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Themed Section: Nitric Oxide 20 Years from the 1998 Nobel Prize

REVIEW ARTICLE

Nitrite and nitrate chemical biology and signalling

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Inorganic nitrate (NO_3^-), nitrite (NO_2^-) and NO are nitrogenous species with a diverse and interconnected chemical biology. The formation of NO from nitrate and nitrite *via* a reductive 'nitrate–nitrite–NO' pathway and resulting in vasodilation is now an established complementary route to traditional NOS-derived vasodilation. Nitrate, found in our diet and abundant in mammalian tissues and circulation, is activated *via* reduction to nitrite predominantly by our commensal oral microbiome. The subsequent *in vivo* reduction of nitrite, a stable vascular reserve of NO, is facilitated by a number of haem-containing and molybdenum-cofactor proteins. NO generation from nitrite is enhanced during physiological and pathological hypoxia and in disease states involving ischaemia–reperfusion injury. As such, modulation of these NO vascular repositories *via* exogenously supplied nitrite and nitrate has been evaluated as a therapeutic approach in a number of diseases. Ultimately, the chemical biology of nitrate and nitrite is governed by local concentrations, reaction equilibrium constants, and the generation of transient intermediates, with kinetic rate constants modulated at differing physiological pH values and oxygen tensions.

LINKED ARTICLES

This article is part of a themed section on Nitric Oxide 20 Years from the 1998 Nobel Prize. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.2/issuetoc

Abbreviations

CBS, cystathionine β-synthase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Cygb, cytoglobin; eNOS, endothelial NOS; Hb, haemoglobin; HfPEF, heart failure with preserved ejection fraction; I/R, ischaemia–reperfusion; mARC-1 and mARC-2, mitochondrial amidoxime-reducing component–1 and 2; Mb, myoglobin; N_2O_3, dinitrogen trioxide; Ngb, neuroglobin; NiR, nitrite reductase; RSNO, S-nitrosothiol; RBCs, red blood cells; sGC, soluble GC; XO, xanthine oxidase

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Introduction

We are celebrating the 20th anniversary of the Nobel Prize awarded for the discovery of **NO** as a physiological signalling molecule with roles in neurobiology, immune defence and the regulation of cardiovascular homeostasis. Canonical *de novo* NO synthesis occurs *via* the oxidation of L-arginine by oxygen catalysed by **NOS**. In the vasculature, NO is generated by the endothelial NOS (eNOS) and then diffuses to smooth muscle where high picomolar to low nanomolar concentrations activate **soluble GC** (sGC), ultimately triggering a signalling cascade leading to vasodilation, described elsewhere in this special issue.

Here, we focus on the chemical biology of a secondary complementary pathway leading to NO generation, the oxygen-independent reduction of nitrate to nitrite to NO. This pathway to NO formation is reductive and NOS-independent, with enhanced activity during conditions of physiological and pathological hypoxia or ischaemia, such as occurs during the arterial-to-venous deoxygenation of blood or during pathological episodes of ischaemia and reperfusion (I/R). While nitrite is readily reduced to form NO by proton and electron transfer reactions in the mammalian vasculature by **haem** and molybdopterin-containing enzymes, efficient nitrate reduction requires more specialized nitrate reductase enzymes typically present in bacteria. Thus, nitrate reduction in mammals largely requires enteral symbiotic bacteria, generating nitrite, which is then further reduced to NO in the stomach or absorbed into the circulation where it is reduced to NO by haemoglobin or members of the molybdopterin family of enzymes. In the context of this review, nitrate (NO_3^-) and nitrite (NO_2^-) both refer to their inorganic forms: anions of various salts with alkali cations (e.g. sodium or potassium).

In vivo metabolism of nitrate and nitrite and regulation of NO-signalling in the vasculature

Inorganic nitrite was long regarded as an inert oxidation product of NO and a biomarker of NO formation, despite some indications to the contrary (Haldane *et al.*, 1897; Brooks, 1937). This thinking was based on the relatively low potency of nitrite as a vasodilator of preconstricted aortic rings; high concentrations of nitrite, in the micromolar to millimolar range, were required to observe significant vasodilation (Furchgott and Bhadrakom, 1953). Murad and Ignarro demonstrated that nitrite can activate GC and vasodilate aortic rings but also at relatively high concentrations or in the presence of thiols (Mittall *et al.*, 1978; Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981). Notably, all of these experiments were conducted at physiological pH values, with high oxygen tensions and in buffered systems without red blood cells (RBCs). More recently, Modin and colleagues showed that vasodilation could occur at approximately 10 μM nitrite concentrations during acidification (Modin *et al.*, 2001). Our group observed arterial-to-venous consumption of nitrite in the human circulation at rest, with exercise, and during NO gas inhalation, the latter associated with vasodilation of the human forearm suggesting that bioactivation of nitrite to a vasodilator (*vide infra*) might be possible *in vivo*. Two studies directly tested this hypothesis with one negative trial showing no vasodilation (Lauer *et al.*, 2001) and a second positive study showing significant forearm circulation vasodilation at both pharmacological and slightly supraphysiological concentrations (Cosby *et al.*, 2003). The latter was later confirmed by other investigators in animal and human studies (Hunter *et al.*, 2004; Webb *et al.*, 2004; Kozlov *et al.*, 2005; Dejam *et al.*, 2007; Ingram *et al.*, 2009; Patel *et al.*, 2011). Further studies established that NO-dependent vasodilation by nitrite can occur in the physiological range (100–200 nM) (Dejam *et al.*, 2005) and afforded insights into the mechanism of bioactivation including the key role of low oxygen (hypoxic) tensions, reactions with deoxyhaemoglobin and accentuation by lower pH (Cosby *et al.*, 2003; Crawford *et al.*, 2006; Dalsgaard *et al.*, 2007; Maher *et al.*, 2008; Pinder *et al.*, 2009). The oxygen and pH effects are elegantly illustrated in exercise, in which plasma nitrite consumption is higher compared with resting values and linked to increases in local blood flow (Bailey *et al.*, 2017).

The half-life of NO in whole blood is calculated to be less than 2 ms (Crawford *et al.*, 2006), whereas that of nitrite is approximately 42–52 min (Dejam *et al.*, 2007; Pluta *et al.*, 2011; Rix *et al.*, 2015). As reductive mechanisms discussed here lead to NO generation, nitrite is considered a 'stable' reserve of NO. Nitrite thus signals *via* a number of mechanisms (Figure 1), including the canonical NO–sGC pathway, by activating sGC *via* iron nitrosylation and stimulating vasodilation (Jeffers *et al.*, 2005). NO/nitrite are also involved in generation of nitrosating agents such as dinitrogen trioxide (N_2O_3), leading to nitrosation of thiols (*vide infra*) such as those in complex I of the mitochondrial electron transport chain. This action is cytoprotective, as nitrosation of these critical thiols occurs during I/R injury resulting in inhibition of complex I activity, preventing generation of damaging ROS as well as cytochrome *c* export following reoxygenation (Shiva *et al.*, 2007; Chouchani *et al.*, 2013). Additionally, nitrite oxidation (*vide infra*) to the radical nitrogen dioxide ($NO_2^\bullet$), an oxidant, results in nitration of several species, including unsaturated fatty acids, generating signalling nitro-fatty acids (Villacorta *et al.*, 2016). All of these topics (the canonical NO pathway, NO in the mitochondria and nitro-fatty acids) are described at length in separate reviews within this special issue.

Nitrate is ubiquitous in leafy green vegetables and beets, while nitrite is not typically found at significant levels in natural foods, although is added in the meat curing process as a preservative (Khatri *et al.*, 2017). Therefore, humans can ingest high levels of nitrate, especially when consuming vegetable-rich diets. The source of intravascular nitrite *in vivo* emanates from oxidation of eNOS-derived NO and from reduction of the otherwise stable dietary nitrate, as it has a plasma half-life of 5–6 h (Lundberg *et al.*, 2008). Although human nitrate reductase activity has been posited as a secondary function of **xanthine oxidase** (XO, *vide infra*), especially for organic nitrates ($R–ONO_2$) (Millar *et al.*, 1998; Li *et al.*, 2005; Khambata *et al.*, 2015), inorganic nitrate reduction is largely attributed to our oral microbiome (Lundberg *et al.*, 2008). When ingested, nitrate is reduced to nitrite by facultative anaerobes inhabiting the salivary glands *via* nitrate reductase enzymes akin to those found in soil bacteria. Further, nitrate is actively taken up from circulation by the salivary glands, resulting in nitrate concentrations 10-fold higher in the oral cavity than in the plasma (Ahluwalia *et al.*, 2016). Depletion of oral microbes attenuates

