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Serial Review: Biomedical Implications for Hemoglobin Interactions with Nitric Oxide

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S-NITROSOHEMOGLOBIN: AN ALLOSTERIC MEDIATOR OF NO GROUP FUNCTION IN MAMMALIAN VASCULATURE

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Abstract—Since the discovery of NO as the endothelium-derived relaxing factor, there has been considerable interest in how NO interacts with hemoglobin (Hb). Numerous investigations have highlighted the possibility that rather than operating as a sink to consume NO, the vasculature can operate as a delivery mechanism for NO. The principal hypothesis proposed to explain this phenomenon is that Hb can transport NO on the conserved cysteine residue β 93 and deliver that NO to the tissues in an allosterically dependent manner. This proposal has been termed the *S-Nitrosohemoglobin (SNO-Hb) Hypothesis*. This review addresses the experimental evidence that led to development of this hypothesis and examines much of the research that resulted from its generation. Specifically it covers the evidence concerning NO in the vasculature, the SNO-Hb Hypothesis itself, the biochemical and biophysical data relating to NO and Hb interactions, SNO-Hb in human physiology, and alternative vascular forms of NO. Finally a model of NO in the vasculature in which SNO-Hb forms the central core is proposed. © 2004 Elsevier Inc. All rights reserved.

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INTRODUCTION

Studies of structure–function relationships in hemoglobins (Hbs) have served the medical and scientific community for more than a century. The physiological role of Hb depends on its ability to reversibly bind oxygen and carbon dioxide. This binding and release are regulated through the intermediacy of a number of alternative ligands, many of which do not associate with the heme moiety of Hb, such as phosphates, chloride ions, and protons. Respiratory demands make it essential that the binding of these heme and nonheme ligands be thermodynamically linked in a physiologically relevant manner. This model was first espoused by Jeffries Wyman who, along with many colleagues, developed the concepts of homotropic and heterotropic allosteric linkage [1]. In seemingly unrelated studies in the 1970s, Murad discovered that soluble guanylate cyclase was activated by the gaseous free radical nitric oxide [2]. Hb researchers

apparently took little note of this. Nitric oxide (NO), a diatomic molecule like oxygen and carbon monoxide, was known to bind both ferrous and ferric hemes of Hb and had been used mostly as an interesting ligand for esoteric structure–function studies. In 1987, Ignarro [3] and Furchgott [4] and their colleagues proposed that an elusive vasoactive compound, endothelial-derived relaxing factor, was NO. By analogy with Hb and the in- and out-of-plane movements of the heme iron associated with ligand binding, Ignarro and co-workers had earlier proposed that the activation of soluble guanylate cyclase, also a heme protein, occurred via a similar structural alteration triggered by ligand binding [5]. In addition, because of the well-known reaction of nitrogen monoxide (i.e., NO) with hemoglobin, both the Furchgott and Ignarro groups adduced the high reactivity of NO with oxyHb as demonstrable “proof” of its functional effectiveness in their studies. Still, most hemoglobin researchers paid scant attention.

In 1992, Stamler and colleagues demonstrated that the single reactive sulfhydryl group of human serum albumin could become S-nitrosylated and that, in fact, S-nitrosoalbumin (SNO-albumin) was present in human serum [6]. Hb, one of the first proteins to be sequenced, has one cysteine in its α (α 104) and two in the β (β 93 and β 112) chains. In the 1960s, the Rome Group of Hb researchers (Wyman, Antonini, Brunori, and colleagues) discovered that the β 3 group of Hb was freely reactive but that the other free cysteines were more buried and less reactive, unless the tetramer was dissociated [7]. Additionally, the Rome Group discovered that the reactivity of β 93 cysteine was linked to oxygen binding, namely, that oxyHb reacted much more rapidly with the SH reagent *para*-hydroxymercuribenzoate than did deoxyHb [8].

This knowledge led researchers to investigate the possibility that Hb, like serum albumin, could form covalent adducts with NO groups. It is important to remember that NO, although frequently referred to as a free radical gas, is, in fact, a redox-active molecule which also occurs as one-electron oxidized (i.e., nitrosonium cation) and one-electron reduced (i.e., nitroxyl anion) species. In a surprising discovery it was found that, beyond merely existing *in vivo*, SNO-Hb showed significant differences in concentration between arterial and venous blood, implying a cyclic metabolism of SNO-Hb during its circulation in the mammalian body [19]. Concurrent with the arteriovenous differences in SNO-Hb concentration, a reversed complementarity in concentration of iron-nitrosyl (FeII(NO)-Hb) was revealed. This article became the basis for what is now referred to as the SNO Hemoglobin Hypothesis. It is the purpose of this review to examine the evidence that led to the construction of this hypothesis and, further, to demonstrate how this hypothesis led to a reexamination of the

interactions of NO with Hb as far as they may relate to the physiological function of the control of blood flow.

NO BIOACTIVITY IN THE VASCULATURE

The maintenance of adequate organ blood flow is a function of cardiac output (i.e., force and frequency with which the heart beats) and the systemic vascular resistance against which that heart pumps. Poiseuille's law informs us that the resistance to flow in an individual blood vessel is inversely proportional to the fourth power of its radius. By extension, *systemic vascular resistance* is proportional to the cross-sectional area of the entire arterial vascular bed. As such, modulators of vascular smooth muscle diameter and tone play a critical role in determining blood flow. These “modulators” can range from locally produced vasodilators (e.g., EDRF/NO), to catecholamines released by the adrenal glands, to autonomic nervous system-mediated vasoactivity and beyond.

A cardinal feature of mammalian circulation is its ability to increase blood delivery to areas of increasing oxygen demand in a prompt, coordinated manner. *Hypoxic vasodilation* describes the increased skeletal muscle blood flow that accompanies a rise in metabolic demand. During periods of heavy exercise, for example, the blood flow to the skeletal muscles can rise 20-fold [10]. The categories of mechanisms proposed to explain the phenomenon of exercise-induced hyperemia include: metabolic, myogenic, mechanical, and endothelial-derived vasodilators [11]. However, the biochemical mechanisms underlying hypoxic vasodilation (and its sister phenomenon, hyperoxic vasoconstriction) remain largely a mystery.

