Molecular Basis of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension

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1 Introduction

Pulmonary hypertension (PH) is a progressive condition that was responsible for 5.5 deaths per 100,000 people in the USA in 2001, a number that increased to 6.5 deaths per 100,000 people in 2010 (National Vital Statistics System, Centers for Disease Control and Prevention, USA) [1]. The pathogenesis of PH is characterized by progressive increases in pulmonary vascular resistance and pulmonary artery pressure (>25 mmHg at rest). Features of PH include pulmonary vascular remodeling, endothelial dysfunction, impaired vasoconstriction, and intravascular thrombosis [2–4]. Causes of PH have been classified in an expert consensus as class I to V (Table 1) [2]. In severe cases of PH (e.g., idiopathic pulmonary arterial hypertension, IPAH), treatment options are limited, and lack of treatment can lead to right heart failure and premature lethality. Current pharmacological therapies targeting abnormalities in the prostacyclin, nitric oxide, and endothelin pathways can improve IPAH symptoms and lead to modest survival benefits, but do not reverse the disease pathogenesis [5, 6]. Lung transplantation remains the best option for this devastating disease.

In healthy individuals, the prevention of pulmonary vascular remodeling and the preservation of a normal pulmonary tension appear to be controlled by cyclic guanosine monophosphate (cGMP)-dependent activation of protein kinase G (PKG); this occurs downstream of nitric oxide (NO) production and subsequent activation of soluble guanylate cyclase (sGC) (Fig. 1). Genetic deletion of PKG-1α induces PH in mice [7], demonstrating the causal role of PKG dysfunction in the pathogenesis of PH. Therapeutic agents targeting this pathway by either inhibiting phosphodiesterase type 5 (PDE5)-dependent cGMP degradation or activating sGC-derived cGMP production have been shown to be effective in improving the symptoms of PH in humans. Early PH typically involves dysregulation of vasoactive pathways including reduced vasodilator pathway signaling (e.g., through impaired bioavailability

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of NO and downregulation of prostaglandin signaling), and enhanced vasoconstrictor pathway signaling (e.g., through increased production of endothelin-1 and reactive oxygen species, ROS) [8]. Nevertheless, the precise molecular mechanisms that are responsible for aberrant pulmonary vascular remodeling and vasoconstriction in PH patients have not been fully defined.

Reactive nitrogen species (RNS) are usually unstable nitrogen-centered free radicals containing unpaired electrons (Table 2). RNS regulate many physiological processes including differentiation, metabolism, migration, and proliferation. These messenger molecules are also heavily involved in the nitrative modification of proteins that regulate PH pathogenesis. NO, for example, is formed by the NO synthase (NOS) enzymes and is stable under anoxic conditions. In the presence of excessive superoxide anion ($O_2^{\bullet-}$) (i.e., oxidative stress), however, NO is converted to peroxynitrite ($ONOO^-$) (i.e., nitrative stress) (Fig. 2a). In general, when superoxide formation occurs at a threefold greater rate than NO synthesis, NO is being quantitatively converted to peroxynitrite, which leads to decreased NO bioavailability, and induces posttranslational modifications (e.g., nitrates tyrosine residues) of proteins and resultant dysregulation of protein functions [9]. Nitrative stress-induced dysregulation of molecular signaling pathways have been implicated in the pathogenesis of PH in both animal models and patients [10–12].

Given that the lung tissue of patients with severe PH demonstrate prominent levels of nitrative as well as oxidative stress [13, 14], delineation of the mechanistic role of oxidative/nitrative stress in the pathogenesis of PH and the signaling pathways regulating oxidative/nitrative stress in the pulmonary vasculature has become an actively pursued area of research. The aim of this chapter is to review mechanisms that mediate nitrative stress-induced PH, delineate molecular sources of ROS and RNS in the context of PH, and describe evidence of nitrative stress in PH patients.

