previously stated, the enzyme arginase hydrolyzes arginine to urea and ornithine, therefore competing with eNOS for arginine. In certain forms of PH, such as sickle cell anemia, arginase I levels are increased which result in impaired pulmonary vasorelaxation due to alterations in the NO pathway (Morris et al. 2005, 2008). Additionally, decreased plasma levels of arginine have been reported in infants with PPHN (Vosatka et al. 1994), which correlates with decreased NO levels (Pearson et al. 2001). Increased bioavailability of L-arginine through exogenous administration is a possible therapeutic target in the treatment of some types of pulmonary hypertension.

In chronically hypoxic rats, L-arginine restored endothelial-dependent vasorelaxation to acetylcholine within the pulmonary circulation (Eddahibi et al. 1992). Dietary L-arginine supplementation in hypoxia-induced PH maintained ameliorated PH by preventing hypoxia-induced increase in resistance of the precapillary pulmonary vessels, possibly be inducing angiogenesis (Howell et al. 2009).

Mehta et al. (1995) evaluated the short-term vasodilatory effects of an infusion of L-arginine in ten subjects with various forms of pulmonary hypertension. Compared to hypertonic saline L-arginine infusion resulted in decreases in pulmonary

and systemic pressures, pulmonary vascular resistance, and an increase in cardiac output. Compared to an infusion of the potent vasodilator, prostacyclin, L-arginine produced a similar reduction in pulmonary artery pressures; however, prostacyclin infusion resulted in a significant reduction in pulmonary vascular resistance. The first study to evaluate the short-term oral administration of L-arginine in 19 subjects primarily with Group 1 PAH and CTEPH resulted in a decrease in pulmonary hemodynamics (16 % reduction in pulmonary vascular resistance) and demonstrated improvement in exercise capacity (Nagaya et al. 2001). Similar reductions in pulmonary artery pressures were shown in a sickle cell population with pulmonary hypertension (Morris et al. 2003). Despite the hemodynamic responses that the above clinical trials demonstrated, L-arginine therapy has not been in the forefront of treatment for pulmonary hypertension.

3.2 Therapies That Increase cGMP Levels

3.2.1 Phosphodiesterase-5 Inhibitors

Therapies that increase cGMP levels, such as the phosphodiesterase-5 inhibitors, sildenafil and tadalafil, have been reviewed in detail in a previous chapter. We briefly review the available data pertaining to the selective cGMP-specific phosphodiesterase type 5 inhibitors.

Phosphodiesterase type 5 enzyme blockade prevents the normal hydrolysis of cGMP and increases the effects of NO including pulmonary vasodilation and inhibition of smooth muscle cell growth (Friebe and Koesling 2003). Sildenafil and tadalafil are the only FDA approved PDE-5 inhibitors (PDE5i) for the treatment of moderate–severity PAH. Sildenafil, which was approved in 1998, is available as a thrice daily oral formulation for iPAH and connective tissue disease-associated PAH and has recently been made available as an IV formulation (Vachieri et al. 2011). A 12-week, double-blind trial of moderate Group 1 PAH (Galie et al. 2005) compared sildenafil and placebo and met the primary endpoint of improved 6MWD in the sildenafil group as well as hemodynamic endpoints of decreased mPAP and PVR.

Tadalafil approved 5 years after sildenafil is available as once a day oral therapy for iPAH, heritable PAH, and connective tissue disease-associated PAH. PHIRST 1, a 16-week, randomized, double-blind, double-dummy, placebo-controlled multicenter trial studying the efficacy and tolerability of tadalafil on Group 1 PAH subjects with and without background bosentan therapy (Galie et al. 2009). Tadalafil significantly decreased time to clinical worsening when compared to placebo and improved exercise capacity. An evaluation of a hemodynamic subgroup demonstrated improved cardiopulmonary hemodynamics, in particular decreased mPAP, PVR and improved CI in the tadalafil group. A double-blind uncontrolled 52-week extension study of PHIRST-1 demonstrated that prolonged

tadalafil treatment was well tolerated and initial improvements noted in 6MWD noted at 16 weeks were preserved at week 52 (Oudiz et al. 2012).

There has been great interest in investigating novel treatment options for patients with interstitial lung disease (ILD)-associated pulmonary hypertension as there are limited therapies available for this population. A randomized, open-label study of 16 subjects with severe pulmonary hypertension (mPAP >35 mmHg) and pulmonary fibrosis compared the acute cardiopulmonary response to IV epoprostenol to oral sildenafil (Ghofrani et al. 2002). Both epoprostenol and sildenafil reduced pulmonary vascular resistance; however, sildenafil had a reduction in pulmonary to systemic vascular resistance; additionally, epoprostenol increased ventilation perfusion (V/Q) mismatch of the lung and decreased arterial O₂ saturations. The sildenafil group maintained V/Q matching and partial pressure of arterial O₂. The likely explanation for this is that eNOS requires molecular oxygen as a substrate for the L-arginine oxidation to NO, so more NO and cGMP are produced in well-oxygenated regions of the lung. Therefore, the inhibition of PDE-5 will increase cGMP levels more in oxygenated lung vasculature.

Based upon the finding that sildenafil preferentially induced vasodilation in well-ventilated regions of lung tissue, the investigators of Sildenafil Trial of Exercise Performance in Idiopathic Pulmonary Fibrosis (STEP-IPF) evaluated the use of sildenafil in 180 subjects in a double-blind, randomized, placebo-controlled trial of 20 mg sildenafil thrice daily (Zisman et al. 2010). The study did not meet the primary endpoint of a 20 % increase in 6-min walk distance from baseline. However, compared to the placebo group treatment, subjects had a significant improvement in the diffusing capacity for carbon monoxide, arterial O₂ saturations, and partial pressure of arterial O₂ and maintained a stable degree of shortness of breath at week 12. This study did not evaluate subjects for the presence of pulmonary vascular disease. It is unclear if the beneficial effects can be attributed to that of a particular subgroup. Additional large clinical trials targeting patients with ILD and pulmonary hypertension are needed.

3.2.2 sGC Activators and Stimulators

As previously reviewed, guanylyl cyclase (GC) is a heterodimeric enzyme that catalyzes the conversion of cGTP to cGMP following stimulation with NO in vascular smooth muscle. The impaired signaling of the NO-sGC-cGMP pathway has been implicated in the pathogenesis of pulmonary hypertension. The activators of sGC bind to and activate the oxidized or heme-free enzyme and do not require NO (Evgenov et al. 2006). The stimulators directly sensitize sGC to low levels of nitric oxide in the presence of a reduced or ferrous synthetic heme (Stasch et al. 2001). These agents are potent vasodilators in the pulmonary and systemic circulation (see Fig. 4).

sGC activators such as BAY 58-2667 and HMR 1766 have been evaluated in animal models and preclinical studies of PH. The sGC activators have been shown to have potent and selective pulmonary vasodilator properties without significant

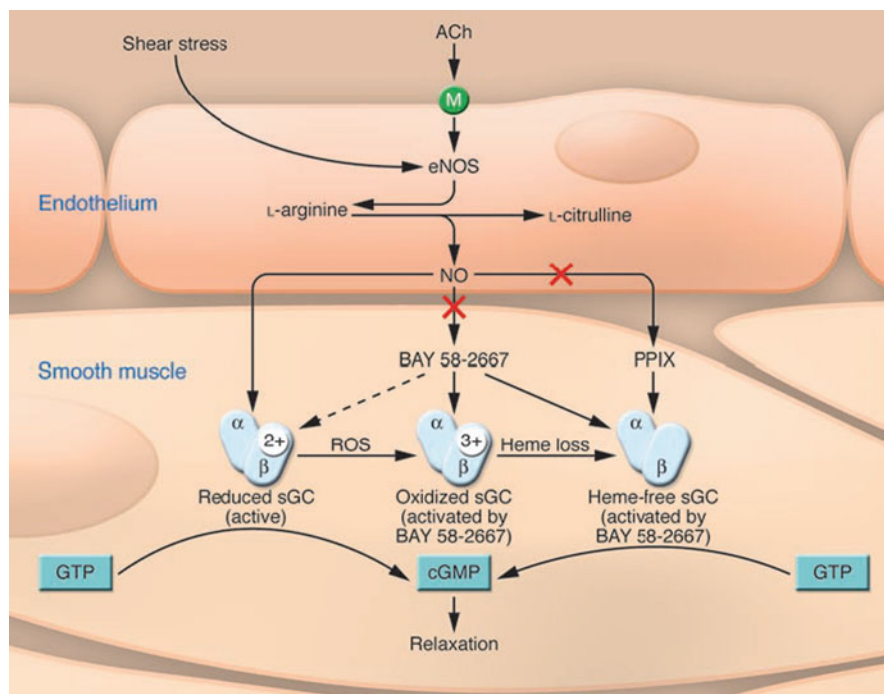


Fig. 4 BAY 58-2667 activates oxidized and heme-free sGC, which is unresponsive to NO. After activation either by shear stress or acetylcholine (ACh), endothelial NOS produces NO, which diffuses to smooth muscle and binds to the reduced hemes (Fe^{2+} ferrous heme) on the α/β heterodimer sGC. This converts GTP to cGMP and leads to smooth muscle relaxation with resultant vasodilation. Endothelial dysfunction can be associated with the accumulation of oxidized (Fe^{3+} ferric heme) and heme-free sGC that is not activated by NO. *M* muscarinic receptor. Reproduced with permission Gladwin (2006)

abnormalities in pulmonary gas exchange (Evgenov et al. 2006). Stimulators of sGC such as BAY 41-2272 and BAY 63-2521 have been shown to both potently vasodilate the pulmonary vascular bed and decrease the workload of the right ventricle (Mittendorf et al. 2009). The evaluation of BAY 41-2272 in a high renin, low-NO rat model of hypertension (transgenic renin rats with additional renin gene, TG(mren2)27) showed an improvement in oxygenation, potent vasodilator effects, augmented response to inhaled nitric oxide, decreased blood pressure, and improved survival in drug treated subjects (Stasch et al. 2001).

BAY 63-2521, also known as riociguat, is one of the first sGC stimulators in clinical development to be evaluated for the treatment of pulmonary hypertension (Griminger et al. 2009). The safety profile of a single dose of riociguat was evaluated in Group 1, 3, and 4 pulmonary hypertension. Despite the lack of pulmonary vasodilator selectivity, riociguat significantly improved pulmonary

hemodynamics, increased cardiac index better than inhaled NO, and did not cause clinically significant systemic hypotension (Grimminger et al. 2009) As a follow-up, Ghofrani et al. (2010) completed an open-label phase II study in patients with chronic thromboembolic PH as well as Class I PAH patients and found that riociguat reduced pulmonary vascular resistance and increased the 6-min walk distance. Side effects related to therapy with riociguat included hypotension, shortness of breath, and headache. Larger randomized trials are currently underway and include evaluation of combination therapy of riociguat with the PDE-5 inhibitor sildenafil and the use of riociguat in non-Group 1 PAH, such as COPD.

3.3 Therapies Targeting ROS Signaling

As ROS has been implicated in the pathogenesis of PAH, there is an increasing interest in diminishing ROS levels as potential therapeutics for PAH. Scavengers of ROS, such as recombinant human MnSOD, increased eNOS expression levels and function in the ductal ligation model of the pulmonary hypertensive lamb through superoxide metabolism and restoration of BH₄ levels (Farrow et al. 2008b). The downregulation of SOD2 precedes the development of PAH in fawn-hooded rats. SOD2 regulates the production of H₂O₂ within the mitochondrion during respiration, which is less toxic than superoxide. Treatment with the SOD mimetic Manganese(III)tetrakis(4-benzoic acid) porphyrin chloride in fawn-hooded rats (a mutant strain that spontaneously develops PAH) reduces mean pulmonary artery pressure, improves exercise capacity, reduces right ventricle free wall thickness, and decreased medial wall thickness in the precapillary resistance arteries (Archer et al. 2010).

Endothelin-1 stimulates the production of H₂O₂ in pulmonary artery smooth muscle cells (PASMC) isolated from the fetal lamb model of pulmonary hypertension. Exposure of the PASMC to H₂O₂ decreases sGC levels and NO-dependent GMP expression. The addition of the ROS scavenger polyethylene glycol catalase, which is specific to H₂O₂, normalizes the vasodilator response to NO, a finding that was not observed in the control arm (Wedgwood et al. 2005)

Apart from ROS scavengers, other strategies have been aimed at the inhibition of ROS generation. Cav1-deficient mice show evidence of heart failure and pulmonary hypertension demonstrate increase in superoxide production related to uncoupled eNOS. The acute inhibition of uncoupled eNOS with L-nitro-arginine methyl ester (L-NAME) reduced vascular superoxide production (Wunderlich et al. 2008). Phosphodiesterase-5 inhibitors, a current standard treatment for PAH, have also been reported to lower superoxide levels in the mouse model of bleomycin-induced pulmonary fibrosis and pulmonary hypertension (Hemnes et al. 2008).

Increased superoxide production in the ductus arteriosus ligation model of persistent pulmonary hypertension of the newborn (PPHN) derived from NADPH oxidase impairs vascular tone and contractility of the pulmonary arteries. Pretreatment of the isolated pulmonary arteries with superoxide scavengers tiron and PEG-SOD and with NADPH oxidase inhibitor diphenyliodonium enhanced the relaxation of pulmonary arteries to the NO donor *s*-nitrosyl-acetyl-penicillamine (SNAP) (Brennan et al. 2003).

Transgenic rat (mRen2)27 models characterized by the overexpression of renin in extrarenal tissue have increased synthesis of angiotensin II, resulting in increased angiotensin II ROS formation with systemic and pulmonary hypertension. This occurs as a result of Nox2-induced oxidative stress through the upstream activation of the renin–angiotensin system in the pulmonary vascular tissue and parenchyma (DeMarco et al. 2008, 2009). Human iPAH subjects have increased renin–angiotensin–aldosterone (RAAS) activity and shown to have increased systemic levels of circulating renin and angiotensin I and II, which are associated with disease progression and mortality and pulmonary (de Man et al. 2012). Angiotensin-converting enzyme inhibitors (ACEi) have been shown to reduce pulmonary artery pressures and thickening of pulmonary arteries (Nong et al. 1996). The effectiveness of the ACEi in PH models likely lies in the upregulation of NO production and a decrease in oxidative stress related to angiotensin II-dependent Nox2 activation (Kanno et al. 2001).

An inhibitor of fatty acid oxidation, the anti-anginal drug trimetazidine, attenuated cardiac fibrosis in a transverse aortic constriction rat model. Trimetazidine acted as an antioxidant reducing ROS generation and directly affected NADPH oxidase in the fibroblasts of cardiac tissue, rather than serving as a free radical scavenger (Liu et al. 2010).

There has also been recent interest in the role of PPAR gamma in pulmonary hypertension (Hansmann and Zamanian 2009; Tian et al. 2009; Sutliff et al. 2010). Patients with idiopathic pulmonary arterial hypertension have been shown to have reduced mRNA expression levels of PPAR-gamma (Ameshima et al. 2003), a ligand-activated nuclear receptor and transcription factor that regulates glucose metabolism and adipogenesis (He et al. 2003). Rosiglitazone is a synthetic PPAR-gamma agonist that reverses pulmonary hypertension by attenuating Nox4 induction and ROS generation in the lungs of chronic hypoxic mice (Nisbet et al. 2010). Furthermore, activation of PPAR-gamma with rosiglitazone attenuated H₂O₂ production, Nox4 expression and cellular proliferation in human pulmonary artery cells exposed to hypoxia (Lu et al. 2010). Together, the inhibition of fatty acid oxidation and the stimulus of PPAR-gamma support other findings that metabolic modulators can be selective for the pulmonary circulation (Sutendra et al. 2010). Since fatty acid oxidation inhibitors and agonists of PPAR-gamma are readily available, clinical trials to evaluate their effect on PAH are needed. Metabolic modulators are new and exciting avenues to pursue in the therapy of pulmonary hypertension.

3.4 Therapies Targeting *S*-nitrosoglutathione Reductase Inhibitors

Decreased *S*-nitrosothiols (RSNO) have been initially demonstrated in pulmonary hypertension (Foster et al. 2003) in particular hypoxic newborns (Gaston et al. 1993) or infants with presumed PPHN (Gaston et al. 1998). As previously mentioned RSNO are produced by the *S*-nitrosylation of the cysteine thiol of proteins by nitrosonium (NO^+), with *S*-nitrosoglutathione (GSNO) representing one of these intracellular *S*-nitrosothiols (Liu et al. 2004). Through *S*-nitrosylation of proteins, NO has been shown to posttranslationally regulate protein function (Benhar and Stamler 2005; Kelleher et al. 2007; Whalen et al. 2007). Denitrosylation pathways within cells can terminate the *S*-nitrosothiols (SNOs) pathways *in vivo*, in particular the enzyme GSNO reductase (GSNOR), previously characterized as formaldehyde dehydrogenase (Liu et al. 2001, 2004). To date, only a few studies have evaluated the therapeutic benefit of *S*-nitrosoglutathione reductase inhibitors (GSNORi) in pulmonary hypertension. The role of GSNOR in the regulation of pulmonary vascular tone and remodeling has been evaluated in mice models of hypoxic pulmonary hypertension (Wu et al. 2010). Wu et al. (2010) showed that hypoxic mice with PH significantly increased GSNOR mRNA and protein expression in lung tissue compared to normoxic mice with resultant interference of RSNO metabolism.

Currently, there are no clinical trials evaluating the use of GSNORi in pulmonary hypertension. However, a small number of basic and preclinical studies evaluating GSNOR inhibitors have been conducted including a study by Sanghani et al. (2009), which identified three novel GSNOR inhibitors through a high-throughput screening approach. One of the novel GSNOR inhibitors, C3, demonstrated properties that suppressed NF- κ B activation (a master regulator of the inflammatory response), increased cGMP formation, and promoted the relaxation of the murine aorta. The first-in-class drug N6022, a potent inhibitor of GSNOR has shown safety and efficacy in animal models of asthma and COPD and is currently being evaluated in early phase human clinical studies. The results of these human safety studies have not been published. Further evaluation of GSNOR inhibitors such as N6022 in the pulmonary hypertensive models and clinical PH trials is required.

4 Summary and Future Directions

As demonstrated in this chapter, the pathogenesis of pulmonary arterial hypertension of various clinical and experimental forms have been associated with endothelial dysfunction, reduced NO signaling, and increased oxidative stress. Therapies outlined in this chapter that enhance NO signaling, recouple eNOS, scavenge ROS, directly inhibit Nox2 and 4 and xanthine oxidoreductase, and restore mitochondrial function share a promising role as future therapeutics for pulmonary arterial hypertension.

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Rho-Kinase Inhibitors

Yoshihiro Fukumoto and Hiroaki Shimokawa

Abstract Pulmonary arterial hypertension (PAH) is a fatal disease with poor prognosis characterized by progressive elevation of pulmonary arterial pressure and vascular resistance due to pulmonary artery hyperconstriction and remodeling; however, the precise mechanism of PAH still remains to be elucidated. Although anticoagulant agents, pulmonary vasodilators, and lung transplantation are currently used for the treatment of PAH, more effective treatment needs to be developed. Rho-kinase causes vascular smooth muscle hyperconstriction and vascular remodeling through inhibition of myosin phosphatase and activation of its downstream effectors. In a series of experimental and clinical studies, it has been demonstrated that Rho-kinase-mediated pathway plays an important role in various cellular functions not only in vascular smooth muscle hyperconstriction but also in actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expressions, all of which may be involved in the pathogenesis of arteriosclerosis. Rho-kinase is activated in animal models of PAH (monocrotaline and chronic hypoxia) associated with enhanced pulmonary vasoconstriction and proliferation, impaired endothelial vasodilator functions, and pulmonary remodeling. Therapeutic application of Rho-kinase inhibitors reverses established experimental pulmonary hypertension. Further, administration or inhalation of Rho-kinase inhibitors exerts acute pulmonary vasodilation in patients with PAH who were refractory to conventional therapies. Taken together, Rho-kinase is a novel and important therapeutic target of PAH, and Rho-kinase inhibitors are a promising new class of drugs for this fatal disorder.

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Keywords Rho-kinase • Pulmonary arterial hypertension • Pulmonary arteriopathy • Pulmonary arterial hyperconstriction

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1 Introduction

In mid 1990s, two Japanese groups and one Singapore group independently identified Rho-kinase/ROK/ROCK as an effector of the small GTP-binding protein Rho (Leung et al. 1995; Ishizaki et al. 1996; Amano et al. 1997), which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expressions (Narumiya 1996; Shimokawa and Takeshita 2005; Loirand et al. 2006). The Rho/Rho-kinase pathway has recently attracted much attention in the cardiovascular research field for several reasons (Fig. 1). First, the Rho/Rho-kinase pathway plays an important role in various cellular functions that are involved in the pathogenesis of a variety of cardiovascular diseases (Shimokawa and Takeshita 2005; Shimokawa 2000). Second, this intracellular signaling pathway is substantially involved in the effects of many vasoactive substances that are implicated in the pathogenesis of cardiovascular diseases (Shimokawa and Takeshita 2005; Shimokawa 2000). Third, the so-called pleiotropic effects of statins, especially those of high-doses of statins, may be mediated, at least in part, by their inhibitory effects on Rho with a resultant inhibition on Rho-kinase (Shimokawa and Takeshita 2005; Shimokawa 2000). Fourth, the important roles of the Rho-kinase pathway have been recently demonstrated in the pathogenesis of pulmonary arterial hypertension (PAH) (Fig. 2) (Fukumoto et al. 2005, 2007; Shimokawa 2002; Abe et al. 2004, 2006; Do e et al. 2009; Fukumoto and Shimokawa 2011).

In this article, we will briefly review the role of Rho-kinase pathway on PAH, in terms of pathophysiology and treatment.

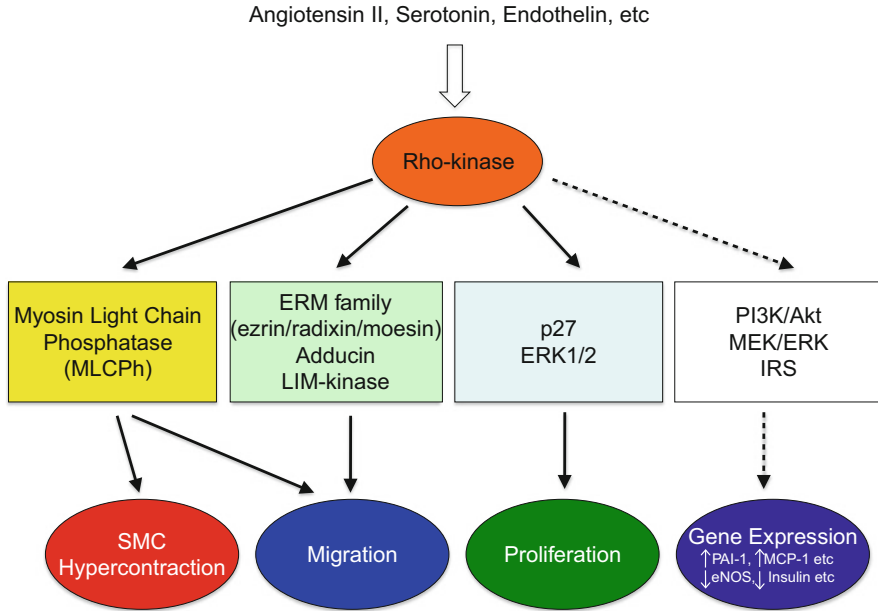


Fig. 1 Pathophysiology of Rho-kinase pathway. Rho-kinase pathway plays an important role in various cellular functions that are involved in the pathogenesis of a variety of cardiovascular diseases

2 Novel Pathophysiological Pathways of PAH

The pathological changes of the pulmonary arteries in PH include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMC), and migration of inflammatory cells (Fig. 2) (Fukumoto and Shimokawa 2011; Humbert et al. 2004; McLaughlin and McGoan 2006).

2.1 Important Role of Rho-Kinase in the Cardiovascular Fields

Recent advances in molecular biology have elucidated the substantial involvement of intracellular signaling pathways mediated by small GTP-binding proteins (G proteins), such as Rho, Ras, Rab, Sar1/Arf, and Ran families (Fukata et al. 2001; Takai et al. 2001). The Rho/Rho-kinase pathway has recently attracted much attention in various research fields, especially in the cardiovascular research field (Shimokawa and Takeshita 2005; Shimokawa 2000, 2002; Shimokawa and Rashid 2007a).

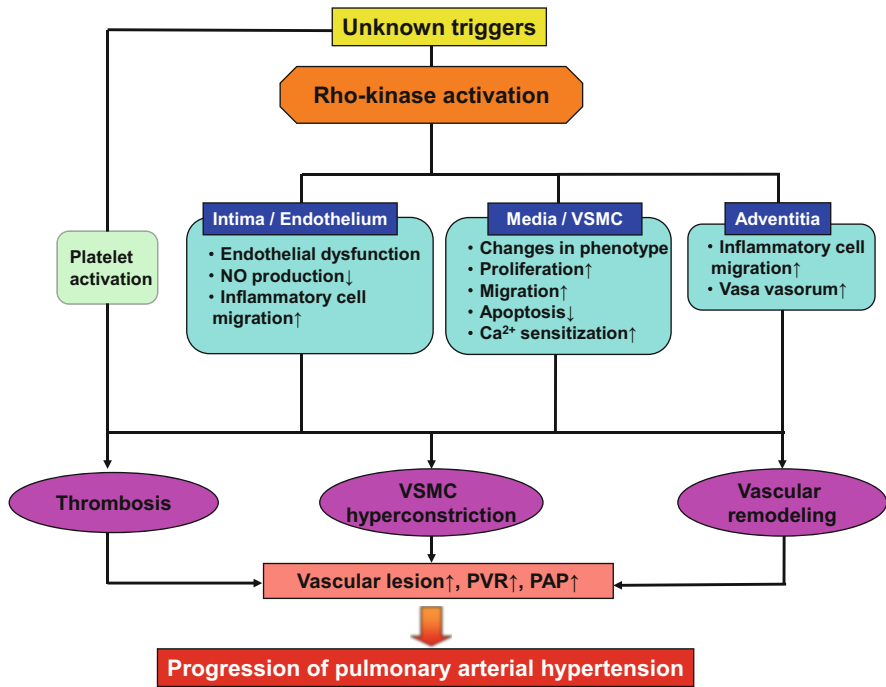


Fig. 2 Roles of Rho-kinase pathway in the pathogenesis of pulmonary hypertension. Rho-kinase activation has been confirmed to be substantially involved in the pathological changes of the pulmonary arteries. *NO* nitric oxide, *PAP* pulmonary arterial pressure, *PVR* pulmonary vascular resistance, *VSMC* vascular smooth muscle cell [from Fukumoto and Shimokawa 2011 with permission)

It has been previously demonstrated that Rho-kinase is a novel therapeutic target in ischemic heart disease (Shimokawa and Takeshita 2005; Shimokawa 2002). Rho-kinase suppresses myosin phosphatase activity by phosphorylating the myosin-binding subunit of the enzyme and thus augments VSMC contraction at a given intracellular calcium concentration (Fig. 3) (Uehata et al. 1997; Somlyo and Somlyo 2000). It has been also demonstrated that the Rho-kinase pathway is associated with enhanced myosin light chain (MLC) phosphorylations at the hyperconstrictive artery segments in animals (Shimokawa and Takeshita 2005; Shimokawa 2002). The activity and the expression of Rho-kinase are enhanced at the hyperconstrictive coronary segments, thereby suppressing myosin phosphatase through phosphorylation of its myosin-binding subunit with a resultant increase in MLC phosphorylations and hyperconstriction (Shimokawa and Takeshita 2005; Shimokawa 2002). Thus, VSMC hyperconstriction mediated by activated Rho-kinase plays a key role in patients with coronary artery spasm (Shimokawa and Takeshita 2005; Shimokawa 2002; Masumoto et al. 2001, 2002), suggesting that Rho-kinase inhibition is an important therapeutic strategy for vasospastic angina (Shimokawa 2002; Masumoto et al. 2002). Moreover, it has recently been

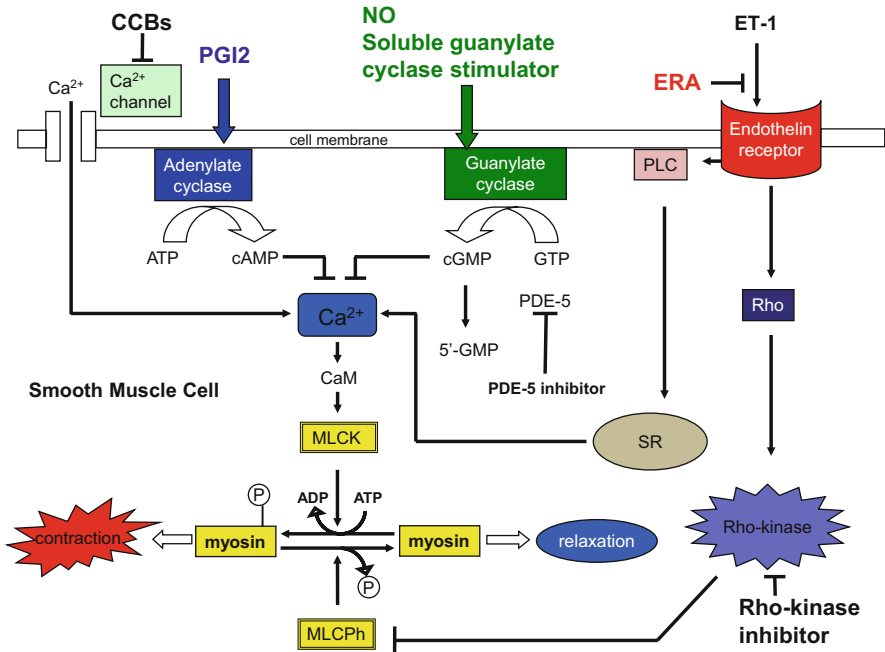


Fig. 3 Mechanism of pulmonary vasodilatation in response to conventional drugs and Rho-kinase inhibitors. *5-HT* serotonin, *CaM* calmodulin, *CCBs* calcium channel blockers, *ERA* endothelin receptor antagonist, *ET-1* endothelin-1, *MLCK* myosin light chain kinase, *MLCKp* myosin light chain phosphatase, *NO* nitric oxide, *PDE-5* phosphodiesterase-5, *PLC* phospholipase C, *VSMC* vascular smooth muscle cell, *PGI₂* prostacyclin, *PLC* phospholipase C, *SR* sarcoplasmic reticulum

demonstrated that Rho-kinase inhibition increases eNOS expression and decreases inflammatory cell migration and angiotensin II-induced upregulation of atherogenic molecules [e.g., monocyte chemoattractant protein (MCP)-1, plasminogen activator inhibitor (PAI)-1, and NADPH oxidase] and cardiovascular hypertrophy both in vitro and in vivo (Shimokawa and Takeshita 2005; Shimokawa 2002; Higashi et al. 2003).

2.2 PAH and Rho-Kinase Pathway

Increased pulmonary vascular resistance in PAH is caused by both pulmonary vascular remodeling and sustained pulmonary vasoconstriction (Giaid and Saleh 1995; Higenbottam and Laude 1998; Yuan et al. 1998), in which endothelial dysfunction and VSMC hyperconstriction may be involved through Rho-kinase activation (Fig. 2) (Higenbottam and Laude 1998; Yuan et al. 1998). Indeed, in patients with PAH, eNOS expression is reduced and pulmonary VSMC are

hyperreactive (Giaid and Saleh 1995; Yuan et al. 1998). It is thus conceivable that the Rho-kinase pathway plays an important role in the pathogenesis of PAH (Figs. 2 and 3).

Rho-kinase suppresses myosin phosphatase activity by phosphorylating the myosin-binding subunit of the enzyme, thus augmenting VSMC contraction at a given intracellular calcium concentration (Uehata et al. 1997; Somlyo and Somlyo 2000). VSMC hypercontraction mediated by activated Rho-kinase plays a key role not only in coronary artery spasm but also in PAH (Fukumoto et al. 2005, 2007; Shimokawa 2002; Fukumoto and Shimokawa 2011; Masumoto et al. 2002; Mohri et al. 2003; Fujita et al. 2010). Rho-kinase inhibition may be preferable to calcium channel blockers because of its selective spasmolytic effect on vascular hyperconstrictive segments (Fukumoto et al. 2007; Shimokawa 2002; Masumoto et al. 2002).

2.2.1 Rho-Kinase and Inflammation

A number of studies have suggested that inflammation may be involved in the pathogenesis of PAH (Fukumoto et al. 2007; Dorfmueller et al. 2003). Some patients with idiopathic PAH have immunological disturbances (e.g., circulating auto-antibodies, such as antinuclear antibodies) and elevated circulating levels of pro-inflammatory cytokines (e.g., interleukin-1 and -6) (Dorfmueller et al. 2003). It has been demonstrated that Rho-kinase is upregulated by inflammatory stimuli (Shimokawa and Takeshita 2005; Hiroki et al. 2004; Liao et al. 2007) and that Rho-kinase inhibition increases endothelial nitric oxide synthase (eNOS) expression and inhibits inflammatory cell migration and angiotensin II-induced upregulation of monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1 *in vivo* or *in vitro* (Shimokawa 2002), in which the Rho-kinase pathway may play an important role in the development of PAH.

2.2.2 Rho-Kinase and PAH

Indeed, it has been demonstrated that the long-term inhibition of Rho-kinase ameliorates monocrotaline (MCT)-induced PAH and hypoxia-induced PAH in animal models (Fukumoto et al. 2007; Abe et al. 2004, 2006; Fukumoto and Shimokawa 2011; Oka et al. 2008; Dahal et al. 2010). In those studies, Rho-kinase activity of pulmonary arteries was enhanced irrespective of the different etiologies and long-term treatment with Rho-kinase inhibitors ameliorated endothelial dysfunction and suppressed hypercontraction and proliferation of VSMC and migration of inflammatory cells (Abe et al. 2004, 2006; Oka et al. 2008). In clinical studies, it also has been demonstrated that a Rho-kinase inhibitor, fasudil, acutely improves pulmonary hemodynamics in patients with PAH (Fukumoto et al. 2005; Fujita et al. 2010; Ishikura et al. 2006).

3 Novel Therapeutic Target as Rho-Kinase Pathway in PAH

3.1 *Potential Importance of Rho-Kinase Inhibitors for the Treatment of PAH*

We have It has recently been demonstrated that Rho-kinase is a novel therapeutic target not only in ischemic heart disease and essential hypertension but also in PAH (Shimokawa 2002; Abe et al. 2004, 2006; Fukumoto et al. 2005; Jiang et al. 2007; Tawara et al. 2007; Yasuda et al. 2011). Potent and selective inhibitors of Rho-kinase like fasudil and azaindole-1 are available possessing an inhibitory effect on Rho-kinase being 100 times and 1,000 times more potent than on other protein kinases C and myosin light chain kinase, respectively (Shimokawa 2002; Shimokawa et al. 1999; Shimokawa and Rashid 2007b; Kast et al. 2007). Rho-kinase inhibition ameliorates VSMC hyperconstriction, increases eNOS expression, and decreases inflammatory cell migration and angiotensin II-induced upregulation of MCP-1 and PAI-1 (Shimokawa 2002; Morishige et al. 2001).

In the rat model of monocrotaline (MCT)-induced PAH, fasudil markedly suppressed the development of PAH when started simultaneously with monocrotaline and even induced a regression of the disorder when started after the establishment of PAH (Abe et al. 2004). Further, the highly selective Rho-kinase inhibitor azaindole-1 has shown to reverse MCT-induced pulmonary hypertension (Dahal et al. 2010; Lohn et al. 2009). Oral treatment with fasudil and azaindole-1 is also effective to inhibit the development of PAH induced by chronic hypoxia in mice, in which both eNOS-dependent and -independent mechanisms were involved (Abe et al. 2006; Dahal et al. 2010; Lohn et al. 2009; Pankey et al. 2012; Peng et al. 2012). Inhalation of fasudil is effective to reduce pulmonary vascular resistance in animal models of PAH with various etiologies (Nagaoka et al. 2005). Rho-kinase inhibitors appear to have pulmonary vasodilator effects through mechanism different from conventional drugs (Fig. 3). Therefore, it is possible that the combination of a Rho-kinase inhibitor and conventional drugs exerts additive or synergistic effects. Indeed, prostacyclin lacks direct inhibitory effects on Rho-kinase and the combination of oral prostacyclin analogue, beraprost, and a Rho-kinase inhibitor, fasudil, is more effective than each monotherapy for ameliorating monocrotaline-induced PH in rats (Tawara et al. 2007; Abe et al. 2005). Finally, the acute beneficial effects of intravenous fasudil have been shown in patients with PAH (Fukumoto et al. 2005; Fujita et al. 2010).

We have performed a clinical trial to examine the mid-term effects of Rho-kinase inhibitor, addressing the efficacy and safety in patients with PAH. (Fukumoto et al. 2013). Through those trials, we expect to learn whether the long-term inhibition of Rho-kinase is a novel therapeutic strategy for the treatment in patients with PAH.

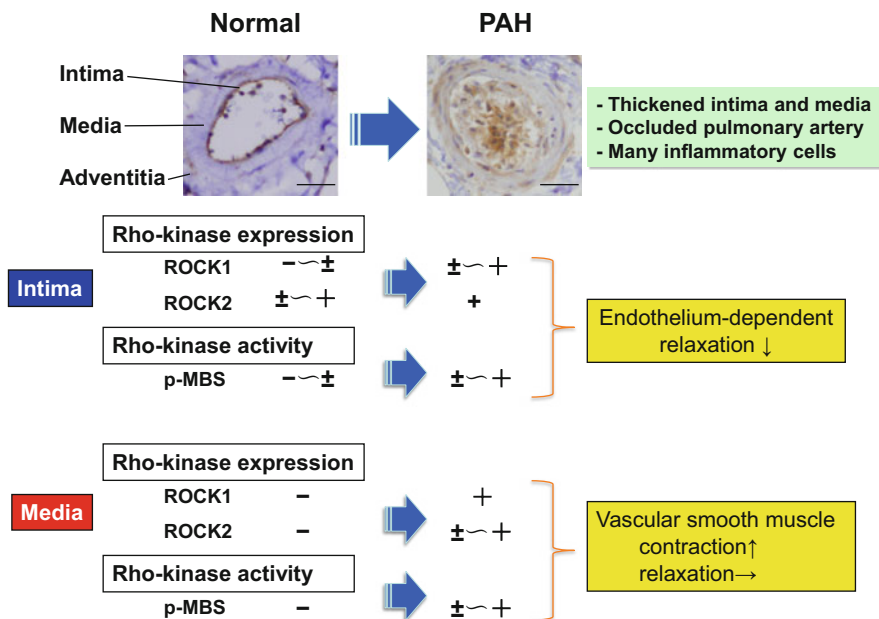


Fig. 4 Enhanced Rho-kinase expression and activity of pulmonary arteries from patients with pulmonary arterial hypertension. Immunohistological findings of Rho-kinase expression (both isoforms, ROCK 1 and 2) and activity in pulmonary arteries from patients with pulmonary arterial hypertension. Activated Rho-kinase expression and activity causes impairment of endothelium-dependent relaxation and enhances vascular smooth muscle contraction. *Scale bar*, 50 μ m (from Fukumoto and Shimokawa 2011 with permission)

3.2 Enhanced Rho-Kinase Expression and Activity in Patients with PAH

Recently, the direct evidence for Rho-kinase activation has been demonstrated in patients with PAH (Do e et al. 2009), where Rho-kinase activity is enhanced in circulating neutrophils and the pulmonary arteries from patients with PAH, resulting in hypercontractions of the artery (Fig. 4). These findings support the previous findings in animal models of PAH and during right heart cardiac catheterization in patients with PAH (Shimokawa and Takeshita 2005; Abe et al. 2004, 2006; Fukumoto et al. 2005; Fujita et al. 2010; Oka et al. 2008; Ishikura et al. 2006). Thus, increased pulmonary vascular resistance may be caused, at least in part, by activated Rho-kinase pathway (Do e et al. 2009). In addition, in patients with PAH, eNOS expression is reduced and pulmonary VSMC are hyperreactive (Do e et al. 2009; Giaid and Saleh 1995; Xu et al. 2004). Indeed, activated Rho-kinase causes several important abnormalities, including eNOS downregulation in endothelial cells, VSMC hypercontraction through inhibition of myosin phosphatase, VSMC proliferation and migration, and inhibition of VSMC apoptosis (Figs. 1 and 2)

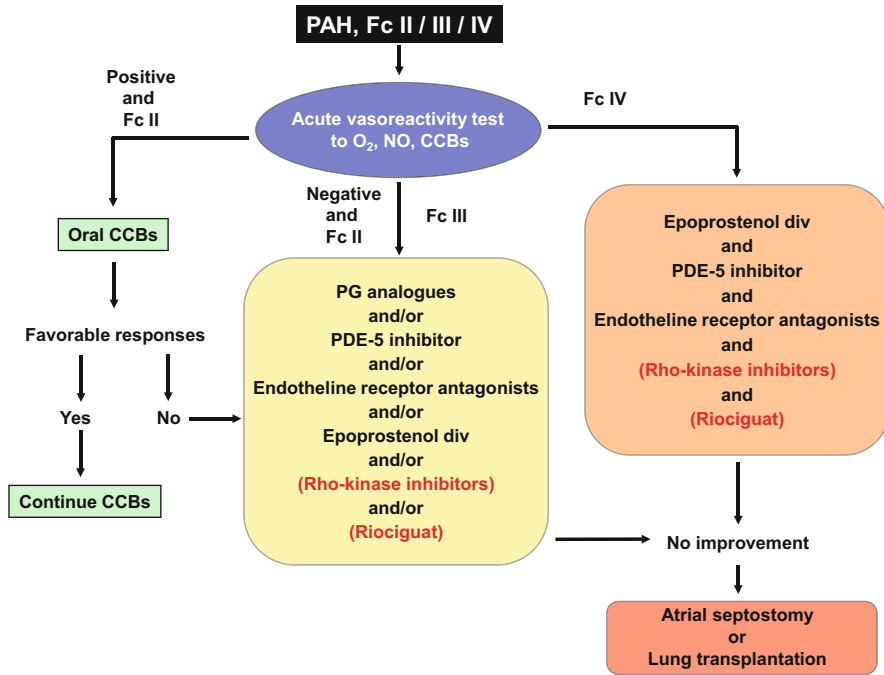


Fig. 5 Potential treatment algorithm for pulmonary arterial hypertension. In Fc II/III, the combination therapy can be beneficial compared with the mono-therapy. In Fc IV, the maximum use of medicines can be required. CCBs calcium channel blockers, Fc functional class, NO nitric oxide, PAH pulmonary arterial hypertension, PG prostaglandin, PDE-5 phosphodiesterase-5 (from Fukumoto and Shimokawa 2011 with permission)

(Shimokawa and Takeshita 2005; Shimokawa 2002; Liao et al. 2007; Shimokawa and Rashid 2007b; Takemoto et al. 2002). Also, the direct evidence has been recently demonstrated that endothelial vasodilator function is impaired and VSMC contraction is enhanced in pulmonary arteries from patients with PAH (Fig. 4) (Do e et al. 2009). These findings are consistent with the previous studies with MCT-induced PH in rats and hypoxia-induced PH in mice and previous clinical studies with PAH patients (Abe et al. 2004, 2006; Fukumoto et al. 2005; Fujita et al. 2010; Oka et al. 2008; Ishikura et al. 2006). Furthermore, the inhibition of Rho-kinase abolishes VSMC hypercontraction of pulmonary arteries from idiopathic PAH patients (Do e et al. 2009), which is also consistent with the previous clinical study that acute inhibition of Rho-kinase improves pulmonary hemodynamics in PAH patients (Fukumoto et al. 2005; Fujita et al. 2010; Ishikura et al. 2006). However, it still remains to be examined whether those functional abnormalities of pulmonary arteries could be ameliorated by the long-term treatment with a Rho-kinase inhibitor in patients with PAH. For this purpose, the effects of long-acting oral form of fasudil in PAH patients are examined in a clinical trial.

4 New Developments of Novel Rho Kinase Inhibitors

Although a clinical trial with long-acting oral form of fasudil was done for the mid-term treatment, further clinical trials are required. Ahead of cardiovascular fields including PAH (Fukumoto et al. 2013), clinical use of Rho-kinase inhibitors has begun for glaucoma and ocular hypertension in ophthalmology (K-115 from D Western Therapeutics Institute/Kowa, AR-12286 from Aerie Pharmaceuticals Inc, AMA-0076 from Amakem NV) (Williams et al. 2011). As Rho-kinase pathway can be a new therapeutic target in various fields, new developments are required to establish safe and effective Rho-kinase inhibitors.

5 Future Perspectives and Conclusions

PAH still remains a fatal disease, leading to right ventricular failure and premature death. Although significant research progress has been made on the pathogenesis of PAH, especially with regard to Rho-kinase, the detailed mechanisms of the disorder still remain to be elucidated. In the clinical practice, significant progress has also been made for new treatment (e.g., endothelin receptor antagonists, Rho-kinase inhibitors, and riociguat, Fig. 5). The usefulness of those new drugs remains to be fully examined in future studies.

It is expected that clinical trials with long-term oral treatment with Rho-kinase inhibitors will elucidate their effectiveness and safety for the treatment of PAH in humans.