A considerable fraction of the overall vascular resistance is provided by the small arteries and large arterioles. Compared with small arterioles (and their susceptibility to the metabolic signals of their surrounding, dependent cells), both large arterioles and small arteries are further removed from tissue beds. Accordingly, their vasoregulation seems to depend on factors such as shear stress and the local production of endothelial factors, including NO. Intravital microscopic studies and measurements of endothelial response to changes in shear stress support this conclusion [12].

It is increasingly clear that skeletal muscle blood flow regulation is not simply a matter of vessels sensing changes in blood oxygen content. A growing body of evidence indicates an important role for the erythrocyte in detecting and responding to changing tissue oxygen demands. Saltin and co-workers exploited carbon monoxide's avidity for Hb binding to manipulate P_{aO_2} levels and oxyHb concentrations more-or-less *independently* [13]. Knocking out oxygen binding sites with CO resulted in increased blood flow that was associated with *reduced* Hb oxygen-carrying capacity. Under all

conditions, perfusion correlated closely with reductions in levels of oxyHb *but not with* PaO_2 , despite a greater than 10-fold difference in oxygen tension (47–538 mm Hg) across the range of conditions. The observed changes could not be explained by significant differences in blood pH, temperature, or lactate or bicarbonate levels. Apparently, hypoxic vasodilation is better understood in terms of a linkage between Hb oxygen saturation rather than oxygen tension.

There are a number of potential mechanisms whereby red blood cell (RBC) desaturation can produce vasodilation. Changes in adenosine release, RBC flexibility, and altered NO metabolism have all been proposed. The last of these is highlighted by the work of Cannon *et al.* [14] with inhaled NO. In these studies, forearm blood flow was measured after blockade of local NO production with L-NAME. At rest and during exercise, inhalation of 80 ppm of NO in air decreased forearm vascular resistance and increased both blood flow and oxygen delivery [14]. This group has consistently demonstrated that NO inhalation can produce positive changes in the peripheral circulation [15–17]. How the NO is maintained in a bioactive state is not clear, but these studies conclusively show that NO is not immediately consumed on contact with the blood. This finding contradicts theoretical calculations predicting an immediate consumption [18]. Therefore, we are in need of a mechanism to explain the way in which RBCs in general and Hb in particular can serve to control NO bioavailability (with particular reference to hypoxia). One explanation for these effects is provided by the SNO-Hb Hypothesis [9].

THE SNO-Hb HYPOTHESIS

After identifying nitrosothiols (SNOs) in plasma, Stamler and colleagues proposed that SNOs could preserve the bioactivity of NO within the vasculature [6]. While this proposal may still hold true (recently SNO-albumin was proposed as a sink for NO equivalents within the vasculature [20]) it was extended by the discovery of SNO-Hb [19]. All mammalian (and 90% of all vertebrate) Hbs contain a cysteine at position $\beta 93$. This cysteine residue is uniquely situated, resting in close proximity to the heme and the salt bridge formed by the aspartate at position 92, which is critical to the Bohr effect. The reactivity of cysteine $\beta 93$ has long been known to be affected by the conformation of the Hb (relaxed, R state vs. tense, T state) [21]. This effect is also confirmed with respect to SNO formation and decomposition. In the R state, Hb's formation of SNO from nitrosothiol donors is more rapid than that in the T state, and the SNO is more stable. That these biophysical observations might be related to a physiological function was indicated by the measurement of SNO-Hb within the

arterial vasculature of the rat [19]. These observations were supported by the work of Rassaf *et al.* [22]. A structural basis for this altered SNO stability within the allosteric states of Hb was proposed in 1997 [23] and later shown by crystallographic studies of SNO-Hb [24]. An SNO-induced alteration in the carboxy-terminal dipeptides of the β subunits was observed, thereby providing a stereochemical basis to the destabilization of SNO with Hb's transition to the T state.

A number of studies have examined the vascular activity of SNO-Hb both *in vivo* and *in vitro*. The earliest experiments used infusion of SNO-Hb into the rat vascular system and measured the effects on brain blood flow. This work showed SNO-Hb to be a potent vasodilator, in contrast to the vasoconstrictive effect of normal Hb (or SNO-Hb under hyperoxic conditions) [23]. Aortic ring bioassays have shown that SNO-Hb's ability to produce vasodilation is dependent on low oxygen tension and the presence of extraneous thiol [25]. These observations provide insight into the mechanisms of SNO-Hb function, namely, that an outlet reaction target is required for the release of NO equivalents from SNO-Hb even on transition to the T state. This may be of key relevance to the biological function of SNO-Hb as it implies that NO equivalents can be delivered only in the presence of a receptive target. Therefore, both a target molecule and an allosteric transition would be required. Such mechanisms may limit NO equivalent delivery by SNO-Hb, which could be crucial in avoiding SNO-Hb-induced hypotension.

Bioassays of human RBCs with both aortic and pulmonary artery rings have shown that erythrocytes produce vasoconstriction under hypoxic conditions. However, at oxygen tensions similar to those seen in arterioles (3–65 mm Hg), RBCs produce an initial vasorelaxation. This initial relaxation is followed by vasoconstriction, as one would predict if a finite store of vasodilator, such as NO, had been consumed [26]. These responses of human RBCs are similar to those seen for SNO-Hb, but distinctly different from those observed for either Hb alone or low-molecular-weight SNOs. Hb produces vasoconstriction at all oxygen tensions, although at low oxygen tensions this response is somewhat muted. S-Nitrosylated glutathione (GSNO), on the other hand, vasorelaxes at all oxygen tensions.