Table 1 Clinical categories of pulmonary hypertension

Class	Name
I	Pulmonary arterial hypertension
II	Pulmonary hypertension owing to left heart disease
III	Pulmonary hypertension associated with lung disease and/or hypoxemia
IV	Pulmonary hypertension due to chronic thrombotic and/or embolic disease
V	Pulmonary hypertension with unclear multifactorial mechanisms

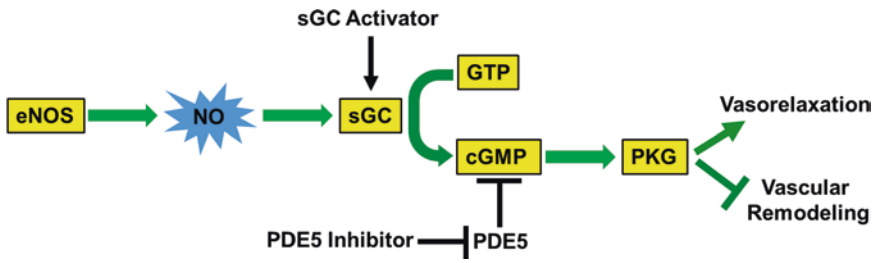

Fig. 1 Nitric oxide signaling maintains vascular homeostasis and inhibits the development of pulmonary hypertension. eNOS generates basal levels of NO, which activate sGC; this in turn leads to cGMP production and subsequent activation of cGMP-dependent PKG. Activated PKG causes vasorelaxation and inhibits pulmonary vascular smooth muscle cell proliferation which underlies the mechanisms of pulmonary vascular remodeling, and thereby preserves normal pulmonary tension. Decreased NO bioavailability leading to impaired PKG activity is a common feature of PH. FDA-approved drugs by activating sGC (Riociguat) or inhibiting PDE5-mediated cGMP degradation (Sildenafil, Tadalafil) have been shown effectiveness in improving the symptom of PH and promoting modest survival, demonstrating the fundamental role of this signaling pathway in maintaining pulmonary vascular homeostasis. *cGMP* cyclic guanosine monophosphate, *eNOS* endothelial nitric oxide synthase, *GTP* guanosine triphosphate, *NO* nitric oxide, *PKG* protein kinase G, *sGC* soluble guanylate cyclase

Table 2 Reactive nitrogen species

Name	Formula
Dinitrogen trioxide	N_2O_3
Nitric oxide	·no
Nitrite	NO_2^-
Nitrogen dioxide	$^{\bullet}NO_2$
Nitronium ion	NO_2^+
Nitrosothiols	RSNOs
Nitrosyl cation	No^+
Nitrosyl chloride	NO_2Cl
Nitrous acid	HNO_2
Nitrous oxide	N_2O
Nitroxyl anion	No^-
Peroxynitrite	$ONOO^-$

2 Molecular Mechanisms of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension

Selectivity for tyrosine nitration by peroxynitrite. As mentioned above, excessive increases in NO and ROS levels give rise to peroxynitrite, a potent, diffusible, and damaging oxidant [15–17]. It has been shown that high levels of peroxynitrite are cytotoxic and induce death of vascular endothelial cells and smooth muscle cells, which may contribute to the pathogenesis of PH [18, 19]. Even at sublethal concentration,

