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CHEMICAL PHYSIOLOGY OF BLOOD FLOW REGULATION BY RED BLOOD CELLS: The Role of Nitric Oxide and S-Nitrosohemoglobin

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■ **Abstract** Blood flow in the microcirculation is regulated by physiological oxygen (O_2) gradients that are coupled to vasoconstriction or vasodilation, the domain of nitric oxide (NO) bioactivity. The mechanism by which the O_2 content of blood elicits NO signaling to regulate blood flow, however, is a major unanswered question in vascular biology. While the hemoglobin in red blood cells (RBCs) would appear to be an ideal sensor, conventional wisdom about its chemistry with NO poses a problem for understanding how it could elicit vasodilation. Experiments from several laboratories have, nevertheless, very recently established that RBCs provide a novel NO vasodilator activity in which hemoglobin acts as an O_2 sensor and O_2-responsive NO signal transducer, thereby regulating both peripheral and pulmonary vascular tone. This article reviews these studies, together with biochemical studies, that illuminate the complexity and adaptive responsiveness of NO reactions with hemoglobin. Evidence for the pivotal role of S-nitroso (SNO) hemoglobin in mediating this response is discussed. Collectively, the reviewed work sets the stage for a new understanding of RBC-derived relaxing activity in auto-regulation of blood flow and O_2 delivery and of RBC dysfunction in disorders characterized by tissue O_2 deficits, such as sickle cell disease, sepsis, diabetes, and heart failure.

INTRODUCTION

Background

The original identification of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) was based in part on the ability of hemoglobin (Hb) to inactivate both substances (1, 2). Earlier work had shown that Hb can react rapidly with NO to form

nitrate from oxy-Hb or a heme-iron nitrosyl adduct with deoxy-Hb, as summarized in Reactions 1 and 2 (3, 4); neither product exhibits bioactivity characteristic of NO.

$$\text{heme Fe(II)} + \text{NO} \rightarrow \text{heme-Fe(II)NO} \quad 1.$$

$$\text{heme Fe(II)O}_2 + \text{NO} \rightarrow \text{heme-Fe(III)} + \text{NO}_3^- \quad 2.$$

In this light, the vasodilatory bioactivity of NO in blood presented conceptual problems: (*a*) Could this activity coexist with Hb, which can rapidly and efficiently scavenge NO (5–7) and (*b*) would red blood cells (RBCs), through this scavenging chemistry, act as relentless vasoconstrictors (8)?

The first question has been addressed on several complementary levels. Liao and coworkers suggested that owing to the flow of blood, RBCs tend to remain centered in the larger vessels and avoid the walls where NO is produced (9). Lancaster provided a rationale (10), later elaborated by others (11–13), for how the cellular packaging of the Hb retards its reaction with NO. More important, the broader chemistry of NO in biology was shown to include the oxidative formation of S-nitrosothiols (thionitrites), which maintain cardiovascular bioactivity in the presence of Hb, circumventing Reactions 1 and 2 (7).

Thionitrites—including both low-molecular-weight nitrosothiol (SNO) derivatives of cysteine and glutathione, and also S-nitrosylated proteins, such as S-nitrosoalbumin (6, 14)—are among the most potent vasodilatory compounds known. Molar potencies of S-nitrosocysteine and S-nitrosoglutathione in bioassays are equal to or higher than NO (15–19), especially when the comparison is made using the smaller resistance vessels that control blood flow (20). Moreover, S-nitrosothiols appear to be the most abundant compounds to exhibit NO-related bioactivity in the blood and blood vessel walls, existing at basal levels orders of magnitude greater than NO (18). SNOs are unique among the various compounds that derive from NO synthase in that their physiological role in vasoregulation has been demonstrated by strict genetic evidence (21). In particular, SNOs contribute to regulation of vascular resistance under basal conditions and its dysregulation in endotoxic shock (21).

An intriguing idea emerged from consideration of the second question: Do RBCs act as vasoconstrictors? A general principle of physiology holds that cells precisely regulate their primary function. For RBCs this primary function is delivery of oxygen (O_2) to tissues. Vasoconstriction implicated by the NO scavenging chemistry (Reactions 1, 2) would impede blood flow and oppose the primary function. In as much as O_2 delivery is determined primarily by blood flow, rather than by oxy-Hb concentration, this line of thinking implies that, far from having a vasoconstricting effect, RBCs should be capable of dilating blood vessels in the microcirculation to regulate blood flow (8). Furthermore, RBC vasodilation in the pulmonary arteries and arterioles could serve to optimize ventilation-perfusion matching, that is, blood oxygenation, and regulate pulmonary artery pressure (18, 22).

Aspects of Blood Flow Physiology

Blood flow in the microcirculation is principally regulated by physiological O_2 gradients: position-to-position variations in O_2 content, which are immediately reflected in changes in Hb O_2 saturation, are coupled to regulated vasoconstriction or vasodilation (23–27). The overall design matches O_2 delivery with metabolic demand. Thus, decreases in the O_2 content of blood lead to increases in blood flow and vice versa (24, 28). While this regulation of blood flow is exerted through local modulation of arteriolar tone, the mechanism through which graded changes in O_2 content evoke the response is a major unanswered question in biology. Hb would appear to be an ideal O_2 sensor in this regulatory process, particularly since it is the O_2 saturation of blood Hb, not pO_2, that determines blood flow (24, 28) (Figure 1*a*). In this context, the fixed ideas about Hb's scavenging NO (Reactions 1, 2) presented a conceptual roadblock for understanding how the O_2 signal, detected by Hb, could be transduced to elicit vasodilation.

SNO-Hemoglobin

The resolution of this problem began with the discovery that Hb itself is among the blood proteins that sustain S-nitrosylation. Specific cysteine residues of Hb, conserved in all mammalian and avian species, form S-nitrosothiols both in vivo and in vitro (8). S-nitrosylation of human Hb is linked in vivo to O_2 saturation (Figure 1*b*) and occurs at Cys-β93 (Figure 2). S-nitrosylated-Hb, or SNO-Hb, has further been characterized by mass spectrometry (29) and X-ray crystallography (30, 31). This work provides direct evidence that the scavenging chemistry (Reactions 1, 2) and concomitant loss of NO bioactivity can be avoided to furnish this previously unsuspected product of Hb interactions with NO reagents: SNO-Hb formation is competitive with and/or circumvents the Fe(II)-NO and nitrate-forming reactions in vivo (18, 22, 32–36) (Figure 1*b*). It has further been demonstrated that the reactivity of these cysteines toward NO reagents is dependent on the quaternary structure of the tetramer (8, 37). SNO-Hb forms preferentially in the oxygenated (or R) structure, whereas conditions favoring T structure, such as low pO_2, favor release of NO groups (8) (Figure 2). The circulating levels of SNO-Hb are thus partly dependent on the O_2 saturation-governing equilibrium between T and R structures, and not on the pO_2 (Figure 1*b*). Crystal structures (30) and molecular models (30, 37) of SNO-Hb provide a rational, "stereochemical" (38, 39) basis for allosterically regulated dispensing of NO bioactivity; thus, whereas β-cys thiol has no access to solvent in R state (and therefore could not dilate blood vessels), it protrudes into solvent in the deoxygenated (or T) structure (Figure 2). Energy-minimization modeling based on the SNO-Hb crystal structure (30) suggests that the entire SNO moiety is folded back into the protein with no solvent access (30) in R state.

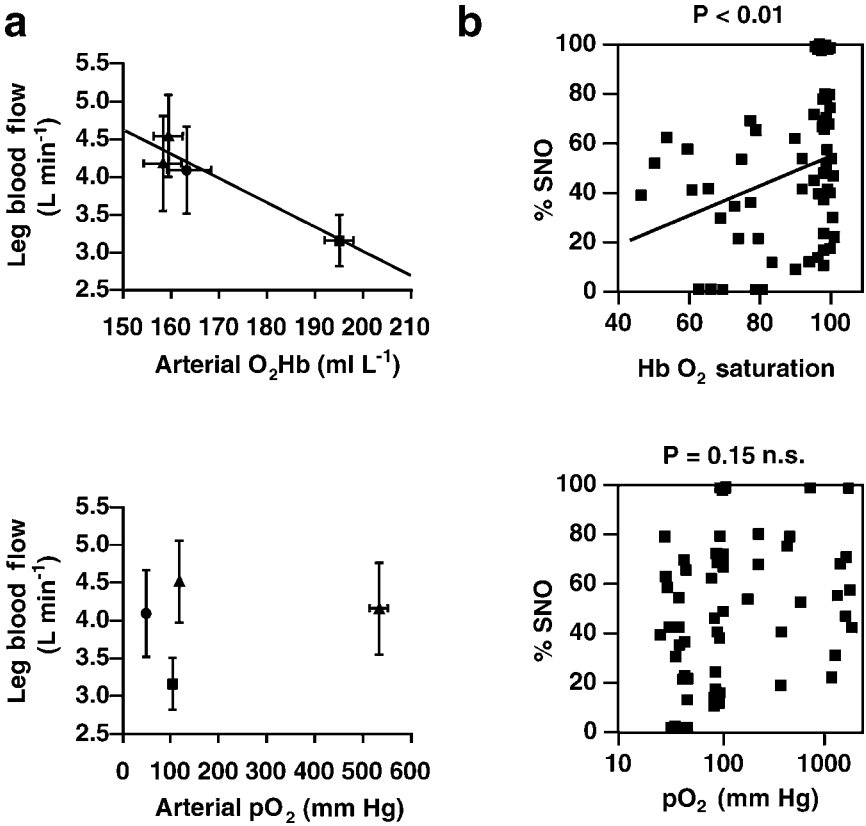

Figure 1 Blood flow and SNO content of Hb are linked to Hb O_2 saturation in humans. Response of limb blood flow (*a*) and Hb S-nitrosylation (*b*) across Hb O_2 saturation (*top*) or pO_2 (*bottom*). Thus blood flow responds to O_2 saturation of Hb and is uncorrelated with pO_2. SNO content of Hb shows a similar behavior, consistent with its role in hypoxic vasodilation [note that many data points overlap at the 100% SNO, 100% sat locus in (*b*)]. In addition to O_2 saturation, other factors including pH, pCO_2, and redox state may influence the O_2-dependent processing of NO by Hb, as discussed below. Panel (*a*) is taken from (24), and (*b*) is from (22), with minor modifications. SNO is presented as a fraction of total NO bound to Hb (%SNO).

