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Therapies that enhance pulmonary vascular NO-signaling in the neonate

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Abstract

There are several pulmonary hypertensive diseases that affect the neonatal population, including persistent pulmonary hypertension of the newborn (PPHN) and bronchopulmonary dysplasia (BPD)-associated pulmonary hypertension (PH). While the indication for inhaled nitric oxide (iNO) use is for late-preterm and term neonates with PPHN, there is a suboptimal response to this pulmonary vasodilator in ~40% of patients. Additionally, there are no FDA-approved treatments for BPD-associated PH or for preterm infants with PH. Therefore, investigating mechanisms that alter the nitric oxide-signaling pathway has been at the forefront of pulmonary vascular biology research. In this review, we will discuss the various mechanistic pathways that have been targets in neonatal PH, including NO precursors, soluble guanylate cyclase modulators, phosphodiesterase inhibitors and antioxidants. We will review their role in enhancing NO-signaling at the bench, in animal models, as well as highlight their role in the treatment of neonates with PH.

Introduction

Inhaled nitric oxide (iNO), the only FDA-approved pulmonary vasodilator, in combination with supplemental oxygen are standard therapies for the treatment of neonatal pulmonary hypertension (PH). Yet the response to iNO is suboptimal in almost one-half of late-preterm and term infants with persistent pulmonary hypertension of the newborn (PPHN)/hypoxic respiratory failure (1). Thus, discovering therapies that enhance NO-signaling have been key objectives in pulmonary vascular biology research.

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In the vasculature, NO activates soluble guanylate cyclase (sGC) to increase guanosine-3'-5'-cyclic monophosphate (cGMP) (2). Through signal transduction pathways, cGMP plays an important role in regulating vascular tone, proliferation, fibrosis and inflammation by lowering intracellular calcium levels and desensitizing the contractile apparatus (3, 4). A second critical second messenger in the pulmonary vasculature is adenosine 3'-5'-cyclic monophosphate (cAMP), which is also involved in the control of pulmonary vascular relaxation (5). The regulatory action of the cyclic nucleotides is mediated by the balance between their rate of synthesis and rate of degradation. Adenylate cyclases (AC) and guanylate cyclases (GC) are the enzymes responsible for synthesis of cAMP and cGMP, respectively, whereas the cyclic nucleotide phosphodiesterases (PDE) degrade these second messengers.

NO precursors

NO synthases catalyze the oxidation of L-arginine to NO and L-citrulline. L-arginine has been reported to improve PH in adult rat models of chronic hypoxia and in rats with monocrotaline-induced PH (6). Administration of L-arginine has also been reported to reverse post-operative pulmonary endothelial dysfunction in children who have undergone cardiac bypass and to reverse pulmonary vasoconstriction in adults with PH (7, 8). The data on L-arginine has been mixed, however, with some studies demonstrating lack of improvement in PH and one study reporting increased mortality in adults taking L-arginine supplementation (9, 10). In addition, L-arginine is quickly metabolized and requires large doses which can lead to issues with patient compliance (10).

L-citrulline is converted to L-arginine and has also been investigated as an alternative PH treatment. L-citrulline has been reported to prevent chronic hypoxia-related PH, decrease established PH in newborn piglets (11, 12), as well as attenuate markers of PH in newborn rats with hyperoxic lung injury (13). In adults with idiopathic PH or Eisenmenger syndrome, L-citrulline was reported to improve exercise capacity and reduce pulmonary arterial pressures (14). Although the use of L-citrulline in a formerly preterm infant with severe bronchopulmonary dysplasia (BPD) and PH has recently been described (15), no clinical trials of this therapy have been reported in the neonatal population.

Soluble guanylate cyclase

Soluble GC (sGC) is a heterodimeric protein consisting of α - and β -subunits. A heme-containing prosthetic group is located on the heme-binding domain of the β -subunit and acts as a functional receptor for NO (2, 4, 16). Binding of NO to the heme domain of the β -subunit of sGC results in its activation and enhances the synthesis of cGMP from guanosine triphosphate (GTP). Two forms of sGC are known to exist, the native, heme-containing form and the heme-free form. Dissociation of the heme group from sGC can occur following oxidation by reactive oxygen species (ROS), rendering sGC less responsive to NO, and leading to its eventual degradation (16, 17).