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Serotonin Transporter and Serotonin Receptors

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Abstract The nature of the primary defect responsible for triggering and maintaining pulmonary artery smooth muscle (PA-SMC) proliferation in pulmonary artery hypertension (PAH) is poorly understood but may be either an inherent characteristic of PA-SMCs or a secondary response to an external abnormality, such as upregulation of growth factors. The serotonin hypothesis of PAH originated in the 1960s after an outbreak of the disease was reported among patients taking the anorexigenic drugs aminorex. The anorexiant dexfenfluramine which inhibits 5-HT neuronal uptake, causes 5-HT platelet depletion, and increases plasma levels of 5-HT, was then shown to increase the relative risk of developing PAH in the adults. More recently, the incidence of persistent pulmonary hypertension of the newborn was shown to be increased by the use of selective 5-HT reuptake inhibitors taken in late pregnancy. Serotonin is a vasoconstrictor and a potent mitogen for pulmonary smooth muscle cells (PA-SMC), an effect which depends upon activity of both the 5-HT transporter (5-HTT) and the 5-HT receptors. Expression analysis of lung tissues from PAH patients undergoing lung transplantation revealed an increased expression of the 5-HT transporter (5-HTT) and an enhanced proliferative growth response of isolated pulmonary arterial smooth muscle cells (PASMC) to 5-HT. Serotonin is contained in platelets but is also synthesized by pulmonary endothelial cells which express tryptophan hydroxylase 1, the rate-limiting enzyme of 5-HT synthesis. While inhibitors of 5-HTT and of 5-HT_{2B} receptors can reverse experimental PH, 5-HTT-overexpressing mice spontaneously develop PH. In patients with chronic lung disease, a close association has been found between a 5-HTT gene polymorphism and the severity of pulmonary hypertension. Agents capable of selectively inhibiting 5-HTT-mediated PA-SMC proliferation deserve to be investigated as potential treatments for pulmonary hypertension. However, the

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5-HT pathway is still studied only on a preclinical level and the usefulness of drugs interacting with the 5-HT pathway remains to be established in human PAH.

Keywords Serotonin • Pulmonary hypertension • Smooth muscle

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1 Introduction

The effects of serotonin (5-hydroxytryptamine, 5-HT) on the pulmonary circulation attracted the interest of researchers when an increased risk of idiopathic pulmonary hypertension (IPH) was reported in patients who had used appetite suppressants that interact with 5-HT (Abenheim et al. 1996). An association between the anorexigen aminorex and IPH was described in the 1960s. In the 1980s, fenfluramine use was shown to be associated with an epidemic of IPH in France and Belgium (Abenheim et al. 1996). The serotonin hypothesis received support from the observation that pulmonary hypertension (PH) develops spontaneously in Fawn-hooded rats, which have an inherited platelet-storage defect (MacLean et al. 2000).

Early studies focused on circulating 5-HT and its potential effects on the pulmonary vascular bed. They showed that patients with PAH had increased circulating serotonin levels even after heart–lung transplantation (Herve

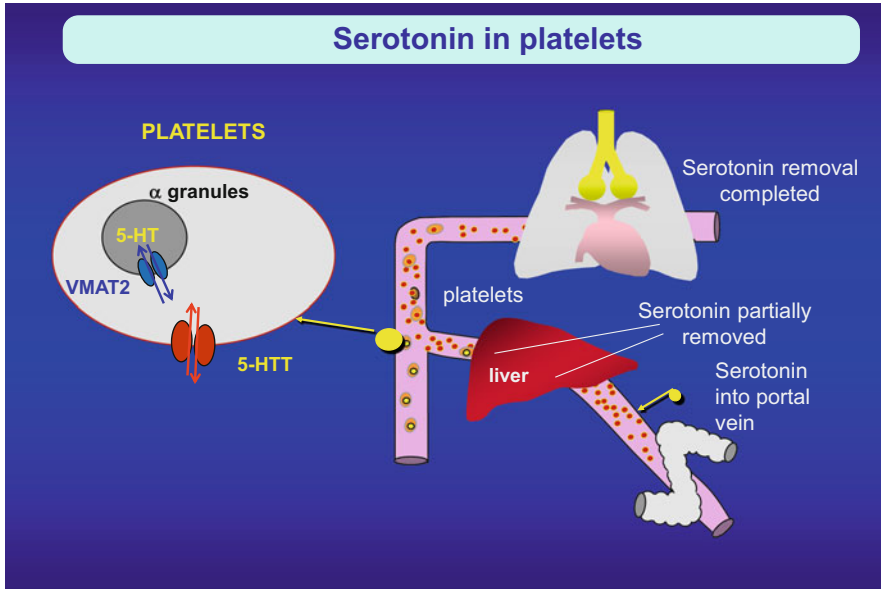


Fig. 1 Besides 5-HT, platelets contain three serotonergic components: the 5-HT_{2A} receptor (5-HT_{2A}), the plasma membrane serotonin reuptake transporter (5-HTT), and the vesicular monoamine transporter 2 (VMAT2). Intact platelets take up 5-HT by the 5-HTT and store the monoamine in vesicles by the VMAT2. Fenfluramine (Fen) is SERT-dependently transported into the cytoplasm and reverses the VMAT2 transport, increasing the cytoplasmic 5-HT concentration [5-HT]_i to levels that reverse the SERT activity, resulting in a continuous slow release of 5-HT. In contrast, selective inhibitors of the 5-HT transporter (5-HTT) block the uptake of 5-HT activity without affecting VMAT2 activity and therefore, do not favor the release of 5-HT from platelet stores. Activation of 5-HT_{2A} receptors potentiates the aggregation response to various platelet activators

et al. 1995). In addition to its vasoactive effects, 5-HT exerts mitogenic and co-mitogenic effects on pulmonary artery smooth muscle cells (PA-SMCs). Convincing evidence indicates that 5-HT-related stimulation of PA-SMC proliferation requires 5-HT internalization through the 5-HT transporter (5-HTT) (Chin et al. 1999; Dennery et al. 1998; Eddahibi et al. 2001; Lee et al. 1991) and activation of 5-HT_{2B} receptors (Launay et al. 2002) in PA-SMCs. In contrast, the constricting action of 5-HT on smooth muscle cells is mainly mediated by 5-HT receptors (5-HT_{1B/D}, 2A, and 2B) (MacLean et al. 2000). Accordingly, drugs that competitively inhibit 5-HTT also block the mitogenic effects of 5-HT on smooth muscle cells (Eddahibi and Adnot 2002). The appetite suppressants fenfluramine, d-fenfluramine, and aminorex differ from selective serotonin transporter inhibitors in that they not only inhibit serotonin reuptake but also trigger indoleamine release and interact with 5-HTT and 5-HT receptors in a specific manner (Eddahibi and Adnot 2002; Rothman et al. 1999) (Fig. 1).

Over the last few years, the role of the 5-HT pathway in the pathogenesis of PH has been extensively investigated, and severe alterations in several critical steps of this pathway have been found during the progression of PH. These alterations include changes in 5-HT synthesis and bioavailability, changes in 5-HT-mediating effects on target cells (PA-SMCs and fibroblasts), and alterations in intracellular signaling. These critical steps represent targets for future therapies and will be discussed individually.

2 Serotonin and Serotonin Synthesis in Pulmonary Hypertension

2.1 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is an endogenous vasoactive indoleamine substance found primarily in enterochromaffin tissue, brain, and blood platelets. The neutral amino acid L-tryptophan is the primary precursor of serotonin, the synthesis of which involving first hydroxylation of the benzene ring of tryptophan by the rate-limiting monooxygenase, tryptophan hydroxylase, to form 5-hydroxytryptophan, and second, decarboxylation of its amino-carboxy terminal group by an aromatic L-amino acid decarboxylase, to form 5-HT. The synthesis and degradation of serotonin are very active processes, the total serotonin pool being replaced every 24 h. In humans, approximately 90 % of the body's total serotonin is located in the intestines where it plays a role in mechano and chemotransduction. The most part of 5-HT that is released from enterochromaffin cells reaches the portal circulation where it is avidly taken up and stored in small electron-dense granules within platelets, the half-life of serotonin in platelets being 33–48 h. Accumulation of serotonin in platelets which are incapable of synthesizing the amine, is accomplished mostly via a sodium-dependent active transport process which is also present in endothelial, smooth muscle, and neural cells. The remaining circulating amine is either catabolized by the monoamine oxidase A (MAO-A) in the liver or taken up by active transport in the lungs and degraded inside endothelial and smooth muscle cells successively by monoamine oxidase B and an aldehyde deshydrogenase to form 5-hydroxyindole acetic acid (5-HIAA). These processes result in very low levels of free 5-HT in plasma under normal physiological conditions.

2.2 Serotonin Synthesis in Human and Experimental PH: Serotonin Synthesis as a Pharmacological Target in Pulmonary Hypertension?

Serotonin production outside the central nervous system occurs chiefly in the enterochromaffin cells, from which indoleamine is released into the bloodstream

before being taken up by circulating platelets. Thus, one hypothesis is that only 5-HT released from platelets acts on pulmonary vessels and that lung 5-HT bioavailability is independent from the rate of 5-HT synthesis (Fig. 1). However, in recent studies, we showed that 5-HT was also produced in the human lung by pulmonary endothelial cells (Eddahibi et al. 2006). The 5-HT synthesis rate was increased in endothelial cells from patients with idiopathic PH, supporting a contribution to pulmonary vascular remodeling of increased 5-HT availability in the immediate vicinity of PA-SMCs.

The functional impact of variations in 5-HT synthesis on PH development was recently examined in animal studies (Izikki et al. 2007; Morecroft et al. 2007). The critical step in 5-HT biosynthesis is catalyzed by the rate-limiting enzyme tryptophan hydroxylase (Tph). Tph activity therefore serves as a marker for 5-HT synthesis. Tph, which was initially thought to derive from a single gene, exists as two isoforms, Tph1 and Tph2, encoded by separate genes. Tph1 is expressed mainly in the gut and pineal gland (Darmon et al. 1988). The recently identified *Tph2* gene appears exclusively expressed in neurons and is known as the neuronal isoform (Walther et al. 2003a). Thus, 5-HT synthesis is thought to be controlled chiefly by Tph2 in the central nervous system and Tph1 in peripheral organs. As mentioned above, peripherally produced 5-HT is synthesized chiefly by enterochromaffin cells in the gut, but small amounts of 5-HT may also be formed at other sites including endothelial cells in the systemic and pulmonary arteries (Eddahibi et al. 2006; Ni et al. 2008). Human pulmonary microvascular endothelial cells produce 5-HT and express Tph1, and both 5-HT synthesis and Tph1 expression are increased in cells from patients with IPH compared to cells from controls (Eddahibi et al. 2006).

In recent studies, we also found that the severity of hypoxic PH in mice was directly linked to the rate of 5-HT synthesis catalyzed by the Tph enzyme isoforms Tph1 and Tph2 (Izikki et al. 2007; Morecroft et al. 2007). In mice lacking the *Tph1* gene and therefore exhibiting marked reductions in 5-HT synthesis rates and contents in peripheral organs, hypoxic PH was far less severe than in wild-type mice (Izikki et al. 2007; Morecroft et al. 2007). Treatment with the Tph inhibitor PCPA further reduced PH severity in *Tph1*^{-/-} mice, normalizing right ventricular systolic pressure and pulmonary artery muscularization, in keeping with persistence of some peripheral Tph activity in *Tph1*^{-/-} mice (Izikki et al. 2007). Evidence for involvement of Tph2 was obtained in mice harboring a polymorphism of the *Tph2* gene and showing differences in brain 5-HT synthesis rates according to their genotype. These animals also showed differences in the severity of hypoxic PH, which were abolished by PCPA treatment, indicating a contribution for neuronal Tph2 activity to the severity of hypoxic PH. In addition, hypoxia was found to increase 5-HT synthesis in the forebrain and lung, although no changes were noted in either Tph1 or Tph2 mRNA levels. Although mRNA levels are not necessarily reflective of protein expression, this finding suggests that the hypoxia-induced increase in 5-HT synthesis may result from enhanced enzyme activity as opposed to increased gene expression. Hypoxia not only increases 5-HT synthesis but also markedly diminishes lung 5-HT contents and blood 5-HT levels, a combination

suggesting a sharp increase in the indoleamine turnover rate during hypoxia exposure and PH development (Izikki et al. 2007).

Dysregulation of serotonin synthesis *in vivo* therefore appears closely linked to the PH phenotype in mice. Moreover, variations in the rate of 5-HT synthesis seem to directly affect the extent of pulmonary vascular remodeling, and, therefore, the severity of PH. Taken together with the observation that both 5-HT synthesis and Tph1 expression are increased in endothelial cells from patients with IPH compared to cells from controls, these results suggest that *Tph1*, and possibly *Tph2*, may deserve consideration as candidate genes for human PH. However, targeting Tph activity does not seem to hold promise for treating human PH, as unacceptable side effects might occur. Recent results, however, provided proof of concept that selectively inhibiting Tph1 may represent a therapeutic option for PAH.

3 Serotonin-Mediated Effects on Target Cells: The Serotonin Transporter (5-HTT) as a Potential Therapeutic Target in Pulmonary Hypertension

3.1 The Serotonin Transporter (5-HTT)

The serotonin transporter (5-HTT) belongs to a large family of integral membrane proteins responsible for terminating the action of neurotransmitters released from presynaptic neurons. This family includes GABA transporter, norepinephrine transporter, and dopamine transporter. In addition to the reuptake of 5-HT into neurons, the 5-HTT is also responsible for the uptake of serotonin by platelets, endothelial, and vascular smooth muscle cells. The 5-HT uptake process is driven by transmembrane gradients of Na^+ which is maintained by the activity of the Na^+/K^+ -ATPase. Transport of serotonin is sensitive to nanomolar concentrations of tricyclic and heterocyclic antidepressants, such as imipramine, fluoxetine, paroxetine, and citalopram which bind to the carrier protein. The identity of the neuronal and non-neuronal 5-HT transporter indicates that these proteins are encoded by the same single-copy gene. The human 5-HT gene mapped to chromosome 17q11.1–17q12 is organized in 14 exons spanning 35 kb. It has been initially cloned from the human midbrain raphe complex and platelets. The coding sequence for the 5-HTT covers about 2 kb which corresponds to approximately 617–630 amino acids. Analysis of the amino acid sequences predicted by the transporter clones suggests the presence of 12 putative transmembrane domains with a large extracellular loop between transmembrane domains 3 and 4 with both the N- and C-termini residing within the cytoplasm. There are multiple sites for NH_2 -linked glycosylation and multiple consensus sequences in the intracellular domains for phosphorylation by protein kinases such as protein kinase A and protein kinase C. The promoter activity also appears to be regulated by an interplay of several positive and negative regulatory elements including AP1, CRE, NF- κ B, and SP1.

3.2 Role of the Serotonin Transporter (5-HTT) in Pulmonary Hypertension

Accumulating evidence suggests that the 5-HT transporter (5-HTT) in the lung may be a key determinant of pulmonary vessel remodeling because of its effects on PA-SMC growth. 5-HTT is abundantly expressed in the lung, where it is predominantly located on PA-SMCs (Eddahibi et al. 2001; Fanburg and Lee 1997). It is encoded by a single gene expressed in several cell types such as neurons, platelets, and pulmonary vascular endothelial and smooth muscle cells. The level of 5-HTT expression in humans seems considerably higher in the lung than in the brain, suggesting that altered 5-HTT expression may have direct consequences on PA-SMC function. The requirement for 5-HTT as a mediator for the mitogenic activity of 5-HT appears specific of PA-SMCs, since no such effect has been reported with other SMC types. Direct evidence that 5-HTT plays a key role in pulmonary vascular remodeling was recently provided by studies showing that mice with targeted 5-HTT gene disruption developed less severe hypoxic PH than did wild-type controls and that selective 5-HTT inhibitors attenuated hypoxia- and monocrotaline-induced PH (Eddahibi et al. 2000; Guignabert et al. 2005). Conversely, increased 5-HTT expression was associated with increased severity of hypoxic PH (MacLean et al. 2004). To investigate whether 5-HTT overexpression in PA-SMCs was sufficient to produce PH, we generated transgenic mice overexpressing 5-HTT under the control of the SM22 promoter. We found that these transgenic mice with selective 5-HTT overexpression in smooth muscle cells spontaneously developed PH (Guignabert et al. 2005). Importantly, PH in these SM22-5-HTT+ mice developed without any alterations in 5-HT bioavailability and, therefore, as a consequence solely of 5-HTT protein overexpression in smooth muscle cells. Interestingly, only female mice that ubiquitously overexpress the human 5-HTT gene develop PH, suggesting that some interactions between estrogens and the 5-HTT gene influence the PAH phenotype (White et al. 2011). Taken together, these observations suggest a close correlation between 5-HTT expression and/or activity and the extent of pulmonary vascular remodeling during experimental PH.

3.3 Overexpression of 5-HTT and PA-SMC Hyperplasia in Idiopathic PH

Evidence that 5-HTT plays an important role in the pathogenesis of human IPH is now clearly established. 5-HTT expression was shown to be increased in platelets and lungs from patients with IPH, where it predominated in the media of thickened pulmonary arteries and in onion-bulb lesions (Eddahibi et al. 2001). Interestingly, the higher levels of 5-HTT protein and activity persisted in cultured smooth muscle cells from pulmonary arteries of patients with IPH, compared to cells from controls

(Eddahibi et al. 2001). Moreover, PA-SMCs from patients with IPH grew faster than PA-SMCs from controls when stimulated by serotonin or serum, as a consequence of increased expression of the serotonin transporter (Eddahibi et al. 2001). In the presence of 5-HTT inhibitors, the growth-stimulating effects of serum and serotonin were markedly reduced, and the difference between growth of PA-SMCs from patients and controls was abolished. The proliferative response of PA-SMCs to various growth factors such as PDGF, EGF, TGF β , FGFa, and IGF did not differ between patients with IPH and controls (Eddahibi et al. 2001). It follows that 5-HTT overexpression and/or activity in PA-SMCs from patients with IPH is responsible for the increased mitogenic response to serotonin and to serum (which contains micromolar concentrations of serotonin).

3.4 5-HTT Gene Polymorphism and Increased 5-HTT Expression in Pulmonary Arteries from Patients with Idiopathic PH

That 5-HTT expression is genetically controlled has been convincingly demonstrated: a polymorphism in the promoter region of the human 5-HTT gene alters the level of transcription (Lesch et al. 1993). This polymorphism consists of two common alleles, a 44-bp insertion or deletion, designated the L and S allele, respectively. The L allele drives a two- to threefold higher level of 5-HTT gene transcription than does the S allele. In studies of PA-SMCs from controls, we found that cells from LL individuals had a twofold increase in 5-HTT mRNA expression, compared to cells from SS subjects, and that LS individuals had an intermediate level of expression (Eddahibi et al. 2001). Accordingly, the growth-stimulating effects of 5-HT or serum were more marked in LL cells than in LS or SS cells, indicating that the ability of PA-SMCs to proliferate in response to serotonin or serum was directly linked to the functional polymorphism of the 5-HTT gene promoter. While studying a population of 89 patients with IPH, we found that the L allelic variant of the 5-HTT gene promoter, associated with 5-HTT overexpression and increased PA-SMC growth, was present homozygously in 65 % of patients and 27 % of controls (Eddahibi et al. 2001). This finding suggested that the 5-HTT gene polymorphism might either convey susceptibility to PH or act as an important modifier of the PH phenotype. However, recent studies investigating larger populations of patients with IPH, associated forms of PH (APH), or familial PH (FPH) did not confirm our previous results (Machado et al. 2006; Willers et al. 2006). This apparent discrepancy might be related to specific characteristics of our cohort, which included a relatively high percentage of lung-transplant recipients. Because patients referred to lung transplantation are selected on the basis of faster disease progression or poor responsiveness to conventional treatment, this may have introduced a bias in our cohort. Support for this possibility comes from our unpublished observation that among 50 patients

with PH who underwent lung transplantation between 1998 and 2005 at the Marie Lannelongue hospital (Le Plessis Robinson, France), 57 % had the LL genotype (Dewachter et al. 2006). Moreover, we previously reported a high frequency of the LL genotype among lung-transplant recipients with APH (Marcos et al. 2004). Thus, one possible explanation for the differences between these studies may be related to differences in the disease progression profiles of the study populations. Therefore, the LL genotype may not deserve to be considered a susceptibility factor for the development of IPH. The possibility that it may affect disease progression remains to be evaluated in a larger population in which transplantation and death are censored.

3.5 Potential Usefulness of 5-HTT Inhibitors in the Treatment of Adult Patients with PH

5-HTT inhibitors in the dosages commonly used in depression may induce therapeutic effects in patients with IPH. Inhibition of in vitro SMC proliferation occurs with micromolar concentrations, which are at the lower end of the plasma level range obtained with the usual dosages of these drugs. Increasing the dosage within the authorized range would produce effective levels in the pulmonary circulation. 5-HTT inhibitors are considered safe, despite recent evidence of an increased risk of persistent pulmonary hypertension (PPHN) in neonates born to mothers treated during pregnancy (Chambers et al. 2006).

At present, the effects of 5-HTT inhibitors on the progression of human PH have not been assessed in a controlled study using appropriate drug dosages. These drugs, however, have occasionally been given to patients with IPH, in the usual recommended dosages, with no major effects detectable in individual patients.

3.6 Serotonin and Lung Development: Persistent Pulmonary Hypertension in the Newborn

Persistent pulmonary hypertension of the newborn (PPHN) is a life-threatening condition that occurs in up to 2 infants per 1,000 live births and that is caused by a failure of pulmonary vascular resistance to decrease after birth. Recent epidemiological studies have linked the use of selective serotonin reuptake inhibitors (SSRIs) for the treatment of maternal depression to a two- to sixfold increase in the incidence of PPHN when taken in late pregnancy (Chambers et al. 2006; Kieler et al. 2012; Delaney and Cornfield 2012). This increased risk seems to be a drug class effect since it appears to be of similar magnitude for each of the specific SSRIs used, including sertraline, citalopram, paroxetine, and fluoxetine (Kieler et al. 2012). The mechanism by which SSRIs may affect the pulmonary vasculature

and cause PPHN remains unknown. Possible contributing factors include the accumulation of SSRIs in the lungs, with some consequences on lung development, as well as some direct effects of serotonin on the pulmonary vasculature. Recently, the hemodynamic effects of 5-HT, 5-HT receptor antagonists, and SSRIs were studied in fetal sheep (Delaney et al. 2012). Infusion of 5-HT was associated with marked pulmonary vasoconstriction which was abolished by ketanserin, a 5-HT_{2A} receptor antagonist, but not by 5-HT_{1B} or 5-HT_{2B} receptor antagonists. Brief infusions of the SSRIs, sertraline, and fluoxetine, caused potent and sustained elevations of pulmonary vascular resistance. Moreover, in the lamb with experimental PPHN, SSRIs and 5-HT caused further elevation of pulmonary vascular resistance. Thus, 5-HT, by causing pulmonary vasoconstriction, may contribute to maintenance of high pulmonary vascular resistance in the normal fetus through stimulation of 5-HT_{2A} receptors. By preventing 5-HT internalization through inhibition of the 5-HT transporter, SSRIs may contribute to increase 5-HT bioavailability in the fetal pulmonary circulation, thereby increasing 5-HT binding to 5-HT receptors. The effects of SSRIs on the developing pulmonary circulation in the neonate may therefore differ to those exerted in adult pulmonary hypertension. Whether it is due to differential effects of SSRIs on 5-HT bioavailability or whether 5-HTT signaling may differ in the neonate and adult pulmonary circulations remain opened questions.

4 Role of the Serotonin Receptors in Pulmonary Hypertension

On release from platelets or nerve endings, serotonin can activate up to 14 structurally distinct 5HT receptors, which are classified into seven families (5HT₁₋₇). Of these, the 5HT_{2A}, 5HT_{2B}, and 5HT_{1B} receptors have been investigated with respect to PH.

4.1 5HT_{2A} Receptor

In most nonhuman mammals, the 5HT_{2A} receptor mediates vasoconstriction in both the systemic and the pulmonary circulation. For example, 5HT_{2A} receptor-mediated vasoconstriction occurs in the mouse and rat lung under control conditions (Keegan et al. 2001; MacLean et al. 1996a). The 5HT_{2A} receptor antagonist ketanserin has proved clinically effective in the treatment of systemic hypertension, especially in the elderly (Frishman et al. 1995). Thus, ketanserin does not act specifically on the pulmonary circulation and its systemic effects limit its use in both IPH and secondary PH, where it fails to significantly improve pulmonary hemodynamics (Frishman et al. 1995). Recent studies performed in fetal animals, however,

reported that 5-HT behaved as a strong pulmonary vasoconstrictor and that this effect was mediated primarily by 5-HT_{2A} receptors. 5-HT_{2A} mediated 5-HT induced pulmonary vascular constriction may therefore contribute to the mechanism by which maternal use of SSRIs increase the risk of PPHN.

4.2 5HT_{2B} Receptor

Strong evidence for the 5-HT_{2B} receptor as a therapeutic target in PAH has emerged. 5-HT_{2B} knockout animals are resistant to hypoxia-induced PH and administration of the specific 5-HTR_{2B} antagonist RS-127445 prevented the increase in pulmonary arterial pressure (Ppa) in mice that were challenged to hypoxia (Launay et al. 2002). Recently, terguride, a potent 5-HTR_{2A/2B} antagonist, was reported to exert anti-remodeling effects upon long-term use in rats with monocrotaline-induced PH (Dumitrascu et al. 2011). Immunohistochemistry against 5-HTR_{2A/B} on human lungs revealed their localisation to the vascular smooth muscle layer and quantitative RT-PCR showed 5-HTR_{2B} upregulation in pulmonary artery smooth muscle cells (PASMC) isolated from PAH patients. Proliferation and migration of cultured primary human PASMC were dose-dependently blocked by terguride (Dumitrascu et al. 2011). Terguride is approved for ovulation disorders due to hyperprolactinaemia by acting as a partial dopamine receptor (DR) D₂ agonist in the pituitary gland (Ciccarelli and Camanni 1996). In addition, it is a strong antagonist of 5-HTR_{2A} and 5-HTR_{2B} (Millan et al. 2002), and, therefore, seems well suited for treatment of PAH. However, terguride doses needed to achieve 5-HT_{2B} receptor antagonistic properties are higher than those required for its dopaminergic agonistic properties. Terguride is well tolerated at dopaminergic doses. Whether terguride or other drugs with 5-HT_{2B} receptor antagonistic properties may be safely used in patients with PH deserve further studies.

4.3 5HT_{1B} Receptor

5HT_{1B} receptors mediate constriction of human pulmonary arteries (MacLean et al. 1996a; Morecroft et al. 1999). There is also evidence supporting a role for the 5HT_{1B} receptor in PH development (Keegan et al. 2001; MacLean et al. 1996b). (1) Inhibition of 5HT_{1B} activity, either by genetic knockout or by antagonism, reduces hypoxia-induced pulmonary vascular remodeling (Keegan et al. 2001). (2) Serotonin is required for the proliferative effect of the calcium-binding protein S100A4/Mts1, and the 5HT_{1B} receptor and 5-HTT are codependent in regulating S100A4/Mts1-induced human PA-SMC proliferation (Lawrie et al. 2005). (3) Cooperation occurs between the 5-HT_{1B} receptor and 5-HTT in mediating pulmonary vascular contraction (Morecroft et al. 2005). (4) The 5HT_{1B} receptor

is increased in mice overexpressing human 5-HTT and in the Fawn-hooded rat, which also exhibits increased 5-HTT expression (Morecroft et al. 2005); both models are particularly susceptible to hypoxia-induced pulmonary vascular remodeling. (5) The 5HT_{1B} receptor and 5-HTT act cooperatively to facilitate serotonin-induced proliferation and rho-kinase-induced nuclear translocation of extracellular regulated kinase (ERK)1/ERK2 in PA-SMCs (Liu et al. 2004). (6) The 5HT_{1B} receptor plays a role in PH development in a pig model (Rondelet et al. 2003). (7) Finally, remodeled PAs from patients with PH overexpress the 5HT_{1B} receptor (Launay et al. 2002). These data suggest that serotonin-induced activation of the 5HT_{1B} receptor may be involved in pulmonary vascular remodeling and that cooperation exists between the 5-HT_{1B} receptor and 5-HTT. As 5HT_{1B} receptor-mediated changes are specific of the pulmonary circulation, this receptor is an attractive therapeutic target for PH.

5 Serotonin Signaling in PH

As mentioned above, 5-HT transport within the cell via 5-HTT is involved in the proliferation of PA-SMCs and fibroblasts. How 5-HT internalization is linked to cell proliferation signaling remains unknown, although several intracellular signaling pathways are known to be involved. ROS generation and ERK1/2 activation have been suggested as potential mechanisms (Lee et al. 1998, 2001). ROS may be generated either via 5-HT breakdown by monoamine-oxidase or via activation of NADPH oxidase. In bovine PA-SMCs, ROS may mediate the phosphorylation of ERK1/2 (Lee et al. 2001). Phosphorylated ERK1/2 may then be translocated to the nucleus, where it may increase the DNA binding of transcription factors such as GATA-4, Egr-1, and Elk-1, leading to the expression of proteins such as S1004/Mts1 that play a major role in both human and experimental PH (Lawrie et al. 2005). Most of these studies, however, did not specify the role for 5-HTT or 5-HT receptors in mediating the activation of these pathways.

More recently, evidence has been provided that activation of the small G protein RhoA and its target Rho kinase may represent a major pathway involved in 5-HT-induced PA-SMC proliferation via 5-HTT (Walther et al. 2003b). First, it was shown that 5-HT internalized in platelets via 5-HTT was covalently linked to RhoA by intracellular type 2 transglutaminase (TG2), which led to constitutive RhoA activation (Walther et al. 2003b). This link was then demonstrated *in vivo* in pulmonary arteries of rats under hypoxic conditions, providing a connection between 5-HTT and RhoA activation (Guilluy et al. 2007). Moreover, we recently provided evidence that RhoA activation by 5-HT occurred both in platelets and in PA-SMCs during PH progression and that this event contributed to PA-SMC proliferation (Guilluy et al. 2009). In mice characterized by 5-HTT overexpression in smooth muscle cells and spontaneous development of PH, treatment with 5-HTT inhibitors limited both PH progression and RhoA/Rho kinase activation. Only partial limitation of PH progression, however, is observed after treatment with the

Rho-kinase inhibitor fasudil, suggesting that activation of the RhoA pathway may generate only part of the intracellular signals originating from internalization of 5-HT via 5-HTT (Guilluy et al. 2009).

In conclusion, direct involvement of the 5-HTT/RhoA/Rho kinase signaling pathway in 5-HT-mediated PA-SMC proliferation and platelet activation during PH progression identifies RhoA/Rho kinase signaling as a promising target for new treatments against PH.

6 Conclusions

In conclusion, the data reviewed here support a crucial role for serotonin synthesis and functional activities of serotonin receptors and transporter in the pathogenesis of pulmonary vascular remodeling. Agents targeting these molecular effectors deserve to be investigated as potential treatments for PH.

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Targeting of Platelet-Derived Growth Factor Signaling in Pulmonary Arterial Hypertension

Eva Berghausen, Henrik ten Freyhaus, and Stephan Rosenkranz

Abstract Despite recent advances in the management of patients with pulmonary arterial hypertension (PAH), this disease remains a devastating condition with limited survival. While the current therapies primarily target the vasoconstrictor/vasodilator imbalance in the pulmonary circulation, there is currently no cure for PAH, and pulmonary vascular remodeling—representing the underlying cause of the disease—is only modestly affected. Hence, novel therapeutic approaches directly targeting the vascular remodeling process are warranted. Recent studies provided compelling evidence that peptide growth factors, which elicit their signals via receptor tyrosine kinases, are important contributors to the development and progression of PAH. In particular, platelet-derived growth factor (PDGF) is a strong mitogen for pulmonary vascular smooth muscle cells and protects these cells from apoptosis, thus representing an important mediator of pulmonary vascular remodeling. PDGF ligand and receptors are upregulated in PAH, and experimental studies have shown that inhibition of PDGF receptor signaling by pharmacological or genetic approaches prevents the development of PAH in animal models and is even able to reverse pulmonary vascular remodeling once it has been established. Consistently, results from phase II and phase III clinical trials indicate that the tyrosine kinase inhibitor imatinib mesylate, which potently inhibits the PDGF receptor, is effective in improving exercise capacity and pulmonary hemodynamics as add-on therapy in patients with severe PAH (i.e., pulmonary vascular resistance >800 dynes s cm^{-5}). Future studies will evaluate the long-term clinical efficacy and safety of imatinib, including patients with less impaired hemodynamics. Based on the current knowledge, targeting of PDGFR signaling is likely to become an anti-proliferative treatment option for patients with PAH and has the potential to at least partially correct the pathology of the disease.

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1 Introduction

Pulmonary hypertension (PH) is characterized by a marked and sustained elevation of pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) (Badesch et al. 2009; McLaughlin et al. 2009; Galiè et al. 2009b). It is defined by an elevation of the mean PAP to ≥ 25 mmHg at rest. The hemodynamic definition also distinguishes precapillary PH from postcapillary PH, in which the elevation of the PAP occurs as a consequence of elevated filling pressures in the left heart. Furthermore, pulmonary *arterial* hypertension (PAH) is distinguished from other more prevalent forms of PH that may develop secondary to left heart disease, chronic lung disease, and chronic thromboembolic disease (Galiè et al. 2009a–c; Rosenkranz 2007; Simonneau et al. 2009). The current clinical classification of PH (Dana Point 2008) defines five subgroups (Simonneau et al. 2009): group 1 comprises patients with PAH including idiopathic PAH (IPAH), heritable PAH (HPAH), PAH associated with connective tissue disease, drug- and toxin-induced PAH, portopulmonary hypertension, PAH due to HIV infection, PAH associated with congenital heart disease, and other rare causes of PAH. The other four groups comprise patients with PH owing to left heart disease (group 2), chronic lung disease/hypoxia (group 3), chronic thromboembolic PH (CTEPH; group 4), and PH with unclear or multifactorial etiologies (group 5).

PAH is a severe disorder which is characterized by incremental pulmonary vasculopathy and—without sufficient treatment—a poor life expectancy (Farber and Loscalzo 2004; Rosenkranz 2007). Chronically elevated PAP and PVR represent an increased afterload of the right heart, which over time leads to right ventricular hypertrophy and failure, eventually causing premature death (van Wolferen et al. 2007; Haworth 2007). Data from the US registry that were collected during the 1980s—when there was no efficient treatment available—showed that the median survival was limited to only 2.8 years (D’Alonzo et al. 1991). In the last decade, a number of drugs have been approved for the targeted treatment of PAH, and these compounds have proven effective in improving outcome and quality of life. However, these therapies are not able to cure the disease, as they primarily target the diminished pulmonary vascular relaxation response, while proliferative remodeling of the small pulmonary resistance vessels underlying the disease is only modestly affected. Although the current treatments improve exercise capacity and delay disease progression, their efficacy with regard to improvement of pulmonary hemodynamics is limited, and mortality remains unacceptably high (Benza et al. 2010; Humbert et al. 2010). Given the lack of curative therapeutic options at present, it is essential to develop innovative additional concepts that further improve pulmonary hemodynamics, clinical status, and survival. To this end, a key aim is to reverse or at least to halt pulmonary vascular remodeling, representing the underlying cause of PAH. Recent studies have indicated that peptide growth factors, which elicit their signals through receptor tyrosine kinases (RTK), play a crucial role in the development and progression of pulmonary vascular remodeling. In particular, several studies have indicated that platelet-derived growth factor (PDGF) plays a prominent role in abnormal remodeling of pulmonary resistance vessels (Schermuly et al. 2005; Perros et al. 2008). Hence, inhibition of PDGF signaling by tyrosine kinase inhibitors (TKI) appears as a promising concept for the treatment of PAH.

2 Pathobiology of Pulmonary Arterial Hypertension

PAH is a complex and multifactorial disease, and three key mechanisms are involved in its development and progression: (1) inappropriate pulmonary vasoconstriction, (2) vascular remodeling, and (3) in situ thrombosis. Various cell types residing in all layers of the vessel wall such as endothelial cells, vascular smooth muscle cells (VSMC), fibroblasts, platelets, and inflammatory cells contribute to the pathobiology of PAH (Hassoun et al. 2009; Morrell et al. 2009; Galiè et al. 2010). While a genetic background may predispose individuals for PAH, associated diseases and/or trigger factors are necessary for manifestation of the disease. Genetic alterations include modifications in the genes that comprise the signaling pathways of transforming growth factor- β (bone morphogenic protein receptor-2, BMPR2; activin receptor-like kinase-1, ALK1) and serotonin (5-hydroxytryptamine transporter; HTT) (Newman et al. 2004; Machado et al. 2009). Since only 15–20 %

of individuals with such a genetic condition develop PAH, the genetic variants are recognized as predisposing factors, and associated diseases, environmental factors, and/or gene–gene interactions are required for the manifestation of PAH (Farber and Loscalzo 2004; Machado et al. 2009).

A key feature in the pathobiology of PAH is a vasoconstrictor/vasodilator imbalance, which results in abnormal pulmonary vasoconstriction. While the bio-availability of pulmonary vasodilators such as nitric oxide (NO) and prostacyclin is decreased, the expression and release of potent vasoconstrictors such as endothelin (ET)-1 and serotonin are enhanced. These abnormalities are accompanied by the dysregulation of further modulators of vascular tone, including overexpression of the serotonin transporter and phosphodiesterase type 5 (PDE5) in the media, reduced expression and activity of voltage gated potassium channels (Kv), and alterations in the cross talk of vascular cells (Hassoun et al. 2009; Morrell et al. 2009). The current targeted PAH therapies all target on the imbalance between pulmonary vasodilators and constrictors.

A second key feature of the vascular pathology in PAH is abnormal remodeling of the small pulmonary resistance vessels. In fact, these structural alterations occurring in all layers of the vessel wall are believed to represent the main underlying cause of the disease. Disorganized endothelial cell proliferation may lead to the formation of plexiform lesions in the vicinity of obstructed arterioles, which contain monoclonal endothelial cell conglomerates and inflammatory cells (Morrell et al. 2009; Tuder et al. 2009). Additional pathological characteristics are owing to pulmonary arterial smooth muscle cell proliferation and migration, which leads to medial hypertrophy of the muscular resistance vessels, and an extension of pulmonary arterial smooth muscle cells into the distal, normally non-muscular peripheral pulmonary arterioles (Hassoun et al. 2009; Morrell et al. 2009). Further changes include intimal fibrosis, chronic inflammatory responses, increased connective tissue deposition in the medial layer, hypertrophy/thickening of the adventitia, and prothrombotic abnormalities causing in situ thrombosis (Hassoun et al. 2009; Morrell et al. 2009; Tuder et al. 2009; Stenmark et al. 2002; Tuder and Voelkel 2010). Both concentric and eccentric pulmonary vascular remodeling lead to progressive vascular narrowing and thus a marked loss of the cross-sectional luminal area. This results in an increase of PVR and right ventricular pressure load, which in turn causes right heart hypertrophy and failure and eventually premature death.

3 Targeted PAH Therapies and Therapeutic Limitations

The current treatment for PAH consists of supportive therapies (e.g., long-term oxygen therapy, oral anticoagulants, diuretics) and targeted drug treatment (Badesch et al. 2007; Barst et al. 2009; Galiè et al. 2009a–c). In few patients who demonstrate a favorable response in acute vasoreactivity testing, high-dose calcium channel blockers may be effective. However, most patients fail to meet the

vasoresponder criteria and do not benefit from such treatment (Sitbon et al. 2005). Hence, the vast majority of patients requires targeted PAH therapy.

In recent years, there has been considerable progress in our understanding of the pathobiology and the targeted treatment of PAH. A number of pharmacological interventions have been developed which have provided symptomatic relief and improved outcome. All currently approved therapies for PAH belong to three classes of drugs: Prostacyclin analogues (prostanoids; epoprostenol, iloprost, treprostinil), endothelin receptor antagonists (ERA; bosentan, ambrisentan), and phosphodiesterase type 5 inhibitors (PDE5i; sildenafil, tadalafil). The efficacy and safety of each of these drugs were demonstrated in randomized, placebo-controlled trials (Barst et al. 1996; Galiè et al. 2005, 2009a; Olschewski et al. 2002; Oudiz et al. 2009; Rubin et al. 2002; Simonneau et al. 2002), and all above compounds have been approved by the European Medicines Agency (EMA) and/or the Food and Drug Administration (FDA). According to current guidelines, members of these drug classes may be prescribed for the treatment of PAH alone or in combination, depending on the patient's functional status, hemodynamic parameters, and the individual response to therapy (Barst et al. 2009; Galiè et al. 2009b).

While the advent of targeted PAH therapies has significantly improved the clinical situation and outcome of affected patients, the current treatment of PAH is not satisfactory. Prostanoids require subcutaneous/intravenous administration or frequent inhalations, and adverse reactions include flushing, headache, diarrhea, injection site pain, and catheter-related complications such as sepsis and pump failure (Galiè et al. 2009b; Simonneau et al. 2002). ERA are associated with liver toxicity requiring periodic monitoring of liver enzymes (Humbert et al. 2007), and PDE5i are not effective in all patients while dosing remains uncertain (Chockalingam et al. 2005; Galiè et al. 2005; Simonneau et al. 2008). Although the current treatment options have markedly improved quality of life and survival (Galiè et al. 2009c, 2010), their efficacy is still limited. Data from randomized, controlled trials have shown that the improvements in exercise capacity and mean PAP and PVR are only moderate. Many patients remain in functional class III, and the mortality of treated PAH patients remains unacceptably high. Recent data from large registries demonstrate that the annual mortality rate in the modern treatment era remains at approximately 10 % (Benza et al. 2010; Humbert et al. 2010).

Since the current PAH therapies primarily act as pulmonary vasodilators and do not markedly affect the underlying morphological changes of the small pulmonary arterioles, it is apparent that these treatments are only partially effective in providing symptomatic relief and in delaying disease progression. Hence, it is essential to establish additional therapies that more effectively target pulmonary vascular remodeling. While some data suggest that prostanoids, PDE5i, and ERA may elicit modest anti-proliferative effects (Chen et al. 1995; Falcetti et al. 2010; Wharton et al. 2000, 2005), this is most likely secondary to their vasodilatory properties and decreased shear stress, which itself represents a mitogenic stimulus (Haga et al. 2003; Sterpetti et al. 1992). Direct targeting of vascular remodeling by anti-proliferative approaches provides a promising strategy which holds the potential to reverse the remodeling process and to take a step closer to more efficiently affect or even cure PAH.

4 Pathogenic Role of Platelet-Derived Growth Factor in PAH

Compelling evidence reveals that PDGF is a major contributor to the pathobiology of vascular disorders including PAH (Raines 2004; Grimminger and Schermuly 2010). PDGF is expressed in multiple cell types including endothelial cells, smooth muscle cells, and macrophages and acts as a strong mitogen and chemokine. In fact, PDGF represents the most potent mitogenic factor for VSMC (Heldin and Westermark 1999; Kazlauskas 1994; Rosenkranz and Kazlauskas 1999; Schwartz 1997). It exerts its actions via the activation of two PDGF receptor (PDGFR) subtypes, PDGFR α and PDGFR β . Both are expressed in numerous cell types of mesenchymal origin and promote various cellular responses including proliferation, migration, cellular survival, and transformation (Rosenkranz and Kazlauskas 1999; ten Freyhaus et al. 2006).

In the normal vessel wall, both PDGF ligand and the PDGFR α and PDGFR β subtypes are expressed at very low levels. However, increased expression of PDGF ligands and receptors was found in atherosclerotic plaques and in neointimal lesions following balloon injury (Abe et al. 1998; Raines 2004; Tanizawa et al. 1996). Interventional studies in animals have shown that inhibition of PDGF signaling by TKIs, neutralizing antibodies, or oligonucleotides against the β PDGFR was able to reduce neointima formation and the accumulation of VSMCs in intimal lesions (Raines 2004; Levitzki 2005). Furthermore, genetic modification of the β PDGFR in F3/F3 mice also resulted in reduced neointima formation following balloon injury (Caglayan et al. 2011).

A role for PDGF in pulmonary vascular remodeling was first suggested by the observation that PDGF ligands (A and B chains) are upregulated in pulmonary cells and lung tissue in monocrotaline (MCT)- and hypoxia-induced experimental PH (Arcot et al. 1993; Cai et al. 1996; Huang and Sun 1997; Katayose et al. 1993). Upregulation of PDGF-B was also observed in a microarray-based expression study in laser-microdissected intrapulmonary arteries in hypoxia-induced PH in the mouse (Kwapiszewska et al. 2005), and a marked shift in the expression of PDGF ligands (A/B) and receptors was found in progenitor smooth muscle cells developing in microvessels of experimental PH (Jones et al. 2006). In humans, similar alterations with increased expression of PDGF ligand and the β PDGFR were found in lung tissue of patients with PAH (Humbert et al. 1998; Lanner et al. 2005). In small pulmonary arteries showing signs of vascular remodeling, upregulation of PDGF ligands (A/B) was mainly found in VSMC and endothelial cells, whereas increased expression of the β PDGFR was restricted to VSMC (Schermuly et al. 2005; Perros et al. 2008).

Several interventional experimental studies suggest that PDGF is causally involved in the development of PH. While chronically hypoxic mice carrying a constitutively active PDGFR- β (D849N) developed exaggerated pulmonary vascular remodeling and PH as compared to wild-type mice (Dahal et al. 2011a), inhibition of PDGF signaling in animal models of PH was effective in perinatal PH in the fetal lamb (Balasubramaniam et al. 2003), as well as in MCT- or

hypoxia-induced PH (Schermyly et al. 2005), and ablation of PDGF-dependent downstream signaling in a genetic model prevented hypoxia-induced PH in the mouse (ten Freyhaus et al. 2012). Importantly, inhibition of PDGF signaling by imatinib reversed pulmonary vascular remodeling once it had been established (Schermyly et al. 2005). In summary, the above studies collectively indicate that PDGF is an important contributor to the pathobiology of PAH, and targeting of PDGFR signaling is therefore considered an important therapeutic target.

5 PDGF Ligands and Receptors

PDGF is a dimeric protein consisting of two of the four known homologous chains (A–D), which are linked by disulfide bonds, and exists as homo- (PDGF-AA, -BB, -CC, -DD) or heterodimers (PCGF-AB) (Bergsten et al. 2001; Gilbertson et al. 2001; Heldin and Westermark 1999; Kazlauskas 1994; LaRochelle et al. 2001; Li et al. 2000; Rosenkranz and Kazlauskas 1999). Whereas PDGF-A and -B are secreted as active ligands, PDGF-C and -D need to undergo cleavage of the N-terminal CUB domain in order to become activated (Bergsten et al. 2001; Gilbertson et al. 2001; LaRochelle et al. 2001; Li et al. 2000).

