Pulmonary vascular disease can be defined as either a disease affecting the pulmonary capillaries and pulmonary arterioles, termed pulmonary arterial hypertension, or as a disease affecting the left ventricle, called pulmonary venous hypertension. Pulmonary arterial hypertension (PAH) is a disorder of the pulmonary circulation characterized by endothelial dysfunction, as well as intimal and smooth muscle proliferation. Progressive increases in pulmonary vascular resistance and pressure impair the performance of the right ventricle, resulting in declining cardiac output, reduced exercise capacity, right heart failure, and ultimately death. While the primary and heritable forms of the disease are thought to affect over 5,000 patients in the U.S., the disease can occur secondary to congenital heart disease, most advanced lung diseases, and many systemic diseases. Multiple studies implicate oxidative stress in the development of PAH. Further, this oxidative stress has been shown to be associated with alterations in reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide (NO) signaling pathways, whereby bioavailable NO is decreased and ROS and RNS production are increased. Many canonical ROS and NO signaling pathways are simultaneously disrupted in PAH, with increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidoreductase, uncoupling of endothelial NO synthase (eNOS), and reduction in mitochondrial number, as well as impaired mitochondrial function. Upstream dysregulation of ROS/NO redox homeostasis impairs vascular tone and contributes to the pathological activation of anti-apoptotic and mitogenic pathways, leading to cell proliferation and obliteration of the vasculature. This manuscript will review the available data regarding the role of oxidative and nitrosative stress and endothelial dysfunction in the pathophysiology of pulmonary hypertension, and provide a description of targeted therapies for this disease.
is increased at the pre-capillary arteriolar level. Elevated pulmonary pressure results from a
decrease in arterial lumen through a combination of: a) endothelial dysfunction and
increased contractility of small pulmonary arteries, b) proliferation and remodeling of
endothelial and smooth muscle cells, and c) in situ thrombosis. To diagnose this condition, a
procedure called a right heart catheterization is required. Measurements are made by placing
a balloon tipped catheter in the pulmonary artery, advancing to the small pulmonary arteries
were the balloon “wedges” in the arterioles, thereby allowing for selective measurement of
the back pressure coming from the pulmonary veins and left atrium (Figure 1). If this
pulmonary artery occlusion pressure is less than 15 mmHg and the pulmonary artery mean
pressure is greater than or equal to 25 mmHg, PAH may be diagnosed. If, on the other hand,
the pulmonary artery occlusion pressure is higher than 15 mmHg, the diagnosis is
pulmonary venous hypertension, suggesting that the high pressure originates from elevations
in the left atrial pressure, not from disease of the pulmonary arterioles.

The normal pulmonary vasculature is a low resistance, high flow system. In the case of
PAH, the pulmonary arterioles are progressively occluded such that the pulmonary vascular
resistance and pulmonary pressures increase, ultimately leading to a drop in cardiac output
as the right heart fails in the face of increasing afterload. Progressive reduction in cardiac
output results in exercise intolerance, shortness of breath, fluid retention, and likely death
from right heart failure. The relationship between vascular obliteration, vascular resistance,
pulmonary pressures, and cardiac output (flow) can be related to Ohm’s law and reviewed in
Figure 2. Patients at the highest risk of death are those with evidence of progressive right
heart failure, elevated right atrial pressures, low cardiac outputs, and episodes of systemic
hypoperfusion or syncope (sudden loss of consciousness). “Sudden death” can occur with a
profound failure of the right ventricle resulting in loss of blood flow to the brain followed by
Cardiac asystole.

Recently, the Dana Point working group published an expert consensus document on
pulmonary hypertension, detailing the classification, epidemiology, natural history, survival,
pathology, pathogenesis, and strategies for diagnosis and treatment [1]. The classification of
diseases that cause pulmonary hypertension are summarized in Table 1 and include: 1)
idiopathic disease where the cause is unknown (which accounts for approximately 50% of
the cases of PAH [2] and occurs with a female/male ratio of 1.7:1 [3]); 2) familial disease
related to hereditable germ-line mutations in three genes encoding transforming growth
factor β (TGF-β) receptor superfamily, namely endoglin 1, the activin-receptor-like kinase-1
(alk 1), and the bone morphogenic receptor (BMPR); 3) disease induced by drugs and
toxins; 4) disease associated pulmonary arterial hypertension, such as that associated with
connective tissue diseases, human immunodeficiency virus, portal hypertension, and
congenital heart diseases; 5) persistent pulmonary hypertension of the newborn and 6)
disease secondary to veno-occlusive disease.

The different forms of PAH share many pathobiological features. For example, familial or
heritable PAH, which is responsible for up to 6% to 10% of the cases of PAH [2, 3], can
occur based on inherited mutations in the TGF-β receptor pathway. This is inherited via
autosomal dominant transmission with incomplete penetrance and genetic anticipation. It is
likely that endothelial and smooth muscle proliferation occur from changes in TGF-β
signaling prior to any observable elevations in pressure or changes in pulmonary blood flow
[4]. Both hereditary PAH and idiopathic PAH are characterized by an overabundance of
vascular smooth muscle in small peripheral arteries, such that the lumens are nearly
occluded, resulting in increased pulmonary vascular resistance and right ventricular overload
[5].
There are other pathologies in which pulmonary hypertension presents as a secondary disease. Some examples include: left heart disease, which is referred to as pulmonary venous hypertension (Class II, Table 1), chronic lung diseases and/or hypoxemia (Class III, Table 1), chronic thromboembolic disease (Class IV, Table 1), or pulmonary hypertension related to other miscellaneous diseases (Class V, Table 1) (e.g., lymphangiomatosis, histiocytosis X and sarcoidosis).

In all forms of PH the progressive vasculopathy is complex with a broad imbalance of vasodilative and vasoconstrictive mediators, likely preceding the development of secondary aberrant cellular proliferation. Classic vasodilator systems are dysregulated with decreases in endothelial NO synthase function (eNOS) and enzymatic “uncoupling”, decreases in production of prostacyclin (cyclooxygenase-2 dysfunction), and increased expression and activity of the vasoconstrictor and mitogenic endothelin-1 signaling system [6–8]. An understanding of the critical imbalances of these three major pathways, NO, prostacyclin, and endothelin-1, has led to the rapid clinical development of three new major FDA approved medications for the therapy of PAH, all targeting these important pathways (See Table 2). While these drugs are considered ‘selective’ pulmonary vasodilators, they do have effects on the systemic circulation and can produce systemic hypotension, as well as adversely affect ventilation and perfusion matching and oxygenation [9–12].

**Dysregulation of ROS and RNS signaling**

In addition to imbalances in NO, prostacyclin, and endothelin-1, it is increasingly apparent that enhanced production and dysregulation of ROS/RNS signaling underlies PAH pathogenesis. These pathways regulate vascular force and tone, cellular proliferation, apoptosis and modulate upstream pathological progression of PAH. A potential role of ROS and RNS in the development of pulmonary hypertension is supported by experimental data showing that several antioxidants prevent some alterations triggered by chronic hypoxia [13–15]. Moreover, it has been shown that diminished pulmonary vasorelaxation to exogenous NO is related to increased levels of ROS [16–18]. Direct scavenging of NO by superoxide can reduce NO levels and drive protein oxidation by secondary generation of peroxynitrite and nitrogen dioxide. Increased superoxide and increased Rho kinase activity can also reduce both synthesis and bioactivity of endothelium-derived NO [19, 20], thereby increasing vasoconstriction related to the development of pulmonary hypertension.