sGC modulators

In pathophysiologic conditions in which either NO bioavailability is limited, NO is ineffective, or tolerance has developed, compounds that act on sGC directly may be beneficial in reducing pulmonary vascular resistance (PVR). There are two categories of pharmacologic modulators of sGC that are divided based on their mechanism of action: stimulators and activators. Both types were developed to work in a NO-independent manner to increase enzyme activity. Stimulators and activators differ based on the presence or absence of the heme group on sGC. Soluble GC *stimulators* require an intact heme group, and directly target the reduced, native form of sGC via a distinct binding site independent of NO. In addition, sGC stimulators stabilize NO-sGC binding, thereby sensitizing sGC to low levels of bioavailable NO (4). Thus, they are able to both directly stimulate the enzyme, and also act synergistically with NO. In contrast, sGC *activators* bind to the unoccupied heme-binding domain and activate the heme-free form of sGC, resulting in increased synthesis of cGMP. These activators function by taking the place of the NO-heme complex, either by binding to the unoccupied heme-binding pocket or replacing the weakly bound oxidized heme. Therefore, sGC activators demonstrate additive effects with NO (17).

sGC stimulators

BAY 41–8543 and BAY 41–2272 are sGC stimulators that have been shown to produce sustained pulmonary vasodilation in multiple animal models (18–22). Studies in neonatal sheep demonstrated that BAY 41–2272 had synergistic effects in combination with iNO to produce a greater decrease in PVR than either agent independently (18, 21). Likewise, co-administration of a NO donor along with BAY 41–8543 decreased pulmonary arterial pressures more significantly than the sum of the responses to either agent administered independently (22). Moreover, in the absence of endogenous NO, attenuation in vasodilator properties was observed (22), suggesting that BAY 41–2272 both directly stimulated the enzyme and caused it to become more sensitive to NO as reported in other studies (21, 23). In a fetal ovine model of PH, infusion of BAY 41–2272 triggered dose-dependent, sustained pulmonary vasodilation with a 75% decrease in PVR and a 3.5-fold increase in pulmonary blood flow. In addition, the observed pulmonary vasodilation was not attenuated by a NO synthase (NOS) inhibitor (20). When compared to the PDE5 inhibitor sildenafil, infusion of BAY 41–2272 demonstrated more pronounced and prolonged pulmonary vasodilation. However, when infused at higher rates and longer times intervals, systemic arterial pressure decreased (20). Furthermore, in a neonatal rat model of chronic hypoxia-induced PH, BAY 41–2272 reduced right ventricular hypertrophy (RVH) and pulmonary vascular remodeling (24). When treated with BAY 41–2272, human pulmonary artery smooth muscle cells (PASMC) exhibited decreased proliferation that was significantly enhanced when combined with sildenafil (25).

In 2013, the FDA approved the oral sGC stimulator, BAY 63–2561 (Riociguat), for use in adults with pulmonary arterial hypertension and chronic thromboembolic PH. A phase 2 clinical study of 75 adult patients with pulmonary arterial hypertension or chronic thromboembolic PH demonstrated significantly reduced PVR with improvement in the median six-minute walking distance (26). Although riociguat was generally well tolerated,

fifteen percent (11/75) of patients developed asymptomatic hypotension. In nine patients, the blood pressure normalized without intervention, whereas doses had to be reduced in two patients. None of the patients required discontinuation of the study drug for the asymptomatic hypotension. In the phase 3 PATENT trial, riociguat was shown to significantly improve exercise capacity, PVR, World Health Organization functional class, and time to clinical worsening compared to placebo in adults with pulmonary arterial hypertension (27, 28).

Although a promising therapeutic option for PH, no clinical data yet exists for the use of sGC modulators in the neonatal or pediatric population. A single case report of riociguat use in a 3.5 year-old boy with suprasystemic pulmonary arterial hypertension resistant to other pulmonary vasodilator therapies including endothelin-1 receptor antagonist and PDE5 inhibition, resulted in decreased PVR-to-systemic vascular resistance, transpulmonary pressure gradients, RVH, pulmonary artery acceleration time and pediatric functional class (29). No adverse effects, including systemic hypotension, were noted on short-term follow-up (29). More studies are needed to elucidate the safety and efficacy of sGC modulation in this population of patients with PH.

sGC activators

sGC activators preferentially increase the activity of the enzyme when it is in the inactive or oxidized state. Therefore, it is postulated that this class of therapies may be most beneficial in disease states associated with oxidative stress in which NO is ineffective. Chester et al. demonstrated significant pulmonary vasodilation following administration of the potent sGC activator, BAY 58-2667 (Cinaciguat), in both healthy fetal lambs and in a PPHN lamb model induced by ductus arteriosus ligation (30). Therefore, in conditions that induce sGC oxidation – including hyperoxia, exposure to the sGC inhibitor ODQ, and hydrogen peroxide – the pulmonary vasodilatory effect by BAY 58-2667 was enhanced (30, 31). Importantly, the sGC activator elicited a more potent reduction in PVR than acetylcholine or iNO. Furthermore, a rat model of acute PH induced with a thromboxane receptor agonist demonstrated significant decreases in PVR, but also displayed a drop in systemic arterial pressure with sGC activator administration (32). Findings were enhanced by the addition of a NOS inhibitor and irreversible oxidation of sGC. Interestingly, a study using inhalation of sGC activator microparticles reported enhanced selective pulmonary vasodilation, transpulmonary cGMP release, and improved arterial oxygenation in a lamb model of acute experimental pulmonary hypertension (33). These data suggest that this inhalational delivery may be effective in decreasing PVR while avoiding systemic hypotension (33).