The biological responses of the various PDGF isoforms are transmitted via activation of two highly specific transmembrane RTKs, termed PDGFR α and β (Heldin and Westermark 1999; Kazlauskas 1994; Rosenkranz and Kazlauskas 1999). Both receptor subunits contain five extracellular immunoglobulin-like domains, a juxtamembrane domain, a kinase domain which is disrupted by a kinase insert, and an N-terminal tail region. Upon binding of the dimeric ligand, two PDGFR subunits merge to form PDGFR homo- ($\alpha\alpha$, $\beta\beta$) or heterodimers ($\alpha\beta$). The two receptor subtypes differ in their binding affinity for the various PDGF isoforms. Whereas the PDGF B-chain associates with both receptor subtypes, the A-chain binds exclusively to the α PDGFR. Consequently, PDGF-AA induces only PDGFR- $\alpha\alpha$ homodimers, PDGF-AB may induce the assembly of PDGFR- $\alpha\alpha$ and - $\alpha\beta$, and PDGF-BB leads to the formation of all possible receptor dimers ($\alpha\alpha$, $\alpha\beta$, $\beta\beta$) (Fig. 1a). The affinity of the PDGFR subtypes for PDGF-C and -D is less well established. Apparently, PDGF-CC assembles both $\alpha\alpha$ and $\alpha\beta$ receptor dimers, whereas PDGF-DD mainly binds to $\beta\beta$, although it may also induce the formation of $\alpha\beta$ heterodimers under certain conditions. This is of relevance, as the two PDGFR subtypes differ from one another in both their signaling properties and biological responses (Rosenkranz and Kazlauskas 1999).

6 Signal Relay Downstream of PDGF Receptors

Binding of PDGF ligands leads to dimerization of two receptor subunits and a subsequent increase of the receptor's intrinsic tyrosine kinase activity, resulting in trans-/autophosphorylation of the receptor. Once PDGFRs are activated,

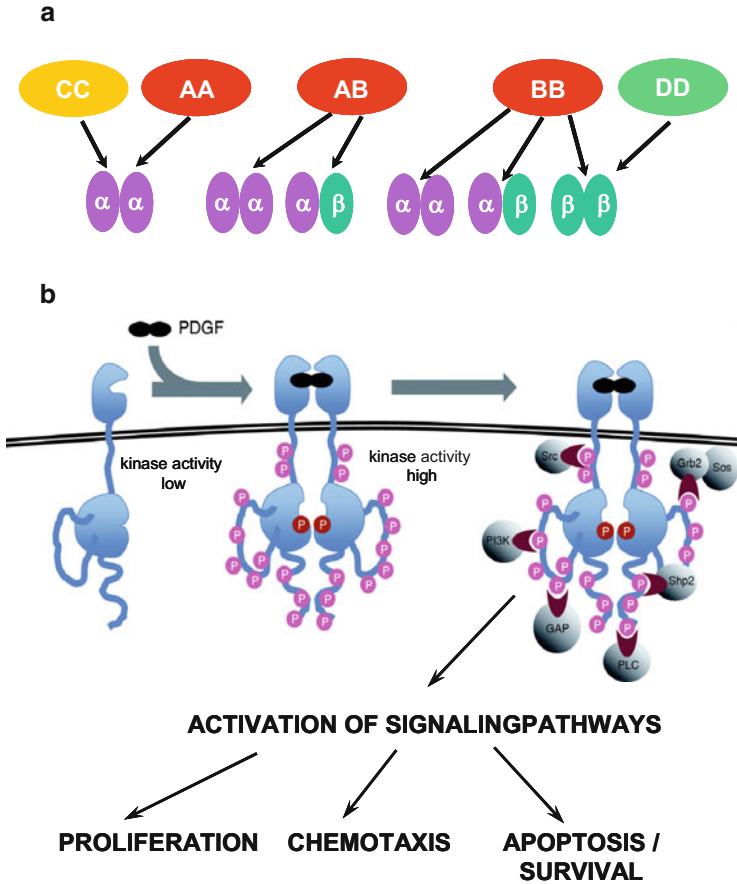


Fig. 1 Signal transduction by PDGF receptors. (a) Binding specificity of the PDGF ligand chains (A, B, C, D) to the α - and β PDGFR subtypes. (b) Receptor dimerization, trans-/autophosphorylation, and cellular effects induced by activated PDGFR

phosphorylated tyrosine residues in the intracellular domain serve as specific docking sites for intracellular signaling molecules, which selectively associate with the receptors at specific sites. Once bound to the receptor, each of the receptor-associated signaling molecules is activated and triggers downstream signal relay cascades which mediate PDGF-dependent cellular responses such as protein synthesis, proliferation, migration, protection against apoptosis, and cellular transformation (Fig. 1b).

Signaling via the β PDGFR subtype is required for the proper development of the vessel wall during embryogenesis and is also essential for maintaining the integrity of vascular structures (Leveen et al. 1994; Lindahl et al. 1997; Mellgren et al. 2008; Soriano 1994; Tallquist et al. 1999, 2000). The activated β PDGFR associates with SH2 (Src homology 2) domain containing signaling molecules such as Src family

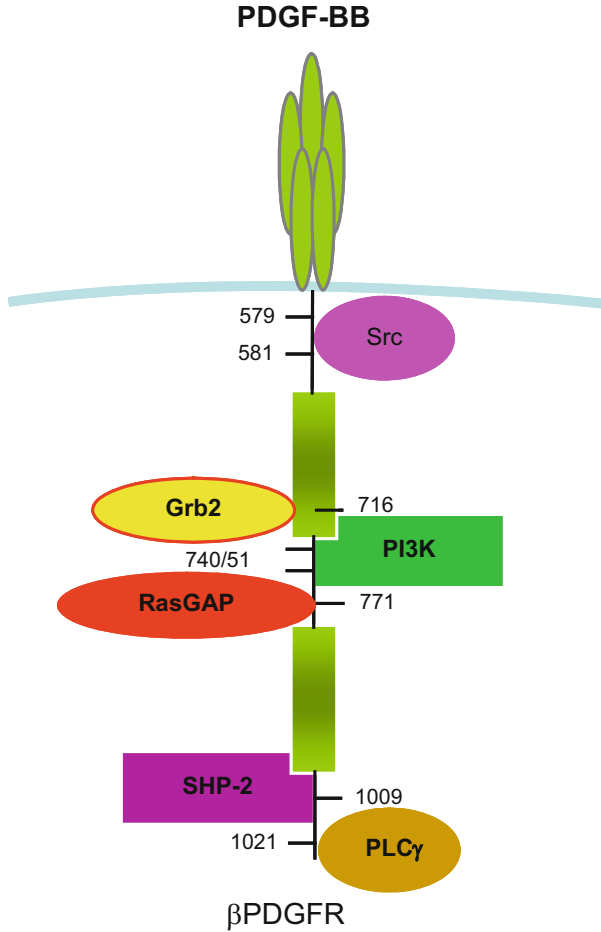


Fig. 2 Signaling molecules that interact with the activated β PDGFR. *Numbers* indicate the amino acid position in the intact human β PDGFR

kinases (Src), phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (RasGAP), the tyrosine phosphatase SHP-2, and phospholipase C γ 1 (PLC γ), which specifically bind to phosphorylated tyrosine residues (Fig. 2) (Rosenkranz and Kazlauskas 1999). In addition, SH2-SH3 domain containing adaptor proteins without enzymatic activity such as Grb2, Grb7, Grb10, Nck, and Shc also associate with the activated receptor, but their role in signal transduction is yet less clear. Each of the receptor-associated signaling molecules has a unique role in the activation of downstream targets such as Erk1/2, Akt, JNK, and FAK, and the induction of immediate early response genes such as *c-myc*, *c-fos*, *egr-1*, and *JunB*.

The mechanisms of signal transduction by PDGF receptors have been extensively studied in cellular systems. In particular, the contribution of

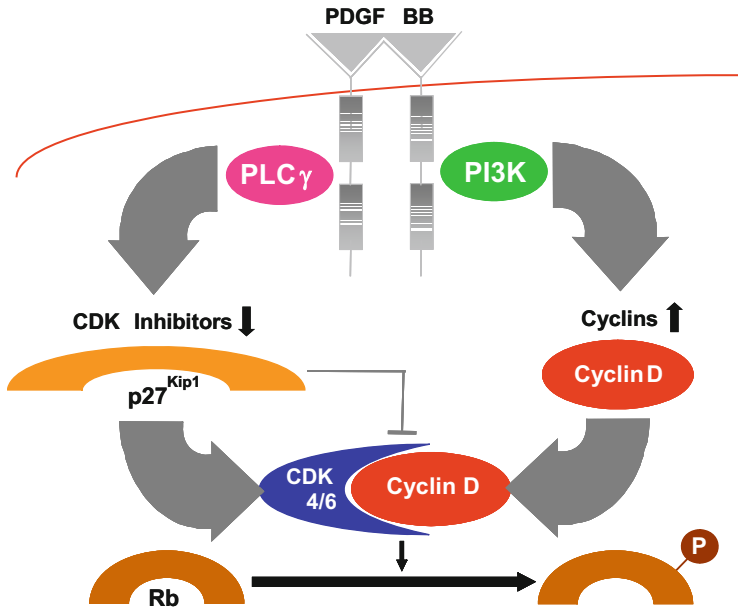


Fig. 3 Schematic diagram illustrating the differential roles of PDGF-dependent PI3K and PLC γ activity for cellular responses. The activated β PDGFR recruits multiple signaling molecules including PI3K and PLC γ . While PI3K is required for efficient upregulation of cyclin D1, PLC γ is mainly responsible for downregulation of p27^{Kip1}. Both events are required for Rb phosphorylation which is crucial for cellular events such as cell cycle progression and chemotaxis (modified from Caglayan et al. 2011)

PDGFR-associated signaling molecules to downstream pathways and cellular responses was investigated by utilizing mutated PDGFRs, in which the selective tyrosine binding sites for intracellular signaling molecules were individually deleted, and that were thus unable to bind and activate single receptor-associated signal relay enzymes. PDGFR mutants have been expressed in PDGFR-deficient cell lines including human HepG2 cells, A431 cells, murine *Ph* cells (3T3-like), and pig aortic endothelial cells (PAE) (Bäumer et al. 2008; Chung et al. 1994; DeMali et al. 1997; Fambrough et al. 1999; Joly et al. 1994; Klinghoffer et al. 1996; Kundra et al. 1994; Montmayeur et al. 1997; Rosenkranz et al. 1999; Valius and Kazlauskas 1993; Valius et al. 1995). These studies have identified relevant signaling pathways and have also shown cell type-specific differences with regard to signal relay of PDGFR-mediated responses. In VSMCs, a critical role of PI3K and PLC γ for PDGF-dependent VSMC proliferation and migration was demonstrated by use of a system of chimeric CSF1R/ β PDGFR mutants (Caglayan et al. 2011). These investigations have shown that PI3K is mainly required for efficient upregulation of cyclin D1, whereas PLC γ is responsible for downregulation of the cdk-inhibitor p27^{Kip1}. Both events are necessary for the activation of cyclin-dependent kinases (cdks) and Rb phosphorylation which are required for both cell cycle progression and chemotaxis (Fig. 3). Disruption of PDGF-dependent PI3K and PLC γ signaling

in mice was sufficient to attenuate neointima formation following balloon injury *in vivo*. Recent evidence indicates that PI3K and PLC γ are also critically involved in PDGF-dependent pulmonary vascular remodeling (ten Freyhaus et al. 2011), and ablation of PI3K and PLC γ signaling downstream of the β PDGFR in F3/F3 mice markedly attenuated the development of hypoxia-induced PH (ten Freyhaus et al. 2012).

7 Control of PDGF Signaling

The control of PDGF signaling is facilitated by several mechanisms. RasGAP directly associates with the activated β PDGFR and serves as a negative regulator of PDGF signaling by inactivating Ras (Rosenkranz and Kazlauskas 1999; Klinghoffer et al. 1996; Valius et al. 1995). A recent study demonstrated that downstream signaling by the PDGFR and other RTKs is amplified by the adaptor protein p130Cas which is upregulated and over-activated in serum and pulmonary vascular cells (PASMC, PEC) in PAH (Tu et al. 2012). The role of Src family kinases, which directly interact with PDGFR, is somewhat controversial. While Src is considered an oncogene, and some studies indicate that it is involved in pulmonary VSMC proliferation and the development of PH (Pullamsetti et al. 2012), Src family members appear to be involved in the termination of PDGF-induced signals, as they promote the ubiquitination and degradation of the active ligand–receptor complex via the phosphorylation of c-Cbl (Miyake et al. 1999; Rosenkranz et al. 2000). PDGFR activity is also negatively regulated by a set of antagonizing protein tyrosine phosphatases (PTPs) including DEP-1, SHP-2, PTP-1B, and TC-PTP, which dephosphorylate and thus inactivate RTKs (Kappert et al. 2005, 2007).

Hypoxia, which is frequently present in lung tissue of patients with pulmonary hypertension, was recently identified as an important modulator of RTK signaling. Hypoxia enhances the proliferative and migratory response of VSMCs to various growth factors including PDGF, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (ten Freyhaus et al. 2011; Humar et al. 2002; Schultz et al. 2006). While the expression of the respective receptors is not affected by hypoxia, it was shown that hypoxia enhances PDGF signaling in the pulmonary vasculature by hypoxia-inducible factor-1 α (HIF1 α)-dependent downregulation of PTP expression and activity, thus resulting in enhanced PDGFR phosphorylation and activity (ten Freyhaus et al. 2011) (Fig. 4). Further studies revealed that PDGF also induces pulmonary vascular remodeling through a 15-LO/15-HETE pathway under hypoxic conditions (Zhang et al. 2012) and that hypoxia induces unique proliferative response in adventitial fibroblasts by engaging PDGFR β /c-Jun N-terminal kinase1 (JNK1) signaling and thus leads to adventitial remodeling in PH (Panzhinskiy et al. 2012).

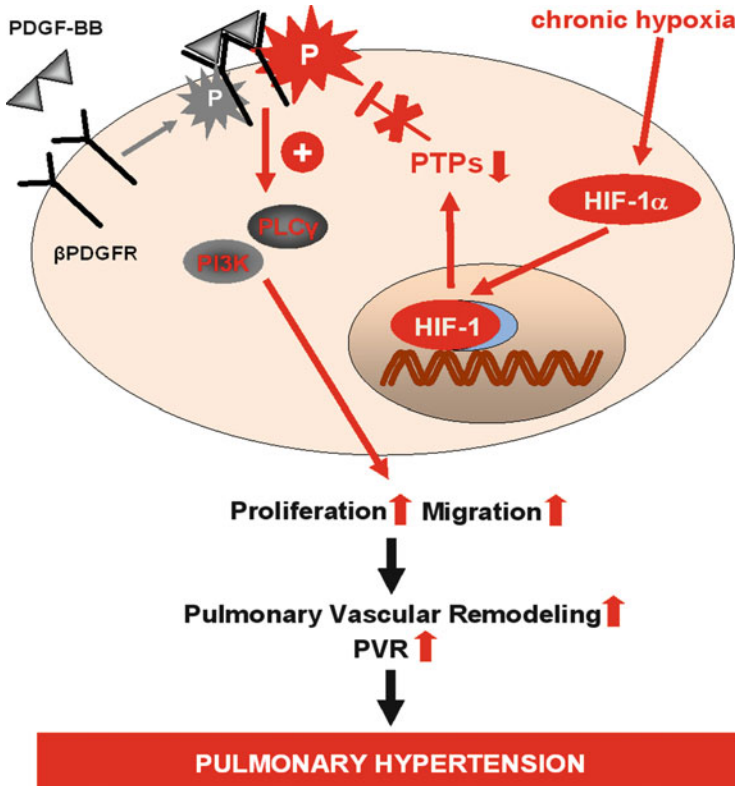


Fig. 4 Schematic diagram illustrating the impact of chronic hypoxia on β PDGFR signaling. In PSMCs, chronic hypoxia induces HIF-1 α , leading to decreased expression and activity of β PDGFR-antagonizing PTPs. This leads to enhanced ligand-induced β PDGFR phosphorylation and cellular proliferation and migration. In vivo, these changes critically impact on pulmonary vascular remodeling, pulmonary vascular resistance (PVR), and the progression of PH

8 Therapeutic Potential of PDGF Receptor Inhibition in PAH

8.1 Tyrosine Kinase Inhibitors: Imatinib Mesylate

Pharmacological inhibition of PDGFRs may be achieved by inhibiting their intrinsic tyrosine kinase activity. Imatinib mesylate (STI571, Glivec) is a small molecule TKI that has been approved for the treatment of various malignant disorders including Philadelphia (Ph) chromosome-positive chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and gastrointestinal stromal tumors (GIST) (Cohen et al. 2005). It is a nonspecific tyrosine kinase inhibitor, interacting with the bcr-abl fusion protein (responsible for efficacy against Ph-positive CML),

both α and β PDGFR, DDR, and the c-kit receptor (Baselga 2006). The inhibitory effect of imatinib is due to binding and thereby blocking of the active site of the phosphate transferring tyrosine kinase. It is a so-called type 2 inhibitor, which binds not only to the ATP binding pocket but also to a site adjacent to the pocket, which enhances specificity and allows binding to the kinase even in an inactive conformation. Imatinib is readily absorbed with maximum blood levels achieved after 1–4 h (Gschwind et al. 2005; Le Coutre et al. 2004; Peng et al. 2005) and is metabolized mainly by the cytochrome P450 enzyme CYP3A4, leading to the formation of biologically active metabolites. Both imatinib and its metabolites are eliminated mostly via the biliary–fecal route and to a lesser extent via the urine (Gschwind et al. 2005). While the invention of imatinib has revolutionized the treatment of the described cancers, recent evidence indicates that it is also effective in the treatment of PAH.

8.2 Preclinical Studies

The observation that PDGF ligand and PDGFR are substantially upregulated in lung tissue of experimental PH and in humans with PAH (Schermlay et al. 2005; Perros et al. 2008) identified PDGF as a potential therapeutic target. On the cellular level, PDGF-dependent proliferation and migration of murine, rat and human PSMCs were potently inhibited by imatinib. Furthermore, imatinib exerts pro-apoptotic effects in PDGF-stimulated PSMCs from patients with IPAH (Nakamura et al. 2011), most likely by antagonizing the anti-apoptotic properties of PDGFR signaling (Vantler et al. 2005, 2010).

In vivo studies have shown that PDGFR inhibition by imatinib ameliorated PH in two animal models by improving pulmonary hemodynamics, reducing RV hypertrophy, and reversing pulmonary vascular remodeling (Schermlay et al. 2005). Furthermore, imatinib was shown to exert immunomodulatory effects on dendritic cells (Sato et al. 2003). In the MCT rat model and in hypoxia-induced PH in mice, the changes in RV pressure and right heart hypertrophy were reversed to near-normal levels, and this was associated with improved survival (Schermlay et al. 2005).

Although some studies suggest that TKIs such as imatinib may also modulate pulmonary vascular tone (Abe et al. 2011), such effects were observed at rather high concentrations (50 mg kg⁻¹ in vivo) which by far exceed the dosages applied in humans. The primary mode of action of imatinib at relevant concentrations is to inhibit the proliferation and migration of vascular cells and to promote apoptosis (Schermlay et al. 2005; Perros et al. 2008). In addition to inhibition of PDGFR, imatinib also inhibits other kinases such as c-kit and Abl. Recent studies have indicated that c-kit may also play a role in the pathobiology of PH (Young et al. 2009), and imatinib was shown to attenuate bone marrow-derived c-kit+ cell mobilization in hypoxic PH (Gambaryan et al. 2010). While specific inhibition of c-kit was recently shown to prevent the development of PH in MCT-treated rats,

it failed to reverse established PH (Dahal et al. 2011b). Although c-kit may contribute to the development of PH to some extent, the main therapeutic effect of imatinib in PH is believed to be via inhibition of PDGFR signaling. This is supported by the large body of data from in vitro findings demonstrating that the expression of PDGF ligands and both receptor subtypes is substantially enhanced in PAH, that PDGF exerts pronounced cellular effects in PSMCs, and that the prevention of PDGFR phosphorylation by TKIs such as imatinib was associated with suppression of PDGF-dependent downstream signaling pathways and cellular responses. Hence, reversal of pulmonary arterial remodeling by imatinib in experimental PH, which is even effective when pulmonary vasculopathy and the impairment of hemodynamics are fully established, may be attributed to inhibition of PDGF-induced proliferation of VSMC and increased apoptosis. The fact that other approaches such as application of the PDGF-B-antagonizing aptamer NX1975 and ablation of PDGF-dependent PI3K and PLC γ activity were also able to prevent PH in animal models further supports the important role of PDGF (Balasubramaniam et al. 2003; ten Freyhaus et al. 2012). In summary, the above studies indicate that inhibition of PDGFR signaling represents a potential causal treatment for pulmonary vascular remodeling and PAH.

8.3 Clinical Studies

In addition to experimental data, several case reports indicated that imatinib is effective as add-on treatment in humans with refractory PAH, and beneficial effects have also been reported during long-term treatment. The first published case describes a 61-year-old patient with rapidly progressing PAH who was insufficiently managed on triple therapy (bosentan, iloprost, sildenafil) and was therefore treated with add-on imatinib. Subsequently, clinical symptoms (WHO class IV–II), exercise capacity, and pulmonary hemodynamics improved dramatically (Ghofrani et al. 2005). Significant improvements after initiation of imatinib were documented from several other groups in a 52-year-old male with refractory idiopathic PAH (Patterson et al. 2006), in two patients with PAH who had received imatinib for the treatment of leukemia (Souza et al. 2006), in a 58-year-old patient with severe scleroderma-associated PAH (ten Freyhaus et al. 2009), and as additional treatment in portopulmonary hypertension (Tapper et al. 2009). Nevertheless, imatinib was reported to be not successful in other cases (Garcia-Hernandez et al. 2008).

Phase II Trial. Based on the promising data from preclinical studies and the encouraging observations from clinical case studies, a double-blind, placebo-controlled, multicenter phase II trial (NCT00477269) was implemented to evaluate the safety, tolerability, and efficacy of imatinib in PAH patients (Ghofrani et al. 2010a). Fifty-nine patients with inadequate response to established therapy with IPAH or HPAH were randomized to receive either imatinib (200–400 mg day⁻¹) or placebo for 24 weeks. Forty-two patients completed the

trial. While the primary endpoint (6 min walk distance, 6MWD) was not met ($+22 \pm 63$ vs. -1 ± 53 m, n.s.), hemodynamic measurements were in favor of imatinib (Ghofrani et al. 2010a). There was a significant decrease in PVR (-300 ± 347 vs. -78 ± 269 dynes s cm^{-5} ; $p < 0.01$) and an increase in cardiac output ($+0.6 \pm 1.2$ vs. -0.1 ± 0.9 l min^{-1} ; $p = 0.02$) (Fig. 5a). A non-prescribed, post hoc subgroup analysis indicated that patients with substantial elevation of PVR ($\geq 1,000$ dynes s cm^{-5}) may in particular benefit from such treatment, as such patients showed a significantly increased 6MWD and a greater hemodynamic improvement on imatinib when compared to placebo than patients with lower PVR (Fig. 5b). Patients who completed the 24-week core study were invited to enter a long-term extension phase. Preliminary results indicate that the beneficial effects of imatinib on exercise capacity and functional class persist for at least 12–24 months (Ghofrani et al. 2010b).

The most common side effects included nausea, headache, and peripheral edema. More severe side effects were equally distributed between groups. While arterial and mixed venous oxygen saturation increased with imatinib treatment, there was a decrease in hemoglobin levels in patients on imatinib which may be relevant. Although there were several limitations (trend towards higher functional class and more patients on triple therapy in the placebo group) and the primary endpoint was not met (most likely due to the small sample size), the study demonstrated that imatinib is safe in the population of PAH patients and that a pure anti-proliferative approach can improve pulmonary hemodynamics (decrease of PVR, increase in cardiac output) in addition to conventional treatment.

Phase III Trial (IMPRES). In consideration of the results of the phase II trial, a randomized, double-blind, placebo-controlled multicenter phase III trial (NCT00902174) was carried out to further assess the efficacy and safety of imatinib in severe PAH. The primary endpoint of the study was the 6MWD, secondary endpoints included pulmonary hemodynamics, pharmacokinetics, and time to clinical worsening. Two hundred and two patients with severe PAH (PVR > 800 dynes s cm^{-5}) despite treatment with at least two targeted PAH therapies were randomized to receive imatinib ($200\text{--}400$ mg day^{-1}) or placebo for 24 weeks. At study entry, 41 % of patients in the imatinib group and 42 % of patients in the placebo group were on a triple combination therapy (ERA, PDE5i, prostanoid), and all remaining patients received dual combination therapy. At 24 weeks, there was significant improvement of the 6MWD (primary endpoint) by 32 ± 10 m in patients who received imatinib as compared to patients exposed to placebo ($p = 0.002$) (Hoepfer et al. 2011a). This was accompanied by substantial hemodynamic improvement, including significant decreases in the mean PAP and PVR, and a rather impressive increase in cardiac output (1.2 vs. 0.3 l min^{-1} , $p < 0.001$) (Hoepfer et al. 2011b, 2012). This was consistent with improvement in a number of echocardiographic measures of right ventricular function (Shah et al. 2012).

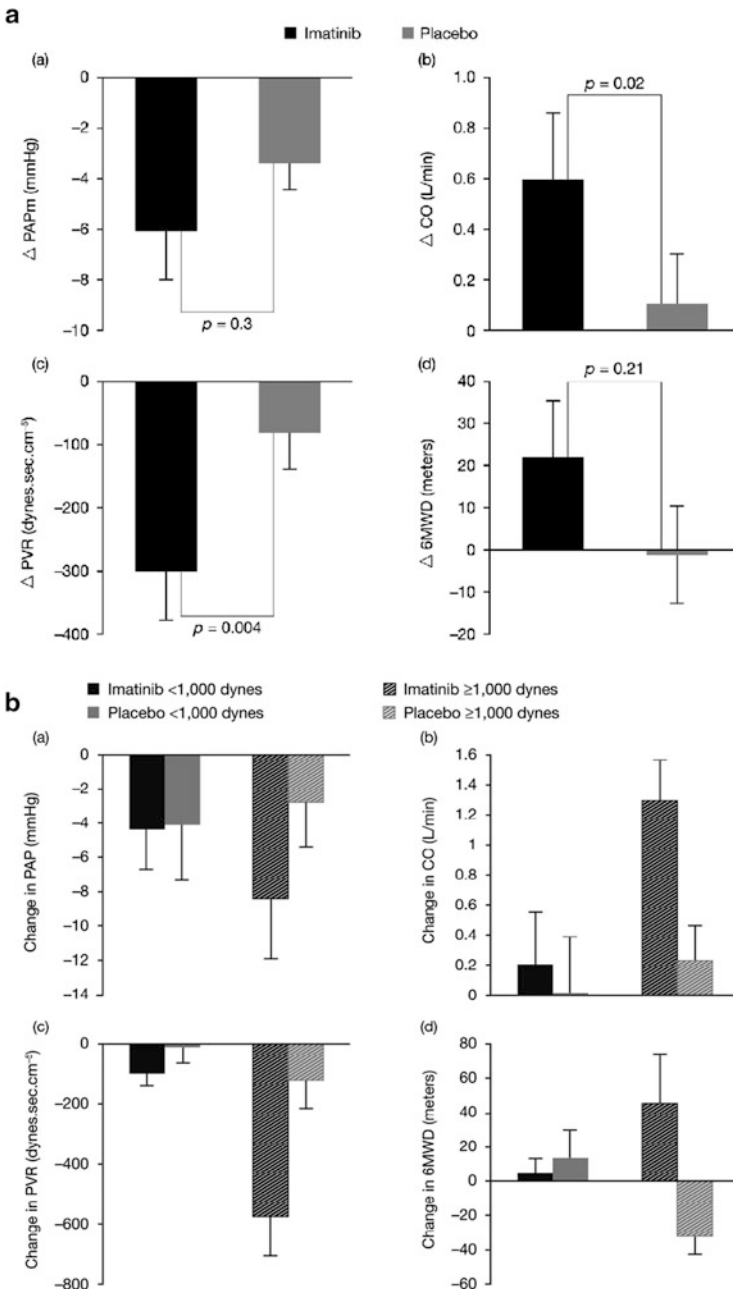


Fig. 5 Results from a phase II study in patients with PAH and inadequate response to established therapy. Effects of imatinib (200–400 mg day⁻¹) on mean pulmonary artery pressure (mPAP), cardiac output (CO), pulmonary vascular resistance (PVR), and 6 min walking distance (6MWD). Shown is the mean change from baseline to study end (24 weeks). **(a)** All patients ($n = 42$). **(b)** Post hoc analyses showed that patients with greater hemodynamic impairment ($PVR \geq 1,000$ dynes; $n = 20$) may respond better than patients with less impairment ($PVR < 1,000$ dynes; $n = 21$) (Ghofrani et al. 2010a)

9 Limitations and Future Aspects

Although the clinical trials with imatinib in PAH have provided encouraging results, this treatment has not been entirely successful thus far and there are a number of findings that deserve critical interpretation. In IMRES, a significant number of patients receiving imatinib (33 %) discontinued the study drug due to side effects (vs. 18 % in the placebo group), and there was no difference in mortality and the time to clinical worsening (TTCW; defined as death, hospitalization for PAH, decrease in the 6MWD by >15 %, or worsening of WHO functional class by ≥ 1 level) between the treatment and placebo groups (HR 1.16; $p = 0.56$). Given the definition of TTCW and the known imatinib side effect of fluid retention, it may be difficult to distinguish between worsening of PAH and imatinib-induced fluid retention in individual cases of peripheral edema. Of note, most of the events defining TTCW occurred during the first 8 weeks of treatment and resolved in the majority of cases despite ongoing drug treatment. Nevertheless, tolerability and the management of side effects have to be improved in PAH patients.

The results from the phase II trial have indicated that only patients with a $PVR > 1,000 \text{ dynes s cm}^{-5}$ benefit from imatinib (Ghofrani et al. 2010a), and the IMPRES trial (phase III) included only patients with a $PVR > 800 \text{ dynes s cm}^{-5}$ (Hoepfer et al. 2011a, b). Thus, current data indicate that imatinib may only be effective in patients with advanced stages of PAH and severe hemodynamic impairment. This is in contrast to the current concept to diagnose and treat PAH as early as possible (Sitbon and Galiè 2010). There is no reason to believe that PDGF-dependent signals are only important at advanced stages of PAH, and it does not make sense to wait until a disease has progressed to an advanced stage before anti-proliferative treatment is initiated. Nevertheless, the current data suggest that imatinib may not be effective in patients with moderate PAH ($PVR < 800 \text{ dynes s cm}^{-5}$). In this context, time may be a critical issue. In contrast to previous randomized, controlled PAH trials that followed up patients for 12 weeks, the observation period in the imatinib studies was extended to 24 weeks, because “reverse remodeling” strategies are believed to require more time than vasodilative approaches. However, even an observation time of 24 weeks may not be enough to capture the full therapeutic effect. At least some observations indicate that continued improvements of clinical status, hemodynamics, and other prognostically relevant parameters persist far beyond 24 weeks (ten Freyhaus et al. 2009; Ghofrani et al. 2010b), which may actually be expected from an effective anti-proliferative approach. Hence, one may speculate that the efficacy of a TKI in patients with moderately impaired hemodynamics and less severely remodeled vessels may require more time and become evident with longer treatment. It will therefore be critical to investigate the efficacy of imatinib in patients with a $PVR < 800 \text{ dynes s cm}^{-5}$, and to monitor patients for a longer period of time.

Besides PAH, the efficacy, tolerability and safety of imatinib may also be evaluated in other forms of PH. Pulmonary vascular remodeling was shown at least in some patients with PH owing to chronic left heart disease (Delgado

et al. 2005), and immunohistochemical analysis indicated high deposition of PDGF and high levels of PDGFR expression in tissue sections from pulmonary endarterectomy (PEA) samples from patients with CTEPH (Ogawa et al. 2009). While surgical PEA represents the first choice of treatment in patients with CTEPH, imatinib may therefore be effective in patients with non-operable CTEPH and those with persistent PH after PEA. However, this has to be assessed with great caution, as subdural hematomas have been observed in some patients with PAH, in whom imatinib was given in addition to oral anticoagulation (Hoepfer et al. 2012). In patients with PAH, the subset of patients with scleroderma-associated PAH may be viewed separately. Stimulatory autoantibodies to the PDGFR have been described in scleroderma patients (Baroni et al. 2006). In scleroderma-associated PAH, the therapeutic effect of imatinib may therefore be attributable to inhibition of PDGFR signaling and reversal of vascular remodeling in the pulmonary vasculature, and/or to interference with stimulatory PDGFR autoantibodies and thus modulation of scleroderma per se.

From the current clinical experience with imatinib, we have learned that it appears not to be equally effective in all patients and that pulmonary hemodynamics are frequently improved but not normalized. A possible explanation for this may be that in addition to PDGF, other growth factors such as EGF and bFGF also contribute to the pathology in pulmonary arterial remodeling (Dahal et al. 2010; Izikki et al. 2009; Merklinger et al. 2005), and that the contribution of each growth factor may differ among individual patients. Therefore, inhibition of central signaling pathways that affect signal relay by all relevant RTKs may represent an even more effective strategy. In this context, PI3K was recently identified as a central mediator of pathogenic responses transmitted by the PDGFR and other RTKs. Selective inhibition of the catalytic class IA PI3K isoform p110 α was shown to potently inhibit PASM C proliferation induced by multiple growth factors and to attenuate PH in mice (Berghausen et al. 2011). However, toxicity certainly remains an issue with TKIs, and—depending on the kinase profile—one has to be aware of unexpected adverse reactions.

10 Safety and Tolerability of Tyrosine Kinase Inhibitors in PAH

Imatinib is a generally safe and well-tolerated drug, particularly when considering that it profoundly interacts with basal cellular processes such as PDGF signaling. Adverse events are usually mild and mostly occur during the first month of treatment. Usual adverse reactions that have been recorded in the initial clinical trials for CML and in long-term observational studies include nausea, headache, skin rash, pruritus, anemia, and fluid retention (Druker et al. 2006). Similarly, adverse drug reactions in the PAH phase II and phase III trials included nausea (approximately 50 % of patients), headache (36 %), and peripheral edema

(25–30 %) (Ghofrani et al. 2010a; Hoeper et al. 2012). In most cases, these adverse reactions did not lead to discontinuation of the drug, and nausea was frequently controlled by taking the drug with food. In the phase II trial, serious adverse events occurred in 39 % of patients receiving imatinib and in 23 % of those receiving placebo (Ghofrani et al. 2010a, b). A potential concern in PAH patients may be liver toxicity, especially in patients on combination therapy with ERAs. However, in patients with CML, imatinib appears to be a safe drug in this respect (Tong et al. 2010). Nevertheless, one has to be aware of potential additive toxicity, and frequent monitoring of LFTs might be advisable. Particular concerns with TKI may be cardiotoxicity, the recent observation of PAH induction by at least one TKI, and the potential occurrence of subdural hematomas when imatinib is given in addition to oral anticoagulants.

10.1 *Cardiotoxicity*

A harmful effect of imatinib on cardiac integrity and function was shown in mice and a small number of patients who had received the drug chronically for CML (Kerkelä et al. 2006). This cardiotoxic effect was not attributed to PDGFR inhibition, but to the inhibitory effect on the non-receptor Abelson tyrosine kinase (c-Abl), leading to mitochondrial dysfunction and an ER stress response via activation of the PKR-like ER kinase (PERK) and IRE1, and to increased expression of PKC δ in cardiac myocytes (Kerkelä et al. 2006; Cheng and Force 2010). The involvement of c-Abl was confirmed by the fact that a redesigned imatinib that no longer inhibited c-Abl did not exert cardiotoxic effects (Fernandez et al. 2007). Despite these experimental data, further analyses of clinical studies revealed that congestive heart failure is a rare event in patients receiving imatinib for CML (Druker et al. 2006), but occurs more frequently in patients with preexisting cardiac conditions (Atallah et al. 2007). The latter fact may be of particular significance in patients with PAH and impaired right ventricular function since the right ventricle responds particularly sensitive to hemodynamic and/or cardiotoxic impairment. However, add-on imatinib was associated with improved right ventricular function as assessed by MRI in a case of scleroderma-associated PAH (ten Freyhaus et al. 2009), cardiac output significantly improved in the phase II and phase III trials of imatinib for PAH (Ghofrani et al. 2010a, b; Hoeper et al. 2011a, b), and in the IMPRES trial, this was associated with a marked improvement of echocardiographic measures of right ventricular function (Shah et al. 2012). Thus, the implemented clinical investigations of imatinib in patients with PAH did not provide any signals that would indicate detrimental effects on cardiac function. Nevertheless, cardiac side effects remain a concern of TKIs that requires close follow-up and regular monitoring of contractile function by echocardiography.

10.2 Induction of PAH by Tyrosine Kinase Inhibitors

Recent observations indicate that—depending on the kinase profile of a TKI—some compounds may actually have the potential to induce PAH. Although dasatinib was recently shown to be therapeutic in experimental PH (Pullamsetti et al. 2012), chronic treatment with dasatinib in several cases of imatinib-refractory CML was associated with an onset of severe PAH that mostly occurred in conjunction with pleural effusions (Dumitrescu et al. 2011; Hennigs et al. 2011; Mattei et al. 2009; Quinta's-Cardama et al. 2007; Rasheed et al. 2009). Consistently, Montani et al. recently published a series of nine cases with dasatinib-induced PAH that had been identified in the French PH registry (Montani et al. 2012). Interestingly, in many of the cases of dasatinib-associated PAH, pulmonary hypertension appeared to be reversible after termination of dasatinib. This is remarkable because complete remission and normalization of pulmonary hemodynamics is usually not achieved by medical treatment in PAH. Therefore, it is assumed that the pathophysiological mechanism leading to increased PVR differs from the vascular alterations typically seen in PAH. While the mechanism remains elusive, it may be related to the additional targeting of Src family kinases by this compound. Since Src is required for degradation of activated PDGFRs and the termination of PDGF signals (Miyake et al. 1999; Rosenkranz et al. 2000), its inhibition may result in increased signaling by PDGF and possibly other growth factors. Alternatively, Src kinase inhibition may directly alter the pulmonary vascular tone through a functional mechanism. Src was recently shown to control the potassium channel TASK-1, thus acting as a cofactor for setting a negative resting membrane potential in human PASMCs and a low resting pulmonary vascular tone. Consequently, inhibition of Src decreased the tyrosine-phosphorylation state of TASK-1 and potassium current density, resulting in considerable depolarization and an increase in PAP in experimental settings (Nagaraj et al. 2013). An additional explanation for the induction of PAH by dasatinib may be related to off-kinase inhibition which may affect pulmonary vascular wall integrity, possibly leading to (peri)vascular edema and subsequent PVR increase. Although such effects have not been reported for imatinib, these observations remind us that one should be aware of potential unexpected side effects whenever a drug of this class is used.

10.3 Subdural Hematomas

A potential concern with the use of imatinib in PAH is the unexpected occurrence of subdural hematomas (SDH), which has recently been reported in some patients who were on concomitant oral anticoagulation with vitamin K antagonists (Hoepfer et al. 2012). While such events have not been reported at increased incidence in patients on imatinib for CML or GIST, the indication for anticoagulation in PAH patients as suggested by current guidelines (Galiè et al. 2009a–c) may represent an

important difference between the populations of CML/GIST vs. PAH patients. A detailed analysis of all cases with SDH and the potential underlying mechanism(s) are warranted.

11 Conclusions

In summary, a series of preclinical studies has identified PDGF as a major contributor to pulmonary vascular remodeling, increased PVR, and subsequent right ventricular failure. Indeed, experimental studies demonstrating that inhibition of PDGF signaling by the TKI imatinib or by genetic approaches is able to prevent or even reverse pulmonary vascular remodeling *in vivo* are complemented by the observation that PDGF ligands and receptors are upregulated and activated in experimental and human PAH. It is therefore not surprising that two randomized clinical trials that have investigated the safety, tolerability, and efficacy of imatinib in human PAH have shown that this anti-proliferative compound is well tolerated in most patients and produces significant improvements of exercise capacity and pulmonary hemodynamics in patients with advanced PAH. Therefore, inhibition of PDGFR signaling, e.g., by imatinib mesylate represents an encouraging therapeutic concept and is likely to become an additional targeted therapy for PAH. In addition to existing therapies, this anti-proliferative approach has the potential to establish a sustained reduction of the underlying pulmonary vascular remodeling, which in turn allows hemodynamic improvement. The possibility to reverse pulmonary vascular remodeling in human disease would indeed represent an important step forward in the endeavor to establish a causal treatment for PAH that is able to correct the pathology of the disease. While the current data provide evidence for therapeutic efficacy only in patients with severe hemodynamic impairment, the role of PDGFR inhibition in less severe PAH and possibly in other forms of PH remains to be investigated. Furthermore, one should be alert for unexpected adverse events including induction of PAH by TKIs, cardiotoxicity, and SDH, and the long-term benefit remains to be documented.

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Emerging Molecular Targets for Anti-proliferative Strategies in Pulmonary Arterial Hypertension

Ly Tu and Christophe Guignabert

Abstract The combination of pulmonary vasoconstriction, in situ thrombosis, and pulmonary arterial wall remodeling is largely responsible for the rise in pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP) in patients with pulmonary arterial hypertension (PAH). Even though several drugs have been developed over the past decades, at this time there is no cure for PAH. The overriding goals of the current therapeutic options seek to compensate for the defects in the relative balance of competing vasoconstrictor and vasodilator influences. Because the past decade has seen great strides in our understanding of the pathogenesis of PAH, interest has been growing in the potential use of anti-proliferative approaches in PAH. Indeed anti-proliferative strategies could offer ways not only to reinstate the homeostatic balance between cell proliferation and apoptosis but also to reverse the progressive pulmonary vascular obstruction in PAH. However, further efforts still need to be made in order to establish the long-term safety and efficacy of those anti-proliferative approaches in PAH and their potential additive benefit with other drugs.

Keywords Pulmonary hypertension • Pulmonary vascular remodeling • Anti-proliferative compounds • Signal transduction • Growth factors

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1 Introduction

Pulmonary arterial hypertension (PAH) is a rapidly progressive disease characterized by sustained elevation of pulmonary vascular resistance (PVR) and pulmonary arterial pressure (PAP) leading to right heart failure and death. Although the exact mechanisms of remodeling of pulmonary arteries, leading to the onset and progression of the disease, are still largely unclear, many disease-predisposing factors and/or contributing factors have been identified, including inflammation, endothelial dysfunction, aberrant vascular wall cell proliferation, as well as mutations in the *bone morphogenetic protein receptor type 2 (Bmpr2)* gene (Humbert et al. 2004; Rabinovitch 2005; Morrell et al. 2009) (Fig. 1).

Development of therapeutic agents that modulate abnormalities in three main pathobiologic pathways for PAH endothelin (ET)-1, prostacyclin (PGI₂), and nitric oxide (NO) has revolutionized our approach to the treatment of PAH and has changed the course of this devastating disease (O’Callaghan et al. 2011). However, although the spectrum of therapeutic options for PAH has expanded in the last decade, available therapies remain essentially palliative.

Irreversible remodeling of the pulmonary vasculature is the cause of increased PAP in PAH and frequently leads to progressive functional decline in patients despite treatment with current available therapeutic options. This process is ascribed to the increased proliferation, migration, and survival of pulmonary vascular cells within the pulmonary artery wall, i.e., myofibroblasts, pulmonary vascular smooth muscle (SMCs), and endothelial cells (ECs). The increasing knowledge on PAH pathogenesis has revealed the complex nature of these structural and functional changes in the pulmonary arteries of patients with PAH and highlighted the need to elucidate the molecular mechanisms involved. Over recent years, special attention has been devoted to the disease-promoting roles of three different growth factors and their corresponding tyrosine kinase

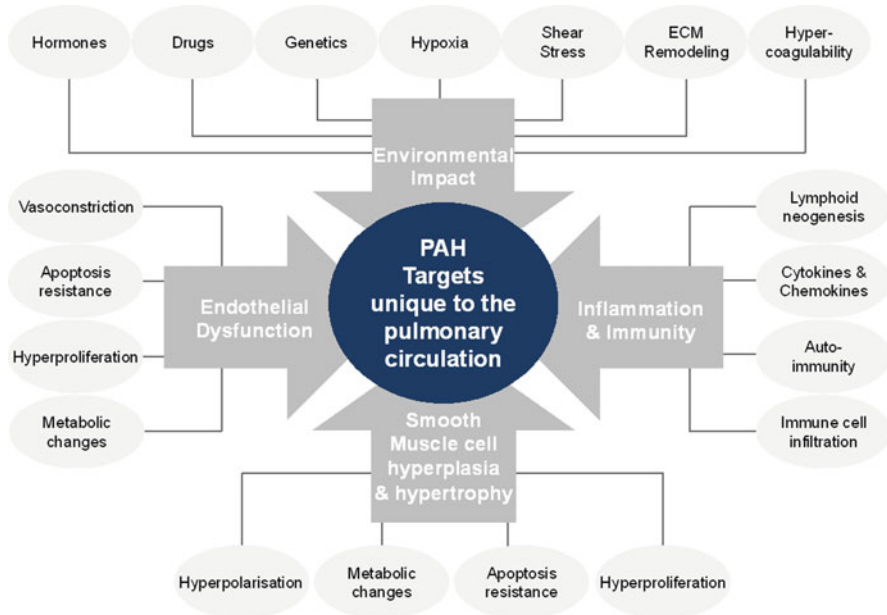


Fig. 1 Conceptual framework for the development of pulmonary arterial hypertension (PAH) therapies. *ECM* extracellular matrix

receptors: platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) receptors. Altered expression and/or increased activity of these three signaling pathways as well as their contribution to the abnormal proliferation and migration of smooth muscle and endothelial cells have been demonstrated in human and experimental models of pulmonary hypertension. Furthermore, their inhibition by specific inhibitors, tyrosine kinase inhibitors (TKIs), has been shown to exert beneficial effects in rodent models (Merklinger et al. 2005; Schermuly et al. 2005; Perros et al. 2008; Izikki et al. 2009; Tu et al. 2011, 2012). Currently, imatinib (a TKI of PDGF receptors), which is widely used for the treatment of chronic myeloid leukemia, is being tested as a new potential therapy in PAH and ongoing studies seek to better evaluate the overall risk benefit ratio of this anti-proliferative molecule in PAH.

