Biological Chemistry of NO

Nitric oxide is a free radical (•NO) endowed with high reactivity in biological systems due in part to its reaction with molecular O₂ (reaction 1) (49, 79, 93).

\[
4\text{•NO} + O_2 + 2H_2O \rightarrow 4\text{NO}_2^- + 4H^+ \\
(\text{•NO autoxidation})
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\[K_{aq} = 3.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \text{ at } 37^\circ\text{C} \] (174)

The great variability of NO half-life in various biological settings owes to the fact that this reaction is third order. The remarkable reactivity of •NO (compared with other small messenger molecules) with proximal targets accounts for some of its autocrine and paracrine effects. For the purpose of this review, •NO stands for the radical nitric oxide, and NO for species whose chemical nature is not elucidated and can be •NO, NO⁺, or NO⁻. To illustrate the importance of this distinction, strictly equating the bioactivity of the radical •NO with NO bioactivity [i.e., equating •NO with endothelium-derived relaxing factor (EDRF) generally] has proven perilous. Recognition of NO’s biochemical versatility has exposed a wider world of NO biology. In living organisms, •NO is in fact in equilibrium with other NO species that constitute stable stores of NO bioactivity. Among these species, S-nitrosylated hemoglobin (S-nitrosohemoglobin; SNO-Hb) is an evolved transducer of NO bioactivity that acts in a responsive and exquisitely regulated manner to control cardiopulmonary and vascular homeostasis. In SNO-Hb, O₂ sensing is dynamically coupled to formation and release of vasodilating SNOs, endowing the red blood cell (RBC) with the capacity to regulate its own principal function, O₂ delivery, via regulation of blood flow. Analogous, physiological actions of RBC SNO-Hb also contribute to central nervous responses to blood hypoxia, the uptake of O₂ from the lung to blood, and baroreceptor-mediated control of the systemic flow of blood. Dysregulation of the formation, export, or actions of RBC-derived SNOs has been implicated in human diseases including sepsis, sickle cell anemia, pulmonary arterial hypertension, and diabetes mellitus. Delivery of SNOs by the RBC can be harnessed for therapeutic gain, and early results support the logic of this approach in the treatment of diseases as varied as cancer and neonatal pulmonary hypertension.

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Transport and Peripheral Bioactivities of Nitrogen Oxides Carried by Red Blood Cell Hemoglobin: Role in Oxygen Delivery

The biology of NO (nitric oxide) is poorly explained by the activity of the free radical NO (•NO) itself. Although •NO acts in an autocrine and paracrine manner, it is also in chemical equilibrium with other NO species that constitute stable stores of NO bioactivity. Among these species, S-nitrosylated hemoglobin (S-nitrosohemoglobin; SNO-Hb) is an evolved transducer of NO bioactivity that acts in a responsive and exquisitely regulated manner to control cardiopulmonary and vascular homeostasis. In SNO-Hb, O₂ sensing is dynamically coupled to formation and release of vasodilating SNOs, endowing the red blood cell (RBC) with the capacity to regulate its own principal function, O₂ delivery, via regulation of blood flow. Analogous, physiological actions of RBC SNO-Hb also contribute to central nervous responses to blood hypoxia, the uptake of O₂ from the lung to blood, and baroreceptor-mediated control of the systemic flow of blood. Dysregulation of the formation, export, or actions of RBC-derived SNOs has been implicated in human diseases including sepsis, sickle cell anemia, pulmonary arterial hypertension, and diabetes mellitus. Delivery of SNOs by the RBC can be harnessed for therapeutic gain, and early results support the logic of this approach in the treatment of diseases as varied as cancer and neonatal pulmonary hypertension.
healthy human adult, *NO production by NOS amounts to ~1.0 mmol/day (63). Among the three NOS isoforms [endothelial (e)NOS, inducible (i)NOS, and neuronal (n)NOS], eNOS is most prevalent in the vascular endothelium and in blood platelets where it controls shear stress-related vascular homeostasis. Endothelial cells and platelets produce similar amounts of *NO (153). *NO has been shown to bind rapidly and with high affinity to ferrous iron (Fe2+). It has a particularly high reactivity with iron within iron-sulfur centers and hemoproteins, especially when the heme contains a free ligand position. A well studied example of *NO interaction with heme-containing proteins is its binding to the ferrous heme of the soluble guanylate cyclase (sGC) in vascular smooth muscle cells (VSMCs) and in platelets (5, 121, 152). *NO binding to the iron heme activates sGC, inducing a burst in cGMP production that leads to a decrease in cytoplasmic Ca2+, vasodilation, and inhibition of platelet aggregation. In these particular systems, the effects of *NO are rapid (occurring within seconds) and transient, in part because the turnover rate of cGMP is high. Although the interaction of *NO with the heme of sGC is indisputable, sGC is certainly not the sole (and probably not the main) target of *NO. In fact, most activities of NOS, including sGC activation (115), could be conveyed by S-nitrosylation of target proteins, in which a reactive thiol sulfur binds an NO moiety at optimal redox conditions (107, 113, 127, 140, 170). Indeed, it has been argued that even the classical EDRF generated from endothelial NOS activation may represent an S-nitrosothiol rather than *NO (115, 130, 154).

Historically, the identification of *NO as a potent endogenous vasodilator was based on the observed ability of cell-free Hb to abolish both *NO- and EDRF-induced vasodilation (80, 135). Hb is a tetrameric RBC protein composed of two α- and two β-chains, each containing a heme prosthetic group. One α- and one β-chain are combined in stable αβ dimers, and two dimers are more loosely associated to form tetramers. The physiological role of Hb depends on its ability to reversibly bind O2 at its iron hemes. This transport capacity is governed by a cycle of allosteric transitions in which Hb assumes the R (relaxed, high O2 affinity) conformation to bind O2 in the lungs and, on partial deoxygenation, the T (tense, low O2 affinity) conformation to efficiently deliver O2 to peripheral tissues. O2 binding to Hb is cooperative, the fully deoxygenated tetramer having low affinity for O2, and the four-O2-bound tetramer having high affinity for O2 so that Hb tends toward all-or-nothing O2 binding. O2 binding is further modulated by allosteric effectors, including pH, PCO2, local redox conditions, and certain RBC anions (8, 94, 109). Furthermore, ~1% of Hb is also normally oxidized to ferric met-Hb [that assumes a quaternary structure similar to T-state Hb (87)] (140). Together, these features illustrate that Hb is not a single entity but a population of molecules that, although related by their protein sequence, are structurally and chemically different and have varying reactivities. A proper deciphering of Hb’s interactions with NO species therefore requires studying Hb in its natural RBC environment at physiologically relevant RBC, O2, and NO levels. Endogenous concentrations are typically in the millimolar range for Hb and O2, but low nanomolar for *NO. Neglecting this simple principle has led to the misinterpretation of Hb chemistry and to the formulation of hypotheses that are at odds with the reality of Hb’s physiology (reviewed in Ref. 158).