Cinaciguat has been used in the experimental treatment of adults with acute decompensated heart failure. In a multi-center phase 2 study, intravenous infusion of cinaciguat significantly reduced pulmonary capillary wedge pressure, mean right atrial pressure, mean pulmonary artery pressure, PVR, and systemic vascular resistance (34). The medication was well tolerated with hypotension as the most common adverse effects. At present, there have been no studies examining the use of sGC activators in the neonatal or pediatric population.

Cyclic Nucleotide Phosphodiesterases

There are more than 20 different PDE genes expressed in mammalian tissues, within a superfamily of 11 PDE enzymes (35). The different families of PDE vary in their tissue and cellular distribution, structure, affinity for specific cyclic nucleotides, and modes of regulation. At least five PDE isozymes have been identified in vascular tissues: 1, 2, 3, 4, and 5 (3). Although other PDEs have been studied, this review will focus primarily on PDE3 and PDE5, given that antagonists against these two enzymes have been utilized in neonatal medicine for their vasodilatory effects, presumably via the downstream actions of cAMP and cGMP.

PDE3

Phosphodiesterase 3 (PDE3) is often referred to as the cGMP-inhibited PDE. While PDE3 hydrolyzes both cAMP and cGMP with high affinity, the velocity for cAMP hydrolysis is 4- to 10-fold higher than that for cGMP, resulting in competitive inhibition of PDE3 by cGMP (36, 37). Hence cAMP and cGMP are mutually competitive substrates for PDE3 (38). Furthermore, some biological effects of endogenous cGMP may be mediated by inhibition of PDE3, thereby resulting in increased cAMP levels to promote vasodilation (38). Moreover, a more detailed investigation of this cross-talk is necessary as alterations in the NO-cGMP pathway, such as via iNO, as will be discussed later, have been demonstrated to alter the cAMP-PDE3 pathway.

Two PDE3 isoforms have been identified: PDE3A and PDE3B, which exhibit cell-specific differences in properties and regulation and serve cell-specific functions. The *Pde3a* gene is located on chromosome 12 and although it is expressed in many tissues, it is most abundantly expressed in the heart, vascular smooth muscle, and platelets (38, 39). The *Pde3b* gene is located on chromosome 11 and its relative abundance is highest in adipose tissue (38, 39). PDE3A has been implicated in cardiac contractility and vascular smooth muscle cell (VSMC) proliferation, whereas PDE3B plays a role in mitochondrial function and energy (40). Consequently, PDE3A is believed to play a larger role in vascular pathology, although PDE3B has more recently been implicated in cardioprotection against ischemia-reperfusion injury in a murine model (41). Although both PDE3A and PDE3B isoforms are expressed in the pulmonary vasculature and have been therapeutic targets in pulmonary hypertensive diseases, their exact role within the pulmonary vasculature is not known and to date, no isoform-specific PDE3 inhibitors exist on the market.

PDE3 and Pulmonary Hypertension

In varying models of PH, the regulation of PDE3 has been shown to be altered. Increased PDE3 activity has been reported in pulmonary arteries from a rat model of hypoxia-induced PH, as well as in pulmonary artery smooth muscle cells (PASMC) isolated from patients with PH (42, 43). Additionally, PDE3 has been implicated in iNO unresponsiveness (44, 45), iNO tolerance (45), and rebound PH upon iNO discontinuation (46, 47).

As previously described, supplemental oxygen and iNO are standard therapies for the treatment of neonatal pulmonary hypertension. NO increases cGMP levels, which would

also be expected to inhibit cAMP hydrolysis by PDE3, resulting in increased vasorelaxation. However, *in vitro* and *in vivo* animal studies have demonstrated an increase in PDE3 expression and/or activity following treatment with NO and enhanced relaxation of the pulmonary vasculature with the addition of the PDE3 inhibitor, milrinone to iNO (44, 45, 48). Healthy newborn lambs exposed to iNO and 100% O₂ (often used concurrently to treat PPHN) demonstrated the highest PDE3 activity and the greatest vasorelaxation response to milrinone. Importantly, this fetal lamb model explored the developmental regulation of the cAMP-PDE3 pathway, with alterations due to mechanical ventilation, oxygen, and NO exposures (44). Similarly, rat PASMOC exposed to NO exhibited an increase in PDE3A gene and protein expression and cAMP PDE enzyme activity which was inhibited following the addition of milrinone (45). Furthermore, in a rabbit model of PH, the combination of iNO and milrinone had additive effects with a more substantial reduction in PVR than when given individually, suggesting synergistic effects of these medications in the pulmonary vasculature (48).

