How do red blood cells cause hypoxic vasodilation?
The SNO-hemoglobin paradigm

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Allen, Barry W., and Claude A. Piantadosi. How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. Am J Physiol Heart Circ Physiol 291: H1507–H1512, 2006. First published June 2, 2006; doi:10.1152/ajpheart.00310.2006.—One of the most intriguing areas of research in erythrocyte physiology is the interaction of hemoglobin with nitric oxide (NO). These two molecules independently fulfill diverse and complex physiological roles, while together they subtly modulate microvascular perfusion in response to second-by-second changes in local metabolic demand, contributing to hypoxic vasodilation. It is through an appreciation of the temporal and structural constraints of the microcirculation that the principal requirements of the physiological interplay between NO and hemoglobin are revealed, elucidating the role of the erythrocyte in hypoxic vasodilation. Among the candidate molecular mechanisms, only S-nitrosohemoglobin (SNO-hemoglobin) directly fulfills the physiological requirements. Thus, NO is transported by red blood cells to microvascular sites of action in protected form as an S-nitrosothiol on the highly conserved hemoglobin β-93 Cys residue, invariant in birds and mammals. SNO-hemoglobin dispenses NO bioactivity to microvascular cells on the release of oxygen, physiologically coupling hemoglobin deoxygenation to vasodilation. SNO-hemoglobin is the archetype for the role of S-nitrosylation in a newly identified class of biological signals, and disturbances in SNO-hemoglobin activity are associated with the pathogenesis of several important vascular diseases.

nitrite; nitrite reduction; S-nitrosylation; S-nitrosohemoglobin

THE RED BLOOD CELL (RBC) has the marvelous ability to facilitate the matching of capillary perfusion with local metabolism by means of hypoxic vasodilation—the relaxation of vascular smooth muscle in regions of low tissue PO₂—which enhances oxygen delivery. Although a subject of ongoing research and a diversity of interpretations, many studies show that a major pathway to this phenomenon involves the release of nitric oxide (NO) bioactivity by RBCs (7, 9, 11, 17, 22, 23). But how?

Any mechanism attempting to link the release of NO bioactivity to hypoxic vasodilation must satisfy the physiological requirements and the architectural constraints of both the systemic and pulmonary circulations and must act within the brief time that an erythrocyte traverses a capillary from arteriole to venule. Capillary transit time is 1 s or less in resting tissue, and a fraction of that in exercise, as shown schematically in Fig. 1A, for a capillary in skeletal muscle. In fact, the real temporal window may be even narrower because the vascular smooth muscle cells that are the effectors of this dilation cluster near the arteriolar end of the capillary (41). Thus a metabolic signal must be detected by a physiological sensor, transduced to a chemical signal, and then transported to target effector cells, all within a few hundred milliseconds.

The seminal observations, in 1996 and 1997 (23, 37), implicating hemoglobin in the RBC in NO-mediated local vasodilation met with skepticism at first. Although the reaction of blood with NO has been investigated for more than a century (18) and the chemistry of NO with heme-iron has been of scientific interest for nearly 50 years (15), a conceptual obstacle was that free hemoglobin consumes NO and is therefore a vasoconstrictor (24). The idea that NO and hemoglobin could coexist is based on the protein biochemistry of S-nitrosothiols (R-SNO) and has shifted a central paradigm of NO biology. Thus the terms “S-nitrosylation” and “S-nitrosation” first appeared in the biomedical literature in 1992 and 1993, respectively (36, 39). Today, however, a literature search of these topics reveals hundreds of articles that support diverse physiological roles for the binding of NO to the cysteine thiols of many proteins, modulating their conformation, function, and interactions. Most investigators no longer think of free NO as an exclusive physiological entity but instead use the term “NO bioactivity” to signify chemical species with one or more nitrosyl groups that can exert the physiological effects of NO, often mediated by apparent NO⁺ activity.

Indeed, molecular NO is evanescent in biological systems, particularly in the presence of free hemoglobin. However, when stabilized for transport or storage as an R-SNO, it can move through extracellular fluids and into cells, or signal at cell surfaces, by means of transnitrosation, the ability of one thiol to transfer NO to another. Most researchers in the field now agree that nitrosothiols are capable of transducing NO bioactivity far from their location of formation (10, 17, 27), readily crossing cell boundaries. It has been shown that NO binds heme in a small fraction of available deoxyhemoglobin, which on reoxygenation transfers the NO to a cysteine on

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β-chains of the hemoglobin tetramer as an S-nitrosothiol, in amounts sufficient to convey bioactivity (27). This mechanism does not invoke free NO; instead NO bioactivity is transported by RBCs in a protected form (on β-93 Cys) and is dispensed on hemoglobin’s oxygen-dependent allosteric transformation. This idea has strong support from multiple laboratories (2, 11, 13, 29, 34, 35) but has only slowly achieved general acceptance, in part because exacting experimental and analytical techniques are required to capture the fugitive intermediates of NO bioactivity, mediated by conformational transitions in native hemoglobin, and because the most sensitive method commercially available for measuring NO (reductive chemiluminescence using tri-iodide) does not directly distinguish NO from its higher oxides (NOx) (11, 34) nor does it measure Fe(III)NO, which may be the prevalent iron nitrosyl in vivo (30).