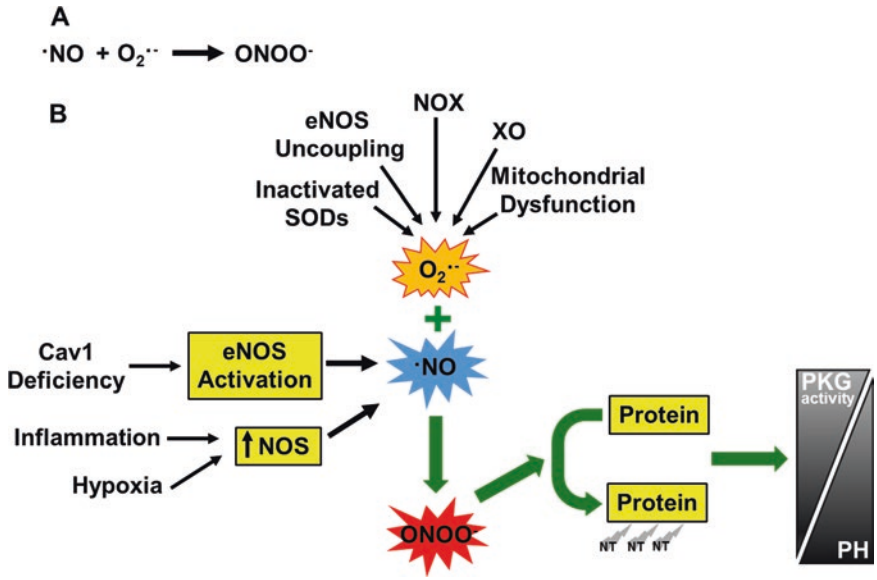

Fig. 2 Molecular basis of nitrative stress in the pathogenesis of PH. (**a**) Generation of peroxynitrite. In the presence of excessive superoxide anion (i.e., oxidative stress), nitric oxide and the reactive oxygen species, superoxide anion, react to form the potent, diffusible and damaging oxidant peroxynitrite. $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite. (**b**) Peroxynitrite induces tyrosine nitration of proteins leading to endothelial dysfunction, vasoconstriction and vascular remodeling and contributes to the pathogenesis of PH. Under various conditions, such as tissue inflammation, hypoxia, and Cav1 deficiency, excessive NO is generated because of increased expression of NOS or activation of eNOS (secondary to Cav1 deficiency). Under these conditions, excessive superoxide is also generated, which is attributable to increased activities of NOX (mainly NOX2 and NOX4) and xanthine oxidase (XO), and decreased antioxidant enzyme (SODs) activities, as well as eNOS uncoupling and mitochondrial dysfunction. Excessive NO and supeorxide anion leads to generation of peroxynitrite which induces tyrosine nitration of proteins and resultant dysfunction. For example, PKG nitration leads to impaired PKG activity which in turn induces vasoconstriction and vascular remodeling and thereby PH. The roles of nitration of other proteins in the pathogenesis of PH are listed in Table 3. *NT* tyrosine nitration

Table 3 Proteins modified by peroxynitrite and involved with pulmonary hypertension

Name	Action of nitration	Pathological function of nitration	Refs
PKG	Inhibition	Pulmonary vasoconstriction and remodeling	[10, 11]
Prostacyclin synthase	Inhibition	Decreased production of vasodilator prostacyclin and increased production of vasoconstrictors	[25, 26]
eNOS	Inhibition	Induction of eNOS uncoupling generating superoxide and decreasing NO production	[11, 55, 59]
Mitochondrial SOD	Inhibition	Increased oxidative and nitrative stress	[30, 31]
ERK	Activation	Vascular cell proliferation: Vascular remodeling	[33, 39]
PKC	Activation	Vascular cell proliferation: Vascular remodeling	[36, 39]
p85PI3K	Inhibition (of PI3K)	Endothelial cell apoptosis and endothelial dysfunction	[40]
Src kinase	Activation	Vascular cell proliferation and migration: Vascular remodeling	[41]

eNOS endothelial nitric oxide synthase, *ERK* extracellular signal-related kinase, *PI3K* phosphoinositide 3 kinase, *PK* protein kinase, *SOD* superoxide dismutase

