Nitrite in pulmonary arterial hypertension: therapeutic avenues in the setting of dysregulated arginine/nitric oxide synthase signalling

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Abstract

Pulmonary arterial hypertension (PAH) is an insidious disease of the small pulmonary arteries that is progressive in nature and results in right heart strain/hypertrophy and eventually failure. The aetiologies may vary but several common pathophysiological changes result in this phenotype, including vasoconstriction, thrombosis, and vascular proliferation. Data suggest that nitric oxide (NO) signalling is vasoprotective in the setting of PAH. The classic arginine–NO synthase (NOS)–NO signalling pathway may represent an adaptive response that is eventually dysregulated during disease progression. Dysregulation occurs secondary to NOS enzyme down-regulation, enzymatic uncoupling, and arginine catabolism by vascular and red cell arginases and by direct NO inactivation via catabolic reactions with superoxide or cell-free plasma haemoglobin (in the case of haemolytic disease). The anion nitrite, which has recently been recognized as a source of NO that circumvents the arginine–NOS pathway, may serve as an additional adaptive signalling pathway that is now appreciated to have a vasoregulatory role in the pulmonary and systemic vasculature. Inhaled nebulized sodium nitrite is a relatively potent pulmonary vasodilator in the setting of hypoxia and is also anti-proliferative in multiple experimental models of pulmonary hypertension. Multiple nitrite reductases have been shown to be relevant in the conversion of nitrite to metabolically active NO, including deoxy-haemoglobin and myoglobin in the circulation and heart, respectively, and xanthine oxidoreductase in the lung parenchyma.

Keywords

Nitric oxide • Pulmonary arterial hypertension • Sodium nitrite • Nitrite reductase • Smooth muscle cell

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1. Introduction

1.1 Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a disease marked by increased vascular resistance in the small pulmonary arteries. This results in increased pulmonary arterial pressures and subsequently an increase in right ventricular pressures (RVPs). The right ventricle compensates by muscular hypertrophy, but eventually the right ventricle will fail, ultimately leading to death. The nomenclature of pulmonary hypertension has been updated over the years, reflecting different categories of pulmonary hypertension that may require unique treatments.1,2 Although the aetiologies vary, the development of increased pulmonary vascular resistance is a common phenotype that results from shared pathophysiological phenomena at the vascular level upon which these diverse aetiologies converge.3 These common features include pulmonary vasoconstriction, vascular remodelling, and vascular thrombosis. Additionally, despite the varied aetiologies that result in these pathophysiological changes, there are several common molecular pathways and signalling molecules that are altered in PAH and commonly implicated in the development of this phenotype. One such major implicated signalling molecule is nitric oxide (NO).

1.2 Nitric oxide

NO was originally recognized as a free radical signalling molecule that was naturally produced in the human body in the 1980s. Early investigations identified this molecule as the ‘endothelium-derived relaxing factor’, and the discovery gave way to a myriad of studies characterizing the roles of NO as a vasoregulatory molecule in the late 1980s and early 1990s.4 The endogenous source of NO in the body was early
discovered to be secondary to metabolism of L-arginine by a family of enzymes known as NO synthases (NOSs). These enzymes utilize the substrates L-arginine, molecular oxygen, and NADPH to produce L-citrulline and NO (Figure 1). Two of the NOS isoforms are expressed constitutively (endothelial NOS, NOS-III, or eNOS and neuronal NOS, NOS-I, or nNOS), whereas one isoform is inducible in multiple cell types and tissues (inducible NOS, NOS-II, or iNOS). eNOS, as the name implies, is produced by the vascular endothelium and is the isoform that is most important in regulating NO production to influence the cardiovascular system.

NO is a highly reactive molecule that is diffusible and has a short half-life, thus making this an ideal signalling molecule to act in an autocrine or paracrine fashion. NO regulates vasodilation via effects on soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP).5–8 Other important vasoregulatory properties of NO include regulation of smooth muscle cell proliferation and migration, platelet aggregation, and leucocyte adhesion to the endothelium.