Red Blood Cells

NO BIOACTIVITY In addition to the major pool of cytosolic Hb, which serves in the bulk transport of O_2, a second pool of Hb is localized to the plasma membrane through interaction with the N-terminal cytoplasmic tail of the band 3 protein (anion exchanger 1:AE1). Nitric oxide and related congeners that enter the RBC

first encounter the membrane Hb (40): Not only is a major fraction of SNO-Hb directly associated with the membrane, but transfer of NO from β-cysNO of Hb to cysteine thiols of band 3 protein at the RBC membrane was also shown to be necessary and sufficient for robust vasodilation by RBCs under relevant physiological conditions. Moreover, RBCs were shown to actuate a unique, rapid, and graded vasodilator or vasoconstrictor response across a physiological range of pO_2 (22). The primary data that illuminate this response are presented in Figure 3*a–d* and the mechanistic details are shown in Figure 3*e*. Note that there is some evidence that GSNO may serve as an intermediate in the inport or export from RBCs of NO bioactivity (Figure 3*e*) (8, 18, 21). The biological activity of SNO-Hb (40, 41) and RBCs (22) is thus seen to exhibit the requisite dependence on Hb O_2 saturation, apparently through the allosteric behavior of Hb. RBCs were shown not only to dilate blood vessels, but to do so in a manner that recapitulated the autoregulation of vessel tone by the physiological O_2 gradient.

Although hypoxic vasodilation by RBCs can be partly blocked by inhibitors of guanylate cyclase (33), cGMP-independent effects have also been reported (42). Vasodilatory effects of RBCs are observed in endothelium-denuded vessels (G. Ahearn, J.R. Pawloski, T.J. McMahon & J.S. Stamler, unpublished results) and are potentiated by the pretreatment of RBCs with NO (33, 40) or SNO (8), consistent with the observation that hypoxic vasodilation in vivo can be entirely endothelium independent (44).

TISSUES, LUNG, AND BRAIN Whereas arteriolar blood flow in peripheral tissues subserves O_2 delivery (Figure 1*a*), in the lung it is regulated to optimize O_2 uptake. For example, alveolar hypoxia results in constriction of blood vessels that perfuse alveolar units to preserve V/Q matching. NO counteracts this hypoxic pulmonary vasoconstriction (45), thereby mitigating excessive increases in pulmonary artery pressure and creation of alveolar dead space (46). RBCs entering the lungs contain significant amounts of SNO-Hb (see below), and emerging evidence indicates that dispensing this vasodilator activity may contribute to NO homeostasis; RBC-derived NO bioactivity may thus serve in V/Q matching and maintainance of basal pulmonary arterial tone (Figure 4*a*). Other studies, by contrast, suggest that by sequestering endothelial NO, RBCs enable hypoxic pulmonary vasoconstriction (47). The extent to which this effect of (infused) RBCs may be an artefact of reduced endogenous SNO-Hb levels is undetermined (Figure 4*a*). As discussed below, RBCs rapidly lose SNO ex vivo, and RBCs depleted of SNO may accentuate pulmonary hypertension and impair oxygenation. Also of note, RBC-derived SNO can stimulate centers in the brain that control the hypoxic drive to breathe (48), and vasodilation by RBCs within these highly vascularized centers may play a regulatory role (Figure 4*b*). Thus, although a respiratory cycle for NO is not yet fully understood (49), RBCs may affect essential control mechanisms, not only in peripheral tissue but also in the lungs and brain.

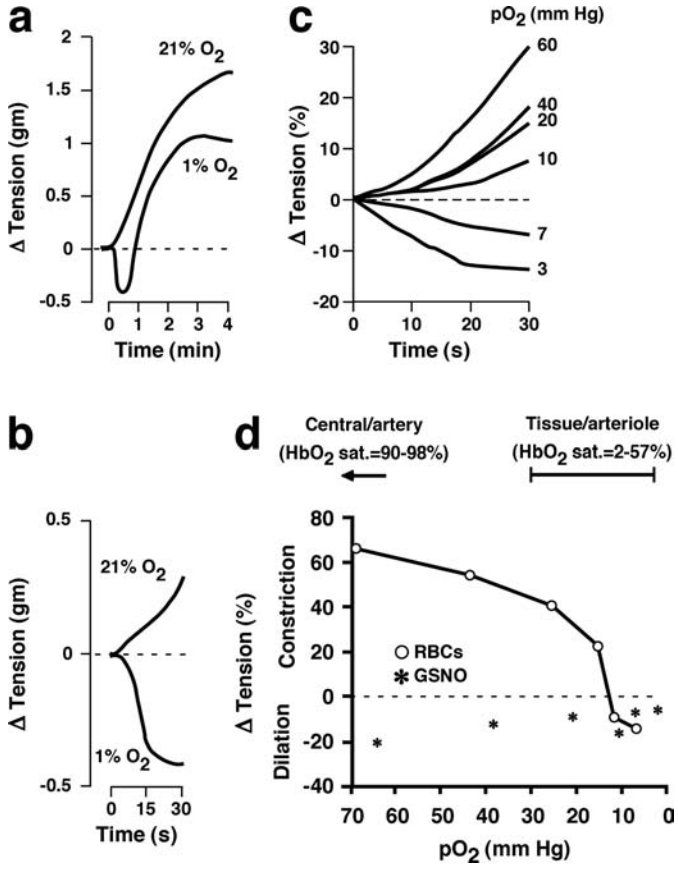

Figure 3 Graded oxygen-dependent vasodilation and vasoconstriction by RBCs, and erythrocytic NO trafficking. (*a*) In organ chamber bioassays, RBCs dilate blood vessels at low pO_2 (1% O_2), which is characteristic of tissues, but are vasoconstrictive in room air. The hypoxic vasodilator response is followed by vasoconstriction in vitro (representing scavenging of endothelial NO), which starts at approximately 1 min following addition of RBCs and therefore has no physiological relevance. (*b*) Responses to RBCs occur over seconds, commensurate with arterial-venous transit times. (*c*, *d*) The effects of oxygen tension on the activity of RBCs compared with that of the simple endogenous vasodilator S-nitrosoglutathione (GSNO) (*d*). Aortic rings were pre-equilibrated at 2 g and the indicated oxygen pressures. Oxygenated human RBCs were then added at low hematocrit. GSNO (3 nM) evokes a dilatory response independent of oxygen tension. In contrast, RBCs elicit a graded vasodilator response beginning at pO_2 of approximately 60 torr and across the physiological range of hemoglobin O_2 saturations. One should not deduce from panels *c* and *d* that RBCs constrict blood vessels at pO_2 greater than 10 torr, but rather that vasodilatory activity is seen below pO_2 of 60 torr; the data in aortic tissue bioassays cannot be extrapolated to the microcirculation in vivo.

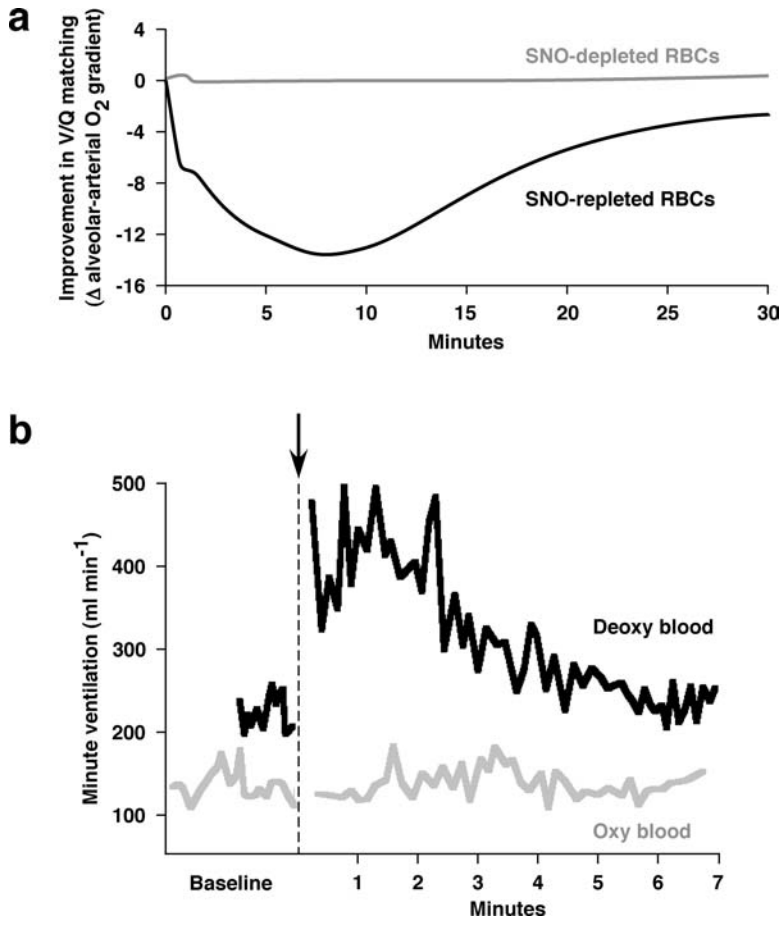

Figure 4 Red blood cell-derived NO bioactivity subserves blood oxygenation in the lungs and mediates central nervous system control of ventilation. (*a*) Infusion of RBCs (50 cc/< 30 s, 30% hematocrit) into the pulmonary artery of an anesthetized pig had little effect if SNO content had been reduced (∼ 20% of basal SNO-Hb) by prior deoxygenation and storage, but produced a rapid improvement of ventilation (V)/perfusion (Q) matching (decrease in the alveolar-arterial oxygen gradient) if the SNO content was reconstituted ex vivo (to within twofold of endogenous SNO-Hb content) by exposure to NO. (*b*) Following pretreatment with glutathione, the low-mass fraction from oxygenated (left heart) or deoxygenated (right heart) blood was microinjected into the brainstem nucleus tractus solitarius of conscious rats (*arrow*). Deoxygenation (but not oxygenation) generated low-mass S-nitrosothiol (S-nitrosoglutathione; identified by mass spectrometry), which rapidly and potently stimulated the respiratory drive, as revealed by increased minute ventilation. Figure taken from Lipton et al. (48).

TABLE 1 Characteristics of physiological flow responses

	SNO-Hb	ATP	EDRF-NO
Dependence			
RBC	✓	✓	—
Endothelium	—	✓	✓
Shear	—	—	✓
O_2	✓	✓	—
NO bioactivity	✓	✓	✓

Oxygen concentration also influences vessel tone through a direct effect on the vessel wall; the mechanism is dependent on the duration of change in the O_2 concentration, and is largely independent of EDRF-NO.