PDE3 as a therapeutic target

Milrinone, a selective PDE3 inhibitor, is utilized clinically for its inotropic, lusitropic, and vasodilatory properties (49). In the adult population, it is FDA-approved for use as short-term treatment in acute decompensated heart failure, as its long-term use was shown to increase both morbidity and mortality in adult patients with severe chronic heart failure (50). In the neonatal and pediatric population, milrinone is well established as a first-line agent for the prevention and treatment of low cardiac output syndrome and PH in patients who have undergone cardiac surgery (51–54). However, less is known regarding its efficacy in the treatment of PPHN/hypoxic respiratory failure. Although milrinone is being increasingly utilized in the treatment of neonates with PH, data to support its safety and efficacy is limited. In neonates, several case series reported improved oxygenation after the addition of milrinone in those infants with PPHN and poor response to iNO (55, 56). A prospective study of 11 neonates with PPHN with suboptimal response to iNO therapy was performed to determine the pharmacological profile of milrinone. The investigators found that the addition of milrinone improved oxygenation, decreased fraction of inspired oxygen (FiO₂) requirements, and improved hemodynamic parameters. Importantly, no significant changes in heart rate or systemic blood pressure were observed (57). These findings support the hypothesis that increased PDE3 activity plays a role in neonatal PH and implicates PDE3-inhibition as a potential therapeutic intervention for this disease.

Despite the animal and clinical data to support milrinone use in PH, there have been no published RCTs to date for the use of milrinone in neonates with PPHN, although at present ongoing enrollment is taking place as part of a pilot study in Europe (58). Similarly, a clinical trial evaluating the use of milrinone in neonates with congenital diaphragmatic hernia is underway as part of the Neonatal Research Network (59). The delay in performing larger RCTs to evaluate the use of PDE3 inhibitors, or in general other vasodilatory therapies in this population has been due to the difficulty of adequate enrollment of the relatively few infants with PPHN or BPD-associated PH (60, 61). Additionally, the use of milrinone may have inconsistent beneficial effects observed in the clinical realm that perhaps may be secondary to the nonspecific inhibition towards both PDE3A and PDE3B

isoforms in the pulmonary vasculature. Our unpublished studies suggest a differential role of each PDE3 isoform in the regulation of vascular resistance within the pulmonary circulation, which may demonstrate a need for isoform-selective inhibition of PDE3 in varying pulmonary vascular diseases. More *in vitro* and *in vivo* studies are necessary to understand the role of each isoform in the varying cell types, their developmental regulation, and their specific interactions with the NO-cGMP pathway within the pulmonary vasculature.

PDE5

PDE5 is the most prevalent phosphodiesterase in the pulmonary vasculature where it contributes to vasoconstriction through hydrolysis of cGMP. Alternative splicing of the human *Pde5* gene, located on chromosome 4, results in three isoforms. These isoforms differ in their tissue distribution with PDE5A1 and PDE5A2 being ubiquitous in many tissues while PDE5A3 is localized to smooth muscle (62). Expression of all three PDE5 isoforms is elevated in lungs of patients with PH as compared to controls, with PDE5A1 being expressed at the highest levels, particularly in intimal lesions and muscularized distal vessels (25).

The role of PDE5 in neonatal diseases associated with PH has been demonstrated in various animal models, including piglet and lamb models of PPHN. Lambs with PPHN exhibit increased PDE5 expression and activity and decreased cGMP concentrations at baseline, as well as exaggerated PDE5 activation during periods of hyperoxia (63–65). An increase in PDE5 protein expression and activity have also been described in healthy neonatal lambs ventilated with 100% O₂ (63), suggesting that increased oxidative stress plays a role in PDE5 activation even in the absence of lung pathology. In addition to the importance of PDE5 in PPHN models, aberrant cGMP signaling has been described in murine models of hyperoxic lung injury. Neonatal mice exposed to chronic hyperoxia demonstrate decreased sGC activity and cGMP levels and increased activation of PDE5 that persists even after room air recovery (66, 67). Finally, studies in mouse hyperoxic lung injury models have also suggested that aberrant PDE5 signaling may be a contributor to hyperoxic myocardial injury as demonstrated by increased PDE5 activity and decreased cGMP concentrations in the right ventricle of neonatal mice with hyperoxia-induced PH. PDE5 inhibition, on the other hand, is associated with attenuated RVH, reduced right ventricular PDE5 activity, as well as improved right ventricular cGMP concentrations (68). Similar PDE5 upregulation has been described in failing and hypertrophied human right ventricles (69, 70).