More recently, it has been discovered that NO could be produced endogenously in the body via an L-arginine and NOS-independent mechanism from the anion nitrite (NO2\(^-\))9,10 Nitrite was thought to be a relatively stable end-product of NO metabolism that had no significant biological action. Quite the contrary, the body can convert nitrite back to NO via both non-enzymatic and highly regulated enzymatic mechanisms, with enhanced rate of reduction under physiological or pathological hypoxia (Figure 2). The recognition of a number of enzymes that possess nitrite reductase activity to convert nitrite to biologically active NO has suggested new pathways to NO formation and signalling and identified nitrite as an endogenous circulating ‘endocrine reservoir’ of NO.11,12 Interestingly, circulating nitrite is produced endogenously from the eNOS/NO pathway, but other important sources include dietary consumption of nitrates, which are mainly converted to nitrite via an elegant enteral-salivary pathway.5,13

This review will focus on our current understanding of the NOS/NO pathway in the healthy pulmonary vasculature and in the setting of PAH. In addition, our evolving appreciation and understanding of the nitrate-to-nitrite-to-NO pathway and characterization of cellular nitrite reductases will be reviewed. Finally, current and promising experimental therapeutic modalities for PAH related to NO and NO biology, including nitrite, will be summarized.

2. Arginine/nitric oxide synthase/nitric oxide in pulmonary arterial hypertension

The arginine/NOS/NO pathway appears to be very important in regulating vascular tone and remodelling in PAH. Changes in NOS expression and increased NO generation are generally interpreted to be a protective compensatory response to the underlying disease processes that increase pulmonary vascular resistance. The vasodilatory properties of NO are well characterized and are clearly important in the setting of PAH. NO can also decrease smooth muscle cell proliferation, but may also protect via increased apoptosis or autophagic signalling to limit the progression of vascular lesions and to remodel the vascular wall.14,15

Investigations that have sought to determine the contribution of the arginine–NOS–NO axis in PAH demonstrate somewhat contradictory results. Several studies have illustrated that eNOS levels are increased in the pulmonary arteries in multiple animal models of pulmonary hypertension, including chronic hypoxia, monocrotaline, and fawn-hooded hypertensive rats.16–20 In addition to eNOS, some authors have demonstrated that inducible NOS (iNOS) is increased in the chronic hypoxia model of pulmonary hypertension.17,20 We have made similar observations, demonstrating that iNOS protein is

Figure 1 The classic arginine–nitric oxide synthase–nitric oxide pathway. This figure illustrates the ‘classic’ nitric oxide pathway and both cyclic guanosine monophosphate-dependent and -independent signalling. Furthermore, the figure highlights the multiple levels of this pathway that can be taken advantage of for therapeutic benefit. One strategy is to increase nitric oxide synthase substrate availability via L-arginine supplementation or arginase inhibitors. Alternative strategies are to increase nitric oxide synthase enzymes via gene or protein therapy as well as direct delivery of nitric oxide gas via inhalation or pharmacological donors. Additionally, therapeutics take advantage of cyclic guanosine monophosphate-dependent signalling including phosphodiesterase inhibitors, such as sildenafil, and the direct guanylate cyclase activators such as riociguat.
increased in the microvasculature in the rat monocrotaline model (unpublished data). However, studies utilizing specific iNOS inhibitors have not demonstrated major influences in vascular tone, and the role of iNOS in this setting remains uncertain.17,18

Although animal studies consistently demonstrate increased eNOS in the setting of pulmonary hypertension, data from human tissues are more difficult to resolve. Xue and Johns21 reported increased eNOS immunostaining and Giaid and Saleh22 demonstrated decreased eNOS immunostaining, whereas Tudor et al.23 illustrated unaltered eNOS levels compared with non-diseased pulmonary arteries. Several issues may account for the discrepancy between the animal models and human tissue results. Animal models allow for the investigation of NOS proteins during the development of pulmonary hypertension, whereas human tissue samples are generally obtained after the disease is clinically significant or post-mortem. Additionally, experimental pulmonary hypertension in animal models does not result in the classic plexiform vascular lesions that are seen in human disease. Furthermore, as PAH is more of a phenotype than an individual disease process, there may be variations that occur depending on the aetiology and context in which the disease develops.

Increased mRNA expression and/or protein levels do not necessarily result in increased NO production. Several studies have attempted to measure NO production in the aforementioned animal models of pulmonary hypertension. Using chemiluminescence to measure NOx (NO and NO2−) accumulation in the perfusate of isolated perfused lungs, several studies have demonstrated increased NO2− levels in the chronic hypoxia model.19,24 Utilizing the citrulline assay to measure NOS activity, Rengasamy and Johns25 illustrated that NOS activity was decreased in the chronic hypoxia model. These authors hypothesized that this decrease in NOS activity could be explained by the requirement for oxygen as a substrate for the reaction in converting L-arginine to NO.

