Chapter 15
Pulmonary Arterial Hypertension and Oxidative Stress

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15.1 Introduction

Pulmonary arterial hypertension (PAH) is a complex and multidisciplinary disorder comprising a series of diseases that result from restricted blood flow through the pulmonary arterial circulation [213, 232]. All of these conditions share a common arterial histopathology characterized by medial hypertrophy, eccentric and concentric intimal fibrosis, and plexiform lesions [114, 213]. The pathophysiology of PAH is not completely understood. Many factors have been shown to be involved in the pathogenesis of PAH, including growth factors, pro-inflammatory molecules, vascular tone mediators, genetic mutations, microRNAs (miRs), and oxidative stress [5, 221, 284]. Currently, the treatment for PAH remains limited and the disease is still associated with a poor long-term prognosis [221]. Growing evidence suggests that reactive

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oxygen species (ROS) and oxidative stress play a pathogenic role in PAH and some antioxidants appear to be useful in various forms of pulmonary hypertension (PH) [373].

15.2 Pulmonary Arterial Hypertension

15.2.1 Epidemiology

PAH was previously considered a rare disease with an unknown frequency, but in 2006 a French registry reported a prevalence of 15 per million [158, 232]. The most common cause found in this study was idiopathic pulmonary arterial hypertension (IPAH) accounting for 39.2 % of the cases, followed by anorexigen exposure, connective tissue disease, congenital heart diseases (CHDs), portal hypertension, and HIV infection [158]. The Scottish morbidity record found a prevalence of 52 cases per million in an adult population [273]. In both studies, PAH was more common in the female population [158]. According to the Centers for Disease Control and Prevention (CDC), deaths attributed to PH varied between 11,000 and 16,000 per year between 1980 and 2002 [159].

15.2.2 Diagnosis and Pathological Findings

15.2.2.1 Signs and Symptoms

The main symptoms found in patients with PH are dyspnea on exertion (around 60 % of patients), fatigue, angina pectoris, syncope, palpitations, and lower extremity edema [232]. Clinical signs include accentuated pulmonary component of S2 audible at the apex (90 % of patients with IPAH), early systolic click, mid-systolic ejection murmur, left parasternal lift, right ventricular (RV) S4, and increased jugular "a" wave [232]. In more advanced stages of the disease, other signs may be seen, including a holosystolic murmur that increases with inspiration, increased jugular "v" waves, pulsatile hepatomegaly, hepatojugular reflex, peripheral edema, ascites, low pulse pressure, and cool extremities [232]. These usually indicate right ventricular (RV) failure [230]. The main chest X-ray finding suggesting PH is enlargement of main and hilar pulmonary arterial shadows accompanied by attenuation of peripheral pulmonary vascular markings [213, 230]. Electrocardiographic findings that should raise the suspicion of PH include right axis deviation, signs of RV hypertrophy (tall R wave in RV leads and R/S ratio <1 in V5 and V6), and right atrial enlargement (tall p wave in leads II, III, and aVF and frontal p axis of more than 75°) [213, 230, 232].

Table 15.1 Arbitrary criteria for estimating the presence of PH based on tricuspid regurgitation peak velocity and Doppler-calculated PA systolic pressure at rest (assuming a normal right atrial pressure of 5 mmHg) and on additional echocardiographic variables

	Class[a]	Level[b]
Echocardiographic diagnosis: PH unlikely		
Tricuspid regurgitation velocity ≤2.8 m/s, PA systolic pressure ≤36 mmHg, and no additional echocardiographic variables suggestive of PH	I	B
Echocardiographic diagnosis: PH possible		
Tricuspid regurgitation velocity ≤2.8 m/s, PA systolic pressure ≤36 mmHg, but presence of additional echocardiographic variables suggestive of PH	IIa	C
Tricuspid regurgitation velocity 2.9–3.4 m/s, PA systolic pressure 37–50 mmHg with/without additional echocardiographic variables suggestive of PH	IIa	C
Echocardiographic diagnosis: PH likely		
Tricuspid regurgitation velocity >3.4 m/s, PA systolic pressure >50 mmHg with/without additional echocardiographic variables suggestive of PH	I	B
Exercise Doppler echocardiography is not recommended for screening of PH	III	C

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[a]Class of recommendation

[b]Level of recommendation

15.2.2.2 Diagnosis and Classification

PAH is defined as a mean pulmonary arterial pressure (mPAP) greater than 25 mmHg at rest with a normal pulmonary capillary wedge pressure (PCWP) of 15 mmHg or less and a pulmonary vascular resistance (PVR) greater than 3 Wood units [232]. Screening is crucial in all patients with risk factors for PAH, such as bone morphogenetic protein receptor 2 (BMPR2) mutation, first-degree relative with BMPR2 mutation, history of anorexigen intake (fenfluramine), HIV infection, portal hypertension, CHD with systemic-to-pulmonary shunt, systemic sclerosis, recent acute pulmonary embolism, and sickle cell disease (SCD) [232]. If clinical, radiologic, and electrocardiographic findings raise the suspicion of PH, a Doppler echocardiogram is the screening test of choice, providing an estimate of the RV systolic pressure and RV function, as well as allowing identification of potential cardiac causes of PH [230, 232]. Common echocardiographic findings seen in patients with PAH include enlargement of right-sided chambers, abnormal surface of the interventricular septum, and underfilled left atrium and left ventricle [232]. The European Society of Cardiology (ESC) and the European Respiratory Society (ERS) proposed a series of arbitrary criteria for establishing the presence of PH based on echocardiographic findings that have been shown to correlate with PH on right heart catheterization (RHC) (Table 15.1) [125]. In cases where a tricuspid regurgitation profile cannot be determined by conventional echocardiography, intravenous saline or encapsulated microbubble contrast agents can be administered to enhance the signal [147, 232]. Patients with abnormal echocardiograms, including RV systolic pressure greater than 40 mmHg, should be further evaluated [232].

Table 15.2 WHO 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. Drugs and toxin-induced
   - 1.4. Associated with
     - 1.4.1. Connective tissue disease
     - 1.4.2. HIV infection
     - 1.4.3. Portal hypertension
     - 1.4.4. Congenital heart disease
     - 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 disease
   - 2.1. Systolic dysfunction
   - 2.2. Diastolic dysfunction
   - 2.3. Valvular disease
3. Pulmonary hypertension owing to lung disease 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 altitudes
   - 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 disease, thyroid disorders
   - 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

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When continuing evaluation of these patients, all causes of PH (including PAH and non-PAH causes) must be considered in order to guide proper management [232]. The revised WHO classification of PH (Dana Point 2008) is shown in Table 15.2 [317]. Although all of the secondary causes of PH should be evaluated before establishing the diagnosis of PAH, excluding chronic thromboembolic pulmonary hypertension (CTEPH) is particularly important because the management of these patients is very different, as some patients may be eligible for surgical treatment

[248], and this condition may coexist in the presence of other risk factors for PAH such as scleroderma [232]. The screening test of choice for ruling out CTEPH is the ventilation/perfusion lung scan, since a normal result virtually rules out this condition [148, 232, 248]. Despite the usefulness of the V/Q scan in patients without underlying lung disease, pulmonary multidetector CT angiography (MDCTA) is now considered the gold standard for the diagnosis of CTEPH because it allows identification of thrombosis, concomitant lung changes, and can aid in the diagnosis of pulmonary embolism in patients with preexisting lung disease [176]. Even though Doppler echocardiography aids in the detection of possible PH, the only way to confirm the diagnosis is through RHC [232, 248]. Once left ventricular or valvular disease (Group 2), lung disease (Group 3), and CTEPH (Group 4) are excluded, a RHC showing a mPAP greater than 25 mmHg and a PVR greater than 3 Wood units with a normal PCWP <15 mmHg confirm the presence of PAH, which means that it remains a diagnosis of exclusion [232].

The most recent classification of PH was established in the fourth World Symposium on Pulmonary Hypertension that was held in Dana Point in 2008 [317]. Patients with PAH should be classified into one of the five groups shown in Table 15.2 [317].

Idiopathic Pulmonary Arterial Hypertension and Heritable Pulmonary Arterial Hypertension: Groups 1.1 and 1.2

IPAH is sporadic and unrelated to any family history or identified risk factor [317]. Heritable PAH is diagnosed when there are mutations of genes that have been identified as having a strong association with the PAH phenotype, such as the *BMPR2* gene, which is present in 70 % of heritable cases. Other mutations that have been identified in patients with PAH are located in the activin receptor-like kinase type 1 (*ALK1*) or endoglin (*ENG*) genes [248, 317]. Some studies have also suggested that mutations in the Smad proteins and caveolin-1 (*CAV1*) genes may also predispose to PAH [9, 18, 28, 259, 316]. It is critical that these patients get involved in a comprehensive program that includes genetic testing, counseling, and discussion of risks and benefits [21, 317].

Drug and Toxin-Induced PAH: Group 1.3

Drug and toxin-induced PAH is further classified depending on the strength of the association between the exposure and the presence of disease, but the main substances that have been found to have a strong association with PAH are anorexigens (aminorex, fenfluramine) and toxic rapeseed oil. Other agents that have been related to PAH include cocaine, phenylpropanolamine, St. John's Wort, chemotherapeutic medications, selective serotonin reuptake inhibitors (SSRIs), and amphetamines [317]. However, further studies are needed to establish the true association of these latter substances.

Associated with PAH: Group 1.4

Associated with PAH (APAH) includes connective tissue disorders, congenital systemic-to-pulmonary shunts, portal hypertension, HIV infection, schistosomiasis, and chronic hemolytic anemia [232].

PAH Associated with Connective Tissue Diseases: Group 1.4.1

The presence of PAH has been well established in systemic sclerosis, with an estimated prevalence of 7–12 % and is associated with poor prognosis in this group of patients [138, 248, 253]. The presence of PAH has also been reported in systemic lupus erythematosus (SLE) and mixed connective tissue disease, but the exact prevalence has not been determined [317]. Other mechanisms may be involved in the induction of PH in these patients, such as left heart dysfunction, lung fibrosis, and primary cardiac involvement, which highlights the importance of determining the true cause of PH with RHC.

PAH Associated with HIV Infection: Group 1.4.2

The presence of PAH in patients with HIV infection is rare, with a prevalence of 0.5 % [28, 317]. Clinical, hemodynamic, and histological findings are very similar to those seen in IPAH patients [28, 317]. Concomitant PAH in patients with HIV significantly worsens their prognosis [243].

Porto-pulmonary Hypertension: Group 1.4.3

PAH associated with an increase in the pressure of the portal circulation is classified as porto-pulmonary hypertension (POPH) [248]. Some prospective studies have shown a prevalence of 5–6 % in patients with advanced liver disease [303]. POPH is also a predictor of poor prognosis, since these patients are usually not eligible for liver transplantation due to the high perioperative morbidity and mortality that have been documented in this population [303]. RHC should be performed to accurately diagnose PAH, since other factors, such as fluid overload and diastolic dysfunction, may elevate the pressure of the pulmonary vasculature in patients with portal hypertension [317].

Congenital Heart Diseases: Group 1.4.4

PAH is a fairly common complication of CHD in patients that have left-to-right shunts [81, 317]. It is estimated that 4–15 % of patients with CHD will develop PAH [81] and the most common anomalies associated with PAH are ventricular septal defects (VSD) [104]. Patients with CHD who develop PAH are classified into four groups: Eisenmenger's syndrome, PAH associated with systemic-to-pulmonary shunts, PAH with small defects, and PAH after corrective cardiac surgery [81, 317]. Eisenmenger's syndrome is the most severe form of PAH in this context, where there is a reversal of the initial shunt to a right-to-left shunt, where deoxygenated

blood is being returned to the systemic circulation and cyanosis ensues along with other potential complications such as blood hyperviscosity, hemostasis, stroke, and endocarditis [81].

Schistosomiasis: Group 1.4.5

Before the Dana Point classification of PH, schistosomiasis was listed under the subgroup of chronic thrombotic or thromboembolic disease. Nevertheless, recent evidence has shown that the obstructive mechanism of schistosoma eggs plays a minor role in the induction of PH in this group of patients, and clinical and pathological findings resemble those of IPAH [248, 317]. Although the exact mechanisms responsible for the induction of PH in patients with schistosomiasis remain largely unknown, the inflammatory response to the schistosoma antigens with the release of cytokines that have also been proven to be upregulated in IPAH, as well as the presence of hepatosplenic disease and portal hypertension likely plays an important role [133]. For these reasons, schistosomiasis is now listed under Group 1 of the Dana Point Classification [248, 317].

Chronic Hemolytic Anemia: Group 1.4.6

PAH has been identified as a complication of many hemolytic anemias including SCD, thalassemia, hereditary spherocytosis, stomacytosis, and microangiopathic hemolytic anemia [317]. Histological findings seen in IPAH have been commonly described in patients with SCD [317]. However, the true prevalence of PAH in these patients remains unknown since most epidemiological studies have defined the presence of PH in terms of echocardiography rather than RHC [317]. Such studies have documented a prevalence of 20–30 % in patients with SCD and 10–75 % in patients with thalassemia [219]. The pathophysiology of PAH induced by hemolysis is not entirely understood, but mechanisms such as inactivation of nitric oxide (NO) by free hemoglobin, depletion of L-arginine in the presence of elevated arginase, and increased endothelin-1 (ET-1) responses have been described [110, 250, 301].

Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) are rare conditions that were included in Group 1 of the most recent WHO classification of PH (Dana Point 2008) [317]. This inclusion was based on the similarities of PVOD/PCH and PAH regarding histologic findings, clinical presentation, risk factors, and potential for inheritance [317]. Nevertheless, they are still considered separate conditions classified as 1′ (Table 15.2) [317].

As discussed above, PAH is a diagnosis of exclusion and both PAH and non-PAH causes of PH may overlap. Therefore, it is crucial to evaluate and classify patients based on their etiology of PH and WHO group (Table 15.2), and confirm that the elevated pressure is limited exclusively to the pulmonary arterial system [90]. This can only be accomplished with a RHC, which remains an indispensable tool in the assessment of patients with PH [90]. Additionally, this test gives further information that is useful to determine prognosis, such as the severity of the hemodynamic impairment and the vasoreactivity of the pulmonary circulation [125]. The diagnostic PH algorithm established by the American College of Cardiology Foundation/American Heart Association Task Force (ACCF/AHA) experts can be found in Fig. 15.1 [125, 232].