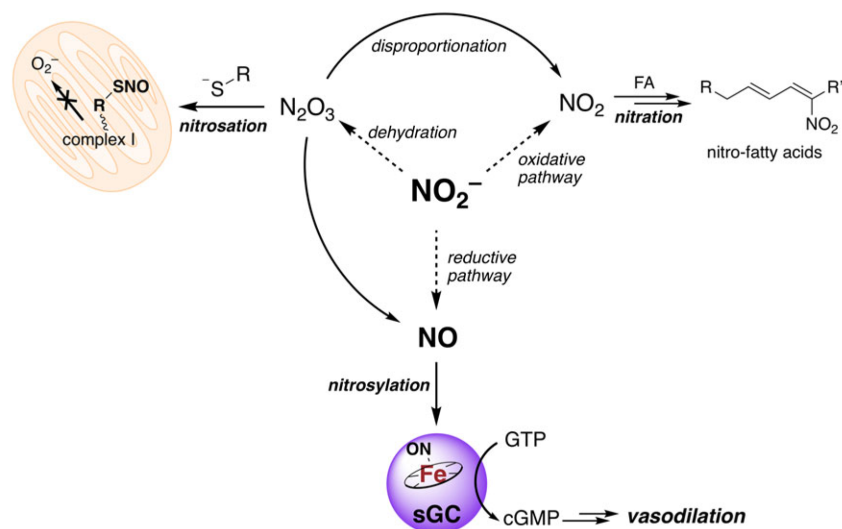


Figure 1

Myriad chemistries of nitrite (NO_2^-) leading to protective and signalling effects. Nitrite can be reduced to NO (mechanisms discussed below), which nitrosylates a ferrous haem in sGC, triggering the production of cGMP from GTP. cGMP triggers a signal cascade resulting in vasodilation. Alternatively, nitrate can be oxidized (conditions described below) to yield the oxidant $NO_2^{\bullet}$. $NO_2^{\bullet}$ reacts with a number of targets, but under conditions of excess $NO_2^{\bullet}$, can participate in nitration chemistry of fatty acids (FAs). The generated nitro-fatty acids are electrophilic and modify critical redox active thiols to mediate signalling, for example, by activating transcription factors like Nrf-Keap1. Nitrite can also be dehydrated catalytically or in the presence of acid (*vide infra*) to generate the strong nitrosating agent N_2O_3. N_2O_3 can react with nucleophiles such as thiols resulting in S-nitrosothiol (RSNO) formation. Here, this activity is depicted on a critical thiol of complex I of the mitochondrial electron transport chain, preventing formation of damaging superoxide (especially under I/R injury). N_2O_3 can also disproportionate into $NO_2^{\bullet}$ and NO. Dotted arrows represent reactions that are also catalysed.

nitrite formation from nitrate and abolishes the vasodilatory response of nitrate, implicating nitrite as the central active agent (Webb *et al.*, 2008b). When swallowed, some nitrite is subsequently reduced to NO in the stomach by a non-enzymic, low pH-dependent mechanism where it modulates gastric fluid production and confers defence against infections (Lundberg *et al.*, 2011). The rest of the nitrite is absorbed *via* the gastrointestinal (GI) tract and becomes part of the 'nitrate-nitrite-nitric oxide pathway' responsible for vasodilatory control under low oxygen conditions. It is important to note that other, yet-to-be elucidated mechanisms are likely to exist in the context of this pathway as the proton pump inhibitor esomeprazole, which lowers gastric acidity, blunts the effects of only orally ingested nitrite (Montenegro *et al.*, 2017). Such findings imply a gastric activation step in swallowed nitrite, though probably not directly to short-lived NO by a non-enzymic path. Additionally, a recent clinical trial reported that the vasodilatory effects of oral nitrite were also blunted during co-administration of conjugated linoleic acid (Hughan *et al.*, 2017). As mentioned above, nitrite potently vasodilates during direct intra-arterial infusion, so more work is required to understand the mechanisms for how gastric reactions can modulate its bioactivity.

A few investigators have reported that mammals reduce nitrate to nitrite through direct action of native XO (Li *et al.*, 2003; Jansson *et al.*, 2008; Piknova *et al.*, 2015, 2016). In 2003, Li *et al.* characterized the nitrate reductase activity of XO using electron paramagnetic resonance (EPR), chemiluminescence and an NO electrode. The reduction was found to be acid-catalysed. Jansson *et al.* (2008) observed nitrate reductase activity of rodent and human tissues that was dramatically blunted by the XO inhibitor, **allopurinol**. In addition, plasma nitrite was increased *in vivo* after nitrate infusions in rodents and, when allopurinol was also administered, these nitrite increases were blunted. Importantly, increases in plasma nitrite were observed in germ-free mice as well, supporting the existence of a eukaryotic nitrate reductase (Jansson *et al.*, 2008). Interestingly, the Schechter group has observed that nitrate is present in about threefold higher concentrations in skeletal muscle than in blood and is reduced to nitrite in the muscle (Piknova *et al.*, 2015, 2016). Thus, there exists evidence of a mammalian nitrate reductase. Such mammalian nitrate reductase activity may be important in certain tissues and conditions such as ischaemia.

While this body of research suggests that the reduction of nitrate may occur by mammalian enzymes, placebo-controlled crossover studies in normal volunteers, examining the effects of the mouthwash chlorhexidine to eliminate commensal oral bacteria, have reported that salivary, plasma and urinary nitrite levels were lowered and BP was increased (Kapil *et al.*, 2013), supporting a major role for bacterial nitrate reduction occurring in the oral cavity. Several mouse and rat studies have also shown that effects of nitrate can be blocked in germ-free mice or with oral antiseptic treatment (reviewed in Koch *et al.*, 2017). Thus, the weight of evidence suggests that the major pathway for nitrate reduction is through the oral microbiome, with lesser contribution from mammalian tissue xanthine oxidoreductase enzyme systems.

The physiological role for the nitrate–nitrite–NO pathway in the regulation of blood flow has been supported by the elegant studies by the Ahluwalia group (Kapil *et al.*,

2010, 2013). As noted, antiseptic mouthwash use results in increased BP, accentuating the importance of our oral microbiome for the reduction of nitrate. The corollary is also true: supplementing with exogenous nitrate increases plasma nitrite levels, decreases BP and improves exercise performance (Larsen *et al.*, 2006; Kapil *et al.*, 2014; Mills *et al.*, 2017). Thus, the chemical biology implicates a delicate interplay featuring many nitrogen oxidation states. This 'nitrate–nitrite–NO pathway' exists complementary to the traditional NOS-derived NO-signalling mechanism and postulates a large nitrate pool as a stable store of nitrite and nitrite, providing a stable vascular reserve of ephemeral NO.

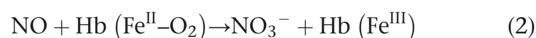
Chemistry of NO

No discussion about nitrite or nitrate is possible without briefly remarking on the chemistry of NO, as it is the recognized bioactive downstream product of both salts. Critically, NO is a stable free radical in aqueous/physiological conditions and does not dimerize due to the delocalization of the unpaired electron over both the nitrogen and oxygen atoms. Thus, NO can only form a partial ON–NO bond (Fukuto *et al.*, 2012). The exhibited stability of NO is paramount to the molecule's unique biology as interactions between diamagnetic (most biological substrates) and paramagnetic molecules (like NO) react very slowly. Simply put, NO diffuses freely until it encounters other paramagnetic molecules such as free radicals like superoxide (O_2^-) and transition metals (Heinrich *et al.*, 2013). The very high reaction rates and affinities for these molecules allow for specific signalling at very low NO concentrations. Finally, it is important to note that the reaction of aqueous NO with paramagnetic oxygen (i.e. autoxidation of NO) is a kinetically slow trimolecular reaction, which is second order in NO (Equation 1, $4k_{aq}$ = ~8×10^6 $M^{-2} \cdot s^{-1}$) (Ford *et al.*, 1993). An interested reader in the relevant chemistry of NO should consult reviews by Fukuto *et al.* (2012; Heinrich *et al.*, 2013) and Ford (2010) as well as other articles in this issue.

$$-\frac{d[NO]}{dt} = 4k_{aq}[NO]^2[O_2] \quad (1)$$

In vivo nitrate generation

The amounts of excreted nitrate greatly exceed that of what is typically ingested (Khatri *et al.*, 2017), and levels found in the plasma are between 20 and 40 μM under fasting conditions (Khambata *et al.*, 2015), implicating endogenous production. Nitrate is rapidly generated from the NO reaction with oxygenated-haemoproteins in a process called NO-dioxygenation ($k_2 = 9 \times 10^7$ $M^{-1} \cdot s^{-1}$, pH 7, 25°C) (Doyle and Hoekstra, 1981). Effectively, the oxy-ferrous (Fe^{2+}–O_2) haem serves as an electron donor and catalyses the oxidation of NO (Equation 2).

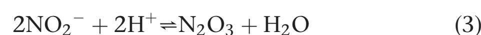


$$NO + Hb\left(Fe^{II}\text{–}O_2\right) \rightarrow NO_3^- + Hb\left(Fe^{III}\right) \quad (2)$$

While protein environment affects the rate of NO-dioxygenation, this reaction is nearly diffusion limited in most haemoproteins (Mishra and Meuwly, 2010). NO-dioxygenation is viewed as a crucial detoxifying reaction to scrub cardiovascular tissue of excess toxic NO. The very short (>2 ms) intravascular half-life of NO is due predominantly to these reactions, but the simultaneously generated nitrate functions as a stable nitrite source, which is then further harnessed for NO-generation as discussed below. Another biological route to nitrate formation will also be considered later.