Accumulating evidence indicates that oxidase systems, such as NADPH oxidase (Nox) and xanthine oxidoreductase, are involved in changes of the pulmonary vasculature during PH, specifically in the long-term responses of the pulmonary vasculature to hypoxia. For example, the phagocytic “respiratory burst oxidase”, Nox2, plays an important role in hypoxia-induced endothelial NO-dependent dysfunction in pulmonary arteries, which results in reduced activation of soluble guanylate cyclase (sGC) or cyclic guanosine monophosphate (cGMP)-dependent protein kinase activity following chronic hypoxia [21, 22]. Increased expression of NADPH oxidase isofrom 4 (Nox4) in the vasculature of patients with PAH may disrupt canonical NO signaling through a number of pathways [23]. Increased oxidative stress from Nox4 can critically alter the balance of NADP+:NADPH and tetrahydrobiopterin to dihydrobiopterin (BH₄:BH₂), which are both necessary to maintain eNOS in a NO producing “coupled” state. In pulmonary artery adventitial fibroblasts exposed to hypoxia, a significant upregulation of Nox4 expression at the mRNA and protein levels has been described, whereas silencing of Nox4 expression by siRNA reduces ROS levels and decreases cellular proliferation [24]. In a murine hypoxia-induced model of PAH, the development of the disease has been linked to increases in Nox4 expression in vascular smooth muscle cells [25]. Similarly, in hypoxic human pulmonary
smooth muscle cells, an increase in Nox4 expression has been reported [26] and TGF-β-induced Nox4 expression and ROS production have been linked to proliferation [26, 27].

In this review we will provide an overview of some of the functions of NO, ROS, RNS, their signaling pathways, and the relationships between them, as well as how they contribute to pulmonary endothelial dysfunction and pathological proliferative remodeling in pulmonary hypertension. We also review new therapeutic approaches targeting these pathways. Throughout this review we will refer to pulmonary hypertension as PH in the context of pre-clinical animal models as well as Class II–V disease. We will refer to pulmonary arterial hypertension as PAH, which will specifically indicate Class I human disease (Table 1).

Pulmonary hypertension and endothelial dysfunction: Dysregulation of NO and endothelin-1 signaling

Despite a multitude of inciting factors, the final common outcome in PAH is the elevation of pulmonary vascular resistance mediated by vasoconstriction and obstructive cellular proliferation of the pulmonary vasculature. Through the synthesis and release of vasoactive factors, the endothelium plays a critical role in maintaining the delicate and precise balance between vasoconstrictor (endothelin-1, thromboxane A$_2$) and vasodilator (prostaglandin I$_2$, NO) molecules. In PAH, endothelial damage and dysfunction change this homeostasis, altering the balance to favor vasoconstriction. An increasing number of studies suggest that dysfunction in the NO-related signaling pathway represents an important element in the pathological remodeling of the pulmonary vasculature during the development of PAH [28, 29]. One of the early events in the pathogenesis of PAH is impaired endothelium-dependent vasodilation, as evidenced by a lack of vasodilator response to acetylcholine, bradykinin or calcium ionophore [30]. Increased superoxide production in the vessel wall and the subsequent decrease in NO bioavailability also characterize endothelial dysfunction [31–33].

NO in PAH

NO, an endothelium-derived relaxing factor; is an important signaling molecule involved in the regulation of basal vasomotor tone, blood pressure, and inhibition of vascular cell growth. NO acts by binding to soluble guanylate cyclase which in turn converts guanosine triphosphate (GTP) to cGMP, which then activates downstream cGMP-dependent protein kinases [34–36]. Another important vasoregulatory property of NO includes regulation of vascular smooth muscle proliferation and migration [37, 38].

While the protein expression level of one member of the nitric oxide synthases (NOS), endothelial NO synthase (eNOS), has been ambiguously reported as up-regulated or down-regulated in patients with PAH and in animal models of PAH [39–42], there is general consensus that the NO signaling is impaired. Numerous studies have implicated decreased bioavailability of NO and/or decreased responsiveness to NO during PAH [43–45]. Furthermore, it has been shown that this decrease in bioavailability may occur secondary to impaired formation or increased consumption [46–48]. In this section we attempt to summarize the current status of NO signaling in PAH, which is thought to be related to multiple signaling mechanisms, including: a) reduction, dysfunction, or uncoupling of eNOS, b) downstream effects on NO-cGMP signaling, c) catabolism of NO by ROS or hemoglobin. Finally, we describe the use of NO and agents that augment NO signaling as they relate to specific PAH therapies in clinical and experimental models.

Sources of NO

NO is primarily formed in the metabolism of L-arginine by a family of enzymes known as oxide NOS, or through a NOS-independent mechanism from the anion nitrite (NO$_2^-$) [49–
There are 3 isoforms of the enzyme: inducible NO synthase (iNOS), endothelial NO synthase (eNOS), and neuronal NO synthase (nNOS), all of which are expressed in the lung. These enzymes utilize the substrate L-arginine, molecular oxygen, and NADPH to produce L-citrulline and NO. Perhaps the most important enzyme in regulating NO production in the endothelium is the isoform eNOS. In animal models, studies have reported increased eNOS expression associated with PAH [39, 53]. Other animal studies suggest that chronic PH increases lung eNOS mRNA and protein expression, and total lung NOS activity. However, data from human lung tissue studies have shown a lack of consistent results. For example, Xue et al. found an increase in eNOS immunostaining [40], Giaid and Saleh found a decrease in eNOS [41] and Tuder et al. reported unaltered levels compared to healthy pulmonary arteries [42]. Some of the conflicting results within both animal models and human tissue studies may be due to the differences in time points being sampled. In the case of animal models, the NOS proteins are measured during the progression of the disease, whereas human tissues are harvested very late in the progression of the disease or even post-mortem. Another important point is that the animal models of PAH do not develop the classic plexiform vascular lesions, which are characteristic of the human disease.

Some studies have also demonstrated increased iNOS in the microvasculature of a rat monocrotaline model of PAH [54]. However, further studies of the role of iNOS in PAH are needed and are beyond the scope of this review.

**eNOS uncoupling**

While the levels of eNOS in lung appear to vary, a functional reduction in NO signaling has been consistently reported in pre-clinical models and in patients with PAH. Reduced NO formation, measured through NO enzymatic synthesis and its rate of consumption, has been found in patients with primary PH [55] and in the hypoxic rat model of PH. This decreased NO production leads to reduced vasodilation in the setting of normal or increased eNOS protein levels [56]. It is now increasingly appreciated that this paradox of normal or increased eNOS expression and diminished NO signaling is potentially explained by a process termed “eNOS uncoupling,” a dysfunctional state of the enzyme in which electrons transferring from the NOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine [57], forming superoxide rather than NO. eNOS normally forms homodimers, with each monomer containing an oxygenase domain in the N-terminal that is comprised of binding sites for BH₄, heme iron and L-arginine. (Figure 3, for more detailed recent review, see ref [6]). Uncoupling of eNOS is associated with changes in the quaternary structure of the enzyme, which is experimentally observed as a reduction in enzyme homodimer assembly and increase in the monomer.

eNOS uncoupling can be triggered *in vitro* through depletion of the co-factors, L-arginine and BH₄ [58]. Recent studies suggest that oxidation of tetrahydrobiopterin co-factor to dihydrobiopterin, measured as decreases in the ratio of BH₄:BH₂, or perhaps more importantly, increases in BH₂, are central to enzyme coupling [59]. The eNOS enzyme generates superoxide when BH₄ levels decline or when it is replaced by the oxidized BH₂ [45, 60]. Mice that have very low levels of BH₄, or decreased BH₄:BH₂ ratios, exhibit PH [45]. Using an experimental animal model of hypoxia-induced PH, it was reported that the oxidized form, BH₂, is increased in the lungs [32].

As further evidence that eNOS uncoupling may participate in the pathogenesis of PH, administration of BH₄ has been reported to decrease pulmonary artery pressure and reduce muscularization of distal pulmonary arteries in rats with monocrotaline-induced PH [61]. 6-acetyl-7,7-dimethyl-7,8-dihydropterin, a BH₄ analogue, has been shown to improve NO-mediated pulmonary artery dilation and enhanced expression of eNOS in the vascular
pulmonary endothelium in an *in vivo* rat model of hypoxia-induced PH [58]. This topic has been recently reviewed in more detail [6].