Other Vasodilatory Responses

Hb-mediated vasoregulation must be distinguished from related effects, involving ATP and EDRF, with which it might be confused. The distinguishing features are summarized in Table 1. Recent studies support the view that RBCs also release the endothelium-dependent vasodilator, ATP, to regulate blood flow (50, 51). With sustained changes in O_2 saturation, blood levels of ATP rise or fall within minutes. This type of sustained control is to be contrasted with the Hb-regulated (endothelial NO-independent) response in which vasoregulation can be effected over seconds, commensurate with arterial-venous transit times. Thus Hb and ATP may serve complementary roles, respectively, in acute local and prolonged systemic hypoxia. At even longer timescales, transcription-mediated processes, among others, influence blood flow.

The vasodilator effect of RBCs also needs to be distinguished from that of EDRF. Indeed, inherent to this proposition of Hb-mediated vasodilation by RBCs is the idea that endothelial cells and RBCs play complementary roles in the regulation of blood flow. It had been recently argued that EDRF would overwhelm any vasodilation mediated by RBCs, thus eliminating a role for RBCs in vasodilation (34, 52). This contention, which has engendered much controversy, was based on a fundamental misconception of the relevant physiology. EDRF mediates shear- and hormonally induced vasodilation but has no significant role in hypoxic exercise-induced vasodilation (24). Conversely, RBCs dilate in response to low pO_2 (22, 24, 50) but have no direct role in shear-mediated vasodilation.

The proponents of this latter hypothesis have recently reversed their position. They now concur both that Hb/RBCs, through an Hb-allostery regulated, NO-dependent process mediate hypoxic vasodilation (53), and that SNO-Hb can mediate RBC vasodilation (54). Still in dispute are details of the molecular mechanism

by which Hb carrries out this function. Accordingly, with this nascent consensus on the basics of RBC regulation of blood flow, it is an especially attractive time to review the progress in this field, to consolidate core ideas, to identify areas that have been and remain in dispute, and to examine critically the experimental results that underlie these disputes, in order to set the stage for a new understanding of the role and function of RBC-derived relaxing factor activity, and of diseases of RBC vasodilator-dysfunction.

BASICS OF SNO-HEMOGLOBIN-MEDIATED RBC-INDUCED VASODILATION

The core elements of Hb's mediation of RBC-induced vasodilation are (*a*) the sensing of oxygen levels by Hb (influenced by allosteric effectors and iron oxidation/spin state), (*b*) the intermediacy of SNO-Hb in RBC vasodilator activity, and (*c*) the release of NO bioactivity in response to reduced oxygen tension (and/or to changes in allosteric effectors, iron oxidation, and spin state). In this paradigm, SNO-Hb is identified as the active species through which oxygen (and oxidation/spin state)-responsive NO-group transfer occurs (7, 8, 41, 42). Thus, this model requires that SNO-Hb can be formed and turned over in amounts sufficient for regulated dilation of constricted vessels. Given the great potency of SNO-Hb (vasodilatory response detected in vitro at <10 nM) (see below) and the typical values reported for its concentration in blood (generally >10 nM, and typically >0.3 μM), Hb clearly dispenses NO in limited quantities—in contrast to its high-throughput delivery of oxygen. It is, moreover, reasonable that Hb interacts with NO in a fashion that tends to avoid the dead ends of nitrate and the putatively unrecoverable heme-Fe(II)NO. The only real requirement in this context, however, is that the NO budget is balanced: the rate of NO loss cannot exceed the daily NO production, which, from NOS, amounts to $\sim$1.0 mmol/day in a human adult (55). The second requirement is the transduction of the ambient oxygen signal to release NO-bioactivity through reactions of SNO-Hb. We have proposed that this process is connected to Hb allostery—the changes in quaternary structure of Hb associated with changes in oxygen saturation, oxidation, spin state, etc. (56).

These requirements, and their correlates, suggest an intriguing principle of this biochemistry. Hb serves as a sensor and reactor that adaptively modifies the chemistry of its interaction with NO to regulate NO bioactivity, blood flow, and ultimately oxygen delivery. This adaptive chemical response presumably includes dispensing of NO-bioactivity in hypoxic vasodilation, capture of NO in hyperoxic vasoconstriction, and, potentially, trapping and/or elimination of NO under conditions of NO overproduction that characterize, for example, septic shock (57, 58). Moreover, at the very high NO levels used in the early in vitro studies, Hb chemistry must faithfully reflect the predominant production of nitrate and heme-Fe(II)-NO. Hb's NO chemistry is complex.

TABLE 2 Salient features of NO binding to Hb

1. Bi-tropic effector (homotropic and heterotropic interactions)
2. Binding of oxidized and reduced hemes
3. Reactions dependent on both NO concentration and NO-to-Hb ratio (physiological: $\ll$1 micromolar, NO/Hb $<$1:250)
4. Promotion of either T or R structure
5. Pronounced subunit selectivity in reactions (high NO, α; low NO, β)
6. Reactions coupled to heme/thiol redox

COMPLEXITY

In the familiar example of Hb's oxygen-binding function, the presence of interacting subunits gives rise to a distinctive, sigmoidal binding isotherm that is readily distinguished from the simple behavior shown by monomeric myoglobins (and by dimeric Hbs that form at low concentrations). Representative oxygen-binding isotherms are illustrated in Figure 5. This behavior reflects a suppression of the affinity of the tetramer for the first oxygen molecule bound, as compared with the relatively high affinity for the fourth oxygen bound, and it likewise implies a strong tendency toward all-or-nothing binding (zero or four oxygen ligands), and thus a substantial, but not complete, suppression of species with intermediate numbers of ligands. The oxygen-binding function is, moreover, modulated by so-called allosteric effectors, other ligands including protons and certain anions whose binding is thermodynamically "linked" (59) to and affects oxygen binding.

These characteristics provide some lessons on the interactions of NO with Hb. Molecular properties are adaptive: They are coordinated functions of the concentrations and/or saturation of oxygen and the various allosteric effectors, with implicit coupling of the adaptive responses. This latter characteristic, well-evidenced in Hb's oxygen binding (56), suggests how it conducts its adaptive NO chemistries.

NO introduces additional complexity that requires elaboration of the paradigms used in describing the oxygen-binding function. NO attaches both at the heme site and the β-93cys. It is thus both a homotropic and a heterotropic allosteric effector; it is a bi-tropic effector. In addition to binding at the heme iron, in place of oxygen, and coupling to thiol, it reacts to form higher oxides. In its chemistry with Hb, oxidation-reduction plays a central role. It coordinates to both oxidized and reduced heme irons. In further contrast to most heme ligands, NO expresses substantial subunit non-equivalence in its reactions, which are themselves NO-concentation dependent. These distinctive characteristics of NO important to its interactions with Hb are summarized in Table 2.

The products of interactions of Hb with NO, and with related NO-reagents, is dependent not only on the saturation of NO and of O_2 (and concentrations of allosteric effectors) but also on their subunit disposition and the oxidation

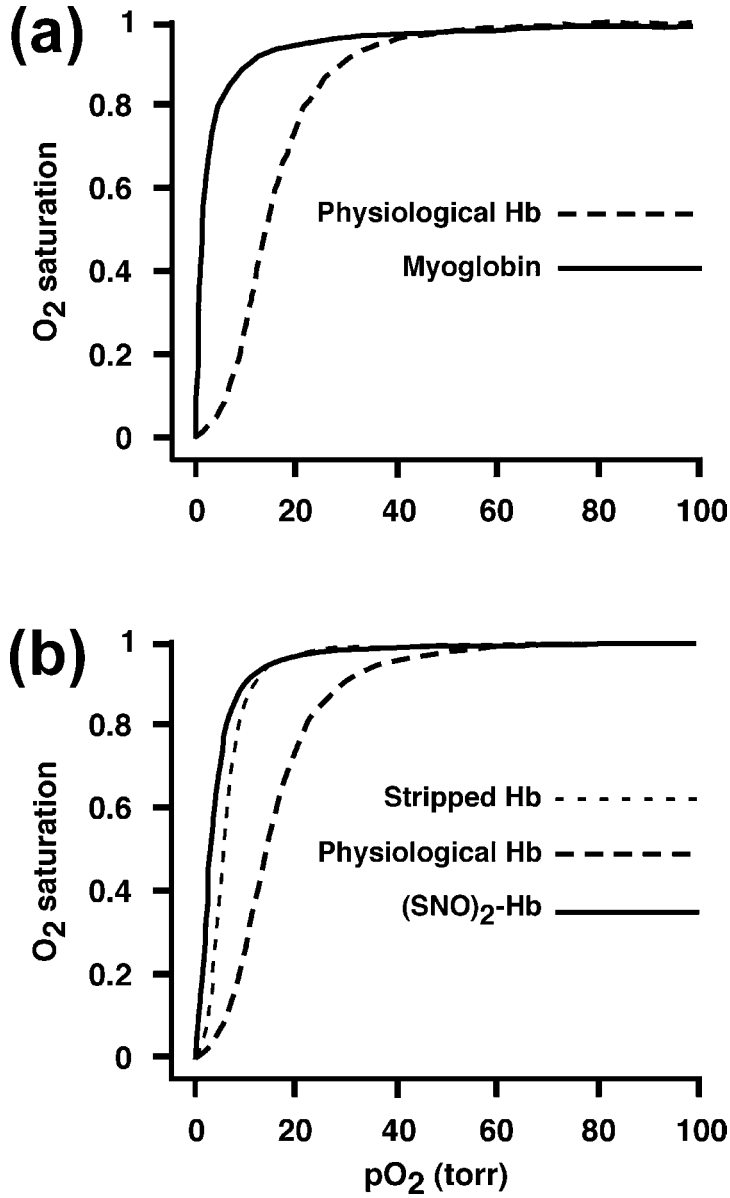

Figure 5 Representative O_2-saturation curves. (*a*) Comparison of monomeric myoglobin and human Hb [pH 7.4 in 100 mM NaCl with 2 mM diphosphoglycerate (DPG)] O_2-saturation curves (56). (*b*) Comparison of different human Hb preparations, with physiological Hb as in Figure 3*a*, stripped Hb without DPG in 7 mM NaCl pH 7.4 (56), and SNO-Hb pH 7.4 phosphate buffered saline (41).

state of the accompanying hemes. The landscape of these micropopulations remains to be fully understood, but their differential reactivities are clearly evident. From this perspective, Hb senses ambient levels of oxygen tension, pH, anion levels, etc. It processes this information through structural alterations of the protein that modulate reactive behavior and thus, overall, adaptively modulates NO chemistry (input/output) to yield products that provide the optimal response to the ambient conditions. Salient aspects of these responses are indicated in Figure 6.