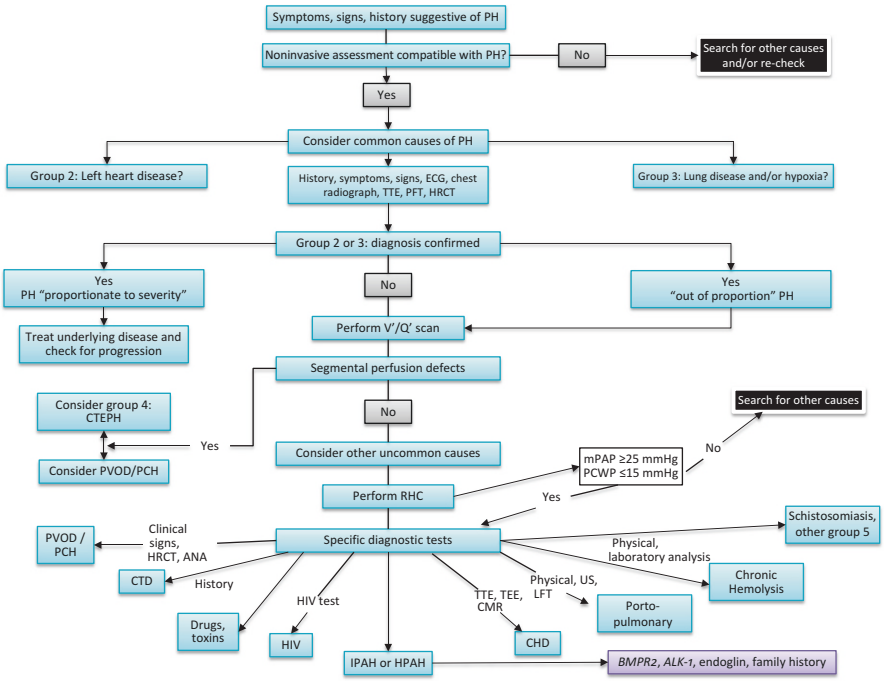

Fig. 15.1 Diagnostic algorithm for pulmonary hypertension. Reproduced with permission from [125]

15.2.2.3 Gold Standard and Pathological Findings

The gold standard for the diagnosis of PAH is the RHC since it is defined by hemodynamic criteria [125, 232]. Lung biopsy in patients with PAH is not recommended, since it has a high morbidity and mortality in this group of patients and is unlikely to change the diagnosis or treatment [125]. Therefore, the natural history of vascular lesions that occur in PAH is not entirely known because biopsies are not regularly obtained in these patients [232]. Arterial abnormalities seen in histological studies of patients with PAH include intimal hyperplasia, inflammation, adventitial proliferation, medial hypertrophy, thrombosis in situ, abnormal muscularization of non-muscular precapillary arteries, and plexiform arteriopathy [232, 284].

15.2.2.4 Prognosis

Despite a better understanding of the pathophysiological mechanisms involved in PAH and the improvement in treatment options, the long-term prognosis remains poor [232]. Data from the French Network on Pulmonary Hypertension Registry revealed a survival rate of 83 % (95 % CI 72–95 %) at 1 year, 57 % (95 % CI 57–79 %) at 2 years, and 58 % at 3 years [158].

15.2.3 Pathophysiology

PH results from an increase in PVR and restriction in blood flow through the pulmonary vascular circulation, finally leading to altered right heart function [232]. Elevation of PVR and decreases in pulmonary vascular compliance cause increased RV afterload, which ultimately results in adaptive RV hypertrophy [221]. If the pressure overload persists, the RV eventually dilates and becomes dysfunctional, leading to increase in RV contraction time, asynchrony, and decreased RV stroke volume [221]. All of the latter changes result in underfilling of the left ventricle (LV) and subsequent reduction in cardiac output [126, 221, 223].

The main cause of elevated PVR is the reduction in luminal cross section due to vascular remodeling, which results from altered cell growth, apoptosis, migration, and production of extracellular matrix [5, 232]. Various stimuli can induce vascular remodeling, including mechanical forces (changes in transmural pressure, stretch, shear stress), inflammatory cytokines, serotonin (5-hydroxytryptamine [5-HT]), hypoxia, growth factors, angiotensin II (AT-II), endothelin-1 (ET-1), increased serine elastase activity, and increased production of ROS [5]. All of these stimuli induce changes in different cells that are responsible for the changes seen in vascular remodeling, mainly endothelial cells (EC) and smooth muscle cells (SMC) [5].

15.2.3.1 Pulmonary Arterial Endothelial Cells

Pulmonary arterial endothelial cells (PAEC) that are exposed to injury caused by the various stimuli mentioned above may become dysfunctional and respond in ways that contribute to vascular remodeling [5]. This remodeling occurs through the release of agents that stimulate proliferation of pulmonary arterial smooth muscle cells (PASMC), such as platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2) and/or failure to produce factors that suppress proliferation of PASMC, such as apelin [284]. Furthermore, PAEC from patients with IPAH have increased expression of the Tie2 receptor, which results in increased production of 5-HT and subsequent PASMC proliferation (Fig. 15.2) [5, 91, 284]. Moreover, dysfunctional PAEC seen in PH generate less nitric oxide (NO) as a result of uncoupling of endothelial NO synthase (eNOS), which ultimately leads to an increase in the production of ROS, particularly superoxide (Fig. 15.2) [5]. The effect of ROS in pulmonary vascular remodeling is further discussed in the next section. Uncoupling of eNOS is related to low levels of enzymatic cofactors L-arginine and tetrahydrobiopterin (BH_4) [200]. L-Arginine depletion results from the upregulation of arginase, which has been documented both in animal and human EC exposed to different stimuli, including hypoxia, lipopolysaccharide (LPS), shear stress, and inflammatory cytokines [105]. Increased asymmetric dimethylarginine (ADMA) has also been found to be elevated in patients with PH [5, 312]. ADMA is an endogenous analogue of L-arginine and competes for the substrate binding site of eNOS, which can further contribute to the uncoupling of the enzyme [5, 284].

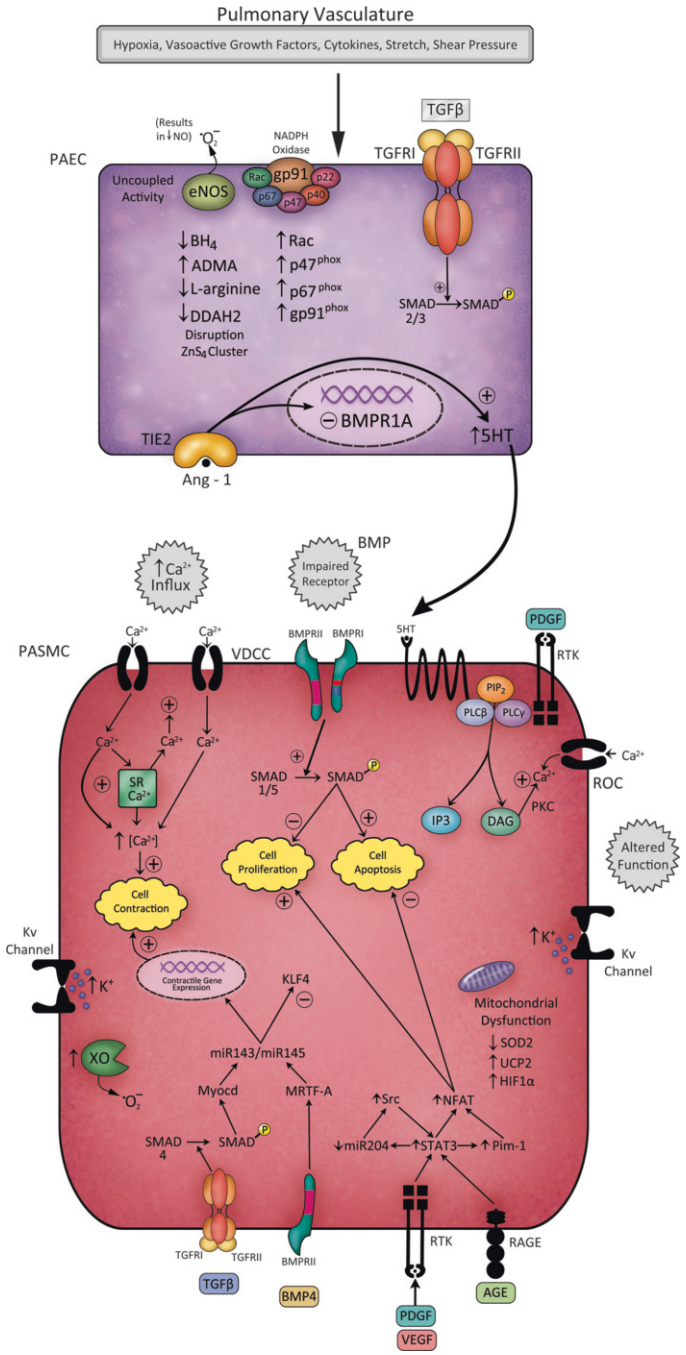

Fig. 15.2 Overview of mechanisms involved in the pathogenesis of PAH. Diverse stimuli result in endothelial dysfunction and abnormal PASMC proliferation. Decreased NO production in PAEC due to eNOS uncoupling attenuates relaxation of PASMC and promotes vasoconstriction. Factors that contribute to eNOS uncoupling include decreased arginine, increased ADMA, enhanced arginase activity, low BH_4, and disruption of the zinc tetrathiolate (ZnS_4) cluster.

ADMA has also been shown to contribute to mitochondrial dysfunction through the increase of uncoupling protein-2 (UCP2), which leads to augmented mitochondrial ROS (mROS) production and decreased ATP synthesis (Fig. 15.2) [5, 329].

In addition to decreased synthesis of the vasodilator NO, dysfunctional endothelial cells also produce lower levels of prostacyclin, and higher levels of vasoactive substances such as ET-1, AT-II, and thromboxane A_2 (TXA_2), and growth factors, namely PDGF, transforming growth factor β (TGF-β), FGF-2, and vascular endothelial growth factor (VEGF) [5, 100, 227, 361]. All of these may stimulate PASMC proliferation in vascular remodeling [5]. Finally, PAEC from patients with PAH seem to have increased glycolytic activity and a highly proliferative response to growth factors, which contributes to the formation of plexiform lesions [5, 284, 382]. PAEC seen in these lesions exhibit increased levels of hypoxia-inducible factor (HIF) subunits (HIF-1α and HIF-1β), which induce VEGF under hypoxic conditions [5, 342].

Elevated expression of VEGF and VEGF receptor 2 (VEGFR2) has been documented in plexiform lesions of patients with PAH [221, 342]. VEGF promotes survival and suppresses apoptosis in PAEC [221, 305]. However, mice and rats exposed to hypoxia combined with the VEGFR2 inhibitor, SU5416, develop PAH [221, 353]. Moreover, VEGF is decreased in the monocrotaline (MCT) rat model of PAH,

Fig. 15.2 (continued) The eNOS uncoupling not only results in lower NO levels but also increases ROS production. Upregulation of NADPH oxidase subunits further contributes to the generation of ROS. Altered function of potassium Kv channels in PASMC leads to membrane depolarization and opening of voltage-dependent calcium channels. Influx of calcium ions stimulates additional release of Ca^{2+} from the SR. Increased $[Ca^{2+}]_{cyt}$ and upregulated membrane receptors (5-HT, ET-1, leukotrienes) decrease apoptosis and stimulate cell proliferation. Increased Ang-1 downregulates BMPR1A in PAEC and enhances 5-HT production, promoting PASMC contraction and proliferation. As a result of BMPRII mutations, PASMC display dysfunctional BMP signaling pathways, which normally inhibit cell proliferation and stimulate cell apoptosis. Mitochondrial dysfunction leads to increased ROS production and is evidenced by the low levels of SOD2, high levels of UCP2, and impaired function of complexes I and II. Increased activity of XO also results in higher production of ROS. Increased expression of the STAT3/Pim1/Src/NFAT axis and suppression of miR-204 also promote cellular proliferation and reduce apoptosis. TGF-β and BMP4 increase the expression of miR-143/miR-145 through the stimulation of Myocd and MRTF-A, respectively. These miRNAs inhibit KLF4 which ultimately results in enhanced contractile gene expression. *PAEC* pulmonary arterial endothelial cells, *eNOS* endothelial nitric oxide synthase, *NADPH* nicotinamide adenine dinucleotide phosphate, *TGF-β* transforming growth factor β, *TGFRI* type I receptor for TGF-β, *TGFRII* type II receptor for TGF-β, BH_4 tetrahydrobiopterin, *ADMA* asymmetric dimethylarginine, *DDAH2* dimethylaminohydrolase-2, *TIE2* tyrosine protein kinase receptor, *Ang-1* angiopoietin, *BMP* bone morphogenetic protein, *BMPR1A* BMP receptor 1A, *BMPRI* BMP type I receptor, *BMPRII* BMP type II receptor, *5-HT* 5-hydroxytryptamine, *PASMC* pulmonary arterial smooth muscle cells, *VDCC* voltage-dependent calcium channel, *PIP2* phosphatidylinositol 4,5-bisphosphate, *PLC* phospholipase C, *IP3* inositol triphosphate, *DAG* diacylglycerol, *PKC* protein kinase C, *ROC* receptor-operated calcium channel, *SR* sarcoplasmic reticulum, *Kv channel* voltage-gated potassium channel, *SOD2* superoxide dismutase 2, *UCP2* uncoupling protein-2, *HIF-1α* hypoxia-inducible factor α, *XO* xanthine oxidase, *RAGE* receptor for advanced glycation endproducts, *AGE* advanced glycation endproducts, *RTK* receptor tyrosine kinase, *PDGF* platelet-derived growth factor, *VEGF* vascular endothelial growth factor, *STAT3* signal transducer and activator, *NFAT* nuclear factor of activated T-cells, *MRTF* myocardin-related transcription factor, *Myocd* myocardin, *KLF4* Krüppel-like factor 4

which correlates with early endothelial injury. Overexpression of VEGF also protects against chronic hypoxia and MCT exposure, and VEGFR inhibition results in initial EC apoptosis with subsequent selection of EC clones that are resistant to apoptosis and form angio-obliterative lesions [221, 353]. Therefore, VEGF appears to play a crucial role in angiogenesis and EC growth after vascular injury. Other factors associated with plexiform lesions are angiopoietin 1, 5-lipoxygenase, survivin, and Ki-67 [5, 129, 131, 375]. However, the exact mechanisms responsible for the formation of plexiform lesions are not completely understood [5].

15.2.3.2 Pulmonary Arterial Smooth Muscle Cells

Many pathologic changes take place in the SMC layer of PAs during vascular remodeling. Proximal vessels usually undergo significant hypertrophy, while smaller resistance vessels commonly show hyperplasia [5, 231, 238]. Matrix protein deposition is also a characteristic feature of the muscular layer of PAs in PAH, where SMC seem to acquire a more synthetic, rather than contractile, phenotype, with larger endoplasmic reticula and Golgi apparatus, and increased production of collagen and elastin [5, 238]. Muscularization of otherwise nonmuscular blood vessels results from differentiation of pericytes into SMC and hypertrophy of SMC precursor cells [5, 284].