A role for eNOS uncoupling has been proposed as a mechanism for the development of PH observed in the Caveolin-1 (Cav1) knock-out mouse. Caveolin-1 plays an essential role in binding eNOS in the caveoli and limiting its activity [62, 63]. Dissociation of Cav1 from eNOS thus plays a regulatory role in NO signaling by promoting its activation and allowing eNOS protein movement to the cytosol. The paradoxical finding that Cav1 knock out mice develop PH appears to be related to the release and activation of an uncoupled eNOS that produces superoxide [33, 64, 65]. Indeed, eNOS inhibition using L-NAME reverses PH development in the Cav1 mouse [66], and re-expression of Cav1 can rescue the PH phenotype in the knock-out [67]. Furthermore, treatment of neonatal Cav1 knockout mice with BH$_4$ prevents PH [66]. However, in other studies, eNOS uncoupling alone did not cause PH. For example mice overexpressing eNOS in the endothelium are protected from developing hypoxia-induced PH [68], even though these mice exhibited eNOS uncoupling [69].

More recently, investigators have generated Cav1 and eNOS double knock-out mice and demonstrated the essential role of eNOS-dependent peroxynitrite formation (NO and O$_2^\cdot$ reactions) in the mechanism of PH in Cav1$^{-/-}$ mice. In these studies the downstream formation of peroxynitrite from NO and superoxide reactions resulted in nitration and loss of function of protein kinase G (PKG) and the development of PH [70]. These studies appear to support the hypothesis that in a setting of increased superoxide generation, from either uncoupled eNOS or other oxidases, NO formation may increase peroxynitrite formation and drive pulmonary vasculopathy.

An additional mechanism for eNOS dysfunction that could have relevance during the development of PAH, is eNOS S-glutathionylation. This reversible protein modification could explain effects of oxidative stress on eNOS activity [71]. An association between eNOS S-glutathionylation and endothelial dysfunction has been observed [72].

**Dysfunction of the NO signaling pathway down-stream from eNOS**

Downstream NO signaling events may also be impaired in PAH. Murine studies have suggested that increased superoxide generation may further limit NO bioactivity [73]. In addition, altered levels of one or both sGC units (α1 and β1) as well as reduced NO-dependent sGC activity results in a decreased generation of cGMP and reduced vascular relaxation to NO stimulation [74, 75] (Figure 4 shows a possible interplay of eNOS uncoupling and sGC). The mechanisms for altered sGC expression or activity in PAH are not entirely clear. An upregulation of sGC in pulmonary arterial smooth muscle cells from PAH patient lungs has been shown [76]. Changes in sGC expression seem to be linked but not exclusively to oxidative stress [77].

Several studies have shown an increase in cGMP-specific phosphodiesterase type 5 activity in pulmonary hypertensive animals [78, 79]; suggesting that rapid cGMP hydrolysis may limit cGMP-dependent pulmonary vasodilation [74]. In summary, it seems that chronic PAH disrupts NO-cGMP signaling by decreasing eNOS expression or activity, increasing NO catabolism, reducing sGC responsiveness, and increasing phosphodiesterase type 5 activity.

**Hemolysis- associated pulmonary hypertension**

Patients with chronic hereditary or acquired hemolytic anemias, such as sickle cell disease, thalassemia intermedia, paroxysmal nocturnal hemoglobinuria, and other hemoglobinopathies or red cell membranopathies, develop PH as they age [80, 81]. A
central mechanism underlying this disease progression is the process of intravascular hemolysis, which releases red blood cell hemoglobin into plasma. Cell-free plasma hemoglobin reacts with and catabolizes NO at a very high rate; more than 1000x faster than hemoglobin within the red blood cell. This reaction oxidizes NO to nitrate and inhibits its ability to vasodilate [82, 83]. In addition to the release of hemoglobin, hemolysis releases red cell arginase 1, an enzyme that metabolizes L-arginine to ornithine, reducing its availability for de novo NO synthesis [83, 84]. It is also likely that hemolysis activates vascular oxidases, such as xanthine oxidase and NADPH oxidases, and hemolysis has been shown to drive eNOS uncoupling [43] (see Figure 5). These sources of ROS further reduce NO bioavailability at the endothelial and smooth muscle level. This process has been termed hemolysis-mediated endothelial dysfunction and leads to hemolysis-associated PH.

Therapies using NO or NO generating drugs

NO gas

Inhaled NO is currently utilized as an effective adjuvant therapy that improves gas exchange in newborns with severe PH [85]. However, its usefulness as a therapeutic treatment for other forms of PH is currently limited [86]. In both animal and human studies, inhaled NO (5–80 ppm) induces rapid and selective pulmonary vasodilation [87–90], but its therapeutic value is limited by the dose and duration of exposure. Several concerns regarding the safety of inhaled NO remain, with one of the most important issues being the safety of NO withdrawal. Several studies have noted a potentially life-threatening increase in pulmonary vascular resistance on acute withdrawal of inhaled NO [47, 91–93]. This kind of “rebound pulmonary hypertension” is manifested by an increase in pulmonary vascular resistance, compromised cardiac output, and severe hypoxemia. Exogenous NO exposure inhibits endogenous eNOS activity [48], suggesting that a transient decrease in endogenous eNOS activity during inhaled NO therapy may be a mechanism for rebound pulmonary hypertension. It has been shown that co-administration of a phosphodiesterase 5 inhibitor (sildenafil) in children ameliorates this rebound effect [94], suggesting that inhaled NO may prove useful in the current era where many patients are taking oral sildenafil or other phosphodiesterase 5 inhibitors (see below). New delivery systems that allow for pulsed NO delivery combined with pulsed oxygen from liquid oxygen sources may allow for small portable devices of inhaled NO. Large clinical trials of inhaled NO in patients with PAH on background phosphodiesterase 5 inhibitor therapies are required to address these questions.

NO donors

Administration of organic nitrates, such as nitroglycerin, stimulates vasodilation, although development of nitrate tolerance shortly after treatment limits their usefulness. The underlying mechanisms of action are unclear and are likely to be multi-factorial [95]. Munzel and colleagues demonstrated an increase in vascular superoxide production after nitroglycerin treatment [96]. Furthermore, tolerance was prevented by co-treatment with superoxide dismutase (SOD), highlighting the role of ROS in this inhibition [96]. A later study identified a membrane-bound nicotinamide adenine dinucleotide dehydrogenase (NADH) oxidase as a likely source of superoxide production [97], and more recent studies demonstrated a role for endothelin-1 in the pathway [98]. It is likely that the increase in superoxide inactivates the NO released from nitroglycerin, increasing the formation of peroxynitrite. This may further promote supersensitivity to vasoconstrictors secondary to a tonic activation of protein kinase C, resulting in nitrate tolerance [99]. The proposed superoxide sources are NADPH oxidase, uncoupled eNOS, and mitochondria. Superoxide and NO rapidly form peroxynitrite, which aggravates tolerance by promoting NO synthase uncoupling and inhibition of sGC and prostacyclin synthase [95]. In the case of mitochondria, recent work has defined a new tolerance mechanism through the inhibition of...
mitochondrial aldehyde dehydrogenase, which is the enzyme that bioactivates nitroglycerin [100].