**Functional Interactions of NO with Hb in Physiological Settings**

**Hb as a sink for *NO**

RBCs were initially characterized to be scavengers of *NO that can effectively block NO bioactivity. In simple experimental settings, RBCs indeed induce the contraction of isolated vessels mounted in a myograph and maintained at high Po2. Moreover, vasoconstriction in vivo by exogenous cell-free oxy- or deoxy-Hb produces hypertension (42, 73, 101, 161). In these systems, *NO is quenched as a ferrous heme-nitrosyl adduct (reaction 2) that does not exert direct vasodilatory activity (42, 84, 110, 138) and can further be degraded to inactive nitrate through the dioxygenation reaction (reaction 3) (43). These reactions occur as efficiently at vacant hemes of either the α or β chains and when Hb is in solution or in RBCs (14, 42).

\[ \text{Hb}[\text{Fe(II)}] + \text{NO} \rightarrow \text{Hb}[\text{Fe(II)NO}^\cdot] \]  
2) \hspace{1cm} \text{ferrous heme-nitrosylation}

\[ \text{Hb}[\text{Fe(II)NO}^\cdot] + \text{O}_2 \rightarrow \text{Hb}[\text{Fe(III)}] + \text{NO}_3^- \]  
3) \hspace{1cm} \text{dioxygenation reaction}

The formation of Hb[Fe(II)NO] has been extensively investigated using spectroscopic [absorption, electron paramagnetic resonance (EPR), and nuclear magnetic resonance (NMR)] techniques. The physical and chemical properties of the complex depend on the redox state of the heme iron, the degree of O2 saturation of the Hb tetramer, and the presence of allosteric effectors, such as 2.3-diphosphoglycerate (DPG), normally available in RBCs. Structural and kinetic studies demonstrate that the incubation of deoxy-Hb with *NO leads to the formation of at least three different Hb[Fe(II)NO] species: 1) pentacoordinated α-nitrosyl ferrous heme in deoxy (deoxygenated) T-state of the α-subunit, 2) 6-coordinated α-nitrosyl ferrous heme in oxy R-state, and 3) 6-coordinated β-nitrosyl heme Hb-NO. The rate constants of the association of NO with α ferrous hemes are very high (k = 2.6 × 10^10 M^−1 s^−1) (21), but the mean rate of Hb[Fe(II)NO]^− formed after NO inhalation is about 40 min in human blood (141). This discrepancy could be explained, at least in part, by the fact that a measurable population of ferric (met-)
Hb is always present in human blood, together with the kinetics of the reaction between ferric Hb subunits and *NO: ferric heme binds *NO more slowly (k = 6.4 × 10^3 M⁻¹ s⁻¹) than does ferrous heme but liberates *NO faster. In addition, from Hb[Fe(II)NO⁺], alternative Hb-NO adducts form, including SNO-Hb (see below).

*NO binding with met-Hb hemes has also been proposed to cause *NO inactivation (reaction 4) (2). Met-Hb β-subunits react with *NO 3 to 4 times faster than do the α-subunits (156). However, the affinity of *NO for oxidized hemes is too low to lead to massive *NO inactivation through that reaction (Kₜ = ~0.5 mM).

4) Hb[Fe(III)] + *NO→Hb[Fe(II)NO⁺]⇔ Hb[Fe(II)NO⁺] (ferric nitrosylation)

The vascular production of *NO in close proximity to Hb, which acts as a sink for *NO, raises an apparent paradox if one accounts for EDRF activity. Indeed, rapid inactivation of *NO at the luminal side of endothelial cells would prevent *NO from reaching a functional concentration at the abluminal side where VSMCs transduce *NO-mediated vasodilation (18, 100, 168). An understanding of two complementary phenomena resolves this paradox: 1) the existence of physiological barriers that limit (but do not eliminate) the interaction of *NO with Hb and 2) the active preservation of a significant part of NO bioactivity by Hb. The latter mechanism explicitly formulates that *NO per se is not the sole “EDRF” and, therefore, does not account for all NO-related bioactivity.

### Physiological barriers

Although Hb readily inactivates *NO through reaction 3, irreversible *NO consumption is limited by a factor of ~1,000 because of Hb’s packaging into RBCs (20, 55, 77, 108, 167). Substrate availability is thus the rate-limiting factor in the dioxygenation reaction, which otherwise possesses an extremely high rate constant (k = ~9 × 10⁷ M⁻¹ s⁻¹) (71, 108). *NO produced by eNOS at the vascular endothelium encounters four successive barriers before reaching Hb in RBCs: 1) the endothelial cell membrane, 2) an RBC-free zone at the vascular edge of the migrating RBC column created by blood flow velocity gradients (104, 167, 168), 3) an unstirred layer of blood around moving RBCs (7), and 4) the RBC membrane and cytoskeleton.

The relative importance of these physiological barriers is the subject of continuing investigation. It is generally accepted that cell membranes do not represent important obstacles for *NO diffusion because *NO is equally soluble in aqueous and lipid compartments and, therefore, freely permeates membranes. However, chemical and physical modifications of the RBC membrane result in significant changes in the rate of RBC *NO uptake (66, 77). These changes could be due to changes in essential Hb-protein interactions that modulate *NO uptake by RBCs rather than changes in the nature of the membrane. By contrast, the cell-free zone at the vascular wall, with a width of ~5 μm, accounts for a necessary *NO transit time of ~7.5 ms, during which its derivatives can bind to albumin or glutathione for instance (18). The rate-limited

### Table 1: Milestones in the study of NO species interactions with Hb

<table>
<thead>
<tr>
<th>Year</th>
<th>Nature of the Finding</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1865</td>
<td>First investigation of the reactions of blood with NO</td>
<td>70</td>
</tr>
<tr>
<td>1937</td>
<td>Hb inactivates *NO (dioxigenation reaction)</td>
<td>91</td>
</tr>
<tr>
<td>1953</td>
<td>Nitrites are vasoactive at high concentration</td>
<td>52</td>
</tr>
<tr>
<td>1957</td>
<td>Physiological barriers limit *NO interaction with Hb in RBCs</td>
<td>20, 55</td>
</tr>
<tr>
<td>1967</td>
<td>The reactivity of Cysβ93 is modulated by Hb allostery</td>
<td>16</td>
</tr>
<tr>
<td>1970</td>
<td>Cysβ93 in Hb is freely reactive, other cysteines are sterically buried</td>
<td>24</td>
</tr>
<tr>
<td>1977</td>
<td>NO activates sGC to produce vasorelaxation</td>
<td>5</td>
</tr>
<tr>
<td>1978</td>
<td>Identification of NO in human blood</td>
<td>50</td>
</tr>
<tr>
<td>1981</td>
<td>NO₂ reacts with human deoxy-Hb</td>
<td>44</td>
</tr>
<tr>
<td>1981</td>
<td>NO, NO₂, and organic nitrates may act as vasodilators via SNO formation</td>
<td>81</td>
</tr>
<tr>
<td>1987</td>
<td>*NO is an EDRF</td>
<td>80, 135</td>
</tr>
<tr>
<td>1990</td>
<td>SNO-cysteine is a possible EDRF, and is as potent as *NO</td>
<td>127</td>
</tr>
<tr>
<td>1992</td>
<td>SNO-albumin is a reservoir of NO bioactivity</td>
<td>165</td>
</tr>
<tr>
<td>1993</td>
<td>GSNO transduces NO bioactivity in humans</td>
<td>54</td>
</tr>
<tr>
<td>1996</td>
<td>SNO-Hb hypothesis: discovery of a physiological vehicle of NO bioactivity</td>
<td>87</td>
</tr>
<tr>
<td>1996</td>
<td>SNO-specific recognition sites are suggested by the stereoselectivity of SNO actions</td>
<td>31</td>
</tr>
<tr>
<td>1997</td>
<td>SNO-Hb levels reflect (in rats and humans) and respond to (in rats) the physiological O₂ gradient</td>
<td>51, 164</td>
</tr>
<tr>
<td>1998</td>
<td>Human SNO-Hb crystallized</td>
<td>23</td>
</tr>
<tr>
<td>1999-1999</td>
<td>Novel NO-Hb chemistry identified that underlies SNO-Hb formation and action</td>
<td>63, 64</td>
</tr>
<tr>
<td>2001</td>
<td>NO bioactivity is exported from RBC SNO-Hb through AE-1 transnitrosylation</td>
<td>138</td>
</tr>
<tr>
<td>2001</td>
<td>Identification of an evolutionarily conserved GSNO-reductase</td>
<td>106</td>
</tr>
<tr>
<td>2002</td>
<td>Cardiorespiratory cycling of Hb-(S)NO in human blood in vivo</td>
<td>119</td>
</tr>
<tr>
<td>2003-present</td>
<td>RBC SNO derangements in human diseases, and response to therapy</td>
<td>29, 41, 83, 107, 116, 126</td>
</tr>
</tbody>
</table>

AE-1, anion exchanger-1/band 3 protein; Cysβ93, Hb’s β-chain cysteine residue 93; EDRF, endothelium-derived relaxing factor; GSNO, SNO-glutathione; Hb, hemoglobin; *NO, radical nitric oxide; NO, nitric oxide species; RBC, red blood cell; sGC, soluble guanylate cyclase; SNO-, S-nitroso(thiol)-.
diffusion of *NO in the static plasma layer around RBCs is essentially similar to the rate-limiting diffusion at the blood-endothelium interface. There, rapid *NO uptake by the RBC creates inhomogeneous *NO concentrations (96). Interestingly, *NO itself may influence the rate of RBC uptake of further *NO, as demonstrated by Liao and coworkers (67, 77).