Factors that have been identified in the induction of SMC hypertrophy include bone morphogenetic protein 4 (BMP4), TGF-β1, 5-HT, ET-1, inhibition of glycogen synthase kinase 3β (GSK-3β), and activation of p70S6 kinase [5, 174]. Abnormal activation of transcription factors (HIF-1α and nuclear factor of activated T-cells [NFAT]), increased expression of survivin and PDGF, calcium overload, mitochondrial hyperpolarization, and decreased expression of voltage-gated potassium channels (Kv) all contribute to the increased survival and decreased apoptosis of PASMC seen in PAH (Fig. 15.2) [221, 232].

Finally, in vitro studies have shown that PASMC from PAH patients have higher mRNA and protein levels of Notch 3 and HES-5 [221]. Notch participates in vasculogenesis, angiogenesis, and differentiation of vascular SMC [11, 221]. HES-5, a target gene for Notch 3, is exclusively expressed in adult SMC and may be involved in SMC maturation and proliferation [53, 96, 221, 279].

15.2.3.3 Neointima Formation

The formation of a layer of cells and extracellular matrix between the endothelium and the internal elastic lamina occurs in severe PH [5, 387]. The neointima is composed of myofibroblasts that express SM markers such as smooth muscle α-actin and vimentin [5]. These cells lack markers of highly differentiated SMC, such as SM-myosin heavy chain, and do not exhibit EC markers either [5, 387]. The exact origin of these cells is unclear. They may originate in stem cells, transdifferentiation of endothelial cells, migration of SMC from the media, or migration of adventitial fibroblasts [5, 284]. This currently remains a subject of intense study [284].

15.2.3.4 Changes in the Adventitia

PAH is associated with thickening and disorganization of the pulmonary adventitia, with excessive activation of adventitial metalloproteases [232]. In patients with PAH related to collagen vascular diseases such as scleroderma, the adventitia appears markedly remodeled [5]. Activation of fibroblasts by different stimuli can induce a phenotypic change in these cells, altering their structure and functional behavior [5]. An example of this is the induction of a contractile phenotype in fibroblasts by TGF-β1 and TGF-β2 [5, 387]. The activation and proliferation of fibroblasts and myofibroblasts result in thickening of the adventitia in PH, and some studies have shown that these changes precede remodeling of the intima and SMC layer, which suggests that the initial detection of vascular injury might take place in the adventitia [5, 146].

15.2.3.5 Genes and Transcription Factors Involved in PAH

Genes associated with PAH have helped to identify potential mechanisms involved in the pathogenesis of the disease. Studies have shown that approximately 70 % of patients with heritable pulmonary arterial hypertension (HPAH) and 10–20 % of patients with IPAH are heterozygous for a mutation in *BMPR2*, which is a member of the TGF-β superfamily of growth factor receptors [284]. HPAH is inherited in an autosomal dominant fashion with incomplete penetrance and genetic anticipation [232]. The impaired function of the BMPR2 results in a loss of function of the SMAD signaling pathway, causing proliferation and decreased apoptosis of PASMC in response to TGF-β and BMP2 (Fig. 15.2) [232]. On the other hand, BMPR2 impairment in EC results in increased susceptibility to apoptosis, which alters the normal migration and survival of EC needed in angiogenesis and regeneration of damaged blood vessels (Fig. 15.2) [85, 284]. Abnormal BMPR2 signaling has also been associated with increased ET-1 production in human lung microvascular EC [221, 324].

Recently, signal transducer and activator of transcription 3 (STAT3) has been shown to participate in aberrant PASMC proliferation [221, 272]. IL-6, TGF-β, PDGF, VEGF, ET-1, and AT-II can activate STAT3, which in turn increases the expression of Pim1 (Fig. 15.2) [221, 272, 390]. PIM1 promotes the activation of NFAT, increasing cytokine secretion, enhancing PASMC proliferation, and suppressing PASMC apoptosis (Fig. 15.2) [221, 287]. STAT3 has also been implicated in induction of survivin expression through activation of Krüppel-like factor 5 (KLF5) and in downregulation of eNOS expression (Fig. 15.2) [74, 221].

Moreover, studies have shown that mice with deletion of the peroxisome proliferator-activator receptor gamma (PPAR-γ) gene develop spontaneous PAH [136], and mutations in this gene have also been identified in patients with severe PH [5, 12, 284]. PPAR-γ participates in the antiproliferative effect of BMP2 signaling in PASMC, which is BMPR2/PPAR-γ/ApoE dependent [8, 140, 221]. The receptor of advanced glycation end products (RAGE) is an upstream target of PPAR-γ in PAH, and has been shown to activate STAT3 and downregulate BMPR2

and PPAR-γ in PAH-PASMC (Fig. 15.2) [221, 236]. Furthermore, BMP2-mediated survival of PAEC depends on the formation of a nuclear complex between β-catenin and PPAR-γ [8]. One of the transcriptional targets of this complex is apelin, which is reduced in patients with IPAH [8]. Apelin promotes PAEC survival and migration, and suppresses PASMC growth [284]. Apelin-deficient PAEC have increased apoptosis and promote PASMC proliferation [8, 221]. Other genes that have been associated with the PAH phenotype include *ALK1*, *ENG*, and *CAV1* [18, 59, 142, 218, 221].

15.2.3.6 MicroRNAs Involved in PAH

miRs are now of great interest in the study of diseases that display abnormal cell growth, since they are involved in various posttranscriptional regulatory mechanisms [221]. In PAH, only few miRs have been identified as being abnormally expressed [221]. Downregulation of miR-204 in PAH-PASMC was found to correlate with PAH severity and higher cell proliferation [74]. It was shown that downregulated levels of miR-204 enhance a constitutive activation of Src and STAT3, leading to an increase in PASMC proliferation (Fig. 15.2) [74]. Additionally, downregulation of miR-204 appears to upregulate IL-6 secretion, which in turn downregulates BMPR2 and further contributes to the proliferative phenotype of PAH-PASMC [221, 272]. IL-6 is a potent activator of STAT3, which means that these interactions result in a feed-forward loop between miR-204 downregulation and STAT3 (Fig. 15.2) [221].

Src and p53 pathways regulate the organization of miR-145 and miR-143, which are involved in SMC differentiation and proliferation [221, 283]. TGF-β and BMP4 stimulate the expression of myocardin (Myocd) and Myocd-related transcription factor A (MRTF-A), respectively. These factors in turn activate miR-143 and miR-145 transcription, resulting in decreased KLF4 expression and promotion of contractile gene expression in SMC (Fig. 15.2) [83, 221]. Plexiform and concentric lesions seen in patients with PAH display abnormal expression of miR-143/miR-145 and mice exposed to hypoxia show elevated levels of miR-145 [54, 221].

In PAEC, expression of miR-126 appears to be dysregulated specifically in plexiform lesions [36, 221]. This miR plays an important role in neovascularization, EC proliferation, and vascular integrity, and regulates factors involved in apoptosis and modulation of cell cycle arrest [221, 355, 391]. Other miRs that have been found to contribute to the pathogenesis of PAH include miR-150, which is reduced in patients with PAH and is associated with decreased NK cells and B1 cell expansion; miR-210, the miR most highly upregulated by hypoxia [195, 221]; miR-21, which is highly upregulated in hypoxia and appears to participate in abnormal proliferation and migration of PASMC [221]; and miR-17, which is also upregulated in hypoxia, and targets p21 and Janus kinase (JAK1) impairing angiogenic functions of endothelial cells [221]. miRs remain a subject of intense study, since they are regarded as useful biomarkers, prognostic tools, and potential targets for future therapies [221].

15.3 Oxidative Stress and PAH

Several studies have implicated oxidative stress in the pathogenesis of PAH. Oxidative and nitrosative stress are characterized by an imbalance between oxidant and antioxidant production that can lead to downstream cell and tissue damage. Oxidative stress in PAH is associated with increased production of ROS and reactive nitrogen species (RNS), decreased nitric oxide (NO) levels, and mitochondrial dysfunction. Dysregulation of ROS/RNS/NO homeostasis can impair vascular tone and lead to activation of antiapoptotic and mitogenic pathways resulting in cell hyperproliferation and obliteration of the vasculature in PAH.

ROS are produced from oxygen during normal metabolic processes. ROS can be characterized as either free radicals, reactive molecules with one or more unpaired electrons, or nonradicals, molecules which share unpaired electrons between two free radicals [34] (Table 15.3). Hydroxyl radical ($^{\bullet}OH$) is considered the most reactive free radical in biological systems [335]. In the lung, ROS can be generated by alveolar epithelial cells, endothelial cells, alveolar macrophages, neutrophils, and eosinophils. In the pulmonary vasculature, ROS can be produced by complexes in the cell membrane, within mitochondria and peroxisomes, and from within the cytoplasm. The major enzymatic sources of ROS include uncoupled eNOS, xanthine oxidase (XO), nicotine adenine dinucleotide phosphate (NADPH) oxidase (NOX), and mitochondrial electron transport enzymes (Fig. 15.3). RNS are various nitrogen-containing species (Table 15.3) that can alter protein function via S-nitrosylation, tyrosine nitration, and glutathionylation. NO is the predominant source of nitrosative stress and, at high concentrations, can react with ROS to generate other RNS, including peroxynitrite, $ONOO^{-}$.

Table 15.3 Major oxidants

Oxidative stress			
Free radicals		**Nonradicals**	
Hydroxyl radical	$OH^{\bullet}$	Hydrogen peroxide	H_2O_2
Superoxide anion	$O_2^{\bullet -}$	Hypochloric acid	HOCl
Peroxyl radical	$ROO^{\bullet}$	Ozone	O_3
Hydroperoxyl radical	$HOO^{\bullet}$	Lipid peroxide	LOOH
Lipid peroxyl	$LOO^{\bullet}$		
Nitrosative stress			
Nitric oxide	$NO^{\bullet}$		
Peroxynitrite anion	$ONOO^{-}$		
Nitrogen dioxide	NO_2		
Nitrite	NO_2^{-}		
Nitrate	NO_3^{-}		

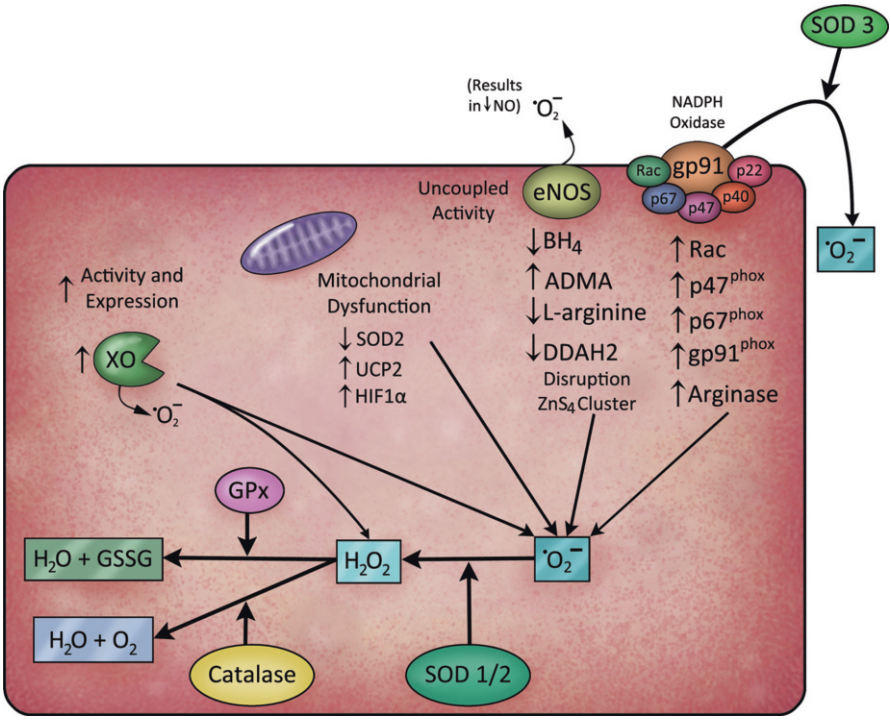

Fig. 15.3 Overview of the mechanisms involved in ROS production and antioxidant mechanisms that counterbalance this oxidative stress. eNOS uncoupling due to decreased arginine, increased ADMA, enhanced arginase activity, low BH_4, and disruption of the zinc tetrathiolate (ZnS_4) cluster results in increased production of superoxide. Upregulation of NADPH oxidase subunits and xanthine oxidase further contributes to the generation of ROS. Superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide. Hydrogen peroxide is reduced by catalase and glutathione peroxidase. *XO* xanthine oxidase, *SOD2* superoxide dismutase 2, *UCP2* uncoupling protein-2, *HIF-1α* hypoxia-inducible factor **α**, *BH_4* tetrahydrobiopterin, *ADMA* asymmetric dimethylarginine, *DDAH2* dimethylaminohydrolase-2, *NADPH* nicotinamide adenine dinucleotide phosphate, *SOD* superoxide dismutase, *GPx* glutathione peroxidase, *GSSG* glutathione disulfide

15.3.1 Mediators and Molecular Mechanisms of Oxidative Stress in PAH

15.3.1.1 Nitric Oxide Dysregulation

NO is a gaseous lipophilic free radical and primary pulmonary vasodilator produced and released by the endothelium. In addition to regulating vascular tone, NO attenuates platelet aggregation and inhibits vascular SMC proliferation and migration within the vascular wall [404]. NO is biosynthesized during the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes called nitric oxide synthases (NOS). Three different isoforms of NOS have been identified including

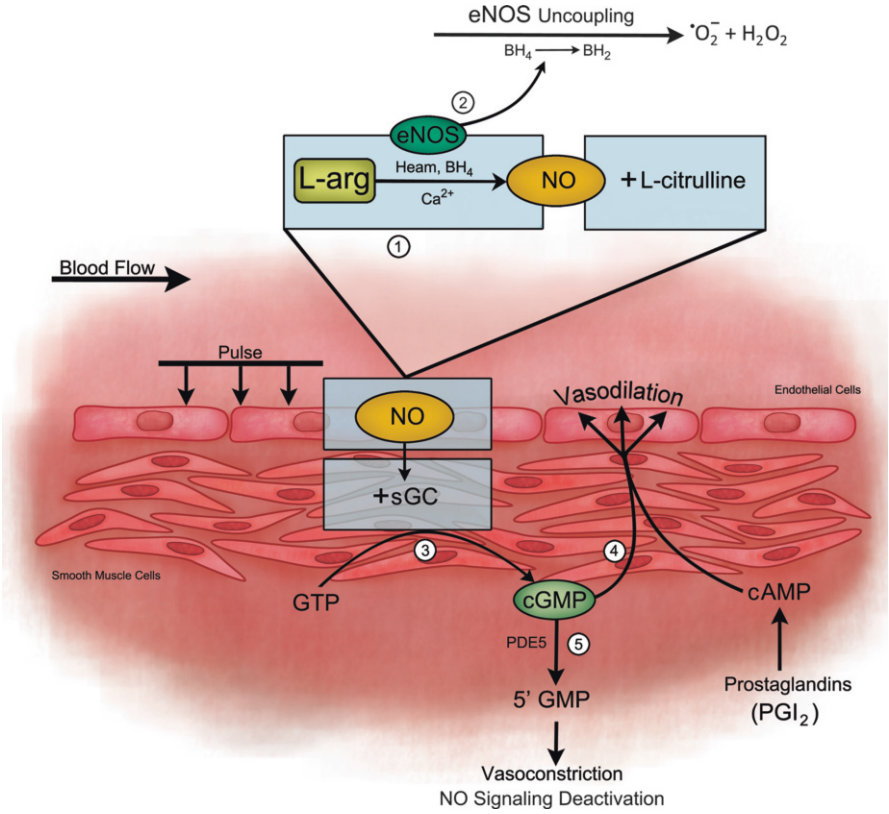

Fig. 15.4 Nitric oxide signaling in PAH. Oxidative stress and nitric oxide (NO) dysregulation in the pathogenesis of PAH. (1) Biosynthesis of NO from the amino acid L-arginine by the enzyme endothelial nitric oxide synthases (eNOS) with L-citrulline as a side product and important cofactors such tetrahydrobiopterin (BH_4), calcium, and heme. (2) Uncoupling of eNOS—when cofactors are limited and there is production of ROS, superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). (3) Binding of NO to its target protein, soluble guanylate cyclase (sGC) and conversion of guanosine triphosphate (GTP) to cGMP resulting in blood vessel dilation (4). (5) Cleavage of cGMP by PDE5 into 5′GMP leading to inhibition of NO signaling resulting in vessel contraction

neuronal NOS (nNOS), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS). The production of NO by NOS requires NADPH and O_2, as well as the cofactors tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and Ca^{2+}/calmodulin (CaM) [52, 220] (Fig. 15.4).