Another mechanism that decreases NO bioavailability, leading to reduced vasodilation that can occur in the setting of normal or increased eNOS concentrations, is ‘eNOS uncoupling’. This paradoxical decrease in NO bioavailability is secondary to increased production of superoxide anions by eNOS itself.26 eNOS uncoupling can occur secondary to decreased tetrahydrobiopterin (BH4) bioavailability. This is demonstrated by the findings that BH4 supplementation or gene transfer of GTP cyclohydrolase 1, the rate-limiting enzyme of BH4 synthesis, restores eNOS-mediated NO generation and endothelial function.27,28 eNOS uncoupling has been demonstrated in pulmonary hypertension secondary to defects in the synthesis of BH4.27–29 Uncoupling in these studies is associated with disruption of eNOS dimers and monomerization.

An additional factor that may limit NO production and contribute to the development of PAH is the substrate availability of arginine. Arginase utilizes L-arginine as a substrate to produce ornithine and urea.30,31 Since NOS and arginase compete for the same substrate, increased expression of arginase can affect NOS activity and NO generation by depleting the substrate pool of L-arginine that would otherwise be available to NOS enzymes. Acute release of arginase has been associated with pulmonary vasoconstriction in the setting of liver transplantation and reperfusion of the transplanted organ, which can cause an acute release of hepatic arginase protein into the pulmonary circulation.32 In sickle cell disease and other haemolytic disorders, chronically increased circulating arginase released from red blood cells has been implicated as a major cause of decreased NO bioavailability and the development of PAH.33,34 Morris33 demonstrated increased plasma arginase activity as well as decreased arginine: ornithine ratios in patients with sickle cell disease. Furthermore, lower arginine: ornithine ratios were associated with greater PAH severity and mortality in this population. Plasma L-arginine levels have been shown to be strongly correlated with right atrial pressure, cardiac index, and 6 min walk distance in patients with idiopathic PAH.35 A number of animal studies have shown associations of increased pulmonary and pulmonary vascular arginase activity in models of PAH, suggesting that this enzyme contributes to the development or progression of PAH.36,37 Although several of these studies illustrate that increased arginase is associated with decreased NO production, arginase may contribute to the vascular pathophysiology of PAH through NO-independent pathways. Arginase and the production of ornithine and subsequent polyamine synthesis may promote vasculo-proliferation and remodelling.38–41 The contribution of arginase to PAH requires further elucidation.

Additional mechanisms may be responsible for limiting NO bioavailability in PAH. This would include endogenous NO scavengers, such as cell-free haemoglobin, as well as endogenous inhibitors of NOS, such as asymmetric dimethylarginine.42,43

3. Nitric oxide and nitric oxide-related therapeutics in pulmonary arterial hypertension

Given the potency of NO as a vasodilator, therapies that target this pathway are of great interests in the treatment of PAH, with some having achieved mainstream success (Figure 1). Basic NO

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Figure 2 Nitrite (NO2−) can be reduced to nitric oxide along a pH and oxygen gradient. Additionally, multiple enzymes possessing nitrite reductase activity, including xanthine oxidoreductase, aldehyde oxidase, myoglobin, haemoglobin, and cytochrome c oxidase can act as nitrite reductases to produce nitric oxide. Nitric oxide can then have a myriad of biological effects, including modulation of smooth muscle cell proliferation, hypoxic vasodilation, and cytoprotective effects.
pathway-based therapeutic strategies can be characterized as those that enhance the enzymatic metabolism of L-arginine by NOS enzymes, direct administration of NO, and those that act downstream from NO on increasing levels of cGMP, an important effector molecule of NO signalling that induces vasodilatation and can inhibit smooth muscle cell proliferation.

Inhaled NO is a potent and selective pulmonary vasodilator. Although modern pulmonary vasodilator therapies have displaced inhaled NO as a primary therapy for PAH, there are some instances in which it has been demonstrated to be effective, such as treatment of persistent pulmonary hypertension of the newborn, and in acute peripartum settings, such as cardiopulmonary bypass complicated by PAH, and post-cardiac transplant.

