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Forum Review

Nitric Oxide Redox Reactions and Red Cell Biology

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ABSTRACT

Three hypotheses explain a role for red blood cells (RBCs) in delivering NO to the vasculature: (a) "the SNOHb hypothesis" involves the uptake of NO by RBCs with NO transferred from the heme to the β-93 thiol in the R quaternary conformation, followed by the release to membrane thiols in the T quaternary conformation; and (b and c) "the nitrite hypotheses" bypass the intrinsic difficulties of transporting the highly reactive NO, by reutilizing the nitrite formed when NO reacts with oxygen. Deoxyhemoglobin reduces this nitrite back to NO. The distinction between the two nitrite mechanisms depends on the importance of intermediate species formed during nitrite reduction. Without bioactive intermediates, the NO must be immediately released to avoid binding to deoxyhemoglobin. The "nitrite intermediate hypothesis" enables the RBCs to store a pool of potentially bioactive NO until it is released from the cell. In this review, we critically compare these different proposals for the transport/delivery of NO by RBCs. We also compare the redox properties in the RBCs associated with NO with the redox properties associated with oxygen. *Antioxid. Redox Signal.* 8, 1193–1203.

INTRODUCTION

Nitric oxide is synthesized *in vivo* by the reaction of arginine with nitric oxide synthase. The three isoforms of nitric oxide synthase are the constitutive neuronal enzyme (n-NOS), the constitutive endothelial enzyme (e-NOS), and inducible enzymes (i-NOS). NO acts as a second messenger and has been implicated in a wide range of biologic functions (Fig. 1). In the circulation, it regulates vascular tone, inhibits platelet aggregation, and is involved in the inhibition of leukocyte adhesion to the vascular endothelium. NO also has antimicrobial activity and plays an important role in neurotransmission (4, 25, 38).

Nitric oxide is, however, a highly reactive unstable molecule (Fig. 1) that can be oxidized to NO^+, the nitrosonium cation, and reduced to NO^-, the nitroxyl anion (30, 57). Although the estimated half-life of NO in phosphate-buffered solutions at pH 7.4 is 130 s (14), the physiologic half-life of NO in the presence of oxygen in aqueous solutions is of the order of seconds (24, 38), and in the circulation, it is thought to have a half-life of <0.1 s (58). The reaction with oxygen is a complex second-order reaction that initially produces NO_2 and N_2O_3, but eventually results in the formation of nitrite (8, 61).

NO also reacts with the superoxide anion very rapidly ($k = 6.7 \times 10^9\ M^{-1}s^{-1}$), producing peroxynitrite ($ONOO^-$). The superoxide radical ubiquitous in mammalian cells and constitutively produced by NOS (59) is thus expected to react with plasma NO, generating peroxynitrite (42, 55). Although peroxynitrite is stable in alkaline pH, peroxynitrous acid with a pK_a of 6.8 present, even at neutral pH, is a strong oxidant and nitrating agent that is responsible for thiol and tyrosine nitration. Peroxynitrous acid is rapidly isomerized to nitrate, whereas an adduct formed from peroxynitrite and peroxynitrous acid decomposes to form nitrite and oxygen. Peroxynitrite also reacts with CO_2 (18), forming $ONOOCO_2^-$, a stronger nitrating agent than peroxynitrite, which decomposes producing $CO^{\bullet}_3^-$ and $^{\bullet}NO_2$.

The NO generated by e-NOS can diffuse into the smooth muscle cells where it reacts with the heme group of guanylate cyclase, generating cyclic guanosine monophosphate (GMP), which is ultimately responsible for NO-induced vasodilation. However, this NO also diffuses into the lumen. In the lumen, the NO is taken up by platelets and red cells but will also react with oxygen and superoxide.

It has been shown that the NO in human plasma is quantitatively oxidized to nitrite and nitrate (60). The reaction pro-

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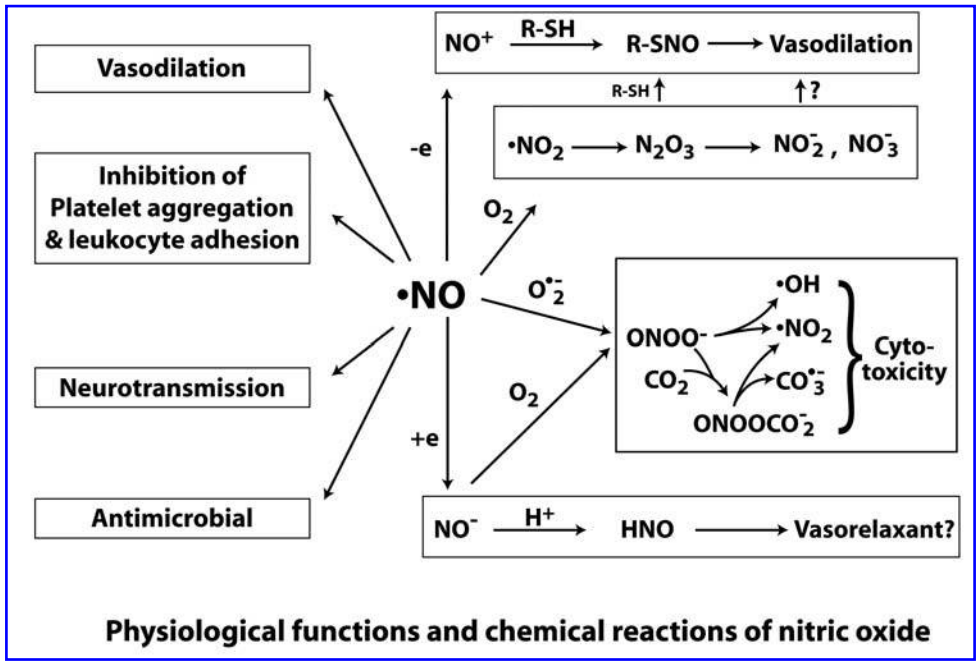

FIG. 1. The multiple reactions involving nitric oxide result in a wide range of biologic functions as well as its instability. Included are the various physiologic functions attributed to NO as well as the products formed by the oxidation and reduction of NO and the products formed when NO react with oxygen and superoxide.

ducing nitrate is associated with NO that is taken up by the red cell and reacts with oxyhemoglobin. This reaction is largely favored in arterial blood, where hemoglobin is almost completely oxygenated. However, in venous blood, the formation of nitrite via the formation of N_2O_3 is more pronounced, resulting in an overall nitrite-to-nitrate ratio in human plasma of 1:5. The N_2O_3 formed during the reaction with oxygen (Fig. 1) reacts with plasma thiols, producing *S*-nitrosothiols. This reaction is thought to be responsible for the 50–100 n*M* *S*-nitrosothiols in plasma.