After release from endothelial cells, NO binds to soluble guanylate cyclase (sGC) in vascular cells and converts guanosine triphosphate (GTP) to cGMP, which leads to activation of downstream cGMP-dependent signaling [77, 270]. cGMP is a transient signaling molecule, as it is rapidly cleaved by phosphodiesterases (PDEs), predominantly PDE5, into 5′GMP, thereby inhibiting NO signaling (Fig. 15.4). Although eNOS-derived NO is primarily responsible for endothelium-dependent vasodilation, iNOS has also been shown to regulate pulmonary vascular tone [111, 113].

In mice, deletion of eNOS results in systemic hypertension [157] and mild PH [326], while eNOS overexpression leads to systemic hypotension [130, 265]. Exposure of eNOS-deficient mice to chronic hypoxia exacerbates PH and right ventricular hypertrophy (RVH) [327] and administration of inhaled NO attenuates hypoxia-induced PH, RVH, and vascular remodeling in rats [192, 297, 300]. In addition, recent findings demonstrate that endothelial-like progenitor cells (ELPC) expressing eNOS reverse MCT-induced PH [395] and attenuate right ventricular systolic pressure (RVSP) and pulmonary arterial muscularization in a lung lobectomy model of PH [366]. Taken together, these findings suggest a critical role for dysregulation of eNOS-derived NO in the pathogenesis of PAH.

While there is general consensus that NO signaling is impaired in PAH, it remains unclear whether this is primarily due to reduced synthesis, decreased bioavailability, decreased responsiveness, or increased consumption of NO. Some studies have demonstrated attenuated bioavailability of NO via hemoglobin and superoxide scavenging [154] or by increased hemolysis in fatal PAH [156].

15.3.1.2 eNOS Regulation

NO synthesis and bioavailability in the pulmonary vasculature are dependent upon the regulation of eNOS [60]. eNOS expression is controlled by two regulatory regions, the positive regulatory domains I and II, and its transcription is regulated by many cofactors acting by complex *cis* and *trans* interactions [309]. Additionally, methylation of nucleotides in those regions specifies vascular endothelial cell expression of eNOS [55]. Following eNOS protein translation, its compartmentalization activity is regulated by phosphorylation of specific serine and threonine residues [42, 43, 69, 194, 252], as well as additional posttranslational modifications (myristoylation and palmitoylation) which allow for eNOS localization to the plasma membrane and subsequent targeting to caveolae [263], where caveolin-1 (Cav-1) regulates intracellular NO signaling [255].

In addition to the Cav-1/caveolae trafficking system [145, 255, 302], the chaperon Hsp90 has also been identified as a regulator of eNOS activity by its rapid binding upon EC activation [386]. One possible mechanism of this regulation is through interaction of eNOS and Hsp90 with CaM. Following VEGF stimulation of EC, there is disruption of the Ca^{2+}/CaM-dependent eNOS/Cav-1 complex and promotion of Hsp90 and eNOS association. The Hsp90/eNOS complex is then triggered for VEGF-activated Akt-dependent phosphorylation of eNOS [49, 336]. Prolonged exposure of cells to Ca^{2+} results in degradation of eNOS and Hsp90, followed by a decrease in NO production [19]. It has also been shown that Hsp90 as an adaptor protein binds eNOS to sGC, allowing cGMP signaling to take place and facilitating responses to NO donors [350, 386].

15.3.1.3 Uncoupling of eNOS in PAH

In addition to impaired NO signaling in the pathobiology of PAH, "eNOS uncoupling" in conditions of substrate/cofactor deficiency or RNS production in the setting of NO excess can lead to decreases in NO bioavailability and increases in

oxidative stress with downstream alterations in vascular tone and aberrant vascular remodeling. eNOS uncoupling can occur in the setting of BH_4 or L-arginine deficiency [196, 200] and results in a shift from NO synthesis to other ROS production with resultant endothelial dysfunction [72] (Fig. 15.4). All three isoforms of NOS contain an oxygenase and a reductase domain, each of which has its own catalytic activity. The oxygenase domain has binding sites for heme and BH_4, while the reductase domain has binding sites for FAD, FMN, and NADPH. Both domains are linked by the binding site for CaM, an important regulator of NOS function.

For the formation of NO from L-arginine, eNOS requires the critical cofactor BH_4, which stabilizes the dimeric structure of eNOS and facilitates binding of L-arginine [73]. When BH_4 levels are insufficient, "eNOS uncoupling" may result with activation of the reductase domain and transfer of electrons to O_2, rather than L-arginine, and production of superoxide ($O_2^{\bullet -}$) [51] (Fig. 15.4). BH_4 can be oxidized by ROS to BH_2, a competitive BH_4 antagonist [130], which shifts eNOS enzymatic activity towards superoxide production [183]. Deficiency of BH_4 in a mouse model led to spontaneous development of PH under normoxic conditions as well as exaggerated hypoxia-induced PH, vascular remodeling, and RVH, which was secondary to reduced NOS activity and increased superoxide production associated with reduced BH_4 levels [183]. Furthermore, overexpression of GTP-cyclohydrolase 1, the rate-limiting enzyme in BH_4 biosynthesis, prevented PH in mice, and exogenous supplementation of BH_4 attenuated MCT-induced PH and muscularization of distal pulmonary arteries in rats [120, 180]. Additionally, the BH_4 analogue, acetyl-7,7-dimethyl-7,8-dihydropterin, improved NO-mediated pulmonary artery dilation and induced eNOS expression in the endothelium of rats with hypoxia-induced PH [196].

Further support for eNOS uncoupling in the pathogenesis of PAH comes from Cav-1-deficient mice that develop PH [222, 396] due to increased superoxide [179] and peroxynitrite production and tyrosine nitration-dependent impairment of protein kinase G (PKG) activity secondary to increased eNOS activity and NO levels [398]. Importantly, PH in Cav-1-knockout (KO) mice can be reversed with NOS inhibition and prevented with BH_4 administration in Cav-1-deficient neonatal mice [376, 377].

Uncoupling of eNOS can also occur in the setting of limited L-arginine availability. Although intracellular concentrations of L-arginine typically far exceed what is necessary for NO production [60], arginase can metabolize L-arginine to L-ornithine and urea, and compete with NOS for substrate. Arginase is upregulated in the lungs of mice exposed to hypoxia [173], as well as in hypoxia-exposed SMC [61], and is increased in EC of PAH patients [381]. Increases in arginase lead to endothelial dysfunction [306, 381], increases in EC and SMC proliferation [205], as well as increases in collagen deposition [186]. Inhibition of arginase decreases SMC and EC proliferation [67], and attenuates pulmonary vascular remodeling in an animal PH model [67]. Increased levels of L-arginine have also been implicated in the development of PAH in patients with SCD [154]. In addition to limiting NO availability, increased arginase and enhanced synthesis of ornithine have also been implicated in SMC remodeling and PH [144, 266].

L-Arginine availability can also be influenced by endogenous methylarginines, specifically L-monomethlyl arginine (L-NMMA) and ADMA, which are produced through posttranslational methylation of amino acids in arginine [14, 372] and compete with L-arginine for the binding site on eNOS [51]. Both L-NMMA and ADMA

are eliminated largely through active metabolism by dimethylarginine dimethylaminohydrolase (DDAH) [204]. Levels of ADMA are increased in animal models of PH [17, 241] and have been associated with increased oxidative stress and endothelial dysfunction [334]. Furthermore, DDAH levels are reduced in animal models of PH [17, 241] and DDAH1 overexpression in mice has been shown to decrease the sustained phase of hypoxic pulmonary vasoconstriction (HPV) via activation of the NO-sCG pathway [24]. Additionally, levels of ADMA are increased in the plasma of patients with pediatric and idiopathic PAH [132, 280] and also have been associated with increased pulmonary vascular pressures in decompensated heart failure patients in the intensive care unit [312].

15.3.1.4 NO Reactions with Other ROS: Formation of RNS

Nitrosative stress has also been implicated in the pathogenesis of PAH. NO is the main RNS produced within cells and can react with other ROS such as superoxide to generate peroxynitrite anion ($ONOO^-$). Peroxynitrite is a potent oxidant that nitrates tyrosine residues and can lead to formation of other extremely reactive RNS such as nitrogen dioxide, nitrosoperoxycarbonate anion, nitrite, and nitrate. These RNS can lead to significant alterations in protein structure and function, lipid peroxidation, nucleic acid damage, and cell death. Nitrotyrosine, a product of tyrosine nitration and marker of peroxynitrite, is upregulated in the endothelium and PASMC of rats subjected to chronic hypoxia [87, 167] and hypoxia-induced peroxynitrite production has been shown to increase proliferation in PASMC [3]. Peroxynitrite-mediated tyrosine nitration has also been shown to inactivate prostacyclin synthase leading to reduced levels of prostaglandin I_2 [401], eNOS uncoupling, as well as inhibition of PKG [4, 397]. In addition, peroxynitrite can activate many signaling pathways involved in cell proliferation including ERK and protein kinase C [3]. Moreover, treatment of newborn rats with a $ONOO^-$ decomposition catalyst, 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) (FeTPPS), attenuated chronic hypoxia-induced PH and decreased proliferation in neonatal PASMC [32].

In addition to tyrosine nitration, RNS can also induce S-nitrosylation and glutathionylation of regulatory proteins that may alter protein function and downstream signaling. Notably, NO can induce S-nitrosylation through formation of dinitrogen trioxide that can covalently link NO to free thiol groups on cysteine residues within proteins leading to formation of *S*-nitrosothiols. Several S-nitrosylation targets may play an important role in modulating oxidative stress and vascular remodeling in PAH including eNOS, sGC, hemoglobin, mitochondrial complex I, NOX, and cyclooxygenase (COX)-2 [224]. The functional effects of S-nitrosylation of several of these key proteins promote vasodilation and decrease oxidative stress, although S-nitrosylation of sGC and eNOS may inhibit NO-mediated effects on vascular tone. In red blood cells (RBC), hypoxia impairs S-nitrosylation of hemoglobin and deficiency of *S*-nitrosohemoglobin (SNO-Hb) is associated with exaggerated HPV and increased pulmonary arterial pressures [233]. Furthermore, restoration of

SNO-Hb levels by ethyl nitrite inhalation enhanced vasorelaxation and improved hemodynamics and oxygenation in PAH patients [233]. Although S-nitrosylation-induced vascular alterations appear to be protective in PAH, the role of *S*-nitrosothiols in the pathogenesis of PAH remains incompletely understood.

15.3.1.5 Xanthine Oxidase

Xanthine oxidoreductase (XOR) is a critical source of intracellular ROS. It catalyzes the terminal two steps of purine degradation, from hypoxanthine to xanthine and then to uric acid, with release of $O_2^{\bullet -}$ and H_2O_2 (Fig. 15.3). It primarily exists in cells as a dehydrogenase reducing NAD^+ to NADH, but in the setting of inflammation, oxidation of cysteine residues or limited proteolysis converts xanthine dehydrogenase into xanthine oxidase (XO). XO transfers substrate-derived electrons to O_2, generating $O_2^{\bullet -}$ and H_2O_2. H_2O_2 is a major ROS product of XOR action under normal and pathophysiological conditions [7, 335] and has been shown to regulate many pathways involved in vascular remodeling including proliferation and Ca^{2+} signaling [143, 356, 389]. H_2O_2 has also been shown to contribute to superoxide production and decreased NO via activation of NOX [208, 400], eNOS uncoupling in an NOX-dependent manner [16, 46], and limiting access to BH_4. Furthermore, H_2O_2 has been shown to inhibit the activity of extracellular superoxide dismutase (EC-SOD) in PASMC and treatment with catalase (which catalyzes decomposition of H_2O_2) enhances EC-SOD activity and decreases superoxide levels in a model of persistent pulmonary hypertension of the newborn (PPHN) [363].

XOR is upregulated in the lung and serum of rats exposed to chronic hypoxia and treatment with allopurinol, an XO inhibitor, attenuates hypoxia-induced PH, pulmonary vascular remodeling, and RVH [151, 167]. In addition, XO activity is increased in the plasma of patients with IPAH [124, 321], suggesting a role for XOR-mediated ROS in the pathogenesis of PAH.