The last few years have seen the emergence of the concept that anti-proliferative strategies could offer a novel approach for the treatment of PAH, by downregulating the progression of the disease and reversal of pulmonary vascular remodeling. However, further studies are needed to better understand the mechanisms underlying this abnormal over-activation of some growth factor-stimulated signaling pathways in PAH and to identify novel pharmacological targets for the development of new, better-tolerated, and more powerful therapeutic tools. This chapter aims to provide an overview on current status and future perspectives of target-based anti-proliferative therapies in pulmonary hypertension.

2 Growth Factor Signaling Pathways as Targets for Anti-remodeling Therapies in Pulmonary Hypertension

Growth factors are diffusible proteins that act through activation of diverse signaling pathways to regulate a myriad of critical cellular functions for normal lung development and homeostasis. As growth factors dictate growth, proliferation, and survival, there has been an increasing interest in the cellular biology of growth factor signaling in PAH in recent years. Excessive release of growth factors that are encrypted in the extracellular matrix, and/or modification of growth factor production, and receptor expression, and/or alterations in the intracellular mitogenic signals, have been reported to have a critical role in the disease; however, it is unknown whether or not this imbalance is the cause or the consequence of PAH. Many molecular therapies that target aberrant growth factor signaling pathways are being investigated, with some agents in the late stages of clinical testing.

2.1 *The Platelet-Derived Growth Factor Signaling System in PAH*

The PDGF family consists of PDGF-A, -B, -C, and -D, which form either homo- or heterodimers (PDGF-AA, -AB, -BB, -CC, -DD). The four PDGFs are inactive in their monomeric forms. The PDGF-AA, -AB, and -BB dimers are processed intracellularly and secreted as active dimers. In contrast, PDGF-CC and PDGF-DD differ from the others in that they are secreted as an inactive form until their N-terminal complement C1r/C1s, Uegf, Bmp1 (CUB) domain is cleaved. There are two receptor subunits α and β that dimerize upon binding one PDGF dimer, leading to three possible receptor combinations, namely, PDGFR- $\alpha\alpha$, $\beta\beta$, and $\alpha\beta$. PDGF-AA binds exclusively to PDGFR- $\alpha\alpha$, while PDGF-BB is the only PDGF that can bind all three receptor combinations with high affinity. PDGF-AB and -CC could assemble and activate PDGFR- $\alpha\alpha$ and $\alpha\beta$. PDGF-DD activates PDGFR- $\beta\beta$ with high affinity, and under certain conditions the PDGFR- $\alpha\beta$. The PDGFR autophosphorylation in the kinase insert region regulates interactions with different cell proteins involved in the initiation of the intracellular signaling pathway.

Since PDGF is an important autocrine and paracrine mitogen for vascular smooth muscle cells, mediating both hyperplasia and migration for pulmonary vascular remodeling, studies on animal models of PH as well as in human tissues have been undertaken (Tanabe et al. 2000; Berk 2001; Yamboliev and Gerthoffer 2001). Balasubramaniam et al. (2003) were the first to note a PDGF upregulation in a lamb model with chronic intrauterine PH and to suggest a pathogenic role of the PDGF signaling system for the disease. This hypothesis was tested and validated by a study from Schermuly et al. (2005), showing that the PDGF receptor antagonist imatinib reverses pulmonary vascular remodeling in the monocrotaline-induced PH in rats and the chronic hypoxia-induced PH in mice. They also reported a

substantial increase in the protein levels of PDGFR- β in total lung homogenates of patients with PAH as compared with controls. Finally, by investigating abnormalities within human tissue of patients with idiopathic PAH, Perros et al. (2008) have reported increased levels of mRNA encoding PDGF-A, PDGF-B, PDGFR- α , and PDGFR- β in microdissected small pulmonary arteries of patients with idiopathic PAH. In addition, they demonstrated a marked increase in the protein levels of PDGFR- β in total lung homogenates of patients with PAH as compared with controls. Consistent with these observations, intense immunoreactivity of the phosphorylated form of PDGFR- β has been shown in vascular lesions in lungs of idiopathic PAH patients. In addition, it has also been demonstrated that PDGFR, specifically PDGFR- β , is transactivated by sphingosine-1 phosphate (Baudhuin et al. 2004), G protein-coupled receptors (GPCRs) stimulated by lysophosphatidic acid (LPA) (Herrlich et al. 1998), angiotensin II (Saito and Berk 2001), and serotonin transporter (5-HTT or SERT) (Ren et al. 2011). Furthermore, Tie2-mediated loss of peroxisome proliferator-activated receptor (PPAR)- γ in mice causes PDGFR- β -dependent pulmonary arterial muscularization (Guignabert et al. 2009a).

2.2 *The Fibroblast Growth Factor Signaling System in PAH*

FGFs are a group of at least 23 structurally related heparin-binding polypeptide mitogens that are expressed in almost all tissues and constitute a signaling system conserved throughout animal evolution. FGFs interact with a family of four distinct, high-affinity RTKs, designated FGFR 1 to 4. The FGF system has a broad range of biological activities that not only stimulate cell proliferation, migration, and differentiation but also inhibit cell death. FGFRs are protein tyrosine kinase receptors, which consist of three extracellular immunoglobulin-type domains (D1–D3), a single-span transmembrane domain and an intracellular split tyrosine kinase domain. FGFRs 1, 2, and 3 undergo an alternative splicing event in which two alternative exons (IIIb and IIIc) can be used to encode the carboxy terminal portion of the third immunoglobulin-like loop. The splicing variant IIIa of FGFRs is a secreted FGF-binding protein. In addition, other types of alternate mRNA splicing events have been described. Each alternatively spliced variants binds to a specific subset of FGFs with variable affinities and have distinct tissue-specific expression patterns. Upon binding of FGF, FGFR monomers dimerize, and subsequently the TK domains autophosphorylate, which initiates the intracellular signaling pathway.

Since FGF2 is a known potent pro-angiogenic stimulator of endothelial and smooth muscle cell proliferation, migration, and synthesis of various extracellular components (Goncalves 1998), there is a strong interest for this growth factor in PAH. In addition, FGF2 is up regulated in response to hypoxia and shear stress in pulmonary vascular cells (Quinn et al. 2002; Li et al. 2003). Lung and circulating FGF2 levels are increased in both experimental and human PH. Abnormally high levels of FGF2 were found in the blood of 51 % and in the urine of 21 % of patients

with idiopathic PAH (Benisty et al. 2004). FGF2 levels are increased in two animal models, a lamb model of PH developed by inserting an aorto-pulmonary vascular bypass graft (Wedgwood et al. 2007) and the rat model of monocrotaline-induced PH (Arcot et al. 1995; Izikki et al. 2009). In PAH patients, we have shown that FGF2 is markedly overproduced by pulmonary endothelial cells in walls of distal arteries and in isolated primary cells than in controls. Furthermore, we have demonstrated that this FGF2 overexpression contributes significantly not only to smooth muscle hyperplasia by a paracrine action, but also to the abnormal endothelial phenotype by an autocrine action (Izikki et al. 2009; Tu et al. 2011, 2012). In idiopathic PAH, dysregulation of the FGF2 signaling pathway was associated not only with FGF2 overproduction, but also with altered expression of the FGF receptor. We found increased FGFR2 expression in pulmonary endothelial cells derived from idiopathic PAH patients as compared to control cells. Interestingly, Matsunaga et al. (2009) recently demonstrated that overexpression of a constitutively active FGFR2 in endothelial cells in vitro enhanced migration and survival, and augmented autocrine FGF2 production. Selection of endothelial cells that naturally overexpress FGF2 in early stages of the disease and/or deficient activity of PPAR γ may also explain the augmented FGF2 production by endothelial cells of idiopathic PAH patients (Tian et al. 2009). Moreover, FGF2 can be sequestered and stored as a complex in the extracellular matrix and then released by proteolytic processes to bind and activate cell targets, thereby promoting mitogenesis (Benezra et al. 1993; Thompson and Rabinovitch 1996; Buczek-Thomas and Nugent 1999; George et al. 2001). Recently, we reported that daily treatment with the FGFR inhibitor dovitinib started 2 weeks after a subcutaneous monocrotaline injection substantially attenuated the abnormal increase in p130^{cas} and ERK1/2 activation and regressed established PH (Tu et al. 2012).

2.3 The Epidermal Growth Factor Signaling System in PAH

The EGF family consists of EGF, transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor (HB-EGF), epiregulin, amphiregulin (AR), epigen, beta-cellulin (BTC), and neuregulin 1, 2, 3, and 4. Each ligand displays overlapping but distinct binding affinities toward ErbB receptors and subsequently induces the formation of homo- and heterodimers of these receptors. There are four types of EGF receptors (EGFR) including ErbB1, also referred to as EGFR or Her1, ErbB2 (Neu/Her2), ErbB3 (Her3), and ErbB4 (Her4). All EGF receptors have a common extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain. EGF, HB-EGF, TGF- α , AR, BTC, and epiregulin all bind ErbB1. HB-EGF, epiregulin, and BTC are known to bind to ErbB4 as well as ErbB1. The other group includes the neuregulins, which are ligands for ErbB3 and ErbB4. Upon ligand binding, EGFR monomers dimerize, and subsequently the TK domains autophosphorylate, which leads to the activation of intracellular signaling pathways.

Multiple lines of evidence suggest that the EGF signaling system contributes to the SMC proliferative response and are involved in the initiation and/or progression of the pulmonary vascular remodeling in PAH. Several studies have demonstrated that EGF co-localizes with Tenascin C, a component of the extracellular matrix (ECM) that is overabundant in obstructive lesions of patients with PAH and thus leads to an EGF-dependent proliferation and migration of pulmonary vascular cells (Jones and Rabinovitch 1996; Jones et al. 1997a, b, 1999; Cowan et al. 1999, 2000b). Consistent with this pathogenic role of the EGF signaling system, transgenic mice that over-express TGF- α under the control of the human surfactant protein (SP)-C promoter (the TGF- α mice) developed severe PH and vascular remodeling characterized by abnormally extensive muscularization of small pulmonary arteries (Le Cras et al. 2003). This phenotype was prevented in bi-transgenic mice expressing both TGF- α and a dominant-negative mutant EGF receptor under the control of the SP-C promoter. In addition, Merklinger et al. (2005) have shown that inhibition of EGFR by PKI166, a dual EGFR/HER2 inhibitor, mediated PASMCM apoptosis and improved survival of monocrotaline-injected rats. However, Dahal et al. (2010) reported no changes in the levels of mRNA encoding of BTC, AR, HB-EGF, ErbB1, ErbB2, ErbB3, and ErbB4 as well as in the protein levels of ErbB1 between lung homogenates from idiopathic PAH (late stage of the disease) and normal subjects. In the same study, Dahal et al. (2010) found that three clinically approved EGFR antagonists (gefitinib, erlotinib, and lapatinib) substantially reduced the EGF-induced proliferation of PSMCs isolated from healthy or monocrotaline-injected rats. In this rat monocrotaline model of PH, an upregulation of EGF, TGF- α , ErbB1, ErbB2, and ErbB3 mRNA levels has been noted in lung homogenates from monocrotaline-injected rats as compared with control rats. However, no differences were noted between the two groups regarding the levels of mRNA encoding for HB-EGF, epiregulin, AR, and ErbB4 in this rodent PH model. They also found in rats that daily treatment with gefitinib and erlotinib but not with lapatinib started 3 weeks after a subcutaneous monocrotaline injection substantially regressed established PH. In contrast to the beneficial effects of gefitinib and erlotinib treatments in the monocrotaline model, no substantial improvements were observed with these drugs in the chronic hypoxia mouse model of PH. The discordant findings may be attributable to differences in species, disease severity, mechanisms underlying PH induction and/or may suggest a much more complex regulatory network. Indeed, cooperative and synergistic signaling exists between EGF and other factors including TGF- β 1 and FGF2 (Kelvin et al. 1989; Ciccolini and Svendsen 1998; Park et al. 2000; Murillo et al. 2005; Ding et al. 2007; Grouf et al. 2007; Uttamsingh et al. 2008).

2.4 *The Serotonergic System in PAH*

Serotonin (5-hydroxytryptamine or 5-HT) is an endogenous vasoactive indolamine found mainly in enterochromaffin tissue, brain, and blood platelets. 5-HT is

synthesized from amino acid tryptophan in two steps: the hydroxylation of tryptophan to form the 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (TPH), and then the decarboxylation of this intermediate by the aromatic L-amino acid decarboxylase. 5-HT is predominantly synthesized in the enterochromaffin cells of the intestine, representing more than 95 % of total body 5-HT. In the circulation, 5-HT is actively incorporated into platelets and stored in platelet dense storage, keeping the free circulating 5-HT in low levels (Nilsson et al. 1985; Vanhoutte 1991; Brenner et al. 2007). 5-HT is also synthesized in the raphe nuclei of the brain, pineal gland, and in pulmonary vascular endothelial cells. In addition to SERT, 5-HT concentration is regulated by the mitochondrial enzyme monoamine oxidase (MAO) and by 5-HT storage. 5-HT is metabolized by monoamine oxidase (MAO) in 5-hydroxyindole acetic acid (5-HIAA). There is one 5-HT transporter (5-HTT or SERT) encoded by the *SLC6A4* gene, and seven known families of serotonin receptors: 5-HT1A-E, P, 5-HT2A-C, 5-HT3, 5-HT4, 5-HT5, 5-HT6, and 5-HT7. The serotonergic system is particularly important for promoting pulmonary arterial smooth muscle cell proliferation, pulmonary arterial vasoconstriction, and local microthrombosis.

The serotonergic system has long been suspected of playing important roles in the pathogenesis of idiopathic PAH. Plasma 5-HT levels are elevated in patients with PAH and remain high even after lung transplantation, indicating that this condition is not secondary to the disease (Herve et al. 1995). 5-HTT belongs to a large family of integral membrane proteins and is responsible for 5-HT uptake (e.g., by platelets, endothelial and vascular SMCs). Analysis of distal pulmonary arteries of patients with PAH and their cultured PA-SMCs indicates that 5-HTT is overexpressed and that the level of expression correlates with PAH severity (Eddahibi et al. 2001, 2002; Marcos et al. 2004, 2005). Tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT biosynthesis, is also expressed at abnormally high levels in pulmonary endothelial cells from patients with idiopathic PAH and therefore raises 5-HT levels locally (Eddahibi et al. 2006). There is evidence that alterations in platelet 5-HT storage and/or increased platelet consumption by the lung may trigger the development of PAH (Herve et al. 1990, 1995; Breuer et al. 1996; Eddahibi et al. 2000b; Kereveur et al. 2000; Morecroft et al. 2005). Furthermore, serotonergic appetite suppressant drugs have been associated with an increased risk of developing PAH (Douglas et al. 1981; Gurtner 1985; Loogen et al. 1985; Brenot et al. 1993; Abenhaim et al. 1996; Perros et al. 2008). Serotonylation of RhoA was also proposed as a possible risk factor of pulmonary vascular remodeling in PAH (Guilluy et al. 2007, 2009). Similarly, findings from another recent study from Wei et al. (2012) indicate increased serotonylation of fibronectin in human and experimental PH. During the last few years, direct evidence for a molecular interplay between 5-HTT signaling and Kv1.5 expression/activity has emerged (Guignabert et al. 2006, 2009b; Guignabert 2011).

Additionally, studies on animal models of PH consolidate all these observations obtained from human subjects. Plasma 5-HT levels are elevated not only in rodents treated with the anorectic agent dexfenfluramine (Eddahibi et al. 1998), but also in

the progression of monocrotaline- and chronic hypoxia-induced PH. The chronic infusion of exogenous 5-HT via osmotic pumps can potentiate the development of PH in rats exposed to chronic hypoxia (Eddahibi et al. 1997). A BMPR-II deficiency increases susceptibility to PH induced by 5-HT in mice (Long et al. 2006). In the fawn-hooded rat, a strain with a genetic deficit in platelet 5-HT storage that causes elevated plasma 5-HT concentrations, PH develops when the animals are exposed to mild hypoxia but not in control rats (Sato et al. 1992). An abnormally high level of 5-HTT in the lungs was reported for fawn-hooded rats (Sato et al. 1992; Morecroft et al. 2005). Furthermore, rodents engineered to constitutively express angiopoietin 1 in the lung develop PH. This effect was found to be directly related to the elevated production and secretion of 5-HT by stimulated pulmonary endothelial cells (Sullivan et al. 2003). It has also been shown in the monocrotaline model that 5-HTT expression levels increased prior to the onset of PH, which strongly supports a role for 5-HTT overexpression in disease development (Guignabert et al. 2005). Treatment with selective serotonin reuptake inhibitors (e.g., fluoxetine) abrogates the disease in chronically hypoxic mice and rats with monocrotaline-induced PH (Wang et al. 2011; Marcos et al. 2003; Guignabert et al. 2005, 2009b; Jiang et al. 2007; Zhai et al. 2009; Zhu et al. 2009). Furthermore, mice carrying null mutations at the 5-HTT locus are protected from developing PH induced by prolonged hypoxia (Eddahibi et al. 2000a). Similarly, hypoxia-induced PH in mice lacking the *tph1* gene, which exhibit marked reductions in 5-HT synthesis rates and contents in their peripheral organs, was less severe than in wild-type mice (Izikki et al. 2007). More recently, direct evidence that elevated levels of 5-HTT gene expression can promote pulmonary vascular remodeling and spontaneous PH was obtained with the creation of two different types of transgenic mice: (1) SM22 5-HTT+ mice that selectively express the human 5-HTT gene in smooth muscle at levels close to that found in human idiopathic PAH; (2) SERT+ mice that ubiquitously express high levels of the human 5-HTT gene from a yeast artificial chromosome (YAC) construct. SM22 5-HTT+ mice undergo pulmonary vascular remodeling, develop PH, and exhibit marked increases in right ventricular systolic pressures (RVSPs), right ventricular hypertrophy (RVH), and muscularization of pulmonary arterioles. One major point is that PH in these mice developed without any alterations in 5-HT bioavailability and therefore occurred as a sole consequence of the increased 5-HTT protein levels in SMCs. Compared to wild-type mice, SM22 5-HTT+ mice exhibited increases of three- to fourfold in lung 5-HTT mRNA and protein, together with increased lung 5-HT uptake activity. However, there were no changes in platelet 5-HTT activity or blood 5-HT levels. PH worsened as the SM22 5-HTT+ mice grew older (Guignabert et al. 2006). Consistent with these observations, female SERT+ mice housed in normoxic conditions developed a threefold increase in RVSP values compared to those of their wild-type controls (MacLean et al. 2004).

Of the 14 distinct 5-HT receptors, the 5-HT-2A, -2B, and -1B receptors are particularly relevant to the pathogenesis of PAH. High levels of 5-HT-1B, -2A, and -2B receptor immunoreactivity were reported in remodeled pulmonary arteries

from patients with various forms of pulmonary hypertension, but only the 5-HTT was found to be overexpressed in pulmonary artery smooth muscle cells (Marcos et al. 2005). Several lines of evidence support the notion that functional interactions exist between some of these 5-HT receptors and 5-HTT and thus have encouraged studies to better understand these complex relationships (Lawrie et al. 2005; Launay et al. 2006). Antagonism of the 5-HT-2A receptor inhibits not only monocrotaline-induced pulmonary hypertension in mice (Hironaka et al. 2003) but also the 5-HT-induced pulmonary vasoconstriction in vessels from normoxic and hypoxic rats (Morecroft et al. 2005; Cogolludo et al. 2006). However, the 5HT-2A receptor antagonist ketanserin is not specific for pulmonary circulation, and systemic effects have limited its use in PAH (Frishman et al. 1995). 5-HT-2B knockout mice are resistant to hypoxia-induced pulmonary hypertension and administration of the specific 5-HT-2B receptor antagonist RS-127445 prevented an increase in pulmonary arterial pressure in mice challenged with hypoxia (Launay et al. 2002). Furthermore, the 5-HT-2B receptor may control 5-HT plasma levels in vivo (Callebert et al. 2006), and its functional loss may predispose humans to fenfluramine-associated PAH (Blanpain et al. 2003). A very recent study showed that terguride, a potent 5-HT-2A/5-HT-2B receptor antagonist, inhibits the proliferative effects of 5-HT on PA-SMCs and prevents the development and progression of monocrotaline-induced PH in rats (Dumitrascu et al. 2011). The 5-HT-1B receptor mediates 5-HT-induced constriction in human pulmonary arteries (Morecroft et al. 1999) and has been shown to be involved in the development of PH in rodents exposed to chronic hypoxia (Keegan et al. 2001). Recently, Morecroft et al. have reported that co-inhibition of the 5-HT-1B receptor and 5-HTT with a combined 5-HT-1B receptor/5-HTT antagonist (LY393558) is effective at preventing and reversing experimental PH in animal models and 5-HT-induced proliferation in PA-SMCs derived from idiopathic PAH patients.

2.5 Other Growth Factor Signaling Systems in PAH

Many other growth factor signaling pathways are suspected to be involved in the pathogenesis of the disease and may serve to identify potential target candidates for anti-proliferative strategies in PH; however, further studies on their pathogenic roles are important.

2.5.1 The Vascular Endothelial Growth Factor Signaling System

The vascular endothelial growth factor (VEGF) signaling system is a potent and selective endothelial cell mitogen implicated in vascularization and angiogenesis and has been shown to be abnormal in PAH. VEGF family consists of five members, VEGFA, B, C, D, and placenta growth factor (PlGF) that show different binding specificities for two tyrosine kinase receptors, VEGFR1 (also called Flt-1)

and VEGFR2 (also called KDR or Flk-1). The binding of VEGF to its receptors induces receptor dimerization and autophosphorylation, which activates several downstream kinases, including protein kinases C and D (PKC and PKD), phosphatidylinositol 3-kinase (PI3K), and MAPK. (Hirose et al. 2000; Voelkel et al. 2006). The VEGF signaling system plays a crucial role for endothelial cell proliferation and promotes the release of endothelial mediators including nitric oxide and prostacyclin (Wheeler-Jones et al. 1997; Dimmeler and Zeiher 1999; He et al. 1999; Nagy et al. 2012). In the lungs of PH patients, VEGF and its receptors are upregulated with a close correlation to the plexiform lesions, suggesting a potential association with the severity of the remodeling process (Cool et al. 1997; Geiger et al. 2000; Hirose et al. 2000; Tuder et al. 2001; Voelkel et al. 2006). Furthermore, patients with PH have an increase in platelet VEGF content (Eddahibi et al. 2000b). The results from the animal models of PH are less clear with differences with the human data and also between the different models studied. On the one hand, Partovian et al. (1998) have reported reduced VEGF mRNA levels in lungs and right ventricles of monocrotaline-injected rats. On the other hand, although no changes in VEGF mRNA levels were noted in lungs from chronically hypoxic rats, an increase in VEGF mRNA levels in right ventricles was observed. Increased VEGF production in the lungs of rats with hypoxia-induced PH was also noted by other investigators (Christou et al. 1998; Laudi et al. 2007). Finally, it has been reported that the *VEGF* gene transfer in the monocrotaline model (Campbell et al. 2001) and in the chronic hypoxia model (Partovian et al. 2000; Louzier et al. 2003) is beneficial. The VEGFR2 is also increased in two other models of PH: in a canine experimental heart failure (Ray et al. 2008) and in overcirculation-induced PH in piglets (Rondelet et al. 2003). However, it is well known that VEGF signaling is required for maintenance of the alveolar structures (Kasahara et al. 2000) and VEGFR-2 blockade with SU5416 in combination with chronic hypoxia causes PH in rats (Taraseviciene-Stewart et al. 2001) and in mice (Ciuclan et al. 2011). In conclusion, further studies are required to identify the precise function of the pathogenic role of VEGF in early stages and during disease progression.

2.5.2 The Insulin-Like Growth Factor Signaling System

The insulin-like growth factor (IGF) signaling system consists of three different ligands (insulin, IGF1 and IGF2), two receptors (IGF1R, IGF2R), and from at least six high-affinity IGF-binding proteins (IGFBP 1-6). IGF1 is mainly secreted by the liver and binds with high affinity to IGF1R and with low affinity to the insulin receptor and IGF2R. IGF2 binds with high affinity to IGF2R and with low affinity to IGF1R and insulin receptor. IGFR1 and IGFR2 share 70 % homology in their protein sequences, but they present differences in signaling and functions. The binding of a ligand to IGF1R leads to activation of distinct downstream signaling pathways such as the MAPK signaling and the phosphatidylinositol 3-kinase-Akt (PI3K-Akt) pathway. Since IGFR2 does not have an intra-cytoplasmic signaling

domain, the binding of a ligand to IGFR2 doesn't initiate downstream signaling events. Different subtype of IGFs can result in enhancement or inhibition of IGF-mediated cellular effects. The IGF signaling system plays a pleiotropic role in normal cell metabolism, growth, proliferation, differentiation, cell-cell and cell-matrix adhesion, and survival. Recently, Guo et al. (2012) have found a substantial increase in IGFR1 in pulmonary arteries from rats exposed 9 days to hypoxic environments, suggesting a potential involvement of the IGF signaling system in the disease.

2.5.3 The Protein Kinase B/Mammalian Target of Rapamycin Signaling Pathway

Mammalian target of rapamycin (mTOR) is a 289-kDa serine/threonine protein kinase and a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family. mTOR consists of several conserved functional domains: a catalytic kinase domain, a FKBP12-rapamycin-binding (FRB) domain, a putative autoinhibitory domain (repressor domain) near the C-terminus and up to 20 tandemly repeated HEAT (Huntington, EF3, A subunit of PP2A, TOR1) motifs at the amino terminus, and FAT (FRAP-ATM-TRRAP) and FATC (FAT C-terminus) domains. In fact, mTOR is found to associate with different cofactors and form two distinct multiprotein complexes, mTORC1 and mTORC2 (Wullschleger et al. 2006; Dann et al. 2007). mTORC1 is sensitive to rapamycin and regulates ribosome biogenesis, autophagy, translation, transcription, and mitochondrial metabolism. mTORC1 is activated by mitogens via the PI3K-Akt and ERK1/2 signaling pathways that promote activation of S6 kinase 1 (S6K1), cell growth, and proliferation (Krymskaya and Goncharova 2009). In contrast, mTORC2 is insensitive to rapamycin and functions as an important regulator of actin cytoskeleton through stimulation of F-actin stress fibers. mTORC2 is activated by insulin in a PI3K-dependent manner and phosphorylates protein kinase B (Akt) at Ser-473, which promotes cell cycle progression and increases cell survival (Laplane and Sabatini 2009). The development of monocrotaline- and hypoxia-induced PH in rats has been found to be associated with marked activation of the Akt/glycogen synthase kinase (GSK)-3 axis (Gary-Bobo et al. 2010). In addition to being a central regulator of cell growth, proliferation, apoptosis, and metabolism, mTOR is also linked to the phosphatidylinositol 3 kinase (PI3K)/phosphatase and tensin homolog (PTEN)/Akt/tumor suppressor complex (TSC) signaling pathway, where genetic mutations of many components in this pathway result in the development of a wide variety of cancers (Fig. 2).

Recent evidence demonstrated that mTOR activation in both mTORC1 and mTORC2 due to chronic hypoxia exposure is required for pulmonary arterial smooth muscle cell proliferation (Krymskaya et al. 2011). Similarly, Gerasimovskaya et al. (2005) showed that hypoxia-induced adventitial fibroblast proliferation requires activation and interaction of PI3K, Akt, mTOR, p70S6K, and ERK1/2 and provided evidence for hypoxic regulation of protein translational

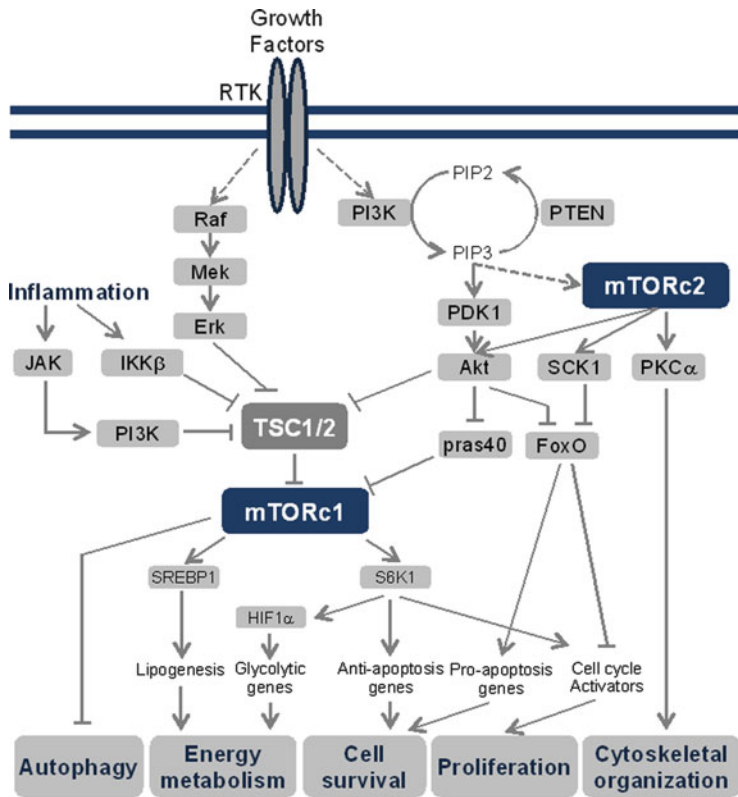


Fig. 2 Schematic representation of the mTOR-signaling pathway. *Akt* protein kinase B; *Erk* extracellular signal regulated kinase; *FoxO* Forkhead box, class O; *HIF1 α* hypoxia-inducible factor 1 α ; *IKK β* inhibitor of nuclear factor kappa β ; *JAK* the Janus kinase; *Mek* mitogen-activated protein kinase; *PDK1* phosphoinositide-dependent kinase-1; *PI3K* phosphoinositide 3-kinase; *PIP2* phosphatidylinositol 4,5-bisphosphate; *PIP3* phosphatidylinositol 3,4,5-trisphosphate; *PKC α* protein kinase C α ; *pras40* proline-rich AKT substrate of 40 kDa; *PTEN* phosphatase and tensin homolog; *RTK* receptor tyrosine kinase; *SCK1* Sck1/2, suppressor of loss of cAMP-dependent protein kinase 1; *S6K1* S6 kinase 1; *SREBP1* sterol regulatory element-binding protein 1; *TSC1/2* tuberous sclerosis complex 1/2

pathways in cells exhibiting the capability to proliferate under hypoxic conditions. Furthermore, Ogawa et al. (2012) reported that PDGF-induced phosphorylation of Akt/mTOR signaling pathway in normal pulmonary arterial smooth muscle cells and that inhibition of Akt/mTOR signaling pathway by either rapamycin or Akt inhibitor attenuates PDGF-induced increase in store-operated calcium entry in these cells (Ogawa et al. 2012). Further studies into the role of Akt signaling are important, and the Akt/mTOR pathway may be a potential target for the anti-proliferative strategy in PH.

2.5.4 The Janus Kinase/Signal Transducers and Activators of Transcription Signaling System

Four JAK family kinases, including JAK1, JAK2, JAK3, and TYK2, and seven STAT family members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, have been identified. JAK1, JAK2, and TYK2 appear to be ubiquitously expressed, while JAK3 expression is normally limited to lymphoid cells. In addition, different isoforms of several STATs have been identified. STATs are latent cytoplasmic transcription factors that become activated after recruitment to an activated receptor complex. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is activated by a wide variety of cytokines, growth factors, interferons, and some hormones. Following the binding of cytokines to their cognate receptor, STATs are activated by members of the JAK family of tyrosine kinases. Once activated, they dimerize and translocate to the nucleus and modulate the expression of target genes. STATs are not only activated by cytokine receptors that associate with JAKs but also by RTKs and G protein-coupled receptors.

Over the last few years, increased evidences have supported the role of inflammation as well as the involvement of immunologic disorders in idiopathic PAH (Humbert et al. 1995; Dorfmuller et al. 2003; Tamby et al. 2005; Terrier et al. 2008; Kherbeck et al. 2013; Tamosiuniene et al. 2011; Huertas et al. 2012; Perros et al. 2012; Price et al. 2012). An abnormal activation of the JAK/STAT signaling system was firstly noted by Mathew et al. (2004) in the monocrotaline-induced PH and by Masri et al. (2007) in human tissues of patients with idiopathic PAH. Besides its central role in chronic inflammation, dysregulated activation of the JAK/STAT3 signaling pathway was shown to play an important role in pulmonary arterial endothelial cell proliferation and survival in response to growth factors as demonstrated by pharmacological inhibition of STAT3 phosphorylation by AG-490 (Masri et al. 2007). More recent evidences of the pathogenic role of the JAK/STAT signaling system were documented by the group of Bonnet et al. (Courboulin et al. 2011, 2012; Paulin et al. 2011a, b) showing close interrelationships with Pim1 (proviral integration site for Moloney murine leukemia virus) kinase and NFATc2 (nuclear factor of activated T-cells). It is now well established that among inflammatory cytokines interleukin-6 (IL-6) plays an important role in the development of PH. Overexpression of IL-6 promotes PH in mice (Steiner et al. 2009), while IL-6-deficient mice are protected from hypoxia-induced PH (Savale et al. 2009). In addition, elevated serum IL-6 levels have been reported in patients with idiopathic PAH or PH associated with inflammatory diseases such as scleroderma and lupus (Nishimaki et al. 1999; Pendergrass et al. 2010; Soon et al. 2010). All known members of the IL-6 cytokine family (IL-6, IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), and oncostatin M (OSM)) through their receptors comprised of the signal transducer gp130 in combination with IL-6R, IL-11R, LIF-R, or OSM-R are known to potently activate STAT3 (Fischer and Hilfiker-Kleiner 2007). A better knowledge of the molecular processes by which JAK-STAT

signaling can be turned off will certainly help to identify new targets for drug treatment in PH.

2.5.5 The RhoA/Rho-Kinase Signaling System

RhoA, a small GTPase protein, and its immediate downstream target, RhoA/Rho-kinase (ROCK), control a wide variety of signal transduction pathways. ROCKs are kinases belonging to the AGC (PKA/PKG/PKC) family of serine–threonine kinases that exist in two isoforms: ROCK1 and ROCK2 (Nakagawa et al. 1996). ROCK is comprised of an amino-terminal kinase domain, followed by a coiled-coil region that contains the ρ -binding domain. The carboxy-terminal consists of a plexstrin-homology domain, which contains an internal cysteine-rich domain. ROCK1 and ROCK2 are highly homologous, sharing an identity of 65 % in their overall amino acid sequences and 92 % in their kinase domains. In addition to their effect on actin organization, or through this effect, ROCKs have been found to regulate a wide range of fundamental cell functions such as contraction, motility, proliferation, and apoptosis.

Recent pharmacological studies suggest that activation of RhoA/ROCK signaling system is an important event in the pathogenesis of PH. In vivo, beneficial effects of treatment with Rho kinase inhibitor fasudil have been demonstrated in several animal models of PH (Nakagawa et al. 1996; Abe et al. 2004; Fagan et al. 2004; Nagaoka et al. 2004, 2005; Guilluy et al. 2009). In addition, the beneficial effect of sildenafil on PH is mediated, at least in part, by the inhibition of the RhoA/Rho kinase pathway (Guilluy et al. 2005). Serotonylation of RhoA by intracellular type 2 transglutaminase (TG2), leading to constitutive RhoA activation was also proposed as a possible risk factor of pulmonary vascular remodeling in PAH (Guilluy et al. 2007, 2009). Similarly, findings from another recent study from Wei et al. (2012) indicates increased serotonylation of fibronectin in human and experimental PH.

2.5.6 Other Growth Factor Signaling Systems

Several other studies have similarly found that other growth factor signaling might be involved in the pathogenesis of PAH such as angiotensin II (AngII) (de Man et al. 2012), connective tissue growth factor (CTGF) (Lee et al. 2005), hepatocyte growth factor (HGF) (Ono et al. 2004a, b; Hiramane et al. 2011), nerve growth factor (NGF) (Ieda et al. 2004; Kimura et al. 2007), and placenta growth factor (PIGF) (Sundaram et al. 2010; Sands et al. 2011).

3 Restitution of the Aberrant Extracellular Matrix Remodeling in PH

The proteolytic ECM remodeling not only causes qualitative and quantitative changes in the ECM, but is also actively involved in the creation of a permissive pericellular/extracellular environment for cell proliferation, survival and migration. Indeed, an aberrant ECM remodeling can (1) generate the excessive releases of growth factors and various molecules that are encrypted in the ECM; (2) expose functionally important cryptic sites in collagens, laminins, elastin, or fibronectin; (3) generate fragments of various ECM components (Giannelli et al. 1997; Rabinovitch 2001; Shang et al. 2001; Xu et al. 2001; Ma et al. 2011; Wei et al. 2012).

Various studies demonstrated an imbalance between proteases and protease inhibitors in PH which, among others, include defects in several proteolytic enzymes: elastases (Rabinovitch 1999; Kim et al. 2011), matrix metalloproteinases (Lepetit et al. 2005; George et al. 2012), chymase (Mitani et al. 1999), and tryptase (Kwapiszewska et al. 2012). In addition, impairments of both the urokinase-type plasminogen activator (uPA)—plasmin or the tissue-type plasminogen activator (tPA)—plasmin systems have also been reported in PH (Huber et al. 1994; Christ et al. 2001; Katta et al. 2008; Kouri et al. 2008). Beneficial effects of serine elastase inhibitors in several experimental models of PH have been obtained (Ilkiw et al. 1989; Maruyama et al. 1991; Cowan et al. 2000a; Zaidi et al. 2002). Similar results were found with MMP inhibitors in the monocrotaline model (Vieillard-Baron et al. 2003), but deleterious effects were found with these MMP inhibitors in the chronic hypoxia model (Vieillard-Baron et al. 2000). Differences between both animal models might partially explain the different outcome obtained with MMP inhibitors. Collectively, these findings support that restitution of the aberrant ECM remodeling in PAH may represent another strategy for inhibition of pro-migratory and pro-proliferative signaling pathways.

4 Restitution of the Dysfunctional BMPR-II Signaling System in PAH

Bone morphogenetic proteins (BMPs) are a large family of secreted molecules that belongs to the transforming growth factor (TGF) β family. To date, over 20 BMP family members and 10 antagonists have been identified and characterized. They operate with varied duration, distance, and affinity. There are two classes of transmembrane receptors, type I receptors (ACVRL-I, ACVR-I, BMPR-IA, and BMPR-IB), and the type II receptors (BMPR-II, ActR-IIA, and ActR-IIB). Following ligand binding, the kinase domain in BMPR-II phosphorylates the type 1 receptor, which then phosphorylates Smad proteins 1, 5, and 8. Following activation of Smad 1, 5, and 8, a complex with the common partner Smad4 is generated and it migrates into the nucleus and transactivates specific target genes involved in cell

proliferation, survival, migration, and differentiation. *Bmpr2* gene mutations confer a reduction in the BMPR-II signaling activity (Foletta et al. 2003) resulting from a dose-dependent modulation of BMPR-II oligomerization with its co-receptor, most commonly, BMPR-IA (Gilboa et al. 2000). BMPR-II expression is also substantially reduced in patients with various form of PH without a mutation, as well as in experimental animal models (Takahashi et al. 2006; Reynolds et al. 2012). Steady-state levels of BMPR-IA are also reduced in the pulmonary vasculature of patients with pulmonary hypertension (Du et al. 2003), suggesting that disrupted BMP signaling contribute to the pathogenesis of PAH and/or represent a genetic susceptibility of developing the disease. Recently, a study by Reynolds et al. (2012) suggested a therapeutic potential for upregulation of the BMPR-II axis in PAH.

It has been shown that the BMPR-II signaling system plays pleiotropic roles, depending on the cell types: on the one hand, BMPs inhibit proliferation of smooth muscle cells; on the other hand, they promote pulmonary arterial endothelial cell survival (Teichert-Kuliszewska et al. 2006; Nasim et al. 2012). In addition, a constitutive activation of p38MAPK has been shown in primary cultured pulmonary arterial smooth muscle cells harboring a mutation in BMPR-I, a phenomenon that could contribute partly to the failure to suppress cell proliferation (Yang et al. 2005). Although further studies are required to determine the importance of these abnormalities for the initiation/progression/reversal of the disease, restitution of the dysfunctional BMPR-II signaling system may represent another anti-proliferative strategy.

5 Recovery of Oxidative Metabolism in PAH

In the presence of oxygen, normal cells completely oxidize glucose to CO₂ and H₂O, and generate ATP through aerobic oxidation. The Warburg effect is defined as an increased dependence on glycolysis for ATP synthesis, even in the presence of abundant oxygen. The Warburg effect has been found in a wide spectrum of human cancers as well as in PH (Xu et al. 2007), however the underlying mechanisms are still unclear. In cancer cells, this metabolic change has been found to be regulated by both oncogenes and tumor suppressor genes including hypoxia-inducible factors (Goda and Kanai 2012), p53 (Puzio-Kuter 2011), E2F transcription factor-1 (Puzio-Kuter 2011), and phosphatase and tensin homolog (PTEN) (Garcia-Cao et al. 2012). Interestingly, several groups have shown abnormalities in these different signaling pathways in PAH (Bonnet et al. 2006; Natali et al. 2011; Ravi et al. 2011). Restitution of oxidative metabolism with the use of dichloroacetate has been shown to be efficient in several animal models of PH (Michelakis et al. 2002; McMurtry et al. 2004; Guignabert et al. 2009b). Inhibition of pyruvate dehydrogenase kinase (PDK) by dichloroacetate frees up the mitochondrial gate-keeping enzyme pyruvate dehydrogenase (PDH), which is then able to convert pyruvate to acetyl-CoA and initiate normal oxidative phosphorylation via the Krebs cycle.

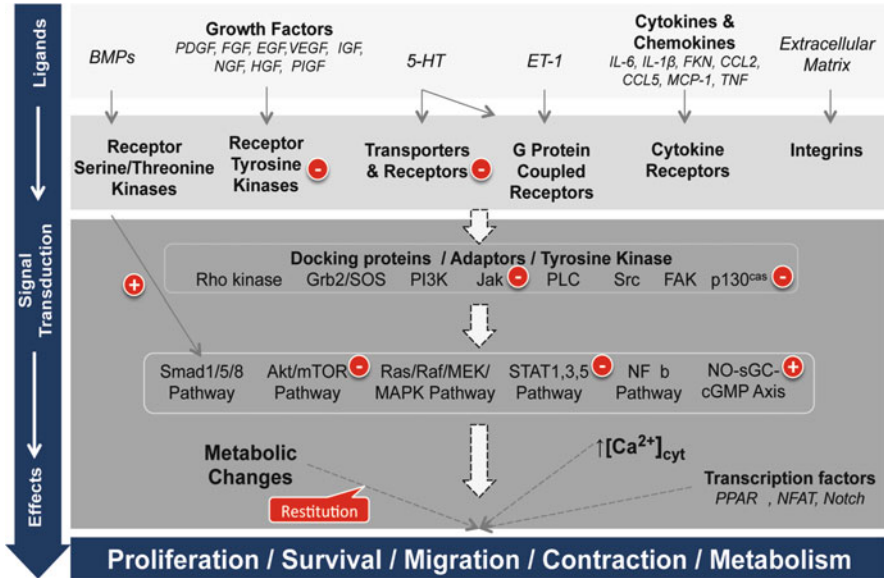


Fig. 3 Schematic representation summarizing the emerging molecular targets for the development of novel anti-proliferative therapies in pulmonary hypertension. *5-HT* serotonin; *Akt* protein kinase B; *BMPs* bone morphogenetic proteins; *CCL* CC chemokine ligands; *cGMP* cyclic guanosine monophosphate; *ET-1* endothelin-1; *FAK* focal adhesion kinase; *FKN* fractalkine; *GRB2* growth factor receptor-bound protein 2; *IL* interleukin; *Jak* the Janus kinase; *MAPK* mitogen-activated protein kinase; *MCP-1* monocyte chemotactic protein-1; *MEK* MAPK kinase; *NFκB* nuclear factor-kappaB; *NO* nitric oxide; *PI3K* phosphoinositide-3-kinase; *PLC* phospholipase C; *sGC* soluble guanylyl cyclase; *SOS* son of sevenless; *STAT* signal transducers and activators of transcription; *TNFα* tumor necrosis factor α

Since mitochondrial fatty acid oxidation (FAO) contributes to the “Randle cycle” inhibition of glucose utilization, the FAO inhibition prevents also this metabolic shift and limits the proliferative and anti-apoptotic cell phenotype observed in PH (Sutendra et al. 2010).

6 Conclusions and Challenges

In this chapter, we summarize several different emerging molecular targets for anti-proliferative strategies in pulmonary hypertension (Fig. 3).

However, growth factors, RTKs, BMPR-II, energetic and metabolic adaptation, as well as the proteolytic ECM remodeling control various aspects of normal cellular physiology, including cell growth, differentiation, motility, and death. Both the potency and selectivity of TKIs as well as of other anti-proliferative molecules are therefore important considerations, particularly as these agents are

being tested as a potential therapeutic approach to PAH. Another important aspect concerns the potential impacts of these anti-proliferative molecules on the adaptive response of myocardial hypertrophy that need to be further evaluated. Therefore, a better understanding of how these factors act in the lung as well as in the heart under normal and pathologic conditions will provide a stronger rationale for their use in specific therapeutic interventions and minimize the adverse effects of less focused treatments.

Substantial work remains to be done to discover and/or develop a new, better-tolerated, and more powerful therapeutic tool for PAH that combines promotion of vasorelaxation, suppression of cellular proliferation, and activation of apoptosis within the pulmonary-artery wall. Therefore, further studies are needed to better evaluate the overall risk benefit ratio of the available and future anti-proliferative molecules in PAH as well as their efficacies in experimental models of PH.