It has been shown that measuring the excretion rate of nitrate in urine reliably assesses long-lasting changes in whole-body NO synthesis. However, even though the plasma levels of nitrate are greater than the plasma levels of nitrite, it is the plasma nitrite level that is sensitive to short-term changes in endothelial NO synthesis and has been used as a measure of e-NOS activity (26, 52).

The validity of plasma nitrate as a measure of e-NOS activity is relatively unaffected by dietary nitrite (44). Most of the ingested nitrate is converted to nitrite in saliva. However, under the acidic conditions in the stomach, this nitrite is converted to nitrous acid and other reactive nitric oxide species, and does not show up in the plasma as nitrite. It has thus been shown (44) that a high level of nitrate in the diet results in a significant sevenfold increase in nitrate, but no significant increase in nitrite.

SCAVENGING OF NITRIC OXIDE BY THE RED CELL

NO in the red cell can directly bind to deoxygenated chains producing the iron nitrosylhemoglobin [Hb(II)NO] or oxidizing oxygenated chains producing nitrate and methemoglobin that no longer binds oxygen. The nitric oxide oxidation product, nitrite, is also taken up by the red cell (35) where it oxidizes hemoglobin.

The early literature considered the role of red cells *vis-à-vis* nitric oxide as an NO scavenger (31), and its oxidation products producing nonfunctional methemoglobin, tightly bound Hb(II)NO and nonfunctional nitrate. With rate constants $>10^7$ $M^{-1}s^{-1}$ for the reactions of NO with both oxyhemoglobin and deoxyhemoglobin, the red cell readily uses up the entire NO generated by the endothelium limiting any vasodilation attributed to NO and resulting in vasoconstriction. However, recent studies indicate that the physiologic effects of nitric oxide were attributed to a red blood cell–free zone near the endothelium and an unstirred layer around red cells that limit the access of the NO generated at the endothelium to the red cell (32, 33).

THE SNOHB HYPOTHESIS

A study in 1996 (27) found that in rat blood NO is found both on the heme and reacted with the β-93 cysteine thiol group, an invariant residue in mammalian hemoglobins. Furthermore, the ratio of the heme complex [Hb(II)NO] and the *S*-nitrosated hemoglobin (SNOHb) was found to be different in arterial and venous blood. In arterial blood, the SNOHb was greater than heme-NO, whereas in venous blood, heme-NO was greater than SNOHb. This observation has been subsequently shown to be found also in human subjects (37).

A dynamic transfer of NO between the heme and the thiol is the basis for the proposal whereby arterial blood binds NO to the few unliganded heme sites and, in the R-state of pre-

dominantly oxygenated hemoglobin, the red cell transfers NO from the heme to the cysteine thiol. At reduced oxygen pressures in the systemic microcirculation, the hemoglobin conformation switches to the T-state. Under these conditions, the linkage with the thiol is destabilized, facilitating the release of some of this NO (19, 56) (Fig. 2).

This "SNOHb Hypothesis" has generated a great deal of interest in a role for the red cell in transporting NO. It helps explain a dynamic role for the red cell in controlling blood vessel tone, with hypoxia releasing NO and causing vasodilation and hyperoxia scavenging NO and constricting blood vessels.

The reaction of NO with hemoglobin had been extensively studied before the identification of NO as an important secondary messenger. However, the SNOHb hypothesis requires a completely new perspective in our understanding of the reactions of NO with hemoglobin. The earlier studies emphasized the rapid reaction of NO with oxyhemoglobin and deoxyhemoglobin (see earlier). However, the conformationally induced changes in the reaction of NO were for the most part neglected, because of the very high affinity for NO, and the reaction with the β-93 cysteine thiol was not considered. These early studies could not explain (a) the binding of NO to the few percent deoxygenated chains in arterial blood in the presence of a large excess of oxyhemoglobin, (b) the release of the very tightly bound Fe(II)NO, (c) its transfer to a thiol on the β-chain when the sample is oxygenated, (d) the release of this NO from the thiol when the sample is deoxygenated, and finally (e) the transport of this NO from the red cell. The Stamler group has done extensive studies trying to establish the feasibility/validity of this hypothesis. This hypothesis has also resulted in a large number of studies from other groups, some of which are consistent with the hypothesis, but many of these studies raise serious questions about the hypothesis (17, 45, 54, 62–64).

