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Nitrate- nitrite- nitric oxide pathway in pulmonary arterial hypertension therapeutics

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Pulmonary arterial hypertension (PAH) is a disorder characterized by elevated vascular resistance in pulmonary arterioles. Progressive increases in pulmonary vascular resistance and pulmonary artery pressures result in right heart failure and reduced cardiac output. Patients experience progressive exertional dyspnea, right heart failure, syncope, and ultimately death. The common pathophysiological features of PAH include pulmonary vasoconstriction, intimal and smooth muscle proliferation, *in situ* thrombosis, and pathological remodeling of pulmonary arterial circulation. While the origin of PAH is multifactorial, impairments in vasodilator (nitric oxide and prostaglandin signaling) and vasoconstrictor (endothelin-1, reactive oxygen species, angiotensin II) pathways underlie the evolution of early disease.[1] Based on this knowledge, drugs that enhance the NO signaling pathways (phosphodiesterase 5 inhibitors), the prostenoids, and endothelin receptor blockers, have been developed and approved for PAH specific therapy.

Inhaled nitric oxide (NO) gas can alleviate vasoconstriction and may modulate cellular proliferative responses, but NO therapy is limited by the need for continuous inhalation, NO reactions with oxygen to form nitrogen dioxide, and special delivery devices. It is now appreciated that inorganic nitrite and nitrate are bio-transformed to NO via the nitrate-to-nitrite-to-NO pathway,[2] leading to studies with inhaled nitrite as an alternative to NO gas inhalation.[3, 4] In this issue of Circulation, Baliga and colleagues investigate the nitrate-to-nitrite-to-NO pathway by studying the effects of oral nitrite and nitrate on pre-clinical mouse and rat models of PAH, and then attempt to characterize the enzymes that regulate bioconversion of nitrite to NO. They find that both nitrate and nitrite delivered in drinking water can prevent and reverse experimental PAH in the hypoxic and bleomycin mouse models; consistent with published models for in vivo conversion of nitrite to NO.[5-7] They also provide unexpected evidence that eNOS may have nitrite reductase activity in the diseased lung, with experiments demonstrating that eNOS null mice with hypoxic PH do not significantly respond to oral nitrate and nitrite treatment.

The therapeutic effects of nitrate and nitrite have been investigated in several disease models (recently reviewed[2]). Nitrite can induce vasodilation, reduce blood pressure, act as a cytoprotectant in ischemia-reperfusion induced injury, and modulate mitochondrial respiration, energetics and exercise efficiency.[2, 8, 9] Nitrate also mediates these effects via

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conversion to nitrite in the oral cavity by commensal bacteria containing efficient nitrate reductase enzyme systems.[10-12]

In PAH sheep, mouse, and rat models nitrite acts as a pulmonary vasodilator and produces therapeutic vascular remodeling. Inhalation of nebulized nitrite decreases pulmonary arterial pressure induced by hypoxia and thromboxane[4] and can prevent or reverse established right ventricle (RV) pressure increases, RV hypertrophy, and pulmonary vascular remodeling in hypoxia- or monocrotaline- induced PAH.[3] Similarly, injection of sodium nitrite attenuates PAH induced by monocrotaline,[13] hypoxia, and/or thromboxane.[2, 14] Baliga and colleagues now report the effect of oral nitrite and nitrate on hypoxia-induced PAH. Their data demonstrate that oral nitrate or nitrite treatment decrease RV pressure, RV mass, and pulmonary vascular remodeling induced by hypoxia and bleomycin. The protective effects of oral nitrate and nitrite administration correlate to elevated plasma nitrite and cGMP concentrations, providing a potential orally-active therapy for PAH via the nitrate-nitrite-NO pathway.

Mammals can utilize two pathways to produce NO and regulate blood pressure and blood flow: the well-established arginine-to-NO pathway and the more recently characterized nitrate-to-nitrite-to-NO pathway.[2] In the arginine-to-NO pathway, the nitric oxide synthase (NOS) enzymes catalyze NO production. The nitrate-to-nitrite-to-NO pathway is less completely understood, as the molecular mechanisms involved in each interconversion and in each organ system are yet to be fully elucidated. However, the upstream pathways for conversion of nitrate to nitrite have been clearly defined since early studies first described this pathways involvement in stomach NO level regulation, shown to be important in regulating stomach mucosal integrity, mucosal host defense, and later mucosal blood flow.[10, 12, 15] The two NO biosynthesis pathways respond to oxygen concentration differently: the arginine-to-NO pathway requires oxygen as a substrate for NO formation; conversely, the nitrate-to-nitrite-to-NO pathway is oxygen independent and is actually potentiated under hypoxic conditions (Figure 1). Because the nitrate-to-nitrite-to-NO pathway exhibits increased potency under lower oxygen tensions, a role for nitrite as an effector of hypoxic signaling and vasodilation has been considered.[2, 5, 7]