**Nitrite**

While nitrite has historically been considered an inert oxidation product of NO and oxygen, increasing evidence over the last ten years has supported a role for this molecule as a potent physiological vasodilator [52, 101–104]. Nitrite is reduced to NO by a variety of enzyme systems as oxygen tension drops and intracellular pH decreases along the physiological and pathological hypoxic gradient (Figure 6). Enzymes that have been identified with nitrite reductase activity include hemoglobin, myoglobin, neuroglobin, eNOS, xanthine oxidoreductase, and acidic disproportionation [105–112]. Nitrite has been proposed as a therapy for PH based on inhaled studies in newborn sheep with hypoxia- and thromboxane-induced PH [51]. Recent studies show that inhaled, low-dose nebulized sodium nitrite can prevent and reverse experimental PAH and heart failure in the chronic hypoxia mouse model and monocrotaline rat model [113]. Zuckerbraun and colleagues showed that a low dose of sodium nitrite has a potent antiproliferative effect that is dependent on nitrite reduction by the enzyme xanthine oxidoreductase [113]. Nitrite in this context signals through cGMP to increase the levels of the cell cycle checkpoint inhibitor P21. Animal toxicology studies and phase Ia and Ib human studies have been completed with plans for a proof of concept phase II study over the next two years. However, further characterization of the dosing, toxicity, and mechanism of action are required.

**Therapies that increase cGMP levels**

**Phosphodiesterase inhibitors**

Selective inhibitors of the cGMP-specific phosphodiesterase type 5 enzyme, such as sildenafil and tadalafil, have shown clear efficacy in the treatment of PAH. The blockade of the phosphodiesterase type 5 enzyme prevents the normal hydrolysis of cGMP thus increasing the effects of NO, such as pulmonary vasodilation and inhibition of smooth muscle cell growth [114]. In a murine model of hemolysis-mediated PAH [44], where enhanced platelet activation and aggregation are associated with reduced vascular NO bioavailability, it has been shown that treatment with sildenafil produces an inhibitory effect on platelet activation and inactivation of the coagulation pathway [43]. Sildenafil, which potentiates NO-dependent signaling, has a relatively favorable side-effect profile; the most common being headache (16%) and skin flushing (4–8%). Sildenafil has been shown to improve symptoms of PAH, such as exercise tolerance and hemodynamics, and to improve overall quality of life [115, 116], and is currently an FDA-approved therapy for PAH.

Another phosphodiesterase inhibitor approved by the FDA is tadalafil. An initial report in 2004 by Palmieri et al showed improvements in hemodynamics and gas exchange [117]. Later studies by Galie and colleagues reported positive effects for tadalafil alone or in combination with bosentan (PHIRST trial) [118]. The results indicated improvements in time to clinical worsening, health related quality of life, pulmonary hemodynamics, and improvements in exercise tolerance. Tadalafil has generally been well tolerated with the most common side effects being headache, myalgias, and flushing [118].

There remain several unanswered questions regarding the use of phosphodiesterase type 5 inhibitors in PAH. A major one being: whether these drugs will improve survival of patients with PAH. Because of small sample sizes (PAH is a very rare disease, so large studies are difficult to perform), short trial duration, and the addition of second and third drugs to patients with clinical worsening, we still do not know if these drugs improve survival.
Longer-term studies examining the durability of phosphodiesterase type 5 inhibitor benefits are still needed.

**sGC activators**

Because impaired signaling on the sGC-cGMP pathway has been implicated in the pathogenesis of PH, recent efforts have focused on finding pharmacological activators and stimulators of sGC. The activators are pharmacological substances that, in a preferential manner, activate sGC in its oxidative or heme-free state but do not require NO for this activation [119], while the stimulators directly sensitize sGC to low levels of NO in the presence of a reduced (ferrous) synthetic heme [120].

BAY 58-2667 and HMR-1766 are examples of sGC activators that have been studied in animal models and in preclinical studies, producing potent selective pulmonary vasodilation (lambs) without adverse effects on pulmonary gas exchange [119]. Examples of the stimulators are BAY 41-2272 and BAY 63-2521 that stimulate sGC on an NO-independent but heme-dependent site [120, 121]. Recent studies have shown that BAY41-2272 causes potent vasodilation, improves oxygenation independently of endogenous NO release, augments the response to inhaled NO, decreases blood pressure, and improves survival in hypertensive adult rats [120]. BAY 63-2521 has been shown to be as potent of a vasodilator in the pulmonary vascular bed as BAY41-2272, and both show decreases in workload of the right heart [121].

One of the sGC stimulators, Riociguat (BAY 63-2521), is currently in clinical development. A recently completed open-label (non-randomized or blinded) phase II study in patients with chronic thromboembolic PH and in patients with Class I PAH (defined in Table 1) found that this drug reduced pulmonary vascular resistance and increased the distance walked in six-minutes [122]. Side effects included hypotension, shortness of breath and headache. Larger randomized trials are currently underway.

**Endothelin-1**

Another important protein produced by vascular endothelial cells and important in PAH pathogenesis is endothelin-1 [123]. During PAH, an increase of endothelin-1 has been demonstrated in both plasma and lung tissue in human patient samples, as well as in animal models of PAH [124–126]. Endothelin-1 is a 21 amino acid vasoactive polypeptide that is mitogenic for vascular smooth muscle cells and stimulates the generation of other local mediators of vascular tone. The complex pulmonary vasoactive effects of endothelin-1 may include vasoconstriction as well as vasodilation, with contrasting effects determined by at least two different G-protein-coupled receptors, ET\textsubscript{A} and ET\textsubscript{B}. Endothelin-1 exerts opposite effects under normal physiological conditions based on the receptor types being expressed and cellular distribution of the receptors [127, 128]. ET\textsubscript{A} receptors are located predominantly on smooth muscle cells and mediate vasoconstriction, proliferation, hypertrophy, cell migration, and fibrosis [129], whereas ET\textsubscript{B} receptors are found predominantly on vascular endothelial cells where they promote vasodilation by increasing NO synthesis, prostacyclin production, and generally enhances endothelium-dependent vasodilators, which exhibit anti-proliferative properties and prevent apoptosis [130, 131]. ET\textsubscript{B} in smooth muscle generates a vasoconstrictive signal, similar to ET\textsubscript{A} [132, 133]. As PAH progresses, the cellular distribution of the endothelin-1 receptors changes, with decreased expression of vasodilatory endothelial ET\textsubscript{B} and increased expression of both constrictive ET\textsubscript{A} and ET\textsubscript{B} in the vascular smooth muscle cells. This observation helps explain the apparent efficacy of both selective ET\textsubscript{A} receptor blocking medications (ambrisentan, Table 2), as well as the dual ET\textsubscript{A} and ET\textsubscript{B} receptor blockers (bosentan, Table 2).
Several studies suggest that NO and endothelin-1 regulate each other through autocrine feedback loops [68, 134, 135]. An example of this regulation is that activation of ET-1 receptors stimulates eNOS activity, whereas NO-cGMP production inhibits endothelin-1 peptide secretion and reduces ET<sub>A</sub> receptor gene expression in vascular endothelial cells [136, 137]. In addition, it has been shown that peroxynitrite, formed in the reaction between superoxide and NO, can irreversibly inhibit eNOS activity in vitro [138] and increase levels of nitrated eNOS protein in vivo [31]. This nitration is significantly reduced by ET<sub>A</sub> receptor blockade [138]. These data suggest that endothelin-1-ET<sub>A</sub> receptor-mediated increases in superoxide production with a resultant increase in vascular smooth muscle cell proliferation and NOS inhibition, coupled with ET<sub>A</sub> receptor-mediated vasoconstriction, may play a significant role in the development of endothelial dysfunction and PH.