Nitrite bioactivation

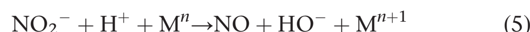
Inorganic nitrite is the conjugate base of nitrous acid, which has a much higher pK_a [3.11 at 37°C (da Silva *et al.*, 2006)] than nitric acid (conjugate acid of nitrate, $pK_a = -1.3$), consistent with nitrite protonation under certain physiological circumstances such as in the low pH of the stomach and, thus, increased lipid solubility. Nitrous acid generates NO *via* dehydration to N_2O_3, a strong nitrosating agent, followed by disproportionation (Equations 3 and 4) (Fukuto *et al.*, 2012). The standard reduction potential (E^0) at low pH is quite favourable and nitrite is a strong oxidant, where HNO_2, H^+/NO, E^0 = 0.98 V versus NHE (Bratsch, 1989), but this drops to 0.37 V at pH 7 (Ford, 2010). Thus, NO production from nitrite in this manner requires both relatively high concentrations of nitrite and acid. A few biological conditions support this possibility, including, critically, ischaemic events where reduced blood flow leads to tissue acidosis (Feelisch *et al.*, 2008).



$$2NO_2^- + 2H^+ \rightleftharpoons N_2O_3 + H_2O \quad (3)$$

$$N_2O_3 \rightleftharpoons NO_2^{\bullet} + NO \quad (4)$$

The reduction of nitrite to NO is catalysed at less acidic pH values by certain metalloproteins that provide an electron and facilitate proton donation as shown in Equation 5, where M is a redox-active metal (e.g. iron) in the *n* oxidation state (Doyle *et al.*, 1981b; Huang *et al.*, 2005b; Li *et al.*, 2008; Webb *et al.*, 2008a).



$$NO_2^- + H^+ + M^n \rightarrow NO + HO^- + M^{n+1} \quad (5)$$

The most prominent reductases contain porphyrin-chelated iron-cofactors known as haem. Haem readily cycles from the reduced ferrous (Fe^{2+}) valance to the oxidized ferric (Fe^{3+}) state and *vice versa*. Various haems are found in mammalian systems and are used in transporting and biosignalling processes, most notably as the oxygen-binding component of haemoglobin (Hb) and myoglobin (Mb). As noted previously, at low partial pressures of oxygen (i.e. hypoxia), ferrous globins exhibit nitrite reductase (NiR) activity, binding and reducing nitrite while generating the met-globin (ferric) and an equivalent of NO (with Hb, Equation 6) (Lundberg *et al.*, 2008).

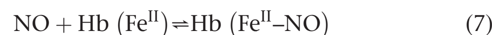
$$NO_2^- + H^+ + Hb\left(Fe^{II}\right) \rightarrow NO + Hb\left(Fe^{III}\right) + HO^- \quad (6)$$

An inner-sphere electron transfer mechanism of nitrite reduction is supported by the work of Doyle and co-workers (i.e. nitrite binding before electron transfer), where oxidation of ferrous deoxygenated-Mb (deoxyMb) by alkyl nitrites, R–ONO, trigger facile homolytic cleavage of the RO–NO bond, resulting in the Mb–OR species and liberating NO (Doyle *et al.*, 1981a, 1984). This chemical activation of nitrites by haemoproteins has been studied in great detail, and

the binding mode appears to be relevant to the kinetics of NiR activity. Nitrite has relatively high nucleophilicity and can bind metal centres in different fashions, but only η^1-coordination (i.e. one atom bound) is observed with haem (Ford, 2010). However, the manner of binding has been a subject of debate. Most ferric haem proteins and models favour *N*-bound 'nitro', though the *O*-bound 'nitrito' form is stabilized in native metmyoglobin and methaemoglobin, attributed to hydrogen-bonding of the distal histidine, though the linkage isomers are close in energy (Yi *et al.*, 2008). Mutation of the distal histidine H64 in metmyoglobin to a hydrophobic valine residue triggers linkage isomerization to the *N*-nitro species, while also significantly slowing NiR activity by a factor of 16 (Yi *et al.*, 2009). Nitrite reduction depends on protonation (Equation 5), and the histidine delivers the requisite proton(s) *via* a hydrogen-bonding network of water molecules, analogous to bacterial NiRs (Cutruzzolà *et al.*, 2001; Perissinotti *et al.*, 2008). Unlike bacterial reductases, mammalian globins have only one distal histidine and the ferrohaems are also thought to favour *O*-nitrito coordination. *O*-bound nitrite to ferrous globins allows for a single protonation step and facile loss of NO with concomitant formation of the ferric-hydroxo complex (Figure 2), stabilized by the hydrogen-bonding of the distal histidine to the metal-bound oxygen of nitrite (Silaghi-Dumitrescu, 2004). A crystallographic analysis claiming a solid state ferrous Mb further indicates an *O*-nitrito configuration (Copeland *et al.*, 2006), though disagreements still exist on the bonding mode as a computational study exploring NiR mechanisms favours *N*-nitro coordination in ferrous Hb (deoxyHb) (Perissinotti *et al.*, 2008). Regardless, while the ferrohaem may not follow the same binding scheme as the ferrihaem, in either case the distal histidine and its protic environment is paramount as its absence in H64V Mb appears to interrupt the stepwise proton transfer(s), resulting in decreased NiR activity (Yi *et al.*, 2009). Finally, it is important to note that perturbation of the metal to other redox-active *3d*-block metals cobalt or manganese (in Mb) greatly reduces the NiR activity. Iron is essential to nitrite reduction (Heinecke *et al.*, 2012).

NiR activity of Hb

Physiological nitrite gradients from artery-to-vein (arterial, 176 ± 10 nM and venous, 143 ± 7 nM) (Dejam *et al.*, 2005) are temporally associated with the formation of NO in the red cell (measured as an increase in venous iron-nitrosyl-Hb). Moreover, erythrocytic ferrous nitrosyl-Hb is formed during intra-arterial infusion of nitrite in the arm (Cosby *et al.*, 2003). In this case, nitrite is reduced to NO by deoxyhaemoglobin, which also rapidly binds NO forming ferrous nitrosyl-Hb [Equation 7, $K_{NO}^{T\text{-}state} = 9 \times 10^9 M^{-1}$, $K_{NO}^{R\text{-}state} = 1 \times 10^{11}\ M^{-1}$ (Moore and Gibson, 1976); $k_{NO}^{T,R} = 2.6 \times 10^7\ M^{-1} \cdot s^{-1}$ at 20°C, pH 7 (Cassoly and Gibson, 1975)]. These observations strongly support the model that Hb is the principal metalloprotein involved in reducing nitrite to NO in the vasculature.



$$NO + Hb\ (Fe^{II}) \rightleftharpoons Hb\ (Fe^{II}\text{–}NO) \quad (7)$$

Brooks initially characterized the reaction of deoxyhaemoglobin with nitrite under anaerobic conditions (Brooks, 1937). In the proposed mechanism (Equations 6 and 7), equimolar amounts of methaemoglobin and ferrous nitrosyl-Hb are produced and have been subsequently observed for Mb (Huang *et al.*, 2005b). If the process is governed by a single reaction rate constant, this reaction should be a simple second-order process, determined by the concentration of nitrite and deoxy-haem. However, while the reaction of deoxyhaemoglobin with excess nitrite under stringently anaerobic conditions has confirmed Brooks' stoichiometry,

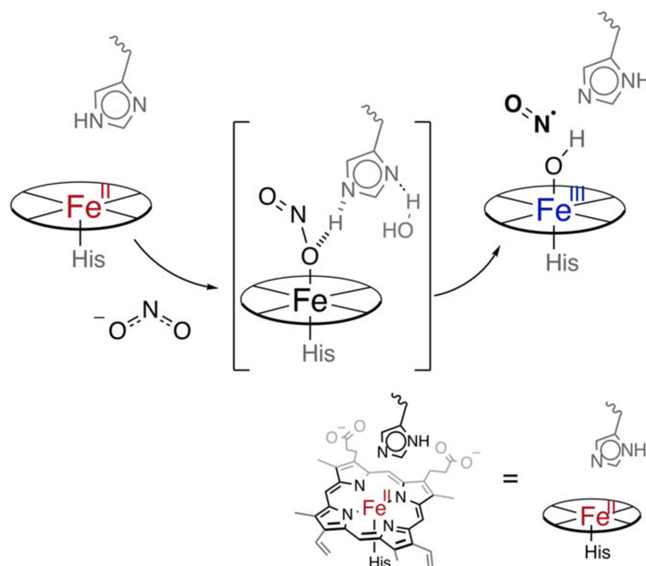


Figure 2

NiR activity of haemoproteins in *O*-nitrito nitrite coordination (i.e. Mb and Hb). Addition of a proton either directly from the histidine (shown) or water yields the nitrous acid bound species. After protonation and subsequent inner-sphere electron transfer from the ferrohaem to the bound nitrous acid, NO dissociates leaving the hydroxo-ferrihaem complex which can then be protonated to the aquomet form.