Given its role as a substrate in NO production, arginine supplementation is one target for potential PAH therapy. Supplementation of L-arginine and citrulline has reduced pulmonary pressure in animal models of pulmonary hypertension. L-arginine has been shown to reduce pulmonary vascular resistance in humans with PAH, although in a trial comparing sildenafil with oral L-arginine in patients with sickle cell disease, only sildenafil improved pulmonary pressures and 6 min walk distances. Of note, however, a subsequent study of sildenafil in patients with sickle cell disease and PAH was halted early due to increased frequency of vaso-occlusive pain crises. Another potential strategy would be to limit arginine activity; however, these studies are only in early experimental stages. In addition to enhancing substrate availability through supplementation, another potential therapeutic target is through increasing NOS activity via gene transfer. Early experiments in the monocrotaline rat model and in the flow-induced rabbit model have indicated a potential role for gene transfer of NOS in the treatment of PAH.

Agents that increase the concentration of cGMP, the end-product of the NO pathway, have also been targeted as potential therapeutic agents, with notable successes. Phosphodiesterase-5 inhibitors prevent the degradation of cGMP, ultimately decreasing intracellular calcium concentrations within smooth muscle cells, leading to increased vasodilation. These medications, most notably sildenafil citrate and tadalafil, have been shown in randomized clinical trials to be effective both as monotherapy and in combination therapy in the treatment of PAH. Two early randomized, double-blind trials of sildenafil as monotherapy showed improvement in exercise capacity, haemodynamics, time to clinical worsening, and quality of life. The Pulmonary Arterial Hypertension and Combination Study of Epoprostenol and Sildenafil (PACES) study, treatment with sildenafil as a second agent for 12 weeks improved exercise capacity (most notably in patients with a baseline 6 min walk distance over 325 m), haemodynamics, time to clinical worsening, and quality of life. Tadalafil has been studied in the Pulmonary Arterial Hypertension and Response to Tadalafil (PHIRST) trial and was shown to improve exercise capacity and quality of life and decrease clinical worsening when used as a single agent as well as when added to bosentan. More recent studies have begun to examine guanylate cyclase agonists. Riociguat has been shown to improve haemodynamics and exercise capacity in patients with PAH more than inhaled NO. Phase III trials using riociguat in patients with PAH are ongoing.

4. The nitrate–nitrite–nitric oxide signalling pathway

Both nitrate and nitrite are generated from endogenous and dietary sources. The primary endogenous source of nitrate and nitrite is the L-arginine–NO pathway, where NO is rapidly oxidized in blood and tissue into these anions. NO reacts with oxyhaemoglobin in red cells to form nitrate and reacts with plasma ceruloplasmin and oxygen to form nitrite. Dietary sources also contribute significantly to circulating nitrate and nitrite. The main dietary source of nitrate is leafy green and root vegetables, accounting for up to 90% of dietary nitrate intake. Nitrite is also found in the diet as a food additive and preservative in meat, primarily to prevent the growth of Clostridium botulinum, as well as to enhance the colour and taste. Approximately half of our plasma nitrite comes from dietary nitrate and half from oxidation of endogenously produced NO.

The conversion of dietary nitrate to nitrite and NO is a quite elegant pathway that involves a symbiotic cycle with the mammalian microbiome. Nitrate is both produced endogenously and absorbed in the gastrointestinal tract into the bloodstream. Nitrate is concentrated in saliva by the salivary glands to high levels (low millimolar concentrations), and in the oral cavity, commensal bacteria that express nitrate reductase enzymes convert nitrate to nitrite. This is known as the enteral-salivary circulation of nitrate. It is not well appreciated why nitrate is excreted into the saliva at such high levels, but up to 25% of plasma nitrate is taken up by the salivary glands. As a result of this reaction, salivary nitrite levels commonly exceed 1 mM after a typical serving of nitrate-rich vegetables.

Nitrite is then swallowed and can undergo complex biological reactions that are highlighted below.

