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Current perspectives and challenges in understanding the role of nitrite as an integral player in nitric oxide biology and therapy

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Abstract

Beyond an inert oxidation product of nitric oxide (NO) metabolism, current thinking posits a key role for nitrite as a mediator of NO-signaling, especially during hypoxia. This concept has been discussed both in the context of nitrite serving a role as an endogenous modulator of NO-homeostasis, but also from a novel clinical perspective whereby nitrite therapy may replete NO-signaling and prevent ischemic tissue injury. Indeed, the relatively rapid translation of studies delineating mechanisms of action to ongoing and planned clinical trials has been critical in fuelling interest in nitrite biology and several excellent reviews have been written on this topic. In this article we limit our discussions to current concepts, and what we feel are questions that remain unanswered within the paradigm of nitrite being a mediator of NO biology.

Our understanding of the functions of nitrite in biological systems has evolved considerably over the last twenty years. Typically thought of as an inactive end product of nitric oxide (NO) metabolism, initial studies documented the potential for nitrite reduction to NO under anoxic and / or acidic conditions [1-4]. The more recent interest in nitrite biology stems largely from the realization that the generation of NO from nitrite reduction can occur under conditions that span a range of oxygen tensions and pH observed over the pathophysiological spectrum [5-7]. In this paradigm, nitrite is a source for NO, with more NO being produced the more ischemic and acidic the tissue becomes. The potential for nitrite to be an hypoxic source for NO has also opened the door for nitrite therapeutics for various conditions characterized by ischemic injury, the pathogenesis of which is mediated in part by a loss of NO-signaling as exemplified by ischemia-reperfusion injury [8-11]. Interestingly, anecdotal therapies for ischemic disorders have utilized nitrite based therapies for over a century [7, 12]. It is only now that these long-standing insights are being integrated into systematic basic and clinical studies which have developed as the molecular mechanisms by which nitrite can mediate NO-signaling are being unraveled. These concepts and how perspectives on nitrite biology have evolved over the last century have been reviewed elegantly in a number of recent articles and will not be discussed in detail here [5-7, 9-20]. In this article we focus our discussions on key questions that we feel remain unanswered in the context of the paradigm of nitrite being an endogenous and therapeutic

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mediator of NO-homeostasis. Central amongst these are the mechanism(s) by which nitrite is reduced to NO and what biological parameters regulate these mechanisms and in turn how these may change during the transition from physiology to pathology.

Nitric oxide formation from nitrite reduction- role of metalloproteins

Despite early studies by Zhang et al [21, 22] and Millar et al [23] demonstrating that xanthine oxidoreductase can mediate the one-electron reduction of nitrite to NO under anoxic conditions, the potential for nitrite to serve as a substrate for NO generation remained largely ignored until recently. The resurgent interest in nitrite biology is predominantly associated with the elucidation of nitrite reduction mechanisms which can operate at physiologic oxygen tensions and pH. Most insights into this paradigm have implicated a key role for heme proteins in mediating nitrite reduction as exemplified by the reactions of nitrite (NO_2^-) with the ferrous heme (Fe^{II}) of either deoxyhemoglobin or deoxymyoglobin. In this reaction, nitrite is reduced to NO and the heme is oxidized to the ferric (Fe^{III}) oxidation state (Scheme 1).

Scheme 1

Interestingly, nitrite also reacts with hemoglobin or myoglobin when oxygenated ($\text{Fe}^{\text{II}}\text{O}_2$), but in this case nitrite oxidation to nitrate occurs (not shown, see [24]). This differential reactivity of nitrite reduction versus oxidation being controlled by oxygen tension (i.e. presence of deoxygenated vs oxygenated hemoglobin / myoglobin) illustrates the biochemical mechanism underlying the proposed model in which nitrite can selectively produce NO during graded hypoxia [5, 25-28]. However, proposing a biological role of pentacoordinated heme centers in stimulating NO-signaling via nitrite reduction comes with a significant problem arising from the fact that both ferrous deoxy and oxyheme react relatively rapidly (rate constants $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$) with NO to produce nitrosyl heme (Scheme 1) or nitrate respectively [29, 30]. Furthermore, the high concentrations of hemoglobin and myoglobin in red blood cells (20 mM) and cardiac/skeletal muscle (300-900 μM) [31, 32], determines that the reaction between their hemes and NO will be fast and might inhibit NO-signaling. That said, a variety of experimental evidence ranging from in vivo to isolated organ- and organelle-based studies all indicate that the combination of hypoxia, nitrite and either RBC, hemoglobin or myoglobin can stimulate NO-gas formation and / or NO-signaling. A notable example is data showing that protection afforded by nitrite administration in a model of ischemia-reperfusion in the heart is lost when myoglobin knock-out mice are used instead of wild type animals and this coincides with a loss of NO formation detected by EPR [33]. How is it possible then for heme proteins to function as sources of bioactive NO via nitrite reduction, when kinetic predictions strongly indicate that any NO produced should be scavenged?

How could 'NO-signaling activity' escape the clutches of ferrous heme?

Rifkind and colleagues have proposed that nitrite and deoxyhemoglobin reactions first yield an adduct that eventually gives rise to the formation of an $[\text{Hb}^{\text{II}}\text{-NO}^+ = \text{Hb}^{\text{III}}\text{-NO}]$ intermediate where the electron is delocalized between the heme and NO. The transformation of this intermediate into non-delocalized $\text{Hb}^{\text{III}}\text{-NO}$ precedes NO release from the heme center and has been shown to be favored when hemoglobin is in the T-state, a conformation populated during low oxygen conditions [34-36]. Whilst this reaction scheme may provide an additional degree of refinement in the regulation of nitrite-deoxyhemoglobin interactions, the fundamental kinetic obstacles associated with NO being generated in the presence of excess ferrous hemoglobin still remains. Compartmentalization of nitrite

reduction away from excess ferrous heme is one potential strategy by which kinetic barriers discussed above are bypassed. In this context, the preferential association of T-state deoxyhemoglobin with the red blood cell membrane within the context of a multi-protein metabolon has been proposed as a mechanism to facilitate NO escape from heme-mediated scavenging [15, 36-38]. However, considering that the net direction of NO diffusion in space is dictated by its concentration gradient [39], then the presence of such an important intracellular sink as hemoglobin should still prevent a significant portion of NO from escaping regardless of its site of production within the cell. In this regard, one hypothetical condition that could facilitate NO export from the red blood cell would be to have a high proportion of methemoglobin bound to those sites in the membrane at which nitrite is being reduced, as this would provide a diffusional barrier that would shield NO from scavenging by ferrous heme [40].

A second model suggests the reaction shown in scheme 1 is more complex than written and in fact proceeds via the formation of a diffusible intermediate that is less reactive with the heme than NO and thereby able to escape [41]. Recent evidence points towards the formation of the nitrosating agent dinitrogen trioxide (N_2O_3) as one such plausible intermediate for the generation of NO downstream from nitrite reduction (Scheme 2) [42-45].