Although physiological barriers certainly limit *NO uptake by RBCs and thereby prevent chronic systemic hypertension, they do not preclude the interactions of *NO with Hb. Alternative, stable NO stores formed in and ferried by the RBC could ensure that NO bioactivity reaches the tissues when needed (see below).

**Evidence for systemic preservation of NO bioactivity**

In vivo, Hb O2 saturation was shown to bring tissue blood flow in line with O2 demand through a dynamic regulation of the vasodilation-vasoconstriction balance (61). The fact that Hb O2 saturation (and not free plasma O2) modulates blood flow reveals that RBCs act as O2 sensors in the systemic microcirculation and suggests that they transduce not only vasoconstrictor but also vasodilator responses (61, 119, 172). They do it, at least partially, by modulating the availability of NO (25, 29, 41, 83, 119). Arguments for such biology are numerous. For example, Datta et al. (29) showed that RBCs dilate preconstricted aortic rings at low Po2 (1% O2) but not at high Po2 (95% O2). In fact, the vasodilator response invoked by RBCs across a physiological range of Po2 is rapid, graded, and unique, and implies a depletion of endogenous vasodilatory stores at fixed Po2 (119). Cannon et al. (19) further observed that NO gas inhalation significantly lowered forearm resistance in human volunteers undergoing NOS inhibition. These selected examples illustrate that NO bioactivity can be exported from the site of NO production/delivery through circulating RBCs to peripheral tissues where it acts. Such shuttling suggests that NO bioactivity is preserved in stable, mobile stores.

**Loading Hb with NO bioactivity**

Interestingly, Hb contains a highly conserved cysteine residue at position 93, and Cys 93’s persistence throughout avian and mammalian evolution (24) may owe to its reactions with NO (87). Binding and release of NO equivalents are under allosteric control, because the reactivity of the Cys 93 residue is conforma-
dependent: it is reactive toward NO in the R (oxygenated) state of Hb and relatively unreactive in deoxygenated T state (FIGURE 1). The allosteric transition in Hb from T (tense, deoxygenated) to R (relaxed, oxygenated) structure promotes the transfer of an NO group from Hb’s hemes to its thiols (Cys 93), forming HbCys 93NO (SNO-Hb, reaction 5) (48, 87, 119). The hemes furnishing the NO equivalents are likely to be the β-chain hemes, owing in part to the proximity of the β heme and Cys 93 (4, 109). The Hb-SNO-forming reaction requires an electron acceptor; O2 can serve this function, in this case leading to the concurrent formation of superoxide (O2 •−) (64, 72). In R-state Hb, Cys93NO is protected from solvent through confinement into a hydrophobic pocket (16, 23, 24). The Hb-SNO formation reaction requires an electron acceptor; O2 can serve this function, in this case leading to the concurrent formation of superoxide (O2 •−) (64, 72).

5) Hb[Fe(II)NO]+Cys93-SH + 2 O2→ Hb[Fe(II)O2]+Cys 93-SNO + *O2 •− + H+ (Hb S-nitrosylation)

Superoxide dismutase (SOD), abundant in the RBC, ensures that the superoxide generated in this reaction is eliminated and may also participate in facilitating the transfer of nitrosylating equivalents from GSNO to Hb in the RBC, as demonstrated by English and coworkers (149).

*NO is not the only NO-related reactant competent for Hb S-nitrosylation. The reaction of RNOS with deoxy-Hb can lead to the formation of met-Hb and *NO (reaction 6) (162). Although this reaction is relevant to mechanisms underlying some conventional methods for synthesizing SNO-Hb by reacting Hb with NO-glutathione (GSNO) or SNO-Cys (59, 87) in the laboratory, its physiological significance is dubious, since neither glutathione (GSH) nor GSNO freely crosses membranes, and GSNO has not been definitively detected within RBCs (118).

6) Hb[Fe(II)] + RSNO + H+→Hb[Fe(III)] + *NO + RSH

In a succession of reactions, *NO generated in reaction 6 directly nitrosylates a vicinal ferrous heme (reaction 2) and, on R-state transition, S-nitrosylates Hb (reaction 5) (158).

Nitrites, which have long been viewed as inactive degradation products of *NO (reaction 1), in fact represent another endogenous route to NO bioactivity: they can dilate blood vessels but must be converted to NO or SNO to exert this activity (Refs. 25, 27, 44, 128, 146, and P. Sonveaux and O. Feron, unpublished data). Systemic NOS activity (reaction 1) and, to a lesser extent, food intake determine plasma NO−2 supply, and NO−2 clearance is primarily by its oxidation to inactive NO−3, which is excreted by the kidneys. Similarly to GSNO, NO−3 does not freely cross membranes. However, the discovery of the interaction of anion exchanger-1 (AE-1; also known as band 3 protein) with (SNO)-Hb at RBC membrane locales supports the existence of a transportation pathway that could facilitate NBC NO−3 uptake (96). The reaction of NO−3 with deoxy-Hb generates iron-nitrosyl adducts (reaction 7) that are dislodged on reoxygenation to produce SNO-Hb (reaction 8) (4, 128, 129, 158).

\[ \text{Hb}[\text{Fe(II)}] + \text{RSNO} + \text{H}^+ \rightarrow \text{Hb}[\text{Fe(III)}] + \text{*NO} + \text{RSH} \]
7) \( \text{Hb}[\text{Fe}(II)] + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Hb}[\text{Fe}(III)\text{NO}]^- + \text{OH}^- \rightarrow \text{Hb}[\text{Fe}(II)\text{NO}]^- \)

8) \( \text{Hb}[\text{Fe}(II)\text{NO}]^-\text{-Cys}/\text{SH} + \text{O}_2 \rightarrow \text{Hb}[\text{Fe}(II)\text{O}_2^-\text{-Cys}/\text{SH} + \text{NO} + \text{H}^+ \)

**Hb auto-S-nitrosylation**

A dominant NO\(_2^-\) reduction intermediate, with an electron delocalized between the heme iron and the bound NO was suggested, and the delocalization may include the Cys \( \beta 93 \) residue. Because these hybrid species are EPR-silent, they will be missed by laboratories using EPR and assuming that the heme portion of "NO" mass balance between hemoglobin and thiols can be assessed by following Hb[Fe(II)NO\(^-\)] alone (4). Of note, under conditions of low O\(_2\) and pH (such as in tumors), NO\(_2^-\) can also be reduced to NO by acidic disproportionation and by the reductase activity of xanthine oxidase (122, 176). The relative importance of these nitrite-dependent paths to NO bioactivity are unknown.