15.3.1.6 NADPH Oxidases

ROS produced by oxidases such as NOX are considered a major contributor to oxidative and nitrosative stress in the lungs and pulmonary vasculature [7, 82], and have been shown to play an important role in dysregulation of vascular tone in the setting of hypoxia [118, 211]. The parenchymal family of NOXs includes NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2 and the phagocyte NOX includes gp91phox (NOX2). Only NOX1, NOX2, and NOX4 are found in the human vasculature and generate ROS by electron transfer from NADPH to oxygen to generate $O_2^{\bullet -}$ that can be further converted to H_2O_2 by cellular superoxide dismutases (SODs). For enzymatic function, each NOX requires several adaptor subunits. In endothelial cells, NOX2 is constitutively associated with $p22^{phox}$ and, after stimulation, $p47^{phox}$ is phosphorylated followed by recruitment of $p67^{phox}$, $p40^{phox}$, and Rac1 to the NOX2 complex where it is then able to generate $O_2^{\bullet -}$ [20] (Fig. 15.3).

In the pulmonary vasculature, NOX1, NOX2, and NOX4, as well as the subunits $p22^{phox}$ $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are expressed in the lung and pulmonary arteries of mice [246]; however, NOX4 is the predominant NOX upregulated by hypoxia in PASMC [245, 246], PAEC [260], and pulmonary artery adventitial fibroblasts [207]. In addition, $p22^{phox}$ and NOX4 have recently been shown to be upregulated in PASMC in a lamb model of pulmonary hypertension of the newborn (PPHN) [362]. Knockdown of NOX4 decreased ROS production and attenuated proliferation in PASMC and pulmonary artery adventitial fibroblasts [207, 245, 246], as well as increased apoptosis in adventitial fibroblasts [207]. In addition, knockdown of NOX4 increased EC-SOD activity as well as attenuated increases in cyclin D1 and NF-κB in PPHN-PASMC [362]. Furthermore, NOX4-derived ROS have been shown to mediate hypoxia-induced decreases in Kv channel current and increase Kv1.5 channel oxidation in PASMC [245].

NOX4 has also been shown to be upregulated by TGF-β in PASMC [328]. TGF-β significantly induced NOX4 expression and ROS in human PASMC in a Smad2/3-dependent manner that was attenuated by diphenylene iodonium, an NADPH inhibitor, knockdown of NOX4 by siRNA, and transfection of dominant negative Smad2/3 plasmids. In addition, TGF-β stimulation induced NOX4-dependent increases in proliferation in PASMC and, furthermore, led to increases in contractile protein expression that was redox- but not NOX4 dependent. Furthermore, NOX4 has been shown to be significantly upregulated in the lungs of PAH patients compared with healthy donor control lungs [246].

NOX1 and NOX2 have also been shown to play a potential role in the pathogenesis of PAH. In a chronic hypoxia-induced PH model in mice, deficiency of NOX2 reduced hypoxia-induced ROS production, pulmonary artery vasoreactivity, and attenuated hypoxia-induced increases in RVSP, pulmonary vascular remodeling, and RVH [211]. Interestingly, in a rat MCT-induced PH model, PASMC isolated from MCT-treated rats had increased expression of NOX1 and enhanced superoxide production. Knockdown of NOX1 reduced superoxide production as well as attenuated MCT-induced increases in SOD2, cyclin D1, and phosphorylation of ERK. Furthermore, knockdown of NOX1 attenuated proliferation and migration of PASMC from MCT-treated rats [348].

NOXs have also been shown to play an important role in the endothelium in response to hypoxia [122, 405]. PAEC exposed to hypoxia-reoxygenation had significant release of H_2O_2 compared with control cells and inhibition of NOX with diphenyliodonium attenuated H_2O_2 production in response to hypoxia-reoxygenation [405]. In addition, acute hypoxic vasoconstriction (HPV) was attenuated in $p47^{phox}$-deficient mice and ex vivo treatment with an NOX inhibitor significantly reduced HPV in isolated perfused rabbit lungs [371]. Although human data on the role of NOX regulation in the pathobiology of PAH is limited, there is strong animal data supporting an important role for NOX-derived ROS in the pathogenesis of PAH. Further study in patients is warranted to elucidate the role of NOX in human PAH and to determine whether NOX represents an effective pathway for therapeutic targeting in PAH.

15.3.1.7 Mitochondria-Derived ROS

Mitochondria are an additional source of ROS production that may play a role in the pathogenesis of PAH [99]. PAH has been reported in patients with genetic alterations in mitochondrial genes [322, 349] and there is growing recognition that metabolic aberrations and mitochondrial dysfunction exist in PASMC and PAEC isolated from patients with PAH [40, 117, 235, 382]. ROS are generated in mitochondria during the electron transport chain when electrons flowing down the redox gradient prematurely react at complexes I and III with O_2 to generate $O_2^{\bullet -}$ [98, 103, 370]. There is also data to suggest that complex II may be a source of mROS generation in the lungs from hypoxic mice and the hearts isolated from MCT-treated rats [267, 292]. Additional ROS can be generated in mitochondria from superoxide by manganese SOD2 that catalyzes rapid conversion of $O_2^{\bullet -}$ to diffusible H_2O_2 (Fig. 15.3), which can serve as a signaling molecule and regulate transcription factors such as HIF-1α [57, 137, 235] and sulfhydryl-rich voltage-gated potassium Kv channels [155], which have been shown to play a critical role in PAH.

Debate exists as to whether hypoxia increases or decreases mROS and, furthermore, whether mROS promote or protect against pulmonary vascular remodeling [98, 368]. Previous work has demonstrated that hypoxia increases mROS, Ca^{2+} influx, and PASMC contractility and that inhibition of the electron transport chain attenuates increases in Ca^{2+} and HPV [56, 106, 290, 359]. In addition, hypoxia-induced increases in mROS have also been shown to enhance PASMC proliferation via opening of mitochondrial K^+_{ATP} channels and overproduction of H_2O_2 [155]. Furthermore, a recent study demonstrates that redox signaling in PASMC in response to hypoxia is dependent upon subcellular mitochondrial compartment location [358].

While supraphysiologic levels of mROS can lead to oxidative damage and cellular dysfunction, mROS are critical regulators of vascular tone and sustained decreases in mROS may lead to upregulation of transcription factors and signaling pathways that promote aberrant vascular remodeling in PAH. Emerging data suggest that mitochondrial function is impaired in PAH and that cellular metabolism is shifted towards glycolysis leading to enhanced cellular proliferation and resistance to apoptosis, similar to cancer cells (i.e., the Warburg effect) [39, 347]. This has been attributed to decreased mROS production, inhibition of Kv channels with subsequent increases in Ca^{2+} signaling, and activation of HIF-1α and NFAT which promote proliferation and suppress apoptosis [40, 41, 240, 369].

Reduced levels of mROS have been found in animals models of PH including the fawn-hooded rat (FHR) that spontaneously develops PAH [40] and MCT-treated rats [235]. Additionally, PASMC isolated from PAH patients have decreased Kv1.5 expression, increased intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$, increased mitochondrial membrane potential, and activation of NFAT [41]. Inhibition of NFAT with VIVIT or cyclosporine restored Kv1.5 expression, decreased $[Ca^{2+}]_i$, and reversed mitochondrial hyperpolarization leading to decreased proliferation and increased apoptosis in PAH-PASMC [41].

In addition, treatment with dichloroacetate (DCA), a pyruvate dehydrogenase kinase (PDK) inhibitor that enhances oxidative phosphorylation, improved mortality and hemodynamics, as well as reversed vascular remodeling and RVH in MCT-treated and chronic hypoxia-exposed rats [235, 239]. DCA was found to reverse MCT-induced vascular remodeling by restoring Kv1.5 expression, depolarizing mitochondria, increasing H_2O_2 production, and inducing apoptosis in PASMC [235, 239]. Furthermore, mitochondrial survivin, a cytoprotective protein that promotes tumorigenesis and inhibits apoptosis in cancer cells [94], has also been shown to be upregulated in MCT-treated rats and in pulmonary arteries of PAH patients [234]. Adenoviral transfection of a dominant negative survivin mutant increased Kv channel current, depolarized mitochondria, attenuated proliferation, and increased apoptosis in PASMC. Intratracheal administration of the survivin mutant in vivo improved hemodynamics and survival and attenuated vascular remodeling in MCT-treated rats [234]. Although conflicting data exists in animal models, mitochondrial-derived ROS clearly play an important role in the pulmonary vasculature and mitochondrial dysfunction is increasingly recognized as contributing to the pathobiology of PAH. Future studies are necessary to evaluate whether mitochondrial-based therapies have efficacy in animal models of PH and patients with PAH.

15.3.1.8 Lipid Peroxidation and Isoprostanes

Lipid peroxidation has recently been recognized as an additional source of ROS during pulmonary vascular dysfunction [251]. Isoprostanes, chemically stable isomers of prostanoids, are formed when ROS products (particularly peroxynitrite) react with unsaturated bonds of membrane lipids such as arachidonic acid [168]. As isomers of prostaglandins (PG), they can act on several cell types within the pulmonary vasculature via specific prostanoid receptors, including the thromboxane A_2 receptor (TP), and PGE_2 and $PGF_2\alpha$ receptors (EP and FP) [109, 169]. In PASMC and EC, isoprostanes can be released in response to stimulation with growth factors (PDGF, TGF-β), pro-inflammatory cytokines (TNF-α, interferon-γ, IL-1β), as well as by ROS (H_2O_2 and $O_2^{\bullet -}$) [168]. This can lead to activation of signaling pathways downstream of prostanoid receptors including RhoA/ROCK, phospholipase C (PLC), and cyclic AMP/protein kinase A [168], resulting in vasoconstriction and release of other vasoconstrictors, including endothelin-1 (ET-1) from endothelial cells and PASMC [167, 388].

Isoprostane levels have been shown to be elevated in the lung in animal models of hypoxia- and hyperoxia-induced PH [166, 178]. In addition, inhibition of the TP receptor has been shown to reduce ET-1 production in PASMC, as well as attenuate RVH and lung smooth muscle-α actin expression in a hyperoxia neonatal rat model [166]. Urinary levels of isoprostaglandin $F_2\alpha$ type-III ($iPF_2\alpha$-III), a stable lipid peroxidation product indicative of oxidative stress [298], are significantly elevated in patients with PAH compared with controls [75, 296], as well as in patients with *BMPR2* mutations regardless of disease status [201]. Furthermore, while urinary levels of $iPF_2\alpha$-III inversely correlate with vasoreactivity to inhaled NO [75],

increased urinary iPF$_{2\alpha}$-III levels directly correlate with hemodynamic and clinical response to epoprostenol [296], and recently have been found to be independently associated with mortality in PAH patients [76]. Although future studies in animal models and patients will be necessary to further elucidate the role of isoprostanes in PAH, emerging data suggest that isoprostanes may play a role in the pathogenesis of PAH and may serve as a possible lipid peroxidation biomarker in PAH patients.

15.3.2 Oxidative Stress and Animal Models of PH

15.3.2.1 Hypoxia-Induced PH Model

Oxidative stress has been implicated in the pathogenesis of PAH in several animal models of PH (Table 15.4). In the chronic hypoxia model of PH, hypoxia has been shown to induce ROS/RNS production with observed increases in lung superoxide [260], phosphatidylcholine hydroperoxide (PCOOH) [151], isoprostanes [178], nitrotyrosine [87, 167], and oxidized glutathione (GSSG) [261]. Hypoxia has also been shown to increase expression of ROS generators including eNOS [112], NOX2 [211], NOX4 [245, 246], XO [151, 167], and, in some studies, mROS [56, 357, 359]. In addition, hypoxia decreases expression of the antioxidant EC-SOD (SOD3) in the lungs of mice [261] and in pulmonary arteries from calves exposed to chronic hypoxia [143]. Furthermore, several studies have demonstrated efficacy of antioxidants (e.g., *N*-acetyl cysteine) [198], inhibitors of ROS-producing enzymes (e.g., allopurinol) [26, 151, 167], peroxynitrite decomposition catalysts [32], and SOD mimetics [351] in hypoxia-induced PH rodent models [151, 199, 260], suggesting oxidative stress contributes significantly to the pathogenesis of hypoxia-induced PH.

In the hypoxia-induced PH model in newborn pigs, increases in oxidative stress were observed after 3 days of hypoxia with increases in isoprostanes in pulmonary resistance arteries [88]. Additionally, NOX1 and p67^{phox} were increased and SOD1 was decreased in pulmonary arteries from pigs raised in hypoxia for 3 or 10 days. Furthermore, inhibition of NOX with apocynin or treatment with an SOD mimetic + polyethylene glycol-catalase attenuated acetylcholine vascular responses of pulmonary arteries from hypoxia-exposed pigs [88].

15.3.2.2 Monocrotaline-Induced PH Model

In the MCT model, increases in isoprostanes [177] and NOX1 [348] have been observed in rats and increased NOX4 expression was reported in mice exposed to MCT [311]. Additionally, while increases in antioxidants SOD, catalase, and glutathione peroxidase have been reported in the lungs [97, 172], decreases in SOD1 and SOD2 have been observed in RV homogenates from MCT-treated rats [292]. Adenoviral overexpression of EC-SOD in MCT-treated rats decreased lung tissue

Table 15.4 Animal models of PH

Model	Phenotype	References
Chronic hypoxia (rodent)	Increased superoxide, phosphatidylcholine hydroperoxide, isoprostanes, nitrotyrosine, and oxidized glutathione (GSSG) in lung	[87, 151, 167, 178, 260, 261]
	Increased NOX4 expression in PASMC, PAEC, and pulmonary artery adventitial fibroblasts	[207, 245, 246, 260]
	Increased lung expression of eNOS, NOX2, NOX4, XO, and mitochondrial ROS (mROS)	[56, 112, 151, 167, 211, 245, 246, 357, 359]
	Decreased lung expression of EC-SOD (SOD3)	[143, 261]
	Allopurinol attenuated hypoxia-induced PH, pulmonary vascular remodeling, and RVH	[26, 151, 167]
	FeTPPS (peroxynitrite decomposition catalyst) reduced lung nitrotyrosine, attenuated vascular remodeling, and normalized pulmonary vascular resistance	[32]
	MnTE-2-PyP (SOD mimetic) attenuated hypoxia-induced PH, RVH, and pulmonary vascular remodeling	[351]
	N-acetyl cysteine inhibited hypoxia-induced PH, RVH, and muscularization of distal pulmonary arteries	[198]
Chronic hypoxia (pigs)	Increased isoprostanes, NOX1, and $p67^{phox}$ in pulmonary arteries; decreased SOD1 in pulmonary arteries	[88]
	Apocynin (NOX inhibitor) and SOD mimetic + polyethylene glycol-catalase attenuated acetylcholine vascular responses of pulmonary arteries from hypoxia-exposed pigs	
Monocrotaline (rats)	Increased isoprostanes in lung; increased superoxide and NOX1 in PASMC; increased NOX4 in lung (mice)	[177, 348, 311]
	Increased SOD, catalase, and glutathione peroxidase in lung	[97, 172]
	Decreased SOD1 and SOD2 in RV	[292]
	Decreased mROS in PASMC	[235]
	EC-SOD overexpression attenuated MCT-induced PH, RVH, and vascular remodeling	[177]
	Resveratrol attenuated MCT-induced PH, RVH, and vascular remodeling	[269]
	Antioxidant EUK-134 attenuated MCT-induced right heart failure	[291]
PPHN (lambs)	Increased H_2O_2 in PAs; increased superoxide in lung and PAs; increased $p22^{phox}$ and NOX4 in lung, PAs, and PASMC; increased $p67^{phox}$ in lung and PAs; decreased EC-SOD in lung and PASMC	[48, 325, 362]
	Recombinant SOD1 enhanced pulmonary vascular responses to inhaled NO	[325]
Neonatal shunt model (lambs)	Increased superoxide, Rac, $p45^{phox}$ in lung, increased eNOS uncoupling	[135]