Scheme 2

The production of N_2O_3 provides with an alternative pathway for NO formation following diffusion of the uncharged N_2O_3 away from the heme and homolysis to NO and nitrogen dioxide ($\bullet NO_2$) [43]. Another proposal involves ultimate formation of a stable S-nitrosothiol (RSNO) that is capable of diffusing out of the red blood cell [46]. Intermediate N_2O_3 formation may provide a mechanism for RSNO synthesis through nitrite reduction. Consistent with this model, exogenous administration of nitrite correlates with increased levels of S-nitrosothiols in red blood cells and other tissues both in vivo and in vitro [25, 43, 46-53]. Interestingly, per the reaction mechanism presented in scheme 2 deoxyhemoglobin functions as a catalyst for N_2O_3 formation from nitrite, which suggests that first, the amount of N_2O_3 (and possibly NO) produced will be directly dependent on nitrite availability and second, that nitrite reduction by hemoglobin is not necessarily associated with a 1:1 stoichiometry to methemoglobin formation.

Whereas this hypothesis helps circumvent the problems related with NO escape from red blood cells or Mb scavenging in muscle, there are still theoretical concerns regarding the viability of this mechanism [14, 54]. For instance, the dissociation constant for the formation of the methemoglobin-nitrite adduct [55], has been reported to be in the order of 1mM (pH 6.4, 25°C) [56] and more recently reassessed at 50-150 μ M (pH 6.5 at 37°C) or 0.5-2 mM at pH 7.4, 37°C [43, 57]. This suggests that the levels of this complex under physiological conditions (~300nM nitrite, ~100 μ M methemoglobin) will oscillate between 15 and 50nM in the red blood cell. However, if we consider that the formation of this adduct takes place in the presence of excess oxy- and deoxyhemoglobin, then it is likely that the concentration of the methemoglobin-nitrite adduct will be even lower. Furthermore, production of N_2O_3 according to scheme 2 requires the nitrite-methemoglobin adduct to outcompete both oxy- and deoxyhemoglobin in their reaction with NO which overall suggests that formation of N_2O_3 under physiological conditions might require special spatial localization conditions in the red blood cell [14]. Finally, the fact that N_2O_3 reacts with high rate constants with thiols

($k = 6.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ [58]) coupled to the presence of ~2-5mM glutathione in red blood cells may limit the ability of N_2O_3 to reach the vascular compartment. Therefore, although this mechanism alleviates one kinetic constraint (heme scavenging of NO), the kinetic constraint imposed by the high reactivity of N_2O_3 relative to its diffusion is still significant.

Also, when considering the reactivity of nitrite within intraerythrocytic hemoglobin in vivo, it is important to recognize that under physiological conditions both the oxygenated and deoxygenated forms of hemoglobin will co-exist and therefore can be expected to compete for reacting with nitrite. Interestingly, in the case of red blood cells, it has been shown that these two reactions not only occur in parallel under intermediate oxygen fractional saturations, but also recent data suggests that these two reactions interact significantly with each other [59]. More specifically, it has been shown that an intermediate(s) generated during the reaction between nitrite and oxyhemoglobin is capable of reacting with nitrosylhemoglobin produced during the reaction between nitrite and deoxyhemoglobin resulting in NO release in a process termed oxidative denitrosylation [59]. As a result, it is possible that both the formation of N_2O_3 as well as the facilitated release of NO from nitrosyl hemes via oxidative denitrosylation could synergize to allow net NO formation from nitrite in partially deoxygenated red blood cells and possibly in myoglobin-rich muscle.

Understanding the mechanisms by which hemoglobin or myoglobin affect nitrite reduction to mediate NO-dependent signaling remains an important goal and subject of ongoing research efforts. The lack of insights into how NO-signaling can be sustained has raised questions as to the applicability of heme mediated nitrite reduction to NO beyond the test tube. It is important to state then that the mechanism outlined in scheme 2 can explain the product profile, namely NO and nitroso species (e.g. S-nitrosothiols) observed upon addition of nitrite to hypoxic tissue. Moreover, evidence that myoglobin/nitrite interactions can promote NO-signaling in the context of an ischemic insult to the heart has been established using multiple criteria including the use of myoglobin knockout mice [28, 33, 53, 60]. The evidence for nitrite reactions with erythrocytic hemoglobin and stimulation of hypoxic blood flow remains more limited and is the subject of much debate [16, 61]. This question is far from trivial as the design of an experimental system capable of specifically scrutinizing the nitrite reductase activity of hemoglobin without interfering with either NO homeostasis or red blood cell function has proven to be difficult. For example, the reaction between nitrite and hemoglobin cannot be inhibited without also affecting either oxygen binding by hemoglobin or NO production by nitric oxide synthases. A non-invasive strategy capable of modulating nitrite/hemoglobin reactions without using heme blockers, allosteric modulators or NOS inhibitors will be required to shed further light in this particular area. In this context, and for nitrite biology in general, a specific nitrite scavenger that can function at physiologic pH, would be an ideal experimental tool; which to our knowledge does not exist as yet.

What about myoglobin / hemoglobin independent mechanisms of nitrite reduction?

Nitrite reduction is not exclusive to hemoglobin and myoglobin, with a variety of heme-containing and non-heme containing proteins including xanthine oxidoreductase [21, 22], aldehyde oxidase [62], neuroglobin [63, 64], eNOS [65], cytochrome c [47], carbonic anhydrase [66], cytochrome c oxidase [67], complex III [68], and cytochrome P450 [69] being suggested as mediators of NO-formation. Some of these mechanisms are also subject to questions about relevance in vivo. For example, xanthine oxidase dependent reduction of nitrite requires substantial hypoxia (or anoxia) or the presence of high concentrations of SOD to prevent NO scavenging by superoxide produced via oxygen reduction catalyzed by the same xanthine oxidoreductase [70, 71]. That said, nitrite reduction by xanthine oxidoreductase has been shown to have a potentially protective role within the context of cardiac ischemia in an isolated heart model [72]. Furthermore, some therapeutic effects of nitrite are sensitive to xanthine oxidoreductase inhibition [73, 74] again highlighting the lack

of knowledge connecting in vivo observations with biochemical understanding of nitrite reduction mechanisms. Notwithstanding these issues, the observation that multiple proteins may possess nitrite reducing capabilities poses an interesting question as how is nitrite metabolism regulated in the organism. Beyond the general paradigm of lower oxygen tensions and pH facilitating nitrite reduction, very little is known on how these proteins and their ability to reduce nitrite to NO is regulated by the availability of co-factors and substrates, the level of expression, post-translational modification or cellular / tissue localization. Emerging data are shedding some insights however. For example, sub-cellular compartmentalization of distinct nitrite-reducing activities has been reported in the liver [75]. Also the nitrite reductase activity of neuroglobin [63, 64, 70, 76] or cytochrome c [47] is negligible when the heme is hexacoordinated. However, NO-formation by these proteins is increased significantly under conditions that favor transition to a pentacoordinated state. In the case of cytochrome c, transition from hexa- to pentacoordination has been shown to be induced by Met-80 oxidation [77], Tyr-67 nitration [78] and by interaction with anionic lipids such as cardiolipin [79]. Similarly, neuroglobin will increase its penta-coordinated character upon oxidation of Cys46 and Cys55 to form a disulfide bond [64, 80-82]. These mechanisms suggest that nitrite reduction to NO by these proteins might be upregulated under conditions of oxidative stress and potentially be involved in cellular protection analogous to known pathways of anti-apoptotic and anti-inflammatory effects of NO. Delineating how the conversion of nitrite to NO is regulated by redox pathways is likely to reveal key insights into the function of endogenous nitrite and help define the parameters for nitrite therapy (discussed later).