peroxynitrite reacts with amino acids leading to protein modifications such as tyrosine nitration. Nitration of tyrosine residues involves the addition of a nitro group ($-NO_2$) to the hydroxyl group on the tyrosine residue. Peroxynitrite-induced tyrosine nitration is selective for certain tyrosine residues, which is not directed by specific tyrosine-containing signatures within the primary sequences, by the abundance of the proteins, or by the total amount of tyrosine. Instead, it is attributable to the local environment in which the tyrosine residue resides and the proximity of the protein to the nitrating agents [20]. The presence of a proximal negatively charged Glu or Asp residue promotes the selective nitration of tyrosine, provided there is no Cys or Met near the tyrosine residue, as Cys or Met would otherwise preferentially react with nitrating agents [21]. Additionally, the presence of enzymatic metal cofactors near the tyrosine residue is likely to confer specificity to nitration due to the metal-catalyzed formation of $^{\cdot}NO_2$ from $ONOO^-$. Tyrosine nitration can be enhanced by the presence of heme-containing proteins (e.g., prostacyclin synthase) or in the presence of hydrogen peroxide through the generation of the nitrogen dioxide free radical by heme-peroxidases (e.g., myeloperoxidase) [22, 23]. Specific molecules that can be modified by peroxynitrite and could be involved in the pathogenesis of PH are given in Table 3.

Nitration of protein kinase G impairs its activity and induces PH. In the lungs of Caveolin 1 (Cav1)-deficient mice that develop PH, there are enhanced levels of nitrotyrosine (a surrogate marker for peroxynitrite) [11]. Tyrosine nitration of PKG is also enhanced in the lung of these mice versus wild types, while PKG activity is impaired at baseline or with its activator, cGMP [11]. In the same study, authors demonstrated that alterations in PKG activity that are induced by Cav1 deficiency are eNOS-dependent and occur at least partly through nitration of PKG-1α tyrosine residues 345 and 549, which results in decreased kinase activity (Fig. 2b). Genetic deletion of eNOS in Cav1 null mice results in normalization of the hypertensive pulmonary phenotype. Finally, this study also showed that PH phenotypes observed in Cav1 null mice could be reversed by treatment of these mice with either a superoxide dismutase mimetic (MnTMPyP, which scavenges superoxide) or the NOS inhibitor, L-NAME [11]. Additionally, in Cav1 null mice, restoration of PKG activity through increased expression of PKG attenuates PH.

Another genetic study also demonstrated the causal role of impaired PKG activity in the pathogenesis of PH by showing that *Prkg1* (encoding PKG-1) knockout mice develop PH [7]. Decreased PKG activity induces vasoconstriction and vascular remodeling partly through activation of Rho A/Rho kinase signaling [7]. Together, these studies provide unequivocal evidence for the role of nitrative stress-induced tyrosine nitration of PKG and the resultant inhibition of its activity in the pathogenesis of PH.

In another study, it has been shown that nitration of tyrosine 247 in PKG-1α results in decreased cGMP binding and thereby decreased PKG activity in pulmonary artery smooth muscle cells [12]. Tyrosine nitration of PKG has also been shown to occur in ovine fetal intrapulmonary veins in a hypoxia-dependent manner that is endothelial NOS (eNOS)-independent but through increased levels of nitrite and nitrate [10]. As little as 30 min exposure to hypoxia induces PKG tyrosine nitration and inhibition of its activity, which is attributable to hypoxia-induced impairment of pulmonary vessel vasorelaxation [10].

Nitration and inactivation of prostacyclin synthase induces vasoconstriction. Prostacyclin, generated primarily by the vascular endothelium, is a potent vasodilator through activation of adenylate cyclase in vascular smooth muscle cells, which increases synthesis of cyclic adenosine monophosphate (cAMP). It has been shown that prostacyclin synthase can be nitrated at residue 430 and that this results in impairment of its activity [24]. Prostacyclin synthase nitration was also observed to be increased in pulmonary arterial endothelial cells from newborn lambs with persistent PH [25]. Inactivation of prostacyclin synthase through tyrosine nitration impairs production of the potent vasodilator prostacyclin, but also promotes the generation of the vasoconstrictors prostaglandin H2 (PGH2) and thromboxane A2, and thereby contributes to vasoconstriction and development of PH [26, 27].