Sugen hypoxia model (rats)	Increased nitrotyrosine and heme oxygenase 1 (HO-1) in lung; decreased HO-1 in RV	[38, 352]
	Protandim (Nrf2 activator) prevented RV failure and fibrosis	[37]
FHR	Decreased ROS in PAs and PASMC; decreased SOD2 in PASMC; mitochondrial abnormalities, normoxic activation of HIF-1α, and inhibition of Kv1.5 channels in PASMC; metabolism shift from oxidative phosphorylation to glycolysis in PASMC	[40, 293]
	SOD2 overexpression in PASMC restored Kv1.5 expression and inactivated HIF-1α; metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin (SOD mimetic) improved hemodynamics and exercise capacity, decreased vascular remodeling	[15]
Transgenic BMPR2-mutant mice	Increased isoprostanes and isofurans in lung; increased superoxide and peroxide in VSMC	[116, 201]
$ALK1^{+/-}$ mice	Develop spontaneous PH; increased $iPF_2\alpha$-III and H_2O_2 in lungs; increased eNOS uncoupling	[170]
	Tempol (SOD mimetic) prevented PH and RVH	[170]
ET-1 transgenic mouse	Develop hypertrophic vascular remodeling and impaired vascular relaxation; increased NOX activity and $gp91^{phox}$ expression in mesenteric arteries	[13]
SOD2-knockout mouse	Severe mitochondrial injury; central nervous system and cardiac injury; significant postnatal mortality	[202]
SOD1-knockout mouse	Develop spontaneous PH; increased urinary isoprostanes; increased plasma TBARS; increased superoxide in PAs	[286, 310]
	A-285222 (selective NFAT inhibitor) decreased PH, arterial wall thickness, and vasoreactivity	[286]
	Tempol (SOD mimetic) reversed PH, reduced NFAT activity	
SOD3-knockout mouse	Exaggerated hypoxia-induced PH; increased urinary isoprostanes; increased plasma TBARS	[310, 380]
SOD3 mutation (rats)	Exaggerated MCT-induced PH; increased TBARS and nitrotyrosine in lung	[380]
	SOD mimetic Mn(III)TmPyP attenuated MCT-induced PH and RVH	
SOD3 overexpression	Attenuated and reversed hypoxia-induced PH; attenuated MCT-induced PH; attenuated PH secondary to bleomycin-induced fibrosis	[6, 177, 261, 346]
Caveolin-1-knockout mouse	Develops spontaneous PH; increased eNOS and peroxynitrite in lung; tyrosine nitration of PKG in lung	[376, 396, 397]
	L-NAME and BH_4 reverse PH; mice deficient in both Cav-1 and eNOS are protected from the development of PH	[376, 377]

levels of 8-isoprostane and attenuated RVSP and pulmonary vascular remodeling [177]. Furthermore, several antioxidants [291, 393] and resveratrol [269] have shown benefit in the MCT-induced PH model in rats.

15.3.2.3 SU5416-Hypoxia PH Model

In the Sugen hypoxia model, rats treated with SU5416 followed by exposure to chronic hypoxia had significantly increased expression of nitrotyrosine and heme oxygenase 1 (HO-1) in the lung compared with controls [352], in contrast to the RV where levels of HO-1 were decreased following Sugen hypoxia [38]. Treatment with protandim, a nuclear factor erythroid 2-related factor 2 (Nrf2) activator which induces antioxidant expression (e.g., HO-1, SOD), prevented RV failure and fibrosis; however, it did not attenuate pulmonary vascular remodeling [37].

15.3.2.4 Pulmonary Hypertension of the Newborn Model

Increases in oxidative stress have also been demonstrated in the newborn lamb PPHN model where animals undergo prenatal ligation of the ductus arteriosus [48, 325, 362], as well as a CHD model where a surgical shunt between the aorta and pulmonary artery is created in prenatal lambs [135]. In the PPHN model, newborn lambs that had undergone ductus arteriosus ligation in utero demonstrated increased levels of superoxide, decreased SOD expression/activity, as well as increased $p67^{phox}$ expression in pulmonary arteries [48]. Treatment of PPHN lambs with recombinant SOD1 enhanced pulmonary vascular responses to inhaled NO with greater decreases in PVR, suggesting a critical role for NOX-mediated ROS and potential efficacy of SOD in PPHN [325]. A more recent study demonstrated increased NOX4 and $p22^{phox}$ and decreased EC-SOD in the lungs and PASMC from PPHN lambs [362]. Similarly, in the neonatal shunt model, shunted lambs demonstrated elevated superoxide levels and increased expression of Rac and $p45^{phox}$ in the lung, as well as eNOS uncoupling, further supporting the role of NOX and eNOS in ROS generation in animal models of PH [135].

15.3.2.5 Fawn-Hooded Rat Model

The FHR, a strain in which PAH occurs spontaneously, has provided critical information on the role of mitochondrial dysfunction in the pathogenesis of PAH. The FHR has an autosomal recessive disorder similar to Hermansky–Pudlak syndrome characterized by dysfunction of several organs including systemic hypertension, pulmonary fibrosis, renal disease, as well as platelet and coagulation dysfunction [193]. As described above, PASMC isolated from FHR have decreased ROS, decreased SOD2 expression, as well as marked mitochondrial abnormalities, normoxic activation of HIF-1α, and inhibition of Kv1.5 channels [40]. In addition,

PASMC from FHR demonstrate a shift in metabolism from oxidative phosphorylation to glycolysis despite adequate oxygen [293]. Overexpression of SOD2 in PASMC from FHR restored Kv1.5 expression and inactivated HIF-1α, and treatment of FHR with an SOD mimetic (metalloporphyrin Mn(III)tetrakis (4-benzoic acid) porphyrin) improved hemodynamics and exercise capacity, as well as decreased vascular remodeling [15].

15.3.2.6 Genetic Models of PH

Genetic models have offered the opportunity to further evaluate the role of ROS in pulmonary vascular remodeling and the development of PAH. Several genetically modified mice that develop PH have recently been associated with increases in oxidative stress. Transgenic (TG) mice with a mutation in the cytoplasmic tail of *BMPR2* have increased lung levels of lipid peroxidation products, isoprostanes, and isofurans, and transfection of rat vascular SMC with BMPR2 mutants increases superoxide and peroxide production compared with wild type (WT) BMPR2-transfected cells [116, 201]. Mutations in *ALK1*, which encode an endothelial-specific receptor of the TGF-β superfamily and are associated with hereditary hemorrhagic telangiectasia (HHT) and PAH [141, 142], have also been associated with increased oxidative stress [170]. Mice heterozygous for *ALK1*, that develop PH as they age, have increased ROS in the lungs (iPF$_2$α-III, H_2O_2) at 12 weeks of age secondary to increased eNOS uncoupling, and treatment with tempol, an SOD mimetic, prevents increases in RVSP and RVH in ALK1$^{+/-}$ mice [170]. In addition, TG mice overexpressing ET-1 in the endothelium, that develop hypertrophic vascular remodeling and have impaired vascular relaxation, have enhanced vascular NOX activity and increased expression of gp91^{phox} [13], suggesting these TG mice have increased oxidative stress.

Genetic models of SOD have provided additional insight into oxidative stress and ROS scavenging in animal models of PH. Mice lacking mitochondrial manganese SOD (MnSOD, SOD2) have severe mitochondrial injury with central nervous system and cardiac injury leading to significant postnatal mortality [202]. Mice deficient in intracellular copper-zinc SOD (CuZnSOD, SOD1) or extracellular SOD (EC-SOD, SOD3) have increased oxidative stress as measured by urinary isoprostanes and plasma thiobarbituric acid-reactive (TBARS) levels, and mice deficient for both SOD1 and SOD3 have additional increases in oxidant stress markers [310]. The absence of SOD1 has recently been reported to be associated with the development of spontaneous PH and is dependent on NFAT activation in PASMC [286]. SOD1-deficient mice have elevated superoxide levels and develop significant increases in RVSP under normoxic conditions. Spontaneous PH in SOD1-deficient mice is attenuated by selective inhibition of NFAT as well as tempol, an SOD mimetic, which prevents NFAT activation in SOD1-knockout mice [286]. Although SOD3-knockout mice do not develop spontaneous PH, the absence of SOD3 exacerbates hypoxia-induced PH with significant increases in RV pressures, RVH, and vascular remodeling compared with WT mice [380]. Similarly, a loss-of-function

SOD3 mutation in rats leads to increased TBARS and nitrotyrosine in the lung, as well as exaggerated PH and RVH following MCT, which is attenuated by the SOD mimetic Mn(III)TmPyP [380].

Transgenic overexpression of SOD1 [330] and SOD3 [6, 177, 261] protects against oxidative stress and overexpression of SOD3 has been shown to both attenuate [261] and reverse established PH in response to chronic hypoxia [6], as well as attenuate MCT-induced PH [177], and PH secondary to bleomycin-induced fibrosis [346]. Interestingly, in both the chronic hypoxia-induced PH model and in the bleomycin model of secondary PH, overexpression of EC-SOD in the lung attenuated upregulation of the transcription factor early growth factor-1 (Egr-1) [261, 346]. EC-SOD also decreased TGF-β induction in the bleomycin model [346] and prevented eNOS downregulation in the rat MCT model [177]. Additionally, PAs from EC-SOD knockout mice have enhanced vasoconstriction in response to 5-hydroxytryptamine (5-HT), while PAs from transgenic mice overexpressing EC-SOD have decreased superoxide production and attenuated 5-HT-induced vasoconstriction [210].

The caveolin-1-knockout mouse also provides additional evidence that oxidative and nitrosative stress play a role in the pathobiology of PAH. Mice deficient in Cav-1 develop PH spontaneously with significant increases in PA pressures and RVH compared with WT control mice [396], and restoration of endothelial cell-specific Cav-1 in knockout animals rescues the PH phenotype [254]. The absence of Cav-1 leads to increased activation of eNOS [376], NO-dependent peroxynitrite production, and tyrosine nitration of PKG, which can be reversed by PKG overexpression [397]. Furthermore, inhibition of eNOS with L-NAME [376, 398] or BH_4 treatment [377] prevents PH in Cav-1-knockout mice. Additionally, mice deficient in both Cav-1 and eNOS are protected from the development of PH [398].

15.3.3 Oxidative Stress and Human PAH

Several studies have demonstrated increases in oxidative stress in patients with PAH. As described above, elevated levels of urinary $iPF_2\alpha$-III have been demonstrated in PAH patients [75, 296] and recently have been shown to be independently associated with survival in PAH [76]. Additional studies have demonstrated increased levels of plasma malondialdehyde (MDA) [124, 162] and xanthine oxidase [124, 321], as well as decreased EC-SOD [124] and glutathione peroxidase activity [162] in the plasma of PAH patients. Increases in oxidative stress markers have also been demonstrated in plasma from patients with chronic obstructive pulmonary disease (COPD) and secondary PH [175], and in children with congenital portosystemic venous shunts at risk of developing PH [257]. Furthermore, oxidative posttranslational modification of albumin has been shown in patients with both idiopathic PAH and PAH secondary to SCD [262].

Increases in oxidative stress have also been demonstrated in lung tissue from PAH patients [225]. Immunohistochemical staining demonstrated increased staining for nitrotyrosine and 8-hydroxy guanosine, a marker of oxidative DNA damage, in

lung tissue from PAH patients compared with controls [47]. Levels of the eicosanoid metabolites, 5-oxo-eicosatetraenoic acid (5-oxo-ETE) and 5-hydroxyeicosatetraenoic acid (5-HETE), were also found to be elevated in lung tissue from PAH patients not on prostacyclin and secondary PH patients [47]. In addition, lung tissue homogenates from PAH patients had decreased SOD activity and levels of SOD2 compared with control lungs [47]. Furthermore, SOD and glutathione peroxidase activity were also decreased in airway epithelial cells and lysates from bronchial tissue obtained from explanted PAH lungs compared with controls [225]. Taken together, substantial evidence from animal models and human PAH samples suggest that oxidative stress plays a critical role in the pathogenesis of PAH.

15.3.4 ROS and Mechanisms of Pulmonary Vascular Remodeling

Several mechanisms have been identified by which oxidative stress can mediate the vascular alterations observed in PAH. ROS have been shown to alter the balance of vasoactive mediators, enhance calcium signaling, upregulate growth factors, and induce pro-proliferative signaling pathways, all of which can contribute to enhanced vasoconstriction and pulmonary vascular remodeling in PAH. XO-derived O_2 metabolites have been shown to significantly increase thromboxane B_2 levels 30-fold while only minimally increase PGI_2 levels, leading to enhanced vasoconstriction in isolated perfused rabbit lungs [337]. In addition, peroxynitrite has been shown to inactivate PGI_2 synthase and reduce levels of PGI_2 [401]. ROS have also been shown to upregulate endothelin-converting enzyme-1 [215] and induce ET-1 expression in endothelial cells [66] and, furthermore, ET-1 has been shown to stimulate PASMC proliferation via increases in superoxide production [360]. Additionally, H_2O_2 has been shown to promote eNOS uncoupling leading to decreases in NO and further increases in ROS [46, 400]. Taken together, several studies suggest that oxidative stress leads to an imbalance in vascular mediators with release of potent vasoconstrictors that can overwhelm the effects of endothelial-derived vasodilators and promote enhanced vasoconstriction and vascular remodeling in PAH.