the simultaneous formation kinetics of both nitrosylated and ferric Hb exhibit a sigmoidal shape rather than an exponential form, as observed with Mb (Figure 3), implying the reaction rate speeds up and slows down again (Huang *et al.*, 2005b; Grubina *et al.*, 2007). We discovered that this deviation from a simple second-order reaction occurs because the reaction rates of nitrite with Hb are modulated by the allosteric conformation of the tetramer. The deoxygenated tense state or T-state of Hb reacts much more slowly with nitrite than the relaxed or R-state of Hb. Generally, the R-state of Hb is stabilized when at least two of the four subunits of the Hb tetramer are either ferric or bound to a ligand such as O_2, CO or NO due to changes in the proximal histidine. Thus, during anaerobic nitrite reduction (a case where there is no oxygen to determine R- and T-state), the reaction rate increases as the R-state is stabilized by the formation of nitrosyl-Hb and ferric Hb, even though active ferrohaem centres are depleted by their generation. Given that each nitrite yields an oxidized haem and equivalent of NO, for every nitrite that is consumed, two Hb molecules can transition from the T- to the R-state. This process is known as allosteric autocatalysis. The R-state deoxyhaemoglobin that forms during the anaerobic reaction reduces nitrite faster as the T-state is depleted (Huang *et al.*, 2005a). The rate nonetheless slows again as more of the vacant R-state haems are occupied by NO or converted to ferrihaem. The bimolecular rate constants of nitrite reduction for the T- and R-states are 0.2 and 12 $M^{-1}{\cdot}s^{-1}$ respectively (Huang *et al.*, 2005b; Gladwin and Kim-Shapiro, 2008). The observed rate of the reaction is given by Equation 8, where [R] and [T] represent the concentrations of the R- and T-state available haems respectively.

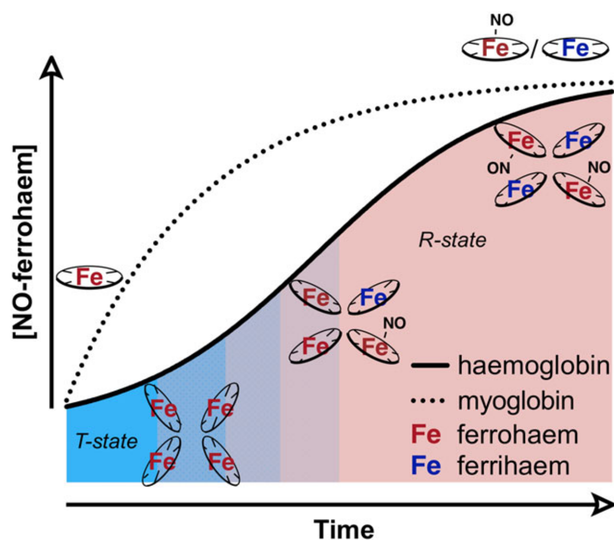


Figure 3

Simulated data representing the NiR activity of deoxymyoglobin and deoxyhaemoglobin under anoxic conditions, specifically tracing the formation of nitrosylated ferrohaem (Equation 6) over time. Mb, which has a constant k_{NiR}, behaves in an exponential manner. Hb exhibits autocatalysis and a sigmoidal shape: tetrameric T-state (blue zone) deoxyHb^{II} reduces nitrite, generating a methaem (3+) and an equivalent of NO. The NO binds a vacant ferrohaem on the same or different tetramer to form a nitrosylated ferrohaem species. Each of these new species stabilizes the R-state (red zone), which has a higher bimolecular rate constant than the T-state, resulting in an increase in apparent rate and propagating nitrite reduction. As the vacant reactive ferrohaem sites are filled, the rate of nitrosylated-ferrohaem formation drops off.

$$-\frac{d[Hb(Fe^{2+})]}{dt} = [NO_2^-]\left(k_{NiR}{}^R[R] + k_{NiR}{}^T[T]\right) \quad (8)$$

While the autocatalytic reaction is observed *in vitro* and caused by NO binding to Hb, the regulation of R- and T-states *in vivo* are determined by oxygen concentrations and Hb oxygen affinity. Therefore, the fastest rate of nitrite reduction to NO paradoxically occurs when the most *deoxy*haems are available on the R-state tetramer: the observed k_{NiR}' is highest in arterial blood, but vacant deoxyhaem sites necessary for NiR activity are maximized in venous blood. This optimized nitrite reduction scenario arises when oxygen is unloaded from R-state Hb in the circulation at about 40–60% oxygen saturation (around the P_{50}) and naturally occurs at the point of arterial-to-capillary transit (Gladwin *et al.*, 2009). Further, physiological studies show the onset of hypoxic vasodilation to occur at these oxygen tensions (Ross *et al.*, 1962). Thus, the Hb maximal reductase activity occurring at the natural P_{50} is also consistent with a role in hypoxic vasodilation. At lower oxygen tensions, oxygen-dependent NOS enzymes function less effectively, consistent with the hypothesis of nitrite serving as a complementary NO-generating pathway. It should be noted that this chemistry is somewhat complicated by nitrite interaction with oxyhaemoglobin, discussed in detail below.

The disparity of bimolecular rate constants between the T- and R-states has been attributed to a difference in redox potential of the haem between the states (Huang *et al.*, 2005b). R-state Hb has a lower redox potential than T-state Hb ($E_{1/2}$ vs. NHE: HbA^R = 42 mV, HbA^T = 154 mV), meaning the R-state is more readily oxidizable (Bonaventura *et al.*, 2013). Canonically, as an exogenous ligand (e.g. oxygen) binds T-state ferrohaem, the pentacoordinate high spin iron (II) (which is displaced from the porphyrin ring), is rendered low spin, decreasing the iron radius, pulling it flush with the porphyrin ring and shifting the proximal histidine and protein backbone. This ‘proximal pull’ is the basis of the T-to-R-state transition and is responsible for the decrease in redox potential (Bonaventura *et al.*, 2013). Thus, the lower redox potential R-state increases the probability of nitrite reduction. Furthermore, this R-state potential is analogous to that of Mb (Taboy *et al.*, 2002). Sickle cell Hb (HbS) has a higher NiR activity than healthy adult Hb (HbA), correlating to its more favourable potential than HbA (Grubina *et al.*, 2008). It is important to note that redox potential is not solely responsible for the effect. A study comparing deoxyhaemoglobin and other modified haemoglobins indicated faster nitrite reduction even as the redox potential *increased* (Bonaventura *et al.*, 2008). Bonaventura *et al.* (2013) ascribe the shift in the kinetics of the reaction to ligand affinity changes driven by the steric and electronic factors of the haem active site, as redox potential is responsible solely for the thermodynamic driving potential. But, as mentioned above, the T-to-R state transition triggers physical rearrangement as well as a concomitant decrease in redox potential in HbA. These changes together can be responsible for the increased k_{NiR}, as the more open R-state pocket is a

better NiR catalyst (i.e. lowers the activation energy of the reaction) and binds nitrite more readily.

The importance of a favourable proton-donating environment and a consequence of the tetrameric nature of Hb is illustrated by the proton dependence of nitrite reduction from pH 6.0 to 8.0 (Doyle *et al.*, 1981b). As expected, nitrous acid formation in the distal pocket is implicated as the catalysed species leading to NO formation. However, the authors observed rate order $[H^+]$ dependence of 0.88 instead of an ideal first-order dependence, deviating from Equation 6. They noted this may result from conformation changes in Hb, and the unexpected sub-unity proton dependence has indeed since been ascribed to the redox Bohr effect (Huang *et al.*, 2005b). Allosteric proton binding results in a more stabilized T-state and consequently a diminished haem redox potential, decreasing NiR activity. As a nitrite protonation step is essential for reduction, the smaller contribution from the Bohr effect works in the opposite direction, resulting in a less than first-order proton dependence on the rate, whereas a close agreement to a slope of 1.00 has been observed for all other haemoproteins apart from Hb (Gladwin *et al.*, 2009).

The ultimate practicality of NiR activity of Hb in RBCs depends on two essential matters. First, the basal levels of nitrite must be sufficient to be physiologically relevant to hypoxic vasodilation. Nitrite is found in plasma (120 nM) but is pooled in even greater amounts in the erythrocytes themselves (300 nM) (Dejam *et al.*, 2005). As such, Hb is proposed as a dominant NiRs in blood, responsible for hypoxic vasodilation (Cosby *et al.*, 2003; Huang *et al.*, 2005b; Crawford *et al.*, 2006), though it does not act alone. Several metalloenzymes in the vasculature have been evaluated for nitrite reduction, notably eNOS and XO (Webb *et al.*, 2008a, *vide infra*). However, these do not appear to generate NO at physiological pH and oxygen tensions. In mice, Mb contributes to nitrite reduction at low oxygen, with the Mb-knockout mice showing reduced nitrite-mediated hypoxic vasodilation and cardioprotection (Hendgen-Cotta *et al.*, 2008).