In the physiological situation, NO-containing Hb molecules are themselves a micropopulation (1 in 1000–10,000 Hbs possesses an NO), that is, the NO saturations do not vary up to nearly complete saturation, as with oxygen, but stay at levels typically less than 1%. In in vitro studies on the physiological interactions of NO and Hb, the importance of adhering to physiological amounts and proportions of the reagents has been underestimated; changes in these proportions can give rise to stark, nonlinear changes in the distribution of NO micropopulations and reaction products. The disposition and reactivity of NO bound to Hb is a function of many variables, including pH, pCO_2, pO_2, amount of NO, and the ratio of their concentrations to heme (55, 57, 60–65). Overall, complexity emerges from the tetrameric nature of the protein, which provides intersubunit couplings; the heterogeneity of the chemical formulation of the Hb adducts (micropopulations) and their concomitant heterogeneity in reactivity; the allosteric behavior of Hb and concomitant effects on reactivity; and the intricate branched network of coupled kinetic equations underlying this rich chemistry.

CONTROVERSIES

Much of the controversy regarding the role of Hb in RBC-induced vasodilation is traceable to a disregard of the complexity inherent to this system and of the biologically relevant conditions. Conditions used and results obtained in various pertinent studies are summarized in Table 3. Experiments with Hb are typically performed under nonphysiological conditions, e.g., at high pO_2 (typical bioassay is 95% O_2; 700 mm Hg) and low Hb (typical concentration in bioassay is 100 nM–1 μM Hb), whereas tissue pO_2 is much lower (~0.5–3% O_2; 4–20 mm Hg) (25, 66–68) and Hb concentration is much higher (millimolar). NO concentrations in recently reported experiments reach unphysiological levels of many tens to hundreds of micromolar (69–73) (whereas NO is nanomolar in vivo; Table 2). Studies with RBCs have involved lengthy exposures to hundreds of micromolar to high millimolar NO or S-nitrosocysteine (54, 70, 74–77)—conditions that not only obfuscate the relevant chemistry by raising the intracellular iron nitrosyl Hb and SNO concentrations to hundreds of micromolar, but also result in indiscriminate oxidation and nitrosylation of cellular constituents. Such experiments have no relevance to and do not illuminate the physiological situation.

TABLE 3

Author (Ref.)	Amount NO/Hb	NO:Hb ratio	Method	Mode of mixing	Yield SNO-Hb	Absolute amount SNO-Hb
			Reaction of NO with oxy Hb			
Gow[a] (55)	0.2 μM ($\times$ 6)/48 μM	1:240	Photolysis	Slow addition	~33%	~0.4 μM
	0.1–0.2 μM ($\times$ 8)/ 25 μM (+ SOD)	1:125	Photolysis/UV	Slow addition	~50%	0.75 μM[d]
Joshi (94)	5 μM/125 μM	1:25	Chem/chemilum[c]	Rapid bolus	~1%	~0.05 μM
	5 μM MAHNO*/ 125 μM	1:25	Chem/chemilum	Continuous slow release	~1%	~0.05 μM
Herold[a,b] (65)	5 μM/12.5 μM	1:2.5	Saville	Slow addition	~20%	1 μM
	25 μM/12.5 μM	2:1	Saville	Slow addition	~6%	1.5 μM
	50 μM/12.5 μM	4:1	Saville	Slow addition	~3.6%	1.8 μM
	5 μM/25 μM (oxy Hb/met Hb)	1:5	Saville	Slow addition	19%	~1 μM
	50 μM/25 μM (oxy Hb/met Hb)	2:1	Saville	Slow addition	8.6%	4.3 μM
Palmerini (1, 140)	1.5 μM/150 μM	1:100	Electrode	Rapid bolus	High	?
Han (70)	100 μM NO/ 1.5–5 mM	1:15–1:50	Chem/chemilum	Rapid bolus	1%	0.5–1 μM
	65 μM DEANO (~100 μM)/5 mM	~1:15	Chem/chemilum	Continuous slow release		0[e]
Han (72)	50 μM / 0.1–1 mM	1:2–1:20	Chem/chemilum	Rapid bolus	~1%	~0.5 μM

(*Continued*)

TABLE 3 (*Continued*)

Author (Ref.)	Amount NO/Hb	NO:Hb ratio	Method	Mode of mixing	Yield SNO-Hb	Absolute amount SNO-Hb
Reaction of NO with deoxy Hb followed by oxygenation						
Gow (64)	2 μM–200 μM/ 200 μM	1:100–1:1	Photolysis Saville	Slow addition Slow addition	~75 → 0%	~1–3 μM (fixed)
Fago (71)	44 μM/175 μM	1:4	?	?	~0–20%	up to 8.8 μM
Herold[a,b] (65)	5 μM/12.5 μM 50 μM/12.5 μM	1:2.5 4:1	Saville Saville	Slow addition Slow addition	16% 1.6%	0.8 μM 1.45 μM
Chen (142)	16 μM/100 μM	1:6	EPR	?	No measurement	No measurement
Reaction of NO with met Hb						
Herold (65)	5 μM/12.5 μM 50 μM/12.5 μM	1:2.5 4:1	Saville Saville	Slow addition Slow addition	42% 13%	2 μM 6.5 μM
Luchsinger (63)	90–660 μM/ 250–450 μM	5:12.5	Saville	—	12–60%	16–210 μM
Palmerini (1, 140)	1.5 μM/150 μM	1:10	Electrode	Continuous	"High"	?

[a]Yields were higher in low phosphate than in high phosphate.
[b]Yields were lower with rapid NO additions than when NO was slowly added.
[c]chem/chemilum = chemical/chemiluminescence.
[d]Numbers were derived from direct nitrosyl Hb measurement and yield of iron nitrosyl Hb by UV-Vis.
[e]Authors subsequently reported low sensitivity of assay. SOD, superoxide dismutase.

NO BIOACTIVE COMPOUNDS IN VIVO: LEVELS AND ANALYSIS

The distribution of various products obtained from the reaction of NO and related compounds with Hb under physiological conditions, and the amounts of these products (including NO, nitrite, iron nitrosyl, and S-nitrosothiol) in the circulation remain subject to debate. These issues are addressed through both in vitro biochemical studies and analyses of reaction products formed in vivo.

Basal Levels in Vivo

On the central point of nitrosothiol and nitrosyl iron formation and levels, convergence to accurate, reproducible values has been impeded by a fundamental problem. While chemical, electrochemical, and spectroscopic methods have proven adequate for analysis of in vitro chemistry conducted at comfortable analyte concentrations, the low NO:Hb ratios in the physiological situation and the complex chemistry of Hb have made quantitative analysis of in vivo samples very challenging. Among the different assay methods, there are no common sets of practices or rigorous standards that ensure accurate, reproducible measurements of the various protein nitrosyl species and avoid artifacts induced by harsh chemical processing. Recent detailed discussion (35) of these difficulties has led to specific recommendations for improved standards (78) and requirements (78, 143) to help close the gap in quantitative results obtained with existing analytical methods.

The current picture, however, is not altogether bleak. If we think in orders of magnitude, there is a broad consensus for finding protein-nitrosyl levels, in the blood of various mammals examined, in the 10^{-5} to 10^{-7} M range. Kirima et al. (79) reported 1–10 μM Hb-NO derived from L-arginine in the blood of rats at basal conditions. Similar results were reported in one mass spectroscopic study on human blood (80). Other recent electron paramagnetic resonance (EPR) measurements in sheep (81), pigs (82), and humans (83, 84) show similar levels (0.3–3 μM). These EPR measurements are also consistent with our own measurements in rats and humans (0.3–3 μM) in which two different techniques—photolysis-chemiluminesence and a modified Saville assay (8, 22, 37)—have been employed. They are also in keeping with the measurements of James using an electrochemical approach ($\sim$5 μM) (32); of Funai et al. using a modified Saville assay ($\sim$3 μM) (36); of A. Doctor & B. Gaston (personal communication) using a novel copper/cysteine based methodology ($\sim$5 μM); and of Nagababu et al. (85), who detected a pool of reactive species assigned as HbFe(III)NO ($\sim$0.5 μM) using a modified chemiluminescence assay and significantly higher amounts (approximately many micromolar) using an EPR-based technique. Collectively, these results establish the existence of nitrosylated Hb in vivo at levels over 10^2 greater than is required for efficacy in vessel relaxation.

Outside of this range are results of Feelisch and coworkers who measured 70 nM nitrosyl Hb in rat blood (10–100-fold lower than measured by EPR) and,

remarkably, zero in human blood (86). These measurements, by the method of Gladwin et al. (74, 87, 88), are thus incompatible with the direct EPR and mass spectroscopic measurements and the photolysis-chemiluminescence and modified Saville and copper/cysteine and electrochemical and chemiluminescence determinations, which agree with the EPR measurements. According to Rifkind (85), Gladwin and colleagues method cannot detect the majority of Hb-NO in human blood, as Hb-NO is readily lost during processing. In our hands, the Gladwin/Feelisch method is highly sensitive to sample aging, processing, and redox status and thus predisposed to imprecision in quantifying and properly discriminating heme-NO and SNO-Hb. Feelisch and coworkers (89) have reported the lack of recovery of certain test samples in their assay that would lead to severe underestimation of levels in actual samples; they ascribe unique chemistry to heme-NO versus nonheme iron-nitrosyl standards that has no precedent. The method of Gladwin et al. (89a) has also markedly underestimated the levels of plasma SNO-albumin compared with that of mass spectrometry measurements (89b,c), as well as other methods (78). The discrepancy between multiple methods, in particular the direct EPR and mass spectrometry assays, and the method of Gladwin et al. calls for great caution in its application.

EPR measurement of paramagnetic heme-NO species is superior to chemical assays, which typically involve various preparative steps, but the EPR method also has its limitations. First, it is limited to the paramagnetic species, and is thus blind to diamagnetic species including SNO-Hb, nitrite, or low-spin heme-Fe(III)NO species (85, 90). Interconversion of paramagnetic and diamagnetic species during sample preparation can give a misleading picture of in vivo levels. Although the detection limit of EPR may be, with typical instrumentation, as low as $\sim$0.5 μM for the α-subunit 5-coordinate heme Fe(II) NO species that predominates with supraphysiological amounts of NO, the sensitivity is worse for the other species that should predominate under more physiological conditions. The 5-coordinate α-subunit heme-Fe(II)NO Hb species, with its sharp hyperfine structure, is most readily distinguished, whereas it would be comparatively difficult to quantify the spectrum of 6-coordinate β-subunit heme-Fe(II)NO Hb species, which has essentially no resolved hyperfine structure, and no field-domain where it alone would contribute to a composite spectrum in a mixture of species. In addition, EPR (and chemiluminescence), because of multiple correction factors introduced into the measurement, has an absolute accuracy probably no better than $\pm$0.5–1 μM (bridging the range of reported physiological levels, 0.3–3 μM in vivo). The claims of sensitivity of assays to 1 nM and reliance on EPR to establish basal NO-Hb levels are thus open to question (for reviews, see 74, 87, 88).