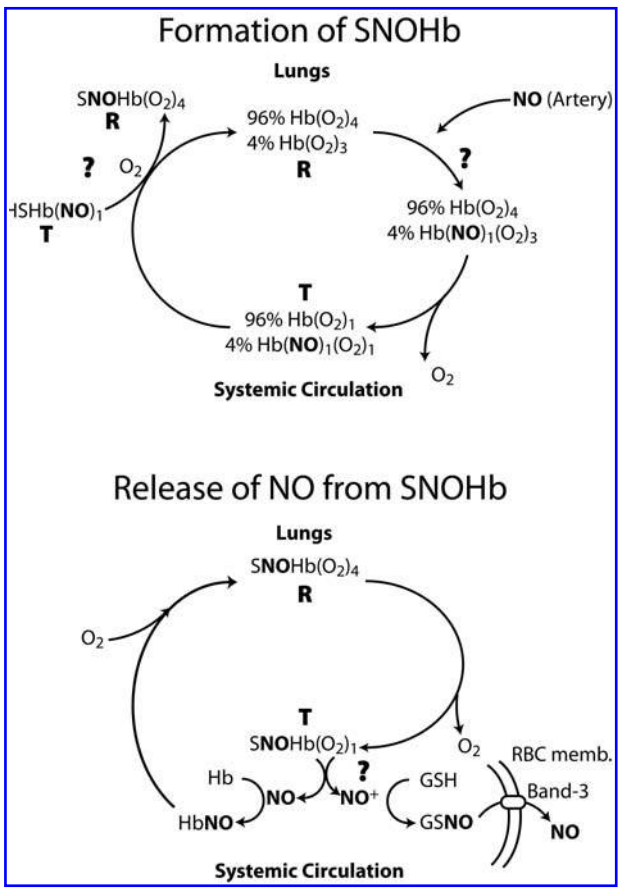

FIG. 2. The SNOHb hypothesis. The initial reaction of NO with the few deoxygenated chains in arterial blood is depicted. This NO remains bound in the systemic circulation, but can be transferred to a thiol when the cells are reoxygenated, forming SNOHb. The release of NO and the transnitrosation reactions involving SNOHb in the T-state formed, when oxygen is removed, is the basis for the ultimate transfer of NO to the vasculature.

The redox reaction required for the transfer of NO from the heme to the thiol

One of the major unresolved issues, central to this hypothesis, involves the shuttling of NO within the red cell between the heme [Hb(II)NO] and the thiol. This process involves a redox reaction, with an electron acceptor required for the formation of a nitrosothiol when NO reacts with the cysteine.

$$RS^- + NO \rightarrow RSNO + e^- \qquad [1]$$

Although the NO can undergo oxidation, no obvious electron acceptor exists for this electron. To resolve this issue, the coupling of the heme-iron valence change with the reaction of NO with the thiol was suggested (34).

Insight into such a process is provided by studies involving the reaction of NO with Hb(III). Unlike carbon monoxide and oxygen, NO binds not only to the Fe(II) heme, but also to the oxidized Fe(III) heme. The NO bound to Fe(III) undergoes a redox reaction, with an electron shared between the NO and the heme iron with properties of both Hb(III)NO and $Hb(II)NO^+$. This reaction results in reductive nitrosylation, with Hb(II) formed when the nitrosonium complex reacts with nucleophiles. This reduced heme then readily binds NO (13, 23). We have shown that the relatively rapid formation of SNOHb, with a first-order rate constant of 0.048 ± 0.018 per second, coincides with the simultaneous formation of one molecule of Hb(II)NO for each molecule of SNOHb (53). This reaction occurs only at low NO/heme ratios in which the NO preferentially binds to the Fe(III) of the β-chains, which have the reactive cysteine on the other side of the heme (Fig. 3).

The simultaneous formation of Hb(II)NO and SNOHb has been explained by an electron-transfer reaction between the NO^+ on the heme and the nearby thiol group on β-93 cysteine.

$$:S\text{-}Hb(III)NO \leftrightarrow :S\text{-}Hb(II)NO^+ \leftrightarrow \bullet S\text{-}Hb(II)NO \qquad [2]$$

Even though the thiol is on the other side of the heme 13.6 Δ from the heme iron, electron transfers requiring the delocalization of electron density over much greater distances have been extensively studied (3, 22, 43) in proteins.

Several studies actually confirm that electron delocalization between the heme iron and the β-93 cysteine takes place in hemoglobin. The release of NO from SNOHb has thus been shown to involve the transfer of an electron from the Fe(II) to the thiol (48). A transfer of electrons between the thiol and the heme was also demonstrated by the carbon monoxide–

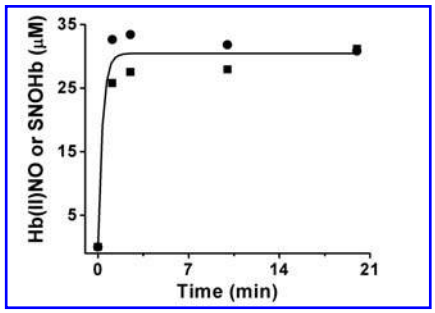

FIG. 3. The addition of a stoichiometric concentration of NO to metHb results in the rapid formation of SNOHb (●), which coincides with the formation of an equal concentration of Hb(II)NO (■). This reactions is faster than reductive nitrosylation and is thought to involve the binding of NO to a thiyl radical substrate formed when NO binds to the β-chain Fe(III) heme.

induced reduction of metHb when Cu(II) is bound to the cysteine (6). The transfer of electron density from the thiol to superoxide on the distal side of the heme has been established during the autoxidation of hemoglobin (2). For the nitrosonium cation, a much stronger oxidizing agent than superoxide, the formation of a thiyl radical and NO bound to the Hb(II) (Eq. 2) is even more likely.

The relatively low affinity (K_{eq} = 2.6 – 4.3 × 10^3 M^{-1}) of Hb(III) for NO (29), even with an excess of hemoglobin, results in free NO when NO reacts with Hb(III). This free NO will rapidly react with the thiyl radical substrate formed, resulting in the simultaneous formation of Hb(II)NO and SNOHb.

$$\bullet\text{S-Hb(II)NO} + \text{NO} \rightarrow \text{NOS-Hb(II)NO} \qquad [3]$$

This same product can be produced without a thiyl intermediate by the transfer of NO^+ or N_2O_3 to the cysteine, followed by the binding of NO to Hb(II). However, much of the released NO^+ or N_2O_3 will be lost before reacting with the β-93 cysteine group 13.6 Å away on the other side of the heme, and the level of Hb(II)NO formed would be appreciably greater than the level of SNOHb.