A similar feedback loop occurs between the ET-1 signaling pathway and NADPH oxidase-dependent ROS formation. While endothelin-1 activation of ET<sub>A</sub> receptors can activate NADPH oxidase-dependent ROS formation, conversely, ROS can regulate cellular levels of endothelin-1 and mediate its secretion. Endothelin-1 release is increased by human umbilical vein endothelial [139] or bovine aortic endothelial cells [140] exposed to cyclic strain, which can be blocked by pretreatment with antioxidants. Some studies also suggest a role for the transcription factor complex activator protein-1 in the ROS-mediated increase in endothelin-1 promoter activity. Interestingly, endothelin-1 has been shown to increase activator protein-1 DNA binding in rat aortic smooth muscle cells via a pathway involving ROS [140]. Furthermore, endothelin-1 stimulates activator protein-1 activation in rat smooth muscle cells via ET<sub>A</sub> receptor binding and ROS production [141]. This evidence suggests that abnormal regulation of endothelin-1 expression in PH may involve a positive feedback loop; ET<sub>A</sub> receptor-mediated increases in superoxide production from smooth muscle cells may result in increased endothelin-1 promoter activity and endothelin-1 secretion in the adjacent endothelial cells.

**Endothelin-1 receptor antagonists**

As discussed above, endothelin-1 is a potent endogenous vasoconstrictor and smooth muscle cell mitogen that is overexpressed in the lungs and blood of PAH patients. Endothelin-1-receptor antagonists have emerged as important therapeutics for PAH. In patients with PAH, bosentan, a combined ET<sub>A</sub> and ET<sub>B</sub> receptor agonist, decreases pulmonary vascular resistance, and pulmonary pressures, increases cardiac output, and improves exercise tolerance [142]. Significant benefits of bosentan treatment have been demonstrated in children with PAH [143], in PAH associated with human immunodeficiency virus [144], and in patients with portopulmonary hypertension [145]. However, bosentan can produce hepatotoxicity and liver enzymes must be monitored during its use.

Ambrisentan blocks only endothelin A-receptors and has also been shown to significantly improve hemodynamics and exercise capacity in PAH [142, 146, 147]. Although endothelin-1 receptor antagonists are generally well tolerated, they are associated with side effects related to their vasodilatory properties, including; peripheral edema, headache, and palpitations. The FDA black box warning for liver toxicity has recently been removed for ambrisentan based on the limited evidence for significant liver toxicity with this agent [148].

Other potential therapeutic uses for endothelin-1 receptor antagonists include pulmonary hypertension associated with congenital heart disease and persistent pulmonary hypertension of the newborn. Endothelin-1 receptor antagonists induced potent pulmonary vasodilation in a lamb model of congenital heart disease with decreased pulmonary blood flow [85]. In addition, ET<sub>A</sub> receptor blockage prevented endothelin-1 induced fetal pulmonary arterial smooth muscle cell proliferation [149].
Oxidative stress: ROS and RNS

Aside from their initially-described role in antimicrobial defense, ROS/RNS generation and redox signaling have emerged as normal physiological responses leading to essential cellular functions. Numerous effector responses that arise from redox signaling include cellular differentiation, proliferation, migration, apoptosis, and antioxidant gene expression. Some of the best studied signaling effectors involved in these phenotypes include the MAPKs, protein tyrosine phosphatases, NF-κB, AP-1 and the Nrf2/keap pathways. ROS/RNS levels reported to effect such responses are generally considered to be log orders of magnitude lower than those generated by the phagocyte respiratory burst. In contrast, ‘oxidative stress’ has been widely used to describe the consequence of an imbalance in ROS and RNS homeostasis due to excessive formation of these species and/or to decreased antioxidant defenses. By one generally accepted measure, cellular “stress” occurs when a shift in the balance of a remarkably stable reservoir of reduced and oxidized glutathione occurs in favor of oxidation and/or when essential reducing proteins with specialized function (i.e. thioredoxin, glutaredoxin) are neutralized or depleted. This imbalance can affect cellular homeostasis either through direct oxidative and often irreversible damage of basic cellular components (proteins, lipids, and nucleic acids), leading to defective cellular function, apoptosis, tissue damage, and vasculopathy. In recent years it has become evident that ROS and RNS play a contributory role in the pathogenesis of PAH [150]. The term ROS encompasses a variety of oxygen-derived moieties with high reactivity towards other biomolecules, including both free radicals (substances containing one or more unpaired electrons, such as superoxide (O$_2^-$), hydroxyl (OH•), peroxyl (RO$_2$•), and hydroperoxyl (HO$_2$•) radicals) and non-radical species such as hydrogen peroxide (H$_2$O$_2$) and other peroxides (ROOH), which are generally capable of oxidizing molecular targets. Increased markers of oxidative stress are found in human patients with PH, as well as in various animal models of PAH [14, 32, 150–152], supporting the notion that ROS and RNS play a major role in the development and progression of PH.

As alluded to above, ROS at the proper locations and concentrations can function as second messengers and activate multiple signal transduction pathways within the cell, facilitating the action of growth factors, cytokines, and calcium, via common or diverse upstream pathways. As an example, ROS activates protein kinase C-ε to regulate specific ion channels, contributing to the increase in intracellular Ca$^{2+}$ and associated contraction in pulmonary artery smooth muscle cells. A positive-feedback mechanism to further enhance intracellular ROS generation has been proposed by Rathore and collaborators, whereby they find mitochondrial ROS-dependent activation of protein kinase C-ε can augment Nox activity and leads to a further increase in intracellular ROS generation [153]. As a consequence, Nox activity contributes to a significant and functionally important increase in intracellular ROS and Ca$^{2+}$ concentration observed in PAH. Another example is the activation of mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) by ROS [141]. JNK is a crucial mitogen-activated protein kinase, which activates key proteins involved in the release of cytochrome c and other pro-death mediators in the cytosol. As further example, the addition of exogenous H$_2$O$_2$ or different pharmacologic agents (i.e. Naphthoquinolinedione LY83583) that can increase ROS generation appear to stimulate the activation of MAPK and cellular growth [154–157].

The effects of ROS in cellular signaling include activation of many major cell signaling pathways such as MAPK, phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor kappa-light chain-enhancer of activated β cells (NF-κB), extracellular signal-regulated kinase (ERK), JNK, and protein 53 (p53).
In chronic hypoxia, some studies have shown an increase in cellular levels of ROS, which are likely due in part to increased numbers of inflammatory cells in the lung vasculature and parenchyma [158, 159]. These increases of ROS also stimulate platelet-derived growth factor [157, 160], and play an important role in signal transduction pathways of growth factors.

RNS, such as peroxynitrite (ONOO⁻), have also been implicated in PH pathogenesis. For example, under hypoxic conditions, the downstream mediator PKG, which plays an important role in vascular relaxation [161], is inhibited by peroxynitrite mediated tyrosine nitration [162, 163]. During PH, peroxynitrite reacts strongly with numerous biologically important molecules, and can cause toxicity and death of endothelial cells and smooth muscle cells. Additionally, peroxynitrite can activate ERK [16–18], p38 MAP kinase [24], and protein kinase C [25, 164], which are key mediators of smooth muscle cell and endothelial cell proliferation. Nitrative stress, through peroxynitrite-induced tyrosine nitration, damages eNOS and prostacyclin synthase, which impairs vasodilation by diminishing the capacity of vessels to synthesize the vasodilators NO and prostaglandin I₂ [165]. Moreover, peroxynitrite damage to eNOS redirects the synthase activity from NO to superoxide generation (uncoupling), which, in turn, has been implicated in eNOS-dependent tyrosine nitration of prostacyclin synthase [166] and PKG [above].

In the next sections we review the sources of cellular ROS in PH and the data suggesting their important role in PH pathogenesis.

What are the sources of ROS?

ROS is produced by all vascular cell types; including, endothelial, smooth muscle, and adventitial fibroblasts [167–171], and can be generated from both metabolic and enzymatic sources. These sources include the mitochondrial electron-transport chain, pathways of arachidonic acid metabolism, uncoupled eNOS, xanthine oxidoreductase, the many isoforms of Nox, cyclooxygenase, and cytochrome P450. In PAH, Nox 2 and 4, xanthine oxidoreductase, uncoupled NOS and the mitochondrial electron-transport chain appear to play a major role in physiological and pathological ROS signaling [25, 172, 173].

