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Nitric oxide production pathways in erythrocytes and plasma*

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Abstract

Nitric oxide (NO) is a potent regulator of vascular tone and hemorheology. The signaling function of NO was largely unappreciated until approximately 30 years ago, when the endothelium-derived relaxing factor (EDRF) was identified as NO. Since then, NO from the endothelium has been considered the major source of NO in the vasculature and a contributor to the paracrine regulation of blood hemodynamics. Because NO is highly reactive, and its half-life in vivo is only a few seconds (even less in the bloodstream), any NO bioactivity derived from the intraluminal region has traditionally been considered insignificant. However, the availability and significance of NO signaling molecules derived from intraluminal sources, particularly erythrocytes, have gained attention in recent years. Multiple potential sources of NO bioactivity have been identified in the blood, but unresolved questions remain concerning these proposed sources and how the NO released via these pathways actually interacts with intravascular and extravascular targets. Here we review the hypotheses that have been put forward concerning blood-borne NO and its contribution to hemorheological properties and the regulation of vascular tone, with an emphasis on the quantitative aspects of these processes.

Keywords

Erythrocytes; quantitative analysis; nitrite reduction; intraerythrocytic hemoglobin; S-nitrosohemoglobin

1. Introduction

Nitric oxide (NO) is a pivotal signaling molecule that regulates blood flow and hemorheology, stimulating vasoactivity, inhibiting smooth muscle cell proliferation, inhibiting platelet adhesion, and regulating cell adhesion, vascular permeability, and erythrocyte deformability [1]. NO can be enzymatically synthesized in a reaction catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Since the discovery of NO as an important signaling molecule more than 30 years ago, the NO molecules synthesized by endothelial NOS present in the vascular endothelium have been considered the dominant source in the vasculature [34, 50].

NOS3 is localized in the endothelial lining of the vascular wall, adjacent to the smooth muscle of the vessel. This enzyme catalyzes the degradation of L-arginine to L-citrulline, with the release of NO molecules [1]. Once formed, NO diffuses out of the endothelial cells

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and enters the extravascular and intraluminal spaces [43]. In the vascular wall, NO reacts with soluble guanylate cyclase (sGC), which catalyzes the formation of 3',5'-cyclic guanosine monophosphate (cGMP) to induce vasodilation. In the extravascular region, NO can inhibit mitochondrial respiration by binding to the heme group of cytochrome c oxidase, further regulating oxygen delivery to tissue [19]. Inside the lumen, NO can reach its cellular targets and regulates certain rheologic factors, such as erythrocyte deformability and platelet-platelet adhesion [9, 11] at least partially through the transient interaction with the heme group of guanylate cyclase expressed on erythrocytes and platelets [4, 48, 56]. Other proposed regulation mechanisms may involve the interaction between NO and ion transport channels on erythrocytes and platelets [21, 55]. Thus, in the vasculature, NO can be consumed as a result of its reactivity with a variety of species in the vasculature, including O₂, reactive oxygen species, reactive nitrogen species, and cytochrome c oxidase, while carrying out its signaling role.

The life-time of NO in the extravascular tissue is relatively longer, on the scale of sub-seconds to a few seconds [43, 65]. However, inside the lumen of the blood vessel, the NO that is formed is rapidly scavenged by hemoglobin contained in erythrocytes and converted into relatively inert nitrite and nitrate [59], and its lifetime can be as short as a few milliseconds [36, 65]. The surviving NO in the lumen has the capacity to regulate hemorheological properties. However, for many years the blood was viewed solely as a sink for NO molecules. Targets of NO signaling in the lumen were thought to be regulated by locally produced NO from the endothelium. One interesting possibility is that NO could be "carried" through the circulation by sources in the blood itself, with the NO bioactivity being released when needed and thereby providing an endocrine regulation of hemorheology. Also, if a significant amount of NO from these sources could eventually reach the smooth muscle and react with sGC, vascular tone could be regulated by the blood-borne NO. However, in vitro experiments and theoretical modeling suggested that most of the free NO in the bloodstream is rapidly converted to relatively inert nitrite and nitrate [28, 39, 42, 43] because of the fast reactivity of NO with hemoglobin contained in erythrocytes and the abundance of such hemoglobin in the blood. These findings appeared to close the door on the possibility that NO bioactivity could be stored in the blood and that blood-borne NO could regulate vascular tone and other biological functions.

The NO concentration around blood vessels has been measured, and the values reported have been in the range of 200–1000 nM [54, 67, 72], with the measured NO generally considered to be endothelial in origin. However, the microelectrodes used in these experiments measured the local NO level (discussed in Ref. [14]) that is derived from all possible NO sources in the vasculature. Furthermore, theoretical biochemical pathway analyses [15, 16] have shown that NOS3-derived NO cannot account for the large concentration of NO measured in the perivascular region, suggesting a discrepancy between the predicted and measured NO concentrations. This discrepancy, coupled with real-time NO concentration mapping evidence, indicated that a significant amount of NO might be produced from the intraluminal sources and might reach the vascular wall [14, 38]. Indeed, several hypotheses have been proposed since the 1990s to account for the possible release of NO from the blood (reviewed below).