In 2003 an alternative to SNO was proposed, the “hemoglobin compartmentalization model,” in which molecular NO in the plasma is protected by diffusional barriers and “neither special preservation nor delivery of NO by hemoglobin is required” (16). Indeed, the renowned physiologists Roughton and Comroe proved long ago that the diffusion of NO is hindered by the erythrocyte membrane (4, 15). The idea of extraerythrocytic barriers in the plasma, however, is based on thought experiments and mathematical modeling, useful to explain the preservation of endothelium-derived relaxing factor (EDRF) but lacking empirical or theoretical connections to hypoxic vasodilation. And the magnitude of these barriers is unknown, for as one eminent theoretician states, “Even though great advances have been made in NO measurements, the spatial resolution of the methods has not been sufficient to measure these steep NO gradients” (25). Lacking reconciliation with the effects of erythrocytes on vascular tone, some former advocates of this model have acknowledged that there are reaction products of NO with blood that conserve its bioactivity and transduce vasodilation (40). A few investigators remain agnostic about any role for NO in hypoxic vasodilation, relying instead on tissue metabolites generated by hypoxia, for example adenosine, or the long-known ability of low PO2 to dilate vessels directly (33). It has also been proposed that venules in proximity to small muscularized arteries could allow diffusion of a hypoxia-generated mediator from veins to influence arterial tone. But one must link these effects to the RBC or risk a struthonian denial of the many observations that vasodilation of systemic blood vessels by hypoxia is always accelerated and augmented in the presence of carefully harvested erythrocytes at the appropriate O2 saturation (7, 9, 11, 17, 22, 23).

A more recent idea is that a nitrite (NO2−) gradient across the arteriovenous bed reflects a role for nitrite in the vasodilation of hypoxia or exercise (5, 21, 30). A later development postulates that nitrite in the erythrocyte is a protected reservoir for NO, the latter mobilized through a heme-mediated process termed “nitrite bioactivation” and is subsequently released.
from Fe(II)NO independently of SNO. This concept has been presented in various accounts, most noticeably in four studies published in *Blood* from 2004 to 2006 (6, 7, 10, 20). It is suggested that nitrite derived from endothelial NO diffuses from the plasma into erythrocytes, where it achieves a concentration twice that in plasma and where some of it is reconverted to NO by deoxyhemoglobin acting as a nitrite reductase.

The idea that bioactivated nitrite has a role as an independent, physiological vasodilator has been vigorously advanced but is difficult to fit into a coherent physiologic RBC dilator mechanism without S-nitrosohemoglobin (SNO-hemoglobin). Although nitrite concentration in RBCs is reported to be nearly 300 nM and adequate for vasodilation (10), a sluggish response in vascular rings in the presence of erythrocytes appears only after exogenous nitrite is added at pharmacological concentrations, and the nitrite effect is not maximal until hemoglobin falls to half-saturation (7). Nonetheless, in an attempt to validate the nitrite effect physiologically, it was paired with “PO2 at onset of relaxation” (7), a parameter that fluctuates with so many factors that it is difficult to see it as a useful basis for understanding hypoxic vasodilation. This variable, however, was used in a model system along with a standard descriptor of hemoglobin’s behavior with respect to oxygen, P50, the PO2 at which hemoglobin is 50% saturated with oxygen (Fig. 1B). That study reported “a linear relationship between P50 and oxygen tension at onset of relaxation . . .” as the foundation for this idea (7). Because a lower P50 means more oxyhemoglobin at any plasma P O2, the nitrite hypothesis would predict vasoconstriction as the amount of deoxyhemoglobin (the “nitrite reductase”) falls.