Nox family in PAH

The Nox family of parenchymal NADPH oxidases includes Nox1, Nox3, Nox4, Nox5, Duox 1 and Duox 2, and, the phagocyte NADPH oxidase gp91phox, also known as Nox2. Four of these- Nox1, Nox2, Nox 4 and Nox5, are found in the vasculature of humans, whereas Nox5 is not present in rodents. The NADPH oxidases are distinct biochemically in their regulation, expression, and activity, but retain their ability to use NADPH as the electron donor to reduce molecular oxygen to O₂⁻ and/or H₂O₂. The production of H₂O₂ by Nox is still controversial and many maintain that it arises from rapid dismutation in or in close vicinity to the Nox catalytic site. Importantly, Nox are shown to be the major source for the generation of ROS in vascular smooth muscle cells [149], endothelial cells [28], and fibroblasts [171]. Accumulating evidence indicates that in the pulmonary vasculature ROS derived from Nox enzymes, in particular Nox1, Nox2 and Nox4, play an important role in the impaired tone and contractile response due to hypoxia [23, 174]. A large number of growth factors and agonists of G-protein coupled receptors, such as serotonin and endothelin-1 have been shown to activate Nox. Activation of Nox by serotonin stimulates proliferation of bovine pulmonary arterial smooth muscle cells via a pathway involving the MAP kinases, ERK1/2 [175, 176]. Furthermore, endothelin-1 stimulates the proliferation of smooth muscle cells isolated from the pulmonary arteries via Nox-catalyzed ROS production [149].
DeMarco and colleagues [150, 177] recently showed that the transgenic (mRen2)27 rat, a model characterized by over-expression of mouse renin in extra-renal tissues and increased local synthesis of angiotensin II, results in angiotensin II-dependent ROS formation, pulmonary vascular remodeling, and systemic and pulmonary hypertension. Data from those studies support the concept that PH can occur as a consequence of Nox2-induced oxidative stress, mediated by upstream activation of the local renin-angiotensin system within the pulmonary vasculature and lung parenchyma [150, 177]. Other laboratories have shown that gene therapy targeting the renin-angiotensin system reverses PH. Specifically, the use of angiotensin-converting enzyme inhibitors or angiotensin receptor reduces right ventricular systolic pressure and wall thickening of small pulmonary arteries [178–181]. The efficacy of these therapies relates to a decrease in angiotensin II-dependent Nox2 activation and resultant oxidative stress in the pulmonary vasculature and in the right ventricle.

Another model of PH that has been used to study the role of Nox is persistent PH of the newborn caused by either prenatal placement of an aortopulmonary shunt or ductal ligation [74, 182]. In this model, elevated levels of superoxide have been found in the pulmonary arterioles [182, 183]. Superoxide has been defined as the primary oxidant causing oxidative stress and may be derived mainly from Nox and secondarily from uncoupled eNOS [74, 182–185]. In the chronic hypoxia-induced PH model, alterations in NO and ROS have been implicated as important mechanisms by which hypoxia induces pulmonary vascular dysfunction. In this model, hypoxia causes increased Nox4 expression in pulmonary artery smooth muscle cells [186], and the inhibition [25] or knock down- silencing [26] of Nox4 reverses PAH through reduction of ROS formation and smooth muscle cell proliferation. Furthermore, pathological changes in this model, such as increased mean right ventricular pressure, medial and adventitial wall thickening of small pulmonary arteries, right ventricular hypertrophy, and increased intrapulmonary generation of superoxide, are also abolished in Nox2 knockout mice [23], suggesting a role for both Nox isoforms in this model. The Nox enzymes are not only the principal generator of O$_2^•$ in the vasculature during PAH, but also regulate the activities of other ROS-generating oxidases such as xanthine oxidase and eNOS [151, 187], and they are important in the recruitment of ROS-generating phagocytic cells [188]. Although the activation of intrapulmonary Nox2 and 4 has been postulated as mediator of PAH in animal models, the extrapolation of the contribution of oxidative stress in the etiology of PAH in humans may be premature because the animal models of PAH do not completely reproduce the pathophysiology observed in the advanced stages of human PAH.

**Xanthine oxidoreductase**

Another critical source of intracellular ROS is xanthine oxidoreductase, which catalyzes the terminal two steps in purine degradation (hypoxanthine $\rightarrow$ xanthine $\rightarrow$ uric acid) and primarily exists in cells as a dehydrogenase where substrate-derived electrons reduce NAD$^+$ to NADH. During inflammatory conditions, oxidation of critical cysteine residues or limited proteolysis converts xanthine oxidoreductase dehydrogenase to xanthine oxidase [189, 190]. Xanthine oxidase transfers substrate-derived electrons to O$_2$, generating O$_2^•$ and H$_2$O$_2$. However, it is important to note three crucial concepts: 1) conversion to xanthine oxidase is not essential for ROS production, as xanthine oxidoreductase dehydrogenase displays partial oxidase activity under conditions in which the NADH/NAD$^+$ ratio is increased, such as hypoxemia [191, 192], 2) the major ROS product (80–95%) of xanthine oxidoreductase under normal and pathophysiological conditions is H$_2$O$_2$ [193], and 3) hypoxia induces xanthine oxidoreductase expression and activity [194]. Therefore, when produced in or distributed to critical sites in the vasculature, xanthine oxidoreductase is a significant source of ROS that modulates vascular function. This is evidenced by reports demonstrating enhanced xanthine oxidoreductase activity in the arteries of patients with PAH [195], in
cultured endothelial cells [196–198], and in a rodent model of PH [14]. As such, xanthine oxidoreductase can serve as a crucial locus of ROS formation driving pathological processes that drive PAH. For example, increased vascular O$_2$$•^-$ production during chronic hypoxia, which has been found to adversely impact endothelial function by impairing NO signaling [199, 200] and to directly contribute to vascular remodeling, has been attributed in part to enhanced xanthine oxidoreductase activity [14], whereas treatment of chronic hypoxia-induced PAH rodents with xanthine oxidoreductase-specific inhibitors normalizes blood pressure [14, 151]. However, there exists an unresolved paradox regarding the role of xanthine oxidoreductase as contributory to PH or as a nitrite reductase that prevents and reverses experimental PH [113]. One explanation for these discordant roles of xanthine oxidoreductase may involve the presence of nitrite, which may divert electrons from oxygen and pathological superoxide formation to nitrite and protective NO (or alternative protective nitrite oxidation reactions).

### Mitochondrial ROS in PAH

Experimental evidence suggests that a small percentage of the O$_2$ used by mitochondria is not completely reduced to water but is converted to O$_2$$•^-$ because of the escape of electrons at complexes I and III of the electron transport chain [201–205]. Indeed, under physiological conditions, in endothelial and smooth muscle cells from pulmonary arteries, mitochondria produce ROS as byproducts of aerobic metabolism by the electron transport complexes, with 1–2% of oxygen converted to O$_2$$•^-$ at any given time [206–208]. This production can be affected by modulation of mitochondrial redox status and biogenesis, fusion and fission processes, and changes in mitochondrial bioenergetics [209]. Superoxide dismutase 2 (SOD2) converts intra-mitochondrial superoxide to diffusible H$_2$O$_2$, which directly or indirectly (via protein phosphorylation) stimulates various signaling pathways, including those involving adenosine monophosphate–activated protein (AMP) kinase (AMPK), nuclear migration of redox-sensitive transcription factors, and regulation of Kv1.5 channel activity (described in detail below). The downstream actions of H$_2$O$_2$ may play a role in the regulation of pulmonary vascular tone or by modulating cellular proliferation and apoptosis. For example, the activation of AMPK, JNK, and nuclear NF-κB may participate in the upregulation of cytoprotective genes, including those related with glucose transporters, antiapoptotic factors, glycolysis, antioxidant defenses, and repair mechanisms [210, 211].

