Plasma nitrite/nitrate levels: a new biomarker for pulmonary arterial hypertension?

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The role of endothelial-derived nitric oxide (NO) and its downstream signalling pathways in modulating vascular tone and remodelling has been one of the most intriguing areas of vascular biology over the last quarter of a century. Its identification as the endothelial-derived relaxing factor responsible for relaxation of vascular smooth muscle led to the designation of NO as the "molecule of the year" by the journal *Science* in 1992 [1], and to a Nobel Prize in Physiology or Medicine for L.J. Ignarro, R.F. Furchgott and F. Murad in 1998. It is now well appreciated that the vascular endothelium, in response to a variety of stimuli, synthesises NO from L-arginine by activation of endothelial-derived NO synthase (eNOS), which then diffuses to adjacent vascular smooth muscle cells and activates soluble guanylate cyclase (sGC) leading to synthesis of cyclic guanosine monophosphate (cGMP). The increase in cytoplasmic levels of this secondary messenger activates a variety of mechanisms that decrease intercellular calcium and dephosphorylate the myosin light chain. The overall result is a potent mechanism by which the endothelium can induce vascular smooth muscle relaxation [2].

Initial interest in the role of NO in the pulmonary circulation focused on this ability to reverse pulmonary vasoconstriction. However, further study revealed that NO signalling also has antiproliferative and anti-inflammatory properties, and important modulatory effects on apoptosis, angiogenesis and endothelin synthesis that play vital roles in pulmonary vascular remodelling as well [3, 4]. Sentinel studies have shown that NO blunts acute hypoxic pulmonary vasoconstriction in isolated rat lungs and human volunteers [5, 6], whereas mice with targeted gene deletion of eNOS have elevated pulmonary artery pressure (PAP) and increased muscularisation of pulmonary arteries in response to chronic hypoxia [7, 8]. However, inhalation of NO or increasing endogenous NO signalling *via* a variety of methods has been shown to lower PAP, and reduce right ventricular hypertrophy and muscularisation of pulmonary arteries in animal models of pulmonary hypertension [9–11]. These studies led to the development of a variety of therapeutic agents that target the NO/cGMP pathway for the treatment of pulmonary arterial hypertension (PAH). These agents include phosphodiesterase type-5 (PDE5) inhibitors that delay the degradation of cGMP [12], sGC stimulators that both stimulate sGC and enhance NO-induced activation of sGC [13], and inhalation of NO itself [14].

Considering the salient effects of enhanced NO/cGMP signalling on attenuating pulmonary hypertensive responses, it is no wonder that many investigators have suspected that deficiency in NO signalling may contribute to the pathogenesis of PAH. A considerable body of evidence has accrued to support this hypothesis, including the following abnormalities found in patients with PAH: 1) decreased expression of eNOS in endothelial cells that line pre-capillary pulmonary arteriole resistance vessels [15], 2) lower levels of the substrates and co-factors needed for eNOS-mediated generation of NO such as L-arginine (the

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primary substrate for eNOS) and tetrahydrobiopterin [16, 17], 3) higher levels of arginase that degrades L-arginine [18], and 4) higher plasma levels of asymmetric dimethyl arginine that competitively inhibits the binding of L-arginine to NO synthase [19]. Together, these findings suggest that patients with PAH have impaired ability to generate NO in the pulmonary circulation. Indeed, the concentration of NO in exhaled air is lower in patients with idiopathic pulmonary arterial hypertension (IPAH) and PAH associated with connective tissue disease than in healthy controls [20, 21].

This strong association between NO signalling and PAH makes it intriguing to consider whether some assessment of NO signalling could be used to assess the severity of pulmonary vascular disease and predict outcome in patients with PAH. Unfortunately, none of the indices of NO synthesis described above is readily attainable or has been found to be amenable to development into a convenient, reproducible measure that could be used as a biomarker. Another approach then may be to examine changes in NO metabolism. Most synthesised NO is metabolised by the progressive oxidation of NO to nitrite (NO_2^-) and nitrate (NO_3^-). Circulating nitrite/nitrate (NO_x) levels not only reflect the level of NO synthesis but also act as a reservoir of alternative substrate for NO synthesis. NO metabolites can be chemically reduced to NO *in vivo* by haem proteins, such as xanthine oxidoreductase and aldehyde oxidase, that exhibit nitrite reductase activity [22]. Thus, measurement of circulating NO_x could serve as an index of vascular NO synthesis and/or reflect the ability of a patient to synthesise NO *via* this alternative pathway.