Despite the theoretical prediction that NO cannot escape the scavenging of erythrocyte-contained hemoglobin, experiments have provided strong evidence of NO bioactivity after its release from erythrocytes. For example, inhalation of NO gas can reduce pulmonary hypertension through NO-related mechanisms [76]. In the 1990s, a novel endocrine signaling pathway was proposed [63] in which NO bioactivity was hypothesized to be preserved, rather than destroyed, by blood proteins, with the NO molecules being released under certain conditions, particularly hypoxia. Furthermore, nitrite, which in the past has

been considered a stable species, has also been proposed to be reduced to NO radicals in low-pH environment or by hemoglobin in erythrocytes (and possibly by other heme-containing globins in other cells beyond the intraluminal region) [33, 60, 78]. Recent studies have also shown evidence of NO release from other sources, such as iron-nitrosyl-hemoglobin (HbNO) and via the activity of NOS3 in erythrocytes. The likely pathways that lead to NO release are summarized in Fig. 1. In addition to in vitro and ex vivo experiments that have focused on the specific pathways for the release of NO, in vivo measurements in animals have been conducted to detect NO in the blood. Kashiwagi et al. [38] observed strong fluorescent signals from intraluminal sources from their in vivo experiments using 4,5-diaminofluorescein diacetate in rat mesentery, suggesting the existence of intraluminal NO within the mesenteric vessels.

The potential release of intraluminal NO poses several conceptual challenges: First, it is not clear how the NO molecules that are formed can escape being scavenged by hemoglobin. The large quantity of erythrocyte-contained hemoglobin acts as a natural sink for the highly reactive NO molecules. Second, we still lack direct and specific measurements of the amount of NO derived from each source, especially from the intraluminal region. Finally, we have only an incomplete understanding of the quantity of NO released from the blood and how this blood-derived NO compares to endothelial NO in terms of physiological activity. The NO synthesized by NOS3 in endothelial cells diffuses to other parts of the vascular wall and to the extravascular region, and its periarteriolar level is reported as several hundred nanomolar [77]. How much NO can reach that region from the blood and there regulate a variety of physiological functions?

In the next section, we will review the current hypotheses regarding the release of bioactive NO from the blood and discuss the likely mechanisms by which NO molecules might survive the scavenging activity of hemoglobin.

2. Mechanisms of NO transport within the intraluminal region

In considering NO transport within the lumen of the blood vessel and its signaling activity, particularly in the case of NO derived from erythrocytes, the first question that arises is how NO molecules can be transported after their formation. Is NO transported as the free nitrogen monoxide itself? Are there more stable intermediate species that are involved in NO production from its original source? Must NO be bound to other species in order to carry out its regulatory function? A thorough understanding of these processes will help us to achieve a complete picture of the physiology of NO signaling and develop NO-targeted therapeutics, such as vasodilating agents.

While it is unclear how the NO that is formed is transported out of erythrocytes, there are several hypotheses to account for the ability of NO to escape from the scavenging effect of erythrocyte-contained hemoglobin [12]. The first potential explanation is that NO freely diffuses inside the lumen once it is formed. As discussed below, computational modeling has shown that a free diffusion mechanism would allow only a negligible amount of NO to reach the vascular wall and carry out its signaling functions [12, 17, 36, 45]. Another hypothesis states that once NO is formed inside the erythrocytes, NO can bind to erythrocyte membrane-bound proteins that can shuttle NO molecules out of the cell, avoiding immediate scavenging by the abundant intracellular hemoglobin [62]. Yet another possibility is that an intermediate species with a longer lifetime in vivo is produced prior to the formation of NO itself. Such an intermediate molecule would not be reactive with hemoglobin and would be converted to NO in the extracellular environment [8, 59].

In addition to these potential transport mechanisms, the unique characteristics of the erythrocyte membrane seem to help prevent rapid scavenging of luminal NO by intracellular

hemoglobin: NO is apparently restricted to some extent in its ability to diffuse freely through the erythrocyte membrane. As an uncharged small molecule, NO was initially thought to be able to diffuse freely through the lipid bilayer of the erythrocyte membrane, but experiments have now indicated that although the reaction between free hemoglobin and NO is rapid and limited only by the diffusion rate [26, 28], the reaction between NO and hemoglobin encapsulated in an erythrocyte is significantly slower [10, 44]. Thus, if NO synthesized in erythrocytes is shuttled out of the cells through a facilitated mechanism, this controlled permeability to NO would help decrease the scavenging of NO by intracellular hemoglobin by slowing the movement of some of the NO molecules back into the erythrocyte. Furthermore, computational modeling has predicted that most of the endothelial NO that is responsible for regulating vascular tone would be rapidly converted to relatively inactive nitrite or nitrate by erythrocytic hemoglobin soon after the NO was formed, as a result of the enzymatic activity of NOS3 in the endothelium [43]. These mechanisms, taken together, suggest that the erythrocyte membrane may provide a barrier that reduces NO scavenging by intracellular hemoglobin. Other experiments, in conjunction with computational modeling [5, 70, 71], have indicated that the membrane of erythrocytes indeed exhibits an intrinsic resistance to NO diffusion, which helps preserve the bioactivity of NO from both the vascular wall and intraluminal sources.