H$_2$O$_2$ has both contractile and relaxant effects depending of the anatomical portion of the pulmonary artery (extra-conduit or intralobar-resistance vessel) and vasodilatory state (basal or pre-constricted state). Under basal tone, H$_2$O$_2$ induces constriction in intrapulmonary arteries. However, in the case of the pre-constricted state, H$_2$O$_2$ causes a transient constriction followed by relaxation in the intralobular pulmonary artery [212]. Formation of ONOO$^-$ results from the reaction between NO and O$_2$$•^-$ [187]. As we explained before, ONOO$^-$ is a reactive molecule that may nitrate or oxidize other biological molecules. Under physiological conditions, the contribution of mitochondrial O$_2$$•^-$ to this process is not clear because of the high levels of SOD present in the mitochondria [209]. However, during PAH, where the systems that have the capacity to dismutate O$_2$$•^-$ are impaired or saturated [182, 213], the formation of O$_2$$•^-$ or ONOO$^-$ by the electron transport chain may be enhanced [214, 215].

Two major theories of mitochondrial dysfunction in PAH have been promoted. The first theory suggests that the mitochondrial electron-transport chain, which is embedded in the inner membrane of the mitochondria and is the main energy source for the cell, may become a source of ROS that promotes cellular senescence, necrosis, or apoptosis [187], leading to vasculopathy. In support of this, investigators have reported that increases in mitochondrial-
derived ROS formation at the sites prior to the semi-ubiquinone site of complex III contribute to hypoxic pulmonary vasoconstriction [216, 217]. Additionally, changes in production of mitochondrial ROS have been observed following activation or stabilization of transcription factors with important roles in PAH, such as the peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α [30, 218] and hypoxia-inducible factor-1 alpha (HIF-1α) [219, 220]. Similarly, the catalase mimetic, tempol, which has the potential to scavenge cellular H₂O₂, has shown beneficial effects in PAH [221].

Alternatively, the second mitochondrial-based theory compares the metabolic theory of cancer with PAH, whereby mitochondrial dysfunction and a reduction in physiological ROS (most likely H₂O₂) levels drive the pathogenesis of the disease (Figure 7). According to this model, based largely on studies in the fawn-hooded rat that develops spontaneous PH, both hypoxia and a PAH disease state result in inhibition of the mitochondrial electron-transport chain and a shift from oxidative metabolism to anaerobic glycolysis. This results in both the accumulation of reducing equivalents of NADH and FADH₂ and a reduction in ROS production from the mitochondria, leading to a net decrease in cellular redox potential. This low redox state inhibits the redox-sensitive voltage-gated potassium channel Kv1.512 activity causing cellular depolarization, increasing intracellular Ca²⁺, and vasoconstriction of pulmonary artery smooth muscle cells [222]. Studies supporting this model show that diseased PH-pulmonary arterial smooth muscle cells exhibit morphological fragmentation, hyperpolarized mitochondrial membrane potentials, and reduced H₂O₂ production compared to healthy pulmonary arterial smooth muscle cells [223–225]. Decreases in mitochondrial H₂O₂ production may in part be mediated by SOD2 deficiency [226]. SOD2 regulates physiological production of H₂O₂ (produced from mitochondrial superoxide during respiration), H₂O₂ is less toxic than superoxide and can freely diffuse through membranes. This greater diffusion radius allows it to serve as a signaling molecule. H₂O₂ inhibits hypoxia-inducible factor-1 alpha (HIF-1α) [222, 223] and activates the sulfhydryl rich voltage-gated potassium channel Kv1.512. Therefore, a reduction in mitochondrial H₂O₂ production would be expected to activate HIF-1 dependent hypoxic signaling pathways and to inhibit Kv1.512, producing vasoconstriction and cellular proliferative responses.

Several downstream molecular changes associated with PAH, such as normoxic decreases in mitochondrial H₂O₂ production, mitochondrial pyruvate dehydrogenase kinase activation, and a shift from oxidative to glycolytic metabolism, are explained by normoxic activation of the oxygen-sensitive transcription factor HIF-1α. This drives a pseudohypoxic state, which is characterized by the use of glycolysis even in the presence of normal oxygen levels. These physiological shifts may also drive resistance to apoptosis through expression of survivin, a cell cycle-regulating protein that regulates mitochondrial function and has anti-apoptotic effects [227]. This resistance to apoptosis has been proposed as an example of the similarities between cancer cells and PAH-pulmonary arterial smooth muscle cells [227, 228].

In the case of PAH-endothelial cells, it has been shown that these cells share similar abnormalities with PAH-pulmonary arterial smooth muscle cells, including increased glycolytic rates and lower mitochondrial density compared to healthy cells [229, 230]. Similar to cancer, this is associated with the suppression of mitochondrial activity, such as glucose oxidation, resulting in a metabolic switch to glycolysis, which is associated with anti-apoptotic behavior [228, 231]. Another shared characteristic is that mitochondria found in malignancies have hyperpolarized mitochondrial membrane potential and reduced mitochondrial ROS production [232].
So, how do we reconcile these two competing theories, one suggesting that increases in ROS underlie PH pathogenesis and the other that decreases in mitochondrial ROS cause PH? The answer likely lies in the enzymatic source, along with the identities and quantities of measured ROS. High levels of NADPH and xanthine oxidoreductase-derived superoxide and secondary ONOO$^-$ in the setting of hypertension and inflammation are likely pathologic, while the physiologically regulated flux of H$_2$O$_2$ by mitochondria may tonically activate the potassium channel Kv1.512 and limit intracellular calcium levels.

**Therapies targeting ROS and RNS signaling**

Due to the role of ROS in PAH, multiple strategies intending to diminish ROS levels have been proposed as potential therapeutic treatments for PAH. Scavengers of ROS have shown beneficial effects in animal models of PAH. Indeed, treatment with recombinant human MnSOD is able to enhance eNOS expression and function in animal models of PH through superoxide metabolism and restoration of BH$_4$ levels [182]. The augmentation of MnSOD with Manganese(III)tetrakis(4-benzoic acid) porphyrin chloride, a SOD mimetic, reverses PAH, improving exercise capacity and reducing right ventricle hypertrophy in fawn-hooded rats [222].

Another strategy has been to inhibit the ROS generating sources. Inhibition of uncoupled eNOS with L-NAME decreases ROS and reverses PAH in caveolin-1 knockout mice in which superoxide is produced by the uncoupled eNOS [65]. Studies have detected evidence that increased heme-oxidase-1 may increase extracellular superoxide dismutase expression, decrease superoxide, and attenuate PH [213]. Use of ROS scavengers [183], inhibitors of NADPH oxidase [23, 172], BH$_4$ [41], and statins [177, 233, 234] prevent or reverse pulmonary hypertension through normalization of the vasodilator response to exogenous NO. Furthermore, genetic deficiency in ROS-generating enzymes protects some animals from experimental PAH [23]. For example, in NADPH oxidase knockout mice the pathological changes associated with hypoxia-induced PH are completely abolished [23]. Current standard treatments of PAH, such as phosphodiesterase type 5 inhibitors, have also been reported to lower ROS levels [235].

Therapies causing a regression of the mitochondrial abnormalities that cause the shift from oxidative to glycolytic metabolism constitute promising alternatives against PAH. As an example, dichloroacetate, an inhibitor of mitochondrial pyruvate dehydrogenase kinase, restores normal oxidative metabolism in fawn-hooded rats, a mutant strain unique in spontaneously developing PAH [236], thereby shifting them away from the proliferative/apoptosis-resistant glycolytic state [224, 232, 237]. Another potential treatment lies in targeting the fatty acid oxidation pathway, since inhibition of this pathway indirectly promotes glucose oxidation [238]. In vivo, trimetazidine, an inhibitor of the fatty acid oxidation pathway, depolarizes the mitochondrial membrane potential and increases mitochondrial ROS without affecting systemic blood pressure in rodent models. Recent studies have evaluated PPAR gamma activation using rosiglitazone and nitrated fatty acids, reporting that this reduces Nox4 activation and prevents and reverses experimental PH [239–244]. Collectively, these data support the view that metabolic modulators may be selective for the pulmonary circulation [238]. Since fatty acid oxidation inhibitors are clinically available, more studies of their effect on PAH are needed in order to have a rapid translation to clinical trials.