These studies explain the redox coupling between the heme and the thiol and the formation of SNOHb. Such a process, however, requires that the heme iron first be oxidized to act as an electron acceptor for the thiol reaction. The enhanced rates of autoxidation of hemoglobin reported at reduced oxygen pressures (1) may contribute to this process. Another pathway for increased hemoglobin oxidation is suggested by the report that at low levels of NO in the T-state, the NO bound to the heme in a reaction analogous to that of autoxidation producing superoxide, readily oxidizes the heme, producing NO^- (20). This reaction, which argues for dramatically altered properties of NO bound to Fe(II) at low NO molar ratios, may instead reflect spectral changes that occur with the formation of pentacoordinated NO known to form when NO binds to the α-chain in the T-state (12).

Even with increased levels of Hb(III), SNOHb requires that the low levels of NO bind to the Fe(III) chains, which have a much lower affinity and slower rate than the reactions involving Fe(II) heme, oxyhemoglobin, or deoxyhemoglobin.

The reaction of NO with arterial blood

The SNOHb hypothesis requires that NO generated in the artery react with hemoglobin without being inactivated to form nitrate by the reaction with oxyhemoglobin. This behavior requires an appreciably higher rate of formation for Hb(II)NO than the ~10^7 $M^{-1}s^{-1}$ for the reaction of oxyhemoglobin with NO (19, 21). To explain this phenomenon, it was argued that data on the reactions of relatively high concentrations of NO neglected the allosteric properties of hemoglobin, which involve an increase in the affinity of ligands in the liganded R-state. Studies involving the reactions of NO with hemoglobin, therefore, should be performed at physiologic levels of NO in which only one NO molecule binds to hemoglobin, and the other three chains would have oxygen already bound.

It was further pointed out that the results obtained at high NO are complicated by the unique properties of NO binding to α-chains, where the proximal histidine bond is disrupted in the T-state (15). The initial reaction of NO in the T-state involves binding to the β-chain. However, when samples are kept under hypoxic conditions (36), the NO migrates to the α-chain, disrupting the proximal histidine and binding very tightly. Once the NO is on the α-chain, it is no longer available for the dynamic conformationally induced shuttling between the heme and the thiol.

Allosteric interactions found for the binding of oxygen and carbon monoxide should also be present with NO, resulting in different equilibrium and kinetics when three of the hemoglobin subunits are oxygenated (R-state) than when the entire molecule is deoxygenated (T-state). It should, however, be possible to mimic the physiologically relevant properties of hemoglobin as long as NO binds to only one of the four hemoglobin chains. It is, however, not reasonable to completely discount results obtained at relatively low, but still nonphysiologic NO concentrations. An additional difficulty is related to the fact that it is the "on rate" for binding NO to arterial blood that must differentiate between the oxygenated chains and the few percentage of unliganded chains. However, because the "on rate" is already at the diffusion limit (5), the allosteric increase in affinity for R-state hemoglobin will predominantly decrease the "off rate," which will not explain the preferential binding to deoxygenated chains in the presence of a large excess of oxygenated chains.

Allosteric transfer of NO from heme to thiol

The SNOHb hypothesis involves the transfer of the NO bound to the heme to the thiol when the hemoglobin is oxygenated and the conformation is shifted from the T-state to the R-state. We discussed earlier the redox issues associated with this reaction. It is also necessary to appreciate that even with the coupling between the heme and the thiol, resulting in the formation of a thiyl radical, the actual transfer of NO from the heme to the thiyl 13.6 Å away on the other side of the heme is much less efficient than electron delocalization. This reaction is also distinct from the metHb reaction where excess NO is present to react directly with the thiyl radical (see earlier), and no transfer of NO from the heme to the thiol is required.

It is also necessary to recognize that the transfer requires the release of NO from the Fe(II)heme. The ferrous heme is

known to have a very high affinity for NO. The allosteric factors discussed earlier predict an affinity after oxygenation (in the R-state) that is even higher than for 6-coordinated T-state hemoglobin. The argument that in the R-state, the nitrosated thiol is more stable is irrelevant unless the NO can be released from the heme.

The release of NO from the thiol in the T-state

It is known that the availability of the β-93 cysteine thiol is dependent on the quaternary conformation with greater accessibility in the oxygenated R-state than in the deoxygenated T-state (5). We have, however, shown (see later) that even in the absence of oxygen in the T-state, SNOHb is formed when nitrite reacts with deoxyhemoglobin (53). Furthermore, once formed, SNOHb has been found to be stable in both oxygenated samples and deoxygenated samples in the absence of metal ions and reductants. These results are inconsistent with a dynamic conformationally linked release of NO from SNOHb. However, coupled to interactions involving metal ions and/or reductants, it is feasible that NO can be released from SNO in a conformationally linked fashion.

The final issue that is relevant to the SNOHb hypothesis as well as the other more recent proposals (see later) for the delivery of NO by the red cell involves the mechanism for the release of NO from the red cell. This issue will be discussed later.

THE NITRITE HYPOTHESIS

The SNOHb hypothesis has completely changed our perspective regarding the red cell. Although earlier studies emphasized the red cell as a scavenger of NO and its role in causing vasoconstriction, the SNOHb hypothesis has emphasized a role for the red cell in regulating and modulating vascular NO levels and the ability of the red cell deliver NO to the vasculature under hypoxic conditions when it is needed. This hypothesis directly uses the NO produced by the endothelium, and therefore must accomplish this goal by modifying our prior understanding of the reactivity of hemoglobin with NO.

A new perspective to these issues was provided by our article (40) and subsequently further developed by Gladwin and associates (9). Instead of the red cell picking up NO produced by the endothelium, this hypothesis reutilizes the nitrite produced by the oxidation of the NO in the plasma. The reutilization occurs under hypoxic conditions when hemoglobin is partially deoxygenated and the reduction of nitrite to NO is turned on (Fig. 4). By generating NO where it is needed, the difficulties involved in the red cell transporting the highly reactive NO is avoided. Plasma is known to contain ~100–600 n*M* nitrite (50). Even though the nitrate level in plasma is greater than that of nitrite (60), the nitrate is an end product that is eventually removed from the circulation by the kidneys, whereas the nitrite is available to be taken up by the red cell (35).