It should be noted that this potential unidirectional high resistance of the erythrocyte membrane to NO is under debate. Some studies [45, 68] have suggested that the membrane permeability may be higher than previously reported, because the slow rate of NO binding to intraerythrocytic hemoglobin that was reported in previous studies [27, 70] might be due to other hematocrit-dependent mechanisms (i.e., extracellular unstirred solution layer [45]). Thus, the nature of this membrane resistance to diffusion of uncharged NO requires further investigation if we are to gain a more complete understanding of endocrine NO-mediated signaling.

Next, we review the possible sources of NO inside the erythrocyte.

3. Preservation of NO bioactivity by S-nitrosothiols

Under both normoxic and hypoxic conditions, NO can rapidly react with erythrocytic hemoglobin by binding to the iron group in the heme pocket, with a kinetic rate that is limited only by the rate of diffusion [28, 44]. This rapid binding has meant that erythrocytes have long been regarded as a potent NO sink. In addition to reacting with the iron group in the heme pocket, nitrosonium cation (NO molecule after being oxidized) binds to the thiol group on certain proteins through S-nitrosation [62, 63]. Thus, the formation of S-nitrosohemoglobin (SNOHb) is another potential reaction between hemoglobin and NO.

The SNOHb hypothesis proposes that NO binds to T-state (deoxygenated) hemoglobin to form Fe^{2+}NO ; the bound NO group is then transferred during hemoglobin oxygenation from the ferrous iron group to the cysteine residue at position 93 in the β chain (β -93 cysteine) of the hemoglobin molecule in the R-state, forming the relatively stable species, SNOHb [57, 63]. Then, under hypoxic conditions, SNOHb undergoes allosteric changes that result in the transfer of the NO group to the thiols of the anion exchange protein present on the membrane. The NO group also could be transferred to glutathione via transnitrosation. This bioactivity of NO could then be exported from the erythrocytes and regulate blood flow [2, 62]. According to the SNOHb hypothesis, in addition to playing a role in decreasing NO bioactivity, hemoglobin is also able to sense a change in the ambient O_2 and release NO under hypoxic conditions, thereby regulating O_2 delivery and hemorheological properties through an endocrine mechanism. It is not yet clear whether the nitrogen oxides released from erythrocytes are present in the form of nitrogen monoxide (free NO) [58, 74] or other

NO equivalents [47]. It is likely that NO bioactivity is preserved in the form of S-nitrosothiols; a variety of physiological regulations are dependent on the intracellular uptake of these compounds [62].

Because of the complex chemical reactions involved in the binding of NO to the thiol group of hemoglobin, it is difficult to distinguish any change in NO derived from this source from that of NO released from other sources. Thus far, there have been no direct experimental measurements in vivo of the amount of NO delivered to smooth muscle by this mechanism (or by other possible intraerythrocytic mechanisms). We have recently constructed a computational model that simulates NO production and transport within the framework of SNOHb hypothesis, with the assumption that free NO is finally released [17]. The model predicted that the amount of NO delivered by intraerythrocytic SNOHb to the vascular wall is in the neighborhood of 6 pM, according to the SNOHb hypothesis; this NO availability would be as low as 0.25 pM if the SNOHb concentration were lower (~50 nM), as has been reported in some studies. It should be noted that if there is no protected NO transport mechanism (i.e., if only free diffusion occurs), the value of the NO released from this source would be three orders of magnitude lower than the 6-pM value, according to our computational simulation. Moreover, computational modeling showed that the amount of NO present in the smooth muscle depends strongly on facilitated membrane transport, membrane resistance to NO diffusion, and the physiological level and half-life of SNOHb.

Although intraerythrocytic SNOHb could be an important NO source in the intraluminal region, the role of SNOHb in hypoxic vasodilation is currently under debate [25, 32, 35, 57]. A complete understanding of how much NO comes from intraerythrocytic SNOHb requires further investigation. S-nitrosothiol (SNO) itself can also induce vasodilation, independent of NO and regardless of the NO release rate [49, 52, 53, 64]. NO bioactivity may be present in the form of other small molecules, such as S-nitrosoglutathione (GSNO).

4. Reduction of nitrite by hemoglobin to form NO

Nitrite has been considered one of the stable metabolic end-products of NO. Recent experimental evidence suggests that nitrite acts as a reservoir that preserves NO bioactivity and that it can be enzymatically reduced to NO by hemoglobin under hypoxic conditions [33]. The maximal nitrite reductase activity occurs around the P50 of hemoglobin because of the balance between the enzymatic activities of R-state (oxygenated) and T-state (deoxygenated) hemoglobin. R-state hemoglobin is thermodynamically favorable for nitrite reduction, with a lower redox potential for the hemes, while T-state hemoglobin provides the more ligand-free hemes as the reaction site with nitrite [29, 33]. The chemistry involved in this reaction can be expressed as