**Summary and future directions**

Pulmonary hypertension is associated with a generalized state of reduced NO signaling and enhanced oxidative stress. Current therapies targeting endothelial dysfunction and
vasoconstriction have shown efficacy in terms of reducing pulmonary pressures, increasing right heart function (cardiac output), and improving exercise performance. The large body of evidence indicates a role for ROS and RNS in PH, and the development of therapeutic treatments directly targeting these molecules remain promising. In this review we show that dysregulated NO signaling and oxidative stress plays a significant role in the pathogenesis of this disease. New therapeutic approaches are being developed to directly enhance NO signaling, recouple eNOS, scavenge ROS, directly inhibit Nox 2 and 4 and xanthine oxidoreductase, and restore mitochondrial function.

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Highlights

- PAH is characterized by endothelial dysfunction and enhanced cellular proliferation
- Worsening in patients with PAH is related to development of right heart failure
- Many reactive oxygen species and NO signaling pathways are disrupted in PAH
- Novel therapeutic strategies use nitrite and recoupling of eNOS to enhance NO
- Other strategies include inhibition of Nox, xanthine oxidase, and scavenging $\text{O}_2^-$
Figure 1.
Swan-Ganz Standard Thermocilution Pulmonary Artery Catheter permits measurement of right atrium, right ventricle, pulmonary artery and pulmonary artery wedge pressure (also called pulmonary artery occlusion pressure). To measure the pressure in vivo a balloon is inflated in the tip of this flow-directed catheter. The balloon is inflated to occlude a small distal branch of the pulmonary artery, and then the pressure is measured during occlusion and captures the reflected pressure coming from the left atrium.
Figure 2.
The electrical principles found in Ohm’s law are applicable to the pulmonary circulation. Electrically, with a given current flow (I), the voltage (V) that is generated across the resistance is given by I x R. In the case of the pulmonary circulation, for any given blood flow (CO), the blood pressure (BP) that will be generated by this flow through the pulmonary vascular resistance (PVR) is given by the same relation: BP = CO x PVR. BP is defined by the difference between mean pulmonary artery pressure (mPAP) and the pulmonary artery occlusion pressure (PAOP).
Figure 3.
The uncoupling of eNOS is a dysfunctional state of the enzyme. In the presence of sufficient L-arginine and BH$_4$, eNOS produces NO (thick arrow) and limited superoxide (thin arrow). When BH$_4$ is oxidized to BH$_2$, uncoupled electrons transferring from the NOS reductase domain to the oxygenase domain are diverted to oxygen (thick arrow) rather than L-arginine (thin arrow).
Figure 4.
A schematic representation of the possible interplay of eNOS uncoupling, reactive oxygen species (ROS) and soluble guanylate cyclase (sGC) in the pathogenesis of pulmonary arterial hypertension. Adapted with permission from [245].
Figure 5.
Representation of the pathogenesis of hemolysis-associated pulmonary hypertension. Intravascular hemolysis releases red blood cell hemoglobin into plasma, which reacts with NO. Furthermore, hemolysis releases arginase 1, which reduces L-arginine availability to synthesize NO. Xanthine oxidase and NADPH oxidase are upregulated and produce superoxide, which also inhibits NO. Reduced NO bioavailability and bioactivity promotes vasoconstriction, the development of pulmonary hypertension, and activation platelets and the hemostatic system. The pulmonary vascular lesion shown is taken from an autopsy specimen from a 35 year old male patient with sickle cell disease who died of sudden death, and shows severe concentric intimal and smooth muscle hyperplasia characteristic of advanced pulmonary hypertension.
Figure 6.
A schematic representation of the process and consequences of nitrite reduction. Nitrite can be metabolized to form NO by various enzymatic and non-enzymatic pathways as oxygen tension and pH decrease. The formation of NO and NO related signaling molecules drives hypoxic signaling and protective effects in a number of disease models. The figure is modified and reproduced with permission from [246].
Figure 7.
Schematic showing how changes in the mitochondria cause PAH based on a cancer analogy theory. In this theory, changes in the mitochondria cause a decrease in the production of mitochondria-derived reactive oxygen species, inhibiting redox-sensitive potassium channels in the plasma membrane, causing depolarization, opening of voltage-gated Ca$^{2+}$ channels, influx of Ca$^{2+}$ and constriction.
### Table 1
Clinical classification of pulmonary hypertension from Dana Point, 2008.

<table>
<thead>
<tr>
<th>Class</th>
<th>Name</th>
<th>Categories</th>
</tr>
</thead>
</table>
| I     | Pulmonary arterial hypertension (PAH) | Subdivided into:  
  - Idiopathic  
  - Familial or heritable  
  - Induced by drugs and toxins  
  - Associated  
  - Persistent pulmonary hypertension of the newborn  
  - PAH secondary to veno-occlusive disease and/or pulmonary capillary hemangiomatosis |
| II    | Pulmonary hypertension owing to left-heart disease |  
  - Systolic dysfunction  
  - Diastolic dysfunction  
  - Valvular disease |
| III   | Pulmonary hypertension owing to lung disease and/or hypoxia |  
  - Chronic obstructive pulmonary disease  
  - Interstitial lung disease  
  - Other pulmonary diseases with mixed restrictive and obstructive pattern  
  - Sleep-disordered breathing  
  - Alveolar hypoventilation disorders  
  - Chronic exposure to high altitude  
  - Developmental abnormalities |
| IV    | Chronic thromboembolic pulmonary hypertension |  
  - Hematologic disorders  
  - Systemic disorders  
  - Metabolic disorders  
  - Others: Tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis |
| V     | Pulmonary hypertension with unclear multifactorial mechanisms (Miscellaneous conditions) |  
  - Hematologic disorders  
  - Systemic disorders  
  - Metabolic disorders  
  - Others: Tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis |
Table 2

Approved medications for the therapy of PAH.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Characteristics</th>
</tr>
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<tbody>
<tr>
<td>Calcium-channel blocker</td>
<td>• Disrupt the movement of calcium through the calcium channels. Examples of this group are nifedipine, diltiazem and amlodipine.</td>
</tr>
<tr>
<td>Prostacyclin and its derivatives</td>
<td>• Effects on the pulmonary vasculature, including inhibition of smooth muscle cell constriction and proliferation, as well as platelet aggregation. Examples of this group are epoprostenol, treprostinil and iloprost.</td>
</tr>
<tr>
<td>Cyclic guanosine monophosphate (cGMP)-binding cGMP-specific phosphodiesterase inhibitors</td>
<td>• Sildenafil (phosphodiesterase type5 inhibitor): Recommended for patients with advanced PAH.</td>
</tr>
<tr>
<td></td>
<td>• Tadalafil: The first once-daily phosphodiesterase type 5 inhibitor has shown favorable results.</td>
</tr>
<tr>
<td>Endothelin receptor antagonists</td>
<td>• Bosentan: Recommended for patients with advanced PAH.</td>
</tr>
<tr>
<td></td>
<td>• Sitaxentan: Selective ET\textsubscript{A} receptor.</td>
</tr>
<tr>
<td></td>
<td>• Ambrisentan: Selective ET\textsubscript{A} receptor which improves exercise capacity and delays clinical worsening [247].</td>
</tr>
<tr>
<td>Combination therapy</td>
<td>• Option for patients not responding to the initial therapy.</td>
</tr>
</tbody>
</table>