In vitro experiments have shown that both SNO-containing RBCs and Hb alone are capable of dilating blood vessels (though SNO-Hb requires the addition of extraneous thiol) [25,26]. These data and the proposal that SNO-Hb within the RBC can act as a physiological regulator of vascular tone raise the question of how SNO exits the red cell. A potential mechanism was identified by Pawloski *et al.*, who showed that the membrane

protein band III, otherwise known as anion exchanger 1 (AE1), can serve as a transnitrosation target for SNO-Hb [27]. The N terminus of AE1, the cytoplasmic domain, is a negative allosteric effector for Hb. Its area of allosteric influence, referred to as the “DPG binding site” or “anion binding site,” bridges the β chains of the tetramer and is close to the β 93 cysteine. The association of AE1 with Hb lowers oxygen affinity, thereby favoring the off-loading of SNO. The potential physiological consequence of these observations was demonstrated by the effectiveness of DIDS, an AE1 channel blocker, in inhibiting the ability of SNO-RBCs to mediate hypoxic vasodilation of aortic rings [27]. Importantly, the facility with which NO equivalents can be delivered via AE1 will be controlled by a complex interplay of equilibria involving Hb, AE1, oxygen tension, NO levels, and the presence of regulators such as CO₂ and 2,3-diphosphoglycerate. In conclusion, it can be seen that SNO-Hb can operate within the vasculature to modulate blood flow.

BIOCHEMICAL AND BIOPHYSICAL STUDIES EXAMINING NO AND Hb INTERACTION

A historical perspective

The history of the study of NO interactions with Hb is long and varied and begins in the first half of the 20th century with the work of Keilin and Hartree on heme oxidation and reduction [28]. For the next 50 years, NO's unique spectral properties and isoelectronic structure allowed for its use as a surrogate ligand for oxygen. Such experiments were generally performed at saturating NO concentrations, and often with invertebrate Hbs. Understandably, the dominant reactions identified were “the oxidation reaction” (the reaction used in pharmacological experiments as the acid test for NO involvement) and the “addition reaction.” The former reaction produces metHb plus nitrate, and the latter, FeIIINO-Hb. More complicated and subtle chemistry, like *S*-nitrosylation of Hb thiols, was largely overlooked. Additionally, some of the potential allosteric linkages were not considered, particularly those involving partial ligand occupancy states and their potential for controlling heme reactivity. The importance of such states and their thermodynamic analysis has been highlighted by the elegant work of Ackers et al. [29,30]. Understanding that NO and NO equivalents are important physiological regulators and that Hbs present them with many possible sites of interaction forces consideration of the presently known facts about Hb's allosteric properties.

More recently, with the identification of physiologically important SNOs, appreciation of *S*-nitrosylation biochemistry has intensified greatly. The *S*-nitrosylation of protein sulfhydryl groups, forming SNOs, preserves

and stabilizes NO's bioactivity. SNO formation not only prevents loss of NO from oxidative degradation but also creates bioactive, low-molecular-weight nitrosothiols, such as GSNO [31]. A growing body of evidence shows that *S*-nitrosylation of protein sulfhydryl groups can affect signaling proteins in a manner analogous to phosphorylation [32,33]. Finally, and most relevant to the SNO-Hb hypothesis, SNOs can chaperone NO equivalents in mammalian circulation, protecting them from scavenging by heme proteins and preserving them for subsequent targeted release and delivery [20].

Reactions of NO at the heme iron

Much of our present understanding of NO-Hb interactions arises from the observation of the oxidation and addition reactions of NO with heme. These two reactions occur at approximately equal rates ($\sim 10^7 \mu\text{M s}^{-1}$) and are thought to be essentially irreversible; their products, FeIII and FeIIINO, are stable and relatively unreactive with either NO or oxygen [34,35]. What evidence has brought us to this line of thinking? Initial work in this area showed that, in comparison to oxygen, NO binding to Hb was noncooperative, i.e., the binding constant for NO did not increase with increasing heme occupancy [36]. Furthermore, these studies showed that the off rate of NO from Hb(NO)₄ is minuscule ($\sim 10^{-5} \text{ s}^{-1}$, $t_{1/2} \sim 2 \text{ h}$). These observations seem difficult to reconcile with our understanding of other homotropic ligands, until one appreciates that NO binding to the α -chain heme cleaves the bond to the proximal histidine [37], which is critical to the stereochemical mechanism of cooperativity [38]. The cleavage of the proximal histidine bond gives α -FeIIINO a pentacoordinate structure and produces the characteristic hyperfine splitting observed by EPR analysis [39]. NO is a stronger ligand than O₂, and it is this strength that causes the proximal histidine bond to be broken when NO is the sole ligand, whereas it is maintained in O₂ binding. Pentacoordinate α -FeIIINO has an extremely low off rate for NO ($\sim 10^{-14} \text{ s}^{-1}$, $t_{1/2} \sim \text{years}$) and is thought to be responsible for the overall slow off rate for FeIIINO in general [40]. Interestingly, when manganese is substituted for iron in the heme moiety, NO binding becomes cooperative [41]. Mn-NO is isoelectric with Fe-CO. On binding of CO, the proximal histidine bond is maintained in both the α and β chains. Presumably it is a lack of cleavage of the proximal histidine in this manganoglobin that allows for cooperative binding of NO. Therefore, there is nothing inherent about NO that precludes it from binding hemoglobins cooperatively; it is the structure of the α chain that dictates this noncooperativity.