In summary, the currently proposed mechanisms for nitrite reduction (largely elucidated in vitro) are consistent with the biochemical fingerprints of nitrite metabolism (elucidated in vivo), namely hypoxia dependent formation of NO and nitrosated thiols and amines. The major challenges remain theoretical and trying to rationalize NO-formation and bioactivity in the presence of ferrous heme. The above discussion can be summarized by stating that although nitrite-derived NO formation might be inefficient [75], it is still biologically and therapeutically important, which could simply reflect the fact that activation of NO-dependent signaling pathways requires relatively low concentrations of this mediator.

Nitrite-mediated vasodilatation: How can a hypoxia-activated NO donor function under normoxic conditions?

The observation that in vivo administration of nitrite either via direct infusion [25, 37, 83], dietary supplementation [84-86] or in the form of its two-electron oxidized precursor nitrate [87-89] leads to a decrease in blood pressure in normal and hypertensive patients with a concomitant increase in markers of NO formation played a pivotal role in establishing nitrite as a physiological NO-donor [6, 15]. In this scenario, nitrite dependent stimulation of NO-signaling occurs under normal or non-ischemic stress conditions. As mentioned earlier however, most literature reports show that metallo-protein mediated nitrite reduction becomes more prominent with hypoxia [5], raising the question are the proposed hypoxia sensitive mechanisms of nitrite reduction the same as those operating to mediate nitrate and nitrite dependent decreases in blood pressure under normal conditions. The answer is most likely yes since, the oxygen tension under non-stressed conditions at the level of the resistance pre-capillary arterioles is sufficiently low to induce hypoxemia encompassing oxygen tensions (30-40mHg) that result in significant hemoglobin deoxygenation [90]. In other words, hypoxemia is an integral part of vascular physiology which is further enhanced during ischemic stress. As mentioned above hemoglobin is one putative nitrite reductase that could mediate hypotensive effects of nitrate / nitrite and which would operate within the physiologic range of oxygen tensions and pH (hemoglobin dependent nitrite reduction is maximal at fractional saturations attained in resistance arterioles[26, 27, 90, 91]). Other

candidate circulating nitrite reductases include carbonic anhydrase [66], xanthine oxidoreductase, aldehyde oxidase [62, 71, 92, 93] although the role of these may be limited to more acidic environments or select vascular beds (e.g. pulmonary [74, 93, 94]) as xanthine oxidoreductase inhibition did not attenuate human forearm blood flow responses to nitrite under non-stressed conditions[95].

Regulation of nitrite metabolism: Reductase activity, oxygen levels or transport?

The sensitivity of nitrite dependent NO-formation to hypoxia is a common aspect of all the protein-dependent pathways that have been proposed to be involved in nitrite reduction. Many reports show that nitrite supplementation to intact organisms is associated with a widespread increase in the concentration of this anion and production of NO-derived species such as S-nitrosothiols or nitrosylhemes in several tissues[25, 43, 46, 48, 49, 96]. Therefore, it becomes apparent that when the levels of oxygen are sufficiently low, the production of NO by the different nitrite reductases is modulated at least in part by substrate (i.e. nitrite) availability. This concept is clear in the case of xanthine oxidoreductase, where the K_M for nitrite binding to the molybdenum site is approximately 2.5mM, two or three orders of magnitude higher than normal tissue levels[71]. A direct consequence of this line of reasoning is that nitrite reduction in vivo is not an on/off process but is occurring continuously within tissues. This suggests that both hypoxia and nitrite supplementation function by increasing the availability of substrates thereby increasing the flux through the nitrite reduction pathway. In other words, it is possible that beyond being a hypoxia-activated reservoir for NO, nitrite could in fact be functioning more as an NO-buffer, continually matching decreases in oxygen tension with increased NO formation. By coupling decreased oxygen supply to higher levels of NO formation a given organ can activate increases in blood flow, modulate oxygen diffusion within the tissue via the controlled inhibition of mitochondrial respiration and at the same time maintain an anti-apoptotic and anti-inflammatory environment.

The question then becomes whether, and if so how, nitrite substrate availability is controlled under basal conditions. Interestingly, intraperitoneal administration of nitrite in rats results in a rapid (≤ 5 min) redistribution of this anion to all organs [50, 96]. It is tempting to speculate that modulating nitrite transport into different tissues might be an effective way of controlling nitrite metabolism in the organism under different conditions, especially when you consider that the predominant form of nitrite (>99.9%) at pH 7 is the nitrite anion ($pK = 3.4$). Unfortunately, there have been relatively few studies interrogating the mechanisms by which nitrite is taken into different cell types and how this process might be regulated. Current perspectives postulate that nitrite diffuses across the plasma membrane by becoming protonated to nitrous acid [97] and, in the context of red cells from some species, by interacting with the anion exchanger 1 (AE-1) [98, 99]. In this regard, we recently suggested that whereas nitrite entry into the red blood cell is likely to be mediated mostly by passive diffusion in the form of nitrous acid, AE-1 might be involved in controlling net fluxes of nitrite transport across the red blood cell membrane by mediating nitrite export via an oxygen-sensitive process coupled to the hemoglobin oxygenation state [99]. Whilst these results still await confirmation, the idea that transport processes might be modulated by oxygen tension provides an appealing mechanism that would facilitate nitrite diffusion preferentially into tissues where it can be promptly reduced to yield NO.

sGC -dependent and independent mechanisms of nitrite action

Are all effects ascribed to nitrite mediated by NO formation? Beyond academic curiosity, this question is important to address when trying to understand whether or not endogenous

nitrite plays a role in NO-homeostasis and also in trying to selectively target or modulate a response by therapeutic nitrite. To date the majority of studies have shown that nitrite effects are NO-dependent and can be either sGC dependent or independent consistent with current paradigms of how nitric oxide synthase derived NO works. Evidence for a role for NO is dependent largely on attenuation of responses by the NO-scavenger CPTIO [25, 26, 52, 100] and measurement of nitrite-dependent increases in cGMP levels [26, 101]. Moreover, both in vitro and in vivo studies using inhibitors and transgenic mouse models that target the classical NO-dependent activation of the sGC-cGMP-PKG signaling pathway further show that the hypoxic effects of nitrite are mediated in large part through this mechanism and include vasodilation, protecting against ischemia reperfusion injury or LPS-induced endotoxemia [101]. Equally important are other studies showing the potential for nitrite administration to elicit effects that are NO-dependent but sGC independent, suggesting that other pathways are involved in nitrite-mediated responses. For instance, nitrite supplementation has been shown to inhibit smooth muscle proliferation by induction of p21^{Waf1/Cip1} [74], decrease HO-1 levels in non-ischemic mice [50], stimulate of hsp70 expression and activate the estrogen receptor alpha in a human breast cancer line [102]. Furthermore, a recent study has shown that nitrate supplementation to healthy humans at doses that increase circulating nitrite levels increase mitochondrial efficiency, likely via mechanisms involving down-regulation of proteins involved in proton leakage in the respiratory chain [103].