The reaction shown above provides an interesting pathway that could regulate blood flow and hemorheology under conditions of hypoxia. As previously mentioned, hemoglobin is abundant in erythrocytes; on the other hand, experiments have shown that erythrocytes are the major intravascular storage sites of nitrite in human blood [23], although there are considerable levels of nitrite in the plasma as well (~100 nM). Thus, erythrocytes are assumed to export NO molecules, particularly at the pre-capillary arterioles where the P50 of hemoglobin can most likely be achieved, provided NO bioactivity can be transported through protected mechanisms. Physiological experiments from a number of labs have shown that the addition of nitrite can cause downstream vasodilation and nitrite does indeed

act as a vasodilator under hypoxic conditions [6, 20, 24, 41]. In addition to the nitrite reduction by hemoglobin, certain proteins in the extracellular region, such as xanthine oxidoreductase and mitochondrial proteins, can reduce nitrite to NO to regulate tissue oxygenation and other physiological functions [7, 22, 46].

However, there have been no direct experimental measurements in vivo of the amount of NO delivered through nitrite reduction, in part because of the complex chemistry involved in this mechanism. Computational modeling has been applied to simulate the reduction process and predict the amount of NO released via this pathway. Jeffers et al. [36] reported that the NO concentration in smooth muscle is 0.08 pM after the intraerythrocytic nitrite is reduced to NO, which is then transported through free diffusion. We also simulated the NO transport that occurs after nitrite reduction in erythrocytes, considering the three possible transport mechanisms discussed above [12]: free diffusion, a membrane-associated mechanism, and a mechanism involving an intermediate product that only liberates NO as it reaches the vascular wall (Fig. 2). Our calculation for the intraerythrocytic sources, which predicted a level of ~0.04 pM NO (Figs. 2A and 2B), was in agreement with the value of Jeffers et al. Our model also predicted that NO is present in smooth muscle at 43 pM (Figs 2C and 2D) if the NO that is formed can be released from erythrocytes through a facilitated mechanism. This value could be increased to around 260 pM (Figs. 2E and 2F) in an extreme case in which an intermediate reaction exists, which would carry the NO and liberate it before it reached the vascular wall. From these results, we can see that, as opposed to free diffusion, a transport mechanism that protects NO bioactivity and facilitates its export out of erythrocytes is potentially important for delivering significant amounts of NO from erythrocytes.

It is also clear that cell-free hemoglobin, which can be produced by hemolysis in sickle cell anemia or as a result of the administration of hemoglobin-based oxygen carriers, can either scavenge NO or react with nitrite to produce NO. Experimental evidence has indicated that hypertension and vasoconstriction are associated with a significant presence of cell-free hemoglobin in the blood [20, 66]. Furthermore, computational modeling has shown that the reaction of cell-free hemoglobin with nitrite generates an insignificant net amount of free NO (~0.02 pM) that reaches the vascular wall because of the lack of a possible protected transport mechanism (thus, there is a strong self-capture effect) [12]. Therefore, erythrocytes, rather than the plasma in which a certain amount of cell-free hemoglobin is present, should be considered the major site for nitrite reduction to NO.

It is also interesting to consider that hemoglobin could potentially synthesize SNOHb by utilizing nitrite as a substrate, as an adjunct to the binding of free NO to the heme group via the cysteine in the hemoglobin β chain [3, 58]. This finding provides a link between the two hypotheses. Thus, it seems that there is a complex interaction among the hemoglobin iron group, the hemoglobin thiol group, free nitric oxide, and nitrite in erythrocytes.

5. NOS3 expressed on erythrocytes

As was discussed above, NOS3 in the endothelial cells has been considered the major source of the bioavailable NO in the vasculature. In addition to its presence in endothelial cells, functional NOS3 has been detected in erythrocytes, offering a possibility that hemoglobin-containing erythrocytes are indeed NO generators. In 1998, Chen and Mehta [18] reported the presence of the L-arginine-nitric oxide pathway in human erythrocytes, suggesting that NO-producing enzymes are indeed present in erythrocytes. However, the enzymes were later suggested to be inactive [37]. Recently, Kleinbongard et al. [40], using multiple experimental approaches, have shown that erythrocytes express a functional NOS3 that can potentially serve as an intraluminal NO source. This NOS3 was located on the plasma

membrane as well as in the cytoplasm of the erythrocytes. Note that NO from this source has been shown to be able to regulate the erythrocyte deformability and platelet aggregation, which are important factors that determine the blood flow in the microvasculature.

The controversy regarding the existence of NOS3 in erythrocytes is not yet resolved: Several recent studies have confirmed the presence or function of NOS3 in erythrocytes [69, 73], but others have questioned its existence [31]. NO from this source has been thought to regulate the function of erythrocytes and platelets [40, 75]. If the NO synthesized by intraerythrocytic NOS3 can escape scavenging by hemoglobin as does other blood-borne NO, it could serve as another important source regulating vascular tone and hemorheological properties.