The stability of this pentacoordinate FeIIINO is unique, and a number of studies have shown that other forms of FeIIINO are not as stable. Kosaka et al. demon-

strated that NO on the β chain of Hb migrates to the α [42,43], and it has been shown that the second-order combination velocity constant is higher in β than in α chains [44]. Furthermore, it has long been known that the NO group of FeIIINO is capable of reacting with oxygen to produce ferric heme and nitrate [42]. More recently, it was shown that with additions of NO to deoxyHb in low molar ratios, an oxidation reaction can occur in the absence of oxygen, resulting in the generation of ferric heme and nitroxyl anion [45]. This reaction is analogous to the autoxidation of oxyHb wherein the oxygen bound to the ferrous iron exits the heme pocket as the superoxide anion, leaving the iron oxidized to the ferric form [46]. These studies showed that NO and heme, like oxygen and heme, are capable of both reversible binding reactions and complicated redox chemistry. Armed with at least a rudimentary understanding of these interactions, one can appreciate that not all FeIIINO molecules are the same and that in such situations one must consider all of the microstates involved [30].

It must be appreciated, too, that singly ligated hemoglobin tetramers are very different from the fully nitrosyl molecule. Consider the stability of the two molecules: Incubation of mononitrosyl Hb at room temperature under anaerobic conditions, for even a short period, results in heme oxidation (a process greatly accelerated by the addition of oxygen). However, Hb in which all the heme sites are NO-bound can be stored for weeks and is stable even in the presence of oxygen. Thus, when performing experiments involving FeIIINO, one must be very clear as to which molecule is being considered. Simply adding a small mole fraction of *fully* nitrosylated Hb to a large amount of Hb is substantially different from adding a small amount of NO to deoxygenated hemoglobin. In the former case the NO will be stably bound to the heme iron as the molecule is $\text{Hb}(\text{NO})_4$, whereas in the latter NO can be readily displaced. In addition, the length of time that substoichiometric amounts of NO are incubated with Hb will alter the type of FeIIINO formed, as eventually all NO will migrate to form five coordinate α species [42]. Unfortunately these differences are not always fully appreciated [48].

Proximal histidine cleavage in the α chain provides a thermodynamic basis for the absence of cooperativity seen in decades of NO–Hb binding research. But does this apply to NO reactions with Hb when NO is not the primary homotropic ligand? Perhaps the question is best asked this way: Is NO–Hb binding subject to the laws of cooperativity? Logically, the answer to this question should be yes; there is no homotropic ligand in Hb biology that is not subject to these laws. Clearly, however, the answer is not so simple. NO is unique. NO is not just a homotropic ligand; it is also capable of oxidizing the heme moiety, nitrosylating Hb's cysteine $\beta 93$ residue, and forming a

stable intermediate (α –FeIIINO). NO, therefore, can be a homotropic ligand [36], a redox partner [28,45], a positive heterotropic effector [25], or a negative homotropic effector [49].

To investigate this question of cooperativity, we chose to study the reaction of Hb and NO at a variety of oxygen concentrations.¹ Importantly, these experiments were performed under conditions where free NO concentration was always less than 200 nM. This minimized side reactions and ensured that oxygen was the primary homotropic ligand for the heme. At oxygen saturations greater than 60% the yield of FeIIINO was higher than would be predicted by simple competition [50]. These observations can be explained either through: (1) increases in oxygen concentration that are paralleled by increases in the binding rate constant for NO, or (2) formation of FeIIINO via an alternate reaction pathway that increases in importance with increasing oxygen concentration. The first of these options suggests that NO is subject to the rules of allostery and cooperativity, a conclusion supported by research employing negative allosteric regulators such as phosphate [50] and inositol hexaphosphate (personal observation). Running the experiments with unstripped Hb [51], which contains 2,3-diphosphoglycerate, is fraught with difficulty. These studies demonstrate that the cooperativity of NO binding is reduced in the presence of negative allosteric effectors, as is the case for oxygen. As for the second possible explanation, FeIIINO formation by an alternate reaction, it is difficult to see how it would occur given that it would require the kind of oxidative interaction between NO and oxygen that only occurs when NO is added as an unmixed bolus [18]. Furthermore, such reactions would be expected to result in SNO formation, not nitrosylation of heme, which is a reductive reaction [52]. Therefore the formation of FeIIINO under such situations would have to result from thiol-to-heme transfer of NO, which would be *disfavored* at higher oxygen tensions.

Reactions of NO with $\beta 93$ cysteine

The interactions of NO with Hbs are complex and critically dependent on the relative concentrations of the reactants and the presence of allosteric effectors. Allosteric control of the reactions at $\beta 93$ cysteine with NO equivalents was clearly demonstrated in the original

¹ It is important to note that, due to the difficulties in making these kinds of measurements, such experiments must be performed using final concentrations of NO that are high enough that accessory reactions with oxygen can occur [18]. However, when NO is added in a stepwise fashion at much lower concentrations (<200 nM) with vortexing, these accessory reactions are reduced in propensity. Interestingly adding NO at a reduced rate produces an increase in SNO formation when reacted with oxyHb, indicating that accessory reactions with oxygen are less efficient in SNO formation [9].

article on the SNO Hypothesis. SNO formation is favored in oxygenated Hb over deoxygenated Hb [19]. In other words, transnitrosation of the β 93 cysteine is more favorable in R-state Hb than T-state Hb, an observation confirmed by demonstrating that nitrosylation of this cysteine residue has a positive allosteric effect (i.e., the presence of SNO increased the oxygen affinity of the Hb) [25]. Additionally, the stability of SNO-Hb is significantly greater for the R-state molecule than the T-state molecule.