Nitrite has been known to be slowly reduced by deoxyhemoglobin to NO (11). It is generally accepted that nitrite reduction involves an intermediate with NO^+ associated with the Fe(II) heme (49). However, the finding that metHb and the Hb(II)NO are the only spectral species detected during nitrite reduction by deoxyhemoglobin (11, 51) led to the assumption that only trace levels of any intermediates formed are present during the reaction.

Both the slow rate of reaction (2.69 $M^{-1}s^{-1}$ at pH 7) and the formation of relatively inert Hb(II)NO seemed to rule out any physiologic importance for this reaction. It was, however, argued that even at this relatively slow rate the formation of NO under hypoxic conditions where hemoglobin is partially oxygenated could provide enough NO to influence vascular activity. This hypothesis was supported by *in vivo* and *in vitro* nitrite-infusion studies coupled with measurements of the formation of Hb(II)NO and SNOHb (9, 10, 39).

The nitrite intermediate hypothesis

In the red cell under hypoxic condition, nitrite directly reduced to NO will rapidly react with the deoxygenated chains. A physiologic role for such a mechanism requires a mechanism to release the NO from the cell as it is formed (see later). This inherent difficulty in the nitrite hypothesis was resolved in our studies, which found appreciable levels of intermediates during nitrite reduction by deoxygenated hemoglobin (40).

These intermediates were originally detected by comparing the NO signal obtained using chemiluminescence to measure the total non-thiol hemoglobin associated NO and electron paramagnetic resonance to measure the final product, Hb(II)NO (Fig. 5). The clear difference established the presence of relatively stable intermediates during the nitrite reduction processes. Further studies on the nitrite reaction actually established appreciable concentrations of two intermediates formed during nitrite reduction of deoxyhemoglobin.

The binding of nitrite to deoxyhemoglobin and the oxygen-labile intermediate. The initial binding of anionic nitrite to deoxyhemoglobin, which usually binds hydrophobic neutral molecules like oxygen, carbon monoxide, and nitric oxide, is very weak. We thus find that by filtration essentially all of the initial nitrite added to the deoxyhemoglobin is in the eluant (Nagababu and Rifkind, unpublished results). This observation is consistent with an increase in the rate for nitrite reduction at low pH where the nitrite is protonated, forming the neutral HNO_2. However, even this hydrophilic molecule is not expected to have a high affinity. Binding of nitrite, as found for the heme-based nitrite reductase, presumably involves interactions with histidines. Any appreciable interaction of the nitrite, even before reduction takes place, should be associated with hydrogen bonding to the distal histidine.

The distal heme pocket must be properly configured for such an interaction. Interactions with the distal histidine, therefore, explain the conformational restrictions associated with the nitrite reduction. We thus find that the addition of DPG or IHP reduces nitrite reduction (28). The reaction is also much slower for α-chains than β-chains and finally the reaction is faster in the R-state produced by cleaving the terminal histidine and tyrosines of the α-chains with carboxypeptidase-A before deoxygenating the hemoglobin

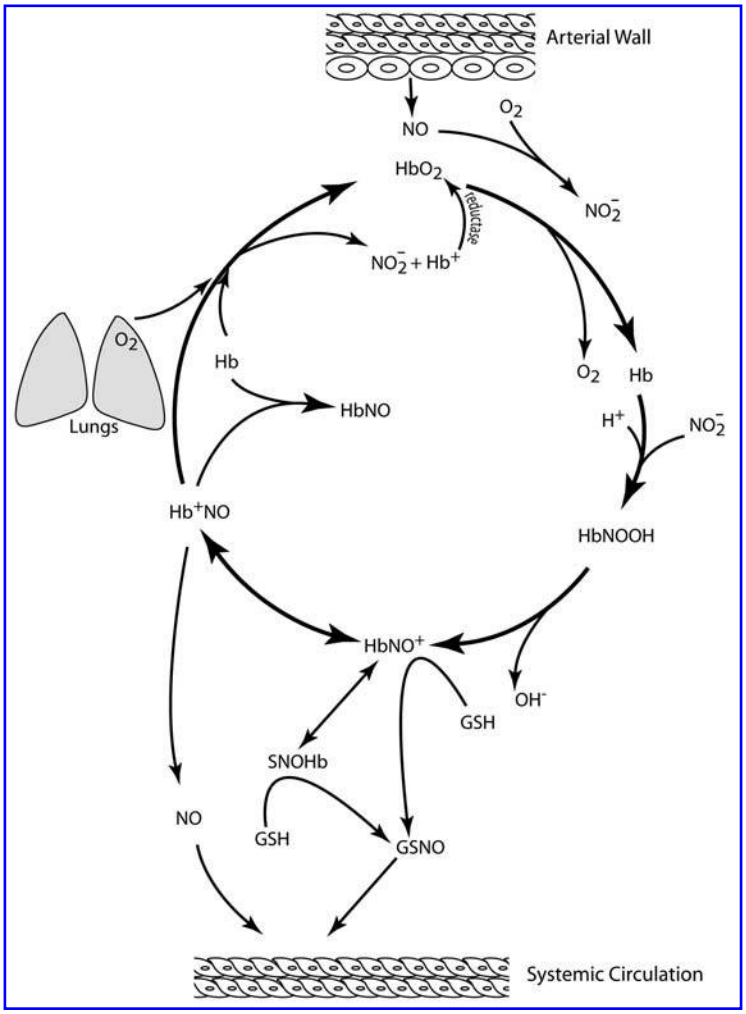

FIG. 4. The Nitrite Hypothesis: The reaction of nitrite with the deoxygenated hemoglobin chains formed when the partial pressure of oxygen is reduced in the systemic circulation. This reaction results in an intermediate with properties of $Hb(II)NO^+$ and Hb(III)NO. The $Hb(II)NO^+$ can react with GSH or produce SNOHb, whereas the Hb(III)NO can release NO. As the blood is oxygenated in the lungs, some of these intermediates decompose, limiting the build-up of reactive NO species.