The reverse is actually observed in the circulation: at constant oxygen content and metabolic rate, blood flow increases as P50 falls, reflecting the greater difficulty for mitochondria to extract oxygen from hemoglobin (42). Conversely, as P50 rises, there is less hypoxic dilation, for example, in myocardium and brain (8, 12). The P50 is also sensitive to pH, shifting to the left (a lower value) with alkalosis and to the right (a higher value) with acidosis (see Fig. 1B). Thus, as a RBC passes through a capillary, P50 increases because of the fall in pH, which facilitates oxygen unloading (Bohr effect). As P50 increases, the amount of deoxyhemoglobin increases more rapidly, and capillary PO2 falls more slowly than at a constant P50. In other words, the slope of the relationship between hemoglobin saturation and PO2 increases during capillary transit as P50 shifts to the right. This is illustrated in Fig. 1B by the slopes of two arrows, showing that as oxyhemoglobin (HbO2) saturation falls from artery to vein, the rate of fall is greater for acidosis (the dotted curve to the right) than at normal pH (the solid curve in the center). If NO generation by deoxyhemoglobin did control the rate of vessel relaxation, it would not physiologically connect PO2 with “onset of relaxation.”

According to this model, NO2- entering the erythrocyte has one of three fates: some persists, some is oxidized to nitrate (NO3-), and some is reduced to Fe(II)NO (not molecular NO). Because these three nitrogen species are said to derive from a single source, endothelial NO (10), stoichiometry would dictate that erythrocytes, like hemoglobin, would be net scavengers of NO. This would favor vasoconstriction, not the vasodilation proposed. Indeed RBCs do constrict blood vessels in certain physiological conditions, but not in all. Thus, in hypoxic regions of the microvasculature, RBCs enhance vasodilation, a fact that nitrite alone cannot explain. In contrast, as indicated in Fig. 2, SNO-hemoglobin is a potent vasodilator in vitro and in vivo on the physiological time scale at low PO2, but not at high PO2 (Fig. 2).

The reliance on pharmacological doses of nitrite to investigate physiological vasodilation adds to the difficulty of drawing firm conclusions. For example, nitrite is used to induce relaxation in aortic rings and is added to solutions of RBCs to enhance the effect of hypoxia at 30 to 130 times the level reported as physiological (7). However, such effects of nitrite must be compared with those of RBCs and hypoxia without nitrite to determine whether hypoxic relaxation is due to nitrite plus RBCs or to RBCs alone (for example, a SNO effect). Data...
that NO\textsubscript{3} plus hemoglobin dilate blood vessels only when NO synthase is inhibited to prevent RBCs from causing constriction are not physiologically relevant. Those studies do confirm, however, that “... SNOHb is formed to some extent from the reaction of nitrite with Hb...” (7). We view this as perhaps the main role of nitrite in the erythrocyte.

Values for cGMP are often used as an index for the activation of soluble guanylate cyclase by NO in vascular smooth muscle, which one would also expect to see in hypoxic vasodilation mediated by nitroso species dispensed by RBCs. However, such an effect is not physiological, for instance, when cGMP doubles 10 min after 3 μM nitrite is added to RBCs (7)—because 10 min is hundreds of times greater than capillary transit time—and when no relaxation is seen and successive additions of nitrite, far above physiological levels, do not produce graded relaxations. In other words, it is hard to agree that “RBCs, hypoxia and nitrite constitute an integrated system for hypoxic vasodilation” (7). Instead, nitrite appears to be influencing the Po\textsubscript{2} settling point of the erythrocyte-vasodilator system, most plausibly by increasing SNO-hemoglobin content.

The idea that nitrite may be an independent physiological vasodilator was originally inferred from the observation that there is a nitrite gradient across the arteriovenous bed (5). However, this inference disregards the more straightforward explanation that such a gradient reflects the oxidation of EDRF to nitrite as blood transits the microcirculatory unit from artery to vein (31). Also, it is mistaken to formulate a hypothesis that does not account for the observation made in the same study that nitrite infusion increases venous O\textsubscript{2} saturation (5) and therefore decreases deoxyhemoglobin, the nitrite “reductase.” Simply put, this does not equate to hypoxic vasodilation because there is no observed coupling of nitrite vasodilation to deoxygenation of hemoglobin. Finally, there is no vasodilation at physiological concentrations of nitrite or in the time scale in which RBCs transit the microcirculatory unit. Such physiological discrepancies argue against a SNO-independent role for nitrite in the dynamic regulation of perfusion during hypoxia.

Questions that remain about the SNO-hemoglobin paradigm do not involve physiological incongruities. Although the exact fate of NO released from SNO-hemoglobin and the particular nitroso species that transduce vasodilatation are not yet delineated, these are subjects of ongoing research that are derivative of the original observations that RBCs do dilate blood vessels in a physiological time scale and that NO is released from Cys-93 on β-hemoglobin by hypoxia (37). In addition, SNO has been detected from RBCs coincident with the relaxation of vascular smooth muscle in seconds or less, with nothing added (35). The rapid transfer of NO from β-heme to β-93 Cys has also been demonstrated (11, 23, 26, 32). Another study reported vasodilation using a mutant hemoglobin in which alanine was substituted for β-93 Cys, thus appearing to challenge a vital part of the SNO-hemoglobin mechanism (7). However, this experiment is not interpretable because the degree of vasodilatation was neither quantified nor correlated with time on a physiological scale.