Interestingly, it has also been documented that Hb S-nitrosylation is potentiated by the presence of met-Hb (64, 72). *Reaction 4*, which was initially proposed to inactive *NO*, could thus rather constitute the initial step of an integrated mechanism yielding SNO-Hb. On T \( \rightarrow \) R transition, the population of either of two species in equilibrium, Hb[Fe(III)NO\(^-\)] and Hb[Fe(II)NO\(^-\)], could thus induce Hb S-nitrosylation wherein ferric heme functions as an internal electron acceptor (*reaction 8*) (109, 128, 129). SNO-Hb is a product in the reaction of *NO* with met-Hb regardless of the sequence of *NO* addition and heme oxidation, but with preference for the \( \beta \) vs. \( \alpha \)-heme subunits. This confirms the high intramolecular reactivity of the nitrosyl-\( \beta \) subunit (97).

A "nitrite reductase" hypothesis posits that Hb converts nitrite to bioactive form by producing an Hb[Fe(II)INo] product. However, this species acts as a vasoconstrictor rather than a vasodilator (110). Bioactivity may derive from Hb[Fe(II)INo] only to the extent that it is further converted into SNO-Hb, since *NO* cannot escape the heme-rich RBC (86). Indeed, under physiological conditions [namely, physiologically relevant nitrite and Hb concentrations and stoichiometry (ratios) of the two] and within PO\(_2\) ranges and cycling times like those encountered by the RBC in the cardiorespiratory cycle, the activity of Hb toward nitrite is revealed to be a "SNO-synthase" function when all products are accounted for (4, 109). A further problem with the nitrite reductase hypothesis, as recently formulated (27, 78), is that the apparent coincidence of its maximal activity with Hb's P\(_{50}\) was mistakenly construed as ideally serving hypoxic vasodilation. In reality, hypoxic vasodilation, which serves to redistribute blood flow to regions in greatest need, predictably increases as PO\(_2\) falls within the full physiological range (down to 5-10 mmHg), which extends well below the P\(_{50}\) (PO\(_2\) ~27 mmHg in vivo) of Hb (3, 45, 61, 119).

Hb S-nitrosylation (by the mechanisms outlined in *reactions 5–8*) is competitive with the dioxygenation reaction (*reaction 3*) and accounts for a physiological concentration of \( 10^{-7}\text{–}10^{-5.5} \) M SNO-Hb in human blood (87, 119, 123, 147, 164). Although the preponderant role of O\(_2\) (vs. other electron acceptors) in *reaction 5* is still under debate (62, 109), crystallographic studies have unequivocally demonstrated both the existence of SNO-Hb and the allosteric control of its generation (22, 23). X-ray crystallography has indeed demonstrated that the exposure of quaternary-T human Hb to NO (i.e., exposition of deoxy-Hb to NO) followed by exposure to air leads to NO binding to Cys \( \beta 93 \). This modification disrupts the intrasubunit salt bridge between His \( \beta 146 \) and Asp \( \beta 94 \), a key feature of the T-state Hb structure. No evidence for this reaction was detected under anaerobic conditions (22). Furthermore, the ability of heme-to-Cys \( \beta 93 \) NO transfer to form SNO-Hb is fully supported by biochemical studies having shown that \( \beta 93 \) Cys \( \rightarrow \) Ala mutation results in nitrosylated-heme accumulation (64). The consensual view is thus that Hb S-nitrosylation is linked in vivo to \( O_2 \) saturation that, in turn, controls Hb's allostery (87). The details of cycling of NO between Cys \( \beta 93 \) and Hb(Fe) in vivo remain to be clarified.

In the physiological situation, Hb S-nitrosylation incorporates a brake, and the NO-containing Hb is by far a minority population, representing \( \sim 1 \) in \( 10^{-7}\text{–}10^{-4} \) Hb molecules (158). It is well documented that the reactions of *NO*, RSNO, or NO\(_2^-\) with Hb are critically dependent on the NO-to-Hb ratio, e.g., yields of SNO-Hb are inversely related to the NO reactant concentration (64, 72). In fact, SNO-Hb formation is most efficient with physiological (nanomolar) NO levels and reaches a plateau of \( \sim 10^{-10} \) M. Over that threshold, additional NO reacts increasingly with \( \alpha \)-hemes to form inert \( \alpha \)-nitrosyl Hb, and the subsequent generation of pentacoordinate \( \alpha \)-Fe(II)NO\(^-\) (through the cleavage of a proximal histidine) modifies Hb allosteroy to prevent additional \( \beta \)-heme-to-Cys \( \beta 93 \) NO transfer (26, 64, 97, 120). Thus, in addition to physiological signaling by Hb-derived SNO, the interactions of Hb with supraphysiological NO are characterized by a chemistry that serves to divert *NO* from toxic reactions. Hb may be viewed as a sensor not only of O\(_2\) but also of the prevailing NO "tone," dispensing or sequestering NO equivalents accordingly in defense of the organism's health.

**Release of NO bioactivity by SNO-Hb**

The existence of an arteriovenous gradient of SNO-Hb (29, 51, 58, 87, 119, 128) places Hb-NO interactions in a dynamic dimension in which Hb sustains S-nitrosylation at high PO\(_2\) and transfers the SNO group at low PO\(_2\) (FIGURE 1). Interestingly, the R \( \rightarrow \) T confor-
mational transition that occurs on SNO-Hb deoxy-
genation (or oxidation) coincides with a shift in β-
chain Cysβ3NO location from its hydrophobic niche
toward the aqueous cytoplasmic solvent (23, 87).
There, Cysβ3NO is chemically available to transnitro-
sylate target thiol-containing proteins (reaction 9) (87,
118, 140).

![Diagram of Hb transitions](image)

**FIGURE 1.** Allosteric transitions of Hb during circulatory cycling regulate the delivery of NO bioactivity to preserve vascular and O2 homeostasis

RBC Hb senses (O2) and responds via allosterically governed (S)NO binding, formation, and release. At high PO2 in the lung venous system, Hb is in the R-state and the reactive Cys93 residue of the β chain is protected in a hydrophobic pocket in the vicinity of β hemes. On partial RBC deoxygenation in the peripheral circulation, Hb adopts the T structure and Cys 93 is exposed to solvent. These allosteric transitions control the reactivity of Cys 93. In the T-state, a population of Hb reacts with \(^{15}NO\), \(NO_2^–\), and/or low-mass S-nitrosothiols to produce nitrosylated heme at the β-chain (bottom left). Transition to R-state that brings Cys 93 close to nitrosylated heme (top left) is fol-
lowed by a nitrosylation reaction between NO heme and Cys 93. This results in the formation of a covalently bound S-nitrosocysteine (top right). In the R-state, SNO-Cys 93 is protected from further reactions in a hydrophobic pocket. A potential direct nitrosylation of Cys 93 by higher oxides of nitrogen (NOx) or by metal catalysis, including by dinitrosyl iron complexes, is also possible (12, 173). When Hb adopts the T conformation on partial deoxygenation (bottom right), SNO-Cys 93 readily reacts with target nitrosothiols and initiates a cascade of transnitrosations (for example with plasma) leading to the formation of SNOs outside RBCs. The precise identity of the SNOs formed remains unresolved, and the candidate GSNO is depicted here. SNO is at least as potent as \(^{15}NO\) as a vasodilator and ensures \(^{15}NO\)-independent functions aimed at preserving vascular homeostasis. RBCs thereby function as oxygen sensors to accurately regulate O2 supply.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Details</th>
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<tbody>
<tr>
<td>A.</td>
<td>(Hb[Fe(II)O_2^-] - Cys\beta93-SNO \rightarrow Hb[Fe(II)] - Cys\beta93-SNO + O_2)</td>
</tr>
<tr>
<td>B.</td>
<td>(Hb[Fe(II)] - Cys\beta93-SNO + R-SH \rightarrow Hb[Fe(II)] - Cys \beta93-SH + R-SNO)</td>
</tr>
</tbody>
</table>

Importantly, the SNO moiety transferred in this reaction is formally analogous to NO\(^+\) (not to \(^{15}NO\)) (28). It is therefore protected from heme recapture (scavenging, reaction 2) and inactivation (reaction 3) and insensitive to pharmacological \(^{15}NO\) chelators used as probes (28). Deoxygenation (or heme oxida-
tion) increases the release of NO bioactivity by RBCs, which provokes vasodilation and inhibits platelet aggregation (29, 87, 138, 140).