A potential mechanism by which nitrite could induce cGMP-independent effects is S-nitrosation of cysteine residues, which is being increasingly recognized as a potential nitrite-induced post-translational modification that can alter protein function [17, 43, 46, 47, 51, 96]. This is exemplified by the nitrite-induced S-nitrosation of complex I of the electron transport chain which decreases mitochondrial reactive species formation and subsequently confers protection against ischemia-reperfusion injury [104] [33, 86, 105, 106]. However, the mechanism by which nitrite mediates S-nitrosation of complex I is unclear. There are two likely pathways by which nitrite may mediate S-nitrosation in vivo [107], one involves the abstraction of a hydrogen atom from the target thiol followed by nitrite-derived NO addition (Scheme 3a) and the other is the direct nitrosation of the thiol by nitrite-derived dinitrogen trioxide (Scheme 3b).

As it can be observed from Schemes 1 and 2 above, nitrite can function as a source of both NO and N₂O₃ in the presence of heme proteins (and acidic conditions). These reaction mechanisms further support the potential for nitrite to be an endogenous and therapeutic mediator of S-nitrosation. Further data combining modulation of nitrite levels together with identification of protein thiol targets for nitrite-mediated nitrosation will be key in developing this paradigm further and likely provide new insights into how nitrite can affect NO-dependent signaling. It should be noted that NO-independent effects of nitrite have been suggested via direct nitrite-mediated nitrosation [75]. However, the underlying biochemical mechanism(s) for such a process remain to be elucidated. If verified, this mechanism may also alleviate concerns about rapid NO-scavenging outlined above.

What about nitrite-dependent protein nitration?

Generation of nitrogen dioxide and subsequent protein nitration from nitrite oxidation by peroxidases such as myeloperoxidase [108], eosinophil peroxidase [109] and also other heme proteins such as cytochrome c [110], hemoglobin [111, 112] and myoglobin [112, 113] was appreciated many years before the biologic potential for nitrite-reduction to NO was realized. Surprisingly, however, the potential role for nitration in responses elicited by nitrite in ischemic tissues has been largely ignored. Protein nitration has usually been considered a deleterious process that either alters protein function or targets it for degradation, which is basis for the use of 3-nitrotyrosine as a marker for oxidative and nitrative stress in vivo. In fact, nitration reactions could also explain the loss of protection observed when higher doses of nitrite are administered in the context of IR injury [52, 114]. An intriguing, yet unexplored possibility is that nitrite-derived nitration may in fact play a role in protecting tissues against inflammation. In this regard, nitration of both fatty acids and guanine nucleotides has been shown to elicit responses that are protective against inflammatory tissue injury [115, 116].

Nitrite therapy

The plethora of studies documenting protective effects of nitrite in cell culture or animal models, together with human use of nitrite in cyanide antidote kits, has laid the foundation for clinical evaluation of nitrite therapy for a variety of ischemia related disorders (summarized in [8, 11, 117]). The data from these and other planned trials, will no doubt play a big role in the level of future interest in nitrite-dependent effects. Several factors should be considered in the design and interpretation of future studies, and in this regard the choice of the targeted dose of nitrite is crucial. Murine based studies of hepatic and myocardial IR injury have shown a 'U' shaped profile for nitrite effects; namely protection increases with increasing dose up to a point and then as the dose is increased further, protection is lost and can -if even higher doses are administered, exacerbate injury [52, 114]. Fortunately, the nitrite dose range encompassing protection versus loss of protection in these studies was greater than two or three orders of magnitude. In principle, this should provide a therapeutic window in which nitrite can be administered safely whilst being effective. However, little is known about whether this therapeutic window changes for different types of ischemic injury (e.g. in different organs or different degrees of hypoxia), or whether it is affected by underlying diseases or patient demography (gender, race age etc). Moreover, we have noticed that the route of nitrite dosing in rats (continuous vs. bolus administration) can differentially affect hemodynamics in the presence or absence of anesthesia (unpublished observations). Additionally, the diet is an important source of nitrite (largely via secondary reduction from nitrate) and several studies have shown that it has the potential to contribute significantly to nitrite-dependent responses [84, 86-89, 118-121]. How this could affect nitrite dosing in a therapeutic setting is currently unknown, but taken together it seems clear that multiple factors are likely to influence the therapeutic window for nitrite in the clinical setting.

One final issue to be considered when assessing the potential of nitrite as a therapeutic agent is whether administered nitrite differs from endogenous nitrite. Specifically, experimental observations show that relatively low doses of nitrite are sufficient to induce dramatic protective effects in vivo. Typically doses of ~50 nmoles have been shown to be protective in models of ischemia reperfusion [52, 114] as well hemorrhagic shock in mice [122] for example. In some cases, increased nitrite levels are measured after administration, but in others no change in nitrite is observed despite clear evidence of NO-dependent signaling induction (a notable example being nitrite dependent stimulation of ischemic angiogenesis) [100, 123]. Furthermore, when the fact that endogenous nitrite levels vary depending on the

tissue [50] is taken into account, it is not readily apparent that when endogenous and exogenous levels are combined, a clear nitrite-dose vs. response relationship exists. This raises the question of whether there is a difference between endogenous and exogenously administered nitrite, and if so, what is the basis of such a difference. These issues remain to be addressed and could potentially have an impact in the determination of the dose of nitrite to use when designing clinical interventions in a tissue-specific manner.

Summary and future perspectives

We have come a long way since the first descriptions of nitrite as a source of NO with a potential role as modulator of blood flow and vascular function. However beyond a key role for lower oxygen tensions and pH many questions remain unanswered (Figure 1). For instance whereas the ability of hemoglobin and red blood cells to generate NO bioactivity via nitrite reduction has been often shown, the relevance of this pathway within the physiological context is still being investigated. Nevertheless, the search for a mechanism that explains how NO could escape from the red blood cell has allowed us to gain significant insights into pathways capable of mediating nitrite-dependent S-nitrosation at physiological pH and oxygen tension.

Another evolving question derives from the realization that there are several proteins that have been shown to be able to reduce nitrite to NO in the test tube. All of these pathways seem to require low levels of oxygen, a certain degree of acidification and an electron donor. How can nitrite reduction be controlled when there are so many seemingly redundant pathways? Are all these nitrite reductases functional enzyme-like proteins or do they just reflect the consequence of containing a metal center capable of mediating redox reactions within the range required for nitrite reduction? Until now the main working hypothesis suggests that these pathways will become gradually activated as the oxygen levels drop below individual thresholds and ischemia-derived acidification ensues [5]. However, only myoglobin and, to a lesser degree, xanthine oxidase have so far been identified as a requirement in order for nitrite to exert cytoprotective functions *in vivo* or *ex vivo*. Notably, the potential for differential transport of nitrite into tissues as a means of targeting nitrite reduction to specific organs has been recognized but it is still waiting to be tested within the context of nitrite-mediated cytoprotection.