PDE5 as a therapeutic target

Because PDE5 activation contributes to vasoconstriction, PDE5 inhibition is a potential therapeutic strategy in a number of neonatal diseases associated with pulmonary vascular disease, including PPHN and BPD-associated PH. One of the first reports of the use of PDE5 inhibitors in neonatology was a case study published in 1995 that reported an enhanced vasodilatory response to iNO in a neonatal patient with congenital diaphragmatic hernia and severe PH who had been treated with a PDE5 inhibitor (71). Subsequent *in vivo* studies demonstrated pulmonary vasodilation with PDE5 inhibitors in various animal models of neonatal PH. Dipyridamole has been shown to increase pulmonary artery flow and to

enhance vasodilatory effects of acetylcholine and iNO in studies of ovine fetuses (72, 73). Similar effects were observed with dipyridamole in newborn lambs with PPHN whether used alone or in combination with low dose iNO demonstrating decreased PVR and increased pulmonary blood flow. Decreases in systemic blood pressure were observed, however, likely limiting usefulness of dipyridamole (74). Finally, work in piglet models of PH demonstrated improvements in PVR, increased cardiac output, and improved oxygenation with the use of sildenafil and tadalafil as compared to control animals, without any untoward effects on systemic hemodynamics (75, 76).

Results from animal studies, combined with the observation that up to 50% of neonates do not respond or sustain their response to iNO (77) and the fact that the PDE5 inhibitors are commonly used in adults with chronic PH, have generated a considerable amount of interest in the potential role of PDE5 inhibitors in neonates and infants with PH. Oral sildenafil, a lower cost vasodilator and potential alternative to iNO in resource-poor areas, has been investigated in three small RCTs that evaluated its efficacy in near- and full-term infants with PPHN. All three studies found improvements in oxygenation and improved survival as compared to placebo (78–80). In addition, an open-label dose-escalation intravenous sildenafil trial found improvements in oxygenation index with higher doses (81). One additional case series reported on the use of intravenous sildenafil in six preterm neonates with refractory PH and found that, while the treatment appeared effective in improving PH, two of the infants developed pulmonary hemorrhage and four patients ultimately died (82).

Compared to the published clinical data on the use of PDE5 inhibitors in PPHN, there are even fewer studies evaluating the efficacy of these drugs in patients with BPD-associated PH. A couple of small retrospective studies noted reductions in PH and potential improvement in right ventricular function in patients with BPD, although sildenafil was associated with systemic hypotension in one of those studies (83, 84). To begin to address this knowledge gap, Gonzalez et al (85), as part of the Pediatric Trials Network Steering Committee, has recently characterized the pharmacokinetics of sildenafil and its active metabolite, N-desmethyl sildenafil, in premature infants and have applied pharmacokinetic modeling to inform dosing for a follow-up, phase II study.

A 2012 FDA warning about the safety of sildenafil in children led to a considerable debate (<https://www.fda.gov/Drugs/DrugSafety/ucm317123.htm>) at the time and resulted in a clarification from the FDA to acknowledge that the use of sildenafil may be appropriate in situations where treatment options are limited (<https://www.fda.gov/Drugs/DrugSafety/ucm390876.htm>). Of note, the study used to justify the initial FDA sildenafil warning did not include any patients under one year of age and, to date, there are no large RCTs of sildenafil use in the neonatal population. Survey data suggests that despite the controversy and lack of RCTs, sildenafil continues to be intermittently utilized to treat patients with PPHN (86) and BPD (87), highlighting the need for more clinical trials in these populations.

PDE4

PDE4 hydrolyzes cAMP with high specificity and affinity. It consists of four isoforms: PDE4A, PDE4B, PDE4C, and PDE4D, which are ubiquitously expressed (88). PDE4 is the

predominant enzyme responsible for cAMP hydrolysis in inflammatory cells and leukocytes, and exhibits a pro-inflammatory effect (88, 89). Multiple experimental animal models have demonstrated anti-inflammatory properties in pulmonary disorders following PDE4 inhibition (90–92). Consequently, PDE4 inhibition has been a therapeutic target for pulmonary disorders characterized by inflammation, including asthma and chronic obstructive pulmonary disease (COPD) (89, 93). Roflumilast, a PDE4-specific inhibitor was FDA-approved in 2011 for use in adults with COPD. A Cochrane meta-analysis that included nine RCTs found a statistically significant improvement in pulmonary function tests and patient-reported exacerbations with the use of Roflumilast in adults with COPD, although rates of gastrointestinal adverse events were high (94).