Because of the technical difficulties involved in attempting to obtain quantitative measurements of intraluminal NO, there are no published data regarding how much NO from this source is available to the intraluminal region or the vascular wall. Computational modeling [13] can simulate NO production from NOS3 in erythrocytes and its transport through an arteriole. Computer simulations indicate that, as has been shown for other intraerythrocytic pathways, whether a significant amount of NO from this source can be delivered to vascular smooth muscle is strongly dependent on the existence of a protected mechanism for NO delivery. If the expression level of NOS3 in erythrocytes is similar to that in endothelial cells, a combined NOS biochemical pathway analysis model [15, 16] and NO transport model [17] would predict a level of ~ 13 pM NO in the vascular smooth muscle from this source, given the existence of such a protected NO transport mechanism [13].

6. NO released from iron-nitrosyl-hemoglobin (HbNO)

Free NO binds to deoxygenated hemoglobin to form iron-nitrosyl-hemoglobin (HbNO), which is present in erythrocytes at a relatively high concentration. This species has been put forward as a possible NO source within the lumen, in particular as an intermediate product during nitrite reduction [20]. However, the dissociation rate of NO from the heme of hemoglobin is slow, in the range of 1×10^3 to 1×10^5 s⁻¹ [51]. This slow dissociation rate would make it possible for the capture of the released NO erythrocytes to occur quickly enough to quench any NO bioactivity from this source. Also, the vasodilation produced by the infusion of nitrogen oxide into the blood would require a faster dissociation rate if the HbNO were to have any regulatory effect on hemorheology.

Recent experiments have indicated that the presence of oxidants in the blood can expedite the release of NO from HbNO [30, 61]. The NO that is generated by this accelerated mechanism may either be transported out of the erythrocyte or be converted back into nitrite ions that can go through the nitrite reduction pathway in response to hypoxia. How much free NO can be released from HbNO under physiological conditions is not yet clear and requires further investigation.

7. Conclusions

Erythrocytes apparently play a dual role in regulating NO availability: they scavenge NO and generate methemoglobin, HbNO, nitrite, and nitrate; and they can serve as a site for NO-related reactions involving SNOHb, nitrite reduction, enzymatic cleavage by NOS3 expressed on erythrocytes, and/or iron-nitrosyl-hemoglobin. These pathways have been identified on the basis of experimental evidence and theoretical modeling; however, the amount of NO that each of these pathways contributes to the regulation of vascular tone and hemorheological properties remains to be elucidated in future theoretical and experimental research. Both the NO carried by and delivered from erythrocytes and the NO present in the vessel lumen may contribute significantly to the regulation of vascular tone and hemorheological properties. It is likely that NO derived from the intraluminal pathways, as

well as that from previously recognized endothelial sources, is of great importance for vascular physiology. A full understanding, particularly at the quantitative level, of the relative contributions of the intraluminal NO pathways and other enzymatic sources of NO is critical to the future development of therapeutics targeting NO signaling in the vasculature.

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References

1. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J*. 2001; 357:593–615. [PubMed: 11463332]
2. Allen BW, Piantadosi CA. How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. *Am J Physiol Heart Circ Physiol*. 2006; 291:H1507–1512. [PubMed: 16751292]
3. 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]
4. Antl M, von Bruhl ML, Eiglsperger C, Werner M, Konrad I, Kocher T, Wilm M, Hofmann F, Massberg S, Schlossmann J. IRAG mediates NO/cGMP-dependent inhibition of platelet aggregation and thrombus formation. *Blood*. 2007; 109:552–559. [PubMed: 16990611]
5. Azarov I, Huang KT, Basu S, Gladwin MT, Hogg N, Kim-Shapiro DB. Nitric oxide scavenging by red blood cells as a function of hematocrit and oxygenation. *J Biol Chem*. 2005; 280:39024–39032. [PubMed: 16186121]
6. Basireddy M, Isbell TS, Teng X, Patel RP, Agarwal A. Effects of sodium nitrite on ischemia-reperfusion injury in the rat kidney. *Am J Physiol Renal Physiol*. 2006; 290:F779–786. [PubMed: 16278276]
7. 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]
8. 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 N(2)O(3) by the concerted nitrite reductase and anhydrase activity of hemoglobin. *Nat Chem Biol*. 2007; 3:785–794. [PubMed: 17982448]
9. Bor-Kucukatay M, Wenby RB, Meiselman HJ, Baskurt OK. Effects of nitric oxide on red blood cell deformability. *Am J Physiol Heart Circ Physiol*. 2003; 284:H1577–1584. [PubMed: 12521942]
10. Carlsen E, Comroe JH. The rate of uptake of carbon monoxide and of nitric oxide by normal human erythrocytes and experimentally produced spherocytes. *J Gen Physiol*. 1958; 42:83–107. [PubMed: 13575776]
11. Cerwinka WH, Cooper D, Kriegelstein CF, Feelisch M, Granger DN. Nitric oxide modulates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules. *Am J Physiol Heart Circ Physiol*. 2002; 282:H1111–1117. [PubMed: 11834510]
12. Chen K, Piknova B, Pittman RN, Schechter AN, Popel AS. Nitric oxide from nitrite reduction by hemoglobin in the plasma and erythrocytes. *Nitric Oxide*. 2008; 18:47–60. [PubMed: 17964300]
13. Chen K, Pittman RN, Popel AS. Hemorrhagic shock and nitric oxide release from erythrocytic nitric oxide synthase: A quantitative analysis. *Microvascular Research*. 2009 in press.
14. Chen K, Pittman RN, Popel AS. Nitric oxide in the vasculature: where does it come from and where does it go? A quantitative perspective. *Antioxid Redox Signal*. 2008; 10:1185–1198. [PubMed: 18331202]
15. Chen K, Popel AS. Theoretical analysis of biochemical pathways of nitric oxide release from vascular endothelial cells. *Free Radic Biol Med*. 2006; 41:668–680. [PubMed: 16864000]
16. Chen K, Popel AS. Vascular and perivascular NO release and transport: biochemical pathways of NOS1 and NOS3. *Free Radic Biol Med*. 2007; 42:811–822. [PubMed: 17320763]