A critical area of interest is the mechanism of SNO formation in hemoglobin. The human hemoglobin tetramer has six reactive cysteines, two from the α chains and four from the β chains. All evidence so far indicates that only the β 93 cysteine becomes S-nitrosylated. As with any other reactive thiol, it is possible for SNO to be formed in many ways, including: direct reaction, so long as an oxidant is present [53]; via interaction with higher oxides of nitrogen [54]; via transnitrosation [55]; or through extraneous metal catalysis [56]. However, additional SNO-producing reactions can occur with Hb, namely, heme–thiol interactions [45] and reductive nitrosylation [52], both of which involve an intramolecular NO group interaction with the heme iron and both of which have been observed. In the former, statistically-distributed FeIINO in low proportion to FeII (1:100 or less), NO was transferred from the heme to the thiol on allosteric transition and in the presence of oxygen (Hb from the T to the R state) [45].² Despite the high concentration of oxygen present during this conformational transition, most of the NO moves from the heme to the thiol, rather than reacting with oxygen to produce nitrate. This reaction requires the abstraction of an electron from the heme iron. It was suggested that a by-product of this reaction may be the superoxide anion. Later it was shown that during this intramolecular transfer a transient radical is generated [26]. It has EPR characteristics significantly different from those generated by the interaction of nitrite with FeII O_2 as it is anisotropic and is centered at 3250 G [48].

The precise nature of this radical remains unclear. It is possible that this reaction pathway can operate in Hb

solutions in which the majority of hemes are oxygen-bound, as addition of low levels of NO to such solutions results in significant production of SNO [9,50]. Heme to thiol transfer was also demonstrated in the formation of SNO-Hb in crystals of R-structured Hb [47]. Recently, this work was extended to indicate that SNO-Hb formed in the absence of an electron acceptor was of a radical nature [57], implying that SNO formation occurred via a direct reaction mechanism [53]. The second reaction mechanism, reductive nitrosylation of ferric heme, appears to occur preferentially within β heme subunits with β 93 SNO-Hb as its product [52]. The diversity of possible reactions between Hb and NO within the physiological milieu is difficult to characterize. Intramolecular NO transfer is the most appealing in terms of both parsimony and the existence of a respiratory cycle for NO [58].

Release of NO equivalents from SNO-Hb

The mechanisms by which NO equivalents can be released from SNO-Hb are also varied. The formation of SNO within Hb acts as a positive, heterotropic allosteric effector [25]. As such, under the principle of thermodynamic linkage [1,59], its own stability is directly linked to the allosteric state of the Hb. In purified Hb, absent any non-Hb targets of NO reactivity, cycling of SNO-Hb between oxygenated and deoxygenated states results in a shuttling of NO between heme and thiol [25,26]. However, these observations can be complicated by the creation of fully nitrosylated Hb or the presence of organic phosphates [48]. In an entirely in vitro system, using purified SNO-Hb, it is possible to release NO equivalents only by a combination of T-state formation and extraneous target (e.g., glutathione) provision [25]. Under these conditions SNO-Hb acts as a vasodilator rather than a vasoconstrictor.

So far we have addressed mostly Hb–NO interactions in vitro. In vivo, SNO occurs within the erythrocyte and the natural question becomes: How can an intracellular protein manifest its properties outside the cell? The original paper on SNO-Hb demonstrated export of SNO from the RBC [19]. One potential mechanism that has been offered proposes that the N-terminal, cytoplasmic domain of the anion channel, AE1, operates as a target for NO equivalent release from SNO-Hb [27]. Other investigators have examined the bioactivity of SNO-Hb in vitro with respect to vessel relaxation [60]. These studies showed that an NO-derived, thiol-dependent vasorelaxant species was released from SNO-Hb. However, they observed that this vasorelaxation was resistant to the addition of oxyHb and suggested that nitroxyl anion might be the released NO equivalent. Unfortunately, much of this work was conducted at Hb concentrations so low as to induce dimer formation;

² It is important to note that the NO transferred in these reactions is less than one-tenth of the heme concentration and optimally less than one-hundredth. In addition the total concentration of NO that has been observed forming SNO via heme based mechanisms is 1.2 μ M in 33 μ M Hb. These experiments were performed using free NO in solution added in a titration (largest single addition used was 200 nM) with vortexing such that these proportions are applicable for an even distribution throughout the solution. It is critical to define what one means by low NO hemoglobin ratio, a term that has been used freely in the literature. These yields of SNO are relatively efficient (50–80% of NO added) but small (typically 1 μ M or less). However, 5 nM SNO is enough to produce a 25% increase in vessel diameter and, therefore, sufficient within the physiological context.

therefore, one cannot be sure if the same processes apply with tetrameric Hb, as dimeric Hb is clearly no longer capable of structural transitions between R and T states.

A review of the preceding discussion highlights the complexity of NO and Hb interactions. However, what is clear is that the reaction pathways required for the SNO-Hb Hypothesis do exist. Furthermore, one can see that these reaction pathways are extraordinarily sensitive to the conditions of the experiment. As such, it is important to be extra cautious in extrapolating from negative observations.

SNOHb AND HUMAN PHYSIOLOGY

The proposal that SNO-Hb is a mediator of blood flow [23] has fostered a large body of research into SNO-Hb in physiology. Subsequent observations have supported the proposition that NO-Hb interactions and Hb's allosteric transitions are involved in the physiologic matching of blood flow to tissue demand. In 2002 Stamler *et al.* reported NO-Hb adduct measurements in healthy adults exposed to normoxia, hypobaric hypoxia, and hyperbaric hyperoxia. They noted a predominance of FeIINO at low pO_2 . This contrasted with the abundance of SNO-Hb, even in mixed venous blood, at the supraphysiologic pO_2 generated by hyperbaric hyperoxia. Manipulations of pO_2 in human subjects (through exposure to hyperbaric hyperoxia and hypobaric hypoxia) revealed shifts in the distribution of NO between the heme iron and Hb thiol, "consistent with reports that NO is exchanged between hemes and thiols as a function of pO_2 " [26]. Although the total amount of NO bound to Hb remained relatively constant across a range of pO_2 , the balance between heme binding and thiol binding changed considerably. Of great interest was the finding that SNO-Hb levels correlated principally with Hb *saturation* rather than pO_2 across the three conditions. There was correlation between pO_2 and SNO-Hb, but only in the nonhyperoxic range of 0–150 mm Hg. This pattern fits with an *allostery-dependent* mechanism for S-nitrosylation, as opposed to one based purely on oxygen tension. Interestingly this matches the observation that tissue blood flow is related to Hb saturation and not pO_2 [13].