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Anti-inflammatory and Immunosuppressive Agents in PAH

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Abstract Pulmonary arterial hypertension (PAH) pathobiology involves a remodeling process in distal pulmonary arteries, as well as vasoconstriction and in situ thrombosis, leading to enhanced pulmonary vascular resistance and pressure, to right heart failure and death. The exact mechanisms accounting for PAH development remain unknown, but growing evidence demonstrate that inflammation plays a key role in triggering and maintaining pulmonary vascular remodeling. Not surprisingly, PAH is often associated with diverse inflammatory disorders. Furthermore, pathologic specimens from PAH patients reveal an accumulation of inflammatory cells in and around vascular lesions, including macrophages, T and B cells, dendritic cells, and mast cells. Circulating levels of autoantibodies, chemokines, and cytokines are also increased in PAH patients and some of these correlate with disease severity and patients' outcome. Moreover, preclinical experiments demonstrated the key role of inflammation in PAH pathobiology. Immunosuppressive agents have also demonstrated beneficial effects in animal PAH models. In humans, observational studies suggested that immunosuppressive drugs may be effective in treating some PAH subtypes associated with marked inflammation. The present chapter reviews experimental and clinical evidence suggesting that inflammation is involved in the pathogenesis of PAH, as well the therapeutic potential of immunosuppressive agents in PAH.

Keywords PAH • Inflammatory mediators • Immunosuppressive agents

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List of Abbreviations

BMPRII	Bone morphogenetic protein receptor 2
CCL	C-C motif ligand
CsA	Ciclosporin A
CTD	Connective tissue disease
CYC	Cyclophosphamide
DHEA	Dehydroepiandrosterone
EC	Endothelial cells
HAART	Highly active antiretroviral therapy
HHV-8	Human herpes virus 8
HIF-1 α	Hypoxia inducible factor 1 alpha
HIV	Human immunodeficiency virus

IL	Interleukin (i.e., IL-6, IL-2)
iPAH	Idiopathic pulmonary arterial hypertension
MCP-1	Monocyte chemotactic protein 1
MCT	Monocrotaline (induced PAH)
MIP-1 α	Macrophage inflammatory protein 1 alpha
MMF	Mycophenolate mofetil
MTX	Methotrexate
NFAT	Nuclear factor for activated T cells
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PA	Pulmonary artery
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cells
PDGF	Platelet-derived growth factor
PH	Pulmonary hypertension
RANTES	Regulated upon activation normal T cell expressed and secreted
RV	Right ventricle/ventricular
SLE	Systemic lupus erythematosus
SSc	Systemic scleroderma
Tc	Cytotoxic T lymphocytes
Th	Helper T lymphocytes
TNF- α	Tumor necrosis factor alpha
Treg	Regulatory T lymphocytes
VEGF	Vascular endothelial growth factor

1 Introduction

Pulmonary arterial hypertension (PAH) is a progressive life-threatening disease characterized by a progressive elevation of pulmonary vascular resistance, right ventricular (RV) failure, and ultimately death (Rubin 1997). According to current classification (Badesch et al. 2009), PAH constitutes the first category of pulmonary hypertension (PH) (Simonneau et al. 2009). This specific group of pre-capillary PH includes idiopathic PAH (iPAH), heritable PAH, and PAH related to drugs and toxins, congenital heart disease, connective tissue disease (CTD), human immunodeficiency virus (HIV) infection, portal hypertension, chronic haemolytic anemia, and schistosomiasis.

Since 1994, when Tuder et al. (1994) identified for the first time inflammatory infiltrates within PAH patients' plexiform lesions, an increasing amount of experimental and human reports supported the crucial role of inflammation in PAH development (Dorfmueller et al. 2003; Hassoun et al. 2009; Price et al. 2012). Numerous inflammatory disorders and autoimmune processes are associated with the

development of experimental and human PAH (Daley et al. 2008; Morse et al. 1997; Negi et al. 1998; Nicolls et al. 2005). Moreover, bone morphogenetic protein receptor type 2 (BMPRII), a pathway largely involved in heritable PAH (Deng et al. 2000; Lane et al. 2000; Machado et al. 2001), also modulates the inflammatory response (Hagen et al. 2007; Song et al. 2008). However, even if inflammation in PAH is well recognized to promote and/or perpetuate pulmonary vascular remodeling (Pullamsetti et al. 2011), its precise relationship with other components of the PAH pathogenesis like endothelial cells (EC) dysfunction, proliferation of pulmonary artery (PA) smooth muscle cells (PASMC) and fibroblast, and in situ thrombosis remains elusive. Furthermore, it is still unclear if inflammation really initiates vascular remodeling (“initial hit”), participates in its progression (“secondary hits”) or represents a reactive response to remodeling only (“bystander phenomenon”) (Price et al. 2012). From a therapeutic perspective, some reports have shown that immunosuppressive or anti-inflammatory drugs may improve PAH, both in animal models (Bonnet et al. 2007; Price et al. 2011; Sutendra et al. 2011; Suzuki et al. 2006; Wang et al. 2011; Zheng et al. 2010) and human cases (Jais et al. 2008; Karmochkine et al. 1996; Ogawa et al. 2011; Tanaka et al. 2002).

This chapter will review the relevance of inflammatory processes in experimental and human PAH, the implication of cells and other mediators in the inflammatory response observed in PAH, the potential targets for immunosuppressive treatment iPAH, and the updated clinical experience with available immunosuppressant agents.

2 Clinical Evidence of Inflammation in Human PAH

Over the past decades, many inflammation processes have been clearly associated with PAH development, including CTD (Asherson 1990; Cool et al. 1997; Fagan and Badesch 2002; Hachulla et al. 2005), HIV infection (Cool et al. 1997; Humbert 2008) and schistosomiasis infection (Butrous et al. 2008; Tuder 2009). PAH may also occur as an ultimate complication of exceptional autoimmune/inflammatory disorders such as POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes) (Lesprit et al. 1998) or Castelman disease (Montani et al. 2005a). Also, several reports indicate an association between Hashimoto thyroiditis and PAH (Thurnheer et al. 1997). Even in iPAH, inflammatory phenomena are present, confirming the key role of inflammation in the global pathophysiology of PA vascular remodeling (Hall et al. 2009; Humbert et al. 1995; Perros et al. 2007; Soon et al. 2010).

2.1 Histological and Cytological Data in Human PAH

Histological data were the first to support the role of inflammation in PAH pathobiology. Tuder et al. (1994) described in 1994 perivascular inflammatory cell

infiltrates composed of T cells, B cells, and macrophages in seven of ten cases of iPAH with plexogenic arteriopathy. Cool et al. (1997) reported a few years later mononuclear inflammatory cells surrounding vascular sites of plexiform growth in CTD-PAH lungs. These common features between iPAH and CTD-PAH underlined a potential role of inflammation in all PAH subtypes. Since these results, many histological and fundamental reports confirmed that immune cells abnormalities are implicated in human PAH pathogenesis (Hassoun et al. 2009; Kherbeck et al. 2011; Perros et al. 2011; Price et al. 2012), including very recent data suggesting that in iPAH, the tertiary lymphoid follicle composed of B lymphocytes, T lymphocytes, and dendritic cells have connections to remodeled PA via a stromal network supplied by lymphatic channels (Perros et al. 2012).

2.2 Inflammatory Mediators and Biomarkers in Human PAH

Increased circulating levels of interleukin (IL)-1 β and IL-6 were initially reported in iPAH by Humbert et al. (1995) in the early 1990s. Elevated serum cytokines have also been observed in other PAH subtypes like CTD-PAH (Gerbino et al. 2008), HIV-PAH (Humbert et al. 1995), as well as PAH associated with sickle cell disease (Niu et al. 2009) and congenital heart disease (Diller et al. 2008). Some authors even suggested inflammatory cytokines (Soon et al. 2010) and C-reactive protein (Quarck et al. 2009) levels predicted survival in PAH, although this remains controversial (Montani et al. 2011; Soon et al. 2010). Different types of chemokines are also increased in human PAH patients' serum, such as chemokine (C-C motif) ligand (CCL) 2 [known as monocyte chemotactic protein (MCP)-1] (Sanchez et al. 2007), CCL5 [known as regulated upon activation normal T cell expressed and secreted (RANTES)] (Dorfmueller et al. 2002), and CXCL3 (known as fractalkine) (Balabanian et al. 2002). In addition, PASMC from PAH patients demonstrate stronger migratory and proliferative response to CCL2 compared to controls (Sanchez et al. 2007). Thus, besides evidences of local inflammation in PAH, these data suggest PAH is associated with a systemic inflammatory state that may correlate with disease severity.

2.3 Inflammatory Conditions Associated with Human PAH

Clinical arguments have been accumulated supporting the role of inflammation in human PAH, especially in the spectrum of CTD and infectious diseases.

2.3.1 CTD and Inflammatory Diseases Associated with PAH

Systemic Scleroderma

The frequent association between two rare conditions is a strong argument for a link between them. Among CTD, scleroderma (SSc) is most commonly associated with PAH, occurring in 5–15 % of SSc patients (Hachulla et al. 2005; Mukerjee et al. 2003b). As in iPAH, EC activation and apoptosis, inflammatory cells recruitment, intimal proliferation, and adventitial fibrosis leading to vessel obliteration are observed in SSc-PAH (Cerinic et al. 2003; Dorfmueller et al. 2007; Le Pavec et al. 2011; Sgonc et al. 2000), supporting the concept that common features take part in the pathobiology of both iPAH and SSc-PAH (Terrier et al. 2008). SSc-PAH patients are also characterized as having an important number of autoantibodies (Fritzler et al. 1995; Grigolo et al. 2000; Morse et al. 1997; Mouthon et al. 2005; Nicolls et al. 2005; Okano et al. 1992; Tamby et al. 2006), including antifibroblast antibodies (Tamby et al. 2006) and anti-EC antibodies (Arends et al. 2010; Tamby et al. 2005), which activate fibroblasts and induce pro-inflammatory and pro-adhesive phenotype, promoting vascular remodeling (Chizzolini et al. 2002). Finally, the impact of the autoimmunity in PAH is further supported by the heterogeneous prevalence of PAH among SSc depending on the precise autoantibodies detected (Steen 2005).

Systemic Lupus Erythematosus

The prevalence of PAH in systemic lupus erythematosus (SLE) varies from 0.5 to 14 % depending on the diagnostic algorithm used to define PAH (Fois et al. 2010). As for other CTDs, the presence of antinuclear antibodies and rheumatoid factor, as well as abnormalities in immunoglobulin G, complement fractions, cytokines, and growth factors suggest a predominant role for immunological mechanisms in SLE-associated PAH (Quismorio et al. 1984). It is important to keep in mind that other mechanisms may lead to PH in SLE like chronic thromboembolism related to antiphospholipid syndrome, diffuse interstitial disease (Pope 2008; Torre and Harari 2011), or more rarely active pulmonary vasculitis (Asherson and Oakley 1986).

Others CTDs and Rare Inflammatory States

Other CTD inflammatory and/or autoimmune disorders such as mixed CTD (Fagan and Badesch 2002; Jais et al. 2008), polymyositis–dermatomyositis (Minai 2009), Sjogren’s syndrome (Launay et al. 2007), rheumatoid arthritis (Chung et al. 2010; Hassoun 2009), Hashimoto’s thyroiditis (Chu et al. 2002; Thurnheer et al. 1997), sarcoidosis (Nunes et al. 2006), and more rarely, POEMS syndrome (Jouve et al. 2007; Lesprit et al. 1998), multicentric Castelman disease (Bull et al. 2003), and systemic vasculitis (Launay et al. 2006) have been associated with PAH.

2.3.2 Infectious Diseases Associated with PAH

Evidence for inflammation in PAH infectious-related forms is also supported by histological, cytological, serological, and clinical data where the infective agent is commonly considered as a potential inflammatory trigger.

Human Immunodeficiency Virus and Human Herpes Virus 8

HIV infection is an independent risk factor for PAH (Opravil et al. 1997), occurring in 0.5 % of infected patients (Barnier et al. 2009; Sitbon et al. 2008). Histological features in HIV-PAH are similar to that in other PAH subtypes (Cool et al. 1997; Humbert 2008; Mehta et al. 2000). The mechanisms by which HIV leads to PA remodeling may involve chronic immune activation and upregulation of proinflammatory cytokines and growth factors (Humbert et al. 1998). Indeed, expression and production of platelet-derived growth factors (PDGF) and vascular endothelial growth factors (VEGF) are increased in lung tissue and HIV-infected T cells (Ascherl et al. 1999; Humbert et al. 1998). Viral proteins like Glycoproteine 120 and Tat are also associated with lung endothelial dysfunction in HIV infection mostly through endothelin-1 secretion (Ehrenreich et al. 1993; Ensoli et al. 1990). Controversial data also suggested Human herpes virus 8 (HHV-8) coinfection could account for the development of HIV-PAH (Hsue et al. 2008; Montani et al. 2005b). The effects of highly active antiretroviral therapy (HAART) on HIV-PAH are controversial as cases of PAH regression (Barnier et al. 2009; Speich et al. 2000) and worsening (Pellicelli et al. 1998) have been reported after initiation of therapy.

As described above, genes coding for the vasculotropic virus HHV-8 proteins have also been identified in plexiform lesions of iPAH patients by Cool et al. in the early 2000s (Cool et al. 2003). HHV-8 is known as Kaposi's sarcoma-associated herpes virus and is usually associated with angioproliferative disorders. HHV-8 could thus have a pathogenetic role in PAH, triggering vascular remodeling and plexiform lesions formation. Moreover, rare case reports documented an association between reversible PAH and HHV-8/HIV-associated multicentric Castelman's disease (Montani et al. 2005a). Although there are indirect evidences from *in vitro* and animal studies in favor of a link between HHV-8 and PAH pathophysiology, HHV-8 has not been detected in recent histological studies in human iPAH (Bendayan et al. 2008; Henke-Gendo et al. 2005; Valmary et al. 2011). Thus, the potential role of this virus on PA remodeling in PAH remains controversial.

Parasites: Schistosomiasis-Related PAH

Among the estimated 200 million people infected by schistosomiasis worldwide (King 2010), between 2 and 5 % are believed to develop PAH (Graham et al. 2010), but their survival remains, in any case, better than in iPAH (dos Santos Fernandes et al. 2010). PAH occurs almost exclusively in patients with hepatosplenic

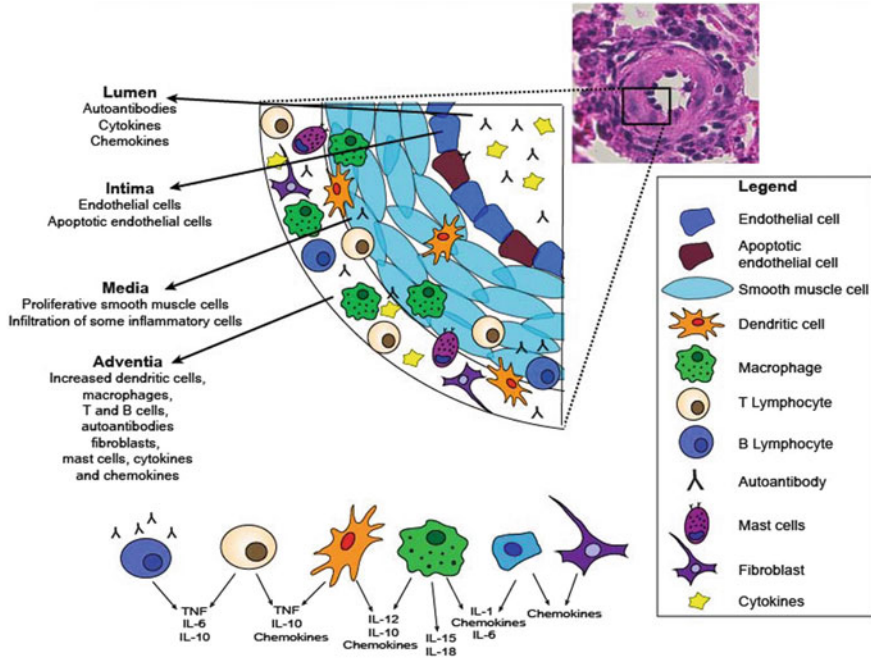


Fig. 1 Large amounts of inflammatory cell infiltrate pulmonary arteries in PAH. There is also vascular wall hypertrophy and presence of apoptotic endothelial cells. Cytokines (interleukins, TNF, MCP, etc.) and autoantibodies (anti-endothelial cells and antifibroblast for example) are present in the vascular wall and blood circulation. These autoantibodies can bind to endothelial cells and fibroblast and enhance cell modification (i.e., apoptosis and increase collagen production). Infiltration of T and B cells as well as macrophages, dendritic cells, mast cells, and fibroblast is observed in PAH. These inflammatory cells produce different cytokines as shown on the *bottom part* of this figure

infection by *Schistosoma mansoni* (Lapa et al. 2009) with subsequent portal hypertension. Histologically, plexiform lesions as well as concentric medial hypertrophy and thrombosis similar to those seen in iPAH are observed in schistosomiasis-related PAH (Tuder 2009). The mechanisms leading to PAH remain elusive, but may be related to mechanical impaction of pulmonary vessels by eggs, focal arteritis, and inflammation and increased pulmonary blood flow as consequence of portocaval shunts. Contribution of inflammation to vascular remodeling is not well understood but could be mediated by the modulation of regulatory T lymphocytes (Treg) activity resulting in the overexpression of a specific transforming growth factor superfamily (Freitas et al. 2007) or by the upregulated IL-13 signaling (Graham et al. 2010). In chronically infected animals without PH, increases in perivascular CD68-macrophages and CD45-lymphocytes have also been reported, suggesting an important switch from a Th1 to a Th2 immune response (Crosby et al. 2010). However, very few data allow extrapolating these experimental findings to human schistosomiasis-related PAH. Taken together, these non-exhaustive data suggest that increased levels of inflammatory mediators are common in human PAH (Fig. 1). Initial or latent inflammatory disorders may be an initial trigger for pulmonary vascular remodeling

or act as a “second hit.” Although the relationship between inflammation and PAH is most obvious for PAH related to autoimmune, inflammatory, and infectious diseases, this association is not limited to a particular type of PAH.

3 Cells and Inflammatory Mediators Implicated in Pulmonary Vascular Remodeling

Different cell types, chemokines, cytokines, and antibodies play a role in the pathogenesis of PAH. This inflammatory process is not specific to a PAH subgroup, suggesting a central role of inflammation in PAH pathology.

3.1 Cell Types Implicated in Inflammatory Processes

3.1.1 T Lymphocytes (or T cells)

T cells are part of the adaptive immune response. They can differentiate into many subtypes according to different stimuli. Major subtypes found in lungs are T helper CD4⁺ (Th), Treg, and T cytotoxic CD8⁺ (Tc) cells. CD4⁺ Th cells are further divided in Th1, Th2, and Th17 according to their activation and cytokine production. Th1 play a role in cellular immunity and clearance of intracellular pathogen by producing interferon- γ . Th2 produce pro-inflammatory cytokines and are mainly implicated in humoral immunity, inflammation, and allergy. Th17 regulate tissue inflammation and autoimmunity by producing IL-17. CD4⁺ Th cells are important regulators of adaptive immune response since they stimulate B cell differentiation and macrophage activation, which are critical in triggering the immune response. CD8⁺ Tc are responsible of killing viral infected cells and tumor cells by binding to major histocompatibility complex class I molecules. Treg suppress autoreactive T cells and, thus, control self-tolerance and autoimmunity by balancing Th1 and Th2 responses. Different evidences support the role of T cells in PAH development. For instance, Treg are able to limit vascular endothelial injury and prevent PAH (Tamosiuniene et al. 2011). In animal models, athymic rats (no mature T cells) develop PAH more rapidly than rats with intact T cell production (Taraseviciene-Stewart et al. 2007), giving a protective role to T cells in PAH. Conversely, depletion of Th cells ameliorates the extent of PAH in other models (Sutendra et al. 2011). Thus, certain subtypes of T cells may confer beneficial effect in PAH, whereas others like Th2 may promote pulmonary vascular remodeling (Daley et al. 2008). Overall, infiltrated T cells within PA wall are increased in iPAH patients. Some studies have shown that Tc are decreased and Treg increased in PAH (Ulrich et al. 2008a) and others showed no difference in

Treg between control and PAH patients (Huertas et al. 2012). The precise role of these T cell subtypes is not yet defined. Studies in cancer demonstrated that T cells inhibit tumor growth, but their functions are often suppressed in tumor microenvironment (Koebel et al. 2007; Zitvogel et al. 2008). A similar pattern could be observed in PAH, as leptin can modulate the hyporesponsiveness of Treg (Huertas et al. 2012). Overall, it is clear that inflammation plays an important role in PAH pathogenesis and that T cells are implicated but the exact mechanism has not yet been completely elucidated.

3.1.2 Dendritic Cells

Dendritic cells are professional antigen-presenting cells, displaying antigen to activate the adaptive immune response (T cell activation). They are responsible for the initiation of the inflammatory response. Recent studies documented the role of dendritic cells in the inflammatory process in different disorders including SLE (Palucka et al. 2005). Furthermore, these cells are present in pulmonary vascular lesions and blood of PAH patients (Wang et al. 2009). Perros et al. (2007) demonstrated that immature dendritic cells are involved in pulmonary vascular remodeling and thus could be involved in PAH immunopathology. Finally, dendritic cells have the ability to differentiate into other cell phenotypes, including EC, expanding their potential role in PAH pathogenesis (Conejo-Garcia et al. 2004).

3.1.3 Mast Cells

Mast cells are derived from bone marrow precursors and reside in tissues adjacent to blood vessels. They are major effector cells of immediate hypersensitivity reactions (allergy). They contain numerous mediator-filled granules, containing histamine and heparin, and a cross-linking of their IgE to a receptor stimulates granules release as well as synthesis and secretion of other mediators leading to hypersensitivity reaction. Accumulation of mast cells is seen in different PAH types (Hamada et al. 1999; Heath and Yacoub 1991). The exact role of mast cells in PAH pathobiology is not yet well established, but they seem to be implicated in direct vasoactive effects (Heath and Yacoub 1991) and in vascular remodeling by releasing matrix metalloproteinases (Vajner et al. 2006). Conversely, mast cells can also produce IL-10, which has important anti-inflammatory and immunosuppressive effects.

3.1.4 Macrophages

Macrophages constitute the first line of immune defense and are part of the innate immune system. In normal lungs, macrophages are mainly present in alveolar airspaces to protect against inhaled pathogens. They play a crucial role in the inflammatory and immune response: they have an antigen-presenting role and

display different antigens to activate T cells. Macrophages are increased in pulmonary vascular lesions and around remodeled PA in human and experimental PAH (Tuder et al. 1994). Macrophages are also responsible for producing a large spectrum of inflammatory mediators such as tumor necrosis factor (TNF), endothelin-1, different interleukins, and chemokines (Hassoun et al. 2009; Humbert et al. 2004). Recent studies demonstrated an interaction between macrophages and T cells in PAH (Gerasimovskaya et al. 2012). Indeed, macrophage migration is suppressed by activated T cells but not quiescent T cells, whereas activated macrophages partly block T cells antitumor growth effect. Activated macrophages are also able to suppress T cell activation, thus inhibiting the anti-migratory effect of T cells on macrophages, sustaining macrophage migration, and inflammation in PAH (Gerasimovskaya et al. 2012; Zitvogel et al. 2008).

3.1.5 B Lymphocytes

B cells are responsible of generating antibodies to specific antigenic epitopes which bind to antigens and tag cells for degradation by complement cascade and phagocytosis. In addition to increased antibody production observed in PAH, B cells are increased in PAH lung vasculature and in plexiform lesions and play a critical role in cell-mediated immune regulation through cytokines production (IL-6, IL-10, TNF), antigen presentation, and lymphoid organogenesis (Ulrich et al. 2008b).

3.2 *Role of Antibodies*

In iPAH, antinuclear antibodies are increased up to 40 % (Rich et al. 1986), whereas autoantibodies such as anti-Scl70, anticentromere, anticardiolipin, and anti-annexin C antibodies are also observed in CTD patients. The specific role of these autoantibodies in PAH pathogenesis is not yet elucidated. More recently, other autoantibodies have been detected in PAH patients.

3.2.1 Antifibroblast Antibodies

Antifibroblast antibodies are detected in 40 % of iPAH and in 30 % of SSc-PAH patients (Tamby et al. 2006). These antibodies enhance fibroblast differentiation and adhesion molecules production. Fibroblast are implicated in collagen production, which plays an important role in both CTD and PAH. Antifibroblast antibodies target different heat shock proteins, glucose-6-phosphate dehydrogenase, PI3-kinase, calumenin, and α -enolase (Terrier et al. 2008, 2009). These targets are implicated in oxidative stress-induced apoptosis resistance

(Efferth et al. 2006), cell energy metabolism, cell growth, and cytoskeleton organization (Shibasaki et al. 1994), leading to increased contractility of myofibroblasts as observed in SSc and PAH.

3.2.2 Anti-endothelial Cell Antibodies

Anti-EC antibodies are detected in over half of patients with SSc and their levels are increased in SSc-PAH compared to SSc patients without PAH (Salojin et al. 1997). Anti-EC antibodies are also present in iPAH patients' serum. A recent study identified lamins, beta tubulins, vinculin, and calumenin as anti-EC antibody targets (Dib et al. 2011). It has been suggested that these antibodies activate EC and induce apoptosis. Further studies would be needed to fully understand their role in the pathogenesis of PAH.

3.3 *Chemical Pro-inflammatory Mediators*

3.3.1 Cytokines and Chemokines

A large number of cytokines and chemokines (soluble cytokines acting as chemoattractants) are elevated in PAH (Balabanian et al. 2002; Soon et al. 2010). They are mainly produced not only by the innate immune system, i.e., dendritic cells and macrophages, but also by cells which form the vascular wall and adventitia (Nathan 2002). Increased IL-1 and IL-6 levels are found in severe PAH (Humbert et al. 1995). Other cytokines such as IL-2, IL-4, IL-8, IL-12p70, and TNF- α are also increased in PAH patients' serum (Fig. 1). Some of these cytokines, like IL-1b, IL-8, and TNF- α , predict outcome in PAH patients (Soon et al. 2010). The implication of these cytokines in PAH development has also been shown in animal models. For example, knock-out mice for IL-6 are resistant to hypoxia-induced PAH (Savale et al. 2009) and mice overexpressing IL-6 develop severe PH (Steiner et al. 2009), suggesting a central role of IL-6 in PAH. Furthermore, circulating IL-6 levels are predictive for the presence of associated PAH among SSc patients (Gourh et al. 2009). IL-6 is known to have many effects on inflammatory and vascular cells (Steiner et al. 2009). In fact, IL-6 stimulates T lymphocytes accumulation, chemokine production (such as CXCL3) by EC (Imaizumi et al. 2004), as well as PASMC and EC proliferation (Savale et al. 2009; Steiner et al. 2009). C-reactive protein is a marker of inflammation and tissue damage and is an active player in vascular wall damage and atherosclerosis (Scirica and Morrow 2006). C-reactive protein levels predict cardiovascular events (Labarrere and Zaloga 2004). Recently, Quarck et al. (2009) demonstrated that C-reactive protein levels are also increased

in PAH patients and correlate with long-term outcomes. Moreover, normalization of its levels with therapy is associated with improved functional capacity and survival in PAH (Quarck et al. 2009; Sztrymf et al. 2010).

An important chemokine potentially involved in PAH pathogenesis is fractalkine (CX3CL1 for C-X3-C motif ligand 1). Fractalkine, which can be detected as a soluble form, is anchored in EC membrane and is overexpressed in severe PAH (Balabanian et al. 2002). This chemokine may be responsible for leukocyte capture from the blood and recruitment (if they express C-X3-C motif receptor) (Balabanian et al. 2002). Circulating levels of monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) are also increased in PAH. MCP-1 is secreted by EC, macrophages, and fibroblasts and stimulates leukocytes and monocytes migration. As described earlier, cytokines can also act as chemokines. Thus IL-8, which is produced by hematopoietic cell and part of the C-X-C class, is chemoattractant for T cells and neutrophils (Schall et al. 1990). Macrophage inflammatory protein-1 α (MIP-1 α , also known as CCL3) promotes migration of monocytes and T and B cells through the endothelial junctions and underlying tissue (Schall et al. 1993). MIP-1 α mRNA expression is increased in PAH lung biopsy specimens compared to control (Fartoukh et al. 1998). RANTES is another important chemoattractant for T cells and monocytes and is increased in PAH (Dorfmueller et al. 2002; Luster 1998; Schall et al. 1990). Dorfmueller et al. (2002) demonstrated that RANTES mRNA levels are increased in EC from small remodeled PA and in plexiform lesions, thus increasing the amount of inflammatory cells in remodeled arteries of PAH patients.

3.3.2 Other Growth Factors Implicated in Inflammation

Growth factors including PDGF, epidermal growth factor, VEGF, and fibroblast growth factor 2 are implicated in the proliferative and apoptosis-resistant phenotype contributing to pulmonary vascular remodeling in PAH. There is an overlap between certain cytokines and growth factors. For example, IL-6 stimulates PASMC proliferation through an increase in VEGF and its receptor VEGFR2 (Steiner et al. 2009). PDGF-like molecules are secreted by many cell types, including PASMC, EC, and macrophages (Heldin 1992) and their expression is increased in PAH patients (Humbert et al. 1998). It has been shown that PDGF is able to induce smooth muscle cell proliferation and migration, which explains its implication in various fibroproliferative disorders such as hypoxic PH (Katayose et al. 1993; Schermuly et al. 2005).

4 Inflammation in PAH: Beyond the Pulmonary Vasculature

Right ventricular failure is an important component of the PAH pathophysiology. Indeed, RV failure is the leading cause of death in PAH. Not surprisingly, many circulating biomarkers currently used in PAH prognosis assessment are in fact cardiac-derived biomarkers (Barrier et al. 2012), such as brain natriuretic peptides (Blyth et al. 2007; Gan et al. 2006; Nagaya et al. 2000), troponins (Hoepfer et al. 2004; Torbicki et al. 2003), and osteopontin (Lorenzen et al. 2011). The RV capacity to adapt to increased afterload is highly heterogeneous among PAH patients: some are adaptive remodelers with RV hypertrophy and preserved RV function, whereas others are maladaptive remodelers and rapidly develop RV failure (Sztrymf et al. 2010). Some studies have suggested the implication of inflammation in RV remodeling and hypertrophy (Overbeek et al. 2008). However, very little is known about the molecular and cellular mechanisms involved in RV adaptation.

Part of the answer could be found through the analysis of SSc-PAH patients, which have a worse prognosis than iPAH patients (Condliffe et al. 2009; Kawut et al. 2003). This difference may be partly explained by the older age of SSc patients and their comorbidities. Nevertheless, recent data suggest that their RV adapts differently compared to iPAH patients (Chung et al. 2010), including higher N-terminal BNP levels and increased neurohormonal activation found in SSc-PAH compared to iPAH despite of less severe hemodynamic abnormalities (Mathai et al. 2009; Overbeek et al. 2008). Indirect evidence of increased RV inflammation in SSc-PAH has been suggested. For instance, an increased signal intensity in T2-weighted sequence (abnormalities commonly associated with inflammatory myocarditis in the absence of coronary artery disease) is documented on cardiac magnetic resonance imaging studies in SSc-PAH patients compared to SSc-patients without PAH (Hachulla et al. 2009). Recently, Overbeek et al. (2008) confirmed that RV from SSc-PAH ($n = 5$) had more neutrophilic granulocytes, macrophages, and lymphocytes than in iPAH ($n = 9$) or controls ($n = 4$), whereas RV interstitial fibrosis was similar in all groups. However, the participation of inflammation to RV failure is also suspected in all PAH types except, perhaps, for patients with left-to-right shunt and Eisenmenger physiology. In this setting, cardiomyocyte contractions may produce a trigger for autocrine, paracrine, and neuroendocrine signaling pathways leading to a vicious circle of RV inflammation and ischemia, leading to cardiomyocytes apoptosis and RV failure (Bogaard et al. 2009a). Nonetheless, observed RV recovery after lung transplantation supports the idea that RV failure is not irreversible and may be amenable to specific interventions in PAH. Interestingly, Bogaard et al. (2009a) recently demonstrated that a mechanical model of chronic progressive RV pressure overload using PA banding did not lead to fatal RV failure. In contrast, the Sugden model, an established model of angioproliferative PAH, showed myocardial apoptosis, fibrosis, decreased RV capillary density, and VEGF expression despite increased nuclear stabilization of

Hypoxia Inducible Factor-1 (HIF-1), ultimately leading to RV dilation and failure. A more recent animal study from this group assessed a potential RV failure molecular signature (Bogaard et al. 2009b). Their results suggest that RV hypertrophy and/or failure phenotypes in Sugen rats are characterized by distinct patterns of gene expression (mRNA and microRNA) related to cell growth, angiogenesis, and energy metabolism (Drake et al. 2011). Additionally, using PA banding and monocrotaline (MCT) rat models, Piao et al. (2010a, b) demonstrated that in RV hypertrophy, there was a mitochondrial metabolic switch from glucose oxidation to glycolysis. This increase in glucose uptake by the RV can be seen on PET scan, compared to a normal RV that is usually invisible (Bokhari et al. 2011; Can et al. 2011). The increase in glycolysis may temporarily preserve the energetic balance, but ultimately becomes maladaptive (Piao et al. 2010a, b). The reversibility of mitochondrial dysfunction in animals with RV hypertrophy, by a oxidative phosphorylation restoration using a prototypic pyruvate dehydrogenase kinase inhibitor (dichloroacetate), may offer selective strategies for improving RV function; this is in addition to known positive effects of dichloroacetate on PA remodeling in multiple experimental PAH models (Guignabert et al. 2009; McMurtry et al. 2004; Michelakis et al. 2002). These data suggest that complex molecular, cellular, and hemodynamic heart–lung interactions may be involved in the transition from compensated RV hypertrophy to failure in PAH. New therapeutic approaches based, among others, on RV inflammation and abnormal glycolytic metabolism may thus be of interest in PAH.

5 Available Immunosuppressive Agents: Mode of Action and Use in Human Diseases Predisposing to PAH

The mechanisms of action of common immunosuppressive agents are summarized in Table 1 and schematized in Fig. 2.

5.1 Glucocorticoids

Glucocorticoids are small lipophilic compounds affecting B cell and T cell development, differentiation, and function. Their major mechanism for immune suppression is through NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) inhibition. NF- κ B is involved in many cytokines and/or chemokines synthesis. Inhibition of this transcription factor, therefore, blunts the immune system capacity to mount a response (Rhen and Cidlowski 2005). Glucocorticoids also suppress cell-mediated immunity by inhibiting genes coding for different cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and interferon- γ . They can also stimulate T cell apoptosis by triggering the transcription of different genes (Leung and Bloom 2003). Finally, glucocorticoids not only suppress immune response but

Table 1 Immunosuppressive agents in pulmonary arterial hypertension

	Cyclophosphamide (CYC)	Mycophenolate mofetil (MMF)	Methotrexate (MTX)	Ciclosporin A (CsA)
Glucorticoids				
Mechanisms of action	<p>Glucorticoids (prednisone, dexamethasone...)</p> <p>Steroid hormones regulate function and numbers of lymphocytes. They also inhibit phospholipase A2 and cyclooxygenase, two important inflammation pathways (Goppelt-Strube et al. 1989; Leung and Bloom 2003; Rhen and Cidlowski 2005)</p>	<p>Inhibitor of purine synthesis, thus inhibiting cells proliferation (Suzuki et al. 2006; Zheng et al. 2010)</p>	<p>Antimetabolite (dihydrofolate reductase inhibitor), leading to antiproliferative and cytotoxic effects (Pope et al. 2001)</p>	<p>Calcineurin inhibitor interfering with T-cell activation and IL-2 production (Grinyo et al. 2004; Kobayashi et al. 2007; Tsuda et al. 2012)</p>
Effects in experimental PAH	<p>Not reported</p>	<p>Reverses PAH in MCT-induced rats (20 and 40 mg/kg). Does not affect T cells (Suzuki et al. 2006; Zheng et al. 2010)</p>	<p>Not reported</p>	<p>Shows beneficial effect in MCT-induced and hypoxia-induced PAH (Bonnet et al. 2007; Guignabert et al. 2006)</p>
Common dosage in inflammatory disorders	<p>1–2 mg/kg p.o. daily or 500–750 mg/m² iv. monthly (Jais et al. 2008)</p> <p>Lowest effective dose for long-term use (Jais et al. 2008)</p>	<p>500–1,500 mg p.o. twice daily (Contreras et al. 2004; Derk et al. 2009; Gerbino et al. 2008; Zamora et al. 2008)</p>	<p>10–25 mg p.o. or im. weekly (Pope et al. 2001; van den Hoogen et al. 1996; Yildirim-Toruner and Diamond 2011)</p>	<p>2.5–5 mg/kg p.o. daily (Clements et al. 1993; Filaci et al. 1999)</p>

Indications, use and clinical reports	Inflammatory manifestations of CTD and other inflammatory disorders (e.g., POEMS, sarcoidosis) (Bertsias et al. 2008; Coker 2007; Dispenzieri 2011; Kowal-Bielecka et al. 2009)	ILD related to SSC Neuropsychiatric involvement of SLE (Hoyles et al. 2006; Marder and McCune 2007; Nadashkevich et al. 2006; Nannini et al. 2008; Tashkin et al. 2006) Lupus nephritis (Barille-Fabris et al. 2005; Stojanovich et al. 2003)	ILD related to SSC (Gerbino et al. 2008; Zamora et al. 2008) SSs-skin manifestations (Derk et al. 2009) Induction therapy in lupus nephritis (Bertsias et al. 2008; Contreras et al. 2004)	Inflammatory arthritis/ myositis in many CTD Unsignificant efficacy on SSs-skin disease and lung function (Pope et al. 2001; van denHoogen et al. 1996) Skin manifestations of SLE (Yildirim-Toruner and Diamond 2011)	SSs-skin involvement (Clements et al. 1993; Filaci et al. 1999) Lupus nephritis : may be effective (Moroni, G, Nephrol Dial Transplant 2009) but not recommended (Bertsias G, Ann Rheum Dis 2008)
Effects in human PAH	Observational studies suggest that 40–50 % of SLE and MCTD-PAH may respond to a combination of CYC and glucocorticoids. (Jais et al. 2008; Ribeiro et al. 2001) Rare cases of POEMS syndrome have responded to steroids (Lesprit et al. 1998)	Observational studies suggest that 40–50 % of SLE and MCTD-PAH may respond to a combination of CYC and glucocorticoids (Jais et al. 2008; Ribeiro et al. 2001)	Not reported	One case of presumed iPAH reported clinical response to MTX coadministered with prednisone (Bellotto et al. 1999)	Not reported
Main side effects	Muscle weakness ^a (may limit physical exertion), hyperglycemia, anxiety, agitation, insomnia, hypokalemia ^a (may be aggravated by	Anemia ^a (may be poorly tolerated in patients with low cardiac output), risk of bleeding ^a in case of thrombocytopenia or hemorrhagic cystitis (may be	Gastrointestinal disturbance (may be relevant for patients treated with prostanoids whose frequently complaint of diarrhea) ^a , risk of	Rare cases of pneumonitis and pulmonary fibrosis Hepatotoxicity and elevated liver function test (may be confounding in	Renal toxicity Systemic hypertension Hirsutism, tremor Gum hyperplasia Increased risk of infection ^b (Bertsias et al. 2008; Denton

(continued)

Table 1 (continued)

	Glucocorticoids (prednisone, dexamethasone...)	Cyclophosphamide (CYC)	Mycophenolate mofetil (MMF)	Methotrexate (MTX)	Ciclosporin A (CsA)
	diuretics), fluid retention ^a (may simulate RV failure signs), weight gain ^a (may exacerbate dyspnea), may increase the risk of renal crisis in SSC patients, increased risk of infection ^b (Drugs.com 2010)	aggravated by warfarin), Infertility, nausea and vomiting, alopecia, increased risk of infection ^b (Drugs.com 2010)	bleeding in case of thrombocytopenia (may be aggravated by warfarin) ^a , leukopenia, increased risk of infection ^b (Derk et al. 2009; Drugs.com 2010)	patients treated with ERA) ^a Stomatitis Nausea and abdominal distress Pericardial effusion ^a Thrombocytopenia Increased risk of infection ^b (Drugs.com 2010)	et al. 1994; Kowal-Bielecka et al. 2009)
Significant interactions with specific PAH therapy and with warfarin	Not reported with PAH therapy Moderate interactions with warfarin (may increase or decrease the anticoagulant activity) ^a (Drugs.com 2010)	Not reported with PAH therapy Moderate interactions with warfarin (may increase or decrease the anticoagulant activity) ^a (Drugs.com 2010)	Not reported with PAH therapy No interactions with warfarin	The use of MTX in combination with potentially hepatotoxic agents (like ERA) is generally not recommended unless the potential benefit outweighs the risk ^a (Drugs.com 2010) No interactions with warfarin	ERA: Contraindication with bosentan ^a (Pharmaceuticals 2011; Venitz et al. 2012) (competitive inhibition of bosentan metabolism via CYP450 3A4 and/or inhibition of intestinal P-glycoprotein secretion by CsA). Ambrisentan dose should be limited to 5 mg ^a (Spence et al. 2010) (CsA inhibition of P-glycoprotein efflux

transporter and organic anion transporting polypeptide, of which ambrisentan is a substrate)

PDE5i: Coadministration with CsA (inhibitors of CYP450 3A4) may increase the plasma concentrations of *PDE5i* (Schwartz and Kloner 2010) (Sildenafil or Tadalafil), dosage adjustments may be appropriate.^a

Moderate interactions with *warfarin* (may increase or decrease the anticoagulant activity)^b (Drugs.com 2010)

PAH pulmonary arterial hypertension; *MCT* monocrotaline; *CTD* connective tissue disease; *POEMS* polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes; *ILD* interstitial lung disease; *SSc* systemic sclerosis; *SLE* systemic lupus erythematosus; *MCTD* mixed CTD; *iPAH* idiopathic PAH; *RV* right ventricular; *ERA* endothelin receptor antagonist; *CYP* cytochrome; *PDE5i* phosphodiesterase type 5 inhibitors

^aOf particular interest in PAH because of disease pathophysiology or drug interaction

^bParticularly of concern for patients with a central venous line for epoprostenol infusion

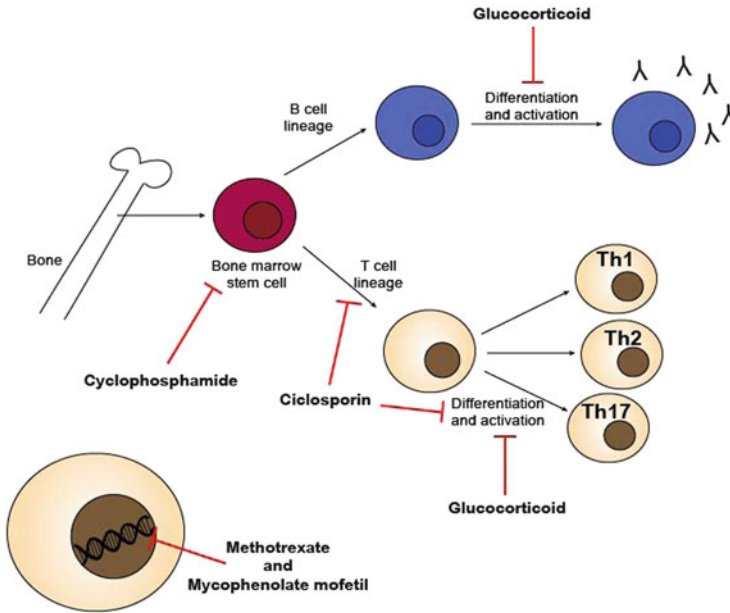


Fig. 2 Mode of action of different immunosuppressive agents as described in text. Cyclophosphamide targets bone marrow stem cells and inhibits their differentiation into B and T cells. Glucocorticoids target B and T cell differentiation and activation. Cyclosporin has a more T cell-specific mode of action as it inhibits differentiation of T cells into Th1, Th2, and Th17. Other molecules such as methotrexate and mycophenolate mofetil target DNA and RNA synthesis, thus blocking proliferation

also inhibit the two main products of inflammation: prostaglandins synthesis at the phospholipase A2 level as well as leukotrienes synthesis at the cyclooxygenase/PGE isomerase level (Goppelt-Struebe et al. 1989). Glucocorticoids are thus potent anti-inflammatory agents, regardless of the inflammation's cause. Therefore, glucocorticoids are often recommended by experts for the treatment of inflammatory manifestations of CTD (Bertsias et al. 2008; Kowal-Bielecka et al. 2009) as well as other inflammatory disorders like POEMS syndrome (Dispenzieri 2011) and sarcoidosis (Coker 2007). Some experimental data also support that glucocorticoids (prednisolone) have an antiproliferative effect on cultured PSMC from patients with iPAH (Ogawa et al. 2005).

5.2 Cyclophosphamide

Cyclophosphamide (CYC) is an alkylating agent used in chemotherapy to slow or stop cell growth. It alkylates or binds to DNA, cross-linking DNA, and RNA strands, thus inhibiting protein synthesis. These actions are neither cell cycle specific nor cell type specific. CYC also exerts its anti-inflammatory function

through direct cytotoxicity of bone marrow precursors and mature lymphocytes (Fig. 2), leading to a reduction of T and B cells as well as the CD4:CD8 T cell ratio (Manno and Boin 2010; Marder and McCune 2007; McCune et al. 1988), but depending on the dose used, it can also act on Treg and in this case stimulate the immune system (Brode and Cooke 2008). CYC is currently used, most commonly with concomitant corticosteroids, to treat some severe manifestations of CTD, including in interstitial lung disease related to SSc (Hoyles et al. 2006; Marder and McCune 2007; Nadashkevich et al. 2006; Nannini et al. 2008; Tashkin et al. 2006) and neuropsychiatric involvement related to lupus (Barile-Fabris et al. 2005; Stojanovich et al. 2003). Because of many side effects of CYC, however, other immunosuppressive agents are generally preferred for less severe complications of CTD or for maintaining remission.

5.3 *Mycophenolate Mofetil*

Mycophenolate mofetil (MMF) is an immunosuppressive drug with antiproliferative effects on inflammatory cells through the inhibition of the 5'-monophosphate dehydrogenase. This enzyme is involved in purines synthesis, an essential step for DNA synthesis in lymphocytes (Fig. 2). This agent is mainly used following organ transplantation. MMF has also been shown to be as effective and better tolerated than CYC for the treatment of lupus nephritis (Bertsias et al. 2008; Contreras et al. 2004). In addition, small series have suggested moderate benefit for interstitial lung disease (Gerbino et al. 2008; Zamora et al. 2008) and skin score (Derk et al. 2009) in SSc.