The second matter is the question of how NO escapes from the RBC. NO generated in a partially oxygenated environment (where NiR activity is maximized) would be expected to be consumed by the rapid dioxygenation activity of oxyhaemoglobin (Equation 2) or captured by the ferrous deoxyhaemoglobin (Equation 7). And yet, even slightly higher than physiological amounts of exogenously supplemented nitrite (2.5 μM) stimulate vasorelaxation, implying that the NO must be escaping from the RBC and stimulating sGC in the smooth muscle (Cosby *et al.*, 2003). Moreover, NO generation has been confirmed directly by experiments with partially deoxygenated RBCs and nitrite (Huang *et al.*, 2005b; Crawford *et al.*, 2006; Shiva *et al.*, 2011; Liu *et al.*, 2015), as well as with aortic ring dilation experiments (Cosby *et al.*, 2003; Isbell *et al.*, 2007). In addition, experiments involving inhibition of platelet activation [a known effect of NO (Radomski *et al.*, 1987; Loscalzo, 2001)] strongly support the notion that nitrite can export NO activity. Physiologically, relevant concentrations of nitrite inhibit platelet activation in the presence – but not absence – of RBCs, and this activity is inhibited by NO scavengers (Srihirun *et al.*, 2012; Wajih *et al.*, 2016, 2017). Numerous mechanisms have been proposed: nitrite reduction being limited to RBC membrane compartments, production of another species such as a nitrosothiol (RSNO) or N_2O_3 formation (Fernandez and Ford, 2003; Basu *et al.*, 2007; Wajih *et al.*, 2016).

Ferrihaem-nitrite disproportionation

Erythrocytic Hb consumes NO *via* Equations 2 and 7, yet NO is exported from RBCs, suggesting a relatively stable intermediate NO_x species is formed. One proposed species is N_2O_3, generated by rapid reaction of radical $NO_2^\bullet$ with NO. N_2O_3 possesses $NO^+NO_2^-$ character and thus in the presence of a nucleophile (e.g. a thiolate, Equation 9) results in nitrosation (Heinrich *et al.*, 2013) and reforms nitrite. Under aqueous conditions, N_2O_3 hydrolyzes to two equivalents of nitrous acid (Grätzel *et al.*, 1970) but may persist in hydrophobic pockets (e.g. the RBC membrane); N_2O_3 is small and uncharged, facilitating diffusion, and exhibits homolytic scission (regenerating NO). Thus, N_2O_3 formation is an attractive supposition for NO escaping from the RBC.

$$N_2O_3 + RS^- \longrightarrow RSNO + NO_2^- \quad (9)$$

The manner in which N_2O_3 is generated is disputed. It can result from autoxidation of NO, transiently generating $NO_2^\bullet$ (Equation 10, back reaction of Equation 4). Though as discussed previously, the second-order dependence on NO (Equation 1) implies N_2O_3 formation from NO autoxidation is too slow to be significant physiologically, although it has been postulated to occur in hydrophobic environments where NO and oxygen levels will be significantly higher (Liu *et al.*, 1998; Vrancken *et al.*, 2016) and conditions of nitrosative stress (Thomas *et al.*, 2008).

$$2NO + O_2 \longrightarrow 2NO_2^\bullet \quad (10)$$

The oxidation of nitrite to $NO_2^\bullet$ is known to occur *via* metal catalysis (Thomas *et al.*, 2008). Specifically regarding RBCs, an EPR-silent NO-modified Hb intermediate was initially hypothesized (Nagababu *et al.*, 2003) but was later determined to be nitrite-bound methaemoglobin (Basu *et al.*, 2007). Consistent with nitrite binding to methaems, the *O*-nitrito-bound nitrite (*vide supra*) favours electron delocalization imparting more iron (II) character (Basu *et al.*, 2007). The newly formed Fe^{2+}-bound $NO_2^\bullet$ has significant enough radical character to react with even low amounts of NO, yielding N_2O_3 (Figure 4, *path a*). Even though nitrite affinity for ferrihaem is 1–2 orders of magnitude lower than NO, experiments on the complex in a glass matrix indicate that NO entering the distal pocket does not displace the nitrite. In fact, a ferrohaem product is formed, consistent with the formation of N_2O_3 (Navati and Friedman, 2009). Pertinent to hypoxic NO generation from nitrite, this N_2O_3 liberation by Hb represents 'ferrihaem-nitrite disproportionation' or a 'nitrite anhydrase' mechanism.

An alternate pathway advanced by Fernandez and Ford involves nitrite acting as a general base (Fernandez and Ford, 2003). The inner-sphere mechanism suggests nitrite directly attacks the nitrosylated-ferrihaem species yielding the Fe^{2+}–N_2O_3 as seen in Figure 4, *path b* (Fernandez *et al.*, 2004; Ford, 2010). This mechanism was favoured in part by the authors due to the relatively high nucleophilicity of nitrite. They could not preclude the possibility of an

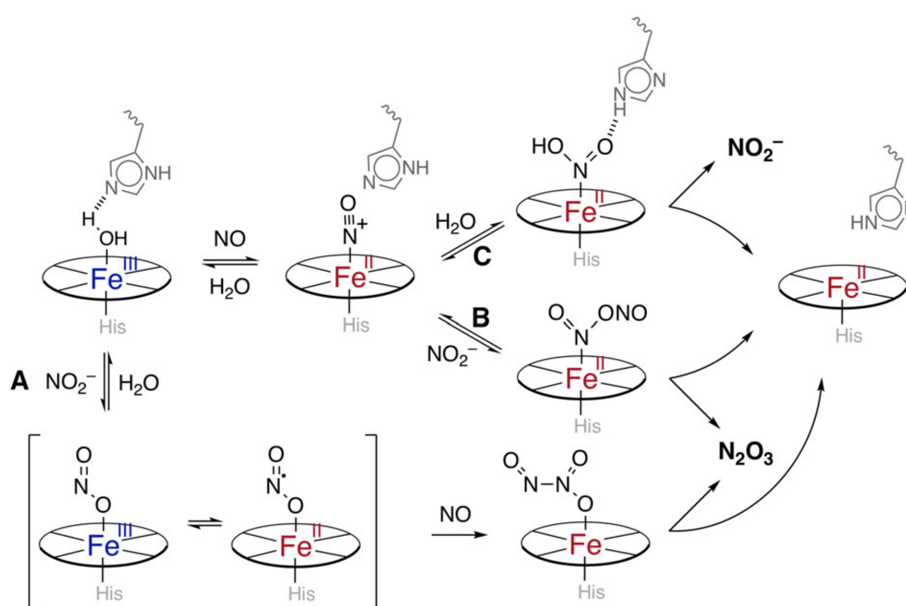


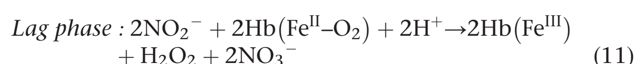
Figure 4

Various mechanisms of non-enzymically induced redox cycling ferrihaem (blue-centres) to ferrohaem (red-centres). The top path represents reductive nitrosylation, where NO displaces water from the aquomet-globin generating a nitrosyl-ferrihaem/nitrosonium-ferrohaem (shown). A nucleophile such as hydroxide/water (*path c*) generates nitrite, whereas when nitrite is the nucleophile (*path b*), N_2O_3 is generated. Importantly, other nucleophiles such as thiolates can participate in this chemistry. Similarly, the nitrite anhydrase mechanism (*path a*) represents a situation where nitrite binds the methaem first. The subsequent nitrito-ferrihaem complex (here depicted as *O*-nitrito, but the *N*-nitro is possible) exhibits some radical $NO_2^{\bullet}$-ferrohaem character, which can readily react with NO to generate diffusible N_2O_3.

outer-sphere mechanism involving nitrite oxidation by nitrosylated-ferrihaem and subsequent reaction of the generated $NO_2^{\bullet}$ radical with the ferrous-nitrosyl species. Unlike 'ferrihaem-nitrite disproportionation', this mechanism is effectively reductive nitrosylation (Figure 4, *path c*, *vide infra*) where the nucleophilic hydroxide/water is replaced by nitrite. Both mechanisms have been shown to be energetically feasible computationally, and both lead to the same products while redox cycling the globin (Hopmann *et al.*, 2011). Either mechanism would be possible under the appropriate conditions. Thus, Hb nitrite anhydrase activity to yield N_2O_3 feasibly explains erythrocytic NO export as well as formation of S-nitrosothiols (Equation 9), important signalling agents themselves (Nagababu *et al.*, 2003; Basu *et al.*, 2007; Thomas *et al.*, 2008).