In the stomach, some of the swallowed nitrite is converted non-enzymatically to NO via protonation and reduction. This reaction is dependent on low gastric pH and can be limited by proton pump inhibitors. The NO produced in the stomach via this reaction has anti-microbial properties and also enhances mucosal blood flow and mucus generation. Nitrite is also absorbed in the proximal gastrointestinal tract and can be further reduced systemically to NO via multiple pathways, including non-enzymatic and enzymatic reduction. Non-enzymatic reduction of nitrite to NO was originally recognized by Zweier et al. in rat ischaemic heart tissue at low pH, noting profound NOS-independent generation of NO. Since then, multiple enzymes that possess nitrite reductase activity have been identified, including enzymes that are present in the pulmonary circulation. Most of these enzymatic reactions are accelerated under hypoxic conditions. This is particularly interesting because it is under these conditions that the classic L-arginine–NOS–NO pathway, which is dependent on oxygen, may be limited. Thus, the nitrate–nitrite–NO pathway may be viewed as complimentary to the NOS–NO pathway, with preferential activity as oxygen tensions decrease. This pathway may also serve a compensatory or adaptive response, especially when NO activity is reduced during oxidative stress or under hypoxic or ischaemic conditions. Our appreciation of the physiological role of nitrite and nitrite reductases is only in its infancy, but the therapeutic potential of this pathway is clearly recognized and has been demonstrated in mul-
tiple pre-clinical models. For example, nitrite has been shown by multiple groups to limit ischaemia/reperfusion-induced apoptosis and cytotoxicity in heart, liver, and brain.\textsuperscript{71–76}

The nitrate–nitrite–NO pathway has been illustrated to have significant vasodilatory effects both acute and chronic. The vasodilating effect of high doses of nitrite has long been appreciated for decades, but a physiological effect at low concentrations was only recently considered. Sodium nitrite at 100\,\mu M concentration was shown by the Nobel laureate Robert Furchgott to relax aortic rings \textit{ex vivo} and by 22\% at 2.5\,mM in the forearm circulation and by 22\% at 2.5\,\mu M, and produced vasodilation during exercise stress even at levels of 900\,nM.\textsuperscript{80} The potent \textit{in vivo} vasodilating effect of nitrite has now been confirmed by a number of investigators.\textsuperscript{76,81–84}

From a physiological standpoint, it is increasingly clear that basal levels of nitrite contribute to blood flow regulation and the cellular resilience to ischaemic stress. In studies of nitrite infusion into the human forearm circulation, concentrations of nitrite of only 200\,nM significantly increased forearm blood flow.\textsuperscript{85}

This vasorelaxation is thought to be attributable to the generation of NO and cGMP signalling. Additionally, observations of a concentration gradient of nitrite in the blood between the arterial and venous systems suggest consumption and utilization in the vascular tree.\textsuperscript{78,80} Multiple enzymes with nitrite reductase capability have been shown to be relevant in different models, including deoxy-haemoglobin, myoglobin, xanthine oxidoreductase, aldehyde oxidase, and cytochrome c oxidase.\textsuperscript{10,67,71,80,86–88}

The dietary contribution of nitrate and nitrite to vasoregulation has also been recently described. Larsen et al.\textsuperscript{89} demonstrated that dietary nitrates significantly decrease blood pressure in humans. Another study similarly demonstrated that nitrate naturally provided by a Japanese traditional diet, which increased plasma and salivary levels of nitrate and nitrite, significantly decreased diastolic blood pressure in normotensive subjects.\textsuperscript{89} More recently, Kapil et al.\textsuperscript{91} illustrated that inorganic nitrate supplementation, via nitrite formation, lowers blood pressures in humans. These data demonstrate dose-dependent decreases in blood pressure after inorganic nitrate ingestion in the form of either supplementation or dietary elevation. The cardiovascular benefits of diets rich in vegetables such as the classic Mediterranean diet are likely secondary to a myriad of nutritional components, but the contribution of the nitrate-to-nitrite-to-NO pathway to the protective properties of these diets is being actively considered.\textsuperscript{88,92} Furthermore, nitrate may contribute to other more chronic aspects of regulating blood flow, including angiogenesis.\textsuperscript{93}