Altered Levels in Disease States

A final, important line of evidence in this context emanates from studies of the correlation of nitrosylated Hb levels in human subjects in health and in diseased states. Systematic alterations in SNO-Hb and Hb(Fe-NO) levels are reported upon

exposure to varied atmospheric oxygen levels (22, 36, 37), and in association with diabetes (84), heart failure (32), pulmonary hypertension (18), sickle cell disease (J.R. Pawloski, D.T. Hess & J.S. Stamler, unpublished results), and septic shock (21). Examination of these relative behaviors provides a means, albeit crude, to mitigate the limitations of assays in determining absolute levels. These studies also raise intriguing ideas concerning the significance of protein-NO function in health and disease.

INTERACTIONS OF NITRIC OXIDE WITH HEMOGLOBIN IN VITRO

SNO-Hb Formation

We have reported the formation of SNO-Hb upon exposure of Hb to thionitrites (8, 37), NO (55, 64), and nitrite (22, 63) in reactions that often involve the intermediacy of iron-nitrosyl Hb (55, 63, 64). SNO-Hb formation through use of both eNOS and iNOS has also been reported (8, 91). As discussed below, these reactions have been confirmed in other laboratories. Details of mechanisms and yields and their dependence on ambient conditions continue to be debated. To date, only James and colleagues have attempted to reproduce our physiological conditions by reaching physiological (submicromolar) levels of NO compounds, and by studying intact RBCs (32, 33).

The first of these reactions presumably involves a simple transnitrosylation process:

$$\mathrm{RSNO} + \mathrm{Hb}[\beta 93\text{-cys}] \rightarrow \mathrm{RSH} + \mathrm{Hb}[\beta 93\text{-cys-NO}] \qquad 3.$$

This reaction, where RSNO is S-nitrosocysteine (8), serves as a standard method for preparing SNO-Hb in vitro [although the $(\mathrm{SNO})_2$-$\mathrm{Hb}[\mathrm{Fe(II)O_2}]_4$ molecule produced in this manner is less reactive than is the predominant form of SNO-Hb found in RBCs (22)]. More complicated mechanisms in which RSNO first generates either NO or a heme iron nitrosyl species are not rigorously excluded in this chemistry. Indeed, formation of SNO-Hb is typically accompanied by production of small amounts of met-Hb, and an NO-based mechanism of SNO-Hb formation has been described (92) for the bulky thionitrite GSNO through the intermediate release of NO:

$$\mathrm{SOD[Cu(I)]} + \mathrm{H^+} + \mathrm{GSNO} \rightarrow \mathrm{GSH} + \mathrm{SOD[Cu(II)]} + \mathrm{NO^\bullet} \qquad 4a.$$

$$\mathrm{Hb}[\beta 93\text{-cys}] + \mathrm{NO^\bullet} \rightarrow \mathrm{Hb}[\beta 93\text{-cysNO}^{\bullet -}] + \mathrm{H^+} \qquad 4b.$$

$$\mathrm{Hb}[\beta 93\text{-cysNO}^\bullet]^- + \mathrm{SOD[Cu(II)]} \rightarrow \mathrm{Hb}[\beta 93\text{-cysNO}] + \mathrm{SOD[Cu(I)]}. \qquad 4c.$$

The cys-NO radical suggested in Equation 4b may involve protonated tautomeric forms (K. Houk, personal communication) and may be related to the free

radical observed by McMahon et al. (22). In our studies, superoxide dimutase (SOD) (55) increased amounts of nitrosylated Hb. An alternative reaction scheme may be possible. Thus

$$\mathrm{Hb[Fe(II)O_2] \leftrightarrow Hb[Fe(III)O_2^-] \leftrightarrow Hb[Fe(III)] + O_2^-} \quad 4d.$$

$$\mathrm{SOD(Cu(II)) + O_2^- \rightarrow SOD[Cu(I)] + O_2} \quad 4e.$$

$$\mathrm{SOD[Cu(I)] + H^+ + GSNO \rightarrow GSH + SOD[Cu(II)] + NO^\bullet} \quad 4f.$$

$$\mathrm{Hb[Fe(III)] + NO^\bullet \rightarrow Hb[Fe(II)\beta 93\text{-}cysNO] + H^+} \quad 4g.$$

$$\mathrm{Hb[Fe(II)]\beta + NO^\bullet \rightarrow Hb[Fe(II)]NO.} \quad 4h.$$

Equation 4g involves heme-iron/NO redox coupling and is discussed further below. These reactions lead to SNO-Hb for R-state Hbs such as oxy-Hb, carbonmonoxy-Hb, nitrosyl-Hb, or met-Hb, but not for T-state Hbs such as deoxy-Hb (8, 30, 31; B.P. Luchsinger & D.J. Singel, unpublished results).

The formation of SNO proteins upon exposure to NO has been recognized since 1992 (6, 7). Electron loss by NO to support the overall chemistry is evidently facile, with numerous electron acceptors (A in Equation 5) (18). In the case of Hb, this chemistry has been reported by numerous laboratories (33, 55, 63, 65, 70–72, 94) with various organic electron acceptors among other electron sinks (95).

$$\mathrm{Hb[\beta 93\text{-}cys] + NO^\bullet + A \rightarrow Hb\,[\beta 93\text{-}cysNO] + A^- + H^+.} \quad 5a.$$

The possible role of O_2 (Equation 5b) or ferriheme (Figure 6) as an acceptor (64, 65) is particularly noteworthy.

$$\mathrm{Hb[Fe(II)NO\beta 93\text{-}cys] + O_2 \rightarrow Hb\,[\beta 93\text{-}cysNO] + O_2^- + H^+} \quad 5b.$$

S-nitrosylation has also been carried out in Hb crystals by exposure to NO; the electron acceptor was not identified (30).

We recently detailed the competence of the heme-iron of Hb itself as a redox partner in several different reaction scenarios that couple Fe(III)/Fe(II) reduction to formal NO oxidation (63).

$$\mathrm{Hb[Fe(III)\beta 93\text{-}cys] + 2NO \rightarrow Hb[Fe(II)NO\beta 93\text{-}cysNO] + H^+} \quad 6.$$

$$\mathrm{Hb[Fe(II)NO\beta 93\text{-}cys] + A \rightarrow Hb[Fe(III)NO\beta 93\text{-}cys] + A^-} \quad 7a.$$

$$\mathrm{Hb[Fe(III)NO\beta 93\text{-}cys] \rightarrow Hb[Fe(II)\beta 93\text{-}cysNO] + H^+} \quad 7b.$$

$$\mathrm{Hb[Fe(II)\beta 93\text{-}cys] + NO_2^- + O_2 \rightarrow Hb[Fe(II)O_2\beta 93\text{-}cysNO] + OH^-.} \quad 8.$$

As with the transnitrosylation reaction (Equation 1), redox-coupled S-nitrosylation of Hb is favored in the R quaternary state. The detailed sequence of bond-breaking, bond-making, and electron transfer in this overall S-nitrosylation

chemistry remans to be elucidated. Ford and co-workers, for example, have highlighted the possible intermediacy of N_2O_3, formed alternatively from the reaction of nitrite with Fe(III)NO or the reaction of NO with NO_2, the latter formed through a Fe(III)/nitrite redox couple (95a,b).

The great surprise in this chemistry is that it occurs at all. It continues to be argued that the rapid reactions to form nitrate and heme-Fe(II)NO species (Equations 1, 2) observed in studies at high NO:heme ratios should also predominate in vivo, even though the relevant reactant concentrations are vastly different. If such reactions were indeed to predominate, more NO would be consumed by Hb than is produced by NOS (55). This continuing controversy is reinforced by the ease in obtaining results similar to those obtained in the early studies, if reaction conditions are maladjusted, mass balance is neglected (i.e., not all products are accounted for), behavioral trends are not tested against simple models to illuminate complex behavior, and relevant in vivo studies and conditions are neglected.

The Oxy-Hemoglobin Reaction

In our studies of reactions of NO with Hb (55), we developed a model that delineated the trend, with oxygen saturation, of the product distribution expected under the assumption of simplicity: that the hemes react independently and exclusively to form met-Hb and nitrate from oxy-Hb, and heme Fe(II)-NO from deoxy-Hb (Reactions 1, 2). Under some conditions, this simple model was adhered to precisely, but in others, a marked deviation was observed. This deviation involved discernible, albeit modest, changes (factors of 3–7) in the relative yields of Fe(II)NO and met-Hb. These changes require an oxygen-dependent shift in the relative rates of Reactions 1 and 2. More significantly, the amount of Fe(II)NO and met-Hb produced was considerably less than the amount of NO added: Additional reaction pathways including SNO-Hb formation, presumably via the reactions summarized in Equations 2–8, clearly were occurring at higher O_2 concentrations. We also showed that these SNO-Hb-forming reaction pathways occur in oxygenated RBCs exposed to submicromolar NO. This observation was recently confirmed by James and coworkers (32, 33).

These results were scrutinized in several other studies. Kim-Shapiro and coworkers (73, 76) examined the oxygen-dependence of the relative reaction rates under experimental conditions very different from ours. They checked for excess protein nitrosylation, above the predictions of our simple model, only in the heme-Fe(II)NO product. They did not verify mass balance, did not quantify SNO-formation, and did not perform any studies with physiological amounts of NO with Hb or RBCs. [As noted above, such data do not provide a sound basis for evaluating physiological chemistry, and thus cannot provide any challenge to the role of NO as the "third gas" in the respiratory cycle (22, 76)]. Working under conditions in which precise quantification was hampered by poor signal-to-noise ratios, Kim-Shapiro and coworkers were unable to discern excess heme-Fe(II)NO product, although the systematic deviations between their experimental values and those computed

on the basis of our simple model are suggestive of some excess heme Fe(II)NO. Further, they show unanticipated β-heme NO predominance, which is at odds with their perspective of simple kinetics. This group also reprised prior work of Moore & Gibson (96), indicating that the on rate for NO recombination after photolysis in a fully heme-ligated Hb is not distinguishable from the initial on rate of NO addition to deoxy-Hb. The relevance to our studies is unclear. A rate acceleration need not imply an increase in rate constant, but could also derive from an increase in reactant concentration. In any case, their result provides no insight into the production of excess Fe(II)NO, or far more importantly, the SNO-Hb now observed in several studies (see Table 3).

Finally, recent work suggests that oxidized heme can be competitive with oxyhemes for NO, or more accurately, that chemistry putatively involving Fe(III)NO intermediates is competitive with the reaction of NO with oxygenated hemes (9, 65, 70). Such competitiveness has evolutionary antecedents in the oxygen-detoxification Hb chemistry of *Ascaris suum* (97), but is surprising in human Hb.