Finally, the use of a wide range of both *in vitro* and *in vivo* models has provided a considerable amount of evidence that the potential of nitrite as a treatment for pathologies characterized by vascular dysfunction and ischemic injury is real. In this regard, the realization that nitrite can both mimic ischemic pre-conditioning and potentiate angiogenesis suggests the possibility that nitrite could be used as an effective treatment to improve the outcome of major procedures such as organ transplants. However, promising as these observations might be, there are questions such as the ones outlined above that still remain unanswered and whose elucidation will not only provide valuable basic knowledge but also will potentially allow us to better approach the design of more effective nitrite-based therapies.

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References

1. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med.* 1995; 1:804–809. [PubMed: 7585184]

2. Modin A, Bjorne H, Herulf M, Alving K, Weitzberg E, Lundberg JO. Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand*. 2001; 171:9–16. [PubMed: 11350258]
3. Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, McKenzie H. Stomach NO synthesis. *Nature*. 1994; 368:502–502. [PubMed: 8139683]
4. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut*. 1994; 35:1543–1546. [PubMed: 7828969]
5. van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J, Gladwin MT. Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev*. 2009
6. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR Jr, Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E. Nitrate and nitrite in biology, nutrition and therapeutics. *Nat Chem Biol*. 2009; 5:865–869. [PubMed: 19915529]
7. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. 2008; 7:156–167. [PubMed: 18167491]
8. Raat NJ, Shiva S, Gladwin MT. Effects of nitrite on modulating ROS generation following ischemia and reperfusion. *Adv Drug Deliv Rev*. 2009; 61:339–350. [PubMed: 19385092]
9. Dezfulian C, Raat N, Shiva S, Gladwin MT. Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc Res*. 2007; 75:327–338. [PubMed: 17568573]
10. Calvert JW, Lefer DJ. Myocardial protection by nitrite. *Cardiovasc Res*. 2009; 83:195–203. [PubMed: 19251721]
11. Calvert JW, Lefer DJ. Clinical translation of nitrite therapy for cardiovascular diseases. *Nitric Oxide*. 2010; 22:91–97. [PubMed: 19909823]
12. Butler AR, Feelisch M. Therapeutic uses of inorganic nitrite and nitrate: from the past to the future. *Circulation*. 2008; 117:2151–2159. [PubMed: 18427145]
13. Kapil V, Webb AJ, Ahluwalia A. Inorganic nitrate and the cardiovascular system. *Heart*. 2010; 96:1703–1709. [PubMed: 20736204]
14. Gladwin MT, Grubina R, Doyle MP. The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxidative denitrosylation, and nitrite reductase/anhydrase. *Acc Chem Res*. 2009; 42:157–167. [PubMed: 18783254]
15. Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol*. 2006; 291:H2026–2035. [PubMed: 16798825]
16. Patel RP, Hogg N, Kim-Shapiro DB. The potential role of the red blood cell in nitrite-dependent regulation of blood flow. *Cardiovasc Res*. 2011; 89:507–515. [PubMed: 20952416]
17. Shiva S. Mitochondria as metabolizers and targets of nitrite. *Nitric Oxide*. 2010; 22:64–74. [PubMed: 19788924]
18. Tang Y, Jiang H, Bryan NS. Nitrite and nitrate: cardiovascular risk-benefit and metabolic effect. *Curr Opin Lipidol*. 2011; 22:11–15. [PubMed: 21102328]
19. Weitzberg E, Hezel M, Lundberg JO. Nitrate-nitrite-nitric oxide pathway: implications for anesthesiology and intensive care. *Anesthesiology*. 2010; 113:1460–1475. [PubMed: 21045638]
20. Kevil CG, Lefer DJ. Review focus on inorganic nitrite and nitrate in cardiovascular health and disease. *Cardiovasc Res*. 2011; 89:489–491. [PubMed: 21183508]
21. Zhang Z, Naughton D, Winyard PG, Benjamin N, Blake DR, Symons MC. Generation of nitric oxide by a nitrite reductase activity of xanthine oxidase: a potential pathway for nitric oxide formation in the absence of nitric oxide synthase activity. *Biochem Biophys Res Commun*. 1998; 249:767–772. [PubMed: 9731211]
22. Zhang Z, Naughton DP, Blake DR, Benjamin N, Stevens CR, Winyard PG, Symons MC, Harrison R. Human xanthine oxidase converts nitrite ions into nitric oxide (NO). *Biochem Soc Trans*. 1997; 25:524S. [PubMed: 9388740]

23. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Letters*. 1998; 427:225–228. [PubMed: 9607316]
24. Keszler A, Piknova B, Schechter AN, Hogg N. The reaction between nitrite and oxyhemoglobin: a mechanistic study. *J Biol Chem*. 2008; 283:9615–9622. [PubMed: 18203719]
25. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Wacławski MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO 3rd, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003; 9:1498–1505. [PubMed: 14595407]
26. Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, Darley-Usmar VM, Kerby JD, Lang JD Jr. Kraus D, Ho C, Gladwin MT, Patel RP. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood*. 2006; 107:566–574. [PubMed: 16195332]
27. Huang Z, Shiva S, Kim-Shapiro DB, Patel RP, Ringwood LA, Irby CE, Huang KT, Ho C, Hogg N, Schechter AN, Gladwin MT. Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J Clin Invest*. 2005; 115:2099–2107. [PubMed: 16041407]
28. Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM, Gladwin MT. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res*. 2007; 100:654–661. [PubMed: 17293481]
29. Cassoly R, Gibson Q. Conformation, co-operativity and ligand binding in human hemoglobin. *J Mol Biol*. 1975; 91:301–313. [PubMed: 171411]
30. Herold S, Exner M, Nauser T. Kinetic and mechanistic studies of the NO^{*}- mediated oxidation of oxymyoglobin and oxyhemoglobin. *Biochemistry*. 2001; 40:3385–3395. [PubMed: 11258960]
31. Bekedam MA, van Beek-Harmsen BJ, van Mechelen W, Boonstra A, van der Laarse WJ. Myoglobin concentration in skeletal muscle fibers of chronic heart failure patients. *Journal of Applied Physiology*. 2009; 107:1138–1143. [PubMed: 19661455]
32. Wittenberg JB, Wittenberg BA. Myoglobin function reassessed. *J Exp Biol*. 2003; 206:2011–2020. [PubMed: 12756283]
33. Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT, Kelm M, Rassaf T. Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A*. 2008; 105:10256–10261. [PubMed: 18632562]
34. Nagababu E, Ramasamy S, Rifkind JM. Intermediates detected by visible spectroscopy during the reaction of nitrite with deoxyhemoglobin: the effect of nitrite concentration and diphosphoglycerate. *Biochemistry*. 2007; 46:11650–11659. [PubMed: 17880185]
35. Rifkind JM, Nagababu E, Ramasamy S. Nitric oxide redox reactions and red cell biology. *Antioxid Redox Signal*. 2006; 8:1193–1203. [PubMed: 16910767]
36. Rifkind JM, Nagababu E, Ramasamy S. The quaternary hemoglobin conformation regulates the formation of the nitrite-induced bioactive intermediate and the dissociation of nitric oxide from this intermediate. *Nitric Oxide*. 2011
37. Rifkind JM, Nagababu E, Cao Z, Barbiro-Michaely E, Mayevsky A. Nitrite-induced improved blood circulation associated with an increase in a pool of RBC-NO with no bioactivity. *Adv Exp Med Biol*. 2009; 645:27–34. [PubMed: 19227446]
38. Salgado MT, Nagababu E, Rifkind JM. Quantification of intermediates formed during the reduction of nitrite by deoxyhemoglobin. *J Biol Chem*. 2009; 284:12710–12718. [PubMed: 19270306]
39. Lancaster JR Jr. A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide*. 1997; 1:18–30. [PubMed: 9701041]
40. Vaughn MW, Huang KT, Kuo L, Liao JC. Erythrocytes possess an intrinsic barrier to nitric oxide consumption. *J Biol Chem*. 2000; 275:2342–2348. [PubMed: 10644684]
41. Jeffers A, Xu X, Huang KT, Cho M, Hogg N, Patel RP, Kim-Shapiro DB. Hemoglobin mediated nitrite activation of soluble guanylyl cyclase. *Comp Biochem Physiol A Mol Integr Physiol*. 2005; 142:130–135. [PubMed: 15936233]