Arteriovenous differences in SNO-Hb measurements have also been reported in blood from umbilical arteries and veins [61]. The mole ratio of SNO-Hb to Hb was significantly higher in umbilical venous blood than in umbilical artery blood ($2.19 \pm 1.22 \times 10^{-3}$ vs. $1.45 \pm 0.66 \times 10^{-3}$). In the fetal circulation the umbilical *venous* blood is relatively oxygen-rich, having just passed through the placenta. This relatively oxygen-rich blood carried higher levels of SNO-Hb, paralleling the finding of increased SNO-Hb levels in oxygen-rich rat arterial blood. Others have confirmed this transplacental

difference in SNO concentrations and extended the observation to include unusually low umbilical artery SNO levels in infants who had abnormal transitions to extrauterine life (e.g., required cesarean section for fetal distress, needed supplemental oxygen) [62].

As mentioned previously, Cannon *et al.* reported on "intravascular biostabilization and delivery of NO" after inhalation of 80 ppm of NO [14]. The accompanying biochemical analyses showed a 10-fold increase in FeIINO concentrations with NO inhalation (compared with previously reported levels), as well as an arteriovenous difference. An 11% increase in arterial nitrite levels, but no significant changes in SNO-Hb, was also reported. These differences in NO equivalent measurements may represent altered mechanisms for handling exogenously added NO or may result from technical difficulties in making these measurements *in vivo*. The quantities of NO bound to Hb *in vivo* are very small (estimates of Hb associated NO have ranged from 50 nM to 2 μ M) and the effects of heme proteins themselves within the assays can vary considerably. Without wishing to present a detailed discussion of NO equivalent measurement techniques (a subject more suitable for an alternative forum), it is important to recognize that many different techniques have been used for measuring NO *in vivo*, including: photolysis-chemiluminescence [19,45], Saville assay [61], fluorescence-adapted Saville assay [26], spectrophotometrically-adapted Saville assay [9], chemical reduction-chemiluminescence (using triiodide) [14,17,63], chemical reduction-electrochemical detection [64], and catalytic cleavage-chemiluminescence (using Cu^+) [65]. The relative efficiencies of these techniques are unclear, as are their abilities to differentiate between iron-bound and thiol-bound NO. Indeed, as techniques are often being revised, the NO species they measure are changing. For instance, the triiodide technique was originally used to measure FeIINO [14], but in a recent publication was shown not to measure EPR-detectable FeIINO [48]. Therefore, caution is again advised when attempting to state whether such measurements "prove" or "disprove" a hypothesis.

Further light on the role of SNO physiology is shed by Gaston and colleagues who explored the role of SNOs in replicating hypoxia-mediated increases in ventilation [66]. They demonstrated the effects of certain low-molecular-weight SNOs (e.g., S-nitrosocysteinyl glycine, S-nitroso-L-cysteine, and GSNO) instilled via a catheter into the brainstems of conscious rats. These injections, administered at the level of the nucleus tractus solitarius (NTS), triggered increases in ventilation equal to those induced by brief periods of hypoxia. The same hyper-ventilatory response was achieved with injections of a low-mass fraction of plasma taken from deoxygenated blood, whereas oxygenated blood-derived plasma had no

effect. In a separate portion of their experiment, Gaston et al. used liquid chromatography/mass spectrophotometry to demonstrate the generation of GSNO on glutathione's combination with venous deoxygenated blood, while no GSNO was formed from arterial oxygenated blood. These findings uphold the proposition that the S-nitrosylation of low-molecular-weight nitrosothiols is involved in hypoxic signaling to the respiratory center of the rat brain and that the saturation state of the blood is critical to the formation of these signaling intermediates.

Gaston et al. also explored potential biochemical modifications involved in this SNO-mediated signaling. Specifically these researchers discovered that pretreating the NTS with acivicin, an inhibitor of the enzyme γ -glutamyl transpeptidase, blunted the hyperventilatory effect of GSNO but not its less stable cleavage product, S-nitrosocysteinyl glycine. They also showed that mice homozygous for γ -glutamyl transpeptidase deficiency exhibit a markedly impaired ventilatory response to hypoxic challenge. In sum their evidence supports a model in which low-molecular-weight SNOs, whose transnitrosylation is favored by Hb's deoxygenation, participate in signaling hypoxia to the brain to trigger increased ventilation. At the molecular level the conveyance of the SNO signal appears to require enzymes (e.g., γ -glutamyl transpeptidase) able to modify low-molecular-weight SNOs for targeting to their site of action.

Another model used to probe the interactions of Hb and glutathione is that of the septic pig [67]. After first establishing free Hb's ability to increase blood pressure, it was demonstrated that glutathione reversed some of Hb's effects. Glutathione demonstrated vasoactivity consistent with NO transfer from SNO-Hb. In addition, across a variety of conditions, the SNO level within the vasculature was found to correlate with blood pressure. These findings are consistent with a model in which Hb thiol groups mediate interactions between heme-bound NO and extraneous thiols.

Alterations in NO and Hb interactions have also been implicated in human pathology. In diabetes there is a progressively increasing glycosylation of circulating Hb which is associated with a reduced perivascular perfusion. When NO metabolism and the levels of heme-bound NO were assessed in type I diabetic patients, it was found that NO was preferentially metabolized to and stored as FeIIINO [64]. Interestingly, this tendency was closely correlated to the degree of hemoglobin glycosylation. This pathology has recently been linked to a reduced ability of red blood cells from diabetic patients to induce a pO_2 -dependent vasorelaxation [68]. In this regard, a pathological situation is associated with disrupted Hb and NO metabolism, which may play some role in the progression of the disease.