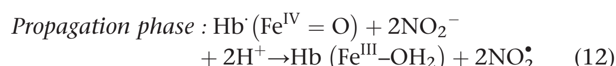
The nitrite oxyhaemoglobin reaction

Nitrite also reacts with oxyhaemoglobin generating nitrate and methaemoglobin. The kinetics follow a complex reaction profile. In excess nitrite, a slower lag or initiation phase is observed that then accelerates to a rapid autocatalytic propagation phase (Kim-Shapiro *et al.*, 2005). Though there are various proposed mechanisms, experiential evidence suggest the rate-limiting initiation reaction generates hydrogen peroxide, methaem and nitrate with an estimated bimolecular rate constant between 0.2 and 0.4 $M^{-1}{\cdot}s^{-1}$ (Equation 11) (Keszler *et al.*, 2008).



$$\textit{Lag phase}: 2NO_2^- + 2Hb(Fe^{II}\text{–}O_2) + 2H^+ \rightarrow 2Hb(Fe^{III}) + H_2O_2 + 2NO_3^- \quad (11)$$

The methaemoglobin and peroxide then form a ferryl ($Fe^{IV}{=}O$)–Hb radical, propagating NO_2 formation from the reaction with additional nitrite (Equation 12). During this autocatalytic propagative phase, $NO_2^{\bullet}$ reacts with additional oxyhaem to regenerate ferrylhaem-radical and more nitrate. The proposed elementary steps are shown in their entirety in Keszler *et al.* (2008).



$$\textit{Propagation phase}: Hb^{\cdot}(Fe^{IV} = O) + 2NO_2^- + 2H^+ \rightarrow Hb(Fe^{III}\text{–}OH_2) + 2NO_2^{\bullet} \quad (12)$$

Grubina *et al.* (2007) also observed at varying oxygen tensions both the deoxy-nitrite reduction and this oxy-reaction occurring simultaneously, but the former (Equation 6) quenched the oxy-reaction intermediates. One possibility is the interaction of NO with $NO_2^{\bullet}$ (the back reaction of Equation 4) generating N_2O_3. Another possibility provides a supporting mechanism of NO escape from RBCs. In this scenario, the stable ferrous nitrosyl-Hb is oxidized under the propagation conditions by $NO_2^{\bullet}$, generating a far more labile ferric nitrosyl-Hb. This process is called 'oxidative denitrosylation', simultaneously liberating NO and limiting the oxy-pathway by consuming $NO_2^{\bullet}$ intermediates, promoting the reductive nitrite pathway to NO.

Physiological nitrite generation

Nitrite is clearly an important NO source under hypoxic conditions, but where does it originate? As our dietary intake of nitrite is limited, most of our nitrite is generated from our microbiome as discussed in the context of the

nitrate–nitrite–NO pathway above and survives passage through the stomach and GI tract into circulation. However, other processes exist which yield biological nitrite and maintain nitrite homeostasis. In the absence of ferrous haemoproteins, nitrite is the chief oxidation product of NO in aqueous solutions with oxygen (Ignarro *et al.*, 1993), but this is kinetically limited. Most ferric haemoproteins can undergo 'reductive nitrosylation' or 'autoreduction' when exposed to excess NO (Figure 4, *path c*), generating nitrite. Reductive nitrosylation first involves production of a nitrosyl-ferrihaem. The NO-moiety is electrophilic as the complex has nitrosonium-ferrohaem character and is subject to nucleophilic attack by water/hydroxide, determined after studies on rate dependence on pH and NO concentration (Hoshino *et al.*, 1996). Reductive nitrosylation depends on the concentration of OH^- and is considered the opposite of NiR activity where the dependence of the redox reaction is on the concentration of the proton. Importantly, unlike Mb, Hb undergoes reductive nitrosylation at low pH values (~6), vital for broader physiological relevance. Additionally, other nucleophiles can react with ferric nitrosylglobins to generate nitrosated species [e.g. S-nitrosoglutathione (Reichenbach *et al.*, 2001)].

Physiological nitrite is also generated by oxidation of NO by ceruloplasmin, a protein that is present in micromolar concentrations in human plasma (Shiva *et al.*, 2006). This hexacopper oxidase catalyses the oxidation of NO to nitrite *via* reduction from Cu^{2+} to Cu^{1+} and is also implicated in RSNO formation as the resulting nitrosonium cation is incredibly electrophilic and reactive. The principal nucleophile in plasma is water yielding nitrite, competing with NO-dioxygenation reaction of globins. After myocardial infarction, ceruloplasmin induction has been observed (Singh, 1992), and mice where the protein is knocked out sustain greater I/R injury than controls (Shiva *et al.*, 2006). Such observations are consistent with the paradigm that nitrite improves outcomes of hypoxic insults, and thus, it has been posited that ceruloplasmin modulates response to ischaemia by generating nitrite. Vrancken *et al.* evaluated species differences in ceruloplasmin levels and activity and found correlations between ceruloplasmin levels and plasma RSNO during NO donor exposure (Vrancken *et al.*, 2013). While they did not see differences in nitrite levels, the oxidation of NO to NO^+ would be expected to form both RSNO and nitrite, so the results of this study remain equivocal.

Other haem-based mammalian NiRs

There are numerous other mammalian enzymes that reduce nitrite under appropriate conditions. Other haem enzymes produce NO from nitrite including the canonical NO-generating eNOS, but under anoxic conditions (Vanin *et al.*, 2007). eNOS may play a role in the regulation of NiR activity in RBCs during ischaemic events as the enzyme is localized within the RBC membrane, thus NO generation is more likely to diffuse away from the cell instead of undergoing autocapture (Webb *et al.*, 2008a). Recently discovered globins such as neuroglobin (Ngb) (Petersen *et al.*, 2008; Tiso *et al.*, 2011) and cytoglobin (Cygb) (Li *et al.*, 2012; Corti *et al.*, 2016) exhibit NiR activity under physiologically relevant conditions, though their respective primary functions are unknown. As with the oxygen-carrying globins, these haem proteins follow Equation 5 for nitrite reduction, where the reductant is the ferrous protein. However, significant differences exist.

Unlike Hb and Mb, the distal histidine in Ngb and Cygb is bound to the iron, which means these proteins do not have an immediately available coordination site for nitrite to bind. However, unlike hexacoordinate cytochromes, the distal histidine is labile and thus interconverts from hexacoordinate to pentacoordinate rapidly. The interconversion is rate limiting: the NiR activity of Ngb increases significantly upon mutation of the distal histidine to a non-hydrogen bonding, non-coordinating amino acid as reflected by a ~2200-fold increase in rate constant (k_{NiR}, Table 1) from the wild-type to H64L (Tiso *et al.*, 2011). The difference stands in stark contrast to that of Mb, the rate constant of which decreases upon mutation to H64V due to the loss of hydrogen-bonding environment (*vide supra*). Mb already contains an available coordination site, whereas in Ngb, removal of the coordinated distal histidine provides a binding site for nitrite, resulting in a large increase in NiR activity upon mutation. Further, the rate-limiting dissociation of the distal histidine confirms an inner-sphere nitrite reduction. Although these studies suggest that Ngb has redox-regulated NiR activity, also recently demonstrated for Cygb, more work is needed to evaluate the physiological roles the six-coordinate globins may play, as functional NiRs and NO signalling molecules.

One intriguing caveat suggesting NiR activity in the native mammalian six-coordinate globins is the presence of two well-conserved cysteines which form intramolecular disulfide bonds (Cys46 and 55 in Ngb, Cys38 and 83 in Cygb), resulting in either reduced (S–H) or oxidized (S–S) forms. In humans, these disulfides affect the position of the E-helix where the distal histidine is located and modulate the haem ligand binding equilibrium: K_{His} (k_{on}/k_{off}) shifts from ~3000 in $Ngb_{S\text{–}H}$ to 280 in $Ngb_{S\text{–}S}$ (Hamdane *et al.*, 2004) and ~2000 to 0.48 in the respective Cygbs (Beckerson *et al.*, 2015). Access to the distal binding site is necessary for nitrite reduction, reflected by a twofold increase in the bimolecular rate constant k_{NiR} in Ngb (Tiso *et al.*, 2011) and a 50-fold increase in Cygb (Reeder and Ukeri, 2018) (Table 1) in proteins with the intramolecular disulfide. This bond may play a pivotal role in the otherwise unknown activity of these two globins, rendering them redox-sensitive to their local environments, as well as creating efficient NiRs under reducing (hypoxic) cellular conditions, controlling NO signalling. It is worth noting that Reeder and Ukeri (2018) point out that the intermolecular disulfide bond of Cygb (dimer) has a k_{NiR} about 140-fold less than that of the intramolecular disulfide monomer.

Cystathionine β-synthase (CBS), a hydrogen sulfide generating enzyme and vital enzyme in sulfur amino acid metabolism, also exhibits NiR activity and has been proposed as a potential NO source *in vivo* (Carballal *et al.*, 2016). Mammalian CBS contains a haem ancillary to the active pocket, but its purpose is relatively unknown; it is suspected to allosterically regulate the active site as ferrous binding of exogenous ligands, such as NO, results in abolition of CBS activity (Carballal *et al.*, 2016). Further, the haem has an unusual electronic structure with a low

Table 1

Kinetic parameters of NO production from nitrite reduction by representative haemoproteins (top) and molybdenum–molybdopterin containing proteins (bottom).

	k_{NiR} ($M^{-1}·s^{-1}$)	T (°C)	Reference
Hb^T	0.12	25	Huang *et al.* (2005b)
	0.2	37	Gladwin and Kim-Shapiro (2008)
Hb^R	6	25	Huang *et al.* (2005b)
	12	37	Gladwin and Kim-Shapiro (2008)
Mb	5.5	25	Yi *et al.* (2009)
	12	37	Shiva *et al.* (2007)
Mb (H64V)	0.35	25	Yi *et al.* (2009)
$Ngb_{S–S}$	0.12	25	Tiso *et al.* (2011)
	0.26	37	Tiso *et al.* (2011)
$Ngb_{S–H}$	0.062	25	Tiso *et al.* (2011)
Ngb (H64L)	259	25	Tiso *et al.* (2011)
$Cygb_{S–S}$ (monomer)	32.3	25	Reeder and Ukeri (2018)
$Cygb_{S–H}$	0.63	25	Reeder and Ukeri (2018)
$Cygb_{S–S}$ (dimer)	0.26	25	Reeder and Ukeri (2018)
CBS	0.66	37	Carballal *et al.* (2016)
Cytochrome *c* (horse)	0.07	25	Li *et al.* (2012)