Effects of Mixing Methods

Lancaster and coworkers (94) hypothesized that SNO-Hb was formed as a result of bolus addition of NO, which reacts rapidly in locales of high concentration before mixing to homogeneity can occur. In probing this idea, they restricted their studies to fully oxygenated Hb (room air; O_2 saturations of $\sim$99.9%) and to supraphysiological total amounts of NO (5 μM) (94), and compared all-at-once addition of saturated NO solution to slow-release of an equivalent amount in a solution of a NONOate (Table 3). Addition of small aliquots of subsaturated NO solutions might represent an intermediate case between their extremes. Additionally, some evidence supports association of NONOates with proteins, including Hb (G. Ahearn, J.R. Pawloski, T.J. McMahon & J.S. Stamler, unpublished results; B.P. Luchsinger & D.J. Singel, unpublished results; 98); such interactions could interfere with mixing homogeneity and present a high effective molarity of NO for reactions with Hb.

Lancaster and coworkers (94) found no bolus effect on SNO-production, and within experimental error they found the same level of SNO-produced regardless of the method of mixing (see Table 3). Their results are not relevant to the SNO-Hb paradigm. They did appear to obtain considerably less SNO than Gow et al. (55) (and other groups, see Table 3) in both their bolus and slow-release additions. This disparity could easily be explained by a lack of recovery since their analyses rely on the problematic Gladwin assay (74).

In these studies, interactions of NO with deoxy-Hb were ignored despite the abundance of deoxy-Hb in vivo; oxygen-dependent trends in behavior were thus not examined. Similarly, the effect of heme to total-NO ratios on the product distribution was not investigated. Overall, the findings of these investigators, albeit not surprising in view of the selected reactions conditions, are not probative of any key tenet of the model of SNO-Hb function. Their work sheds no light on the

complexity—or simplicity—of NO-Hb interactions. Moreover, it has no relevance to physiological situations. In systemic peripheral blood NO is, in fact, undetectable and the pO_2 is much lower than under Lancaster's conditions [tissue pO_2 ~4–20 mm Hg (25, 66–68)]. In the lung capillary, the O_2 saturation is ~99%, but there the NO concentration is four orders of magnitude lower than that employed in Lancaster's experiments (~10 ppb or ~400 picomolar) (99).

More recently, Liao and coworkers (72) again compared bolus additions of saturated NO solutions versus slow release of NO by NONOates, with NO levels corresponding to 50 μM and oxy-Hb concentrations in the range 0.1–1 mM. Predictably, they obtained small SNO-Hb yields, but the reported levels were as much as fivefold greater than those obtained by Lancaster (94). Their most recent study examined, within a limited mechanistic perspective, the possible importance of mixed valence species in SNO-Hb chemistry. Further implications of these species, in particular for intraprotein redox reactions, are discussed below. As with the work of Lancaster and coworkers (94), the connection to physiological conditions is obscure, at best. In neither study is any framework presented through which an extrapolation could be made from the in vitro observations to the physiological situation.

Effects of NO and Heme Concentrations

Such an extrapolation is nontrivial. We have documented that reactions of NO with Hb are critically dependent on both NO concentration and NO:Hb ratio; moreover, the percent yields of the nitrosylated protein are inversely related to NO concentration, with SNO-formation most efficient with nanomolar NO levels (64) (see Table 3). Each aspect of this complex chemical behavior evidenced in our work (55, 64) was recently reproduced in experiments of Herold & Röck (65). Specifically, these authors observed (*a*) production SNO-Hb upon oxygenation of FeNO Hb, (*b*) surprising yields of SNO-Hb relative to met-Hb upon treatment of oxy-Hb with NO, (*c*) potentiation of SNO-Hb forming pathways by met-Hb, (*d*) increased SNO-Hb yields with mixing methods conducive to solution homogeneity, (*e*) increased SNO-Hb production with decreasing NO:Hb ratios, and (*f*) increased SNO-production with decreased phosphate ion concentrations. Indeed, when SNO-Hb yields are viewed as a function of reagent ratio, as illustrated in Figure 7, the quantitative agreement between work from our laboratories and that of Herold & Röck is striking.

These results are difficult to reconcile with those of Lancaster and coworkers (94) and Liao and coworkers (72). They suffice, however, to demonstrate the lack of generality of their results. The trend of increasing SNO-Hb yields with decreasing NO:heme ratios is difficult to reconcile with their work, or with the rationalization of bolus effects by these groups or by Spencer et al. (75).

James et al. (33) recapitulate the trends in behavior observed in vitro by us and by Herold & Röck (65) in experiments on RBCs exposed to physiological NO (240 nM DEA-NO), approximating our results with 200 nM NO (64). The

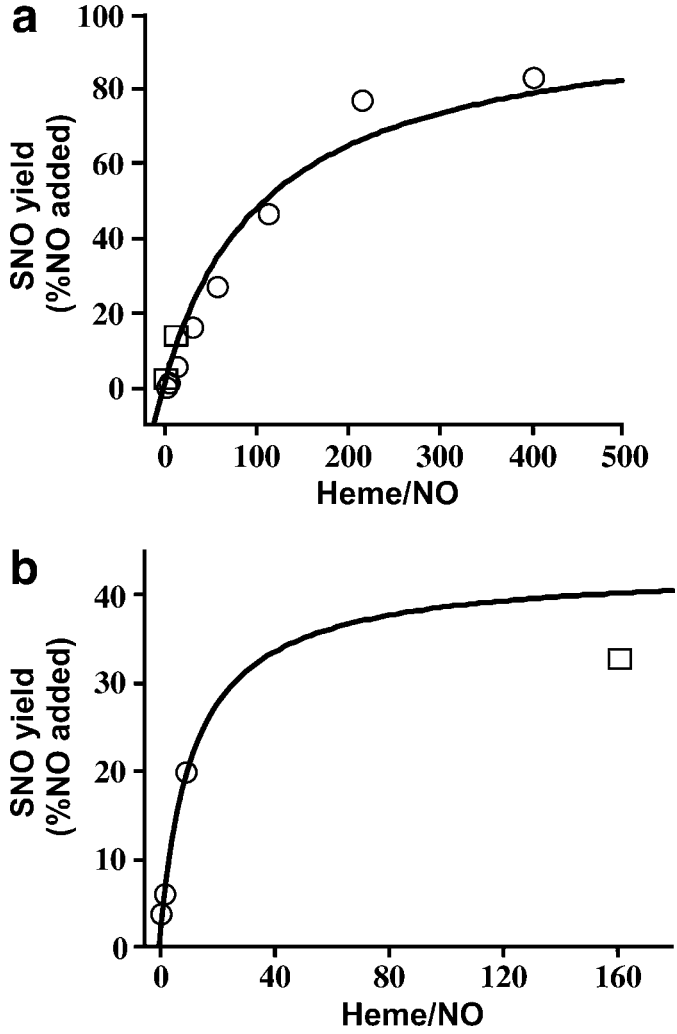

Figure 7 Dependence of SNO-Hb formation on NO:heme ratios in experiments. Comparison of results of Gow et al. (55, 64) with those of Herold & Röck (65). (*a*) SNO-Hb yield upon exposure of deoxy-Hb to NO, followed by oxygenation. Yield is expressed as fraction of added NO. Circles from Gow et al. (64), squares are data from Herold & Röck (65) with best yields (slow mixing, high phosphate). The smooth curve is a best-fit square hyperbola (saturation curve). (*b*) SNO-Hb yield upon exposure of oxygenated-Hb to NO. Circles are data from Herold & Röck (65) with best yields (slow mixing, high phosphate). The square is from Gow et al. (55). The smooth curve is a best-fit square hyperbola (saturation curve). Whereas Herold & Röck (65) called attention to their lower yields compared with those of Gow et al. (55, 64), the data compare agreeably when NO:heme ratios are taken into account.

recent in vivo studies of Mason and coworkers (62) are also noteworthy. Although they introduced sufficient DEA-NO to generate concentrations of NO compounds more characteristic of pathophysiology (e.g., sepsis), they nonetheless observed a striking distribution of products, with heme iron(II) nitrosyl accounting for ~two thirds of the NO released by DEA-NO (or more if less than two NOs are released from each DEA-NO). The fraction of released NO appearing as the heme-nitrosyl product, as predicted by simple competition between the scavenging reactions in Equations 1 and 2, would be a full order of magnitude less. This in vivo result of Mason and coworkers (62) unambiguously underscores the lack of generality of the result of Gladwin et al. in which supraphysiological exposure to NO through inhalation reportedly produced met-Hb as predominant reaction product (87).

Still needed is a complete kinetic/thermodynamic model that would enable prediction of the distribution of products in encounters of Hb with NO under arbitrary reaction conditions. Nevertheless, this biochemistry clearly is complex enough to require caution in interpreting results obtained under different conditions, and the idea that NO-bioactivity survives encounters with Hb can no longer be in doubt.

Effects of Superoxide Dismustase

Recent work from the English group (92) extends the observation by Gow et al. who first reported that SOD (55) increases the yield of Hb nitrosylation (FeNO and SNO) in reactions of oxygenated Hb with NO. In particular, SOD (55, 92) increases the yield of SNO-Hb while decreasing met-Hb accumulation. Most noteworthy is the result, obtained in both laboratories, that under certain physiological conditions, SOD is sufficient to ensure that NO-bioactivity is entirely conserved and channeled to the formation of SNO-Hb, rather than quenched through NO_3^- formation. Romeo et al. (92) provide a specific mechanistic perspective (Equations 4*a–c*) that encourages further study; the abundance of SOD in the RBCs excites particular interest in this effect.

Novel Intramolecular Biochemistry

HEME-TO-THIOL TRANSFER Biochemical studies and mutational analyses (β93 cys→ala) (64) support the interconnection between heme- and thiol-nitrosylation in Hb. SNO-Hb forms via heme-to-thiol NO transfer chemistry under conditions that feature physiological amounts (and ratios) of NO and Hb, whereas NO remains bound to the hemes of a β93-cys→ala mutant (64). The amounts that transfer from the heme depend not only on the amounts of NO (64), but also on the NO/Hb ratio, rate of oxygenation, and redox state of the system (63, 100) (see Table 3). Apart from reactions with highly oxidized Hb, SNO levels plateau at ~1 μM. A critical requirement of these reactions is the formal redox activation of the NO group (or alternative one-electron oxidation of the system). For example, the oxidative requirements of this NO-group transfer chemistry are provided by Hb [Fe(III)NO] intermediaries, which can yield SNO-Hb (63), as depicted by Equation 6. Similarly,

Equation 9 accounts for this chemistry, with initial formation of the ferric nitrosyl species prior to SNO formation:

$$\text{Hb[Fe(II)}\beta\text{93-cys]} + \text{NO}_2^- \rightarrow \text{Hb[Fe(III)NO}\beta\text{93-cys]} + \text{OH}^-. \qquad 9.$$

More generally, O_2 and redox agents, which can both influence the equilibrium between R and T structures and serve as electron acceptors, promote this NO group transfer chemistry as indicated in Equations 5a,b.