Eating nitrate rich foods, such as spinach or beets, increases nitrate levels in saliva, as high as 8 mM. Approximately one quarter of this nitrate is transformed by bacterial nitrate reductases to nitrite, which is subsequently swallowed and then transformed into NO, which is important for host defense, and mucosal integrity and blood flow.[10, 12, 15] The enterosalivary conversion of nitrate to nitrite requires oral bacteria and is inhibited in germ free mice and with antibiotic or antiseptic therapy.[16] Liver xanthine oxidoreductase (XO) has been reported to convert nitrate to nitrite under certain conditions; however, compared to the bacterial transformation, the XO catalyzed reaction is inefficient, and a clear pathway for nitrate reduction in mammalian organs remains uncertain. In the last decade it has become clear that nitrite not only modulates stomach mucosal function, but is an important intravascular source of NO. Arterial-to-venous nitrite gradients within the human circulation lead to early hypotheses that nitrite could be a source of NO in the human circulation.[5] Later studies confirmed that nitrite exhibited potent vasodilatory effects *in vivo*.[7] Since these initial studies, over 10 years ago, the role of nitrite in regulation of blood flow and pressure at physiological levels has been clearly demonstration by many research groups.[17, 18] The signaling properties of nitrite are largely mediated by nitrite reduction to NO, which then activates soluble guanylate cyclase, although the enzyme systems responsible for this concerted redox reaction in humans remains the focus of very active investigation and continued controversy.

Several studies have investigated the possible enzymatic origins for mammalian nitrate and nitrite reduction. Most other genera of life, such as lower eukaryotes (e.g., plants and yeast) and prokaryotes (e.g., bacteria), possess efficient nitrate and nitrite reductase enzymes; however, mammals are believed to lack dedicated homologous enzymes. Still, a number of enzyme systems have been shown to control nitrite reduction to NO under certain conditions. As previously mentioned, xanthine oxidoreductase is a molybdenum-dependent enzymes that has been proposed as a mammalian nitrate and nitrite reductase.[19, 20] Interestingly, xanthine oxidase exhibits a remarkable resemblance to the well-characterized plant nitrate reductases. Furthermore, a number of studies, including the current work by Baliga and colleagues, have clearly shown that the therapeutic effects of nitrite on PH require a functional xanthine oxidase system.[21, 22] Interestingly, in human studies the inhibition of xanthine oxidase using oxypurinol does not inhibit nitrite-dependent blood flow, suggesting that xanthine oxidase does not mediate nitrite-dependent vasodilation in the human circulation.[17]

Several other mammalian nitrite reductase candidates have been proposed; these enzymes can be divided into groups based on active site metal content: molybdenum (xanthine oxidase and aldehyde oxidoreductase), iron (cytochrome c, eNOS, deoxyhemoglobin, deoxymyoglobin, neuroglobin), and copper (carbonic anhydrase).[23-26] It is also possible that additional and potentially more effective (catalytic) human nitrate and nitrite reductases have not been identified.

Nitrite can be converted to nitric oxide by enzyme catalyzed oxidation-reduction (Scheme 1) or anhydrase (Scheme 2) reactions. The majority of nitrite reductase candidates are predicted to act as oxidoreductases (Scheme 1), while carbonic anhydrase has also been proposed to act as nitrite anhydrase. Note that hemoglobin may facilitate both reaction schemes.[27] In Scheme 1, a simple electron and proton transfer reaction, the metal active site of the oxidoreductase enzyme can be oxidized while nitrite is reduced to nitric oxide. Two types of metal enzymes have been implicated in nitrite reduction to nitric oxide: molybdenum and iron. In Scheme 2, two nitrite molecules react to form dinitrogen trioxide (N_2O_3) and water, which then forms nitrite and NO spontaneously (non-enzymatic) by disproportionation.

While the current study suggests that both nitrate and nitrite can prevent and reverse experimental PAH, there are a few caveats and an unexpected result. Firstly, the concentrations of both nitrate (45 mM) and nitrite (15 mM) in water are very high; by our estimates approximately 10 mg of nitrate or 3.5 mg of nitrite was ingested by the mice each day. A 70 kg human would have to ingest 36 grams of nitrate or 9 grams of nitrite each day to achieve these similar levels. Human research studies have been typically administered 500 to 1000 mg of nitrate each day, comparable to drinking about one liter of beet-root juice.[18] Based on the Baliga study, one would have to drink 30 liters of beetroot juice each day to achieve similar results in humans with PAH. Moreover, these doses of nitrate and nitrite may generate significant levels of NO, nitrosothiols and nitrosamines in the stomach and will need to be carefully evaluated for safety and off-target effects.