		Kinetic parameters				
	Type, substrate	k_{cat} (s^{-1})	K_m (nitrite, mM)	Rate of NO formation: V_{max} ($nmol·s^{-1}·mg^{-1}$)	T (°C)	Reference
XO	Bovine, aldehyde	0.693	0.585	–	25	Maia and Moura (2011)
	Bovine, NADH[a]	–	22.9	62	37	Millar *et al.* (1998)
	Bovine, NADH	0.28	2.25	0.92	37	Li *et al.* (2001)
AO	Rat, aldehyde	1.89	9.7	–	25	Maia *et al.* (2015)
	Rat NADH	≥0.331	≥3.99	–	25	Maia *et al.* (2015)
	Rat, NADH	–	2.7	8.5[a]	37	Li *et al.* (2009)
SO	Human, sulfite	0.002	1.6	0.0361	37	Wang *et al.* (2015)
	Human, phenosafranine	1.9	80	34	37	Wang *et al.* (2015)
mARC–1	human, b_5r/b_5	0.1	9.5	3.6	37	Sparacino-Watkins *et al.* (2014)

All bimolecular rate constants are reported for human haemoproteins at pH 7.4. Kinetic parameters of Mo-proteins are determined at pH 7.4 unless noted. Regarding the kinetic parameters of the Mo-containing enzymes, the actual nitrite reduction rate will depend on abundance of the protein. See text and appropriate references for details.
[a]pH 7.2.

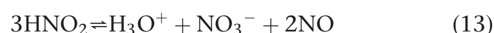
reduction potential (−350 mV vs. NHE). Consistent with this low potential, Carballal and co-workers observed that dissociation of the iron-bound cysteine had little effect on the rate of nitrite reduction, implying that this haem participated in rapid outer-sphere electron transfer to nitrite. Coupled with the observation that NO binding at this haem is weaker than that of Hb or Mb [K_{NO} is 3–5 orders of magnitude lower in CBS (Vicente *et al.*, 2014)], a significant rate of nitrite reduction (Table 1), and an inability to form an oxygen-bound ferrous species (thus excluding NO-dioxygenase activity), CBS may function as a NiR *in vivo*.

Nitrite reduction by molybdenum–molybdopterin containing proteins

Other mammalian metal enzymes exhibit NiR activity, specifically enzymes containing molybdenum (Mo)-bound molybdopterin cofactors. Examples include XO, aldehyde oxidase (AO), sulfite oxidase (SO) and mitochondrial amidoxime-reducing component–1 and 2 (mARC-1 and mARC-2). These typically catalyse oxygen atom transfer reactions, where the molybdenum core cycles between Mo^{6+} and Mo^{4+} (Maia and Moura, 2015). However, each of these Mo-enzymes exhibit one-electron oxidations (Mo^{4+} to

Mo^{5+}) with nitrite, generating an equivalent of NO (Millar *et al.*, 1998; Li *et al.*, 2009; Maia and Moura, 2011; Sparacino-Watkins *et al.*, 2014; Wang *et al.*, 2015; Maia *et al.*, 2015). These enzymes are broadly distributed throughout human tissues and have been implicated as a major source of nitrite-induced vasorelaxation especially as XO is present in the endothelium, as well as in the membrane of RBCs (Webb *et al.*, 2008a). Additionally, XO does not bind NO. However, inhibition of XO does not inhibit nitrite-dependent vasodilation in humans, implying that it is not necessarily the primary NiR in humans (Dejam *et al.*, 2007). Mo-enzyme NiR activity is inhibited by oxygen and is only pertinent at hypoxic or anoxic conditions (Maia and Moura, 2015). The Michalis–Menten kinetic parameters have been assessed by several laboratories under various conditions, and a sampling is included in Table 1, *bottom*. Though the relatively high K_m values are 1–3 orders of magnitude higher than the concentration of nitrite in tissue [1–20 μM (Shiva, 2013)], only nanomolar amounts of NO are needed to carry out vascular functions, meaning Mo-enzymes can act as NiRs at low oxygen tensions in addition to their primary functions.

Mechanistically speaking, the Mo^{5+} species has been observed *via* EPR, indicating that the enzymes are reducing nitrite and not simply catalysing a disproportionation of nitrous acid (Equation 13) (Yang *et al.*, 2015). Metal involvement of NiR activity has been confirmed as tungsten-substituted mARC-1 abolishes all nitrite reduction (Sparacino-Watkins *et al.*, 2014), and oxipurinol (a specific Mo-binding XO inhibitor) similarly prevented NO generation (Okamoto *et al.*, 2008).



$$3HNO_2 \rightleftharpoons H_3O^+ + NO_3^- + 2NO \quad (13)$$

Computational studies by Yang *et al.* (2015) on the postulated mARC active site favour a hydroxyl radical transfer mechanism, in which protonation of the metal-bound oxygen of nitrite is required for facile NO release and is consistent with the pH dependence of Mo-enzyme-catalysed NO formation.

Nitrite and nitrate therapeutics

The discussion above highlights the diverse chemistry underlying how nitrite may regulate NO signalling, especially in low pH and oxygen environments. I/R injury is characterized by the latter and, in many cases, by low NO bioavailability. Thus, nitrite therapy may improve NO signalling in a targeted manner and avoid unwanted, off-target, systemic effects associated with other NO-releasing drugs. Indeed, to date, several preclinical animal model studies have demonstrated that nitrite administered during the ischaemia phase or very soon thereafter affords protection (see more detail in Lundberg *et al.*, 2009, 2015; Calvert and Lefer, 2010; Vitturi and Patel, 2011). Importantly, protection has been observed in all major organ systems including the brain, heart, kidney, liver and lungs with a range of mechanisms from antioxidative, anti-inflammatory, improvement of blood flow and angiogenesis to preventing cell death. While data derived from animal models are largely supportive of nitrite-therapeutics, early phase clinical studies are less clear. Most available human data to date have evaluated effects of nitrite on acute myocardial infarction. Some researchers did not observe any protective effects of nitrite (Siddiqi *et al.*, 2014). However, Jones *et al.* (2015) did observe a reduction in infarct size, a reduction in major adverse cardiac events and a reduction in inflammatory endpoints (Jones *et al.*, 2017) in nitrite treated patients, especially the subgroup with very low coronary perfusion at the time of catheterization. Differences between nitrite dosing may underlie the outcome variability in these studies. Moreover, a similar effect of nitrite-based protection against I/R injury has been proposed in liver transplant patients receiving inhaled NO versus placebo. In this case, inhaled NO gas-derived circulating nitrite was proposed to limit I/R injury and improve the rate of allograft function recovery, especially in patients more prone to I/R injury (Lang *et al.*, 2014). Thus, the therapeutic efficacy of nitrite may be most pronounced and observed in patients with the greatest degree of I/R injury.

In addition to acute disease states, nitrite-therapy is being tested in chronic diseases associated with NO insufficiency, particularly vascular disease such as atherosclerosis, diabetes and systemic and pulmonary hypertension. A number of initial studies suggest positive outcomes for selected endpoints (e.g. BP, improved vascular remodelling and reductions in neutrophil numbers) (Greenway *et al.*, 2012; Bir *et al.*, 2014; Jones *et al.*, 2017). A recently completed study of oral nitrite showed reductions in BP and inhibition of platelet activation with oral doses of 20 mg in normal volunteers (Hughan *et al.*, 2017). Targeted delivery of nitrite *via* inhalation is being evaluated in clinical trials for treating pulmonary hypertension (Sparacino-Watkins *et al.*, 2012; Rix *et al.*, 2015; Simon *et al.*, 2016). Inhaled and oral nitrite are being studied in patients with heart failure with preserved ejection fraction (HFpEF) and have been shown in open label studies to reduce pulmonary and left heart filling pressures and to improve exercise cardiac output (Borlaug *et al.*, 2015, 2016; Simon *et al.*, 2016). However, a recently completed placebo-controlled trial of inhaled nitrite for HFpEF patients was presented at the American Heart Association meetings and reported no efficacy of inhaled nitrite on exercise capacity (Reddy *et al.*, 2017). Our research group is currently performing a placebo-controlled trial of oral nitrite for patients with pulmonary hypertension and HFpEF that is currently enrolling (ClinicalTrials.gov, NCT03015402). At this time, additional randomized placebo-controlled studies are clearly required to determine therapeutic efficacy for cardiovascular diseases.