Nitration of endothelial nitric oxide synthase leads to endothelial nitric oxide synthase uncoupling and endothelial dysfunction. Studies show that eNOS can be modified by peroxynitrite through tyrosine nitration, which results in impairment of eNOS activity and diminished synthesis of the vasodilator, NO [27]. Furthermore, peroxynitrite-mediated damage to eNOS induces eNOS uncoupling, which occurs when eNOS is not coupled with its cofactors or substrate, and the synthase activity is redirected away from generation of NO to superoxide [28, 29]. In eNOS uncoupling, superoxide is generated by dissociation of the ferrous-dioxygen complex from the oxygenase domain [28, 29]. eNOS uncoupling has been shown to be involved in eNOS-dependent tyrosine nitration of prostacyclin synthase. Thus, nitration of eNOS and its resultant uncoupling leads to decreased bioavailability of the vasodilators, NO and prostacyclin, and augmented oxidative/nitrative stress.

Nitration of the antioxidant enzyme manganese superoxide dismutase contributes to oxidative stress and PH. Given that the reaction between NO and superoxide anion leads to generation of the potent oxidant peroxynitrite, dysregulation of antioxidant enzymes that scavenge and reduce levels of superoxide will enhance oxidative and nitrative stress and thereby contribute to the pathogenesis of PH. It has been shown that the mitochondrial manganese superoxide dismutase (MnSOD) is nitrated at residue 34, which results in inactivation of its activity [30]. Tyrosine nitration of MnSOD has been demonstrated in vivo in fetal lambs with persistent PH [31]. This study shows that decreased MnSOD activity contributes to oxidative stress and resultant endothelial dysfunction and thereby facilitates the development of persistent PH.

Nitration of key signaling molecules involved in pulmonary vascular remodeling. Extracellular signal-related kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and protein kinase C (PKC) are important mediators of pulmonary vascular cell proliferation underlying pulmonary vascular remodeling. It has been shown that peroxynitrite can activate these key signaling molecules [32–36]. Peroxynitrite also

activates nuclear factor kappa B (NF-kB), which in turn induces transcription of inducible NOS (iNOS, see Sect. 3). NF-kB is activated in the pulmonary vessels of end-stage IPAH patients [37], while NF-kB inhibition reduces experimental PH in mice [38]. Importantly, another study demonstrates that peroxynitrite activates pulmonary artery smooth muscle cell and endothelial cell proliferation through activation of ERK and PKC [39]. Additionally, it has been shown that peroxynitrite induces nitration of p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [40], and Src kinase [41]. Nitration of p85 inhibits its binding to the catalytic domain of PI3K and thereby attenuates PI3K activity, whereas nitration of Src kinase leads to Src activation. These signaling molecules play an important role in pulmonary vascular cell survival, migration and proliferation. However, tyrosine nitration of these molecules in pulmonary vascular cells or in lung tissue from animal models of PH or from patients with PH has not yet been reported. Future study is warranted to assess the role of tyrosine nitration of these molecules in the regulation of pulmonary vascular remodeling.

3 Molecular Sources of Reactive Nitrogen Species in Pulmonary Hypertension

Induced expression of endothelial nitric oxide synthase. While controversy exists regarding the expression levels of eNOS in the lungs of PH patients compared with controls, eNOS is robustly expressed in the plexiform lesions of IPAH lungs [42], which may contribute to nitrative stress in these lesions. Animal models of PH have also consistently demonstrated increases in the levels of eNOS mRNA, protein, and activity [43–46]. Despite these inconsistent findings, which may be a product of the different stages of disease progression being assessed, it is widely believed that NO signaling is impaired in PH patients. It has been suggested that impaired NO bioavailability and activity during PH is a result of diminished NOS cofactor availability [47] or eNOS uncoupling [48], as opposed to reductions in NOS levels per se. Reducing intracellular tetrahydrobiopterin levels, for example, reduces NO synthesis and enhances superoxide generation in endothelial cells and isolated blood vessels [49], while administration of tetrahydrobiopterin inhibits superoxide production in a dose-dependent manner [50].