Given the role of inflammation in BPD, de Visser et al (95, 96) investigated the effects of both prophylactic and rescue PDE4 inhibition in an experimental BPD model. In this neonatal rat pup model of hyperoxia-induced lung injury with a nine-day exposure, prophylactic treatment with the PDE4 inhibitor, piclamilast, improved mortality, partially improved alveolar development, increased pulmonary vessel density, reduced arteriolar medial wall thickness and fibrin deposition, prevented the development of RVH, and attenuated PH (95, 96). Late treatment of the neonatal rat pups with piclamilast in the last three days of hyperoxic exposure did not improve alveolarization, vascular development and medial wall thickness after a recovery period of nine days. Although there was a significant reduction in medial wall thickness at the end of recovery period on day 18, this reduction was not sustained at day 42 or 51 (96). Late treatment with piclamilast also attenuated hyperoxia-induced RVH. Neither prophylactic nor late treatment improved alveolar enlargement (96). Although some beneficial effects on lung injury have been observed with PDE4 inhibitors, there have been no RCTs on the use of this class of drugs in the neonatal population. Further studies need to be performed to determine if PDE4 inhibition may be of therapeutic benefit in this population.

Antioxidants

Reactive oxygen species and reactive nitrogen species (RNS) are produced under normal physiologic conditions by a variety of cellular sources. These by-products of oxygen metabolism include superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite, as well as other free radicals. While ROS are important second messengers, they can lead to direct injury to protein, lipids, and DNA when the ability of the cells to clear oxidative species is overwhelmed. ROS have been implicated in mitochondrial injury, apoptosis, and increased inflammation, while RNS production can result in protein nitration and subsequent protein dysfunction. In addition, NO can interact with ROS in a number of ways that ultimately increase the oxidative stress burden. For example, NO can combine with superoxide to produce peroxynitrite, a potent and highly reactive nitrogen species, at a rate that is much faster than the dismutation of superoxide by the superoxide dismutase (SOD) enzymes (97). Peroxynitrite can then go on to cause vascular dysfunction through a variety of mechanisms including direct oxidative damage, promotion of vasoconstriction, and endothelial NOS (eNOS) uncoupling, a phenomenon that leads to increased superoxide generation at the expense of NO production (98). eNOS uncoupling has been described

under conditions associated with increased oxidative stress, including in neonatal animal models of cerebral hypoxia-ischemia, necrotizing enterocolitis, and PPHN (99–101).

Animal studies have provided ample evidence of the impact of oxidative stress on the nitric oxide signaling pathways, including effects on PDE5, NO, and sGC. Hyperoxia has been associated with increased PDE5 activity and impaired vasodilation in a sheep model of PPHN (63, 65), where the proposed link between hyperoxia and PDE5 activation is oxidative stress. This link has been supported by studies demonstrating that ventilation of PPHN lambs with 100% O₂ was associated with increases in both ROS production and PDE5 activity, and treatment of isolated PASMC with hydrogen peroxide alone was sufficient for PDE5 induction (63, 102). Moreover, PDE5 induction in the PPHN lamb model has been linked to mitochondrial oxidative stress with rapid increases in mitochondrial ROS and PDE5 activity within 30 minutes of hyperoxia exposure (103). Similarly, PDE5 activation and impairments in cGMP production have been reported in other neonatal models associated with increased oxidative stress, including a swine model of hypoxia-induced pulmonary vascular disease and a neonatal mouse model of HLI (66, 104), as well as in mice with chronic heart failure (70). Findings from animal models of PPHN also demonstrated that increased oxidative stress reduced the bioavailability of NO through eNOS uncoupling and downregulation of eNOS expression and function (101, 105, 106). In addition, studies utilizing extracellular SOD (ecSOD) knockout mice have shown that NO availability is affected by relative abundance of ecSOD, a major antioxidant enzyme in the pulmonary vasculature that increases available NO by reducing the amount of extracellular superoxide thereby decreasing NO-superoxide interactions (107). Extracellular SOD overexpression, on the other hand, appears to be protective as it maintained NO bioavailability in isolated mouse epithelial cells during hyperoxia (108). Finally, in addition to effects on PDE5 and NO, oxidative stress has been reported to be associated with decreased sGC expression and activity, as well as a shift of sGC towards the NO-insensitive oxidized form (109, 110).