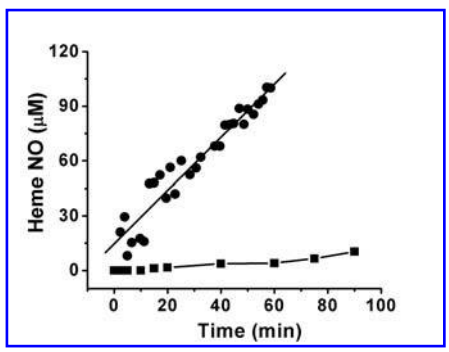

FIG. 5. The reaction of a 1:4 ratio of nitrite to heme can be followed by chemiluminescence (●) and electron paramagnetic resonance (EPR) (■). The EPR measures Hb(II)NO, whereas the chemiluminescence measures all heme complexes that can be released from the heme and reduced to NO in acidic ferricyanide. The chemiluminescence assay does not measure nitrosothiols and, because of the addition of sulfanilamide, does not measure free nitrite. The greater NO measured by chemiluminescence than by EPR establishes the presence of heme-associated intermediate species.

(Ramasamy, Nagababu, Mohanty, and Rifkind, unpublished results).

The H-bonded nitrite in the ligand pocket is presumable the initial intermediate formed that is stable only in the absence of oxygen (Fig. 6). This complex releases NO when injected into the purge vessel of the nitric oxide analyzer, when acetic acid is in the purge vessel. Because the nitrite is bound to the hemoglobin, it does not, however, react with the sulfanilamide in the purge vessel and is detected as NO.

The oxygen-stable intermediate. This nitrite complex subsequently undergoes an electron transfer, forming $Hb(II)NO^+$. This species involves electron delocalization between the iron and the NO with properties of both $Hb(II)NO^+$ and Hb(III)NO. By comparing the oxygen-stable chemiluminescence signal with the electron paramagnetic resonance signal of Hb(II)NO, we have shown that there is also a build up of this intermediate (Fig. 7).

During the reaction of nitrite with deoxygenated hemoglobin chains, this intermediate slowly releases NO that reacts with unliganded hemoglobin chains, producing Hb(II)NO. This intermediate is identical to the intermediate associated with reductive nitrosylation when NO reacts with metHb

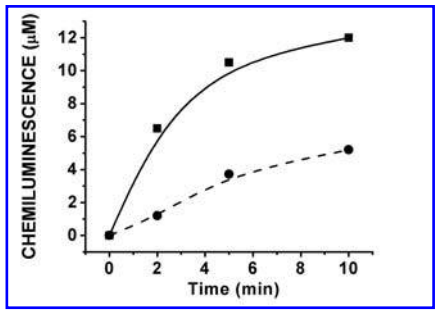

FIG. 6. The chemiluminescence signal, formed when a 1:4 nitrite/heme ratio is reacted with deoxyhemoglobin, contains an oxygen-labile component (■) lost when the sample is oxygenated and an oxygen-stable component (●) that remains after the sample is oxygenated.

(Fig. 8) (see earlier). However, by carefully following the reaction of metHb with stoichiometric NO (not the excess frequently used), we have been able to identify an additional intermediate (53). The initial complex of NO with metHb gives the well-documented visible spectrum associated with Hb(III)NO. Whereas earlier studies attributed this spectrum to the delocalized $Hb(II)NO^{+} \leftrightarrow Hb(III)NO$ species, we find that the Hb(III)NO spectrum disappears within 20 min, producing a species that is not yet reduced Hb(II), but slowly reacts with CO to produce HbCO. It is this second species that is believed to be the delocalized species $Hb(II)NO^{+} \leftrightarrow$ Hb(III)NO, formed both during nitrite reduction and the reaction of NO with metHb (Fig. 8). These results explain the differences between the nitrite intermediate and the reported spectral properties and kinetic properties of the Hb(III)NO complex initially formed when NO reacts with Hb(III).

Nitrite reduction by the red cell in vivo and its utilization during exercise. As mentioned earlier, a number of studies (9, 10, 39) have shown that the infusion of nitrite has vascular affects. It has also been shown that nitrite infused *in vivo* will react with red cells. The question to be resolved was whether, under normal conditions, plasma nitrite reacts with red cells and whether this pool of red cell NO is available to the vasculature. This was addressed by using our data on the intermediates to characterize the reaction of nitrite, in contradistinction with a direct reaction of nitric oxide. A reaction of NO with deoxygenated hemoglobin chains thus produces Hb(II)NO, which is relatively stable, even in the presence of oxygen and can (if the level is above the electron paramagnetic resonance (EPR) detection limit of ~μ*M*) be detected by EPR. However, the nitrite reaction produces a pool of hemoglobin-associated NO that is not SNOHb and not Hb(II)NO (see earlier), and at least some fraction of this NO is oxygen labile (Fig.6). Studies of arterial and venous blood demonstrated that this relatively labile NO species, analogous to that formed during nitrite reduction by deoxyhemoglobin, accounts for most of the red cell NO found in fresh blood samples (Fig. 9). Nitrite reduction by red cells thus occurs *in vivo.*

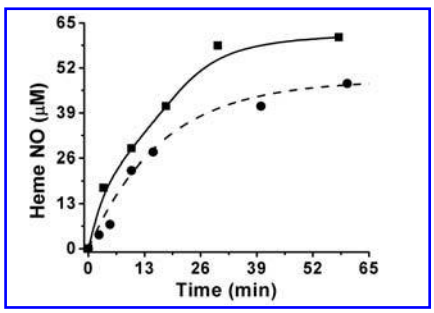

FIG. 7. The reduced chemiluminescence signal, obtained when the reaction mixture formed by a 1:4 nitrite/heme molar ratio of nitrite reacts with deoxyhemoglobin (■), is greater than the Hb(II)NO signal detected by electron paramagnetic resonance (EPR) (●). This observation establishes that during the nitrite reaction, both oxygen-labile and oxygen-stable intermediates are formed before the release of NO.