Another area of investigation is SNO-Hb's ability to oppose hypoxic pulmonary vasoconstriction [69]. In one study oxyHb or oxySNO-Hb was added to the perfusate of an isolated rabbit lung at a concentration of 4 μ M. In both cases infusion augmented the vasoconstrictive response to hypoxia with no opposition to the response, as might have been predicted had SNO-Hb operated as net deliverer of NO. However, on addition of glutathione, NO release and the production of methHb were observed without vascular effect, suggesting that any NO released from SNO-Hb was immediately oxidized by oxyHb. Though these observations may be the result of differences in the responsiveness between pulmonary and systemic vessels, both have previously been shown to respond to SNO-Hb [26]. Another potential complication in these studies was the low concentration of Hb, 4 μ M, which would result in dimer formation and extravasation, both of which confuse the overall picture of NO metabolism. In general the studies examining the effectiveness of SNO-Hb in the physiological milieu have confirmed its potential to act as a controller of hypoxic vascular responses, but not without highlighting the complexity of this biological system and the precise condition dependence of these reactions.

ALTERNATIVE VASCULAR FORMS OF NO

Experimental evidence for SNO-Hb's importance in modulating vascular tone abounds. This is not to suggest, of course, that NO-mediated vasoregulation is predominantly under the control of a single molecular species. Neither is vasoregulation SNO-Hb's sole province. The search for a single, most physiologically relevant, vasoactive NO metabolite in this complicated milieu of NO species has produced many nominees. The evidence of a critical role for SNO-Hb, some of which is presented in this article, is abundant and compelling. Nevertheless, the evidence for SNO-Hb is not necessarily exclusive of other molecules. As such, it is useful to consider some of the extant data.

Nitrite and nitrate anions are the subject of much attention and disagreement in this search. Although "nitrates" continue to be a mainstay in the pharmacologic treatment of angina, nitrate anions specifically are biologically inactive. Nitrite anions, on the other hand, have been reported to vasodilate blood vessels in vitro directly or perhaps through NO donation [70]. The possibility of nitrite-mediated bioactivity via the generation of NO by enzymes such as xanthine oxidoreductase has been reported [17]. Others however, have ascribed nitrite's reported effects to SNO generation [71]. Still other researchers find no convincing evidence of physiologic levels of nitrite causing vasodilation in vivo [72]. Perhaps one of the most intriguing

possibilities for nitrite in mediating hypoxic vascular control was recently proposed by Nagababu *et al.* [79]. They suggested that, through the formation of a FeIIINO species that has the NO reversibly bound, deoxyHb might operate as a donor of NO from nitrite under hypoxic conditions.

One of the first suggested forms of circulating NO equivalents was *S*-nitrosylated albumin in the plasma [6]. Indeed a number of groups have now identified SNO species within the plasma in both human and animal models [17,20,73]. However, it is difficult to conceptualize how plasma SNOs could be used on their own as a mediator of hypoxic vasodilation. Indeed, it has been suggested that SNO in the plasma is formed to a greater extent in the venous rather than the arterial circulation [17]. Dinitrosyl iron complexes (DNICs) have also been identified in biological samples [74]; these compounds have been found to form on activation of NOS and to possess the ability to generate nitrosothiols within the plasma [75]. What role these compounds may play in physiology is as yet unclear, although they clearly represent a potential storage pool of NO equivalents.

A MODEL OF NO IN THE VASCULATURE

The generation of the SNO-Hb Hypothesis has resulted in a vast array of research into the vascular transport of nitrogen oxides [16,17,26,63,67,69,76] and considerable interest in NO and Hb interactions [18,23,45,50,51,77]. Unfortunately much of this research seems to have only generated further confusion into the role of both Hb and NO in the physiology of blood flow control. Thus we are now challenged to understand how these contradictory reports can be resolved. Perhaps it is helpful to begin such a discussion by examining any areas of apparent clarity. First, it would appear that there is a general acceptance of the principle of vascular transport of NO equivalents [15,17,22,26,73,78]. Furthermore, it would appear that this vascular delivery of NO is greatest in areas of hypoxia or restricted blood flow [14,23,26]. Finally, it is clear that the reactions of NO with Hb are considerably more complex than originally believed. This is evidenced by the diversity of reactions that can occur depending on the conditions used *in vitro* [9,18,45,50,51].