The nature of the acceptor nitrosothiol(s) involved in reaction 9 that transmit(s) the NO signal from the RBCs to the vascular wall or to platelets is still unknown. Signal transduction is currently believed to proceed via a cascade of transnitrosylations. AE-1, which physically inter-
acts with SNO-Hb, has been identified as a candidate mediator initiating this cascade (138). The docking of SNO-Hb on AE-1 is indeed followed by the transfer of the SNO group from SNO-Hb, which provokes vasodilation and inhibits platelet aggregation (105, 118, 164). The identification of a conserved GSNO reduct-
ase, involved in the termination of GSNO-mediated signals, supports that view (106). Specifically, trans-
genic mice lacking GSNO reductase show increased SNO-Hb levels (107), which argues in favor of GSNO acting in SNO export rather than importation. At the vascular wall, GSNO elicits vasorelaxation (164). Actions of (G)SNO in target cells may depend on GSNO processing to the cell-permeable dipeptide Cys-Gly-SNO (S-nitrosocysteinylglycine) (75) and/or specific transmembrane SNO-transporters (103, 103a).

Vasodilation is readily detected with concentrations of SNO-Hb of < 10\(^{-8}\) M, which suggests that the endogenous SNO-Hb pool (10\(^{-7}\) – 10\(^{-5.5}\) M) dispenses limited amounts of NO bioactivity in regulated fashion and with an impressively high potency. Deem et al. (33) demonstrated that when grossly supraphysiolog-
cal concentrations of SNO-Hb are studied, the allosteric control of SNO release is obscured. The normally precise regulation involves, at least in part, the increased O2 affinity of SNO-Hb (stabilized R-
state) compared with Hb (14, 118). Although the physi-
ological concentration of SNO-Hb is too low to signif-
icantly impact overall Hb O2 affinity, the more stabi-
lized R state nevertheless allows SNO release in needy, hypoxic tissues while otherwise limiting SNO delivery (87, 137). However, because O₂ loading and unloading from Hb is cooperative, there is always a proportion of deoxy-Hb releasing bioactive SNO at any physiological Po₂ (158). The total level of NO species in RBCs typically does not change during circulatory cycling [because transnitrosylation is partially balanced by heme nitrosylation (87, 119, 164)]. Similarly, the blockage of thiols (alkylation) (162) or hemes (e.g., cyanlation) dramatically alters Hb allostery and prevents its normal functional coupling to NO capture and release. Incorrect conclusions have thus been drawn from experiments using these promiscuous probes (40, 41, 148, 163).

The major findings that have built our current knowledge of the interactions between Hb and NO are placed in an historical perspective in Table 1. An integrated view of Hb chemistry and of its role as an O₂ sensor that regulates vascular homeostasis during RBC cycling in the systemic circulation is provided in Figure 1.

**RBC SNO-Hb Signals at Multiple Loci in the Organism**

**Hypoxic vasodilation**

Hypoxic vasodilation is a response by which falling Po₂ triggers a regional increase in blood flow (3, 158). Although hypoxia potentiates NO-mediated vasorelaxation (28, 29), endothelium-derived NO plays no direct role in hypoxic vasodilation (61), and in fact eNOS activity is impaired in hypoxia because of limitation of substrate O₂ (90). However, eNOS may well provide the endothelium-derived NO (EDNO) substrate ultimately harnessed by RBC Hb to effect hypoxic vasodilation, in most cases distant in time and space from the uptake of EDNO by the RBC. Exploiting the O₂-sensing function of Hb, RBCs drive the graded dilation of blood vessels at decreasing Po₂ to an extent that exceeds by far the more general phenomenon of hypoxic potentiation of the vasorelaxation (29, 60, 61, 119). Hypoxic vasodilation by RBCs is mediated by both cGMP-dependent and cGMP-independent pathways (29, 83, 138). It has been shown to be entirely endothelium-independent (i.e., RBCs dilate endothelium-stripped vessels) and to proceed at least partially through the allosteric mobilization of NO stores from SNO-Hb (FIGURE 2) (25, 26, 83, 87, 140). The export of...
NO bioactivity by hypoxic RBCs occurs within seconds, which contrasts with the release and action of ATP (minutes), another potent vasodilator reported to mediate RBC hypoxic vasodilation (60, 82, 158). Thus SNO-Hb may act preferentially in response to regional and acute hypoxia, in which the vascular transit time of RBCs is not altered. ATP would complement the function of SNO-Hb in episodes of chronic hypoxia in which blood flow is slowed down or stopped and then resumes (e.g., ischemia-reperfusion). Further work is needed to define the relative roles of ATP and SNO release from the RBC in the regulation of blood flow and should be placed into response- and disease-specific context.

**RBC uptake of O_2 in the lung: role of signaling by Hb-derived SNO**

In the lung, the delivery of NO/SNO by RBCs is responsive to hypoxia, but in this case SNO signaling initiated by O_2-sensing Hb serves the uptake of O_2 by the lung at the interface of alveoli and the pulmonary microcirculation (116). In RBCs entering the pulmonary circulation, Hb is ~75% saturated in O_2 and roughly equally represented by T- and R-structure tetramers. Thus, in the normal lung, RBC-Hb is poised to offload the residual SNO it carries. In fact, RBCs likely deliver SNO equivalents in the lung, and in this process blood oxygenation is optimized, reflecting regional alignment of perfusion and ventilation (ventilation-perfusion matching; Figure 2). SNO delivery from RBCs perfusing the lung also dampens the strength of the hypoxic pulmonary vasoconstrictor (HPV) response in the face of global (i.e., nonregional) hypoxia, in which preservation of lung blood flow and protection of the right ventricle from pressure overload are desired (41, 116). The hypoxic potentiation by °NO itself of NO uptake into RBCs has been proposed as a potential mechanism underlying HPV (67). But in the systemic circulation, a similar phenomenon, if operative, would promote vasoconstriction, whereas the physiological response is hyperemia through vasodilation. Thus, the physiological significance of this phenomenon remains unclear.

**Central nervous response to hypoxia**

In addition to its roles in the circulatory reflexes that serve to optimize the uptake of O_2 by RBCs perfusing the lung (HPV) and the peripheral delivery of O_2 by RBCs perfusing tissues, SNO exported by RBCs may also initiate the ventilatory response to hypoxia, by which brain stem centers sensing hypoxia stimulate increases in minute ventilation. This response is mimicked, in a stereospecific manner, by injection of low-mass SNO into the brain stem’s nucleus tractus solitarius, the control center for the hypoxic ventilatory reflex (105). Remarkably, the plasma fraction from deoxygenated blood, but not from oxygenated blood, spiked with GSH also recapitulates the response and predictably contains detectable GSNO (105). In humans, oral N-acetyl-cysteine (NAC) enhanced the hypoxic ventilatory response, underscoring the role of plasma thiols and pointing to a locus for therapeutic intervention (74). The potential influence of RBC-derived SNO signaling in apneic and hypopneic diseases is an area ripe for study.