Pulmonary antioxidant enzyme production increases dramatically during the third trimester in preparation for birth and the relatively hyperoxic extrauterine environment (111–113). It is perhaps not surprising that the ability to metabolize ROS and RNS is limited in premature neonates as compared to their term counterparts, predisposing the preterm infant to oxidative injury (113). Oxidative stress contributes significantly to a multitude of neonatal disease states, including lung disease, necrotizing enterocolitis, retinal, brain, and renal injury (114–116). While the lungs of both term and preterm infants are vulnerable due to their unique position as the organ of gas exchange, the immature lung is particularly at risk of injury from oxidative stress, given the well-described deficiencies in pulmonary antioxidant enzymes in premature neonates (117). The preterm infant is also more likely to be exposed to hyperoxic ventilation than their term counterparts as a result of relative lung immaturity and higher incidence of respiratory distress syndrome. Studies utilizing fetal and neonatal samples have demonstrated elevated markers of oxidative stress and decreased levels of antioxidants in human neonates born preterm and in those who go on to develop BPD (118–121). Similar to antioxidant deficits seen in preterm infants, animal studies have demonstrated deficiencies in antioxidant enzymes in neonatal models of lung injury, including lower levels of the mitochondrial antioxidant, manganese SOD, in PPHN sheep

and impaired ecSOD activity in newborn rats exposed to hyperoxia (122, 123). These data suggest that both prematurity and pulmonary pathology are associated with limited antioxidant capabilities.

Treatment with exogenous antioxidants has been reported to have a protective impact on NO signaling pathways in animal models associated with neonatal oxidative stress. For example, treatment of isolated PASMC from a lamb model of PPHN with N-acetyl-cysteine (NAC) attenuated hyperoxic PDE5 activation and improved cGMP concentrations (63). Similar effects on PDE5 and cGMP were seen with mitochondrial catalase treatment *in vitro* (103) and with recombinant human Cu/Zn SOD (rhSOD) *in vivo* (64) in the same model.

Treatment of PPHN sheep with rhSOD also restored eNOS expression and function and normalized levels of tetrahydrobiopterin, a critical eNOS cofactor (102). Another group of investigators have demonstrated that rhSOD improved vasodilatory responses to a NO donor in pulmonary arteries isolated from PPHN lambs and that rhSOD alone produced pulmonary vasodilation and facilitated the action of iNO *in vivo* (124). While there have been a number of animal studies showing beneficial pulmonary effects of antioxidants, adverse effects have also been described. One study utilizing NAC in rats exposed to hyperoxia demonstrated that while it had protective effects on the pulmonary endothelium, it was also associated with significant systemic side effects (including weight loss and increased respiratory distress) and potentially harmful effects on the pulmonary epithelium with increased apoptosis and DNA damage in cultured type II pneumocytes (125). Another study demonstrated development of PH in mice treated with NAC, similar to a three-week exposure to hypoxia (126).

The effects of antioxidants on NO signaling pathways in human neonates remain largely unknown. Several studies have demonstrated increased nitrotyrosine, a marker of peroxynitrite formation, in bronchoalveolar lavage from premature neonates with respiratory disease, as well as in plasma of infants who go on to develop BPD (127, 128). Another study reported decreased eNOS gene expression in umbilical vein endothelial cells cultured from newborn infants with PPHN (129). The relevance of findings from animal studies that suggest protective effects of antioxidants on the development of pulmonary pathology remain unclear, however, as antioxidants have produced largely disappointing results in neonatal clinical trials. With the exception of vitamin A, studies evaluating the efficacy of either systemic or inhaled antioxidants have failed to produce improvements in rates of BPD (130, 131). Some promising findings have been reported with SOD supplementation in preterm infants with respiratory distress syndrome, including a decreased need for respiratory support, reduced wheezing with subcutaneous SOD (132), and a decline in pulmonary morbidity at one year of age with rhSOD (133). Taken together, studies examining the role of antioxidants in the treatment of infants at risk for the development of BPD highlight the difficulty in translating findings in animal models to complex neonatal diseases with a multitude of etiologies, comorbidities, and clinical presentations.

Conclusions

Despite advances in neonatal respiratory care and increased understanding of the importance of aberrant NO signaling in the pathobiology of pulmonary vascular disease, clinicians

continue to face the dilemma of how best to treat neonates and infants with PH. PPHN remains the most extensively studied neonatal disease associated with PH with clinical trials primarily focused on two types of therapies that enhance NO signaling - iNO and, to a much lesser extent, PDE5 inhibitors. Additional therapies that target the NO pathway have produced promising results in animal models, but have not been adequately evaluated in infants with PH (sGC modulators, PDE3 and PDE4 inhibitors) or have produced disappointing results in clinical trials (antioxidants). Finally, other PH populations, for example infants with BPD, remain understudied, further underscoring the need for more research.