Additionally, nitrate from the diet (*vide supra*) as an NO-repleting therapeutic agent has been evaluated. Numerous, small, blinded, placebo-controlled studies with healthy volunteers or hypertensive patients demonstrate that dietary nitrate can lower BP and platelet reactivity and improve exercise performance and cellular energetics; all endpoints associated with increased NO bioavailability (Lundberg *et al.*, 2009, 2018; Kapil *et al.*, 2014; Gee and Ahluwalia, 2016; Carlström *et al.*, 2018). Animal studies have shown that dietary nitrate prevents I/R injury, improves ischaemic angiogenic signalling with revascularization and prevents metabolic syndrome, all expectedly modulated *via* the formation of nitrite and subsequent stimulation of NO-dependent signalling (Carlström *et al.*, 2010; Hendgen-Cotta *et al.*, 2012). Thus, nitrate clinical trials have been proposed for improving vascular-related endpoints in active coronary artery disease

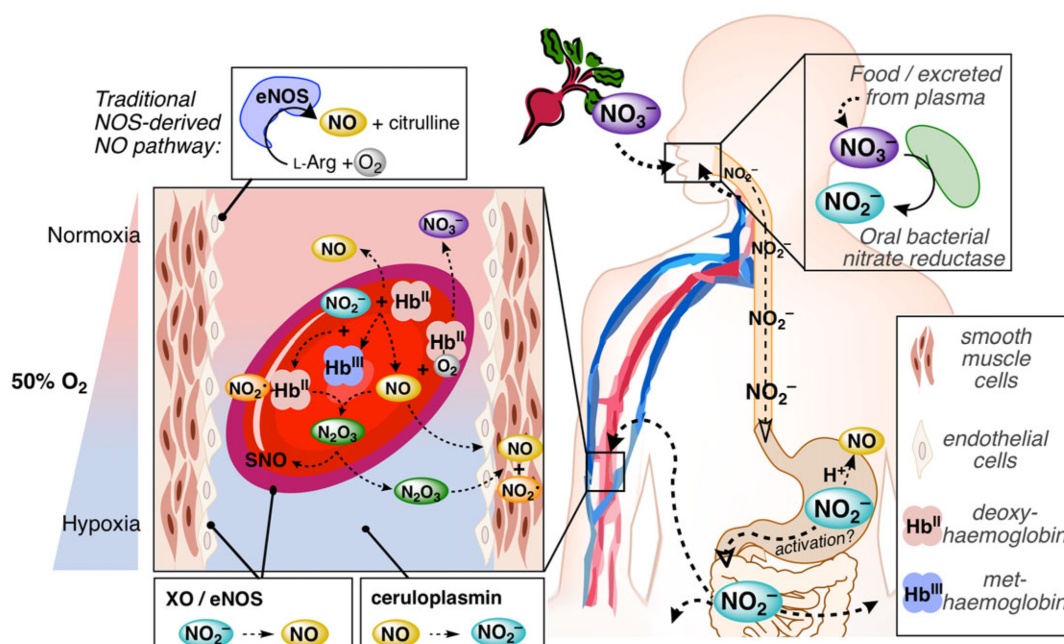


Figure 5

The nitrate–nitrite–NO pathway and simplified nitrite chemical biology in the vasculature. The nitrate–nitrite–NO path is a complementary route for NO generation in the vasculature that occurs under hypoxic conditions, when canonical NO generation at normoxia from eNOS (*upper left*) is less effective. Nitrate is ingested (*upper right*) from various foods including beetroot and leafy greens. Oral bacteria in the salivary glands reduce nitrate to nitrite, which is subsequently swallowed, traversing the oesophagus and into the acidic gastric fluids and into GI tract (*hollow arrowheads*). Some of the nitrite (as nitrous acid) may dehydrate and disproportionate (Equations 3 and 4 in text) to generate NO in the stomach. Orally swallowed nitrite has been suggested to be bioactivated in the stomach under non-enzymic acidic conditions. Nitrite may also escape the GI tract and enter the circulation (*bottom right*). Numerous reactions occur in RBCs (*centre left*), especially at the point of artery-to-vein transport (Hb is ~50% O_2-saturated or P_{50}) where the nitrite reduction rate by deoxyHb is maximized. NiR activity yields metHb and an equivalent of NO (Equation 6). metHb reacts with nitrite to form a radical $NO_2^{\bullet}$-bound ferrohaem, which reacts rapidly with NO to generate N_2O_3 (Figure 4, *path a*), responsible for RSNO formation (Equation 9). N_2O_3 is one mechanism by which NO is proposed to escape from the RBC and generate NO (Equation 4) in the smooth muscle. NO may also be autocaptured by deoxyHb generating ferrous nitrosyl-Hb (Equation 7, *not shown*) and subsequently released *via* oxidative nitrosylation (Equations 11 and 12, *not shown*), or NO may react with oxyHb to liberate nitrate (Equation 2). The nitrate is secreted from the plasma into the salivary glands (*upper right*), starting the process anew. XO and eNOS found in both endothelial cells and the surface of RBCs have been suspected to generate NO from nitrite, but only under hypoxic and more acidic conditions (*bottom left*). Finally, nitrite is generated by the copper plasma protein ceruloplasmin from NO, preventing dioxygenation to nitrate or reductive nitrosylation by ferrihaems (*not shown*).

(Rathod *et al.*, 2016; Schwarz *et al.*, 2016). A number of placebo-controlled randomized trials have evaluated beetroot juice that has high nitrate concentrations compared with placebo nitrate-depleted juice in patients with diabetes and HFpEF. In patients with Type 2 diabetes, there were no improvements in exercise capacity, endothelial function, glucose homeostasis or other measured endpoints (Gilchrist *et al.*, 2013; Shepherd *et al.*, 2015, 2016). Similar studies in HFpEF patients showed mixed results with one trial reporting a significant improvement in BP and exercise endurance after a week of beetroot treatment (Eggebeen *et al.*, 2016), while a second trial found that there was no added benefit observed for any outcomes when comparing beetroot juice to placebo in either hypertensive or HFpEF patients undergoing exercise training ($P \geq 0.14$) (Shaltout *et al.*, 2017).

While these ongoing studies explore the potential for nitrate-therapy in diseased populations, it is important to highlight that some studies have reported no effect of dietary nitrate leading to the concept of 'nitrate-responders' versus 'non-responders'. The existence of these two populations suggest that oral microbiome diversity, abundance of nitrate-reducing bacteria and presence of metabolites that inhibit saliva nitrate transport or reduction are potential variables that underlie discrepant responses (Burleigh *et al.*, 2018). Frequency of mouthwash use and type are obvious factors, but more recent work identifies other lifestyle variables such as smoking: smoking-derived thiocyanate competes with and inhibits nitrate transport into the salivary glands (Bailey *et al.*, 2016), and smokers have oral dysbiosis characterized by lower nitrate-reductase activity (Ahmed *et al.*, 2017). Moreover, as discussed previously, stomach pH is suggested to be an important variable in connecting nitrite from our oral microbiome to the activation of NO signalling systemically. Such factors must be allowed for in the design of any clinical trial.

Concluding remarks

Nitrate and nitrite represent a relatively new paradigm in intravascular NO signalling, where nitrite is a stable intermediate reduced when needed under physiological and

pathophysiological hypoxia and nitrate can resupply the nitrite pool. Consistent with this paradigm, nitrate is protective against I/R injuries and several other cardiovascular disorders in animal models (Lundberg *et al.*, 2011; Kapil *et al.*, 2014; Hezel *et al.*, 2016; Mills *et al.*, 2017), and changes in plasma nitrite are protective down to 200 nM (Duranski *et al.*, 2005). Moreover, nitrate supplementation in human studies show pronounced beneficial effects on BP and overall vascular health as thoroughly reviewed by Kapil *et al*. (2014). These observations are consistent with the nitrate–nitrite–NO postulation and nitrite chemistries described throughout this text and largely summarized in Figure 5, where nitrate is ingested or produced endogenously and converted to nitrite, the chief vascular reserve for NO and other NO_x species. Nitrite is rapidly converted to NO by multiple enzymes; in erythrocytes, this occurs during deoxygenation of Hb from artery-to-vein transit, where the NO in the RBC can form nitrate, generate N_2O_3 or bind ferrohaem and subsequently undergo oxidative denitrosylation regenerating NO. The chemical biology described in this review expounds the physiological and pharmacological observations around this pathway, illuminating an essential endogenous source of NO delivery, especially when the conventional NOS route is compromised under hypoxic conditions.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

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Author contributions

A.W.D. prepared the initial and final drafts and all figures. R.P.P. critically read the manuscript and contributed the section on nitrite therapeutics. D.B.K.-S. and M.T.G. contributed to the preparation and editing of the full manuscript.

Conflict of interest

M.T.G. is a co-inventor of pending patent applications and planned patents directed to the use of recombinant neuroglobin and haem-based molecules as antidotes for CO poisoning, which have recently been licensed by Globin Solutions, Inc. M.T.G. is a shareholder, advisor and director in Globin Solutions, Inc. Additionally, and unrelated to CO poisoning, Dr. Gladwin is a co-inventor on patents directed to the use of nitrite salts in cardiovascular diseases, which have been licensed by United Therapeutics and Hope Pharmaceuticals, and is a co-investigator in a research collaboration with Bayer Pharmaceuticals to evaluate riociguate as a treatment for patients with SCD.

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