17. Chen K, Pittman RN, Popel AS. Vascular Smooth Muscle NO Exposure from Intraerythrocytic SNOHb: A Mathematical Model. *Antioxid Redox Signal*. 2007; 9:1097–1110. [PubMed: 17536957]
18. Chen LY, Mehta JL. Evidence for the presence of L-arginine-nitric oxide pathway in human red blood cells: relevance in the effects of red blood cells on platelet function. *J Cardiovasc Pharmacol*. 1998; 32:57–61. [PubMed: 9676721]
19. Cooper CE, Brown GC. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *J Bioenerg Biomembr*. 2008; 40:533–539. [PubMed: 18839291]
20. 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, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med*. 2003; 9:1498–1505. [PubMed: 14595407]
21. Crane MS, Olsson R, Moore KP, Rossi AG, Megson IL. Novel role for low molecular weight plasma thiols in nitric oxide-mediated control of platelet function. *J Biol Chem*. 2002; 277:46858–46863. [PubMed: 12297511]
22. Daiber A, Shoun H, Ullrich V. Nitric oxide reductase (P450_{nor}) from *Fusarium oxysporum*. *J Inorg Biochem*. 2005; 99:185–193. [PubMed: 15598501]
23. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT, Schechter AN. Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood*. 2005; 106:734–739. [PubMed: 15774613]
24. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO, Schechter AN, Gladwin MT. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation*. 2007; 116:1821–1831. [PubMed: 17893272]
25. Diesen DL, Hess DT, Stamler JS. Hypoxic vasodilation by red blood cells: evidence for a nitrosothiol-based signal. *Circ Res*. 2008; 103:545–553. [PubMed: 18658051]
26. Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, Mathews AJ, Johnson KA, Smith RD, Phillips GN, Olson JS. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry*. 1996; 35:6976–6983. [PubMed: 8679521]
27. El-Farra NH, Christofides PD, Liao JC. Analysis of nitric oxide consumption by erythrocytes in blood vessels using a distributed multicellular model. *Ann Biomed Eng*. 2003; 31:294–309. [PubMed: 12680727]
28. Gibson WH, Roughton FJ. The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *J Physiol*. 1957; 136:507–524. [PubMed: 13429517]
29. 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]
30. 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]
31. Hilarius PM, Goedhart PT, Ince C, Verhoeven AJ. Human erythrocytes contain negligible amounts of endothelial type NO synthase (eNOS). Abstracts of Second International Meeting on the Role of Nitrite in Physiology, Pathophysiology, and Therapeutics. 2007:90–91.
32. Huang KT, Azarov I, Basu S, Huang J, Kim-Shapiro DB. Lack of allosterically controlled intramolecular transfer of nitric oxide from the heme to cysteine in the beta subunit of hemoglobin. *Blood*. 2006; 107:2602–2604. [PubMed: 16339397]
33. 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]
34. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A*. 1987; 84:9265–9269. [PubMed: 2827174]