THIOL-TO-HEME TRANSFER Similar principles apply to the transfer of NO from SNO-Hb to the β-heme. That is, deoxygenation or oxidation of hemes in SNO-Hb decreases β-cysNO stability, thereby promoting NO group release (8, 30, 41, 90). Deoxygenation simultaneously decreases the redox potential of the β hemes, favoring their auto-oxidation (90). Kluger and coworkers (90, 101) described this process as featuring a coupling between heme deoxygenation and β heme/SNO redox that liberates $NO^\bullet$ from the SNO anion radical:

$$\text{Hb[}\beta\text{Fe(II)O}_2\beta\text{93-cysNO]} \rightarrow \text{Hb[}\beta\text{Fe(III)}\beta\text{93-cysNO}^{\bullet-}\text{]} + \text{O}_2 \qquad 10a.$$

$$\text{Hb[}\beta\text{Fe(III)}\beta\text{93-cysNO}^{\bullet-}\text{]} + \text{H}^+ \rightarrow \text{Hb[}\beta\text{Fe(III)}\beta\text{93-cys}^-\text{]} + \text{NO}^\bullet. \qquad 10b.$$

The released NO is then available for adduct formation with vacated, reduced hemes. Ferric heme accumulation is mitigated by met-Hb reductase (41, 90). This type of chemistry echoes early observations of Rifkind linking heme and exogenous copper redox couplings (102, 103), and provides a specific circuitry for the generic chemistry outlined in Equation 5. This chemistry also should serve as a reminder of the subtleties of β cys-93 thiol modification: such modifications immediately impact not only general oxygenation and heme redox properties, but also this particular internal redox circuit.

Auto-oxidation of SNO-oxy-Hb can lead to analogous chemistry that furnishes a heme iron(II) nitrosyl (63):

$$\text{Hb}\{[\text{Fe(II)O}_2]_4\beta\text{93-cysNO}\} \rightarrow \text{Hb}\{[\text{Fe(II)O}_2]_3\beta\text{Fe(III)}\beta\text{93-cysNO}\} + \text{O}_2 + \text{e}^- \qquad 11a.$$

$$\text{Hb}\{[\text{Fe(II)O}_2]_3\beta\text{Fe(III)}\beta\text{93-cysNO]}\} + \text{H}^+ \rightarrow \text{O}_2 + \text{Hb}\{[\text{Fe(II)NO}][[\text{Fe(II)O}_2] + [\text{Fe(III)}]_2\beta\text{93-cys}\}. \qquad 11b.$$

Dynamical loss of the oxy-ligand on an S-nitrosylated β-subunit, with a coordinated electron-NO transfer, can lead to formation of a β-subunit outfitted with thiolate and Fe(III)NO, as in Equation 11a. Reduction of the latter species by reduction coupled with oxidation of an acceptor, possibly a neighbor heme, furnishes the Fe(II)NO, as in Equation 11b. In this process, β-subunit selectivity emerges both from the proximity of the (β cys-93)-NO and heme on the β-subunit and the redox properties of the hemes. The relative stability of doubly oxidized met-Hb hybrids may enhance the favorability of the process (104, 105).

Not all of the NO would be expected to be captured by the hemes. Transfer to the heme of Hb simply allows for NO economy in a situation where bioactivity is to be dispensed in a limited manner (41). The functionally important chemistry is transfer from SNO-Hb to other NO-accepting groups that advance the signal transduction (Equation 3, in reverse; Equation 13, below). Indeed, either deoxygenation or heme oxidation was shown to increase RBC bioactivity (8, 32, 40, 42); in addition, both regulated the disposition of NO bound to hemes and thiols in human blood (22, 63). NO transfer to band 3 protein [and/or perhaps ultimately to glutathione (48)] is central to dilating blood vessels (40).

REDOX AND HYBRIDS The salutary coupling of NO and heme redox/spin states substantially enriches the chemistry of NO as compared with other heme ligands. This coupling is important in understanding how heme Fe(II)NO provides a tappable store of NO bioactivity, rather than a dead-end for NO. Experiments that show an effective loss of NO through the formation of tightly bound 5-coordinate complexes on the α-subunits (61, 106, 107) are often carried out with methods aimed to inhibit oxidative processing. When enabled (55, 106, 107), oxidation of heme (or equivalent redox processes) leads to encounters with NO that produce SNO on (R-state) Hb. The connection between heme redox and NO chemistry again underscores a link to allosteric effectors via their effect on heme redox properties. This coupling also rekindles interest in the intriguing difference in the microstate populations associated with ligand binding (108) versus "hole binding" (i.e., oxidation). Apart from molecules with fully occupied or fully vacant hemes, the former process more strongly suppresses doubly liganded forms, whereas the latter suppresses species with odd numbers of oxidized hemes (104, 105). Analogous chemistry with NO-met hybrids that favors formation of $Hb[Fe(III)]_2[Fe(II)(NO)]_2$ both in vitro and in vivo has also been observed (B.P. Luchsinger & D.J. Singel, unpublished results; 109). Collectively, these reactions are suggestive of facile intramolecular electron-transfer—a chemistry that can be viewed as an emergent property of multimeric Hbs, with fundamental implications for energy landscapes of the NO micropopulation and for Hb reactivity, as suggested in Figure 6.

CRITICISMS Aspects of this chemistry have been criticized. The transfer of NO from SNO-Hb to heme (8, 22, 90, 101, 110) has recently been suggested ad hoc to be a "nitrite artifact" (75). Isotope-labeling experiments that ostensibly support this contrary view, however, miss the mark. Our results, illustrated in Figure 8, show that over the course of the slow loss of SNO, from various SNO-Hb preparations, a comparable amount of heme-Fe(II)NO is formed (22, 110). With concomitant increases in met-Hb, an overall reaction such as indicated by Equation 11 is possible. The remarkable feature of this chemistry is the preferential β-subunit reactivity. To resolve a point of confusion in the literature, we emphasize that no exogenous nitrite was used in these experiments. Although this reaction is slow in in vitro experiments on neat samples, the reaction is potentiated by physiological levels of thiols and by other reductants (8, 22, 41).

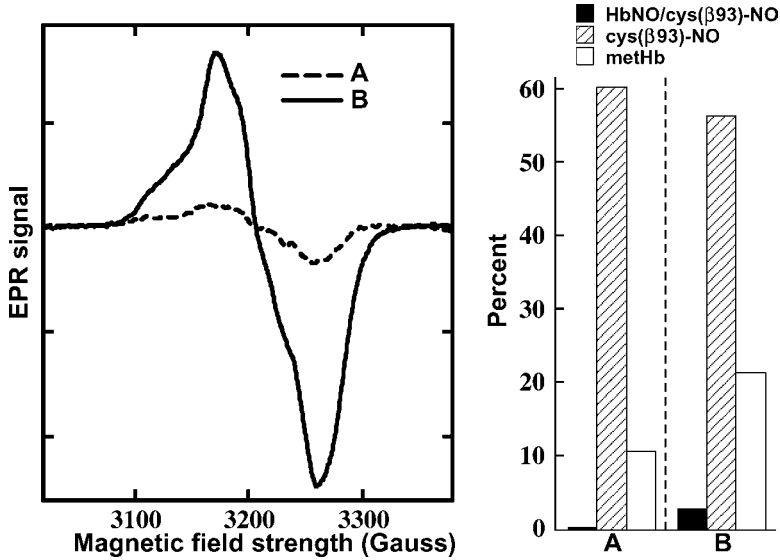

Figure 8 Thiol-to-heme NO-group transfer (22, 110). EPR spectrum of a fresh SNO-oxyHb sample prepared from S-nitrosocysteine (8) (*dashed line A spectrum*, *left panel*) shows a small signal from heme-Fe(II)NO. Further analysis of the sample shows also a small met-Hb component and 60% S-nitrosylation (*right panel, A*). EPR spectrum of the same sample after aging (*solid line B spectrum*, *left panel*) shows large heme-Fe(II)NO with predominat β-character. Further analysis shows a correlate decrease in S-nitrosylation and increase in met-Hb content (*right panel*, *B*).

Nitrite was used to generate heme-Fe(II)NO in other in vitro experiments (22, 63, 110). Again, this reaction can hardly be called artifactual; it represents a specific route to SNO-formation through coupling of heme and NO redox and Hb oxygenation. In these experiments, we demonstrated that the heme-Fe(II)NO species formed from nitrite reaction with deoxygenated Hb was dislodged by oxygenation. This loss is analogous to the oxygenation-induced loss that occurs in samples in which heme Fe(II)NO is formed from reaction of NO with deoxygenated Hb (64, 71). Xu et al. (75) found experimental conditions under which the heme-Fe(II)NO species could not be dislodged by oxygenation, and hastily concluded that our results entailed some "artifact." To clarify this point, Figure 9 illustrates the effect of sample aging on the oxygenation induced loss of heme Fe(II)NO. Immediately after sample preparation, this loss is essentially complete, and the radical signal that accompanies oxygenation is large; some minutes later, however, both the diminution of the iron nitrosyl signal and oxygenation-induced radical signal, as well as the accompanying production of SNO-Hb, are substantially attenuated. At longer intervals (not shown),

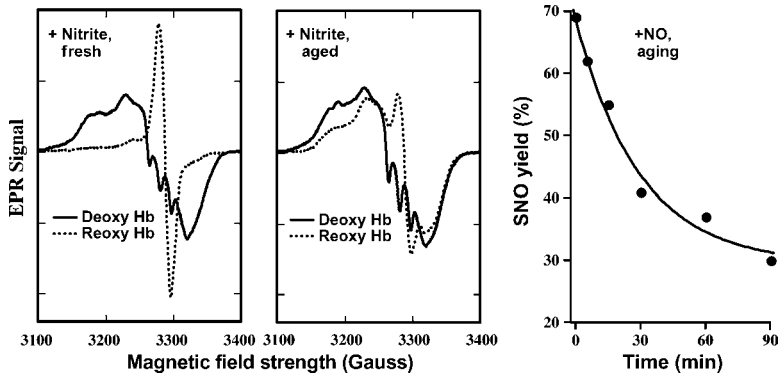

Figure 9 Heme Fe(II)NO displacement accompanying oxygenation (22, 110). Heme Fe(II)NO species revealed by EPR (*left panel*, *solid line*) of a sample freshly prepared by incubation of deoxygenated Hb with nitrite. Upon oxygenation the spectrum is dramatically altered with the Fe(II)NO signal diasappearing and being replaced by a free-radical spectrum (*dashed line*). After aging, repeated cycling, and/or nitrite exhaustion, the behavior is muted; progressively, oxygenation-induced changes are observed (*middle panel*). All EPR spectra shown in the figure are on the same scale. Analogous experiments have also been conducted with heme Fe(II)NO prepared by NO addition (nitrite-free) at low NO:Hb ratio and establish that the efficiency of SNO-formation on oxygenation is also impaired progressively by sample aging (*right panel*). Aging effects are more rapid in native RBCs (T.J. McMahon & J.S. Stamler, unpublished data).

we obtain the results of Xu et al. (75). Our view is that chemical processes, which occur over the longer time intervals, alter the microscopic composition of the samples and contribute to the alteration in oxygenation-linked reactivity.