5. Nitrite as a pulmonary vasodilator and putative lung nitrite reductase enzymes

Nitrite also has biological and therapeutic effects in the setting of the pulmonary vasculature and in PAH. The vasoregulatory contribution of circulating nitrite and nitrite reductase enzymes in the pulmonary circulation under healthy conditions has yet to be clearly defined. Hunter et al.\textsuperscript{83} have demonstrated that exogenously delivered sodium nitrite via nebulization can act as an effective pulmonary vasodilator in the setting of hypoxia or thromboxane-induced vasoconstriction in an ovine model. Inhalation of nebulized nitrite (300\,mg in 5\,mL buffered saline for 20\,min) resulted in a decrease in pulmonary arterial pressure by \textasciitilde 65\% of the increase induced by hypoxia and reduced pulmonary artery resistance by \textasciitilde 70\%, but had no measurable effect on systemic mean arterial blood pressures when compared with control animals. Furthermore, the decrease in pulmonary arterial pressure with nitrite nebulization was associated with a progressive increase in exhaled NO, demonstrating in \textit{vivo} conversion of nitrite to NO in the lung. This study went on to compare the pulmonary vasodilatory effects of inhaled NO gas with inhaled nebulized nitrite. Although both NO gas inhalation and nitrite nebulization resulted in a pronounced reduction in hypoxic pulmonary hypertension, the response to inhaled NO gas was slightly more rapid and more potent. Owing to the relative chemical stability of the nitrite anion compared with NO gas (half-life of nitrite of 30\,min compared with microseconds for NO), the vasodilation was sustained for more than 60\,min after the discontinuation of nitrite inhalation, whereas the termination of NO gas delivery abolished the vasodilating effect in seconds (Figure 3). This is relevant for the potential clinical applications of inhaled sodium nitrite, suggesting that intermittent dosing is possible. Casey et al.\textsuperscript{94} similarly demonstrated in rats that intravenous sodium nitrite reversed pulmonary hypertensive responses to a thromboxane mimetic and hypoxia.

Enzymes that have been shown to have nitrite reductase activity in the human and rodent lung include deoxy-haemoglobin, xanthine oxidoreductase, and aldehyde oxidase. The aforementioned study by Hunter et al.\textsuperscript{83} demonstrated that the pulmonary vasodilation elicited by aerosolized nitrite was augmented at low pH and by hypoxia and associated with systemic formation of NO bound to the haeme of haemoglobin (iron-nitrosyl-haemoglobin). This suggested either a role for deoxy-haemoglobin as a nitrite reductase that generated the NO or that nitrite was reduced by a lung nitrite reductase enzyme with secondary formation of iron-nitrosyl-haemoglobin. The study by Casey et al.\textsuperscript{94} illustrated in a rodent model that the vasodilatory response of intravenous nitrite in both the systemic and the pulmonary circulation was reversible by allopurinol, suggesting that xanthine oxidoreductase, an enzyme with known nitrite reductase activity, was responsible for the conversion of nitrite to biologically active NO in rats. It must be noted that differences between studies may be accounted for by species differences (rodents have a much higher xanthine oxidase activity than humans) as well as modes of delivery of sodium nitrite (aerosolized vs. intravenous).

Hypoxia and other conditions in the local microenvironment can modulate nitrite reductase activity in the lung. Hypoxia has been shown to increase xanthine oxidoreductase activity in rat microvascular endothelial cells \textit{in vitro} or in lung tissue \textit{in vivo}.\textsuperscript{75,94} Furthermore, arginine supplementation has been shown to decrease and pharmacological NOS inhibition has been shown to increase xanthine oxidoreductase activity in these settings.\textsuperscript{95–97} These studies would allow one to hypothesize a possible feedback loop between NO levels and the generation of NO by xanthine oxidoreductase from nitrite in the lung; however, this requires further investigation. Moreover, these \textit{in vivo} studies are performed in the context of chronic hypoxia for 5-day duration and do not localize the site of activity of xanthine oxidoreductase. Li et al.\textsuperscript{98} have demonstrated that during ischaemia pulmonary aldehyde oxidase and nitrite levels were suffi-
cient to result in NO generation that was comparable to or exceeding production by constitutive NOS enzymes. Similar results have been demonstrated investigating cardiac xanthine oxidoreductase. Additionally, under conditions of hypoxia, deoxy-haemoglobin can act as a nitrite reductase to produce biologically active NO resulting thought to contribute to vasodilation. Many other enzymes are currently being studied that may be relevant in the lung vasculature, including nitrite reduction by eNOS, mitochondrial cytochrome c and cytochrome c reductase, and carbonic anhydrase.