42. Robinson JM, Lancaster JR Jr. Hemoglobin-mediated, hypoxia-induced vasodilation via nitric oxide: mechanism(s) and physiologic versus pathophysiologic relevance. *Am J Respir Cell Mol Biol.* 2005; 32:257–261. [PubMed: 15778415]
43. Basu S, Grubina R, Huang J, Conradie J, Huang Z, Jeffers A, Jiang A, He X, Azarov I, Seibert R, Mehta A, Patel R, King SB, Hogg N, Ghosh A, Gladwin MT, Kim-Shapiro DB. Catalytic generation of N₂O₃ by the concerted nitrite reductase and anhydrase activity of hemoglobin. *Nat Chem Biol.* 2007; 3:785–794. [PubMed: 17982448]
44. Fernandez BO, Ford PC. Nitrite catalyzes ferriheme protein reductive nitrosylation. *Journal of the American Chemical Society.* 2003; 125:10510–10511. [PubMed: 12940720]
45. Ford PC. Reactions of NO and nitrite with heme models and proteins. *Inorg Chem.* 2010; 49:6226–6239. [PubMed: 20666383]
46. Angelo M, Singel DJ, Stamler JS. An S-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate. *Proc Natl Acad Sci U S A.* 2006; 103:8366–8371. [PubMed: 16717191]
47. Basu S, Azarova NA, Font MD, King SB, Hogg N, Gladwin MT, Shiva S, Kim-Shapiro DB. Nitrite reductase activity of cytochrome c. *J Biol Chem.* 2008; 283:32590–32597. [PubMed: 18820338]
48. Crawford JH, Chacko BK, Pruitt HM, Pikhova B, Hogg N, Patel RP. Transduction of NO-bioactivity by the red blood cell in sepsis: novel mechanisms of vasodilation during acute inflammatory disease. *Blood.* 2004; 104:1375–1382. [PubMed: 15150083]
49. Nagababu E, Ramasamy S, Rifkind JM. S-nitrosohemoglobin: a mechanism for its formation in conjunction with nitrite reduction by deoxyhemoglobin. *Nitric Oxide.* 2006; 15:20–29. [PubMed: 16545588]
50. Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol.* 2005; 1:290–297. [PubMed: 16408059]
51. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourdain D, Kelm M. Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J.* 2002; 16:1775–1785. [PubMed: 12409320]
52. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest.* 2005; 115:1232–1240. [PubMed: 15841216]
53. Rassaf T, Flogel U, Drexhage C, Hendgen-Cotta U, Kelm M, Schrader J. Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetics and function. *Circ Res.* 2007; 100:1749–1754. [PubMed: 17495223]
54. Schwab DE, Stamler JS, Singel DJ. Nitrite-methemoglobin inadequate for hypoxic vasodilation. *Nat Chem Biol.* 2009; 5:366. author reply 367. [PubMed: 19448598]
55. Yi J, Safo MK, Richter-Addo GB. The nitrite anion binds to human hemoglobin via the uncommon O-nitrito mode. *Biochemistry.* 2008; 47:8247–8249. [PubMed: 18630930]
56. Rodkey FL. A mechanism for the conversion of oxyhemoglobin to methemoglobin by nitrite. *Clin Chem.* 1976; 22:1986–1990. [PubMed: 11898]
57. Goetz BI, Shields HW, Basu S, Wang P, King SB, Hogg N, Gladwin MT, Kim-Shapiro DB. An electron paramagnetic resonance study of the affinity of nitrite for methemoglobin. *Nitric Oxide.* 2010; 22:149–154. [PubMed: 19895897]
58. Keshive M, Singh S, Wishnok JS, Tannenbaum SR, Deen WM. Kinetics of S-nitrosation of thiols in nitric oxide solutions. *Chem Res Toxicol.* 1996; 9:988–993. [PubMed: 8870986]
59. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, Ringwood LA, Jiang A, Hogg N, Kim-Shapiro DB, Gladwin MT. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. *J Biol Chem.* 2007; 282:12916–12927. [PubMed: 17322300]
60. Hendgen-Cotta UB, Kelm M, Rassaf T. A highlight of myoglobin diversity: the nitrite reductase activity during myocardial ischemia-reperfusion. *Nitric Oxide.* 2010; 22:75–82. [PubMed: 19836457]