ROS have also been shown to enhance Ca^{2+} mobilization [209] and Ca^{2+} sensitization in PASMC [50, 171, 185], and therefore may play a critical role in enhanced contraction and proliferation of PASMC in PAH. H_2O_2 leads to release of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-gated sarcoplasmic reticulum stores in PASMC [209] via activation of phospholipase C-γ1 [356] and conversion of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3. Calcium mobilization by H_2O_2 in PASMC [209] and sustained constriction of rat intrapulmonary arteries (IPA) have also been shown to be dependent on ryanodine-sensitive intracellular Ca^{2+} stores [276]. In addition, superoxide has been shown to activate Rho A/Rho-kinase (ROCK) leading to increased phosphorylation of myosin light chain (MLC), Ca^{2+} sensitization, and vasoconstriction in rat pulmonary arteries [185]. Similarly, hypoxia- and ET-1-induced ROS production enhance Ca^{2+} sensitization via activation of Rho A/ROCK signaling in PASMC [50, 171].

mROS production has also been implicated in pulmonary vascular remodeling as discussed above. Numerous studies have demonstrated that hypoxia increases mROS, Ca^{2+} influx, and PASMC contractility [56, 106, 290, 359]. However, more recent studies suggest that decreases in mROS lead to inhibition of Kv channels, membrane depolarization, activation of voltage-gated Ca^{2+} channels, and increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]$) which lead to increased vasoconstriction, enhanced proliferation, and suppression of apoptosis [40, 41, 240, 369].

ROS can also increase expression of several growth factors and enhance pro-proliferative signaling pathways that play a critical role in vascular remodeling in PAH. ROS have been shown to activate latent TGF-β [27] and TGF-β can further induce ROS via induction of NOX4 leading to enhanced proliferation and contraction in PASMC [328]. ROS can also induce PASMC expression of FGF-2 [35] which is upregulated in a lamb model of increased pulmonary blood flow and PH [361]. VEGF expression is also upregulated by ROS in PASMC [31] and is dependent on TGF-β activation of NADPH and ROS generation [226]. In addition, hypoxia has been shown to upregulate VEGF expression in pulmonary artery endothelial cells [212], and both H_2O_2 [249] and hypoxia have been shown to increase PDGF expression in endothelial cells [191].

ROS can also activate signaling pathways and transcription factors that regulate cellular proliferation, growth, and apoptosis leading to enhanced proliferation and growth of PASMC, PAEC, and fibroblasts, as well as matrix deposition in the pulmonary arterial wall. ROS have been shown to activate the G protein Ras leading to recruitment of phosphatidylinositol 3′kinase (PI3K) and activation of downstream signaling pathways involved in cell survival and hypertrophy, including Akt/protein kinase B and ERK1/2 [89, 344]. H_2O_2 has also been shown to upregulate the p38 mitogen-activated protein kinase (MAPK) pathway [343] and induce Src-dependent JNK activation in vascular SMC [389], as well as Src-dependent activation of big MAPK1 (BMK1/ERK5) in fibroblasts [1]. Peroxynitrite can also stimulate proliferation of PAEC and PASMC via activation of the Ras-Raf-MEK-ERK pathway as well as via protein kinase C [3].

ROS have also been shown to modulate key transcription factors that play a role in PAH and that regulate genes involved in the cell cycle and cell growth. H_2O_2 and hypoxia have been shown to upregulate transcription of peroxisome proliferator-activated receptor-γ coactivator-1 protein-α (PGC-1α), a transcriptional coactivator and critical regulator of mitochondrial biogenesis [163]. In PASMC, hypoxia has been shown to induce PGC-1α expression via PI3K/Akt signaling and activate mitochondrial biogenesis via NRF-1 and TFAM [288]. Additionally, knockdown of PGC-1α inhibits hypoxia-induced cyclin expression and proliferation of PASMC [288], suggesting that ROS-induced PGC-1α may play a key role in regulating mitochondrial biogenesis and vascular remodeling in PAH. XO-derived ROS have also been shown to upregulate Egr-1 via ERK1/2 in PASMC, which has been shown to play an important role in animal models of PH [92, 203, 345]. Furthermore, ROS have been shown to induce NFAT expression [181], a critical transcription factor linked to PASMC proliferation and vascular remodeling which plays a key

role in the pathogenesis of PAH [33, 84, 286]. Interestingly, NFAT has recently been linked to the development of spontaneous PH in SOD1-deficient mice suggesting a critical role for NFAT in mediating ROS-induced PAH [286].

15.4 Antioxidants in PAH

Drugs that are currently available for the management of PAH include calcium channel blockers, prostanoids, endothelin-1 receptor antagonists, and PDE5 inhibitors, which lie outside the scope of this review [247]. Even though there have been significant advances in the understanding of PAH pathogenesis and new therapeutic options available for treatment, PAH remains incurable and patients eventually progress to right heart failure and death [247]. Present therapeutic approaches have been developed based on the imbalance in endothelium-derived vasoactive mediators that exists in patients with PAH [247]. Growing evidence of the importance of oxidative stress in the pathogenesis of PAH has led to the identification of new therapeutic targets. Antioxidant strategies for the treatment of PH have been recently classified into four groups: enzymatic ROS scavengers and regulators, small chemical ROS scavengers, inhibitors of ROS generation, and Nrf2 activators [332]. Additional strategies include eNOS uncoupling agents and mitochondria-active agents.

15.4.1 Enzymatic ROS Scavengers and Regulators

Enzymatic ROS scavengers and regulators include SOD, catalase, glutathione peroxidase, glutathione reductase, glutaredoxin, thioredoxin, thioredoxin reductase, peroxiredoxin, and sulfiredoxin. These enzymatic scavengers exist naturally in human cells and act synergistically in order to protect tissues against free radical damage [62].

15.4.1.1 Superoxide Dismutase

SOD is one of the most important enzymatic antioxidants in the body and is ubiquitously expressed [5, 62]. All three isoforms (SOD1, SOD2, SOD3) act by catalyzing the rapid conversion of $O_2^{\bullet -}$ into H_2O_2 (Fig. 15.3) [5]. SOD has been shown to be downregulated in animal models of PH and PAH patients [5], and administration of SOD has been shown to be beneficial in animal models of PH. Steinhorn et al. found that treatment with recombinant human SOD (rhSOD) in sheep with PPHN reduced PVR in vivo and enhanced relaxation responses of pulmonary arteries to exogenous NO ex vivo [325]. Farrow et al. also showed that rhSOD increases eNOS expression and restores its function, decreases generation of ROS, and increases BH_4 in PPHN lambs [115]. The effect of SOD administration in human PAH has not been studied.

15.4.1.2 Catalase

The enzyme catalase is also key in the antioxidant machinery of cells and is of particular importance during high levels of oxidative stress, since it has a very high turnover number [5]. Catalase exerts its antioxidant action by converting hydrogen peroxide into water and oxygen (Fig. 15.3) [5]. Data regarding the role and expression of catalase during PH is variable, with increased activity reported in MCT-treated rats [172], decreased levels in lambs with PH secondary to increased postnatal pulmonary blood flow [313], and no difference reported in humans with IPAH [225]. Studies to evaluate the effect of exogenous catalase in animal PH models have revealed variable results. Goats pre-treated with intravenous catalase and subjected to endotoxin infusions displayed minimal attenuation of PH compared with controls [229]. However, endotoxin-exposed sheep pre-treated with intraperitoneal catalase had attenuated elevation of pulmonary pressures compared to untreated controls [242]. Wedgwood et al. evaluated the effect of catalase on isolated pulmonary arteries from PPHN lambs and found a normalization of the vasodilator responses to exogenous NO [364]. They also demonstrated that intratracheal administration of catalase to PPHN lambs enhanced SOD3 activity and improved oxygenation [363]. Thibeault et al. evaluated the effect of intratracheal injection of liposome-encapsulated catalase in a rat model of hyperoxia, finding reduction in vascular and parenchymal damage caused by oxygen toxicity [340]. The role of catalase in treatment for human PAH is not clear and further studies are needed to determine potential benefit [5].

15.4.2 Small Chemical ROS Scavengers

15.4.2.1 Dietary Antioxidants

Vitamin C

Ascorbic acid is an excellent reducing agent, capable of donating an electron to oxidizing radicals such as hydroxyl, alkoxyl, peroxyl, thiol, and tocopheroxyl [101]. This makes vitamin C a good antioxidant and a substance of interest for the treatment of many diseases. Interestingly, reversible PH secondary to vitamin C deficiency and clinical scurvy has been described [197, 237]. Furthermore, low levels of ascorbate have been observed in patients with high altitude PH [22], suggesting a potential beneficial role of vitamin C in PAH. Xiang et al. investigated the effect of vitamin C supplementation in broilers with pulmonary hypertension syndrome (PHS) induced by low temperatures [379]. Vitamin C supplementation reduced PHS incidence and attenuated the percentage of thick-walled peripheral lung vessels and associated muscularization of pulmonary arterioles [379]. Paradoxically, however, Walton et al. found that broilers with PHS secondary to low temperatures and fed with flax seed oil had higher incidence of PHS when vitamins C and E were

added to the diet [354]. On the other hand, Belaiba et al. showed that vitamin C inhibits the production of ROS and HIF-1α protein, as well as the increase of VEGF mRNA in PASMC stimulated with thrombin or $CoCl_2$ in vitro [31]. No clinical trials have explored the effects of vitamin C on PH in humans. One clinical trial found no benefit of vitamin C supplementation in the prevention of acute mountain sickness [23]. Currently, there are two ongoing clinical trials registered in the NIH that aim to determine the use of antioxidants, including vitamin C, as prophylaxis for acute mountain sickness (NCT01182792, NCT01571687).

Tocopherols

Vitamin E is the most important lipophilic antioxidant in the lung and plays a key role in scavenging hydroxyperoxyl radicals produced during lipid peroxidation [189, 341]. Severe oxidative stress leads to increased concentration of vitamin E in the lung [189]. Patients with IPAH appear to have decreased levels of α-tocopherol in the plasma and vitamin E levels have been shown to correlate with pulmonary function better than other antioxidants [278, 308]. These findings suggest that there is a mobilization of vitamin E from other tissues to reach adequate levels in the lung [189]. There is limited and variable evidence on the effect of vitamin E in models of PH. In a model of broilers with PHS induced by cool temperatures, high dietary vitamin E attenuated mitochondrial dysfunction [161], lowered PHS-induced mortality, and improved antioxidant capacity [44]. However, a subsequent study demonstrated no mortality benefit of vitamin E supplementation in broilers with PHS [45]. Additional studies found that α-tocopherol [182] and vitamin E failed to improve RVH in broilers with PHS, nor improved cardiopulmonary performance or NOS activity in isolated pulmonary arteries [216]. Further studies are needed to further elucidate the effects of vitamin E in PAH.

Carotenoids

The antioxidant activity of carotenoids is due to their multiple conjugated double bonds, which makes them susceptible to oxidative cleavage [314]. The antioxidant properties of vitamin A have been of great interest in the study of many diseases, including lung cancer [123]. The role of retinol in lung development, vasculogenesis, and angiogenesis has been well documented [304, 307]. In PH, it has been demonstrated that patients with IPAH have reduced levels of retinoic acid, and treatment of hPASMC with this vitamin suppressed 5-HT-induced cell growth in vitro [278]. In a rat hypoxia model, treatment with all-*trans* retinoic acid (ATRA) significantly reduced muscularization of peripheral PAs and medial wall thickness of small muscular arteries; however, it did not attenuate PH or RVH [392]. Similarly, in MCT-induced PH in rats, Swamidas et al. found that dietary retinol resulted in less vascular inflammation in the lung and RV, but did not improve RVH [333]. Conversely, Qin et al. found that ATRA treatment in rats

with MCT-induced PH lowered mPAP and inhibited collagen accumulation and MMP1 mRNA overexpression in the lungs [281]. No clinical trials have evaluated the benefits of carotenoids in human PAH.

Flavonoids

The antioxidant properties of flavonoids have been well documented in vitro [217]. They act through different mechanisms including chelation of metal ions, stimulation of antioxidant enzymes, and inhibition of enzymes that increase oxidative stress [80]. The benefits of flavonoids have been evaluated in a wide array of pathologies, including cardiovascular diseases, type II diabetes, neurodegenerative diseases, and cancer [217]. Many investigators have been interested in the effects that flavonoids may have on oxidative stress in PH. In rat models of MCT-induced PH, administration of flavonoids, such as quercetin and genistein, has been shown to decrease mPAP, RVSP, RVH, medial wall thickness, and neomuscularization of PAs, as well as inhibit hPASMC proliferation and progression to right heart failure [127, 150, 228]. In rats exposed to hypoxia, puerarin was shown to lower levels of ET-1 and type I collagen, enhance the activity of SOD, and improve pulmonary vascular remodeling [206]. Similarly, breviscapine was shown to decrease mPAP, RVH, and vascular remodeling as well as decrease fractalkine and Rho-kinase mRNA expression in a rat hypoxia model [63, 383]. In addition, genistein was shown to inhibit the mean change in tension caused by ET-1 in IPA of rats previously exposed to chronic hypoxia [367]. Finally, genistein has been shown to significantly attenuate PH, activate eNOS, restore endothelial function, and decrease vascular remodeling in broilers with PH [384]. No clinical trials have yet explored the effects of flavonoid administration in patients with PAH.

Resveratrol

Resveratrol is commonly found in foods such as grapes, plums, and peanuts, and has become a substance of interest because of its potential benefits in cardiovascular disease and cancer [86]. Resveratrol exerts its antioxidant effects possibly through scavenging superoxide radicals formed in the mitochondria, inhibiting lipid peroxidation, and competing with coenzyme Q to decrease the oxidative chain complex [86]. Other antioxidant mechanisms of resveratrol include upregulation of antioxidant enzymes, decrease in NOX levels, and regulation of GTP-cyclohydrolase 1, which increases BH_4 levels and reverses eNOS uncoupling [378]. In the rat MCT-induced PH model, resveratrol attenuates elevation in RVSP, RVH, and thickening of IPAs [79, 268, 269]. In addition, resveratrol normalizes alterations in BMP receptors and SMAD signaling molecules, upregulates NOX subunits, and attenuates expression of IL-6, IL-1β, TNF-α, PDGF-α, PDGF-β, MCP-1, iNOS, and ICAM-1 in vivo. Furthermore, resveratrol prevented proliferation of PASMC after PDGF

stimulation, and inhibited cytokine-induced NF-κβ activation in PASMC in vitro [79]. Finally, Chun et al. also showed that resveratrol reduced mPAP and monocyte chemoattractant protein-1 expression in rats with PH induced by infusion of autologous blood clot in the PA [68].

15.4.2.2 Gases

Nitric Oxide

Currently, inhaled NO is indicated for the treatment of term or near-term neonates with hypoxemic respiratory failure associated with PH, and is clinically used in acute vasoreactivity testing in the cardiac catheterization laboratory in patients with PAH [2, 29]. Inhaled NO has also been shown to be beneficial in patients that undergo surgery for CHDs or heart transplant [160]. There have been non-controlled observational clinical studies that show improved PVR and PAP and minimal adverse events in patients with PAH treated with long-term inhaled NO [29, 58, 164, 274, 275, 319]. However, there are still concerns about the potential risks of long-term inhaled NO therapy in PAH patients, including rebound PH upon sudden discontinuation, and toxicity due to production of NO_2 and methemoglobin [29, 160]. Further clinical trials are needed to determine the safety profile of inhaled NO in the treatment of PAH.