**Baroreceptor inhibition**

In a recent publication, our laboratory (161) showed that the systemic regulatory effects of SNO-Hb can be extended to the modulation of baroreceptor activity. The function of the baroreceptor is to oppose any increase in the mean arterial blood pressure (MAP) by a reflex decrease in the heart rate (HR). °NO (as a neurotransmitter produced by nNOS) is well known to regulate baroreceptor activity through sympathetic afferent nerve fibers. In contrast, °NO reaching baroreceptor sensors from the bloodstream has no direct action unless delivered at high concentration (>100 μM) directly into the carotid sinus (68). By comparison, we observed that cell-free SNO-Hb, infused at a ~6.5 μM concentration (a [Hb] typical of hemolytic states) into the aortic lumen of anesthetized rats actuated a long-lasting (minutes) inhibition of the baroreflex characterized by a sustained increase in MAP without HR adaptation (161). This activity was preserved when SNO-Hb was delivered via the femoral vein of animals breathing pure O_2 but lost in normoxic animals or on native Hb infusion by either route and at either P_O2. That SNO-Hb stability in vivo (approximately twofold increase in plasma half-life on pure O_2 delivery) and efficient inhibition of the baroreceptor are correlated suggests that RBC SNO-Hb’s responsibilities could also include baroreflex regulation (FIGURE 2). Interestingly, other endogenous S-nitrosothiols (such as SNO-cysteine) suppress baroreceptor activity independently of cGMP generation (114). The existence of stereoselective SNO recognition sites in the baroreceptor vasculature (31) suggests that low-molecular-weight S-nitrosothiols could transport SNO from SNO-Hb in RBCs toward putative SNO receptors in the vascular wall and thereby couple blood O_2 content, as reflected by RBC SNO(-Hb) flux, to changes in MAP.

**Methodological Issues**

Several analytical techniques have been used for purported detection of SNO-Hb in the blood. All of the developed techniques require a denitrosylation step for the final analysis, involving either a spectrophotometric detection of the products of a diazotation reaction with Griess reagent, or chemoluminescence-based NO detection (41, 51, 57, 58, 89, 119). The latter approach, which analyzes released NO in a highly sensitive, gas-phase chemiluminescence reaction with ozone, has the advantage of precisely measuring...
NO concentrations in the low-nanomolar (i.e., physiological) range (41, 57, 58, 89, 119). Assay-dependent difficulties in the interpretation of results, feeding controversial discussions in the determination of the absolute SNO-Hb level in RBC, arose from differences in strategies used for sampling, sample storage, sample pretreatment, and methods used to minimize NO-group loss in SNO-Hb quantification. Reported RBC SNO-Hb concentrations vary from 10 nM to 5 μM (41, 51, 57, 58, 89, 119). Among the main topics of discussion are the possible degradation, or the de novo (artifactual) formation, of SNO(-Hb) during sample processing for NO measurements. Reactions of nitrosylation (reaction 9) could play important roles in both processes and nonphysiological NO2- concentrations could promote artifacts.

Levels of endogenous human SNO-Hb (~2–5 μM) were measured by HgCl2-induced SNO cleavage followed by spectrophotometric NO-derivative detection or by high-energy UV photolysis followed by the detection of liberated NO by gas-phase chemiluminescence with (and without) HgCl2 treatment (51, 119). Importantly, this technique is selective and does not efficiently detect NO2- or nitrate [in the presence or absence of thiols (McMahon TJ, unpublished observations; Refs. 34, 68a)]. Lower estimates of endogenous SNO-Hb (0.5 μM and lower) were derived in RBCs by a number of groups using a triiodide-based chemiluminescence assay developed by Gladwin et al. (57, 58). This method has been shown to be fundamentally flawed, as the technique yields artifacts due to the destructive chemical pretreatment on one hand and to heme autocapture (reactions 2 and 4) of NO by the Hb in the reaction solution on the other hand (46, 68a, 119, 148). The ability of triiodide-coupled chemiluminescence to differentiate SNO from Fe(II)NO is therefore dubious. Some modifications were offered to avoid these difficulties: the analysis of the influence of an acidified-sulfanilamide pretreatment (putatively used to remove NO2-) and a ferricyanide-based reaction (putatively used to block heme nitrosylation) were studied at biologically relevant NO-heme ratios. Unfortunately, these agents have only further obfuscated the picture (68a, 109).

EPR spectroscopy is currently recognized as a selective and reliable quantitative method to analyze nitrosyl adducts under some conditions. The technique is widely used to study the transformation of paramagnetic nitrosyl Hb to the EPR-silent SNO-Hb in the arteriovenous cycle as well as in model chemical investigations (63, 85, 109). An advantage of EPR is that blood can be analyzed immediately after sampling without any pretreatment. EPR detection limit for HbNO (>200 nM) is, however, quite high compared with physiological levels (141). Based on EPR, endogenous levels of nitrosylated Hb in total blood amount to ~0.2 μM in human blood, ~0.6 μM in rat venous blood, and ~0.4–0.5 μM in mouse blood (Refs. 38, 85, 141; and I. I. Lobysheva, unpublished observations). Apart from a shift from paramagnetic 5-coordinate α-HbNO to 6-coordinate α-HbNO (routinely reported as the transition from deoxy-like T-state to oxy-like R-state nitrosylated Hb), no significant change in the concentration or fate of nitrosyl Hb was observed during the arteriovenous cycle in the blood of rats treated with a NO-donor or in human blood after NO inhalation. These results are in apparent contradiction with the proposed transfer within Hb of NO from nitrosylated hemes to Cys β93 on reoxygenation (reactions 5 and 8) (38, 85, 141), unless one proposes that any NO transfer to Cys β93 is immediately balanced by the replenishment of heme nitrosyl stores. The apparent contradiction may derive, in part, from a failure to protect samples from air exposure during assays, resulting in “arterialization” of venous samples and thereby artifactually eliminating arterial-venous differences (38, 85, 119, 141). In addition, failure to demonstrate mass balance in experiments relying on EPR to account for heme-bound NO is inevitable, given the EPR-silent Hb[Fe(III)NO] ↔ Hb[Fe(II)NO+] species studied by Angelo, Luchsinger, and colleagues (4, 109). Numerous problems with selectivity and a propensity to generate artifacts in the latest incarnation of the triiodide assay further complicate the matter (68a).

A final layer of methodological complexity resides in the use of different animal models. Different biochemical and hemodynamic features across species complicate the interpretation of studies focused on NO interaction with Hb in general and with blood in particular (56, 150). The intensive development of studies aimed to elucidate the reactivity and fate of NO2- in RBCs could move the existing controversies toward resolution (25, 129).

**SNO-Hb in Pathophysiology and in Therapy**

Disturbances in the (S)NO delivery function of RBC Hb have now been described in a number of disease states, and the variety of specific lesions underscores the complexity of the RBC SNO system.

**Pulmonary hypertension**

As detailed above, RBC-SNO modulates pulmonary hypertensive responses (such as that to hypoxia), and, indeed, patients with idiopathic pulmonary arterial hypertension (IPAH) were demonstrated to have depressed levels of arterial SNO-Hb (relative to normal human controls), whereas Hb[Fe(II)NO+] and total Hb-bound NO levels did not differ significantly. Sustained exposure of RBCs to modest hypoxia reproduced the deficit and induced an accumulation of α-heme-NO species, from which SNO-Hb formation is strongly disfavored. In parallel to the defect in RBC SNO-Hb, the RBCs from IPAH patients were deficient in their ability to elicit hypoxic vasodilation. In
support of the link between SNO-Hb, RBC-dependent vasoactivity, and regulation by RBC-SNO of pulmonary vascular tone and blood oxygenation in vivo, when IPAH patients breathed ethyl nitrite (ENO, which restored RBC SNO-Hb levels to the normal range), improvements were seen in RBC-dependent vasoactivity, pulmonary vascular resistance, and blood \( O_2 \) uptake (116). Because the RBC lesion in IPAH represents a defect in the processing of NO to SNO by Hb, ENO may have advantages over inhaled NO in that ENO provides NO equivalents with a SNO character (116, 117). By contrast, extrapulmonary actions of inhaled NO, which contribute to its clinical benefit in neonatal lung disease and pulmonary hypertension (24a, 154a), might in fact be channeled by RBC SNO-Hb (117).