35. Isbell TS, Sun CW, Wu LC, Teng X, Vitturi DA, Branch BG, Kevil CG, Peng N, Wyss JM, Ambalavanan N, Schwiebert L, Ren J, Pawlik KM, Renfrow MB, Patel TM, Townes RP. SNO-hemoglobin is not essential for red blood cell-dependent hypoxic vasodilation. *Nat Med*. 2008; 14:773–777. [PubMed: 18516054]
36. 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]
37. Kang ES, Ford K, Grokulsky G, Wang YB, Chiang TM, Acchiardo SR. Normal circulating adult human red blood cells contain inactive NOS proteins. *J Lab Clin Med*. 2000; 135:444–451. [PubMed: 10850643]
38. Kashiwagi S, Kajimura M, Yoshimura Y, Suematsu M. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ Res*. 2002; 91:e55–64. [PubMed: 12480826]
39. Kavdia M, Popel AS. Wall shear stress differentially affects NO level in arterioles for volume expanders and Hb-based O₂ carriers. *Microvasc Res*. 2003; 66:49–58. [PubMed: 12826074]
40. Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch HG, Godecke A, Webe AA, Robenek M, Robenek H, Bloch W, Rosen P, Kelm M. Red blood cells express a functional endothelial nitric oxide synthase. *Blood*. 2006; 107:2943–2951. [PubMed: 16368881]
41. Kozlov AV, Costantino G, Sobhian B, Szalay L, Umar F, Nohl H, Bahrami S, Redl H. Mechanisms of vasodilatation induced by nitrite instillation in intestinal lumen: possible role of hemoglobin. *Antioxid Redox Signal*. 2005; 7:515–521. [PubMed: 15706099]
42. Lamkin-Kennard KA, Buerk DG, Jaron D. Interactions between NO and O₂ in the microcirculation: a mathematical analysis. *Microvasc Res*. 2004; 68:38–50. [PubMed: 15219419]
43. Lancaster JR. Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc Natl Acad Sci U S A*. 1994; 91:8137–8141. [PubMed: 8058769]
44. Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem*. 1998; 273:18709–18713. [PubMed: 9668042]
45. Liu X, Yan Q, Baskerville KL, Zweier JL. Estimation of nitric oxide concentration in blood for different rates of generation. Evidence that intravascular nitric oxide levels are too low to exert physiological effects. *J Biol Chem*. 2007; 282:8831–8836. [PubMed: 17267398]
46. Lu P, Liu F, Yao Z, Wang CY, Chen DD, Tian Y, Zhang JH, Wu YH. Nitrite-derived nitric oxide by xanthine oxidoreductase protects the liver against ischemia-reperfusion injury. *Hepatobiliary Pancreat Dis Int*. 2005; 4:350–355. [PubMed: 16109514]
47. Luchsinger BP, Rich EN, Gow AJ, Williams EM, Stamler JS, Singel DJ. Routes to S-nitroso-hemoglobin formation with heme redox and preferential reactivity in the beta subunits. *Proc Natl Acad Sci U S A*. 2003; 100:461–466. [PubMed: 12524454]
48. Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Fassler R, Ruth P, Krombach F, Hofmann F. Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *J Exp Med*. 1999; 189:1255–1264. [PubMed: 10209042]
49. Mathews WR, Kerr SW. Biological activity of S-nitrosothiols: the role of nitric oxide. *J Pharmacol Exp Ther*. 1993; 267:1529–1537. [PubMed: 7903392]
50. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol*. 2006:213–254. [PubMed: 16999221]
51. Moore EG, Gibson QH. Cooperativity in the dissociation of nitric oxide from hemoglobin. *J Biol Chem*. 1976; 251:2788–2794. [PubMed: 1262343]
52. Morris SL, Hansen JN. Bacterial spore components which enhance the bacteriostatic effectiveness of S-nitrosothiol. *Appl Environ Microbiol*. 1981; 42:958–962. [PubMed: 6797352]
53. Morris SL, Hansen JN. Inhibition of *Bacillus cereus* spore outgrowth by covalent modification of a sulfhydryl group by nitrosothiol and iodoacetate. *J Bacteriol*. 1981; 148:465–471. [PubMed: 6795179]
54. Nase GP, Tuttle J, Bohlen HG. Reduced perivascular PO₂ increases nitric oxide release from endothelial cells. *Am J Physiol Heart Circ Physiol*. 2003; 285:H507–515. [PubMed: 12860561]

55. Pernollet MG, Lantoine F, Devynck MA. Nitric oxide inhibits ATP-dependent Ca^{2+} uptake into platelet membrane vesicles. *Biochem Biophys Res Commun.* 1996; 222:780–785. [PubMed: 8651922]
56. Petrov V, Lijnen P. Regulation of human erythrocyte $\text{Na}^{+}/\text{H}^{+}$ exchange by soluble and particulate guanylate cyclase. *Am J Physiol.* 1996; 271:C1556–1564. [PubMed: 8944639]
57. Reynolds JD, Ahearn GS, Angelo M, Zhang J, Cobb F, Stamler JS. S-nitrosohemoglobin deficiency: a mechanism for loss of physiological activity in banked blood. *Proc Natl Acad Sci U S A.* 2007; 104:17058–17062. [PubMed: 17940022]
58. Rifkind JM, Nagababu E, Ramasamy S. Nitric oxide redox reactions and red cell biology. *Antioxid Redox Signal.* 2006; 8:1193–1203. [PubMed: 16910767]
59. Robinson JM, Lancaster JR. Hemoglobin-mediated, hypoxia-induced vasodilation via nitric oxide: mechanism(s) and physiologic versus pathophysiological relevance. *Am J Respir Cell Mol Biol.* 2005; 32:257–261. [PubMed: 15778415]
60. Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM, Gladwin MT. Deoxyhemoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res.* 2007; 100:654–661. [PubMed: 17293481]
61. Sibmoo N, Piknova B, Rizzatti F, Schechter AN. Oxidation of iron-nitrosyl-hemoglobin by dehydroascorbic acid releases nitric oxide to form nitrite in human erythrocytes. *Biochemistry.* 2008; 47:2989–2996. [PubMed: 18225862]
62. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. *Annu Rev Physiol.* 2005; 67:99–145. [PubMed: 15709954]
63. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, Loscalzo J. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci U S A.* 1992; 89:7674–7677. [PubMed: 1502182]
64. Stamler JS, Singel DJ, Piantadosi CA. SNO-hemoglobin and hypoxic vasodilation. *Nat Med.* 2008; 14:1008–1009. author reply 1009–1010. [PubMed: 18841126]
65. Thomas DD, Liu X, Kantrow SP, Lancaster JR. The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O₂. *Proc Natl Acad Sci U S A.* 2001; 98:355–360. [PubMed: 11134509]
66. Torres Filho IP, Spiess BD, Barbee RW, Ward KR, Oldenhof J, Pittman RN. Systemic responses to hemodilution after transfusion with stored blood and with a hemoglobin-based oxygen carrier. *Anesth Analg.* 2005; 100:912–920. [PubMed: 15781498]
67. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. *Blood.* 2006; 108:3603–3610. [PubMed: 16857991]
68. Tsoukias NM, Popel AS. Erythrocyte consumption of nitric oxide in presence and absence of plasma-based hemoglobin. *Am J Physiol Heart Circ Physiol.* 2002; 282:H2265–2277. [PubMed: 12003837]
69. Ulker, P.; Meiselman, HJ.; Baskurt, OK. Mechanical stimulation of nitric oxide synthesizing mechanisms in erythrocytes. 13th International Congress of Biorheology and 6th International Conference on Clinical Hemorheology; 2008.
70. Vaughn MW, Huang KT, Kuo L, Liao JC. Erythrocyte consumption of nitric oxide: competition experiment and model analysis. *Nitric Oxide.* 2001; 5:18–31. [PubMed: 11178933]
71. Vaughn MW, Kuo L, Liao JC. Estimation of nitric oxide production and reaction rates in tissue by use of a mathematical model. *Am J Physiol.* 1998; 274:H2163–2176. [PubMed: 9841542]
72. Vukosavljevic N, Jaron D, Barbee KA, Buerk DG. Quantifying the L-arginine paradox in vivo. *Microvasc Res.* 2006; 71:48–54. [PubMed: 16316668]
73. 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. *Circ Res.* 2008; 103:957–964. [PubMed: 18818408]