5.4 *Methotrexate*

Methotrexate (MTX) is a folic acid analogue and a potent competitive inhibitor of dihydrofolate reductase. It inhibits both DNA and RNA synthesis (Fig. 2). MTX is frequently used in SSc to treat inflammatory arthritis and myositis. Its efficiency for skin disease and interstitial lung disease is however limited (Pope et al. 2001; van den Hoogen et al. 1996). Similarly, MTX has an uncontested role in the management of arthritis and skin manifestations of SLE (Yildirim-Toruner and Diamond 2011), although it has no role for major organ involvement related to SLE (Carneiro and Sato 1999).

5.5 *Ciclosporin*

Ciclosporin A (CsA) is frequently used for Th1-, Th2-, and Th17-related disorders. It exerts its immunosuppressive function by interfering with T-cell production as

well as Th differentiation and function (Grinyo et al. 2004; Kobayashi et al. 2007) (Fig. 2). CsA inhibits calcium synergic action and suppresses the calcineurin pathway, although the precise pharmacological mechanisms of CsA have not yet been fully elucidated (Tsuda et al. 2012). Calcineurin is a key molecule for the nuclear factor for activated T cells (NFAT) activation, which is the main transcription factor for IL-2. Additional anti-fibrotic effects as well as efficiency in chronic graft-versus-host disease have prompted consideration for SSc treatment. In a small open-label study, CsA was associated with a 36 % decrease in skin scores (Clements et al. 1993). Similar improvements were demonstrated in a small randomized trial testing CsA in combination with iloprost (Filaci et al. 1999). However, side effects like systemic hypertension and renal toxicity (Denton et al. 1994) have limited the clinical development of this calcineurin inhibitor in the spectrum of SSc, and this molecule is not currently recommended as first line immunosuppressive agent in SSc (Kowal-Bielecka et al. 2009). Similarly, CsA is not recommended for the management of SLE whatever its manifestations (Bertsias et al. 2008).

5.6 Anti-infectious Agents

5.6.1 Highly Active Antiretroviral Therapy in HIV-Related PAH

Considering on one hand the absence of apparent correlation between the stage of HIV infection, the degree of immunodeficiency, the CD4⁺ T lymphocytes counts, and the occurrence or severity of HIV-PAH (Mehta et al. 2000; Nunes et al. 2003) and, on the other hand, the heterogeneous evolution of HIV-PAH following antiretroviral therapy initiation, experts do not recommend systematic HAART initiation in case of HIV-PAH (Galie et al. 2009; Hammer et al. 2008). In fact, a large majority of patients are diagnosed for HIV-PAH while already on HAART. Nevertheless, a stable prevalence of HIV-PAH overtime despite HIV patients live much longer than in the 1980s suggest HAART may be associated with a lower incidence of PAH among HIV-infected patients (Opravil and Sereni 2008; Sitbon et al. 2008).

5.6.2 Antihelminthic Treatment in Schistosomiasis-Related PAH

Prazicantel is the most widely used antihelminthic drug to treat schistosomiasis in humans. It is a pyrazinoisoquinoline derivative only active against the adult worms (Ross et al. 2002). In mice, hepatic fibrosis and lung granuloma formation has been shown to be partly reversed by prazicantel and other antihelminthic treatment (de Almeida and Andrade 1983). In a murine model, prazicantel also reversed pulmonary vascular remodeling and prevented the development of schistosomiasis-related PAH in association with a reduction of lung mRNA expression of cytokines IL-13, IL-8, and IL-4 (Crosby et al. 2011). In humans, prazicantel is usually given

to prevent further progression of the disease, even if pulmonary embolism composed of dead worms may follow this specific treatment and lead to a superimposed acute cor pulmonale (Lambertucci et al. 2000). However, antischistosomal therapy has not demonstrated any effect on pulmonary hemodynamics in schistosomiasis-related PAH, despite one case report documenting remission of PAH after prazicantel treatment (Bouree et al. 1990). New therapeutic strategies potentially targeting the inflammatory component of the disease (e.g., IL-13 antagonists) are thus needed for schistosomiasis-related PAH (Fernandes et al. 2011).

6 Immunosuppressive Agents in Experimental PH

6.1 *Presence of Inflammation in Different PH Experimental Models*

To study PH pathobiology, three different models are commonly used: hypoxia model, monocrotaline-induced model (MCT) and the newly developed Sugen-induced model (SU5416, a VEGF receptor inhibitor associated to chronic hypoxia) (Ryan et al. 2011). Other models [e.g., transgenic mice overexpressing 5-HTT specifically in PASM and fawn-hooded rat are also used but at lesser extent (Guignabert et al. 2006)]. The hypoxia model develops mild to moderate PH with modest inflammation. Conversely, the MCT model develops significant inflammation with higher cytokine levels and marked inflammatory cells infiltrates in remodeled vascular wall, with inflammatory cells displaying a strong IL-6 expression (Bhargava et al. 1999; Price et al. 2011). In addition to these changes, the Sugen model shows plexiform-like lesions and, as in humans, many inflammatory cells are present in these vascular lesions. All these models show an increase in T and B cells, macrophages, mast cells, monocytes, and dendritic cells.

In these experimental models, inflammatory cells are not only innocent bystanders of the pulmonary vascular remodeling but also influence its development. For instance, absence of T cells increases vascular remodeling and worsens PH in the Sugen model (Taraseviciene-Stewart et al. 2007). Similarly, euthymic rat develop severe PH when submitted to both SU5416 injection and chronic hypoxia, whereas athymic rat develop severe vascular remodeling even in normoxia. In athymic rats, pulmonary arterioles become occluded by proliferating EC and are surrounded by mast cells, B cells, and macrophages. IL-4, proliferating cell nuclear antigen, and collagen type I levels are also markedly increased. These studies demonstrate the complex interactions between the immune system, the VEGF receptor signaling pathway, and the pulmonary vascular remodeling process. As a result, different immunosuppressive treatments have been tested in experimental PH.

6.2 *Glucocorticoids*

Some experimental data support that glucocorticoid treatments (with prednisolone) have an antiproliferative effect on PASMC isolated from iPAH patients (Ogawa et al. 2005). While PDGF induces iPAH-PASMC proliferation and migration, prednisolone reverses this effect in a dose-dependent manner (Ogawa et al. 2005). Very high dose of prednisolone thus caused cell cycle arrest of PASMCs with subsequent suppression of proliferation, whereas prednisolone has no effect on PASMC from controls.

Dexamethasone treatment (5 mg/kg/day) also significantly improved survival in a rat model of in MCT-induced PH (Price et al. 2011; Wang et al. 2011). Furthermore, Price et al. (2011) demonstrated that dexamethasone not only prevented but also normalized hemodynamics and right ventricular hypertrophy in a dose-dependent manner in established PH. At the vascular level, dexamethasone reversed medial and adventitial thickening, reduced MCT-induced adventitial infiltration of IL-6-expressing inflammatory cells (Price et al. 2011), inhibited pulmonary IL-6 overexpression, reduced CX3CR1 expression (Wang et al. 2011), suppressed perivascular CD8⁺ T cells, and restored the EC integrity (Wang et al. 2011).

6.3 *Cyclophosphamide*

No animal data was found.

6.4 *Mycophenolate Mofetil*

In the past decade, different groups demonstrated the beneficial effects of MMF (20–40 mg/kg/day), a prodrug of mycophenolic acid, on MCT-induced PH (Suzuki et al. 2006; Zheng et al. 2010). MMF decreased PASMC proliferation in both MCT-induced PH and in vitro (with fibroblasts to stimulate proliferation) in a dose-dependent manner (Zheng et al. 2010). MMF was also associated with a decrease in macrophages and IL-6 production in the pulmonary vasculature (Suzuki et al. 2006), whereas T cells or mast cells were unchanged (Zheng et al. 2010). From a hemodynamic point of view, MMF decreased RV systolic pressure and hypertrophy. These beneficial effects were seen with MMF concentrations within clinical applicable range, suggesting potentiality of MMF in the treatment of human PAH (Zheng et al. 2010).

6.5 *Methotrexate*

No animal data was found.

6.6 *Ciclosporin*

Koulmann et al. (2006) suggested that CsA treatment inhibits PH and RV hypertrophy either by inhibiting HIF-1 transcriptional activity in lung, by decreasing calcineurin activity in lung and heart, by direct effects of CsA, or by a combination of these factors. The CsA dose used in this study (25 mg/kg/day) is much higher than in more recent studies. The exact mechanism by which CsA inhibits PH has been determined more recently. Indeed, Bonnet et al. (2007) demonstrated that NFAT was overexpressed both in human and experimental PH and was largely involved in the PH pathobiology. NFATc2 was in fact mostly activated in iPAH PASMCMC (Bonnet et al. 2007). By different techniques, they provided evidence that the inhibition of NFAT using a specific [VIVIT (Aramburu et al. 1999)] or an indirect inhibitor (CsA) had beneficial effect on experimental (in vitro and in vivo) PH (Bonnet et al. 2007). Both CsA and VIVIT decreased PASMCMC proliferation and restored apoptosis levels in vitro. In established MCT-induced PH, CsA (1 mg/kg, similar to doses used clinically) conferred beneficial effects on hemodynamics, RV hypertrophy, and vascular remodeling (Bonnet et al. 2007). In a PH model of mice overexpressing 5-HTT (Guignabert et al. 2006), CsA decreased the pulmonary expression of NFAT and increased Kv1.5 protein levels but did not affect PH itself (mean PA pressure for example) (Guignabert et al. 2006). These results may be explained by the fact that inflammation is not an important component of SM22-5-HTT⁺ PH mice model (Macian 2005). Whether NFAT inhibition using indirect (e.g., CsA) or specific (e.g., VIVIT) inhibitors is effective in human PH or RV hypertrophy and failure remains unknown (McKinsey and Olson 2005).

6.7 *Dehydroepiandrosterone*

Our team demonstrated that dehydroepiandrosterone (DHEA) confers beneficial effects on PH not only due to its vasodilator effects but also by disrupting the IL-6/STAT3/NFAT axis known to potentiate PH (Paulin et al. 2011). In vitro data demonstrated that DHEA treatment inhibits IL-6-induced PASMCMC proliferation. By doing so, it confers anti-inflammatory effects. In MCT-induced PH, DHEA therapy also reverses PH, as assessed by mean pulmonary arterial pressure, right ventricular hypertrophy, and vascular wall thickness.

6.8 *Etanercept*

Tumor necrosis factor alpha (TNF- α) is a key proinflammatory cytokine that is increased in human and experimental PH (Stojanovich et al. 2003). Furthermore, TNF transgenic rats overexpressing TNF- α develop PH demonstrating its implication in PH pathogenesis (Fujita et al. 2001). Etanercept is a recombinant TNF- α receptor which binds to circulating TNF- α and functionally inhibits its action by blocking binding to cell membrane receptors, thus weakening its pro-inflammatory action (Zhou 2005). In MCT-induced PH, etanercept administered at clinically relevant doses (Lovell et al. 2000) at the same time as MCT injection (in prevention) or 3 weeks later of when PH was established (as a reversal therapy) decreased PH severity and RV hypertrophy (Sutendra et al. 2011). Since TNF- α expression is driven by NFAT in T cells, they confirmed that NFAT activation led to increased levels of TNF- α in an autocrine manner in PASMC. Furthermore, NFAT activation (and its downstream target Kv1.5) was similarly decreased by etanercept and VIVIT, a specific NFAT inhibitor. MCT-PH rats treated with etanercept also demonstrated lower TNF- α and IL-6 levels (another NFAT target) and decreased CD8⁺ T cells (Sutendra et al. 2011). Etanercept also reduced PH in a late endotoxin-induced model in pigs (Mutschler et al. 2006). Conversely, etanercept at a significantly lower dose had a nonsignificant effect on RV systolic pressure in MCT-induced PH (Henriques-Coelho et al. 2008).

6.9 *Sirolimus*

Sirolimus (or rapamycin) is an immunosuppressive drug preventing T cells and B cells activation by blocking their response to IL-2. It also blocks the VEGF pathway. Unlike tacrolimus and CsA, sirolimus is not a calcineurin inhibitor but has similar effects on the immune system. While sirolimus may prevent the development of MCT-induced PH, it is ineffective in reversing established PH (McMurtry et al. 2007; Nishimura et al. 2001).

7 Immunosuppressive Agents in Human PAH

Even if preclinical data show some beneficial effects, therapies targeting inflammation have not been formally tested in human PAH. Nevertheless, anti-inflammatory/immunosuppressive therapies are sometimes cautionary recommended by experts in exceptional circumstances. These pharmacological agents, their reported effects on inflammatory disorders and PAH, as well as their potential adverse effects and drug interactions with current PAH therapies are summarized Table 1.

7.1 Updated Experiment of Immunosuppressive Agents in Human PAH

7.1.1 Idiopathic PAH

Rare case reports suggested immunosuppressive therapy could be effective in iPAH. In 1999, Bellotto et al. described the case of an iPAH patient with sustained clinical and hemodynamic improvement following the initiation of prednisone and MTX (Bellotto et al. 1999). Recently, Ogawa et al. (2011) reported the case of a 34-year-old female with iPAH treated with epoprostenol for 3 years, who significantly improved after prednisolone was prescribed for concomitant idiopathic thrombocytopenia. Importantly, both cases of presumed iPAH were characterized by concomitant inflammatory disorders. Apart from these rare cases, no data support the use of immunosuppressive therapy in iPAH.

7.1.2 PAH Related to CTD

According to the EULAR Scleroderma Trials and Research database, PAH is a leading cause of SSc-related deaths even in the era of modern PAH therapies (Mathai and Hassoun 2011; Tyndall et al. 2010). Current guidelines recommend SSc-PAH should follow the same treatment algorithm as in iPAH (Galie et al. 2009). To date, the efficacy of immunosuppressive therapy in SSc-PAH is not supported by observational studies. Indeed, Sanchez et al. (2006) reported that, among a retrospective cohort of 28 CTD-PAH patients, none of the six SSc-PAH responded (defined as patients remaining in New York Heart Association functional class I or II with sustained hemodynamic improvement after at least 1 year without the addition of PAH specific therapy) to monthly cyclophosphamide and glucocorticoids alone. More pronounced fibrotic vascular disease and presence of major comorbidities are proposed reasons to explain the absence of response to immunotherapy in SSc-PAH (Le Pavec et al. 2011). Conversely, cases reported before the advent of specific therapies suggested a positive impact of immunosuppressive therapy on the SLE-PAH course (Groen et al. 1993; Kawaguchi et al. 1998; Morelli et al. 1993; Pines et al. 1982). In the same retrospective study from Sanchez et al. (2006), 5 of the 12 (42 %) SLE-PAH were considered as responder under immunosuppressive therapy alone. Later and from the same group, Jais et al. (2008) reported similar findings, with half of the SLE-PAH patients responding clinically and hemodynamically to immunosuppressive therapy. Patients with less severe PAH at the time of diagnosis were more likely to benefit from immunosuppressive therapy. Normalization of hemodynamic parameters was even observed in some patients with SLE-PAH who received immunosuppressive therapy either alone or in combination with PAH-specific treatment (Heresi and Minai 2007; Jais et al. 2008; Ribeiro et al. 2001). Clinical and hemodynamic improvements have also been observed, although less frequently, in patients with PAH associated with

mixed CTD (Sanchez et al. 2006). More recently, immunosuppression added to specific PAH therapy has been reported effective in a heterogeneous cohort of 13 CTD-PAH as compared to a historical group of 8 CTD-PAH receiving specific PAH therapy alone (Minai 2009). Unfortunately, repartition of CTD subtypes was different between the two groups (more SLE and less SSc in the “combination” group) limiting the interpretation of these results. Despite the limited data supporting the efficacy of immunosuppressive therapy, current guidelines suggest immunosuppression, most commonly in combination with PAH-specific agents, may be considered in SLE-PAH or mixed CTD-PAH (Jais et al. 2008; Ribeiro et al. 2001).

7.1.3 POEMS Syndrome-Related PAH

POEMS syndrome has been occasionally associated with PAH. Recently, Jouve et al. (2007) described two cases of PAH related to POEMS syndrome, including one case ascribed to PAH and one case of post-capillary/high cardiac output PAH. In any case, clinical symptoms disappear and hemodynamic study showed a significant improvement in both patients (Jouve et al. 2007). Mukerjee et al. (2003a) also reported a single case of reversible POEMS-PAH after sequential therapy including initial iloprost therapy followed by immunosuppressive therapy. Finally, among 20 cases of POEMS syndrome, 5 cases of PAH were reported during the follow-up (Lesprit et al. 1998). Of note, however, PAH was confirmed by catheterization in only two of these patients. Overproduction IL-1 β , IL6, TNF- α , and VEGF was found in all cases, suggesting that cytokines may mediate the development of PAH in the POEMS syndrome. However, only two of these five patients presented a sustained improvement under immunosuppressive therapy (Lesprit et al. 1998). Results about the benefit of anti-VEGF were also conflicting (Dispenzieri 2011).

7.2 *Side Effects and Drug–Drug Interactions of Immunosuppressive Agents with PAH-Targeted Therapies*

Potential side effects and drug–drug interactions were detected using the side effect and drug interactions checker within <http://www.drugs.com> database (2010). Few data are currently available about the safety profile of immunosuppressive drugs in the setting of PAH. Nevertheless, among the usual side effects of immunosuppressive therapies (Table 1), some are particularly of concern in the setting of PAH. For example, glucocorticoids promote hypokalemia, which may be aggravated by diuretics and may be associated with an increased risk of arrhythmias. CYC and MMF may reduce red blood cells and platelet production. Anemia may be poorly tolerated, especially in PAH patients with low cardiac output. Patients treated with

warfarin may also be at increased risk of bleeding in case of thrombocytopenia or CYC-related hemorrhagic cystitis. MMF may also worsen the gastrointestinal disturbance associated with prostanoid therapy. More widely, it is important to keep in mind that all immunosuppressive therapy increases risk of infection. This additional issue should be taken into consideration in the management of PAH patients, especially in those with a central venous line for epoprostenol infusion.

The main concern about drug–drug interaction relates to the cytochrome P450 metabolite pathway. Indeed, both bosentan and CsA are metabolized through the CYP450 3A4 isoenzyme. Coadministration of CsA and bosentan is contraindicated due to significant increased bosentan exposure and decreased CsA exposure (Pharmaceuticals 2011; Venitz et al. 2012). Clinicians should also be cautious if tacrolimus (another calcineurin inhibitor) and bosentan are used together. Conversely, coadministration of ambrisentan and CsA or tacrolimus is not contraindicated, although it is recommended to limit the ambrisentan dose to 5 mg once daily (Spence et al. 2010). Limited data are available for phosphodiesterase 5 inhibitors, which are also primarily metabolized by the CYP450 3A4 pathway (Schwartz and Kloner 2010). Therefore, coadministration with inhibitors of CYP450 3A4 may increase plasma concentrations of sildenafil or tadalafil, and possibility of prolonged and/or increased pharmacologic effects of these drugs should be considered. A Spanish renal transplant study was reassuring as sildenafil therapy was safe for the treatment of erectile dysfunction and did not modify CsA and tacrolimus blood levels (Cofan et al. 2002). MMF is not expected to compete with ambrisentan metabolism (Mandagere et al. 2010). Similarly, CYC is not expected to interact with specific PAH treatments because of its multiple metabolic pathways. Finally, no specific data are available concerning immunosuppressive therapy interactions with prostanoids agents.

8 Conclusion

Inflammation is undoubtedly part of the PAH pathobiology. Giving the poor outcomes in PAH despite recently developed treatments, there is an urgent need for new therapies targeting novel pathophysiological pathways. In many animal models of PAH, targeting the inflammation by current or new immunosuppressive agents improves PAH and pulmonary vascular remodeling. Treating the underlying inflammatory condition in human PAH associated with inflammatory disorders such as SLE, mixed CTD, and POEMS syndrome also led, in some cases, to marked clinical and hemodynamic improvements. Nevertheless, further studies are needed to characterize the elusive mechanisms by which inflammation triggers and sustains vascular remodeling and likely RV failure. More importantly, the poor safety profile of existing immunosuppressive drugs and the potential for drug–drug interactions with current PAH therapies mandate that appropriate large-scaled randomized trials are performed before immunosuppression becomes standard of care in any PAH type.

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Vasoactive Peptides and the Pathogenesis of Pulmonary Hypertension: Role and Potential Therapeutic Application

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Abstract Pulmonary hypertension (PH) is a debilitating disease with a dismal prognosis. Recent advances in therapy (e.g. prostacyclin analogues, endothelin receptor antagonists and phosphodiesterase 5 inhibitors), whilst significantly improving survival, simply delay the inexorable progression of the disease. An array of endogenous vasoconstrictors and vasodilators coordinates to maintain pulmonary vascular homeostasis and morphological integrity, and an imbalance in the expression and function of these mediators precipitates PH and related lung diseases. The vasodilator peptides, including natriuretic peptides, vasoactive intestinal peptide, calcitonin gene-related peptide and adrenomedullin, trigger the production of cyclic nucleotides (e.g. cGMP and cAMP) in many pulmonary cell types, which in tandem exert a multifaceted protection against the pathogenesis of PH, encompassing vasodilatation, inhibition of vascular smooth muscle proliferation, anti-inflammatory and anti-fibrotic effects and salutary actions on the right ventricle. This coordinated beneficial activity underpins a contemporary perception that to advance treatment of PH it is necessary to offset multiple disease mechanisms (i.e. the pulmonary vasoconstriction, pulmonary vascular remodelling, right ventricular dysfunction). Thus, there is considerable potential for harnessing the favourable activity of peptide mediators to offer a novel, efficacious therapeutic approach in PH.

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1 Introduction

Pulmonary hypertension (PH) is a multifactorial, progressive disease characterised by increased pulmonary vascular resistance (PVR), remodelling of the pulmonary small arteries, right ventricular hypertrophy (RVH) and ultimately right ventricular failure. The disease carries a substantial morbidity and mortality; even with the introduction of prostacyclin analogues (Barst et al. 1996), endothelin receptor antagonists (Channick et al. 2001) and phosphodiesterase 5 inhibitors (PDE5i) (Galie et al. 2005b), there remains no cure and clinical worsening is merely delayed, not prevented, by therapy (McLaughlin et al. 2009a, b). Advances in the understanding of the disparate aetiologies that underpin different forms of PH [for WHO classification please refer to (Simonneau et al. 2009)] have highlighted new signalling pathways and potentially novel therapeutic strategies that may improve the treatment of patients with the disease. One such area, in which considerable progress has been made in the past decade, is that of polypeptide hormones and paracrine mediators and their contribution to both pulmonary physiology and disease, particularly in the context of PH. These low molecular weight signalling peptides exert a multitude of activities that coordinate to maintain pulmonary vascular homeostasis and lung architectural integrity; moreover, dysfunctional bioactivity of these peptides also appears to underlie many aspect of PH

pathogenesis. This overview provides an account of this family of pulmonary active peptides, with focus on those with vasodilator activity, and the potential for targeting these mediators therapeutically to improve management of this debilitating disorder with an extremely poor prognosis.

2 Peptides and Pulmonary Homeostasis

A plethora of systemic and lung-specific peptides are now recognised to play a role in pulmonary health and disease. PH is a disease of the small pulmonary arteries defined by an abnormal increase in pulmonary artery pressure (PAP) and PVR, which are primarily regulated locally within the lung, and influenced by many endogenous agents, both vasoconstrictors and vasodilators. Indeed, it is likely that an imbalance between the endogenous production and activity of vasoconstrictor and vasorelaxant polypeptides contributes significantly to the haemodynamic aberrations characteristic of PH. This is perhaps best exemplified by the excessive expression, release and actions of endothelin-1 (ET-1), but other endogenous vasoconstrictor peptides have been implicated in lung (patho)physiology including angiotensin II (Ang II) and somatostatin-28 (SS-28). This family will be considered briefly below, but the main focus herein will be to describe the key function played by vasodilator peptides in governing pulmonary vascular and structural integrity and how aberrant activity of this cohort of peptides, including vasoactive intestinal peptide (VIP), natriuretic peptides (NP), pituitary adenylate cyclase activating peptides (PACAP), adrenomedullin (AM), calcitonin gene-related peptide (CGRP) and somatostatin-14 (SS-14), might lead to the development of PH. Moreover, consideration will be given to the possibility of therapeutic interventions targeted to these vasodilator peptides to advance therapy in this area of unmet medical need.

3 Vasoconstrictor Peptides

3.1 *Endothelin-1*

Endothelin-1 (ET-1) is the major cardiovascular isoform of the endothelin family, a 21 amino acid (aa) peptide with potent vasoconstrictor activity in most vascular beds (Yanagisawa et al. 1988). The gene product, prepro-ET, is cleaved by an endothelin converting enzyme (ECE) into big ET and subsequently to the biologically active ET-1 (Webb 1991). The biological activity of ET-1 is exerted via stimulation of two G-protein-coupled receptors; ET_A receptors on vascular smooth muscle cells cause vasoconstriction and proliferation, while ET_B receptors on

endothelial cells stimulate NO and prostacyclin release (and clear ET-1 from the circulation), but on vascular smooth muscle cells induce vasoconstriction and mitogenesis. Plasma levels of ET-1 are significantly elevated and correlate with disease severity in experimental models and patients with PH (Rubens et al. 2001), and pulmonary expression of ET_A is also upregulated in the diseased lung (Motte et al. 2006). Experimental models of PH validated the use of endothelin receptor antagonists (ERAs), including bosentan (dual ET_A/ET_B), ambrisentan (ET_A > ET_B) and sitaxsentan (ET_{1A} ≫ ET_B), to treat PH (Chen et al. 1995; Iglarz et al. 2008; Ivy et al. 1997; Raja 2010; Sato et al. 1995; Underwood et al. 1998; Yuan et al. 2006). This efficacy has translated to the clinical arena where ERAs have been shown to improve pulmonary haemodynamics and exercise capacity (Barst et al. 2004; Channick et al. 2001; Galie et al. 2005a; Williamson et al. 2000). However, it remains to be determined if ERAs produce a positive effect on survival and whether selective ET_A receptor antagonists hold any clinical advantage over mixed receptor blockers (since inhibition of ET_B prevents the release of NO from the vascular endothelium and slows clearance of circulating ET-1, both potentially adverse actions).

3.2 Angiotensin II

The lung is the principal site of conversion of angiotensin I to Ang II under the influence of angiotensin converting enzyme (ACE) found in caveolae on the pulmonary vascular endothelium (Ryan et al. 1975); it is not surprising therefore that Ang II has been reported to contribute to the development of PH. For example, activation of the angiotensin receptor 1 (AT1R) leads to vasoconstriction, vascular smooth muscle proliferation, fibrosis, production of reactive oxygen species and inflammation in the lung, all processes thought to precipitate PH (Cargill and Lipworth 1995b; Morrell et al. 1995). Moreover, AT1R blockade and inhibition of ACE reduce the severity of PH in experimental hypoxia models (Morrell et al. 1995; Nong et al. 1996; Zhao et al. 1996a). Finally, single nucleotide polymorphisms (SNPs) in the angiotensinogen (precursor peptide) and ACE genes are associated with susceptibility to PH (Kanazawa et al. 2000; Solari and Puri 2004). Intriguingly, the recently identified ACE2, which hydrolyses Ang II to the biologically active fragment angiotensin₁₋₇ (Ang₁₋₇), appears to exert an opposing, cytoprotective effect in PH. Both genetic and pharmacological interventions targeted to this isoform of ACE appear to worsen the pathogenesis of experimental PH, suggesting that Ang₁₋₇ is an endogenous protective pathway (possibly via activation of the *Mas* receptor) (Ferreira et al. 2009; Shenoy et al. 2010; Yamazato et al. 2009).

while NPR-C binds with high affinity to all three NPs (Bennett et al. 1991; Koller et al. 1991; Koller and Goeddel 1992).

4.1.2 Natriuretic Peptide Receptors

NPR-A and NPR-B are guanylate cyclase coupled receptors and facilitate the conversion of guanosine-5'-triphosphate to the second messenger cyclic guanosine-3',5'-monophosphate (cGMP), which governs cellular function via activation of specific ion channels, kinases and phosphodiesterases (Hobbs 1997).

The downstream effects of NPR-A stimulated cGMP formation are mediated by the same protein kinases, phosphodiesterases and channel proteins as nitric oxide (NO) and therefore exert complementary functions. NPR-A acts to reduce cardiac load, stimulating natriuresis, vasodilatation, diuresis, endothelial permeability and inhibition of the renin-angiotensin pathway (Dubois et al. 2000; Kishimoto et al. 1996; Lopez et al. 1997). The receptor also exerts a direct inhibitory effect on cardiac growth; mice lacking the NPR-A receptor are mildly hypertensive at rest, but show enhanced basal cardiac hypertrophy and fibrosis disproportionate to the systemic hypertension observed (Holtwick et al. 2003; Kishimoto et al. 2009; Kuhn et al. 2002). NPR-B is preferentially activated by CNP and is abundantly expressed in the brain, lung, bone and heart (Koller et al. 1991; Potter 2011).

NPR-B-mediated CNP signalling is essential for normal bone growth (Tamura et al. 2004). Inactivation of NPR-B causes dwarfism and early death (Chusho et al. 2001; Yasoda et al. 1998) and targeted overexpression of CNP can counteract dwarfism in a mouse model of achondroplasia (Yasoda et al. 2004). However, NPR-B activity is also critical to cardiovascular homeostasis, particularly with respect to cardiac integrity. For example, CNP produces anti-proliferative and anti-hypertrophic effects through NPR-B activation (Dickey et al. 2007; Qvigstad et al. 2010; Tokudome et al. 2004). Rats with a dominant negative NPR-B Δ KC mutation display progressive, pressure-dependent cardiac hypertrophy which is exacerbated by volume overload (Langenickel et al. 2006; Pagel-Langenickel et al. 2007).

The third receptor, NPR-C, is homologous to NPR-A and NPR-B but devoid of guanylyl cyclase activity. This NPR acts as a clearance receptor as binding of NPs to NPR-C leads to internalisation and lysosomal degradation (Maack et al. 1987). However, it has also been demonstrated that NPR-C contains a 37 aa cytoplasmic domain with a G α inhibitory protein-activating sequence and can couple to G $_i$ /G $_o$ -proteins to inhibit adenylate cyclase activity and activate phospholipase-C β 3 (Anand-Srivastava et al. 1996). We have also shown CNP/NPR-C signalling as a key component of EDHF through the opening of G-protein-coupled inwardly rectifying K $^+$ channels (GIRK) (Chauhan et al. 2003; Hobbs et al. 2004; Villar et al. 2007). NPs are also degraded by neutral endopeptidase enzymes (and other peptidases including dipeptidyl peptidase and insulin degrading enzyme), which are found in high concentrations on the luminal surface of endothelial and epithelial cells (Roques 1998; Soleilhac et al. 1992); these are discussed further below.

4.1.3 Natriuretic Peptides and Pulmonary Hypertension

NPs and their cognate receptors have been localised to many areas of the lung, including peripheral lung tissues, airway epithelium and pulmonary circulation (Bianchi et al. 1985; Gutkowska et al. 1987; Gutkowska and Nemer 1989; Sakamoto et al. 1986; Tallerico-Melnyk et al. 1992; Toshimori et al. 1988). Accordingly, the natriuretic peptide axis has been reported to play a key role in lung physiology, regulating several processes including vasodilatation, bronchorelaxation, pulmonary permeability and surfactant production (Hulks et al. 1990; Perreault and Gutkowska 1995). ANP is a potent relaxant of pulmonary arteries (significantly greater than the systemic vasculature) (Angus et al. 1993; Baliga et al. 2008) and a potent bronchodilator in a number of species (Gutkowska et al. 1987; Gutkowska and Nemer 1989; Hulks et al. 1990). This is perhaps not surprising since we were the first to identify this pivotal role of cGMP-mediated control, i.e. the non-adrenergic, non-cholinergic (NANC) innervation of bronchial smooth muscle triggers the NO–cGMP pathway to govern airway tone (Tucker et al. 1990). Indeed, the bronchorelaxant activity of NPs in the lung may be dependent, at least in part, on NO release from the airway epithelium (Matera et al. 2011; Mohapatra et al. 2004).

Evidence for the importance of endogenous NPs in modulating the development of PH is demonstrated by the exacerbated PH and pulmonary fibrosis in numerous transgenic animals with aberrant natriuretic peptide signalling, including ANP (*Nppa*^{-/-}) and NPR-A (*Npr1*^{-/-}) mice (Chen et al. 2006; Klinger et al. 1999, 2002; Zhao et al. 1999). Similarly, chronic blockade of endogenous ANP by infusion of monoclonal antibodies stimulates the development of PH (Raffestin et al. 1992), while exogenously administered or adenovirus-mediated natriuretic peptide supplementation protects against PH and the accompanying RVH (Chen et al. 2006; Hill et al. 1994; Jin et al. 1990, 1988; Klinger et al. 1993; Louzier et al. 2001). Administration of urodilatin, an elongated ANP molecule thought to be expressed exclusively in the kidney, also reduces several indices of severity in experimental PH (Schermyly et al. 2001). Infusion of NPs has also been evaluated in small clinical studies involving healthy volunteers and patients with PH. For instance, ANP reduces the pulmonary vascular permeability index in patients with pulmonary oedema (Sakamoto et al. 2010), counteracts Ang II-mediated vasoconstriction in healthy subjects (Cargill and Lipworth 1995b) and reduces pulmonary pressure in altitude-induced PH (Cargill and Lipworth 1995a; Liu et al. 1989) and *cor pulmonale* (Cargill and Lipworth 1996). BNP administration also acutely reverses the pulmonary vasoconstriction in PH patients (Klinger et al. 2005, 2006). Natriuretic peptide receptors are also highly expressed in the lung. During hypoxia and PH, NPR-C mRNA and protein levels are significantly reduced, whereas NPR-A and NPR-B expression is relatively unaltered (Chen 2005; Sun et al. 2000, 2001). This raises the possibility that clearance of NPs from the circulation is intentionally slowed under chronic hypoxia to optimise the beneficial activity of this family of peptides in PH and other hypoxia-driven pathologies.

The short plasma half-life and negligible oral bioavailability make NPs (in common with most vasoactive peptides) poor candidates for drug therapy. An alternative strategy is to increase endogenous natriuretic peptide levels by (a) blocking clearance via NPR-C and (b) inhibiting neutral endopeptidase (NEP). Although NPR-C antagonists were developed in the 1990s, predominantly for left-sided heart failure (Veale et al. 2000), they have not been evaluated to date in PH. However, the strategy of NEP inhibition has been proven to be effective in animal models (Baliga et al. 2008; Klinger et al. 1993; Zhao et al. 1991). Indeed, our work has shown that PDE5 is pivotal in terminating the bioactivity of natriuretic peptide-induced cGMP signalling in the pulmonary vasculature, whereas this PDE isozyme is less of an important hydrolytic pathway for NPs in the systemic circulation (Baliga et al. 2008). A similar synergistic activity is observed between urodilatin and dipyridamole (Schermyly et al. 2001). Therefore, in PH patients, whose circulating natriuretic peptide concentrations are raised, it is likely that the beneficial effects of PDE5i are dependent on natriuretic peptide, rather than NO, bioactivity [since the latter pathway is deficient in PH (Humbert et al. 2004)]. In addition, this phenomenon accounts for the pulmonary selectivity of PDE5i [this thesis is supported by data from experimental models (Zhao et al. 2003)]. These observations also represent clear evidence that in PH release of NPs represents an intrinsic, host defence mechanism that slows pathogenesis. As such, the therapeutic potential of manipulating natriuretic peptide bioactivity to reverse the haemodynamic abnormalities associated with PH holds great promise. However, this is true not only for the haemodynamic dysfunction, but also for attenuating the pulmonary vascular re-modelling that also characterises the disease. This is exemplified by the fact that NPs inhibit pulmonary vascular smooth muscle proliferation and TGF β -induced extracellular matrix expression *in vitro* and prevent structural changes *in vivo* in animal models of PH (Chen et al. 2006; Jin et al. 1990; Klinger et al. 1998, 1999; Li et al. 2007). Moreover, NPs (and cGMP *per se*) have been shown to play a key role in preventing the development of fibrosis in a number of organ systems, including the heart and kidney, exemplified best in mice with genetic deletions of the natriuretic peptide signalling pathway (Das et al. 2010; Ellmers et al. 2007; Knowles et al. 2001; Nishikimi et al. 2009; Oliver et al. 1997; Pandey et al. 1999; Tamura et al. 2000). Furthermore, administration of ANP or BNP halts the development of hepatic fibrosis (Ishigaki et al. 2009; Sonoyama et al. 2009), and direct activation of soluble guanylate cyclase, the NO receptor, is effective in reducing fibrosis in the heart, lung and kidney (Evgenov et al. 2011; Kalk et al. 2006; Masuyama et al. 2009). Thus, augmentation of natriuretic peptide bioactivity appears to fulfil a contemporary perception that to advance current therapy a multifaceted therapeutic approach is needed that (a) targets the lung, (b) reverses the pulmonary vasoconstriction, (c) prevents pulmonary vascular re-modelling and (d) halts the deterioration of the right ventricle.

4.1.4 Neutral Endopeptidase and Pulmonary Hypertension

Neutral endopeptidase (EC 3.4.24.11, neprilysin; CD10; common acute lymphoblastic leukaemia antigen) is an endothelial membrane-bound zinc metallopeptidase that is the major pathway for degradation of numerous vasoactive peptides, including NPs, AM, substance P, bradykinin, ET-1 and Ang II (Borson 1991; Thompson and Morice 1996; Richards et al. 1991). NEP has a wide organ (e.g. lung, gut, adrenal glands, brain and kidney) and cellular (endothelial cells, vascular smooth muscle cells, cardiac myocytes and fibroblasts) distribution (Erdos and Skidgel 1989; Graf et al. 1995; Llorens-Cortes et al. 1992; Raizada et al. 2002). NEP is also involved in processing big ET to ET-1, and so it is involved in both the activation and degradation of this peptide (Grantham et al. 2000; Murphy et al. 1994). The net effect of NEP inhibition on vascular tone therefore depends on whether the predominant substrate(s) degraded by NEP are vasodilators (e.g. NPs, AM) or vasoconstrictors (e.g. ET-1, Ang II) and the extent of NEP involvement in big ET processing. This critical balance is exemplified by studies in normotensive subjects, in whom NEP inhibitors do not lower systemic pressure, but augment the effects of ANP and lower blood pressure in hypertensive subjects (Richards et al. 1992, 1993; Ando et al. 1995; Laurent et al. 2000). NEP inhibitors have been used with limited success in hypertension and heart failure (Ando et al. 1995; Bevan et al. 1992; O'Connell et al. 1992; Ogihara et al. 1994; Rouleau et al. 2000). Tolerance to NP, altered expression of NPR receptors and activation of the renin-angiotensin-aldosterone system (RAAS) are possible explanations for this loss of chronic efficacy. Since the functional effects of natriuretic peptide augmentation can be limited by the concurrent augmentation of the RAAS system, combined ACE/NEP ('vasopeptidase') inhibitors have also been developed. Experimental studies and early clinical trials showed these drugs to have highly potent antihypertensive and anti-remodelling effects (Xu et al. 2004; Backlund et al. 2003; Blais et al. 2000; Burrell et al. 2000; Klapholz et al. 2001; McClean et al. 2001, 2002; Rouleau et al. 2000; Trippodo et al. 1999; Troughton et al. 2000). However, larger scale studies [e.g. Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events (OVERTURE) and Omapatrilat Cardiovascular Treatment Assessment Versus Enalapril (OCTAVE)] only revealed modest increases in efficacy (composite endpoints of CV death and hospital readmission) above placebo (ACE inhibitor alone); moreover, the vasopeptidase inhibitors were associated with an unacceptably high incidence of angioedema, likely due to the 'double hit' on bradykinin metabolism, which led to their withdrawal from clinical development (Packer et al. 2002; Solomon et al. 2005; Zanchi et al. 2003). Interestingly, recent studies suggest substituting AT₁R antagonists for ACEi can produce similar antihypertensive and anti-remodelling effects without promoting angioedema (Hegde et al. 2011).

Interestingly, though NEP inhibitors are ineffective in lowering after-load in HF, they do lower the pulmonary capillary wedge pressure (Ando et al. 1995). Further, mice lacking NEP are less susceptible to hypoxia-induced pulmonary vascular leak (Irwin et al. 2005). Experimental studies show that inhibition of NEP can decrease

hypoxia-induced PH and vascular remodelling (Klinger et al. 1993; Thompson et al. 1994a, b; Winter et al. 1991) by potentiating the action of NPs (Seymour et al. 1995; Valentin et al. 1992; Yamamoto et al. 2004; Zhao et al. 1992). NEP is abundantly expressed in cardio-pulmonary tissue and can also be upregulated in injured lung and heart failure (Abassi et al. 1995; Borson 1991; Cohen et al. 1998; Hashimoto et al. 2010; Trippodo et al. 1991). Thus targeting NEP produces selective pulmonary effects in PH as a result of bolstering natriuretic peptide activity. The strategy of targeting NEP for the treatment of PH may also have the added benefit of slowing the breakdown of other protective peptides that will contribute to efficacy, including AM and VIP.

In addition to NEP, other peptidases involved in the catabolism of vasoactive peptides might be exploited in a similar strategy. ACE and ECE have been discussed briefly above. Another widely distributed peptidase with multi-potent activity is dipeptidyl peptidase 4 (DPP-4). DPP-4 inhibitors have recently been developed as novel drugs for the treatment of type 2 diabetes and might have efficacy in PH (Chrysant and Chrysant 2012).

4.2 Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase Activating Peptide

4.2.1 Structure and Expression

VIP is a 28 aa peptide originally isolated from porcine duodenum (Said and Mutt 1970a, b). VIP causes vascular and non-vascular smooth muscle relaxation via activation of adenylate cyclase and the production of cyclic adenosine-3', 5'-monophosphate (cAMP) (Desbuquois et al. 1973). VIP is expressed in many organs and tissues, particularly in the central and peripheral nervous systems as a parasympathetic co-transmitter (Fahrenkrug and Hannibal 2004; Larsson et al. 1976; Van Geldre and Lefebvre 2004). The VIP gene is found on human Chromosome 6 (Tsukada et al. 1985) and, akin to the NPs, is expressed initially as a prepro-precursor of 170 aas that is post-transcriptionally pruned to the principal biologically active (28 aa) form, and a related 27 aa variant termed peptide histidine methionine [PHM; peptide histidine isoleucine (PHI) in rodents] (Itoh et al. 1983).

Pituitary adenylate cyclase activating protein (PACAP) is a 38 amino-acid peptide related to VIP (68 % homology; Fig. 1) that was identified following a concerted effort to search for novel hypothalamic/pituitary-derived hormones that activated adenylate cyclase (Miyata et al. 1989). A variant, consisting of 27 aas (PACAP-27), was also isolated during purification (Miyata et al. 1990) and both PACAP peptides were shown to activate adenylate cyclase with a potency far exceeding that of VIP (Miyata et al. 1990). Akin to VIP, (human) PACAP is also transcribed as a prepro-peptide of 176 amino acids (Hosoya et al. 1992, 1993) and then processed into PACAP-38, PACAP-27 and a further fragment PACAP-29

(which has lower homology with the other peptides and VIP; Fig. 1). Expression of PACAP is regulated in a parallel fashion to VIP, with both cAMP and protein kinase C playing key roles (Suzuki et al. 1994; Yamamoto et al. 1998).

4.2.2 VIP and PACAP Receptors

VIP and PACAP both bind and activate G_s -linked G-protein-coupled receptors (GPCRs) to promote adenylate cyclase activation and cAMP formation, and bring about effects on cellular function. The peptides activate the 'B' class of GPCRs, which are also stimulated by a number of other small biologically active 'secretin family' peptides, and which are almost exclusively G_s coupled. The VIP/PACAP receptor family consists of two principal transmembrane proteins: VPAC₁R and VPAC₂R.

VPAC₁R was first cloned from rat lung (Ishihara et al. 1991) and subsequently identified in human and murine tissue (Johnson et al. 1996; Sreedharan et al. 1993). VIP and PACAP fragments all stimulate this receptor with EC₅₀ values (in terms of cAMP production) in the pM range, with a rank order of potency VIP = PACAP-27 = PACAP-38 = PHM > secretin (Dickson et al. 2006; Ishihara et al. 1991; Sreedharan et al. 1993). VPAC₁R has a relatively ubiquitous distribution, found in many central and peripheral organs and tissues, including the heart, lungs, kidneys, liver, spleen, epithelial cells and lymphocytes (Hashimoto et al. 1993; Ishihara et al. 1991; Reubi 2000; Sreedharan et al. 1993).

VPAC₂R was discovered shortly after the cloning of VPAC₁R, again initially in rat cells with human and murine homologues discovered in turn (Inagaki et al. 1994; Lutz et al. 1993; Svoboda et al. 1994). In cell-based assays, the EC₅₀ values tend to be higher than for VPAC₁R (in the low nM range) with a rank order of potency VIP = PACAP-27 = PACAP-38 > PHM ≫ secretin. VPAC₂R also exhibits widespread heterologous tissue distribution, both centrally and peripherally, although it is most highly expressed in vascular and non-vascular smooth muscle (Harmar et al. 2004).

A third, more PACAP-selective receptor, PAC₁R, has also been identified in rat human and murine tissue (Hashimoto et al. 1993; Hosoya et al. 1993; Morrow et al. 1993; Ogi et al. 1993; Pisegna and Wank 1993; Yamamoto et al. 1998). The two PACAP fragments have highest potency at this receptor subtype (EC₅₀ ~ 200 pM) whereas VIP is approximately 50-fold less potent (EC₅₀ ~ 10 nM). PAC₁R is expressed in many central tissues and also in many endocrine organs peripherally (e.g. pituitary, adrenal, testes) (Hashimoto et al. 1993; Ogi et al. 1993). Splice variants of this receptor have also been identified and appear to have a more specific tissue localisation.