In this issue of *European Respiratory Journal* (*ERJ*), ZHANG *et al.* [23] examine the association between plasma NO_x levels and severity of PH and clinical outcome in patients with PAH. They measured plasma NO_x levels in 104 patients with PAH at the time of first right heart catheterisation and in 110 healthy controls, and followed them prospectively for a mean of 26±9 months. They found that circulating NO_x levels in patients with PAH were only about a third of the level found in controls. Furthermore, plasma NO_x levels were lower in 18 of the 104 PAH with a bone morphogenic protein receptor-2 (*BMPR2*) mutation than in the 85 patients without. Plasma NO_x levels correlated inversely with mean pulmonary artery pressure (mPAP) and pulmonary vascular resistance (PVR), and directly with cardiac output and cardiac index. More importantly, PAH patients with lower circulating NO_x levels had higher 1- and 3-year mortality rates. In fact, during the 3-year follow-up period, patients with a plasma NO_x concentration of $<10\ \mu mol \cdot L^{-1}$ had a mortality rate that was nearly four times higher than those with levels $>10\ \mu mol \cdot L^{-1}$ (37.5% *versus* 10.4%). Interestingly, plasma NO_x level had greater ability to predict death than mPAP, PVR, cardiac output and plasma brain natriuretic peptide levels by area under the curve in receiver operating characteristic analysis. The negative prognostic value of low plasma NO_x persisted after controlling for additional factors including age, sex, *BMPR2* mutation, World Health Organization functional class and 6-min walk distance.

These findings add to a growing body of data that suggests that impaired NO synthesis either contributes to the pathogenesis of PAH or is a marker of endothelial dysfunction that reflects PAH severity and disease progression. More importantly, this study identifies a potentially new biomarker that may contribute to the diagnosis and management of PAH. It is particularly interesting to note that plasma NO_x levels were lower in PAH patients with *BMPR2* mutations. These patients had higher mPAP and PVR than those without *BMPR2* mutation, and their lower plasma NO_x may reflect more severe disease. However, *BMPR2* mutations have also been associated with impaired NO synthesis [24] and it is possible that this reduction in NO synthesis contributed to more advanced disease.

Plasma NO_x was measured by a commercially available assay in venous blood collected in standard EDTA-containing tubes with 0.005% butylated hydroxytoluene to improve stability. Samples were frozen for up to a month at −80°C with little deterioration and acceptable inter-assay variation, suggesting that plasma NO_x levels are amenable to routine collection, storage and measurement.

Limitations of the study include its single-centre design and restriction of study subjects to those with IPAH. Patients with IPAH and a variety of comorbidities that could affect plasma NO_x levels were also excluded, including those with systemic hypertension, hyperlipidaemia or type 2 diabetes mellitus, and patients who used tobacco, excessive alcohol or nonsteroidal anti-inflammatory drugs. These comorbidities are not uncommon in patients with IPAH and it remains to be determined if plasma nitrates have similar predictive value for disease severity or outcome in these patients. Further studies will also be needed to examine the prognostic value of plasma NO_x levels in other types of PAH. For example, children with PAH associated with congenital left-to-right shunt have been found to have higher NO_x levels than controls [25]. This may be due to increased pulmonary vascular endothelial NO synthesis caused by increased pulmonary blood flow. Other limitations to the study include the lack of follow-up NO_x levels to determine if changes in plasma NO_x reflect disease progression and the omission of the investigators to examine whether baseline NO_x levels were able to predict clinical response to PAH-specific medications. The latter point is particularly important in that the rapid development of numerous pharmacological therapies for PAH and the absence of direct comparator trials make it difficult to determine which

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patients are best suited for each therapy. It would have been intriguing to see if baseline plasma NO_x is useful in determining which patients are more likely to respond to medications that target the NO/cGMP pathway, such as PDE5 inhibitors or sGC stimulators. It would also be of great interest to see if any of the currently available PAH medications are capable of increasing plasma NO_x levels and if the increase correlates with patient outcome.

Finally, the conversion of inorganic nitrates to NO is one of the mechanisms by which diets that are rich in nitrate-containing vegetables can enhance endothelial function in cardiovascular disease. Previous studies in mice have shown that hypoxic pulmonary hypertension can be attenuated by treating drinking water with NO_2^- or NO_3^- [26]. In these studies, dietary NO_3^- increased plasma and lung concentrations of NO_3^- and cGMP, and these increases mirrored the reduction in right ventricular hypertrophy and pulmonary vascular remodelling. Taken together, these studies and the report by Zhang *et al.* [23] in this issue of the *ERJ* suggest that plasma nitrates may offer a potential biomarker that can be used to assess disease severity, help determine prognosis and perhaps provide us with a valuable tool to assess which patients are most likely to benefit from enhancing NO signalling and monitoring their response to treatment.

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