Most of the rationale behind the studies of inhaled NO in the treatment of PAH are based on the fact that NO is a selective pulmonary vasodilator, rather than the role it may play as antioxidant. However, recent studies have demonstrated that inhaled NO increases antioxidant defenses, decreases DNA damage, and improves lung inflammation in rabbits exposed to conventional mechanical ventilation [119, 299]. In addition, inhaled NO treatment in infants with hypoxemic respiratory failure reduced oxidative stress biomarkers, namely MDA and total glutathione [139]. The potential antioxidant mechanisms of NO are very complex, since this molecule is also involved in the production of RNS and nitrosative stress, as discussed in previous sections. Nevertheless, recent studies have shown that NO participates in scavenging of lipid peroxyl radicals, and some RNS such as $ONOO^-$ might even participate in cell signaling pathways that activate cellular antioxidants resulting in cytoprotective, rather than cytotoxic, effects [271].

Hydrogen Sulfide

The toxic effects of excessive hydrogen sulfide (H_2S) inhalation have been well documented and include pulmonary edema, bronchiolitis, reactive airways disease, pulmonary interstitial fibrosis, and death [64]. Its main mechanism of toxicity is due to inhibition of cytochrome oxidase and other cellular respiratory enzymes, which is dependent on concentration and duration of exposure [64]. However, H_2S is produced endogenously in the lung and studies have now shown potential benefits

of H_2S or H_2S donors in the treatment of chronic pulmonary diseases including COPD, asthma, and PH [64]. Antioxidant mechanisms of H_2S include increasing glutathione levels and activation of Nrf2 with subsequent upregulation of antioxidant response elements [277].

H_2S levels have been shown to be low in rats exposed to hypoxia [282], and in patients with acute exacerbations of COPD who have elevated PAP, compared to those with normal PASP [65]. Treatment of hypoxia-exposed rats with an H_2S donor, sodium hydrosulfide (NaHS), reduces mPAP and RVH [365], decreases vascular remodeling, and enhances total antioxidant capacity compared with controls [282]. Similarly, administration of NaHS to broilers exposed to hypoxia significantly reduced PH compared with untreated controls [385]. In addition, H_2S has been shown to relax rat aortic arteries and inhibit vascular SMC proliferation in vitro [102, 152, 282, 394]. Additionally, H_2S or injected NaHS has been shown to be protective in mouse lung injury models [121]. Investigations of H_2S still remain in a preclinical phase.

Carbon Monoxide

Carbon monoxide (CO) is very well known for its toxic effects both in chronic cigarette smoke exposure or acute intoxication [128]. The interest in the role of CO as a therapeutic gas is relatively recent and has been based on observations that, at low doses, CO may have cytoprotective properties involving inhibition of inflammatory and proliferative signals [128]. The anti-inflammatory effects of CO have been shown in many in vivo and in vitro studies [128, 244], but its antioxidant properties are less known. In fact, some studies have found that CO inhibits cytochrome c oxidase in the mitochondria, increasing accumulation of electrons within the electron transport chain resulting in increased generation of ROS in this organelle [402]. In contrast, other studies have shown that CO inhibits NOX, limiting ROS production [323].

Low dose CO has been shown to be protective in the FHR model, as well as in the hypoxia and MCT-induced PH rat models [403]. Daily treatment with 1 h of inhaled CO at 250 ppm protected FHRs from the development of spontaneous PH and prevented both hypoxia and MCT-induced increases in RVSP, RVH, and pulmonary vascular remodeling [403]. Although effects on ROS were not assessed, CO was found to attenuate PASMC proliferation, decrease apoptosis, and induce eNOS expression in PAEC [403]. In addition, CO has been shown to attenuate PVR elevation in hypoxemic sheep [256], and decrease vascular remodeling in iliac arteries in a porcine model of balloon angioplasty [285]. CO has also been shown to have protective effects in other lung diseases including bleomycin-induced fibrosis [399], lung transplantation [187, 188], and ventilator-induced lung injury [95, 149, 244]. Furthermore, treatment of ex-smoking COPD patients with CO inhalation decreased sputum eosinophils and improved responses to methacholine testing [30]. Further studies are needed to determine the efficacy of CO in patients with PAH, as well as to elucidate the role of CO in modulating oxidative stress in PAH.

15.4.2.3 Antioxidant Enzyme Mimetics

Substances that mimic the functions of antioxidant enzymes can also be used to counteract oxidative stress in the pulmonary vasculature. The antioxidant enzyme mimetics investigated have the same mechanism of action previously described for the enzymes that they emulate.

MnTE-2-PyP

MnSOD mimetics have high selectivity for mitochondria and decrease superoxide levels in the mitochondrial matrix, increasing the levels of diffusible H_2O_2 [98]. The SOD mimetic MnTE-2-PyP has been shown to be protective in a mouse model of hypoxia-induced PH [351]. Treatment of mice with MnTE-2-PyP attenuated hypoxia-induced increases in RVSP, RVH, and pulmonary vascular remodeling [351]. Furthermore, MnTE-2-PyP attenuated hypoxia-induced NALP3 inflammasome activation, caspase cleavage, and IL-1β and IL-18 production [351]. Other Mn porphyrin-based SOD mimetics have demonstrated similar efficacy in the MCT model and FHR [380].

Tempol

Tempol is also an SOD mimetic that has been studied in various animal models of PH. In rats exposed to chronic hypoxia, tempol normalized RVSP and reduced RVH [108], while combined treatment with tempol and tadalafil significantly prevented elevation in RVSP and RV dP/dt(max) and reduced oxidative stress in rats exposed to acute hypoxia [289]. In addition, tempol has been found to inhibit LY83583-mediated constriction of rat IPAs [185], reduce hypoxia-induced SMC proliferation and remodeling in rat PAs, as well as inhibit lung ROS production [184]. Furthermore, treatment with tempol attenuated PH in a sheep model [320], and prevented spontaneous development of PH in $ALK1^{+/-}$ mice [170]. Tempol has not yet been evaluated in any clinical trial.

Ebselen

There is minimal information on the use of the glutathione peroxidase mimetic ebselen in PH; however, recent studies suggest that ebselen may have protective effects in the pulmonary vasculature. Ebselen has been shown to attenuate hypoxia and peroxynitrite-induced proliferation of PASMC in vitro [3]. In addition, ebselen has been shown to decrease the sustained phase of hypoxic vasoconstriction of IPAs in rats [71]. More studies are needed to better understand the effects of ebselen on the pulmonary vasculature and determine whether ebselen has efficacy in animal models of PH.

15.4.3 Inhibitors of ROS Generation

15.4.3.1 Inhibitors of Oxidases

Inhibitors of oxidases include inhibitors of NOX, xanthine oxidase, and monoamine oxidase. These substances function by blocking the main enzymes that produce ROS in cells.

NADPH Oxidase Inhibitors

NOX inhibitors are perhaps the most studied of all the oxidase inhibitors tested in PH. Apocynin, an NADPH inhibitor, attenuates hypoxia-induced PH and vascular remodeling in lectin-like oxidized low-density lipoprotein receptor (LOX-1) transgenic mice that have enhanced ROS in response to hypoxia [264]. In addition, apocynin was shown to attenuate cold-induced PH and PA remodeling in rats [78], and restored pulmonary artery endothelial function and vascular responses in diabetic rats [214]. In lambs with PPHN induced by ductus arteriosus ligation, it has also been shown that apocynin significantly improves oxygenation, enhances PA relaxation and eNOS expression, and improves angiogenic activity of PAEC [339, 363]. Furthermore, in rat PASMC, apocynin reverses hypoxia-induced decreases in Kv current density [245], and suppresses U46619-induced inhibition of Kv currents [70].

Xanthine Oxidase Inhibitors

Allopurinol has been the mainstay of treatment for gout for many years and has recently become of great interest in the study of ischemic heart disease, chronic heart failure, and inflammatory diseases. In mice and rats exposed to hypoxia, allopurinol has been shown to decrease superoxide production, reduce PH, attenuate vascular remodeling, and alleviate the increased RVSP and RVH [26, 151, 167]. In addition, Shen et al. found that isolated rat lungs exposed to hypoxic challenges had attenuated HPV when treated with allopurinol ex vivo [315].

15.4.3.2 Iron Chelators

Iron normally exists in cells in the form of ferric ions (Fe^{3+}), which can react with superoxide releasing highly reactive hydroxyl radicals. These radicals can cause lipid peroxidation, DNA oxidation, and protein oxidation [374]. Based on this rationale, it has been suggested that iron chelation may have a potential benefit on oxidative stress in the lung, but most investigations have failed to support this hypothesis. Treatment of rats exposed to chronic hypoxia with desferroxamine prevented PH and vascular remodeling in vivo, and inhibited human PASMC growth in vitro [374].

However, human studies have demonstrated that healthy volunteers exposed to desferroxamine develop increased PVR [25], and hypoxia-induced pulmonary vasoconstriction is enhanced by desferroxamine in healthy volunteers [318]. In addition, recent studies have found decreased iron levels in patients with IPAH and iron supplementation is now being evaluated as a potential treatment in this group of patients [153, 294]. Further studies are needed to better understand the role of iron in PAH pathogenesis.

15.4.4 Nrf2 Activators

Nrf2 promotes gene expression of antioxidant response element (ARE)-regulated antioxidant enzymes in response to oxidative stress [165]. Nrf2 is held in the cytoplasm by an inhibitor, and activation of the PKC signaling by oxidative stress leads to activation and translocation of Nrf2 to the nucleus with subsequent activation of ARE-regulated genes [165]. Nrf activators act by eliciting this response and increasing the level of ARE-regulated antioxidant enzymes in cells. Protandim, an Nrf2 activator prevented the development of right ventricular failure and fibrosis in the Sugen hypoxia rat model of PH, although it did not prevent the angio-obliterative vascular remodeling [352]. In addition, Nrf2-knockout mice develop exaggerated RVH in response to hypoxia, and the Nrf2 activator olipraz attenuates RVH and vascular remodeling in wild type, but not Nrf2-deficient, mice exposed to hypoxia [107]. Future studies on the potential benefits of Nrf2 activators in the treatment of PAH are necessary.

15.4.5 Tetrahydrobiopterin

The role of tetrahydrobiopterin (BH_4) in oxidative stress and eNOS uncoupling has been reviewed in previous sections. Deficiency of this cofactor has been associated with development of PH and IPF in animal models [10, 183, 338]. Sapropterin dihydrochloride (pharmaceutical preparation of BH_4) has been used in the treatment of hyperphenylalanemia [295]. Interest in the possible benefits of BH_4 supplementation for the treatment of PH is now increasing. Administration of BH_4 to MCT-treated rats attenuated PH and vascular remodeling [120, 180], as well as decreased HPV and increased NO synthesis in isolated lung preparations [120, 190]. In addition, while BH_4 did not improve endothelial dysfunction of IPAs in a porcine model of PPHN [258], treatment of PAEC from PPHN lambs decreased apoptosis, improved angiogenesis, increased NO and eNOS dimer formation, and decreased superoxide production [338]. Furthermore, treatment with sapropterin dihydrochloride, in addition to sildenafil and/or endothelin receptor antagonists, in 18 patients with PAH or inoperable CTEPH was well tolerated and improved 6-min walk distance, although did not significantly alter NO synthesis or oxidative stress [295].

As BH_4 supplements have been proven to be safe in humans, they represent an interesting therapeutic alternative for the treatment of PAH, but further studies are needed to determine their true efficacy.

15.4.6 Mitochondria-Activating Drugs and Mitochondria-Targeting Antioxidants

The hyperproliferative and antiapoptotic phenotype of PASMC observed in PAH is associated with mitochondrial suppression, altered glucose metabolism, and decreased mROS production [98]. These mechanisms are described in detail in previous sections.

15.4.6.1 Mitochondria-Targeting Antioxidants

There has been recent interest in therapeutic strategies that specifically target mitochondria in order to restore their normal function. The fact that this organelle is negatively charged has led to the development of strategies that increase mitochondrial selectivity such as the use of a positively charged ion, namely triphenylphosphonium (TPP^+), to deliver vitamin antioxidants [98]. One of the agents that uses this cation as vehicle and has been studied in vascular diseases is MitoQ, a ubiquinone analogue of the mitochondrial electron transport chain [98]. Treatment of spontaneously hypertensive rats with MitoQ protected against the development of hypertension, improved endothelial function, and decreased cardiac hypertrophy [134]. In addition, the mitochondrial-targeted SOD mimetic mitoTEMPO decreased mitochondrial superoxide production, reduced cellular NOX activity, restored NO expression, improved endothelial-dependent relaxation, and attenuated hypertension in mice exposed to angiotensin II infusion [93].

15.4.6.2 Mitochondrial-Activating Therapies

DCA and trimetazidine (TMZ) stimulate mitochondria and regulate metabolic substrate entry into the TCA cycle [98]. DCA also inhibits PDK, which ultimately results in the inhibition of normoxic HIF-1α production and increases in pro-apoptotic factors, reducing abnormal cell proliferation [98]. Several studies in animal PH models have demonstrated that DCA stimulates glucose oxidation, reduces mPAP, and decreases medial wall thickening of PAs [40, 98, 136, 235, 239, 331]. An early-phase clinical trial of DCA in PAH is currently being completed [98] (NCT01083524). TMZ has also been shown to increase glucose oxidation, suppress fatty acid oxidation, restore perfusion to distal PAs, and reverse established PH in animal models [98, 331]. Finally, phenylbutyrate (PBA), a chemical chaperone which prevents

disruption of the ER-mitochondrial unit, has recently been shown to attenuate PH, vascular remodeling, and RVH in both hypoxia-induced PH in mice and in MCT-induced PH in rats [98].

15.5 Conclusions

This review highlights the important role that oxidative stress and aberrant NO signaling play in the pathogenesis of PAH and emphasizes the mechanisms of ROS-induced pulmonary vascular remodeling in PAH. Although significant progress has been made in understanding the pathogenesis of PAH, currently available therapies that target the imbalance of vasoactive mediators do not improve mortality in PAH patients. Emerging studies implicate oxidative stress as a key mechanism in the pathobiology of PAH and therapies targeting ROS generation have shown efficacy in animal models of PH. Growing evidence of the importance of oxidative stress in the pathogenesis of PAH has led to the identification of potential new therapeutic targets in PAH. New approaches to target oxidative stress include ROS scavengers, inhibitors of ROS generation, Nrf2 activators, mitochondria-activating drugs, and eNOS recoupling agents. Developing novel therapeutics to target oxidative stress in PAH is an active and exciting area of research. Although human data is currently limited, antioxidant therapeutics may hold promise in the future for treatment of PAH.

Conflict of Interest The authors report no conflict of interest.

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