The current study also demonstrated that allopurinol can prevent the beneficial effects of nitrite and nitrate on PAH, which is consistent with the work of other groups.[21] However, the findings that the effects of nitrite are inhibited in eNOS null mice are unexpected and will require further study to understand. It is important to note that inhibition of eNOS does not block nitrite-vasodilatory effects in humans.[7] Previous studies have shown that under anaerobic conditions endothelial NOS can reduce nitrite to NO; although, this is only effective under near-anaerobic conditions.[28,29] One explanation is that oxygen tensions in the diseased lung are sufficiently low to allow nitrite reduction by eNOS, or there is an unexplained interaction in this system. Alternatively, we have reported that nitrite can also

be oxidized by hydrogen peroxide or ferric hemoproteins to form oxidative signaling products, for example nitrogen dioxide. [30] It is possible that XO and eNOS in diseased vessels and tissues function not as nitrite reductases, but as nitrite oxidases via formation of superoxide and hydrogen peroxide. Further studies are required to address these important questions.

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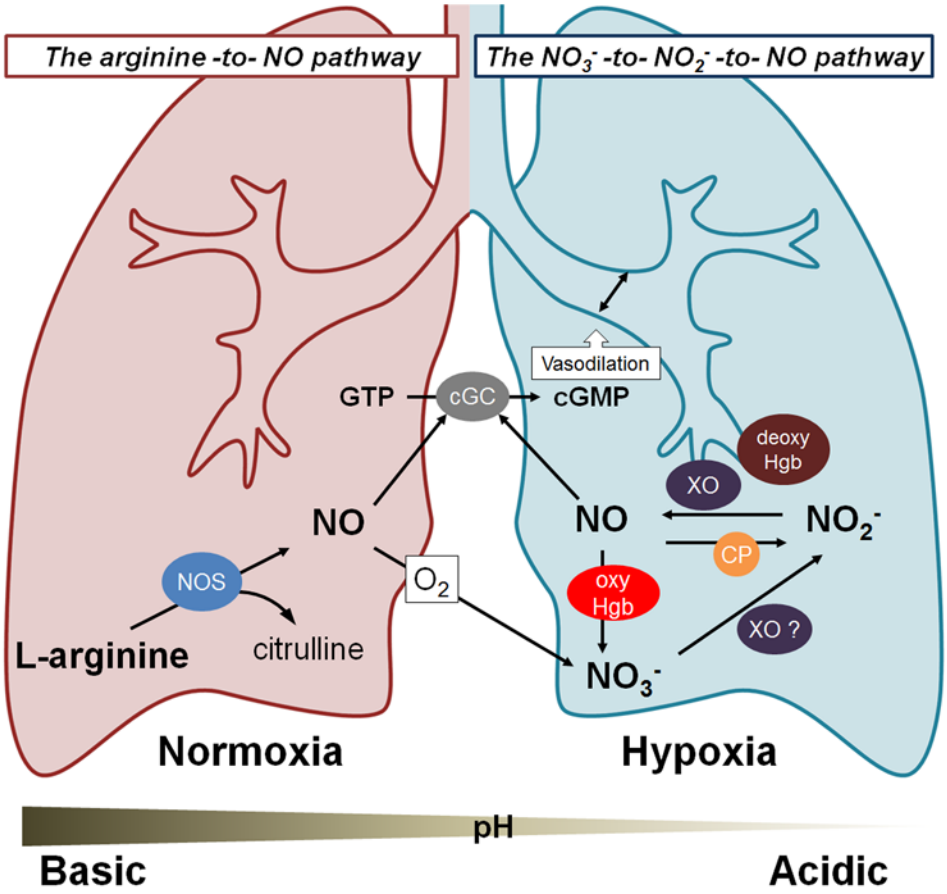

Figure 1.

Nitrite (NO_2^-), and nitrate (NO_3^-) can treat PAH via biotransformation into nitric oxide (NO), a potent vasodilator. The nitrate-nitrite-NO pathway dominates under hypoxia (blue lung), and the arginine-NO pathway under normoxia (red lung). Abbreviations: nitric oxide synthase (NOS); xanthine oxidoreductase (XO); deoxyhemoglobin (deoxyHgb); oxyhemoglobin (oxyHgb); ceruloplasmin (CP); guanosine triphosphate (GTP); cytosolic guanylate cyclase (cGC); cyclic guanosine monophosphate (cGMP).

Scheme 1.

$$2NO_2^- + 2H^+ \rightarrow H_2O + N_2O_3 \rightarrow NO + NO_2$$

Scheme 2.