While VIP effects are principally mediated by the adenylate cyclase AMP pathway, VIP is often found co-localised with nNOS in the parasympathetic nervous system. There is also evidence that NO and VIP can induce each other's release, at least at the presynaptic level (Murthy and Makhoulouf 1994). Based on results obtained in isolated gut smooth muscle cells, a serial post-synaptic VIP/NO model has also

been proposed wherein VIP is the principal neurotransmitter acting primarily through the VPAC/cAMP pathway, but also by induction of muscular NO production, primarily through eNOS (Murthy and Makhoulf 1994; Said and Rattan 2004).

As a consequence of the ubiquitous distribution of VPAC/PAC receptors it is perhaps not surprising that they govern a plethora of cellular functions, particularly in the cardiovascular and respiratory systems. These important roles have been substantiated by the development of an array of transgenic animals with targeted alterations in different components of the signalling pathways, including both endogenous ligands and the receptors subtypes. For example, VIP overexpressing mice have augmented glucose-triggered insulin release (Kato et al. 1994), whereas VPAC₂R knockout mice are significantly leaner than their WT littermates (Asnicar et al. 2002); a similar phenotype is observed in PACAP and PAC₁R KO mice (Gray et al. 2002; Hashimoto et al. 2001). In addition, the circadian rhythm is disrupted in mice deficient in VIP or VPAC₂R (Colwell et al. 2003; Harmar et al. 2002), and PACAP KO mice have impaired neuropathic pain responses (Mabuchi et al. 2004). However, it is perhaps in the cardiovascular system that the physiological and pathological roles of VIP and PACAP have been best characterised. Indeed, it is in the context of PH that the importance of these vasoactive peptides, and the potential of drugs targeted to the signalling pathways they trigger, that has provided the greatest insight.

4.2.3 VIP, PACAP and Pulmonary Hypertension

VIP is found, and often co-localised with acetylcholine and neuronal NO synthase, in the parasympathetic innervation of the heart and lungs. Indeed, it had been known for many years that bronchial tone is regulated by NANC nerves innervating the airway smooth muscle (Belvisi et al. 1993; Tucker et al. 1990). VIP is also found in the myocardium, conductance tissue and vascular endothelial cells (Brum et al. 1986; Della et al. 1983; Henning and Sawmiller 2001) and heavily expressed in the lungs, including both the bronchial smooth muscle and pulmonary vascular tree (Carstairs and Barnes 1986; Dey et al. 1981; Lundberg et al. 1984). This cardiopulmonary distribution of VIP is matched by its cognate receptors. High-density VIP binding sites are found in the smooth muscle of larger airways, with density reducing with airway diameter. Similar binding sites are located in the airway epithelium, submucosal glands and alveolar walls (Carstairs and Barnes 1986; Groneberg et al. 2006).

VIP is also a strong endothelium-independent vasodilator in the pulmonary circulation (and coronary arteries) (Nandiwada et al. 1985; Smitherman et al. 1989) and exerts positive inotropic and chronotropic activity in the heart (Christophe et al. 1984; Kralios et al. 1990), as might be expected from a mediator that raises intracellular cAMP levels (akin to β -adrenoceptor stimulation). In the airways, VIP and PACAP-38 are potent dilators, causing bronchodilatation *in vitro* and *in vivo*, with a potency exceeding that brought about by isoprenaline by approximately 100-fold (Linden et al. 1999; Morice et al. 1983; Palmer

et al. 1986). VIP also appears to counteract airway inflammation, characteristic of PH and other airways disorders including asthma and COPD. For example, VIP dampens the activation of mast cells and lymphocytes and inhibits the production of pro-inflammatory mediators such as IL-16, IL-12 and TNF α . Conversely, VIP enhances the production of IL-10. These anti-inflammatory effects appear to be mediated primarily via modulation of NF κ B activity and a cAMP-responsive element (CRE) that prevents the expression of pro-inflammatory genes (Leceta et al. 2000; O'Dorisio et al. 1989; Udem et al. 1983). VIP also activates VPAC1R and VPAC2R to elicit anti-proliferative effects on pulmonary vascular smooth muscle (St Hilaire et al. 2010).

In the context of PH, analogous to the biological actions of NPs, VIP and PACAP peptides exert a wide range of beneficial effects, including vasodilatation, bronchodilatation, modulation of airway secretion, anti-inflammatory and anti-proliferative activity. In accord, VIP has been shown to alleviate the pathogenesis in a number of models of PH. For instance, in monocrotaline-induced PH, VIP dose dependently reduces pulmonary artery pressure and pulmonary vascular resistance and synergises with endothelin receptor antagonists in preventing pathogenesis and mortality (Gunaydin et al. 2002; Hamidi et al. 2011). Moreover, in VIP KO mice there is pulmonary hypertension accompanied by right ventricular hypertrophy, pulmonary vascular remodelling and immune cell infiltration in the lung; a phenotype that can be reversed by a chronic (4 week) infusion of VIP (Said et al. 2007). VIP infusion in neonatal piglets with PH also produces decreases in the PVR/SVR ratio, thereby executing a relatively pulmonary specific beneficial effect (Haydar et al. 2007). Moreover, expression of VIP but not PACAP receptors is markedly altered in an experimental model of PH, suggesting that abnormal VIP signalling contributes to the pathogenesis of the disease (Vuckovic et al. 2009). PAC1 receptor KO mice also develop a dramatic increase in PAP and RVH shortly after birth, entailing a significant mortality, intimating that lack of this receptor results in a severe PH phenotype (Otto et al. 2004). There is also the rationale to predict that VIP administration may be effective in PH associated with lung diseases such as COPD and IPF. This has again been substantiated with the use of VIP KO mice, which exhibit airway hyper-responsiveness and inflammation (Said 2008). VIP also prevents cytokine-elicited neutrophil accumulation in the lung and promotes epithelial healing and in response to cigarette-induced damage (Guan et al. 2006; Onoue et al. 2004), and a stable VIP analogue attenuates ovalbumin-induced airway hyper-responsiveness (Misaka et al. 2010).

In line with this positive preclinical experimental data, a single-centre (albeit not placebo-controlled) trial in patients with PAH confirmed that VIP decreases pulmonary artery pressure and augments cardiac output and venous oxygen saturation (Petkov et al. 2003). This patient cohort also had lower circulating and lung VIP levels and increased expression of VIP receptors (the latter perhaps as a compensatory mechanism). A second clinical study also reported that inhaled VIP exerts a short-lived decrease in PAP, increases stroke volume and improves mixed venous oxygen saturation in PH patients (Leuchte et al. 2008). However, a more recent multi-centre, randomised, placebo-controlled trial of inhaled VIP in PH patients

with differing aetiologies has dampened enthusiasm for this approach. Here, VIP did not produce any beneficial therapeutic effect on a number of indices of disease severity including PVR, 6MWD and WHO Functional Class (although some evidence of improved 6MWD at 6 months with the higher doses of VIP was apparent)(Galie et al. 2010). Notwithstanding, it is the problem of delivery that likely holds the key to therapeutic potential of VIP and PACAP peptides in treating PH and other lung disease. In the inflammatory environment of lung disease, VIP is rapidly metabolised by degrading enzymes such as NEP. Indeed, this may explain, at least in part, the ineffectiveness of inhaled VIP in the multi-centre trial; inhalation only produced a minor increase in plasma VIP levels and this may not be the ideal route of administration. To surmount this problem, current thinking advocates the development of more stable VIP analogues, or VIP ‘protected’ from hydrolysis by incorporation into chaperones such as phospholipids. Indeed, the latter approach has been shown to prevent the development of PH in experimental models (Rubinstein 2005). Peptidase-resistant VIP analogues (i.e. receptor agonists) are also being evaluated in vitro and in patients with lung disease, with promising signs of efficacy (Linden et al. 2003; Schmidt et al. 2001). Time will tell if these offer a significant advance in therapy, since the inhalational route will probably be required due to the potent effects of VIP on cardiac function and the peripheral vasculature. Yet, genetic variation also provides proof-of-concept evidence that VIP acts as an innate defence mechanism in the pathogenesis of PH and that drugs targeting VIP signalling may be of therapeutic benefit. For instance, the T8129C variant in the VIP gene has been linked to the development of the disease (Zhang et al. 2009), since individuals with this genotype have lowered plasma VIP levels and higher PAP. Moreover, VIP also appears to have direct salutary effects on the right heart, a therapeutic benefit often overlooked. For example, administration of VIP dilates the coronary arteries, reduces coronary vascular resistance and, as a result, can almost double coronary artery blood flow (Brum et al. 1986; Popma et al. 1990). In addition, VIP increases fractional shortening and left ventricular contractility in human volunteers (Brum et al. 1986; Frase et al. 1987).

4.3 Adrenomedullin

4.3.1 Structure and Expression

AM is a potent, long-lasting vasodilator peptide (52 aa) originally isolated from human pheochromocytoma (Kitamura et al. 1993). The peptide shares homology with calcitonin gene-related peptide (CGRP) and amylin, and analogous to NPs and VIP, possesses intramolecular disulphide bonds (Fig. 1). AM is localised to blood vessels, kidneys and lungs and circulates in the plasma with a half-life in the order of 15 min (Ichiki et al. 1994; Sakata et al. 1994). Indeed, circulating AM levels are increased in patients with hypertension, renal failure, heart failure and MI (Ishimitsu et al. 1994; Nishikimi et al. 1995). AM has a number of cardioprotective

effects, and its potent vasodilator and natriuretic/diuretic actions, akin to those exerted by NPs, suggest that the peptide has a key role to play in regulating blood volume and pressure (Kitamura et al. 1993; Majid et al. 1996). The biological actions of AM are mediated by a calcitonin receptor-like receptor (CRLR) which appears to function as a selective AM receptor when associated with receptor activity modifying protein (RAMP) subtypes 2 and 3 (McLatchie et al. 1998). Interestingly, AM appears to be able to activate a number of second messenger systems to bring about a wide array of biological functions, including cyclic nucleotides (e.g. cAMP, cGMP) and PI3K. These mechanisms underpin the ability of AM to vasodilate, inhibit vascular smooth muscle proliferation, promote diuresis and natriuresis whilst inhibiting aldosterone production and facilitate angiogenesis. Intravenous administration of AM causes vasodilatation, diuresis and a positive inotropic action in experimental models (Rademaker et al. 1997) and in patients with congestive heart failure (Nagaya et al. 2000b). AM levels are also regulated by the action of NEP and, additionally, by receptor internalisation, again paralleling the natriuretic peptide system.

4.3.2 Adrenomedullin and Pulmonary Hypertension

AM is one of the most potent dilators of the pulmonary circulation and thought to be mediated by both cAMP- and NO/cGMP-dependent pathways (Heaton et al. 1995; Lippton et al. 1994; Nakamura et al. 1997; Nossaman et al. 1996). The dilator effect of AM appears to be more pronounced in isolated pulmonary vessels (compared to systemic aorta), intimating that it may bring about a somewhat pulmonary selective dilatation (Yang et al. 1996). Circulating AM levels are elevated (two- to threefold) in patients with PH and increase with the severity of disease, mimicking the natriuretic peptide family (Kakishita et al. 1999; Nishikimi et al. 1997; Shimokubo et al. 1995; Yoshiyayashi et al. 1997). Circulating AM levels are also elevated in patients with PH secondary to connective tissue disease (Nanke et al. 2000). Heterozygous AM^{+/-} mice [homozygous AM^{-/-} animals are embryonic lethal (Ando and Fujita 2003)] are more susceptible to hypoxia-induced PH and the consequent pulmonary vascular remodelling, suggesting that this peptide presents another intrinsic defence mechanism that prevents development of the disease (Matsui et al. 2004). Moreover, upregulated expression of RAMP2 and AM by hypoxia facilitates inhibition of ET-1 signalling by AM in PH (Dschiertzig et al. 2007). Since intravenous administration of AM can decrease systemic blood pressure, alternative routes of administration have been investigated to achieve greater pulmonary selectivity. Chronic inhalation of AM reverses many indices of disease severity in monocrotaline-induced PH and increases survival (Nagaya et al. 2003c). Aerosolized AM has also been shown to reduce PAP without exerting significant systemic hypotensive effects (perhaps via inhibition of ET-1 bioactivity) (Kandler et al. 2003; von der Hardt et al. 2004) and has the added benefit of reducing pulmonary TGF β and IL-1 β gene expression (von der Hardt et al. 2002). Inhaled AM also seems to synergise with NO or prostacyclin in preventing PH in

experimental models (Dani et al. 2007). AM gene transfer has also shown some evidence of efficacy in experimental models of PH (Nagaya et al. 2003a). AM infusion produces an acute reduction in pulmonary artery pressure in hypoxic rats and the binding of radiolabelled AM and CGRP is increased in the lungs of these animals (Zhao et al. 1996b). AM also lowers pulmonary artery pressure in patients with PH (Nagaya et al. 2000a), whilst concomitantly increasing cardiac index; the latter response is likely due to an AM-induced increase in myocardial cAMP (Nishikimi et al. 1998), although cAMP-independent mechanisms have been reported to underlie this phenomenon (Szokodi et al. 1998). Infusion of AM has beneficial haemodynamic effects in several PH patient cohorts (Nagaya et al. 2000a, 2003b), and inhalation of AM causes a significant reduction of pulmonary vascular resistance without affecting systemic blood pressure and increases exercise capacity in PH patients (Nagaya et al. 2004).

4.4 *Calcitonin Gene-Related Peptide*

4.4.1 **Structure and Expression**

Calcitonin gene-related peptide (CGRP) is a 33 aa peptide which is arguably the most potent endogenous vasodilator identified to date (Brain et al. 1985). The peptide exists as two forms, α and β , which differ by 3 aa in humans (two in rats) (Amara et al. 1982). α CGRP is expressed abundantly in the lung, whereas β CGRP is more prevalent in the GI tract (Mulder et al. 1988). CGRP belongs to a superfamily of structurally and functionally related peptides (Fig. 1) including AM and amylin (a 37 aa peptide co-synthesised and secreted with insulin from islet β -cells and which reduces hypoxia-induced PH) (Keith 2000; Wimalawansa 1996). The biological effects of CGRP are mediated via interaction with cognate G_s -coupled receptors (Aiyar et al. 1997). Initially, two subtypes of CGRP receptor were identified, CGRP1 and CGRP2, defined by binding of selective CGRP-derived agonists (Aiyar et al. 1996). However, subsequent efforts have identified a calcitonin receptor-like receptor (CRLR) which appears to transduce the signals conveyed by CGRP and AM. The specificity of signalling is achieved by association of CRLR with receptor activity modifying proteins (RAMPs) whereby RAMP1 promotes CGRP binding and signalling, whereas interaction with RAMP2 or RAMP3 facilitates AM binding and triggering of downstream pathways (Fraser et al. 1999; McLatchie et al. 1998). CGRP dilates most arteries in vitro by acting on CGRP1 receptors (Aiyar et al. 1996; McCormack et al. 1989) which promote release of NO from the vascular endothelium, although some NO-independent actions of CGRP on vascular reactivity have also been reported (Brain et al. 1985; Chen and Guth 1995).

4.4.2 CGRP and Pulmonary Hypertension

Endogenous CGRP appears to maintain a cytoprotective function in PH since circulating levels of CGRP are reduced in the disease and inversely correlate with PAP (Keith and Ekman 1992; Tjen et al. 1992). Moreover, the development of experimental PH can be prevented and reversed by infusion of α CGRP (Qing et al. 2003; Tjen et al. 1992). Further still, the intrinsic protective mechanism proffered by CGRP is exemplified by exacerbated PH following infusion of the CGRP1 receptor antagonist CGRP₈₋₃₇ (Tjen et al. 1992). The beneficial activity of CGRP in PH is substantiated by the observation that adenoviral delivery of the prepro-CGRP to murine lungs prior to exposure to hypoxia significantly increases lung CGRP and cAMP levels and reduces the severity of the resulting PH (Champion et al. 2000). Administration of CGRP also alleviates hypoxia-induced PH and the antagonist CGRP₈₋₃₇ augments the pulmonary hypertensive response to Ang II (Janssen and Tucker 1994). The endogenous source of CGRP in the lung in the context of PH appears to be the C fibres, since depletion of this pool with capsaicin augments the increased PAP and RVH in response to hypoxia (Tjen et al. 1998). Moreover, 3 weeks exposure to an hypoxic environment after birth causes a drop in circulating CGRP levels and persistent PH in newborn rats (Keith et al. 2000). Moreover, in vivo gene transfer of CGRP to the lung prevents the increase in PAP, RVH and pulmonary vascular remodelling in chronically hypoxic mice (Bivalacqua et al. 2002). RAMP1 overexpression also occurs in hypoxia which, in combination with RDC-1, appears to form a functional CGRP receptor that mediates the beneficial effects of the peptide against hypoxia-induced PH (Qing and Keith 2003). CGRP also produces a potent anti-proliferative effect on pulmonary vascular smooth muscle cells (Chattergoon et al. 2005), possibly via inhibition of ERK1/2, *c-fos* and *c-myc* signalling (Li et al. 2012). The potential for a CGRP-based therapy in PH has been highlighted by the delivery of CGRP-producing endothelial progenitor cells to rats with left-to-right shunt-induced PH (Zhao et al. 2007), the administered cells lodged in the lung vasculature (visualised by immunofluorescence) reducing the PAP and vascular remodelling.

4.5 Somatostatin

Somatostatin fragments have markedly different effects in the pulmonary circulation and PH. SS-28 has been reported to augment hypoxia-induced PH whereas SS-14 appears to do the exact opposite (Tjen et al. 1992). However, this differential activity is perhaps not surprising based on the receptor pharmacology of the two related peptides, which act at different dimeric GPCRs, SST₁₋₅ (Velez-Roa et al. 2004), and cause opposing effects of potassium currents, leading to dilatation and contraction (Wang et al. 1989). Interestingly, infusion of SS-14 in hypoxic rats causes release of CGRP and SS-14 itself whereas administration of SS-28 in the same fashion reduces CGRP levels. Moreover, CGRP infusion increases the

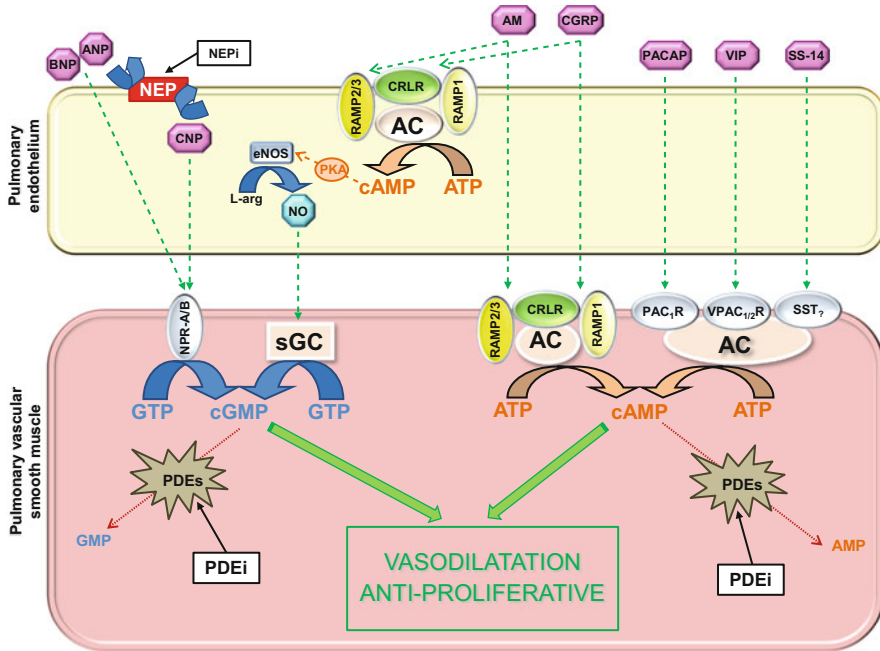


Fig. 2 Schematic representation of the important peptide vasodilator signalling pathways highlighted in this review

production of SS-14 (but not SS-28) levels, raising the possibility of a cytoprotective, positive feedback pathway (Tjen et al. 1992).

5 Conclusions

Advances in the treatment of PH over the past decade have enabled physicians to substantially improve the prognosis, yet the mortality rate remains high. Existing treatments are based predominantly on vasodilatation, whereas many emerging therapies are aimed at cell proliferation and re-modelling. It is likely that each of these processes will need to be targeted to advance therapy for PH. Ideally, a single intervention functioning in a multifaceted manner will be developed. The manipulation of vasodilator peptides holds promise in this regard. Each of those implicated in pulmonary health and disease and described herein augments levels of one or both cyclic nucleotide second messengers, which are established to be clinically effective in treating multi-aspects of PH pathogenesis (Fig. 2). This approach also raises the possibility of combination treatment with existing therapy which is

underpinned by increased cAMP and/or cGMP signalling (i.e. prostacyclin analogues, PDE5i). In sum, harnessing of the cytoprotective activity of vasodilator peptides remains an encouraging strategy to reduce the morbidity and mortality associated with PH, which warrants further investigation and optimisation.

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Pulmonary Hypertension: Novel Pathways and Emerging Therapies Inhibitors of cGMP and cAMP Metabolism

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Abstract Cyclic nucleotides (e.g., cAMP and cGMP) are ubiquitous second messengers that affect multiple cell functions including vascular tone and vascular cell proliferation. After production, different processes can regulate the concentration of cyclic nucleotides. Cyclic nucleotides' degradation by phosphodiesterase (PDE) enzymes has well-known roles in regulating cyclic nucleotides concentrations. Recently, recognition of ATP-binding cassette (ABC) transporter contribution to both local and global regulation of cAMP has been acknowledged. Recent data support an important role of cyclic nucleotide efflux in the pathobiology of pulmonary hypertension, thus suggesting that inhibition of cyclic nucleotide efflux proteins might be a useful strategy to prevent and treat PH.

Keywords Multidrug resistance-associated proteins • Cyclic nucleotides • Pulmonary artery • Efflux

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1 Cyclic Nucleotides and the Pathogenesis of Pulmonary Hypertension

Pulmonary hypertension (PH) refers to a broad group of diseases and conditions that are characterized by persistent precapillary pulmonary hypertension (PH) (increase in mean pulmonary artery pressure (mPAP) ≥ 25 mmHg with a normal pulmonary artery wedge pressure ≤ 15 mmHg), leading to progressive right heart failure and premature death. The pathological mechanisms leading to this progressive and fatal cardiovascular disorder remain partially determined, although pulmonary artery endothelial cell dysfunction and structural remodeling of the pulmonary vessels are early features of PAH. Pulmonary vascular remodeling includes proliferation and migration of pulmonary artery smooth muscle cells (PASMC) leading to medial hypertrophy, increased pulmonary vascular resistance, and PAH progression. Cell hyperproliferation conducts to progressive obstruction of the pulmonary arteries leading to hypertension, progressive right heart failure, and premature death.

Pulmonary arterial hypertension can be idiopathic, hereditary, or associated with other conditions including collagen vascular disease, congenital heart disease, and HIV infection. Different studies have reported that PAH is associated with abnormalities in the homeostasis of cyclic nucleotides, namely, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), that control critical function including vascular tone and smooth muscle proliferation.

Intracellular cGMP is increased by nitric oxide (NO) via activation of intracellular soluble guanylate cyclase (Arnold et al. 1977). Changes in nitric oxide pathways have been detected in patients with PH (Giaid and Saleh 1995; Ghofrani et al. 2004; Kharitonov et al. 1997) with a reduced expression of endothelial nitric oxide synthase, thus supporting a downregulation of cGMP synthesis. In physiological conditions, atrial and brain natriuretic peptides (ANP and BNP) increase intracellular GMP by binding to natriuretic peptide receptor-A. This receptor has a ligand-binding domain on the cell surface connected to an intracellular guanylated cyclase domain (Koeller and Goeddel 1992). Ligand binding results in the cyclization of GTP to cGMP. Overexpression of ANP prevents the development of hypoxia-induced PH, and mice lacking the natriuretic peptide receptor-A

reciprocally develop exaggerated PH in response to hypoxia (Klinger et al. 1999; Zhao et al. 1999). Importantly, a downregulation of ANP-mediated cGMP synthesis has been reported in PAH patients. Taken together, these results suggest that PAH is associated with a decrease in cGMP levels and consequently a reduced activity of the cGMP-downstream targets. The cGMP-dependent kinase PKG mediates most of the cGMP biological effects. In vascular muscle, PKG activation leads to smooth muscle relaxation through a decrease in intracellular calcium (Klinger 2007). PKG might also mediate cGMP-induced inhibition of pulmonary vascular smooth muscle cell proliferation (Lincoln et al. 2001).

cAMP is also a potent regulator of pulmonary vascular tone. The activation of G-protein-coupled receptors, including beta-adrenergic receptors and prostanoid receptors, stimulates the adenylyl cyclase, leads to an increase in the production of cAMP, and eventually reduces the vascular tone. It is generally accepted that cAMP induces vascular relaxation by lowering intracellular calcium levels through inhibition of IP₃ formation, inhibition of Ca²⁺ release from the SR and of Ca²⁺ entry, and stimulation of Ca²⁺ reuptake (Barnes and Liu 1995). cAMP is likely to influence other different targets including potassium channels and different kinase activities. Different studies have also shown that increasing concentrations of cAMP result in a reduction in vascular smooth muscle cell proliferation *in vitro* (Assender et al. 1992) and *in vivo* (Indolfi et al. 1997, 2000). Activation of cAMP signaling typically requires a rise in cellular cAMP concentrations and a specific diffusion in the cell. A spatial organization of cAMP signaling pathways has been proposed and might determine the effectiveness of this pathway (Fischmeister et al. 2006). The role of cAMP in the pathobiology of PH is supported by the reduction in cAMP agonist levels in PAH patients, including PGI₂ in the endothelial wall (Humbert et al. 2004a, b), and by the beneficial effect of agents stimulating cAMP production including inhaled iloprost (Humbert et al. 2004a, b).

2 Usual and Emerging Ways to Modulate cAMP/cGMP Levels

The activation of cAMP and cGMP pathways is generally associated with a rise in their concentrations. These cyclic nucleotides have proved to inhibit pulmonary artery smooth muscle cells' proliferation and to be implicated in the control of pulmonary vascular tone, thus supporting strategies that will eventually sustain an increase in cyclic nucleotides levels. From a general point of view, cyclic nucleotide levels result from their rate of synthesis on the one hand and their rate of elimination on the other hand (Fig. 1). As previously exposed, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are generated by the action of adenylyl cyclase or guanylyl cyclase, respectively. On the other hand, phosphodiesterases (PDEs), by hydrolyzing the cyclic nucleotides, regulate their concentrations and localization. Recently, active export of cyclic nucleotides has become recognized as a process that can impact biological

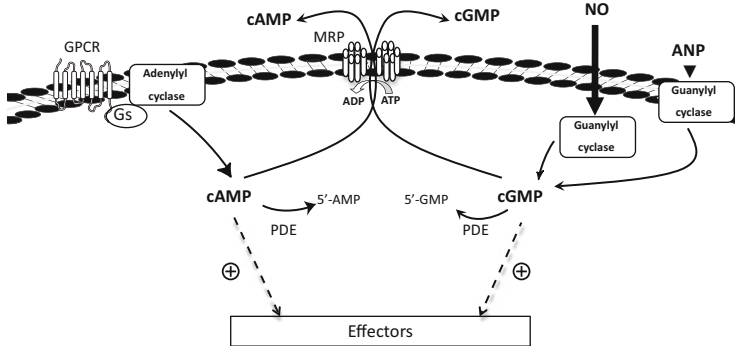


Fig. 1 Schematic representation of the synthesis, degradation, and efflux of cyclic nucleotides. *PDE* phosphodiesterase, *MRP* multidrug resistance-related protein, *GPCR* G-protein-coupled receptor, *NO* nitric oxide, *ANP* atrial natriuretic peptide

processes. Some ABC transporters have been shown to be critical regulator of cyclic nucleotide homeostasis.

2.1 Enhancing Cyclic Nucleotide Production

2.1.1 The Prostacyclin–cAMP Pathway

cAMP is usually produced after activation of the enzyme adenylyl cyclase (AC). AC can be activated or inhibited by G proteins, which are coupled to membrane receptors. There are ten known adenylylated cyclase isoforms, AC2, 5, and 8 being the most important ones for cAMP formation in rat pulmonary artery smooth muscle cells (Jourdan et al. 2001). cAMP levels are decreased during PH. Pulmonary arteries from rats with chronic hypoxia display a decrease in cAMP levels (MacLean et al. 1996). Forskolin (an AC activator) was shown to inhibit DNA synthesis in isolated pulmonary artery smooth muscle cells (Wharton et al. 2000; Davie et al. 2002). Colforsin, a water-soluble forskolin derivative, mediates a vasodilatory response in structurally remodeled pulmonary arteries from rats with PH (Yokochi et al. 2010). Many strategies have been used to increase the intracellular cAMP levels during PH; prostacyclin is one of the used targets in current therapies. Prostacyclin is the main product of arachidonic acid in the vascular endothelium (Moncada et al. 1976) and acts in the smooth muscle cells by binding to a G-protein-coupled receptor (GPCR), the prostacyclin receptor (IP) receptor. IP receptor directly stimulates the adenylyl cyclase (AC) via Gs that converts ATP to cAMP. The prostacyclin receptor agonists increase cAMP in many cell types, including smooth muscle, and the effects of prostacyclin analogs can be potentiated by agents that prevent the breakdown of cAMP. Thus, it has been proposed that cAMP is the main mediator of prostacyclin-induced effects in vascular smooth muscle cells.

Prostacyclin (Prostaglandin I₂) has proved to be a beneficial treatment for patients with severe pulmonary hypertension. In 1985, Bush et al. first reported that PGI₂ might have a role in the treatment of pulmonary hypertension and associated congenital heart defects (Bush et al. 1985, 1986). In 1996, Barst and his colleagues have also shown that the continuous intravenous infusion of prostacyclin plus conventional therapy for primary pulmonary hypertension resulted in better hemodynamics, exercise endurance, quality of life, and survival than conventional therapy alone (Barst et al. 1994, 1996). Since then, many reports confirmed the beneficial effect of prostacyclin (Olschewski et al. 1996, 1999; Rosenzweig et al. 1999; McLaughlin et al. 1998). Many stable prostacyclin analogs have been developed such as beraprost, cicaprost, and iloprost (Narumiya et al. 1999).

2.1.2 The NO-cGMP Pathway

cGMP is generated by particulate or soluble guanylyl cyclases upon stimulation with natriuretic peptides or nitric oxide (NO), respectively. NO, a potent endothelium-derived vasorelaxant substance, inhibits smooth muscle cell proliferation through the cGMP pathway.

NO levels in the pulmonary circulation are decreased in patients with pulmonary hypertension (Cella et al. 2001). Pulmonary hypertension is also associated with diminished expression of endothelial nitric oxide synthase (Giaid and Saleh 1995). A consequence of defect in endothelial NO production during PH is a decrease in cGMP concentrations; for example, pulmonary arteries from rats with chronic hypoxia display a decrease in cGMP levels (MacLean et al. 1996).

NO inhalation has proved to induce vasodilatory effects in PH (Pepke-Zaba et al. 1991; Koelling et al. 1998; Hasuda et al. 2000). An alternative strategy for increasing the intracellular cGMP concentrations is the use of PDE5 inhibitors.

BAY 41-2272 (a soluble guanylate cyclase activator) augments and prolongs the pulmonary vasodilator response to inhaled NO in lambs with acute pulmonary hypertension (Evgenov et al. 2004). BAY-632521, an another soluble guanylate cyclase stimulator, partially reversed the PH, the right heart hypertrophy, and the structural remodeling of the lung vasculature in hypoxic mice and monocrotaline-injected rats (Schermully et al. 2008). Riociguat (BAY-632521) is now in advanced clinical trials for the treatment of PH (reviewed in Schermuly et al. 2011).

2.2 *Blocking Cyclic Nucleotides Degradation*

The concentration of cyclic nucleotides can be regulated by processes within membrane domains (local regulation) as well as throughout the cell. The phosphodiesterase enzymes that degrade cAMP have a well-known role in both these processes.

The phosphodiesterases (PDEs) hydrolyze cAMP and cGMP to their inactive 5' monophosphates. The predominant PDE isoforms in pulmonary arteries are PDE5 (specific for cGMP) and PDE3 (specific for cAMP) which is also inhibited by cGMP. The PDE1 family, activated by the calcium/calmodulin complex, consists of three members (PDE1A, PDE1B, and PDE1C). PDE1A and PDE1B have higher affinity for cGMP than cAMP, whereas PDE1C hydrolyzes cAMP and cGMP with similar efficiency.

Murray et al. detected the presence of PDE1A and PDE1C mRNAs and proteins in PASMC isolated from control human lungs, whereas PDE1B was absent. However, Evgenov et al. detected the presence of PDE1B mRNA in RNA extracted from the main pulmonary artery of healthy sheep (Evgenov et al. 2006). Real-time PCR and immunoblotting demonstrated an increase in the expression of PDE1A and PDE1C in PASMC from patients with idiopathic pulmonary arterial hypertension or secondary pulmonary hypertension compared with control (Murray et al. 2006). An upregulation of PDE1A and PDE1C mRNAs and proteins levels was also detected in PASMC from idiopathic PAH lungs compared with healthy donor, as well as in PASMC from chronically hypoxic mouse lungs and lungs from monocrotaline-injected rats (Schermully et al. 2007). cGMP-PDE1 and cAMP-PDE1 activities are both increased in main pulmonary artery of chronic hypoxia-induced pulmonary hypertensive rats (Maclean et al. 1997).

Many reports proved the PDE1 inhibitor efficacy in preventing or reversing PH. An enhanced cAMP accumulation and an inhibition of cellular proliferation to a greater extent in PH PASMC than controls are observed when cells are transfected with a siRNA designed against PDE1C (Murray et al. 2006).

Inhibition of PDE1, by 8-methoxymethyl 3-isobutyl-1-methylxanthine, reverses structural lung vascular remodeling and right heart hypertrophy in hypoxic mice and monocrotaline-injected rats (Schermully et al. 2007).

Vinopocetine, a PDE1 inhibitor, enhances pulmonary vasodilation and cGMP release induced by NO breathing without causing systemic vasodilation in lambs with acute pulmonary hypertension (Evgenov et al. 2006).

3 Targeting Cyclic Nucleotide Efflux: An Emerging Concept to Modulate cAMP/cGMP Levels

3.1 ABC Transporters and Cyclic Nucleotides Efflux

Besides the classic enzymes involved in cyclic nucleotides elimination (i.e., PDEs), other processes can also alter the intracellular cyclic nucleotide concentrations. Some studies have reported that cAMP and cGMP can be transported from the cytosol to the extracellular space in some specific cell types, including vascular smooth muscle cells and cardiac myocytes (Brunton et al. 1980; Godinho and Costa 2003). Export of monophosphorylated nucleotides was noted by Sutherland when first describing the biosynthesis of the intracellular “second messenger,” 3',5'-cyclic adenosine

monophosphate (cAMP), in response to hormonal stimulation and adenylate cyclase activation (Sutherland and Rall 1958). Sutherland observed not only increased intracellular cAMP but also a concurrent rise in extracellular cAMP. cAMP export has been reported in different cell types including vascular cells (Sassi et al. 2008). This nucleotide extrusion process has general characteristics as being unidirectional, having a saturable kinetics, and being inhibited after depletion of cellular energy [i.e., adenosine triphosphate (ATP)] and after chemical inhibition by compounds such as the organic anion transport inhibitor probenecid and prostaglandins (Campbell and Taylor 1981; Doore et al. 1975; Finnegan and Carey 1998; Rindler et al. 1978).

cGMP export has similarly been reported in different cell types, sharing many of the characteristics observed with cAMP export (Billiar et al. 1992; Sager 2004).

In 1999, it was found that a member of the ATP-binding cassette transporter (ABCC4 alias MRP4) family had the ability to export an antiviral agent [i.e., a purine nucleotide monophosphate analog that resembled cAMP (Schuetz et al. 1999)]. Using membrane vesicle transport systems and altered mammalian cells engineered to stably overexpress MRP4, it was then shown that the vesicular uptake of cAMP and cGMP required ATP and that MRP4 has an affinity for cAMP and cGMP in the low micromolar range (cAMP and cGMP, with affinity constants of 45 μ M and 10 μ M, respectively) (Chen et al. 2001). Collectively these findings provided evidence for energy-dependent export of cyclic nucleotides from cells.

ABC transporters are transmembrane proteins that utilize the energy of ATP hydrolysis to transport various substrates across cellular membranes. In the human genome alone, ABC transporters belonging to the ABC superfamily have 48 representatives. It has progressively been shown that genetic mutations associated with failure to export a specific ligand across a lipid bilayer are the basis of inherited diseases including a neonatal surfactant deficiency (Shulenin et al. 2004), macular degeneration (Allikmets et al. 1997; Martínez-Mir et al. 1998), liver diseases (Strautnieks et al. 1998; Pauli-Magnus et al. 2005), and cystic fibrosis (Riordan et al. 1989; Cutting et al. 1990). Some human ABC transporters also appear to function in a protective capacity by exporting cytotoxic compounds (e.g., dietary cytotoxics and therapeutic drugs) out of the cell.

ABC transporters are usually composed of two distinct domains: the transmembrane domains (TMD) and the nucleotide-binding domain (NBD). The TMD recognizes the substrates and undergoes conformational changes to transport the substrate across the membrane (Dawson and Locher 2006). The TMD is embedded in the plasma membrane and its architecture is variable among ABC transporters, thus reflecting the diversity of substrates that can be translocated. The NBD is the site for ATP binding, is located in the cytoplasm, and has a highly conserved sequence.

As shown in Fig. 2, the domain organization of MRP4 is typical of ABC transporters, with a core structure composed of two TMDs each consisting of six transmembrane helices with two NBDs (Borst et al. 2007; Deeley et al. 2006; Sauna et al. 2004; Lamba et al. 2003). MRP4 is ubiquitously expressed in many tissues, including heart, lung, liver, kidney, brain, intestine, testis, and muscle. Indeed, MRP4 is present in several cell types such as cardiac myocyte (Sassi et al. 2012),

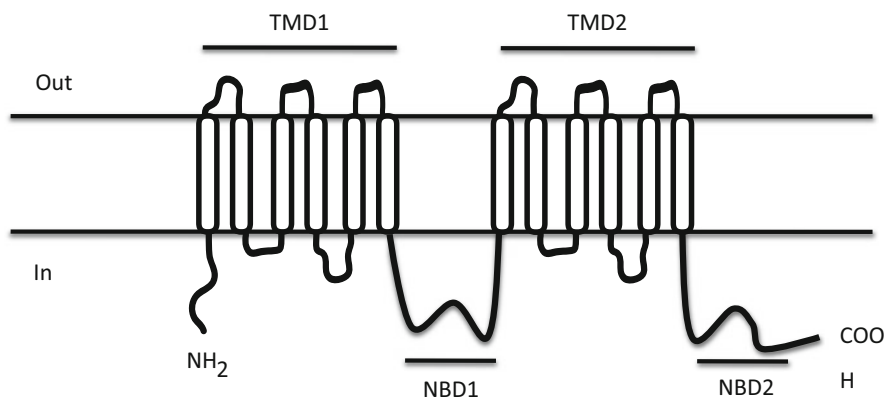


Fig. 2 Membrane topology model for MRP4. The transporter has two transmembrane domains (TMD) and two nucleotide-binding domains (NBD)

erythrocytes (Sundkvist et al. 2002), human kidney proximal tubules (Van Aubel et al. 2002), coronary artery smooth muscle cells (Sassi et al. 2008), endothelial cells (Sassi et al. 2008; Zhang et al. 2004), astrocytes and microglial cells (Hirrlinger et al. 2002), human embryonic kidney cells (Wielinga et al. 2002), hepatocytes (Assem et al. 2004; Rius et al. 2003), platelets (Jedlitschky et al. 2004), and many others cell types.

3.2 Influence of MRP4 on Cyclic Nucleotides Homeostasis

Within the large superfamily of ATP-binding cassette transporters (ABCC), three members of the subfamily of multidrug resistance-associated proteins, MRP4, MRP5, and MRP8 (also known as ABCC4, ABCC5, and ABCC11), have been shown to transport cyclic nucleotides. The affinity of MRP5 and MRP8 mediated for cyclic nucleotides remains however controversial. MRP4 was first identified as a transporter of PMEA (9-(2-phosphonylmethoxyethyl)adenine), a nucleoside monophosphate antiviral agent (Schuetz et al. 1999). MRP4 is able to transport many drugs including antiviral (adefovir, tenofovir, ganciclovir) and cytotoxic (methotrexate, 6-thioguanine, 6-mercaptopurine, topotecan) agents (reviewed in Russel et al. 2008).

In addition to the drugs transport, MRP4 mediates the cellular efflux of several endogenous metabolites. Chen and his colleagues were the first to show an ATP-dependent transport of cAMP and cGMP by MRP4 in membrane vesicles prepared from insect cells (Chen et al. 2001). Since then, many studies confirmed the cellular efflux of cyclic nucleotides by MRP4 (Lai and Tan 2002; Van Aubel et al. 2002; Wielinga et al. 2003; Reichel et al. 2007; Li et al. 2007).

Overall, the studies suggest that MRP4 (and other transporters including MRP5) are low-affinity cyclic nucleotide exporters. This assumption is also supported by the lack of substantial increase in intracellular cyclic nucleotide concentrations after inhibition of MRP4 transport. Reciprocally, overexpression of MRP4 does not lead to a substantial decrease in intracellular cAMP levels, even when the total cAMP concentrations are high (Wielinga et al. 2003). On the other hand, the changes associated with MRP4 inhibition are largely higher in the presence of adenylate cyclase activation or after phosphodiesterase inhibition (Sassi et al. 2008, 2012). These findings fostered the idea that MRP4 is a cAMP overflow pump that may exert important contribution when phosphodiesterases are limiting (Sassi et al. 2008).

Another hypothesis is that MRP4 modulates local membrane concentrations of cAMP and is thus an important contributor in the cyclic nucleotide compartmentalization process. In a recent study (Li et al. 2007), it was reported that MRP4 is physically and functionally associated with the cAMP-regulated chloride channel CFTR. In response to adenosine, the adenosine receptor (a G-protein-coupled receptor) increases intracellular cAMP and activates CFTR chloride currents by compartmentalized changes in cAMP at the apical membrane. At low adenosine concentrations (<20 μM), the MRP4 inhibitor (MK571) potentiated the cAMP-mediated activation of CFTR chloride currents, an effect that was however not observed at high adenosine concentrations (100 μM). Similarly, MRP4 inhibition resulted in moderate changes in the intracellular cyclic nucleotides levels that were however sufficient to induce a significant activation of cyclic nucleotide-mediated signaling pathways (Sassi et al. 2008, 2009). MRP4's ability to modulate local cAMP was evaluated using a FRET-based cAMP sensor, CFP-EPAC-YFP. The adenylate cyclase activation by forskolin produced local intracellular FRET changes in the CFP-EPAC-YFP sensor that reflect local changes in cAMP concentration. Stimulation with lower concentrations of forskolin led to a greater change in FRET in cells transfected with MRP4 silencing RNA as compared to controls. However, the maximal effect was similar regardless of MRP4 activity, supporting the idea that after cAMP has maximally diffused throughout the cell, MRP4 is incapable of modulating local cAMP concentrations. MRP4 inhibition was rather associated with an enhanced signaling response to a minimal cyclic nucleotide production. This supports the assumption that MRP4 physiologically acts by quenching the production of limited amounts of cyclic nucleotides at a local level. This system would be particularly suitable to avoid an inappropriate cellular response to minimal receptor stimulation and minimal cyclic nucleotide production. It is likely that the efflux is maximal in case of higher cyclic nucleotide production which however will not impact any longer the cyclic nucleotides diffusion in the cell. In this model, MRP4 inhibition (and more generally the inhibition of cyclic nucleotide efflux) will result in higher cyclic nucleotide availability after production.

Further support for a role of MRP4 in local regulation of cAMP in membrane domains is the recent finding that MRP4 localizes in caveolin-enriched fractions (Sassi et al. 2008). Because caveolins are integral membrane proteins that act as a scaffold for membrane proteins in microdomains, it is possible that caveolins physically anchor MRP4 in microdomains. Thus, like some PDEs, MRP4 might

promote regulation of local membrane cAMP concentrations that are coupled to some GPCR-mediated processes to accomplish local regulation of cAMP; MRP4 can form a macromolecular complex (Li et al. 2007).

3.3 *MRP4 in the Vasculature*

MRP4 mRNA and protein are present in human coronary artery smooth muscle cells. MRP4 is upregulated in proliferative SMC in vitro and in vivo. Indeed, highly proliferative SMCs in the neointima rat carotid arteries after balloon injury display strong MRP4 protein expression, whereas MRP4 expression in the media is limited. MRP4 knockdown by specific siRNA designed against MRP4 mRNA induces an increase in intracellular cAMP and cGMP levels and an increase in the cAMP-responsive element binding protein (CREB) phosphorylation and activity and thus blocks SMC proliferation in vitro. MRP4 inhibition (using adenoviral vector expressing shRNA against rat *Mrp4* mRNA) prevents neointimal growth in injured rat carotid arteries in vivo (Sassi et al. 2008).

MRP4 mRNA and protein were also detected in preglomerular vascular smooth muscle cells (PGVSMCs). In isoproterenol-treated PGVSMCs, MRP4 siRNA decreased the ratio of extracellular 3',5'-cAMP to intracellular 3',5'-cAMP and the authors conclude that MRP4 is the dominant 3',5'-cAMP transporter in these cells (Cheng et al. 2010).