The buildup of this intermediate, where the NO is not bound nearly as tightly as it is in Hb(II)NO, provides for a source of NO in the red cell that can be released from hemoglobin to supply a potential source of bioactive NO that can affect the vasculature. We have shown that this pool of NO is reduced immediately after exercise (Nagababu, Ravi, and Rifkind, unpublished results). This finding actually suggests that the nitrite-reduced labile NO maybe available to the vasculature during exercise when the demand for oxygen increases.

The formation of SNOHb during nitrite reduction. As indicated earlier when discussing the formation of SNOHb when NO reacts with metHb, the intermediate when formed on the β-chain involves the delocalization of the electron density to the β-93 cysteine residue in addition to the iron and the NO (Fig. 8). This delocalization stabilizes the intermediate and further slows the release of NO and its inactivation with the formation of the final products Hb(II)NO and metHb.

This observation is particularly relevant to nitrite reduction because the nitrite reduction process favors the β-chain (see earlier).

As discussed earlier with respect to the metHb reaction, this delocalization of the electron density to the thiyl facilitates a reaction in which the intermediate can react with any nonbound NO to produce SNOHb. During the nitrite-reduction process, no excess NO is available. We nevertheless observe a slow formation of SNOHb, which is attributed to the transfer of NO from the heme to the thiol (Fig. 10).

This same reaction can produce SNOHb *in vivo*, although a possible direct reaction of this thiyl radical with any NO that gets into the red cell also must be considered. The intermediate thus provides a pool of NO that can be either directly released as NO or transferred to the thiol producing SNOHb. The nitrite intermediate hypothesis thus provides an independent pathway for the formation of SNOHb and the putative bioactivity associated with the SNOHb hypothesis. At the same time, it provides a parallel pathway for the propagation of NO bioactivity that does not involve the thiol.

Although a contribution of SNOHb to any nitrite-induced bioactivity cannot be ruled out, a recent article (10) established a non-SNOHb component to nitrite-induced vasodilation. Thus, a mutant with alanine replacing the β-93 cysteine

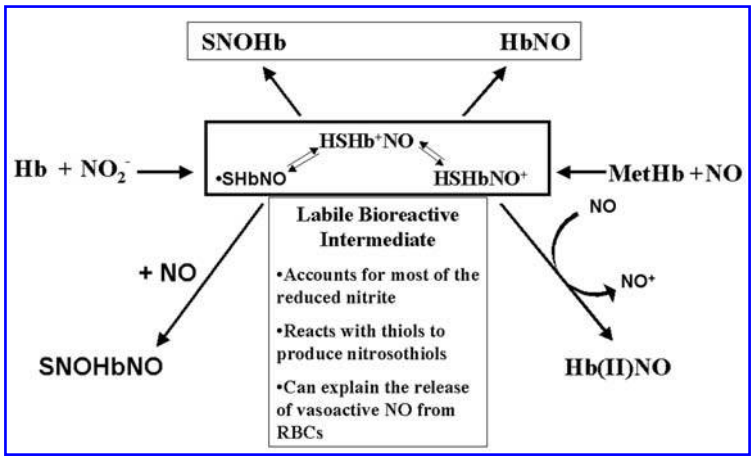

FIG. 8. The electron-delocalized intermediate formed both during the reaction of nitrite with deoxyhemoglobin and during the reaction of NO with methemoglobin. When protonated nitrite reacts with deoxyhemoglobin, a hydroxide ion is cleaved, forming Hb(II)NO^+, and when NO reacts with methemoglobin, Hb(III)NO is formed. These represent two substrates of the same intermediate, with the electron shared by the iron and the NO. The simultaneous formation of Hb(II)NO and SNOHb (Fig. 3) establishes a third substrate in which the electron from the thiol is transferred to NO^+. The presence of these multiple substrates is responsible for the stability of the intermediate and the slow release of NO, forming Hb(II)NO, and the slow transfer of the nitrosonium cation, forming SNOHb, when an excess of NO is not present. In the presence of an excess of NO, a direct reaction with the thiyl radical takes place, forming SNOHb, and the NO^+ can be displaced from the intermediate, forming Hb(II)NO.

actually displays a greater nitrite-induced vasodilation than normal hemoglobin with the cysteine. This mutant, however, alters the hemoglobin conformation, stabilizing the R-state, and cannot be used to argue that SNOHb has no contribution to the vasodilation induced by hemoglobin.

THE RELEASE OF NO FROM THE RED CELL

The entire discussion regarding a role for the red cell in delivering NO to the vasculature depends on the ability of the red cell to release the NO picked up from plasma and/or regenerated from plasma nitrite back to the vasculature.

A role for the red cell membrane must be involved in this process, with a putative role for the hemoglobin association with the cytoplasmic domain of band 3 (theAE1 chloride–bicarbonate anion exchanger) of the red cell membrane. The transport of the NO from SNOHb has been linked to a transnitrosation reaction between SNOHb associated with the membrane and the two reactive cysteine residues on the cytoplasmic domain of AE1 near the hemoglobin binding site (46). It has been shown that SNOHb tends to concentrate on the membrane. The resultant stabilization of the T-state facilitates the transnitrosation reaction. It has also been demonstrated by using vascular strips that these membranes can release NO to the vasculature (47).

Nitrite reduction to NO, without the formation of intermediates, requires that the NO can be shuttled out of the red cell as it is being formed. The possible formation of a membrane band 3/AE1–associated nitrite reductase metabolon has been proposed (15). This process involves bringing together deoxyhemoglobin and carbonic anhydrase, which facilitate nitrite reduction by deoxyhemoglobin (carbonic anhydrase facilitates the nitrite reduction by generating protons necessary for the formation of HNO_2). This NO formed in the hydro-

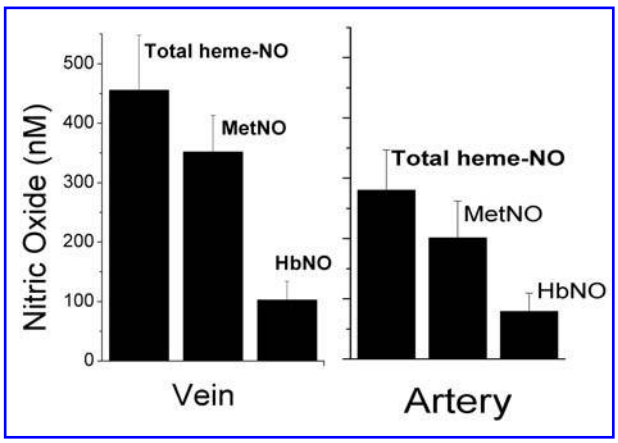

FIG. 9. The oxygen-labile intermediate formed during the nitrite reaction was found to be present in fresh arterial blood and venous blood from human subjects. This supports the contention that nitrite reduction is responsible for at least some of the red cell NO present *in vivo,* and a pool of potentially bioactive NO formed by this reaction is present in blood.