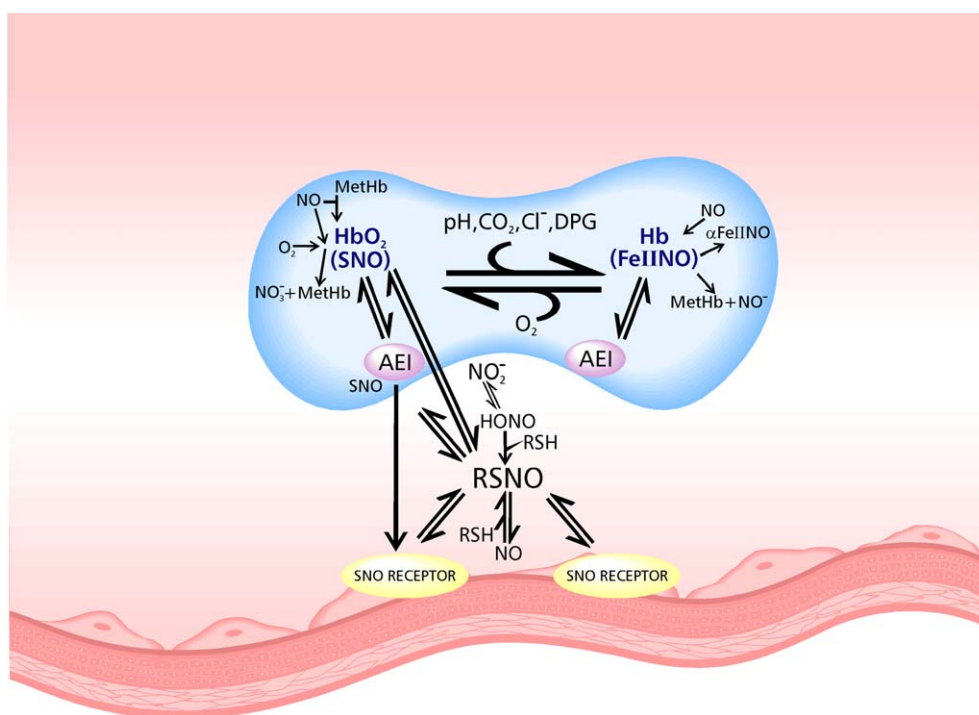


Fig. 1. Model of the potential equilibria that may operate in the movement of NO equivalents within the vasculature. SNO-Hb is in equilibrium with FeIIINO Hb, the proportion of which is determined by the presence of homotropic and heterotropic regulators [45,50]. SNO-Hb can release NO equivalents to AE1 [27] or directly to the surrounding plasma [19]. SNO-Hb can be formed by reductive nitrosylation [52] or by heme-thiol transfer [45]. OxyHb can consume NO by the oxidation reaction [34]. FeIIINO Hb can be formed by the addition reaction [36] and can generate stable α FeIIINO [40] or ferric heme and nitroxyl anion [45] which may operate as a vasodilator whether generated in this manner or from thiol interaction with SNO [60]. AE1 can deliver NO equivalents to the plasma, possibly to glutathione or albumin, or directly to the endothelium [27]. SNO in the plasma may be derived from SNO-Hb or may arise from acidification of nitrite [71], or from direct reaction [53] potentially via metal catalysis [82]. Delivery of NO equivalents to the vessel wall may occur directly or via an SNO receptor such as γ -glutamyl transpeptidase.

For the purpose of explaining the physiologic observations of vascular transport of NO, the SNO-Hb proposal is ideally suited. At the time of this writing, no biochemical evidence exists to preclude the possibility that intraerythrocytic Hb operates as a vehicle for SNO transport and delivery. We can say this in the positive rather than the negative: Intraerythrocytic hemoglobin operating as an allosterically controlled delivery vehicle for extracellular NO action is an attractive hypothetical mechanism that is not excluded by any biochemical evidence. While certain investigators have failed to observe individual aspects of the model, this can generally be attributed to the experimental conditions used. In particular, the list of experimental variances includes: the use of partially dimeric, allosterically incompetent Hb [60,69]; the use of fully nitrosylated Hbs to make solutions of “partially nitrosylated Hb” [48]; the use of unstripped Hb with incompletely removed intraerythrocytic organic phosphates [51]; and the bolus addition of NO [18]. When considering this literature one might be reminded of the maxim that “absence of proof does not constitute proof of absence.” We would all be well advised to refrain from statements of absolutes. Despite the difficulties in understanding all of the chemistry involved in NO and Hb interaction, there remains the clear capability of SNO-Hb within the red cell to produce vasodilation that is hypoxia-dependent [26], a consistently observed physiologic phenomenon.

The complexity of NO and Hb reactivity reveals that many factors can alter the relative yield and the precise nature of the products of such interaction. These include the relative concentrations of the reactants, the presence or absence of allosteric effectors, the rate of addition of the reactants, the presence or absence of alternative targets, and the redox state of the heme iron, the β 93 cysteine, and the NO. We have attempted to summarize the potential interacting equilibria that exist for NO within the vasculature within the model shown in Fig. 1. The basis of this model is that NO can exist in a variety of interrelated redox forms (such as nitrogen monoxide, nitrosonium cation, iron nitrosyl, SNO, and nitrite) and that these forms exist in linked and functionally dynamic equilibria. Immediately, one can see that disruption of any of these interrelated equilibria will result in redistribution within them all. Thus, there is great difficulty in measuring the concentrations of particular NO equivalents within the physiologic milieu. Any such observation will necessarily produce alterations throughout this network, irrespective of the sophistication of the measurement techniques themselves.

Rather than considering these ideas in the abstract, let us consider the concrete example of HbNO measurement. EPR can be used to detect heme-bound NO and provides a nearly unequivocal signal that can be used in

vitro to determine the proportions of α - and β -heme-bound NO and reveal the hexacoordinate and pentacoordinate nature of heme-bound NO. But the EPR detection limit for HbNO is about 1 μ M, 10- to 100-fold higher than both measured and predicted circulating concentrations of free NO. As a result some investigators, using EPR methods, have been unable to detect circulating HbNO. Despite this clear technical limitation, the failure to detect circulating HbNO was recently used as an argument to support consumption of NO by oxyHb as the body's major Hb-NO interaction [48]. The inability to detect free circulating HbNO by EPR is confounded by overlapping signals from the plasma, like those from ceruloplasmin. In an attempt to resolve this problem Kirima et al. showed that by subtracting the NO-depleted signal from whole blood, they were able to detect NO signals as high as 9 μ M [80]. In addition, Jaszewski et al., using slow additions of the NO donor DEANO ($\sim 2.75 \mu$ M min⁻¹) to blood in vivo, found that as much as 30 μ M NO could be delivered to Hb, as detected by EPR, without conversion to nitrate [81]. Hence even with this most definitive technique it is extremely difficult to quantify a single component of the NO equilibria shown in Fig. 1. Biochemical techniques for the measurement of HbNO are similarly problematic, leading to much of the diversity seen in the published data.

In summary the development of the SNO-Hb Hypothesis and the burst of NO-Hb research that followed have taught us much about how NO may operate within the vasculature. Clearly, the system is not fully understood and many of the subtleties involved in NO and Hb interactions both in vitro and in vivo are only now beginning to be understood. Some controversy exists over the levels of NO and Hb equivalents measured in vivo, but even the lowest concentration of SNO-Hb reported (50 nM) is more than sufficient to produce physiologic vasodilation. As such SNO-Hb remains the logical intravascular transporter of NO.

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