**Sepsis**

Sepsis is a complex, systemic inflammatory response to infection that, if unopposed, can lead to septic shock (sustained hypotension resistant to vasopressor therapy), organ failure, and death. It has a 6-month mortality rate of ~50% (35). Vascular dysfunction is seen, with a transition from an initial hyperdynamic phase to a hypodynamic phase characterized by decreased tissue perfusion and loss of vascular homeostasis. At the molecular level, this imbalance is due to an increase in *NO production by iNOS (which is part of the arsenal of inflammatory cells) and vascular desensitization to endothelin-1 and other endogenous vasoconstrictors (76, 95, 112).

In the RBC, the nitrosative stress caused by elevated \*NO production during sepsis results in elevated levels of Hb[Fe(II)NO\*], SNO-Hb, possibly other SNO-proteins, and low-molecular-weight SNOs (26, 41, 88, 99, 111, 133). Interestingly, most of the Hb[Fe(II)NO\*] that accumulates is in the \( \alpha \)-heme-NO form, from which the release of \( O_2 \) is favored and the release of NO bioactivity is essentially impossible (98, 175). The quenching of excessive \*NO in parallel with facilitated \( O_2 \) delivery suggests that septic Hb functions as an \*NO detoxifier to bring tissue \( O_2 \) needs in line with delivery without excessive vasodilation (117). In this sense, septic Hb diverts NO from a vasodilating pathway and recruits it into use as an allosteric effector to regulate \( O_2 \) delivery. Potential therapeutic interventions should thus be targeted at the unbridled NOS activity while preserving Hb function. The treatment of macro- and microcirculatory abnormalities in sepsis could benefit from the further development of selective iNOS inhibitors, such as 1400W, that leave eNOS and nNOS functions intact (143).

**Congestive heart failure**

In contrast to the RBC-SNO elevation seen in the hyperdynamic cardiovascular state of sepsis, in congestive heart failure, transpulmonary gradients of RBC SNO-Hb were increased in direct proportion to the degree of both depression of cardiac index and peripheral Hb \( O_2 \) desaturation, underscoring the role of SNO flux in the RBC’s \( O_2 \) delivery role (29).

**Sickle cell disease**

Sickle cell disease (SCD) is a chronic hemolytic anemia punctuated by clinical events involving recurrent vaso-occlusion. In the absence of appropriate markers of severity, complications (including deep venous thrombosis, stroke, bone pain, and acute chest syndrome) are unpredictable and heterogeneously distributed among the SCD patient population. SCD finds its etiology in a Glu → Val homozygote mutation within Hb \( \beta \)-chains that disturbs Hb allostery and heme redox potential in HbS tetramers (15). Compared with Hb, HbS also has a lower affinity for \( O_2 \) (14) and readily polymerizes on deoxygenation (and the transition to T-state). The resultant sickled cell RBCs are deformed (sickle shape), adherent, and fragile, accounting for episodes of vascular occlusions and hemolysis.

Although the substitution in HbS is distant, its structural consequences influence the heme site, where a decrease in the redox potential of HbS renders it more susceptible to oxidation (15). This redox alteration also prevents efficient heme-to-thiol transfer of NO groups (and thus the formation of SNO-Cys \( \beta \)3 on the allosteric transition from T → R structure) in sickle Hb. In addition, derangements in the critical protein-protein interactions between RBC Hb and acceptor thiols in the cytoplasmic domain of AE-1 reduce the efficiency of transfer of SNO equivalents from HbS to the RBC membrane (139). These alterations may result in part from loss of availability of the cognate thiol in AE-1 secondary to oxidation, for example in membrane-associated hemichrome complexes. For these reasons, sickle RBCs are impaired in their ability to mediate hypoxic vasodilatation (139).

**Hemolytic anemias**

In contrast to sepsis, in which elevated \*NO production by NOS leads to hypotension, various hemolytic diseases are associated with hypertension. Thalassemia, paroxysmal nocturnal hemoglobinuria, and malaria are all characterized by abundant release of Hb from RBCs. Cell-free Hb is unstable at both molecular and functional levels. The exquisite control of Hb allostery that normally occurs in, and depends on, the RBC environment can be viewed as the lesion sustained with hemolysis, and the degradation of functional tetramers to nonfunctional \( \alpha \beta \) dimers further prevents the preservation and transport of NO bioactivity by plasma Hb. The rupture of the chemical balance between NO degradation and preservation results in massive scavenging and inactivation of endogenous \*NO through reactions 2 and 3. \*NO scavenging is aggravated by dimers extravasation along the normal route of \*NO diffusion from its endothelial site...
of production by eNOS to its targets in VSMCs. Furthermore, dimer extravasation causes extensive tissue swelling and renal damage through osmotic effects (17). Most of the clinical consequences of excessive cell-free plasma Hb accumulation during intravascular hemolysis (including pulmonary hypertension, gastrointestinal and cardiovascular dysregulation, and erectile dysfunction) may be partially explained by Hb-mediated *NO scavenging (151). Hemolysis (and *NO scavenging by Hb) accounts poorly for the pathology in some diseases; for example, in sickle cell disease, pulmonary hypertension (PH) has been ascribed to this, but no sound explanation exists for the fact that these patients lack systemic hypertension, even when PH is present (5a, 57a).

**Diabetes mellitus**

The endothelial dysfunction associated with diabetes mellitus has been attributed in part to a lack of bioavailable NO. Besides reduced *NO production from NOS and *NO inactivation by abundant reactive oxygen species (ROS), alterations in NO signaling involve a dysregulation of RBC-SNO delivery that appears to contribute to the associated vasculopathy (123). In diabetes, progressively increasing glycosylation of Hb shifts the O2-binding curve leftward (i.e., raising O2 affinity and promoting R-state), which in turn increases nitrosylation at both the hemes and β93Cys residues (123, 134). Oxygen-sensitive vasorelaxation is thus impaired in response to diabetic RBCs, because the leftward shift disfavors the release from Hb of SNO equivalents. In addition, glycated proteins (including Hb) generate ROS (potentially through reaction 5) and thereby further quench *NO and promote lipid peroxidation (30). Overall, alterations in Hb allostericity in diabetic patients participate in reducing vascular perfusion, which is responsible for the vasculopathies characteristic of the disease (83, 123). In the normal human newborn, abundance of high-affinity fetal hemoglobin apparently does not result in either systemic hypertension or impaired peripheral oxygen delivery; whether developmental compensation is involved, for example regulated at the level of RBC or tissue SNO signal transduction or tissue SNO receptors, is unknown but is an area worthy of investigation.

**Excess morbidity and mortality with RBC transfusion**

Development of blood substitutes is motivated not only by economic considerations but also by the threat of infection borne by blood and blood products and by situations where collection, storage, and maintenance of an adequate blood supply constitute an enormous challenge. Another motivation derives from the observation that even transfusion of serologically compatible, type-matched, banked blood can induce deleterious (and sometimes life-threatening) side effects. Indeed, transfusion-related immunosuppression can promote postoperative infections and tumor recurrence (11); products of passenger leukocytes or degraded RBC membranes can prime proinflammatory agents that trigger neutrophilic responses (132, 157); and substantial shape changes and loss of deformability during storage can lead to RBC entrapment and microvascular obstruction on delivery (9). Given the recent appreciation that the RBC regulates its own function (O2 delivery) by contributing to blood flow regulation (46, 119), a better understanding is clearly needed of the relationship between storage-induced changes in the RBC and dysregulation of blood flow, which may contribute to the excess mortality seen with transfusion in some settings (69, 144).