61. Allen BW, Stamler JS, Piantadosi CA. Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation. *Trends in Molecular Medicine*. 2009; 15:452–460. [PubMed: 19781996]
62. Li H, Kundu TK, Zweier JL. Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. *J Biol Chem*. 2009; 284:33850–33858. [PubMed: 19801639]
63. Petersen MG, Dewilde S, Fago A. Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions. *J Inorg Biochem*. 2008; 102:1777–1782. [PubMed: 18599123]
64. Tiso M, Tejero J, Basu S, Azarov I, Wang X, Simplaceanu V, Frizzell S, Jayaraman T, Geary L, Shapiro C, Ho C, Shiva S, Kim-Shapiro DB, Gladwin MT. Human neuroglobin functions as a redox regulated nitrite reductase. *Journal of Biological Chemistry*.
65. Gautier C, van Faassen E, Mikula I, Martasek P, Slama-Schwok A. Endothelial nitric oxide synthase reduces nitrite anions to NO under anoxia. *Biochem Biophys Res Commun*. 2006; 341:816–821. [PubMed: 16442076]
66. Aamand R, Dalsgaard T, Jensen FB, Simonsen U, Roepstorff A, Fago A. Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. *Am J Physiol Heart Circ Physiol*. 2009; 297:H2068–2074. [PubMed: 19820197]
67. Castello PR, David PS, McClure T, Crook Z, Poyton RO. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: Implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metabolism*. 2006; 3:277–287. [PubMed: 16581005]
68. Kozlov AV, Staniek K, Nohl H. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Letters*. 1999; 454:127–130. [PubMed: 10413109]
69. Li H, Liu X, Cui H, Chen YR, Cardounel AJ, Zweier JL. Characterization of the mechanism of cytochrome P450 reductase-cytochrome P450-mediated nitric oxide and nitrosothiol generation from organic nitrates. *J Biol Chem*. 2006; 281:12546–12554. [PubMed: 16527817]
70. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the Effects of Oxygen on Xanthine Oxidase-mediated Nitric Oxide Formation. *Journal of Biological Chemistry*. 2004; 279:16939–16946. [PubMed: 14766900]
71. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. *J Biol Chem*. 2001; 276:24482–24489. [PubMed: 11312267]
72. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia reperfusion damage. *Proc Natl Acad Sci U S A*. 2004; 101:13683–13688. [PubMed: 15347817]
73. Tripatara P, Patel NS, Webb A, Rathod K, Lecomte FM, Mazzone E, Cuzzocrea S, Yaqoob MM, Ahluwalia A, Thiemermann C. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. *J Am Soc Nephrol*. 2007; 18:570–580. [PubMed: 17202421]
74. Zuckerbraun BS, Shiva S, Ifedigbo E, Mathier MA, Mollen KP, Rao J, Bauer PM, Choi JJ, Curtis E, Choi AM, Gladwin MT. Nitrite potently inhibits hypoxic and inflammatory pulmonary arterial hypertension and smooth muscle proliferation via xanthine oxidoreductase-dependent nitric oxide generation. *Circulation*. 2010; 121:98–109. [PubMed: 20026772]
75. Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, Ashrafian H. Tissue Processing of Nitrite in Hypoxia: AN INTRICATE INTERPLAY OF NITRIC OXIDE-GENERATING AND -SCAVENGING SYSTEMS. *J Biol Chem*. 2008; 283:33927–33934. [PubMed: 18835812]
76. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the Magnitude and Kinetics of Xanthine Oxidase-Catalyzed Nitrate Reduction: Evaluation of Its Role in Nitrite and Nitric Oxide Generation in Anoxic Tissues. *Biochemistry*. 2003; 42:1150–1159. [PubMed: 12549937]
77. Chen Y-R, Deterding LJ, Sturgeon BE, Tomer KB, Mason RP. Protein Oxidation of Cytochrome c by Reactive Halogen Species Enhances Its Peroxidase Activity. *Journal of Biological Chemistry*. 2002; 277:29781–29791. [PubMed: 12050149]
78. Cassina AM, Hodara R, Souza JM, Thomson L, Castro L, Ischiropoulos H, Freeman BA, Radi R. Cytochrome c Nitration by Peroxynitrite. *Journal of Biological Chemistry*. 2000; 275:21409–21415. [PubMed: 10770952]

79. Basova LV, Kurnikov IV, Wang L, Ritov VB, Belikova NA, Vlasova II, Pacheco AA, Winnica DE, Peterson J, Bayir H, Waldeck DH, Kagan VE. Cardiolipin Switch in Mitochondria: Shutting off the Reduction of Cytochrome c and Turning on the Peroxidase Activity. *Biochemistry*. 2007; 46:3423–3434. [PubMed: 17319652]
80. Hamdane D, Kiger L, Dewilde S, Green BN, Pesce A, Uzan J, Burmester T, Hankeln T, Bolognesi M, Moens L, Marden MC. The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin. *J Biol Chem*. 2003; 278:51713–51721. [PubMed: 14530264]
81. Hamdane D, Kiger L, Dewilde S, Green BN, Pesce A, Uzan J, Burmester T, Hankeln T, Bolognesi M, Moens L, Marden MC. Coupling of the heme and an internal disulfide bond in human neuroglobin. *Micron*. 2004; 35:59–62. [PubMed: 15036292]
82. Nicolis S, Monzani E, Ciaccio C, Ascenzi P, Moens L, Casella L. Reactivity and endogenous modification by nitrite and hydrogen peroxide: does human neuroglobin act only as a scavenger? *Biochem J*. 2007; 407:89–99. [PubMed: 17600531]
83. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO III, Schechter AN, Gladwin MT. Nitrite Infusion in Humans and Nonhuman Primates: Endocrine Effects, Pharmacokinetics, and Tolerance Formation. *Circulation*. 2007; 116:1821–1831. [PubMed: 17893272]
84. Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:19144–19149. [PubMed: 18025468]
85. Bryan NS, Calvert JW, Gundewar S, Lefer DJ. Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. *Free radical biology & medicine*. 2008; 45:468–474. [PubMed: 18501719]
86. Raat NJ, Noguchi AC, Liu VB, Raghavachari N, Liu D, Xu X, Shiva S, Munson PJ, Gladwin MT. Dietary nitrate and nitrite modulate blood and organ nitrite and the cellular ischemic stress response. *Free Radic Biol Med*. 2009; 47:510–517. [PubMed: 19464364]
87. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, MacAllister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO. *Hypertension*. 2010; 56:274–281. [PubMed: 20585108]
88. Larsen FJ, Eklblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of Dietary Nitrate on Blood Pressure in Healthy Volunteers. *New England Journal of Medicine*. 2006; 355:2792–2793. [PubMed: 17192551]
89. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 2008; 51:784–790. [PubMed: 18250365]
90. Tsai AG, Cabrales P, Hangai-Hoger N, Intaglietta M. Oxygen distribution and respiration by the microcirculation. *Antioxid Redox Signal*. 2004; 6:1011–1018. [PubMed: 15548898]
91. Bertuglia S, Giusti A. Role of nitric oxide in capillary perfusion and oxygen delivery regulation during systemic hypoxia. *American journal of physiology. Heart and circulatory physiology*. 2005; 288:H525–531. [PubMed: 15650155]
92. Webb AJ, Milsom AB, Rathod KS, Chu WL, Qureshi S, Lovell MJ, Lecomte FM, Perrett D, Raimondo C, Khoshbin E, Ahmed Z, Uppal R, Benjamin N, Hobbs AJ, Ahluwalia A. Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. *Circulation research*. 2008; 103:957–964. [PubMed: 18818408]
93. Badejo AM, Hodnette C, Dhaliwal JS, Casey DB, Pankey E, Murthy SN, Nossaman BD, Hyman AL, Kadowitz PJ. Mitochondrial aldehyde dehydrogenase mediates vasodilator responses of glycyl trinitrate and sodium nitrite in the pulmonary vascular bed of the rat. *American Journal of Physiology - Heart and Circulatory Physiology*. 2010; 299:H819–H826. [PubMed: 20543077]
94. Blood AB, Schroeder HJ, Terry MH, Merrill-Henry J, Bragg SL, Vrancken K, Liu T, Herring JL, Sowers LC, Wilson SM, Power GG. Inhaled Nitrite Reverses Hemolysis-Induced Pulmonary Vasoconstriction in Newborn Lambs Without Blood Participation. *Circulation*. 2011; 123:605–612. [PubMed: 21282501]

95. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO 3rd, Schechter AN, Gladwin MT. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation*. 2007; 116:1821–1831. [PubMed: 17893272]
96. Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, Rodriguez JR, Feelisch M. Cellular targets and mechanisms of nitros(yl)ation: An insight into their nature and kinetics in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:4308–4313. [PubMed: 15014175]
97. Samouilov A, Woldman YY, Zweier JL, Khramtsov VV. Magnetic resonance study of the transmembrane nitrite diffusion. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society*. 2007; 16:362–370. [PubMed: 17306575]
98. Jensen FB, Rohde S. Comparative analysis of nitrite uptake and hemoglobin-nitrite reactions in erythrocytes: sorting out uptake mechanisms and oxygenation dependencies. *Am J Physiol Regul Integr Comp Physiol*. 2010; 298:R972–982. [PubMed: 20130222]
99. Vitturi DA, Teng X, Toledo JC, Matalon S, Lancaster JR Jr. Patel RP. Regulation of nitrite transport in red blood cells by hemoglobin oxygen fractional saturation. *Am J Physiol Heart Circ Physiol*. 2009; 296:H1398–1407. [PubMed: 19286940]
100. Kumar D, Branch BG, Pattillo CB, Hood J, Thoma S, Simpson S, Illum S, Arora N, Chidlow JH Jr. Langston W, Teng X, Lefer DJ, Patel RP, Kevil CG. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc Natl Acad Sci U S A*. 2008; 105:7540–7545. [PubMed: 18508974]
101. Cauwels A, Buys ES, Thoonen R, Geary L, Delanghe J, Shiva S, Brouckaert P. Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase-dependent manner. *J Exp Med*. 2009; 206:2915–2924. [PubMed: 19934018]
102. Veselik DJ, Divekar S, Dakshanamurthy S, Storchan GB, Turner JMA, Graham KL, Huang L, Stoica A, Martin MB. Activation of Estrogen Receptor- α by the Anion Nitrite. *Cancer Research*. 2008; 68:3950–3958. [PubMed: 18483281]
103. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E. Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans. *Cell Metabolism*. 2011; 13:149–159. [PubMed: 21284982]
104. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ, Gladwin MT. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med*. 2007; 204:2089–2102. [PubMed: 17682069]
105. Brown GC, Borutaite V. Inhibition of mitochondrial respiratory complex I by nitric oxide, peroxynitrite and S-nitrosothiols. *Biochim Biophys Acta*. 2004; 1658:44–49. [PubMed: 15282173]
106. Dezfulian C, Shiva S, Alekseyenko A, Pendyal A, Beiser DG, Munasinghe JP, Anderson SA, Chesley CF, Vanden Hoek TL, Gladwin MT. Nitrite therapy after cardiac arrest reduces reactive oxygen species generation, improves cardiac and neurological function, and enhances survival via reversible inhibition of mitochondrial complex I. *Circulation*. 2009; 120:897–905. [PubMed: 19704094]
107. Goldstein S, Czapski G. Mechanism of the nitrosation of thiols and amines by oxygenated NO solutions: the nature of the nitrosating intermediates. *J Am Chem Soc*. 1996; 118:3419–3425.
108. van der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of Reactive Nitrogen Species during Peroxidase-catalyzed Oxidation of Nitrite. *Journal of Biological Chemistry*. 1997; 272:7617–7625. [PubMed: 9065416]
109. Wu W, Chen Y, Hazen SL. Eosinophil Peroxidase Nitrates Protein Tyrosyl Residues. *Journal of Biological Chemistry*. 1999; 274:25933–25944. [PubMed: 10464338]
110. Castro L, Eiserich JP, Sweeney S, Radi R, Freeman BA. Cytochrome c: a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration. *Arch Biochem Biophys*. 2004; 421:99–107. [PubMed: 14678790]
111. Grzelak A, Balcerczyk A, Mateja A, Bartosz G. Hemoglobin can nitrate itself and other proteins. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 2001; 1528:97–100.

112. Herold S. Nitrotyrosine, dityrosine, and nitrotryptophan formation from metmyoglobin, hydrogen peroxide, and nitrite. *Free Radical Biology and Medicine*. 2004; 36:565–579. [PubMed: 14980701]
113. Kilinc K, Kilinc A, Wolf RE, Grisham MB. Myoglobin-Catalyzed Tyrosine Nitration: No Need for Peroxynitrite. *Biochemical and Biophysical Research Communications*. 2001; 285:273–276. [PubMed: 11444837]
114. Jung KH, Chu K, Ko SY, Lee ST, Sinn DI, Park DK, Kim JM, Song EC, Kim M, Roh JK. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. *Stroke*. 2006; 37:2744–2750. [PubMed: 17008610]
115. Akaike T, Fujii S, Sawa T, Ihara H. Cell signaling mediated by nitrated cyclic guanine nucleotide. Nitric Oxide. 2010; 23:166–174. [PubMed: 20601047]
116. Khoo NK, Freeman BA. Electrophilic nitro-fatty acids: anti-inflammatory mediators in the vascular compartment. *Current Opinion in Pharmacology*. 2010; 10:179–184. [PubMed: 20080062]
117. Kevil CG, Kolluru G, Patillo C, Giordano T. Inorganic nitrite therapy: past, present, and future. *Free Radical Biology and Medicine*. 2011 In Press.
118. Tang Y, Jiang H, Bryan NS. Nitrite and nitrate: cardiovascular risk-benefit and metabolic effect. *Curr Opin Lipidol*. 2010; 22:11–15. [PubMed: 21102328]
119. Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim E, Kraus WE, Allen JD. Dietary Nitrate Supplementation Enhances Exercise Performance in Peripheral Arterial Disease. *Journal of Applied Physiology*. 2011
120. Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, Gilchrist M, Benjamin N, Jones AM. Acute dietary nitrate supplementation improves cycling time trial performance. *Med Sci Sports Exerc*. 2011; 43:1125–1131. [PubMed: 21471821]
121. Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O₂ cost of walking and running: a placebo-controlled study. *Journal of Applied Physiology*. 2011; 110:591–600. [PubMed: 21071588]
122. Rodriguez C, Vitturi DA, He J, Vandromme M, Brandon A, Hutchings A, Rue LW 3rd, Kerby JD, Patel RP. Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction. *The Biochemical journal*. 2009; 422:423–432. [PubMed: 19555351]
123. Ingram TE, Pinder AG, Bailey DM, Fraser AG, James PE. Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. *Am J Physiol Heart Circ Physiol*. 2010; 298:H331–339. [PubMed: 19940079]

Figure 1. Current perspectives on regulation and function of nitrite as a mediator of NO-signaling. Blue boxes denote known and potential regulatory mechanisms for controlling nitrite disposition and reduction to NO. Red boxes denote known and potential targets for nitrite derived NO and other reactive nitrogen species that could mediate acute and / or chronic effects of endogenous and therapeutic nitrite.