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Highlights

- Limited pharmacotherapies are available for neonates with pulmonary hypertension
- Nitric oxide is the only FDA-approved therapy for persistent pulmonary hypertension of the newborn (PPHN) that demonstrates a decrease in need for extracorporeal membrane oxygenation, but mortality and poor neurodevelopmental outcomes persist
- ~40% of patients with PPHN do not respond to inhaled nitric oxide therapy
- No FDA-approved therapies exist for bronchopulmonary dysplasia (BPD)-associated pulmonary hypertension
- Nitric oxide precursors, soluble guanylate cyclase, cyclic nucleotide phosphodiesterases, and oxidant pathways, are targets that have been actively investigated to enhance nitric oxide signaling in the pulmonary vasculature as additional treatment options for pulmonary hypertension

Figure 1.

Schematic representing interactions of the NO pathway and the various sites of action of available pharmacologic agents within the pulmonary artery. EC, endothelial cell; SMC, smooth muscle cell; iNO, inhaled nitric oxide; AC, adenylate cyclase; PDE, phosphodiesterase; cAMP, adenosine 3'-5'-cyclic monophosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cGMP, guanosine-3'-5'-cyclic monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; ONOO⁻, peroxynitrite; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; R, reduced; O, oxidized

Table 1.

Therapies that enhance NO signaling in models of pulmonary hypertension. MCT, monocrotaline; DA, ductus arteriosus; U-46619, thromboxane A2 receptor agonist; LPS, lipopolysaccharide; rhSOD, recombinant human Cu/Zn superoxide dismutase; NAC, N-acetyl-cysteine

Agent	Animal	PH Model	Drug	Efficacy	Adverse Effects	References
NO precursors	Adult Rat	Chronic hypoxia; MCT	L-arginine	+		6
	Newborn Piglets	Chronic hypoxia	L-citrulline	+		11, 12
	Human- Adult	PH	L-arginine; L-citrulline	+/-	Decrease in systemic arterial pressure	8, 14
sGC stimulator	Fetal & Neonatal Sheep	Partial ligation of DA; Normal fetal sheep	BAY 41-2272	+	Systemic effects at higher doses	20, 21
	Lamb	U-46619	BAY 41-2272	+	Systemic effects at higher doses	23
	Lamb	U-46619	BAY 41-2272 BAY 41-8543 (nebulized microparticle)	+		33
	Minipig (7 week)	Single-lung ventilation	BAY 41-8543, riociguat	+		18
	Neonatal Rat	Chronic hypoxia	BAY 41-2272	+		24
	Adult Rat	U-46619	BAY 41-8543	+	Systemic hypotension	22
	Adult Dog	Heparin-protamine	BAY 41-2272	+	Exacerbated systemic hypotension	19
	Human - Adult	PH	BAY 41-2272; Riociguat (BAY 63-2521)	+	Dyspepsia, headache, hypotension (asymptomatic)	25, 26
sGC activator	Fetal Sheep	Normal fetal sheep; Partial ligation of DA	Cinaciguat (BAY 58-2667)	+	Minimal decrease in systemic arterial pressure	30, 31
	Adult Rat	MCT, U-46619	BAY 60-2770	+	Decrease in systemic arterial pressure	32
	Lamb	U-46619	BAY 58-2667 (nebulized microparticle)	+		33
PDE3 inhibitor	Fetal & Neonatal Sheep	Normal sheep	Milrinone	+		44
	Adult Rabbit	U-46619	Milrinone	+	Decreased systemic arterial pressure	48
	Human - Neonatal	PPHN, poor response to iNO	Milrinone	+		57
PDE5 Inhibitor	Fetal & Neonatal Sheep	Normal sheep; Prenatal DA ligation	Dipyridamole	+	Increased HR, decreased aortic pressure	72-74
	Piglet	Meconium aspiration; hypoxia	Sildenafil; Tadalafil	+		75, 76
	Human - Neonate	PPHN	Sildenafil	+		78-80
PDE4 Inhibitor	Adult Mouse	Acute Lung Injury (LPS + zymosan)	Rolipram	+		90

Agent	Animal	PH Model	Drug	Efficacy	Adverse Effects	References
Antioxidants	Neonatal Lamb	Prenatal DA ligation	rhSOD	+		102, 124
	Adult Mice	Normal; Hypoxia	NAC	–	Increased RV pressure and hypertrophy, pulmonary vascular remodeling	126