Hb-based O2 carriers are the most promising blood substitute candidates (reviewed in Ref. 1). The allosteric R → T transition of cell-free tetrameric Hb is indeed capable of responsive O2 delivery to O2-depleted respiring tissues. Allostery can be further manipulated to align the cell-free Hb's O2 affinity (which is higher than that of intra-RBC Hb) to physiological values compatible with its clinical use (125, 155). Furthermore, various conjugates and cross-linked tetrameric Hb derivatives potently prevent dimer-associated toxicity of cell-free Hb (1) and may prolong Hb shelf-life (6). O2 affinity is augmented in cell-free Hb not only secondary to dimer formation but also because of the loss of erythrocytic allosteric regulators, such as 2,3-DPG; this difference is important in investigational comparison of cell-free and erythrocytic Hb-NO biology as well as in blood substitute development. Modified Hbs can retain allosteric capabilities. Unfortunately, a major issue remains that simple intramolecular cross-linking does not reduce the vasoconstrictor effects of cell-free Hb produced through reactions 2 and 3 (32). To date, Hb-mediated vasoconstriction represents a major limitation for its clinical development as a blood substitute. S-nitrosylation of Hb constitutes a viable answer to that problem. Indeed, converting Hb to a species that can stimulate O2-sensitive, NO-dependent vasodilation may improve the balance between forces promoting destruction vs. preservation of NO bioactivity and circumvent the marked hypertensive responses otherwise observed on Hb infusion (131, 136, 161).

**Cancer**

Hypoxia is a characteristic feature of solid tumors, resulting from an imbalance between O2 supply and consumption (169). The origins of tumor hypoxia are multiple, including abnormal structure and function of the microvasculature supplying the tumor, increased diffusion distances between blood vessels and tumor cells, and reduced O2 transport capacity of the blood due to anemia. Cancer-related anemia is described as a cytokine-mediated disorder resulting from complex interactions between tumor cells and the immune system. Overexpression of inflammatory
cytokines results, for instance, in inadequate erythropoietin (EPO) production, suppression of erythroid progenitor cells, shortened survival of RBCs, and impaired iron utilization (10). In addition, the rate of anemia rises with the use of chemotherapy and radiotherapy.

Anemia is associated with a poorer quality of life in cancer patients and also with reduced survival (13). There is a close relationship between falling Hb level and worsening tumor oxygenation. Since tumor hypoxia is directly linked to tumor growth, angiogenesis, clonal selection, and the development of treatment resistance, poor prognosis is generally associated with low Hb levels. In fact, of the 90% of cancer patients affected by anemia, more than 60% will require blood transfusion during or after treatment. Recombinant human EPO (rEPO), an alternative to RBC transfusion, is now widely used to treat cancer-associated anemia (13). There is good evidence that rEPO improves quality of life, but it also increases the risk of thromboembolic complications. Furthermore, recent studies indicated that the EPO receptor is expressed in several cancer cell lines, raising the concern of possible stimulation of tumor cell growth by these drugs. In fact, randomized trials recently reported negative outcomes with rEPO (102). The presence of an autocrine-paracrine EPO-EPO receptor system in tumors and its potential effects on the tumor microenvironment and on angiogenesis require further investigation.

The pattern of malignant progression associated with tumor hypoxia highlights the need for effective treatment of anemia as one approach for correcting hypoxia in tumors. Considering the conflicting results obtained so far with rEPO, a better understanding of the relevant Hb biology is required, as well as the optimal manner to exploit it to improve the therapeutic response to conventional anticancer modalities.

Radiotherapy and O2 biology of cancer

O2-dependent stabilization of DNA damage accounts for the efficiency of radiotherapy and many chemotherapeutic treatments. A threshold of 10 mmHg has been determined as the minimal Po2 allowing full tumor radiosensitization (65). Because viable tumor cells accounting for tumor regrowth after treatment are commonly found between 0 (full anoxia) and 10 mmHg, strategies aiming to transiently increase tumor Po2 at the time of treatment constitute an alternative to EPO-based therapies (47). Hyperoxic gas breathing as a single-modality treatment has limited impact on tumor Po2 because Hb is already fully saturated when respiring room air, and increasing O2 delivery will only increase plasma O2 concentration, which is not efficiently retained within the vasculature (36). Moreover, the existence of a longitudinal gradient of blood deoxygenation (that becomes steeper when hyperoxic gas is used) in tumors prevents oxygenation of inner tumor hypoxic zones (37, 39, 142). Toxic side effects were also reported (166). Radiosensitization may also be achieved by exploiting the vasodynamic response of the pool of mature vessels included in the tumor mass (as opposed to immature angiogenic vessels, which are by nature deprived of a VSMC layer) and of mature tumor-feeding arterioles (47). By decreasing resistance to flow, vasodilation was repeatedly shown to increase tumor blood flow and, consequently, tumor Po2 (159, 160). However, to avoid systemic responses (including hypotension and a diversion of blood flow from the tumor to healthy organs), vasodilators must act preferentially at the tumor vasculature.

The hypoxic nature of tumor vessels renders SNO-Hb a potential candidate to achieve full tumor radiosensitization. In a prospective study, we (161) explored that possibility by delivering cell-free SNO-Hb through a systemic route to tumor-bearing rats. In contrast to native oxy-Hb, which decreased tumor perfusion at least in part through vasoconstriction and reaction 3, micromolar SNO-Hb stabilized tumor blood flow. This activity appeared to be exclusively O2-dependent: intravenous SNO-Hb infusion with concomitant delivery of pure O2 or, alternatively, intraarterial SNO-Hb infusion both circumvented the lowering of tumor perfusion by native oxy-Hb. Intravenous SNO-Hb administration alone reproduced the vasoconstrictor effects of oxy-Hb, and pure O2 breathing alone did not influence tumor blood flow. The fact that blood hyperoxygenation prolonged by approximately twofold the half-life of the SNO moiety on Hb, coinciding with increased tumor perfusion, reveals that SNO-Hb bears the intrinsic potential to radiosensitize (via the O2 effect) and chemosensitize (O2 effect + increased drug delivery) tumors. Unraveling these activities would necessitate the delivery of cell-free SNO-Hb at higher doses or the direct loading of Hb with SNO in RBCs. The first strategy would benefit from the development of Hb-based blood substitutes aimed at limiting the toxicity of αβ dimers while preserving the allosteric control of SNO delivery by cell-free Hb tetramers (see before). The latter approach could be based on the delivery of NO-derived molecules that load Hb with NO activity in the lungs and exploit the physiologically controlled allosteric release by Hb of NO bioactivity in the profoundly hypoxic but functional tumor vasculature. For example, the radiosensitizing agent efaproxiral (RSR13), an allosteric modulator of Hb function believed to act by augmenting O2 offloading from RBC Hb, might also be leveraged to manipulate RBC SNO delivery and thus O2 delivery to the target tumor (92).

Concluding Remarks

Signaling through S-nitrosylation has emerged as a pervasive and conserved means by which the biological actions of NO are transduced. The elements required for a relevant and regulated intercellular
signal transduction scheme have been demonstrated, including enzymatic SNO synthesis in response to physiological and pathological triggers, targeting and activation of SNO at sites and times remote from their formation, and appropriate, precise extinction of SNO signals by enzymes specifically evolved for that purpose. Nature has thus endowed the RBC with the ability to actively regulate its own function (O2 delivery) by coupling the O2 sensor Hb to the formation and release of vasodilator SNO, which influences O2 delivery not only through regulation of blood flow to tissues but also by optimizing O2 uptake in the lung and by signaling centrally regulated responses to inadequate O2 supply. Malfunction of any regulatory element of this exquisite activity contributes to several important pathologies, motivating the development of therapeutic approaches aimed at restoring physiological interactions between NO and Hb.

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