Activation of endothelial nitric oxide synthase secondary to Caveolin 1 deficiency. eNOS-derived NO is in general considered to be beneficial and plays an important role in maintaining vascular homeostasis through the activation of PKG (Fig. 1). Activity of eNOS is regulated by its interaction with effector molecules including Cav1. It has been shown that eNOS activity is negatively regulated by Cav1 binding to eNOS [51]. Administration of the Cav1 scaffolding domain inhibits eNOS activity [52]. In Cav1-deficient mice compared with wild type controls, eNOS is activated in blood vessels [53], NO levels in plasma and lung are increased [11, 54]. Cav1 deficiency induces PH as shown by increased pulmonary vascular resistance, medial thickness, and muscularization, along with enhanced right ventricle: left ventricle plus septum weight ratio [11, 54]. PH did not occur, however, when both Cav1 and eNOS were genetically deleted, or when NOS was inhibited pharmacologically by administration of L-NAME to Cav1 knockout mice [11, 55]. These experimental studies together demonstrate the essential requirement for eNOS activation secondary to Cav1 deficiency in the pathogenesis of PH. Cav1 deficiency has been shown in several animal models of PH [56–58] and in the lungs of IPAH patients, which is associated with eNOS activation and tyrosine nitration of other proteins including PKG [11, 57, 59, 60].

Induced expression of inducible nitric oxide synthase and neuronal nitric oxide synthase. Studies of hypoxia-induced PH have shown increased levels of iNOS mRNA and protein in the lungs of rats exposed to hypoxia compared with normoxia [61–64]. Inhibition of iNOS attenuates hypoxia-induced PH in rats [64], indicating the pathogenic role of iNOS-derived NO in hypoxia-induced PH. Another study has shown

similar increases in the levels of neuronal NOS (nNOS) gene and protein expression in the lungs of rats following exposure to hypoxia [47]. Furthermore, nNOS-derived NO is the source of peroxynitrite that causes neuron damage in ischemic stroke [65].

Dietary nitrite. Along with the L-arginine-NO signaling pathway, the diet is a major source of nitrite in humans [8]. The enterosalivary circulation of nitrate originates from intake of leafy greens and vegetables, along with food additives and preservatives. Salivary glands also concentrate nitrates from the plasma and secrete them into the mouth. Symbiotic bacteria in the mouth then reduces nitrate to nitrite, which enters the gastrointestinal tract. From here, nitrite can be absorbed into the circulation or reduced to form NO in the stomach. The enzymatic and nonenzymatic formation of NO from nitrite has been reviewed elsewhere [8]. Nonenzymatic production of NO, for example, is enhanced by copper, ascorbate, and polyphenols, while enzymatic NO generation has been shown in metal-containing proteins such as carbonic anhydrase, hemoglobin, and myoglobin [8]. Little is known, however, about the role of dietary nitrite-derived NO in nitrative stress-induced pathogenesis of PH. In fact, several studies show that inhalation of a low dose of nebulized sodium nitrite is protective from PH in animal models [66, 67].

4 Molecular Sources of Reactive Oxygen Species in Pulmonary Hypertension

Nicotinamide adenine dinucleotide phosphate oxidases. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) exist in numerous isoforms, namely NOX1, NOX2, NOX3, NOX4, NOX5, dual oxidase 1, and dual oxidase 2. NOX enzymes reduce oxygen to superoxide anion ($O_2^{\bullet-}$). NOX5 is not present in rats or mice, and only NOX2 and NOX4 are expressed in the lungs [68]. NOX enyzmes are the predominant source of ROS in endothelial cells [69], fibroblasts [70], and vascular smooth muscle cells [71]. In pulmonary artery smooth muscle cells, for example, NOX4 expression and $O_2^{\bullet-}$ levels are increased by treatment with transforming growth factor β [72]. In chronic hypoxia models of rodent PH, pulmonary vascular remodeling and PH are caused by NOX2- and NOX4-dependent generation of ROS [73, 74], while NOX2 and NOX4 deletion reverses PH through decreases in ROS levels [75]. In a rat model of monocrotaline-induced PH, however, induction of PH was dependent on NOX1 but not NOX4 [76]. Although the type of hypertensive insult may determine which NOX isoform is activated, their dysregulation is well established in the pathogenesis of PH [77].