The appreciation of the roles of the nitrate/nitrite and nitrite reductases in the setting of vasculopathies and PAH is only in its infancy. This newly appreciated pathway for the production of NO may serve as an additional adaptive response in the setting of dysregulated arginine/NOS signalling. As stated earlier, many of the enzymes that act as nitrite reductases are present in healthy lung, including xanthine oxidoreductase and aldehyde oxidase. Additionally, studies have demonstrated increased xanthine oxidoreductase activity in both chronic hypoxia and monocrotaline models of PAH, and hypoxia has been shown to increase xanthine oxidoreductase activity in rat microvascular endothelial cells.

6. Nitrite as a therapeutic for pulmonary arterial hypertension

The development of effective therapeutics for PAH is guided by the common pathophysiological features of vasoconstriction, thrombosis, and vascular proliferation. Nitrite can clearly act as a pulmonary vasodilator as summarized earlier. Together, these studies suggest that nitrite-based therapy could function effectively as a vasodilator to help with symptom relief in patients with PAH. Another aspect of therapy would be targeted at remodelling the vascular lesions associated with PAH to reverse this proliferative process. To date, the development of effective therapeutic strategies has met limited success in reversing or significantly halting the progressive nature of pulmonary vascular hypertrophy. Data from animal models have shown promising early results. We have demonstrated in both chronic hypoxia and monocrotaline models that inhaled nebulized sodium nitrite can prevent or reverse pulmonary hypertension. Exposure of mice to chronic hypoxia (10% oxygen) results in pulmonary vascular remodelling characterized by increased muscularization, leading to increased RVPs and right ventricular hypertrophy. Treatment with nebulized nitrite, either once or three times per
week, significantly prevented the development of PAH as determined by all outcome measurements. Additionally, nitrite treatment 2 weeks into the hypoxic exposure, after the establishment of pulmonary hypertension, halted the progression of pulmonary hypertension and reversed increases in RVPs.

Additionally, the protective effects of nitrite in monocrotaline sodium (MCT)-induced pulmonary hypertension were examined. Nebulized nitrite delivered three times per week during the second half of a 6-week experiment following monocrotaline treatment reduced pulmonary hypertension (Figure 4). Moreover, histological assessment of pulmonary vascular hyperplasia was also determined. Muscularization and hyperplasia of the small pulmonary arteries were assayed by measuring the percentage of the total area of the vessel that comprised media. Monocrotaline treatment increased the percentage to $54 \pm 12\%$ compared with $19 \pm 4\%$ in controls, whereas nebulized nitrite reversed the effects of monocrotaline, decreasing this to $27 \pm 13\%$ ($P < 0.01$). Thus nitrite treatment resulted in a negative remodelling of the vascular wall.

In the setting of experimental PAH in the rat and mouse models of pulmonary hypertension, we found that sodium nitrite undergoes metabolism to biologically active NO via reduction in large part by xanthine oxidoreductase. This was demonstrated in lung tissue homogenates that were made anoxic, and NO generation was measured following the addition of nitrite (1 mM) with or without allopurinol. Upon the addition of nitrite, lung tissue generated a significant amount of NO (Figure 5). Nebulized sodium nitrite reverses monocrotaline-induced pulmonary arterial hypertension. Monocrotaline treatment resulted in pulmonary arterial hypertension as determined by RV:LV + S mass ratio (A), RV:BW mass ratio (B), and right ventricular pressure measurements (C) ($^*P < 0.001$ compared with controls). Inhaled nebulized nitrite (1.5 mg/min for 20 min; three times a week during weeks 4–6) reversed monocrotaline-induced pulmonary arterial hypertension (A–C, $^#P < 0.001$ compared with monocrotaline, vehicle-treated). (D) Cardiac cross-sections demonstrate right ventricular hypertrophy 6 weeks after monocrotaline treatment in nebulized vehicle controls (a) and nebulized nitrite-treated rats (b). An untreated control heart is shown for comparison (c).106 Reproduced with permission from Zuckerbraun et al.106

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/89/3/542/326812/548)