Questions have also been raised about the amount of SNO-hemoglobin produced under physiological conditions and whether it suffices for hypoxic vasodilation. Thus Herold and Rock (19) found yields for SNO-hemoglobin thought to be too low to account for vasodilation, but their results turned out to be similar to those of others who found higher levels, when differences in heme/NO ratios are taken into account (35). These ratios are crucial because SNO-hemoglobin formation requires methemoglobin as an intermediate, and because methemoglobin concentration is normally kept low by regulatory mechanisms, just a small fraction of hemoglobin tetramers is S-nitrosylated. On the other hand, high methemoglobin concentrations will SNO-hemoglobin exceed the detection limit of even the most sensitive analytical method. Conversely, in experimental systems flooded with NO, many thiols and other functional groups will be nitrosylated or nitrated and biological relevance lost. The absolute amount of NO bioactivity released from SNO-hemoglobin is small; otherwise we would die of hypotension while trying to oxygenate our tissues.

However, for Fe(II)NO compounds, the products of nitrite and hemoglobin, no direct mechanism for vasodilation has been found; in fact they are vasoconstrictors. Previous evidence had suggested that most Fe-NO in vivo is Fe(III)NO (30), and newer work has implicated a population of Fe(II)NO or Fe(III)NO in SNO β-93 Cys formation (1). The Fe-NO species derived from nitrite form in T-state hemoglobin, mainly in veins, and therefore cannot induce hypoxic vasodilation in the arterioles; but on the allosteric transition of hemoglobin to the R state at reoxygenation, these species convert quantitatively to SNO-hemoglobin. It is in this context that a possible role for nitrite in hypoxic vasodilation is found, not in the false dichotomy of either nitrite or SNO but in a complementary system in which nitrite and perhaps other factors removed from hypoxic vasodilation support the allosteric coupling mechanism offered by SNO-hemoglobin. The effects of exogenous nitrite are most consistent with this idea that nitrite does not induce hypoxic vasodilation directly but can contribute to the pool of SNO in the erythrocyte (1, 7, 27, 30).

By contrast, the SNO-hemoglobin mechanism, when elicited experimentally, manifests the characteristics of the physiological dilator response: it alters both the rate and extent of vascular relaxation and is fast enough to occur during capillary transit (35). It does not require that nitrite be reduced, which could only occur after O\textsubscript{2} is unloaded from the erythrocytes and deoxyhemoglobin (the “reductase”) becomes more available to reduce nitrite. SNO-hemoglobin ideally fills the need for a protected form of NO in the erythrocyte: it does not require that molecular NO escape from heme that would avidly trap it, nor does it involve Fe(II)NO, the reaction product of nitrite with hemoglobin, which is not a vasodilator.

Thus the ability of SNO-hemoglobin to dispense NO bioactivity on the allosteric release of oxygen fits the physical and temporal constraints of the microcirculation. This accords with the broader concepts that NO reacts readily with sulfhydryl groups (-SH) and that S-nitrosylation modulates the functions of many biomolecules. Hemoglobin is, therefore, the archetype for more than one hundred proteins known to undergo S-nitrosylation (38). A system of protected transport from erythrocyte SNO-hemoglobin to the endothelial-smooth muscle cell unit, most plausibly via low molecular weight nitrosothiols or by cell-to-cell transfer, would comprise the final link in this elegant physiological mechanism. Up to now, attention has centered on the local control of vasodilation by NO bioactivity released from erythrocytes, but this is only a first step toward understanding the role that erythrocytes perform in local reg-
ulation of perfusion. For instance, ATP released from RBCs has also been shown to modulate local vasomotor tone (17), and other physiological messengers may well exist. As the actions and interactions of these control systems are elucidated, we will gain new appreciation of the marvelous phenomenon of local control of perfusion by the RBC.

Finally, the disordered delivery or release of NO bioactivity from RBCs to microvessels, due to defective S-nitrosylation, has been implicated in the pathogenesis of several diseases in which vascular function is impaired. Significant examples include diabetes (22), sickle-cell anemia (3), congestive heart failure (9), sepsis (6, 11), preeclampsia (14), and pulmonary hypertension (28). As better tools become available to measure and distinguish the protected forms of NO bioactivity in the blood under physiological conditions, greater insight will be achieved concerning NO-related hemopathologies. Eventually this will lead to new clinical interventions that will enable therapeutic manipulation of the chemical transport of NO between the erythrocyte and the tissues.

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