Xanthine oxidase. Xanthine oxidase converts purine to uric acid under normoxia, but under hypoxic conditions that commonly occur in PH patients, hypoxanthine is formed from adenosine triphosphate, and oxygen is reduced to hydrogen peroxide and superoxide anion [78]. Xanthine oxidase level is enhanced in the circulation of PAH patients, along with increased xanthine oxidase activity in the pulmonary arteries [79]. Furthermore, xanthine oxidase activity is enhanced in a rodent model of PH and blood pressures can be restored by treatment of these animals with specific xanthine oxidase inhibitors [80, 81].

Endothelial nitric oxide synthase uncoupling. Paradoxical decreases in NO signaling that occur while eNOS expression is unaltered or even raised during PH may be a result of eNOS uncoupling. In this process, electrons being transferred to the NOS oxygenase domain from the reductase domain to form L-arginine are instead diverted to molecular oxygen, which results in the generation of superoxide anion instead of NO (for detailed review, see [48]). eNOS uncoupling is associated with changes in the quaternary structure of the enzyme, i.e., decreased assembly of the homodimer but increased assembly of the monomer. Uncoupling of eNOS is stimulated by depletion of cofactors L-arginine and tetrahydrobiopterin (BH4), or increased dihydrobiopterin (BH2) [82]. Mice that

have low levels of BH4 or decreased BH4:BH2 ratios exhibit PH [83]. Administration of BH4 or a BH4 analogue attenuates PH in rat models challenged with monocrotaline or hypoxia [82, 84]. These studies provide strong evidence for a pathogenic role of eNOS uncoupling-derived ROS in PH.

Mitochondrial electron transport chain dysfunction. Abnormal function of the mitochondrial electron transport chain leads to increased ROS generation in PH [85]. Elevated levels of pyruvate dehydrogenase kinase lead to reduced levels of acetyl-CoA and a shift to aerobic glycolysis, which promotes pulmonary hyperproliferation [86]. It has also been suggested that removal of mitochondrial hydrogen peroxide leads to hypoxic pulmonary vasoconstriction, and nevertheless, increased levels of mitochondrial ROS contribute to hypoxic pulmonary vasoconstriction and hypoxic PH [87, 88].

Antioxidant enzyme dysfunction. Given that the reaction between NO and superoxide anion leads to generation of the potent oxidant peroxynitrite, enzymes that scavenge and reduce levels of superoxide will reduce peroxynitrite-induced injury. While NO reacts with oxyhemoglobin and is rapidly converted to nitrate in erythrocytes, superoxide is removed by SOD enzymes [89]. Mitochondrial SOD is rendered inactive, however, under conditions of nitrative stress when increases in peroxynitrite lead to nitration of tyrosine residue 34 in vitro [30]. Tyrosine nitration of SOD has also been demonstrated in vivo in newborn lambs with persistent PH [31]. Although mitochondrial SOD nitration is seen in human renal allografts [30], it is unknown whether mitochondrial SOD is nitrated and inactivated in lung tissues of patients with severe PH such as IPAH. However, one study has shown decreased activities of SOD isoforms (including CuZnSOD, ECSOD, and MnSOD) in airway epithelial cells and in bronchial tissues of IPAH patients [90], suggesting that levels of superoxide could be increased in IPAH lung tissue.