74. Wolzt M, MacAllister RJ, Davis D, Feelisch M, Moncada S, Vallance P, Hobbs AJ. Biochemical characterization of S-nitrosohemoglobin. Mechanisms underlying synthesis, no release, and biological activity. *J Biol Chem.* 1999; 274:28983–28990. [PubMed: 10506146]
75. Yalcin O, Ulker P, Yavuzer U, Meiselman HJ, Baskurt OK. Nitric oxide generation by endothelial cells exposed to shear stress in glass tubes perfused with red blood cell suspensions: role of aggregation. *Am J Physiol Heart Circ Physiol.* 2008; 294:H2098–2105. [PubMed: 18326799]
76. Yu B, Raher MJ, Volpato GP, Bloch KD, Ichinose F, Zapol WM. Inhaled nitric oxide enables artificial blood transfusion without hypertension. *Circulation.* 2008; 117:1982–1990. [PubMed: 18391111]
77. Zani BG, Bohlen HG. Transport of extracellular l-arginine via cationic amino acid transporter is required during in vivo endothelial nitric oxide production. *Am J Physiol Heart Circ Physiol.* 2005; 289:H1381–1390. [PubMed: 15849232]
78. Zweier JL, Samouilov A, Kuppusamy P. Non-enzymatic nitric oxide synthesis in biological systems. *Biochim Biophys Acta.* 1999; 1411:250–262. [PubMed: 10320661]

Fig. 1.

Major intraluminal pathways of NO release. Intraerythrocytic NO release can potentially come from *S*-nitrosylated blood proteins, nitrite reduction by heme-containing proteins, and iron-nitrosyl-hemoglobin, and NOS3 expressed on erythrocytes. EC, endothelial cells; IS, interstitial space between the endothelium and smooth muscle; RBC, red blood cell; Hb, hemoglobin; B3P, band 3 protein; metHb, methemoglobin; NO_2^- , nitrite; NO_3^- , nitrate; L-arg, L-arginine; L-cit, L-citrulline; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, 3',5'-cyclic guanosine monophosphate. NO sources from the endothelium are NOS3. NOS1 and NOS2 can be expressed in endothelial under pathological conditions.

Fig. 2.

NO concentration profiles when nitrite reduction by intraerythrocytic hemoglobin is the sole source of NO. From top to bottom, the contour plots (A, C, and E) represent the cross-sections of an arteriole that contains erythrocytes and its surrounding tissue. The elevation plots (B, D, and F) show the NO concentration from the center of the lumen to the perivascular region along a path. The starting and ending points of the path are represented as 1 and 2, respectively, in the insets. The small circles in the insets represent erythrocytes. NO delivery by nitrite reductions is calculated under three conditions: 1) NO formation by nitrite reduction occurs uniformly inside erythrocytes and the subsequent transport of NO occurs through free diffusion (A and B); 2) formed NO is transported out of the cell membrane through a facilitated mechanism (C and D); 3) the NO bioactivity is preserved as an intermediate species, which does not liberate NO until it reaches the vascular wall (E and F). The NO concentration inside each erythrocyte was nearly zero (Reproduced from [12] with permission and all parameters used in the simulation can be found therein).