**Figure 4** Nebulized sodium nitrite reverses monocrotaline-induced pulmonary arterial hypertension. Monocrotaline treatment resulted in pulmonary arterial hypertension as determined by RV:LV + S mass ratio (A), RV:BW mass ratio (B), and right ventricular pressure measurements (C) ($^*P < 0.001$ compared with controls). Inhaled nebulized nitrite (1.5 mg/min for 20 min; three times a week during weeks 4–6) reversed monocrotaline-induced pulmonary arterial hypertension (A–C, $^#P < 0.001$ compared with monocrotaline, vehicle-treated). (D) Cardiac cross-sections demonstrate right ventricular hypertrophy 6 weeks after monocrotaline treatment in nebulized vehicle controls (a) and nebulized nitrite-treated rats (b). An untreated control heart is shown for comparison (c).106 Reproduced with permission from Zuckerbraun et al.106

![Figure 5](https://academic.oup.com/cardiovascres/article-abstract/89/3/542/326812/548)

**Figure 5** Sodium nitrite signalling in pulmonary arterial hypertension is dependent on xanthine oxidoreductase (A,B). Nitric oxide generation from nitrite in hypoxic lung tissue. Lung tissue homogenate was incubated with nitrite (1 mM) in the presence and absence of allopurinol (200 μM) or superoxide dismutase (300 U/mL) under normoxic or hypoxic conditions. (A) Representative nitric oxide generation traces over time. (B) Quantitation of at least three independent experiments similar to (A) ($^*P < 0.001$ compared with nitrite). (C) Hypoxia-induced pulmonary arterial hypertension ($^*P < 0.001$ compared with normoxic controls) was inhibited by nebulized nitrite as demonstrated previously ($^#P < 0.001$ compared with hypoxic controls). A tungsten-enriched diet diminished the protective effects of nebulized nitrite ($^§P < 0.001$ compared with nitrite-treated hypoxic mice). Adapted from Zuckerbraun et al.106 and reproduced with permission.
concentration of NO, which was attenuated by allopurinol (200 μM), an inhibitor of xanthine oxidoreductase (Figure 5A and B). Allopurinol treatment also prevented sodium nitrite effects on inhibition of pulmonary artery smooth muscle cell proliferation. In mice in the chronic hypoxia model, sodium nitrite no longer reversed intimal hyperplasia when xanthine oxidoreductase was inhibited by a tungsten-enriched diet (Figure 5C).

Multiple studies clearly demonstrate in the setting of ischaemia/reperfusion injury that nitrite protects against endothelial cell injury and apoptosis, suggesting additional activities that might protect the ischaemic and stressed right ventricle in the setting of advanced PAH. Additionally, sodium nitrite can protect the vasculature against injury induced by a high cholesterol diet. These data together suggest that nitrite would be expected to inhibit endothelial cell activation, platelet adherence, and thrombosis; however, studies have not clearly demonstrated all of these properties of sodium nitrite as of yet.

7. Clinical development of nitrite-based therapeutics for pulmonary arterial hypertension

These aforementioned pre-clinical data suggest that nitrite-based therapy is ready for clinical translation in the care of patients with PAH. In a Phase Ia dose escalation study, inhaled nebulized sodium nitrite increased plasma nitrite concentrations in a dose-dependent manner as expected (Bradley et al., American Thoracic Society, 2009, unpublished data). Furthermore, doses of nitrite were tolerated haemodynamically up to a dose of 125 mg. Inhaled nebulized sodium nitrite, at doses >17 mg, increased exhaled NO, whereas methaemoglobin levels remained <3.5% in all subjects.

8. Summary

In summary, NO is essential for pulmonary vascular homeostasis and may be upregulated as an adaptive response to vascular stress and hypoxia. Progression of PAH clearly occurs in the setting of dysregulated arginine/NOS signalling. Nitrite and nitrite reductase signalling can generate biologically active NO independent of NOS signalling. Nitrite may represent an important adaptive pathway that can be harnessed for therapeutic benefit in the setting of a failed NOS–NO pathway or during cellular hypoxia. Inhaled nebulized nitrite is a potent pulmonary vasodilator and can effectively prevent or reverse PAH in experimental animal models (Figure 6). Early Phase I studies in humans suggest that inhaled nitrite can be delivered safely, but longer trials in normal volunteers and patients with PAH are warranted. Future basic and translational investigations into the role of endogenous nitrite/nitrite reductases in the pulmonary vasculature, effects on proliferative and angiogenic signalling pathways, and therapeutic responses will advance the field.

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