The effects of MRP4 inhibition in experimental hypoxia-induced PH were also studied (Hara et al. 2011). MRP4 was found to be upregulated in human pulmonary arteries from PAH patients compared to control patients as well as in lungs from WT mice exposed to hypoxia compared with normoxia. MRP4 silencing induces an increase in intracellular/extracellular ratios of both cAMP and cGMP in hPASMCS in vitro as well as in PKA and PKG activities. The changes in cyclic nucleotides levels after MRP4 inhibition are enhanced in the presence of the PDE5 inhibitor sildenafil. As a consequence of cAMP and cGMP regulation, PASMCS' proliferation and migration are inhibited by the MRP4 siRNA. MRP4 KO mice are protected from hypoxia-induced PH development. Indeed, exposing WT mice to hypoxia for 4 weeks resulted in an increase in distal pulmonary artery remodeling, right ventricular systolic pressure, and right ventricular hypertrophy (assessed by the Fulton index) whereas no significant changes were detected in these parameters in MRP4 KO mice (Fig. 3). The use of MK571, a pharmacological inhibitor of MRP4, reverses the PH induced by maintaining WT mice under hypoxia for 3 weeks. In fact, mice treated with MK571 (5 or 25 mg/kg/day) displayed a decrease in the medial thickening of small pulmonary arteries and arterioles and a lower right ventricular systolic pressure and Fulton index (Hara et al. 2011). Macrophage and lymphocyte cell recruitment (measured by CD68 and CD45 expression) after hypoxia was significantly reduced in MRP4 KO mice compared to WT. The amount of IL-6 was increased after hypoxia in WT mice but not in MRP4 KO mice. These results suggest a reduction of hypoxia-induced inflammatory response in MRP4 KO mice.

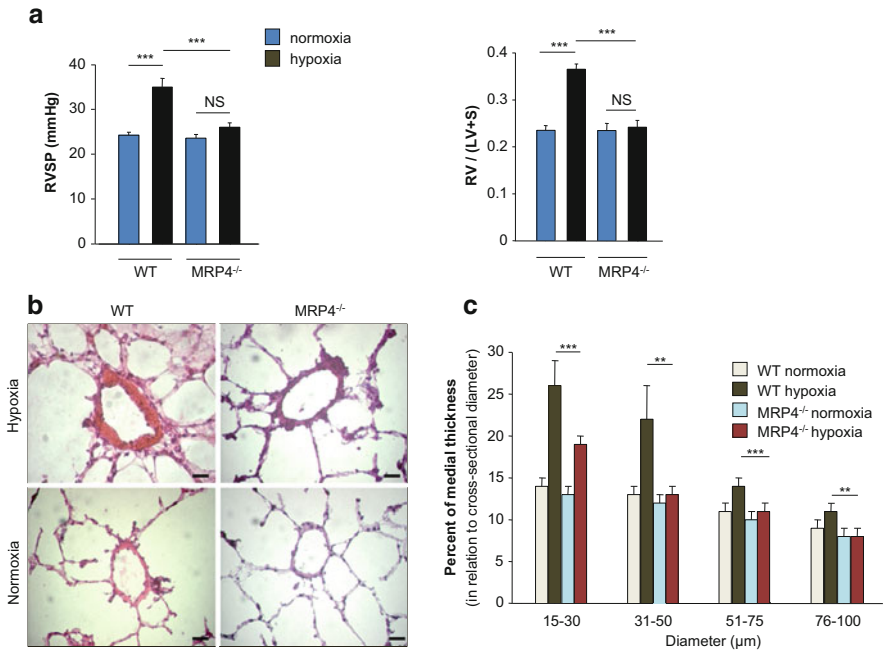


Fig. 3 Prevention of hypoxia-induced pulmonary hypertension in MRP4^{-/-} mice. **(a)** Right ventricle systolic pressure (RVSP, mmHg) and right ventricle hypertrophy reflected by the right ventricle (RV) weight over left ventricle (LV) + interventricular septum (S) weights ratio (RV/[LV + S] = Fulton index) in each group (*n* = 15, ****P* < 0.001). **(b)** Representative H&E-stained sections of small pulmonary arteries from lungs of WT and MRP4^{-/-} mice in normoxia and hypoxia conditions. **(c)** Percentage of medial thickness of small arteries in relation to cross-sectional diameter (***P* < 0.01). Scale bar: 20 μm. Reproduced with permission from Hara et al. (2011)

4 Discussion

Interestingly, changes in cyclic nucleotide levels following MRP4 silencing are further enhanced in the presence of the PDEs nonspecific inhibitor (in human coronary artery smooth muscle cells) or in the presence of the PDE5 inhibitor sildenafil (in hPASCs). In addition, treatment with IBMX or sildenafil led to an increase of MRP4 protein in vitro and in vivo. These results suggest that MRP4 overexpression could act in a manner to compensate for PDE5 inhibition. A combination therapy with PDE5 and MRP4 inhibitors could represent an efficient therapy by attenuating the development of pulmonary hypertension compared with treatment with each drug alone (Fig. 4).

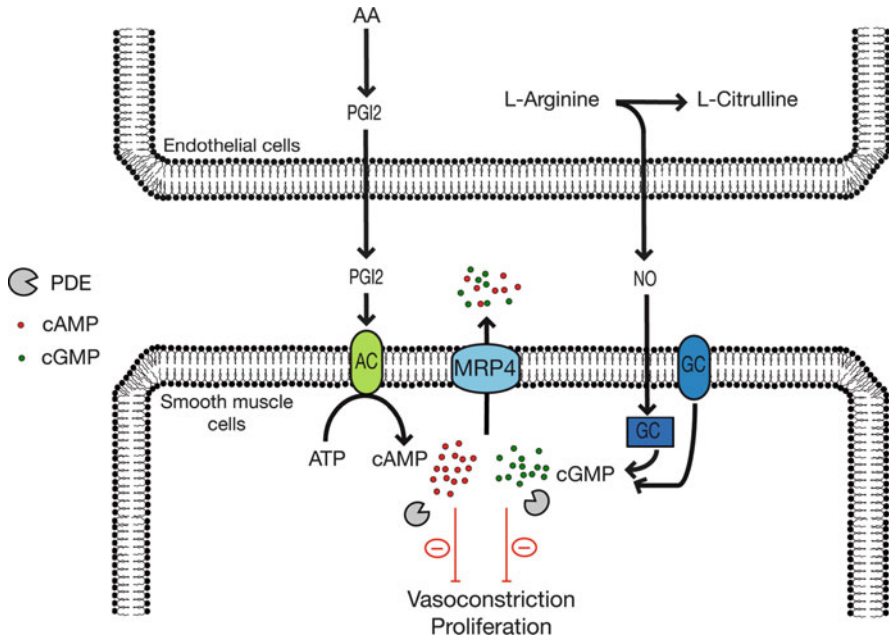


Fig. 4 Schematic representation of MRP in the cyclic nucleotide metabolism during pulmonary hypertension

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Pulmonary Hypertension: Old Targets Revisited (Statins, PPARs, Beta-Blockers)

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Abstract Pulmonary arterial hypertension is a therapeutic challenge. Despite progress in recent years with three drug classes—prostanoids, endothelin receptor antagonists and phosphodiesterase type 5 inhibitors—long-term patient survival remains poor. Importantly, the introduction and commercial success of these new treatments has been accompanied by growing interest in the pathology of pulmonary hypertension. This, in turn, has stimulated a re-evaluation of the molecular factors driving the structural remodelling of pulmonary arterioles and the opportunities to preserve right ventricular function in pulmonary hypertension. Academics with restricted access to new chemicals have turned to existing drugs to investigate new ideas. It is in this context that the role of statins, peroxisome proliferator-activated receptors (PPARs) and beta-blockers are of interest as potential treatments for pulmonary hypertension.

Keywords Treatment • Repurposing • Drug targets • Statins • PPARs • Betablockers

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1 Introduction

The costs and risks associated with the development of new drugs for new targets of therapeutic interest are well documented. The market for the drug has to be substantial to attract investment. Regulators have been prepared to recognise this as a problem for orphan diseases with an unmet clinical need and fast track drugs with therapeutic promise. The success of prostanoids, endothelin receptor antagonists and phosphodiesterase inhibitors as treatments for pulmonary arterial hypertension has stimulated interest in recognising the condition, among academics, practitioners and the pharmaceutical industry. As a result, physicians are recognising the disease more often, the demographics of the disease is changing and its prevalence increasing—but perhaps not enough to make pulmonary hypertension a large commercial market.

A valuable strategy for reducing the cost and risk of drug development is to “repurpose” drugs that have been developed for other diseases for pulmonary hypertension. Experience gained with patient exposure in another disease enables informed decisions on potential dose and safety monitoring in pulmonary hypertension. As new knowledge about the biology of pulmonary hypertension emerges, academics in particular look to the pharmacopeia for agents to investigate. Here we discuss the role of statins, peroxisome proliferator-activated receptors (PPARs) and beta-blockers as treatments for pulmonary hypertension.

2 Statins

Statins have a primary and secondary preventative role in reducing serum cholesterol levels and improving morbidity and mortality in cardiovascular disease (Pedersen et al. 2004). Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is required for cholesterol biosynthesis (Wang et al. 2008). However, there has been growing interest in the pleiotropic, cholesterol-independent effects of this drug class, particularly the role of statins in cell proliferation and apoptosis. The marked vascular remodelling that characterises pulmonary arterial hypertension (PAH) has sparked interest in statins as a treatment for this condition. An impressive body of data from animal

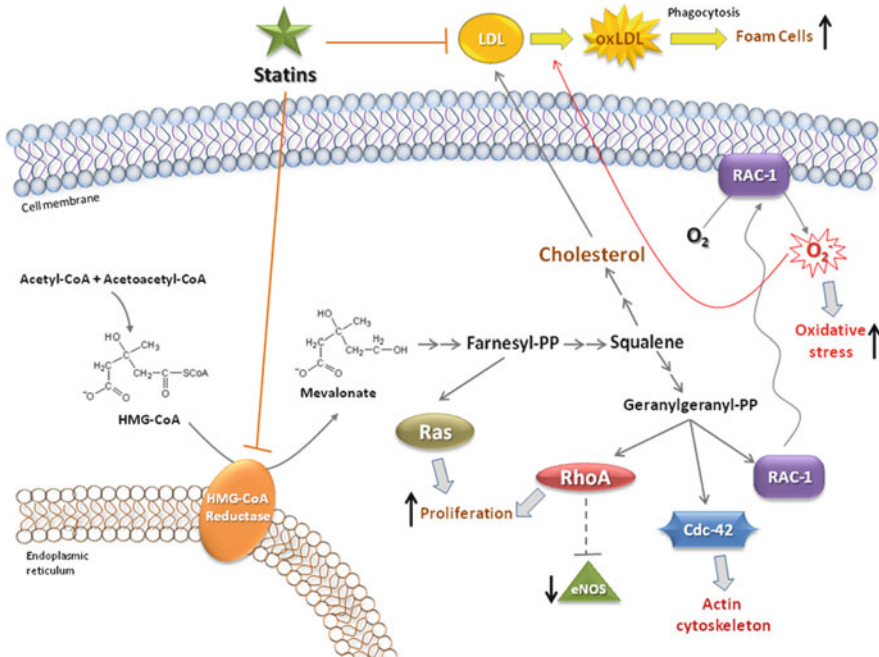


Fig. 1 Cholesterol biosynthesis pathway. Statins’ pleiotropic effect on RhoA, Rac1, Ras, Cdc-42 small GTPases

models has triggered three human studies. But experience in patients has been disappointing and suggests statins are not a therapeutic option for targeting the vascular pathology.

2.1 Molecular Action

Statins inhibit the conversion of HMG-CoA to mevalonic acid, interrupting the synthesis of a number of key downstream molecules, notably the production of isoprenoids, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Goldstein and Brown 1990). These are precursors of cholesterol synthesis but are essential for the post-translational isoprenylation, membrane localization and activation of Rho, Ras and Rac small GTP-binding protein families (Maurer-Stroh et al. 2007) (Fig. 1).

These small GTPases participate in the regulation of diverse cellular functions including smooth muscle contraction, cytoskeletal rearrangement, cell migration, proliferation, apoptosis and gene expression. RhoA and its downstream effector Rho-associated protein kinase (ROCK) are believed to be the most important

regulators of calcium sensitivity in smooth muscle (Nagaoka et al. 2004; Nunes et al. 2010), via inhibition of myosin light chain phosphorylation (Somlyo and Somlyo 2003) and activation of downstream mediators (Sah et al. 2000). Rho/ROCK signalling has been linked with PAH; chronic blockade of ROCK by fasudil or Y-27632 reduces pulmonary vasoconstriction and vascular remodelling in monocrotaline or hypoxia-exposed rodent models (Fagan et al. 2004; Abe et al. 2004; Hyvelin et al. 2005).

Statins have been shown to enhance nitric oxide (NO) production by stabilising endothelial nitric oxide synthase (eNOS) mRNA (Laufs et al. 1997) and augmenting eNOS phosphorylation and catalytic activity (Kureishi et al. 2000). Interestingly, statins have also been reported to increase expression of bone morphogenetic protein receptor type II (BMPRII) (Hu et al. 2006) and have antithrombotic and anti-inflammatory effects. Collectively, this profile of properties makes statins an attractive drug class for PAH.

2.2 *Animal Models*

Most studies have used simvastatin as the probe compound, but other statins have also been investigated, particularly atorvastatin, pravastatin and rosuvastatin. These agents differ in potency and lipid solubility, with pravastatin the most hydrophilic.

Simvastatin (20 mg/kg/day) both prevents and regresses pulmonary hypertension, right ventricular hypertrophy (RVH) and vascular remodelling in chronically hypoxic rats (Girgis et al. 2003). More impressively, simvastatin 2 mg/kg/day orally both prevents and reverses pulmonary hypertension and neointima formation, and improves survival, in a challenging rat model in which pulmonary hypertension is induced by monocrotaline and pneumonectomy (Nishimura et al. 2002, 2003). McMurtry et al. found simvastatin (2 mg/kg/day, gavage) less effective in the monocrotaline model, retarding the progression of pulmonary hypertension and right ventricular hypertrophy (RVH) at 4 weeks but with no effect on survival (McMurtry et al. 2007). Simvastatin (10 mg/kg/day by gavage) led to a significant attenuation of the aggressive pulmonary vascular remodelling induced by vascular endothelial growth factor receptor blocker, Sugen 5416, in combination with chronic hypoxia (SuHx) (Taraseviciene-Stewart et al. 2006). Supporting observations have been made with pravastatin (Guerard et al. 2006), atorvastatin (Laudi et al. 2007), fluvastatin (Murata et al. 2005) and rosuvastatin (Sun and Ku 2008) in the monocrotaline and hypoxia rodent models.

In clinical practice, statins would be given in combination with other targeted treatments. Simvastatin and atorvastatin show a synergistic effect in combination with sildenafil (Satoh et al. 2009; Zhao et al. 2009). The combination of simvastatin and sildenafil augments eNOS expression, leading to a greater increase in lung cGMP levels and reduction in RhoA-GTP expression levels (Zhao et al. 2009).

Upregulation of eNOS and/or NO production has been reported with atorvastatin (Sun and Ku 2008) and pravastatin (Guerard et al. 2006). Changes in expression of

the serotonin transporter (5-HTT), which has been implicated in the pathology of PAH, have been reported with atorvastatin treatment (Laudi et al. 2007).

2.3 *Clinical Studies*

Kao reported his experience with simvastatin in 16 patients with pulmonary hypertension of mixed aetiology. The drug was well tolerated with positive reports of an improvement in exercise capacity (Kao 2005).

The lack of a randomised control group was addressed in the simvastatin as a Treatment for Pulmonary Hypertension Trial (SiPHT) (Wilkins et al. 2010). This was a double-blind, randomised, placebo-controlled study (SiPHT) in 42 PAH patients in functional class II and III. Patients received simvastatin 80 mg/day or placebo, as an add-on to licensed oral treatment, for 6 months and thereafter offered open-labelled simvastatin. The primary outcome was RV mass assessed by cardiac magnetic resonance (CMR). This was based on the observation that a reduction in RV mass was the most consistent finding from the animal studies. Data on exercise capacity and biomarkers were also collected. At 6 months, simvastatin 80 mg in combination with sildenafil and/or endothelin antagonist was associated with a 5 % reduction in RV mass and a 13 % reduction in N-terminal pro-B-type natriuretic peptide (NT-pro-BNP). However, these effects were only transient and both markers returned to baseline in patients who continued the statin for 12 months. There were no significant changes in exercise capacity (6-minute walk test), cardiac index or circulating cytokines. A rise in muscle enzymes was seen in two patients on high-dose simvastatin and sildenafil, likely the result of competitive inhibition of simvastatin metabolism via CYP3A4, leading to a reduction in the dose of simvastatin.

The ASA-STAT trial recruited 65 patients with PAH and randomised them to aspirin or placebo and simvastatin 40 mg or placebo (Kawut et al. 2011). The primary outcome was change in 6-minute walk distance. Activated platelets produce thromboxane leading to platelet aggregation, vasoconstriction and smooth muscle hypertrophy. Aspirin was considered a potential treatment as it reduces prostaglandin–thromboxane (Tx-PGI₂) ratio in PAH and inhibits platelet activity (Robbins et al. 2006). After adjustment for baseline 6-minute walk distance, there was no significant difference in the 6-minute walk distance at 6 months between aspirin and placebo or between simvastatin and placebo.

The absence of haemodynamic data has been addressed by a recent large double-blind study of 220 Chinese patients with PAH or chronic thromboembolic pulmonary hypertension (CTEPH) randomised to receive atorvastatin 10 mg or placebo daily in addition to supportive care (Zeng et al. 2012). At 6 months, atorvastatin 10 mg had no significant effect on pulmonary vascular resistance (PVR), cardiac output or 6-minute walk distance in either PAH or CTEPH.

2.4 *Future Prospects*

The present data do not support the use of statins as a specific treatment for PAH. Statins may be used for cholesterol lowering in patients with PAH, an increasingly common occurrence as the mean age of PAH patients is rising and co-existing diseases, such as coronary artery disease, are more likely in the older age group. Care should be used with statins metabolised by CYP3A4, as sildenafil and bosentan are metabolised by the same enzyme and clinically significant drug interactions may occur.

The discrepancy between the results from animal studies and clinical studies is an important discussion point. There is concern about the failure of current animal models to predict drug response in patients. The animal models do not replicate the human pathology. Other factors include the dose of drugs used, species differences in metabolism and the design of animal experiments (such as the timing of initiating treatment with respect to the course of the disease, the lack of blinding and randomisation).

Could we have missed an important effect from statins? The drugs that make up this class vary in their physiochemical profiles. Lipophilic statins (simvastatin, atorvastatin, mevastatin and fluvastatin) exhibit direct effects on pulmonary artery smooth muscle cells (PASMCs) (Ali et al. 2011). Fluvastatin and atorvastatin have been shown to elicit more potent pleiotropic effects compared to simvastatin in other smooth muscle studies (Turner et al. 2007). But all inhibit mevalonate synthesis and so reduce FPP and GGPP levels. It is possible that specific targeting of FPP with preservation of GGPP levels might be an advantage, but this remains to be explored.

A confounding factor could be high-density lipoprotein cholesterol (HDL-C). HDL-C is linked to survival in PAH. Heresi et al. (2010) reported low plasma levels of HDL-C in patients with PAH, associated with worse clinical outcomes independent of other cardiovascular risk factors. It is acknowledged that patients with advanced PAH may have low HDL-C due to reduced exercise capacity and worsening obesity, but HDL-C was a prognostic marker when adjusted for age and body mass index (Heresi et al. 2010). It has been suggested that HDL-C may have a role in the initiation and/or progression of pulmonary vascular disease. Statins lower low-density lipoprotein (LDL) but do not lower HDL-C; indeed HDL-C increases marginally with some statins. Vickers and colleagues demonstrated HDL transports endogenous microRNAs (miRNAs) and delivers them to recipient cells with both endogenous and exogenous miRNA delivery producing direct targeting of messenger RNA reporters (Vickers et al. 2011). miRNAs are short non-coding RNAs that influence regulatory roles by targeting mRNA for cleavage and translational expression (Bartel 2004). Statin-induced changes in HDL-C could affect miRNA levels, but more extensive studies are needed to understand the role of miRNAs in PAH (Joshi et al. 2011).

3 Peroxisome Proliferator-Activated Receptor Agonists

The PPAR agonists, specifically the thiazolidinediones (rosiglitazone, pioglitazone), have achieved clinical recognition as oral hypoglycaemic agents that improve insulin sensitivity in the management of diabetes mellitus. The broader role of PPARs in regulating the transcription of genes involved in inflammation, proliferation and apoptosis in smooth muscle (de Dios et al. 2003) and the increasing appreciation of metabolic syndrome in patients with PAH have generated interest in PPARs as targets for this condition.

3.1 Molecular Action

PPARs are members of subfamily 1 of the nuclear hormone receptor superfamily (Nuclear Receptors Nomenclature Committee 1999). The three separate PPAR isoforms (α , β/δ , γ) are distributed throughout different tissues. PPAR α is expressed predominantly in liver, heart, kidney, brown adipose tissue and small intestine (Mandard et al. 2004). PPAR β/δ has a broad distribution. PPAR γ has four isoforms. PPAR γ 1 is expressed in virtually all tissues, including heart, muscle, colon, kidney, pancreas and spleen. PPAR γ 2 is expressed mainly in adipose tissue (30 amino acids longer). PPAR γ 3 is expressed in macrophages, large intestine and white adipose tissue. PPAR γ 4 is expressed in endothelial cells. PPAR γ is activated by both natural lipid-derived substrates and synthetic ligands including the thiazolidinediones and fibrates. PPAR γ has received most attention as the target isoform in PH but PPAR β/δ is also of interest

Activated PPARs form heterodimers with the retinoic acid X receptor (RXR) (Fig. 2). The PPAR–RXR heterodimeric complex binds to AGGTCA DNA sequences and regulates multiple genes, including those regulating interleukin-6, adiponectin, endothelin and nitric oxide (Jiang et al. 1998; Maeda et al. 2001; Calnek et al. 2003; Polikandriotis et al. 2005).

PPAR γ is expressed in both endothelial and smooth muscle cells (SMCs) in the pulmonary vasculature of normal lungs. Ameshima et al. (2003) report reduced PPAR γ gene and protein expression in the lungs from patients with severe PH and loss of PPAR γ expression in their complex vascular lesions. Fluid shear stress reduced PPAR γ expression in ECV304 endothelial cells in culture. ECV304 cells that stably express dominant-negative PPAR γ (DN-PPAR γ ECV304) form lumen-obliterating lung vascular lesions when injected into nude mice. The authors concluded that loss of PPAR γ expression characterises an abnormal, proliferating, apoptosis-resistant endothelial cell phenotype in PAH.

PPAR γ appears to be regulated by bone morphogenetic protein 2 (BMP2) and many of the downstream targets of PPAR γ are factors that have been implicated in the pathogenesis of PAH. Hansmann and colleagues have described a BMP2–PPAR γ –apolipoprotein E (apoE) pathway in human and murine vascular

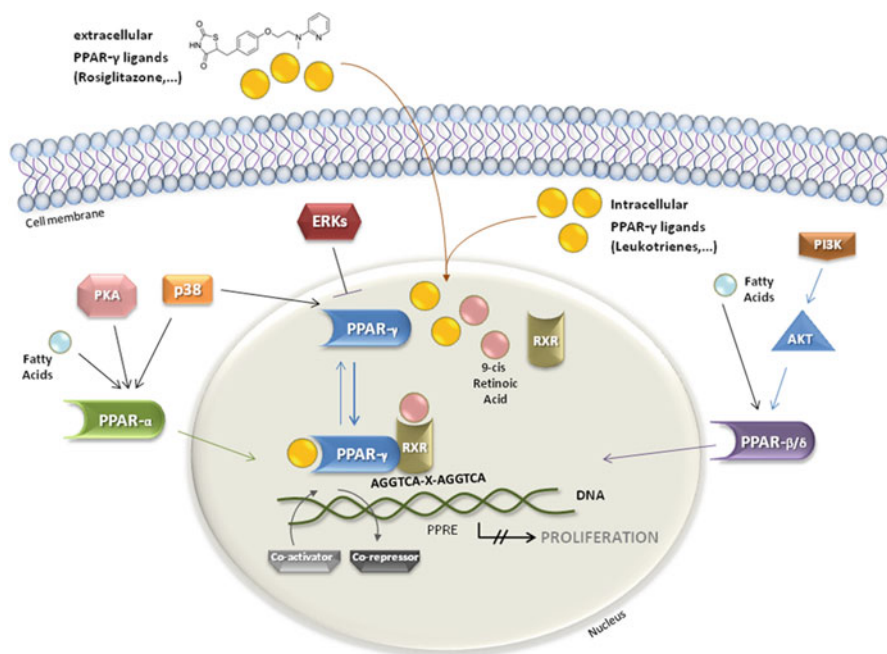


Fig. 2 Mechanism of PPAR activation. *PKA* protein Kinase A, *ERKs* extracellular signal-regulated kinases, *RXR* retinoid X receptor, *AKT* protein kinase B

smooth muscle cells. Pharmacological activation of PPAR γ inhibits PDGF-BB-induced proliferation of human and rat pulmonary artery smooth muscle cell by reducing nuclear ERK phosphorylation. Further investigation by this group revealed that mice with deletion of PPAR γ in vascular smooth muscle cells spontaneously developed PAH. Very recently apelin has been identified as a transcriptional target of the PPAR γ / β -catenin complex (Alastalo et al. 2011).

3.2 Animal Studies

Both pioglitazone and troglitazone have been reported to ameliorate monocrotaline-induced pulmonary hypertension in rodents (Matsuda et al. 2005).

Crossno et al. (2007) investigated the effects of rosiglitazone in the chronic hypoxic rat model of pulmonary hypertension. Rosiglitazone inhibited pulmonary vascular remodelling and, of particular note, reversed remodelling due to prior hypoxic exposure. The decreased remodelling appears to be due to repression of cell proliferation, extracellular matrix deposition and the inhibition of accumulation of c-Kit-positive cells in the pulmonary artery wall. But there was a dissociation between remodelling and pulmonary artery pressure which remained elevated.

Kim et al. (2010) found that the expression of PPAR γ was reduced in the lungs of hypoxic rats, an effect that might be mediated via TGF β signalling (Gong et al. 2011). Administration of rosiglitazone from the onset of hypoxia reduced pulmonary artery pressure, prevented the reduction in PPAR γ levels and decreased the expression of ET-1 and VEGF.

Nisbet et al. (2010) have reported that rosiglitazone both prevents and reverses the development of pulmonary hypertension in the chronically hypoxic mouse. Nox4 expression and superoxide production in the lung were reduced, along with PDGFR β activation and PTEN expression. Rosiglitazone also attenuated hypoxia-induced increases in Nox4 expression in pulmonary endothelial cells in vitro despite hypoxia-induced reductions in PPAR γ expression.

Recently, PPAR β/δ agonists have been shown to relax pulmonary arteries with greater potency than PPAR γ agonists. This effect is likely due to an action independent of target gene induction. Pharmacological studies suggest involvement of RhoA kinase and potassium channels (Harrington et al. 2010). Chronic administration of a PPAR β/δ agonist reduced pulmonary hypertension in the hypoxic rat.

3.3 *Clinical Potential*

The data from animal and in vitro models lend support to the therapeutic potential of PPAR agonists, particularly PPAR γ and PPAR β/δ , in pulmonary hypertension. But clinical experience with PPAR agonists urges caution. Both rosiglitazone and pioglitazone have been under considerable scrutiny recently for their adverse cardiovascular risk profile (Tzoulaki et al. 2009; Sgarra et al. 2012). Of particular concern is fluid retention, the mechanism of which remains to be elucidated but which would not be well tolerated by patients with PAH. It may be that further refinement of agonists to develop an agent with a more favourable safety profile is possible, but this remains to be demonstrated.

4 **Beta-Blockers**

Catecholamines are integral to many physiological and metabolic processes. The effects of both natural and synthetic catecholamines rely on interactions with specific adrenergic receptors located on the cell membrane, resulting in either α - and/or β -mediated effects (Frishman 2008).

4.1 Molecular Action

There are three major adrenoceptors— α_1 , α_2 , and β (Bylund et al. 1994). Three β -adrenoceptor subtypes are recognised: β_1 —comprising around 75 % of all β -adrenoceptors are found predominantly in the heart (Bylund et al. 1994); β_2 —located in vascular and bronchial smooth muscle (Lopez-Sendon et al. 2004); and β_3 —located in adipocytes and of interest in insulin resistance (Pott et al. 2003, 2006) but also in human myocardium (Gauthier et al. 1996). Numerous polymorphisms of beta-adrenoceptors have been described and some are associated with altered response to agonists. β_1 - and β_2 -adrenoceptors couple to Gs and thereby activate adenylyl cyclase and elevate cyclic AMP. β_3 -adrenoceptors exert their effects through Gi coupling to cGMP–NO (Gauthier et al. 1998) (Fig. 3).

Beta-blockers vary in their cardio-selectivity and vasodilatory properties. Cardio-selective agents (bisoprolol, metoprolol, nebivolol) are largely β_1 -adrenoceptor selective but have some β_2 effects at higher doses. Nebivolol is a β_1 -adrenoceptor antagonist but produces vasodilatation through β_3 -adrenoceptor stimulation. Vasodilation can also be effected through α -adrenoceptor blockade (e.g., labetalol) (Ladage et al. 2013). Carvedilol activates both these vasodilatory mechanisms. Non-cardio-selective agents, like propranolol, block β_1 and β_2 without vasodilation (McDevitt 1987).

Chronic heart failure is associated with downregulation of myocardial β_1 - and β_2 -adrenoceptors, perhaps attributable to sustained elevated levels of catecholamines (Bristow et al. 1986; Gaemperli et al. 2010). The reduction in β_1 signalling may be a protective mechanism causing downregulation of the PKA-independent CaMKII-mediated pro-apoptotic pathway. In contrast, β_2 adrenoceptor downregulation may be counterproductive (Bristow 2000). Unlike β_1/β_2 -adrenoceptors, the β_3 -adrenoceptor is upregulated in heart failure (Moniotte et al. 2001). Whether this is a protective mechanism against chronic sympathetic stimulation or a contributor to heart failure is unclear. Recently, β_3 -adrenoceptor stimulation has been reported to reduce cardiac remodelling and improve cardiac function in a mouse model of aortic banding, suggesting a protective function (Niu et al. 2012).

Beta-blockade is one of the cornerstones of the treatment of left ventricular heart failure. The $\alpha_1/\beta_1/\beta_2$ -adrenoceptor blocker, carvedilol, and the selective β_1 -adrenoceptor blockers, bisoprolol and metoprolol, reduce mortality by about one-third. The data suggest reversal of maladaptive cardiac remodelling, improved cardiac function and prevention of arrhythmias (Reiter and Reiffel 1998; Hjalmarson et al. 2000; Packer et al. 2001; Bogaard et al. 2010).

Physicians are much more cautious about using beta-blockers in patients with PAH. Right ventricular function is a major determinant of survival in PAH (D'Alonzo et al. 1991; Sandoval et al. 1994; van de Veerdonk et al. 2011). Increased beta-adrenoceptor stimulation is seen in PAH. This may drive decompensatory changes and predispose to arrhythmias (Bogaard et al. 2009). Supraventricular tachyarrhythmias are a common problem in PAH (Tongers et al. 2007).

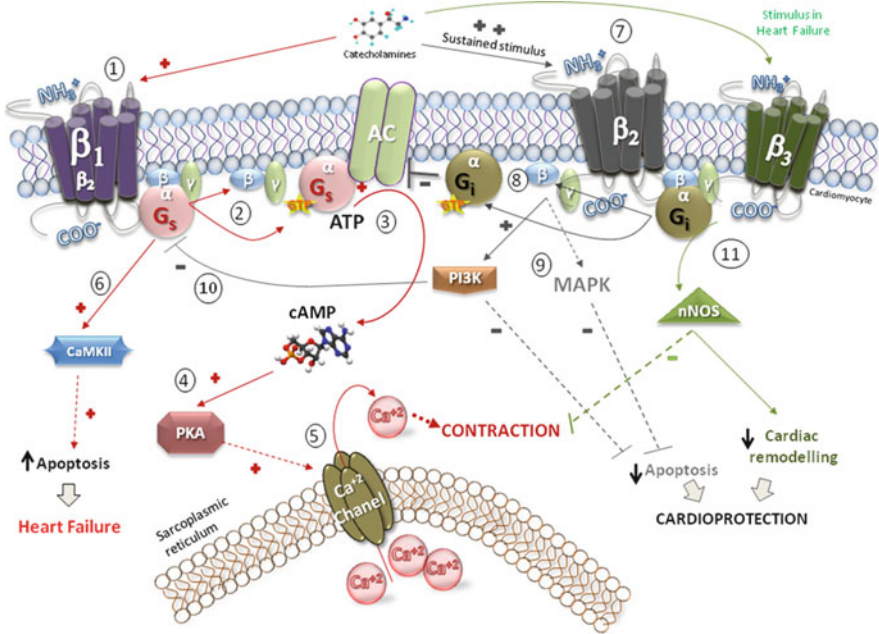


Fig. 3 Intracellular pathway mediated by β -ARs. (1) Main pathway: catecholamines bind to β -adrenoceptors inducing coupling to protein Gs; (2) uncoupling of the $G\alpha$ -GTP subunit and activation of adenylyl cyclase (AC); (3) synthesis of cAMP; (4) protein kinase A (PKA) activation; (5) finally Ca^{2+} release from its stores, leading to contraction of cardiac muscle. (6) Continuous stimulation (as described in chronic heart failure) of β_1 induces apoptosis via Ca^{2+} /calmodulin-dependent protein kinases II (CaMKII) leading to muscular degeneration and heart damage. (7) Continued stimulation of β_2 (increased when using selective β_1 -blockers) induces coupling to protein Gi; (8) AC is inhibited by the $G\alpha$ -GTP subunit of protein Gi; (9) the $G\beta$ subunit of Gi induces both the inhibition of apoptosis [via mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K)] and inhibition of protein Gs deleterious effects (10), leading to cardioprotection. (11) In heart failure, stimulation of the β_3 -adrenoceptor leads to cardioprotection and a reduction in cardiac remodelling via neuronal nitric oxide synthase (nNOS) activation

Cardiac output in PAH is dependent upon the ability of the heart to increase its rate of contraction. Beta-blockers have been contraindicated from concern over their negative chronotropic effects as well as right ventriculo-arterial uncoupling (De Man et al. 2012). This has been supported by individual case studies of life-threatening syncope induced by beta-blockade (Peacock and Ross 2010).

Aside from effects on the myocardium, beta-blockers may also influence pulmonary vascular tone, but this is not well understood. Carvedilol inhibits proliferation of pulmonary artery smooth muscle cells isolated from patients with idiopathic PAH (Fujio et al. 2006). Recent studies in animal models suggest that further investigation of beta-blockers in PAH is warranted.

4.2 *Animal Studies*

The non-selective $\beta_{1,2}$ -adrenoceptor blocker, propranolol, and the α_1 -adrenoceptor blocker, prazosin, blunt hypoxic pulmonary vasoconstriction and hypoxia-induced pulmonary vascular remodelling (Voelkel et al. 1980; Tual et al. 2006; Bogaard et al. 2010). Recently, it was shown that pulmonary vascular remodelling in the rat monocrotaline model of pulmonary hypertension is attenuated by the α_1/β_1 -adrenoceptor blocker, arotinolol (Ishikawa et al. 2009).

Bogaard and colleagues have studied the effects of carvedilol 15 mg/kg, administered for 4 weeks, in the rat SuHx model of pulmonary hypertension (Bogaard et al. 2010). The beta-blocker reduced heart rate and RV mass without changes in pulmonary vascular remodelling. Interestingly, RV systolic pressure showed no significant changes, but this may be a consequence of enhanced cardiac output with improved RV function. At the molecular level, the reversal of maladaptive RV remodelling in this model was associated with normalisation of vascular endothelial growth factor, Serca2a expression and protein kinase G (PKG) activity and enhanced expression of β -MHC, ANP and antihypertrophic MCIP-1. Exercise capacity was significantly improved in the carvedilol-treated group.

In a follow-on experiment, this group assessed whether improvements in right heart disease were due to the direct blocking of β -adrenoceptors or whether these changes were from the pleiotropic effects from the non cardio-selective properties of carvedilol. Carvedilol was compared to the more selective β_1 -adrenoceptor blocker, metoprolol, in both SuHx and monocrotaline-induced PH rats. Echocardiographic assessment revealed improvement in RV function and attenuation of RV hypertrophy with both drugs, but smaller trends in the metoprolol group. Metoprolol had little effect on mRNA expression of Serca2a and MCIP-1 in the SuHx RV.

De Man et al. investigated the role of chronic low-dose bisoprolol (10 mg/kg) in the monocrotaline rat model (De Man et al. 2012). Echocardiographic measurements showed that bisoprolol delayed right heart failure by slowing down RV dilation and deterioration in cardiac output. The small dose of bisoprolol, enough to blunt heart rate response, showed minimal effects on systemic blood pressure. Bisoprolol significantly delayed time to right heart failure. RV load was not affected. Echocardiography indicated better ventriculo-arterial coupling in the bisoprolol-treated group. No changes in RV capillary density were observed, but RV fibrosis was reduced as were the number of inflammatory (CD45+) cells with bisoprolol treatment. Histological and biochemical analysis demonstrated restoration in both cMyBPC and cTnI contractile proteins compared to the controls.

Niu et al. (2012) studied the effects of β_3 -adrenoceptor agonism on cardiac function and hypertrophy induced by pressure overload. C57BL/6J and neuronal NOS (nNOS) knockout mice underwent transverse aortic constriction (TAC) and were given a β_3 agonist (BRL37344) or placebo. At 3 weeks there was an increase in nitric oxide production with attenuation in left ventricular dilation and systolic function in the β_3 agonist group, with partial reduction in cardiac hypertrophy induced by TAC. This raises the therapeutic potential for targeting this receptor as part of heart failure management in the clinical setting.

4.3 *Clinical Trials*

Several reports have demonstrated that sympathetic activity is increased in patients with pulmonary hypertension (Velez-Roa et al. 2004; McGowan et al. 2009). Beta-blocker use is not uncommon in PAH patients. Around 12 % of subjects in the Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL Registry) are recorded as receiving beta-blockers. A small study assessing ten patients with portopulmonary hypertension suggested that withdrawing propranolol or atenolol improved exercise tolerance (Provencher et al. 2006). A recent prospective cohort study of 94 consecutive patients with PAH compared outcomes in subjects who were and were not taking a beta-blocker at baseline (So et al. 2012). Beta-blocker use was common (28 %) in this cohort. After a median follow-up of 20 months, changes in pulmonary haemodynamics and right ventricular size and function were similar between groups. There were no statistically significant differences in adverse events including PAH-related hospitalisation or all-cause mortality ($p = 0.19$), presence of right heart failure ($p = 0.75$) or change in 6-minute walk distance ($p = 0.92$).

The authors conclude that beta-blocker use was not associated with detrimental effects and that a randomised study to evaluate the potential benefits and risks of this drug class in PAH was needed.

4.4 *Future Prospects*

There remains debate about the value and safety of beta-blockers in PAH, but there is sufficient interest to merit cautious investigation. At least two trials are recruiting patients with idiopathic PAH. In one study around 30 patients will be randomised to bisoprolol in gradually escalating doses or placebo in a crossover design and the effect on right ventricular function closely monitored by cardiac MR (clinicaltrials.gov identifier NCT01246037). In another, patients will receive carvedilol (clinicaltrials.gov identifier NCT01586156). The results of these studies will inform the future of beta-blockers as a treatment for PAH.

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Part IV
Conclusions and Outlook

Pulmonary Hypertension: Current Management and Future Directions

Lewis J. Rubin

Abstract Despite major advances in our understanding of the pathobiology of pulmonary vasculopathy and in the treatment of pulmonary vascular disease, pulmonary hypertension remains a severe condition that frequently progresses to right heart failure and death. This chapter will review several novel approaches to treatment, based on recent observations in both patients and experimental models, that may lead to new and more effective treatment approaches to pulmonary hypertension in the future.

Keywords Pulmonary Artery Hypertension • Therapy • Future

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The progress in elucidating the pathobiology of pulmonary vascular disease over the past two decades, and in particular over the past several years, has been both dramatic and gratifying. While all the details have not been pieced together, our basic understanding of the ways by which the pulmonary vessels can become injured, leading to vascular remodeling, has led to seminal investigations into the genetics of pulmonary vascular disease and the development of novel treatment strategies that have improved the quality of life and probably survival for many patients with pulmonary arterial hypertension (PAH).

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It was not that long ago that therapy for PAH was empirical, based on the assumption that vasoconstriction was the predominant pathogenic factor in the development and progression of this disease. Systemic vasodilators were administered in the hope that these drugs may also have pulmonary vasodilatory properties that would provide therapeutic benefit to PAH patients; while some patients improved, particularly with calcium channel antagonists, most either had no benefit or experienced deterioration as a result of systemic vasodilation with no obvious effect on pulmonary vasomotor tone. Anticoagulants were administered based on the assumption that thrombosis, either in situ or as a result of recurrent asymptomatic embolism, may cause or complicate the disease process, and diuretics were given to maintain fluid balance once right heart failure inexorably developed.

Given the orphan nature of PAH, most studies involved small populations and provided limited insight into optimal management. The NIH Registry on Primary Pulmonary Hypertension (D'Alonzo et al. 1991) was a landmark in the field not only by characterizing the demographic and clinical features of this condition, but also by bringing together for the first time a group of physicians representing several disciplines (cardiology, pulmonary medicine, pediatrics, pathology) to jointly study this complex and mystifying disease. Shortly after, others joined, including geneticists, clinical trial methodology experts, biostatisticians, molecular biologists, clinical pharmacologists, and surgeons, among many others. Soon multicenter and, subsequently, international clinical trials were implemented to prospectively evaluate specific drugs or treatment strategies. These trials have led to the demonstration that the perturbations in three endothelial-derived pathways (endothelin, nitric oxide, and prostacyclin) are targets for therapy, resulting in regulatory approval of drugs that specifically target these pathogenic pathways (Humbert et al. 2004).

These global collaborative networks have also resulted in the publication of guidelines for the diagnostic approach and treatment strategies for PAH (Galiè et al. 2004). While all diagnostic modalities and therapies are not universally available, these documents provide general guidance for all physicians who care for patients with pulmonary hypertension and are based on levels of evidence in the medical literature as well as consensus expert opinion. The importance of such guidelines will likely only increase in the future as novel diagnostic and therapeutic modalities make their way into the medical armamentarium for PAH. Unfortunately, it is unclear whether these widely published guidelines have gained entry into medical practice, and recent registries suggest that many physicians in community practice do not adhere to these guidances for management of patients with this complex disease process (McLaughlin et al. 2012). Recent studies have also suggested that patients are referred late in the course of their illness to experienced centers, and this delay in implementing aggressive treatment strategies impacts on outcome (Badagliacca et al. 2012).

1 Future Directions

Despite substantial improvements in the care of PAH patients, this disease remains incurable. Additionally, the optimal treatment strategy for PAH is not well defined, and many patients are unresponsive or incompletely responsive to medical therapy. Accordingly, there is still much work to be done. The following are my thoughts as to the future directions of work in this field:

1. *How can we optimize the use of currently available therapies?* While we have three classes of drugs that have shown efficacy in PAH (endothelin receptor antagonists, phosphodiesterase-type 5 inhibitors, and prostacyclin analogues), the optimal timing and utilization of these compounds remain unclear. More specifically, which drug or class of drug is the best with which to initiate therapy? Where does combination therapy fit into the scheme, early or as add-on? And which combination(s) are best, or is triple combination therapy superior to double? These questions, addressed in rigorous clinical trials, will ensure that the medications currently available are used to their maximum benefit.
2. *By what cellular mechanisms do the drugs currently available exert their beneficial effects?* Remarkably, after 15 years of availability, we still do not fully understand how prostacyclins exert their potent therapeutic effects in PAH. Some argue that it works as a pure vasodilator, but this seems unlikely since other simple vasodilators, such as calcium channel blockers, have little therapeutic benefit in most patients. Others have suggested an antiproliferative effect of both prostanoids and the other classes of drugs, but this is difficult to prove in the clinical setting. I think it is likely that prostacyclin has inotropic effects on the right ventricle that, combined with other effects on the pulmonary vessels, produces both acute and chronic benefit. Tapping into this unknown territory will lead to the development of novel therapies.
3. *What is the role of newer disease targeted therapies?* Recent trials with novel therapies designed to target specific aberrant signalling pathways in PAH have been disappointing, due to both the marginal treatment effect and the high incidence of serious side effects (Hoepfer et al. 2013). Notably, despite impressive effects in animal models of pulmonary hypertension and several case reports demonstrating dramatic results with the tyrosine kinase inhibitor imatinib in a handful of patients refractory to other therapies, the results of large-scale trials failed to demonstrate a beneficial efficacy:safety ratio. Unknown at this time is whether the problem lies with the drug or with the trial design, but some experts believe that newer multikinase inhibitors may prove more effective and safe. No doubt additional potential targets will emerge in the future. Given the precious commodity of patients eligible and willing to participate in clinical trials of new agents in PAH, we must triage these targeted drugs based on the likelihood of efficacy and evidence of safety. Additionally, we need to thoughtfully develop the design of future trials to take into account currently available therapies and meaningful treatment outcomes. Finally, we

should be cautious in interpreting results of treatments in animal models of PAH, since to date many compounds that have exerted dramatic effects in animals with induced PAH, including statins, tyrosine kinase inhibitors, angiotensin-converting enzyme inhibitors, and others, have failed to demonstrate benefit in human PAH.

4. *Can we therapeutically target the right ventricle?* What kills patients with PAH is not the elevated pressures in the pulmonary vascular circuit, per se, but rather the progressive inability of the right ventricle to deal with the increased afterload, ultimately resulting in right ventricular failure. Accordingly, we have two potential targets in PAH—the vasculopathy and the failing right ventricle. The reversibility of right ventricular failure has been demonstrated after lung transplantation for PAH and pulmonary endarterectomy for chronic thromboembolic pulmonary hypertension (CTEPH), making it unnecessary to perform combined heart–lung transplantation in most PAH conditions. A greater understanding of the roles of impaired contractility and myocardial remodeling in PAH could yield novel approaches to targeted therapy of the failing right ventricle.

2 Conclusions

PAH, once a declaration of imminent death, is now a chronic condition which, for many patients, can be treated with improved quality of life and survival. However, PAH is a rare condition and its optimal management is best provided at expert centers. Nevertheless, those physicians who initially evaluate the PAH patient and participate in their ongoing collaboration with the expert centers need to be familiar with the rapid pace of developments in both early diagnosis and treatment. Finally, novel targets for therapy, such as the pathways for disordered vasoproliferation and myocardial adaptation, should be explored to enhance our treatment strategies and, ultimately, to cure this malignant disease.

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