5 Nitrative Stress in Pulmonary Hypertension Patients

Human studies that provide evidence for a role of nitrative stress in the pathogenesis of PH can be observational studies of nitrative stress factors in PH patients or studies showing an impact of therapies that target nitrative stress signaling pathways on clinical outcome. For example, levels of peroxynitrite are elevated in the lungs of patients with severe PH, and these levels are thought to be raised in part by conditions of tissue hypoxia and inflammation [13, 80]. Prominent tyrosine nitration, a hallmark of nitrative stress, is evident in lung tissues of severe PH patients including IPAH patients [13]. Increased tyrosine nitration of PKG, for instance, is observed in lung tissues of IPAH patients [12]. The activity of eNOS is markedly increased in the lungs of IPAH patients compared with healthy controls, without significant change in its protein levels [11]. There is also evidence of high levels of eNOS in the plexiform lesions of lungs from IPAH patients [42], although eNOS levels have been reported to be upregulated [43], unaltered [91], or downregulated [92] in the lungs of PH patients versus control subjects. 8-hydroxyguanosine, the product of the reaction between superoxide and guanine, is markedly increased in the endothelium of plexiform and concentric lesions from IPAH patients but not in control subjects [13]. In the lungs of the same IPAH patients, the amount and activity of MnSOD was lower compared with controls. Another study also shows marked decreases in the expression and activity of all three of the SOD isoforms in lungs of IPAH patients [90]. In contrast, expression and activity of some of the ROS-generating enzymes are markedly increased in lung tissues of IPAH patients compared with controls; NOX4 expression was markedly increased in the pulmonary vasculature of IPAH patients (i.e., predominantly in the thickened medial layer of pulmonary arteries) [93]. Xanthine oxidase level is also enhanced in the circulation of IPAH patients, along with increased xanthine oxidase activity in the pulmonary arteries [79]. Together these studies demonstrate

increased oxidative and nitrative stress in PH patients, especially in IPAH patients.

The presence of nitrative and oxidative stress in PH patients suggests that antioxidant therapy could prove beneficial in the clinic. Despite examples of antioxidant therapy suppressing PH in animal models, such treatments in human PH have proven to be predominantly ineffective [94]. The antioxidant, coenzyme Q, for instance, gave rise to a modest improvement in right ventricle function, without lengthening 6-min walking time [95]. Large-scale randomized clinical studies of therapies that reduce nitrative stress will continue to improve our understanding of the importance of the nitrative stress pathways in the pathogenesis of PH in humans.

6 Conclusions

Increased oxidative/nitrative stress is a hallmark of severe PH in patients including those with IPAH. Peroxynitrite, nitrotyrosine, and nitration of specific proteins are prominent in lung tissues of IPAH patients. Recent studies have demonstrated that increased nitrative stress induces cytotoxicity in pulmonary vascular cells, post-translational modification (tyrosine nitration) of proteins and resultant dysregulation of their functions (Table 3), and decreased bioavailability of vasodilators NO and prostacyclin. Nitrative stress-induced tyrosine nitration of PKG results in impairment of its function (via decreased kinase activity or cGMP binding), which causes vasoconstriction and vascular remodeling leading to PH. Nitration of prostacyclin synthase inhibits its ability to synthesize the vasodilator, prostacyclin, while eNOS nitration induces eNOS uncoupling leading to decreased NO production and increased superoxide generation. Nitration-mediated inhibition of antioxidant enzymes such as MnSOD enhances superoxide levels and oxidative/nitrative stress. Nitrative stress also leads to activation of key signaling molecules such as ERK and PKC, which promotes pulmonary vascular cell proliferation that underlies pulmonary vascular remodeling. Thus, nitrative stress plays an important role in the pathogenesis of PH by inducing vasoconstriction and pulmonary vascular remodeling (Fig. 2b, and Table 3). Although current approaches that restore aberrant nitrative signaling and alleviate nitrative stress can reduce PH in animal models, the potential benefit of such treatments on patient survival is yet to be conclusively proven. Development of novel pathologically relevant animal models of PH [91] and of therapies that inhibit nitrative stress (thereby normalizing the functions of key proteins such as PKG) could ultimately lead to improved clinical outcome in patients with PH.

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