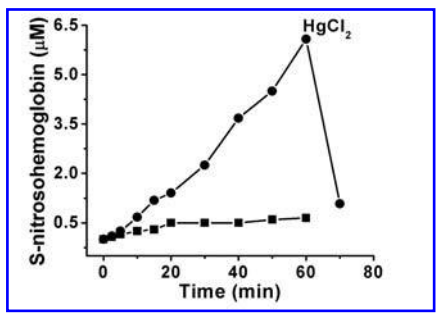

FIG. 10. The slow formation of SNOHb when a 1:10 nitrite/heme molar ratio of nitrite reacts with deoxyhemoglobin (●) is demonstrated. The SNOHb involves a reaction with the β-93 cysteine residue that is not formed when this residue is blocked with NEM (■). Confirmation that the product is a nitrosothiol is demonstrated by the disappearance of the signal when $HgCl_2$ reacts with the product, breaking the S-NO bond.

phobic bilayer is released from the cell without reacting with deoxyhemoglobin (16). The nitrite reduction occurring on the membrane is, however, inconsistent with the recent results that R-state hemoglobin, which has a relatively poor membrane affinity, is primarily responsible for nitrite reduction in the red cell.

The nitrite-intermediate hypothesis bypasses this difficulty by forming a metastable intermediate that can subsequently release its NO at lower oxygen pressures when the hemoglobin membrane association is greater.

An alternative mechanism to release nitrite-reduced NO involving the nitrite anhydrase reaction, whereby the $Hb(II)NO^+$ intermediate reacts with nitrite to produce N_2O_3 (50). The N_2O_3 is a potent nitrosating agent that can form SNOHb. However, its reactivity for thiols is not nearly as great as that of NO for deoxyhemoglobin or oxyhemoglobin. Being a hydrophobic small molecule, it can easily diffuse out of the red cell. Its hydrolysis to form NO and NO_2 (7) once out of the red cell provides a possible mechanism to transfer NO to the vasculature.

An additional factor that should be addressed in any attempt to explain red cell–induced vasodilation is the steep radial velocity distribution (50) in the precapillary arterioles where the pO_2 initially decreases. This phenomenon results in a cell-free plasma zone at the vessel wall, which further complicates the transfer of NO from the red cell to the vasculature. In the smaller capillaries where the red cell is in contact with the vessel wall and NO transfer can more readily take place, smooth muscle cells required to respond to NO are lacking.

The transfer of NO from red cells to the vasculature is essential, if red cell NO plays a physiologic role. Although a number of studies support that such a reaction occurs, at least under certain conditions in which the NO supplied by the endothelium is not adequate, the process for red cell delivery of nitric oxide to the vasculature is clearly not completely understood, and additional studies are necessary to explain this process.

CONCLUSION AND COMPARISON OF NITRIC OXIDE AND OXYGEN

Any role for the red cell in NO function is linked to the red cell redox properties. The SNOHb hypothesis involves the redox reaction required for the transfer of NO from the heme to a thiol. The nitrite reutilization hypotheses are directly coupled to the redox reaction of nitrite to NO, with the Nitrite Intermediate Hypothesis utilizing redox properties of the heme NO and the thiol to stabilize the intermediates formed.

Whereas the oxygen transport role of hemoglobin is facilitated by maintaining the Fe(II) state of the heme, for NO, it is the controlled redox reactions that may play a crucial role in regulating systemic blood flow and thereby facilitating the delivery of oxygen to the tissues, at least under certain conditions.

Analogous redox reactions involving oxygen produce superoxide, hydrogen peroxide, rhombic damaged heme, and fluorescent degradation products (41). These processes, instead of contributing to the functional role of the red cell in delivering oxygen, reflect oxidative damage, which can impair the function of the red cell and can contribute to oxidative stress associated with many diseases.

In comparing the relation between the interactions of oxygen and NO with red cell hemoglobin, it is also necessary to point out that the primary role of oxygen involving the reversible binding of oxygen involves most of the red cell hemoglobin, whereas the related redox reactions involve only a small fraction of the hemoglobin that binds oxygen. Unlike the delivery of oxygen to the tissues, which involves a major fraction of the hemes, the functional role of the red cell in delivering NO requires very low levels of NO, which may, however, have a major impact on blood flow. For this to function, the redox reactions must dominate the reversible binding process so that the function delivery of NO can take place without limiting the ability of hemoglobin to transport oxygen.

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ABBREVIATIONS

EPR, electron paramagnetic resonance; CO, carbon monoxide; CO_2, carbon dioxide; $CO_3^{\bullet -}$, carbonate radical anion; DPG, 2,3-diphosphoglyceric acid; Hb(III), methemoglobin; Hb(II)NO, iron nitrosylhemoglobin; HNO_2, nitrous acid; IHP, inositol hexaphosphoric acid; metHb, methemoglobin; NO, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide; N_2O_3, dinitrogen trioxide; NO^+, nitrosonium cation, NO^-, nitroxyl anion; $ONOO^-$, peroxynitrite; $ONOOCO_2^-$, nitrosoperoxocarboxylate; R-state, relaxed hemoglobin-liganded conformation; T-state, tense unliganded hemoglobin conformation; SNOHb, *S*-nitrosohemoglobin; RSNO, *S*-nitrosothiols.

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