Another aspect of the work of Xu et al. (75) that demands comment is their exposure of RBCs to almost millimolar NO [Fe(II)NO and SNO] levels. Under such extreme conditions, heme-thiol transfer would be expected to be small (55) (Table 3), nowhere near the >100 micromolar values that they errantly impute to our model. Indeed, the imputed values are based on a completely unjustified linear extrapolation of fractional transfer-yields from the quasi-physiological conditions of McMahon et al. (native RBCs), to the extreme conditions of Xu et al. (N.Y. Spencer, personal communication) (RBCs treated with many millimolar NO). Nagababu et al. (85) also dislodge the iron-nitrosyl species derived from nitrite by oxygenation. The results reported by Cosby et al. (53) for nitrite-exposed RBCs also point to this chemistry. Collectively, these results again call attention to condition-dependent chemistry in this complex system.

NITRITE AND HEMOGLOBIN PHYSIOLOGY

It has long been known that nitrite can dilate blood vessels. Nitrite, which is ubiquitous, is by itself ~100–1000 times less potent than NO or S-nitrosothiols and must be converted to either NO or SNO to produce vasodilation. For example, production of nitrite in mitochondria is the first step in the mechanism of nitroglycerin bioactivation (112, 113), and mitochondria support the conversion of nitrite into NO and SNO (113; Z. Chen, M. Foster & J.S. Stamler, unpublished results).

Nitrite Biochemistry

Reutov & Sorokina (114) suggested that the reaction of nitrite with deoxy-Hb, which, as detailed by Doyle and coworkers (107), generates Fe(III) and NO, may represent a source of NO bioactivity. Reutov & Sorokina argued that more NO may be generated through this pathway than though NO synthase, particularly in a low O_2 milieu. Others have recently repeated this idea (53, 85) without addressing its key limitation. Missing from this hypothesis is how the NO could escape the RBC, rather than lodging on vacant Fe(II) hemes that are highly abundant under the low O_2 conditions considered. (Further, most deoxygenated Hb is found in the veins, which is not the region in which blood flow is regulated.) The solution is the (prearteriolar) conversion of FeNO to SNOs, as originally described by McMahon (22) and Luchsinger and coworkers (63). The reaction of deoxy-Hb with limited nitrite leads to production of β FeNO (63), with SNO-Hb formed upon subsequent oxygenation (22, 63) (Equations 5a and 5b, where either O_2 or ferriheme serve as the electron acceptor). Thus Hb/nitrite catalyzes the formation of SNO-Hb through the intermediacy of iron-nitrosyl Hb (nitrite reductase activity). The amounts of SNO-Hb generated correlate directly with RBC bioactivity (G. Ahearn & J.S. Stamler, unpublished results). SNO-Hb formation through interactions of Hb with nitrite—summarized in Equations 8 and 9 and Figure 6, and now observed in several laboratories (22, 53, 63, 85, 115)—overcomes the key limitation of the nitrite reductase hypothesis of Reutov & Sorokina.

An important issue in the utilization of nitrite to generate NO is the possible competition with oxy-Hb reactions to form nitrate and met-Hb (116).

$$4\text{heme-Fe(II)}O_2 + 4NO_2^- + 4H^+ \rightarrow 4\text{heme-Fe(III)} + 4NO_3^- + O_2 + 2H_2O. \quad 12.$$

At physiological nitrite:Hb ratios, however, reaction 12 progresses very slowly compared with the NO-forming, nitrite-reductase reaction, as we have demonstrated (63), owing to lengthening of the cysβ93-dependent lag phase characteristic of the reaction with oxy-Hb (116). The special feature of the nitrite reductase reaction is not the production of an iron-nitrosyl alone but rather its coexistence with a ferric heme within an Hb tetramer, which ultimately, as Hb shifts to its R quaternary state, as discussed above, can support the redox requirements for SNO production. Hybrids of this sort are also likely formed on exposure of partially deoxygenated Hb to NO; under such conditions, reaction with NO inevitably

leads to some heme oxidation. Both UV/VIS and unambiguous EPR spectroscopic measurements support this chemistry.

A recent report by Fago et al. challenges a fundamental part of this latter chemistry (71). They report mixing of NO with deoxyHb under conditions where little or no met-Hb is produced, and claim that the observation of met-Hb by Gow & Stamler (64) derives from an error in spectral deconvolution. However, the spectral analysis method discussed by Fago et al. was not used by Gow & Stamler (64), who focused on spectral changes in the 400- and 630-nm regions where confusion over met- and iron-nitrosyl Hbs is least likely. Previous reports on the production of met-Hb, upon exposure of deoxy-Hb to NO, by Hille et al. (106) and by Doyle et al. (107) indicate that yields are dependent on pH, organic phosphates, and mixing methods. Gow & Stamler (64) emphasize the importance of NO concentration and NO:heme ratios on the product distribution. Conditions used by Fago et al. depart significantly from those of Gow & Stamler (64) (see Table 3). There is no basis provided in Fago et al. for asserting that their results are general. As such, they have no obvious bearing on the results of Gow & Stamler and Doyle et al. (107).

Vascular Effects of Nitrite

Results presented in a recent publication ostensibly amplify ideas on the role of nitrite in regulation of blood flow (53). Specifically, the nitrite reductase activity of Hb alone was invoked to explain the response of hypoxic vasodilation (53). Unfortunately, the vascular physiology implicating nitrite (administered by infusion) in hypoxic vasodilation was misinterpreted in this paper. First, the nitrite-mediated increase in blood flow was associated with an increase in venous O_2 saturation, whereas hypoxic vasodilation is the increase in blood flow that accompanies a decrease in venous O_2 saturation (24–26). Second, the flow increment induced by hypoxic exercise was, in fact, unchanged by nitrite. Third, the vasodilation to hypoxic exercise was not blocked by the NO synthase inhibitor L-N^G-monomethyl-arginine (53, 66), which acutely depletes nitrite (117, 118). Thus hypoxic vasodilation is not directly mediated by nitrite. It is improbable that enough nitrite to enable vasodilation can enter the RBC and be converted to NO, or enough NO diffuse out, within the few seconds that blood transits the arteriolar microcirculation. On closer inspection of the in vitro supporting data, nitrite neither potentiated the rate nor the amount of relaxation by RBCs, which would constitute the necessary signatures of NO effects. Further, what appeared as "release" of NO from RBCs is probably the entrainment of NO in an inert gas that was used to sparge RBCs (moreover, the amounts were too low to dilate blood vessels). Hypoxic vasodilation by native RBCs does not require addition of exogenous nitrite (22, 32). Taken together, the observations of Gladwin et al. (53), who observed copious SNO-Hb formation upon infusion of nitrite, are better interpreted as pharmacologic SNO-Hb-mediated RBC vasodilation, not hypoxic vasodilation, although nitrite could also be acting independently of RBCs.

OXYGEN-DEPENDENT SNO DELIVERY

As noted above, the transduction of the ambient oxygen signal to the release of NO-bioactivity through reactions of SNO-Hb is a fundamental tenent of our model of SNO-Hb function. We propose that this process is linked to the changes in quaternary structure of Hb associated with changes in oxygen saturation, i.e., to Hb allostery.

The final steps by which the NO-signal leads to vessel dilation are not yet fully established. In the bioassay, GSH (glutathione) substantially potentiates the response to cell-free SNO-Hb (Figure 10), but is not required for RBC relaxation (Figure 3*a–d*). Cellular studies demonstrate that transfer of NO groups from β-cysNO to cysteine thiols at the RBC membrane (band 3) is necessary and sufficient for vasodilation by RBCs under representative physiological conditions (40). The means by which S-nitrosylation of band 3 advances vasodilation remain to be elucidated. Clearly, SNO-Hb-mediated bioactivities, analogous to EDRF (119), do not absolutely require free NO (8, 40) or cGMP (42); cGMP involvement has, however, been implicated by James and coworkers (32). There is also evidence that GSH can support RBC-mediated vasodilation. Transgenic mice lacking GSNO reductase, an enzyme that metabolizes GSNO, show increased SNO-Hb levels (21). The addition of GSH to blood under hypoxic conditions, but not normoxic conditions, generates GSNO (48). Collectively, these observations, buoyed by the mechanistic suggestions of English and coworkers (92), suggest an important interplay between GSNO and SNO-Hb. In addition, the chemistry of the oxygen dependencies on the NO reactivities of Hb, although understood in broad strokes, is not yet determined in detail.

Allostery

In a manner reminiscent of Perutz's sterochemical modeling to rationalize ligand-binding, induced allostery, crystal structures (30), and molecular models (30, 37) of SNO-Hb provided a basis for allosterically regulated release of NO bioactivity. The β-cysNO has limited access to solvent in R state (thus hampering release to dilate blood vessels), but it protrudes into solvent in the deoxygenated (or T) structure. McMahon et al. (41) have demonstrated definitively that the transfer of NO groups between SNO-Hb and glutathione is allosterically regulated (Figure 10). They showed that SNO-Hb was stable in air (R structure) in the presence of glutathione, but decomposed upon deoxygenation (Figure 10*a*), with production of GSNO. Both hypoxia and thiol potentiated SNO-Hb-induced relaxations (Figure 10*b*), presumably through their effects on the position of the O_2-dependent equilibrium that produces the vasodilator GSNO (Equation 13, where RSH is glutathione). Consistent with this interpretation, the plasma half-life of infused SNO-Hb increases more than twofold in animals breathing pure oxygen (P. Sonveaux, T.J. McMahon, J.S. Stamler & M.W.Dewhirst, unpublished observations).

$$\mathrm{Hb[[Fe(II)O_2]_x}\beta\text{93-cysNO]} + \mathrm{RSH} \leftrightarrow \mathrm{Hb[[Fe(II)O_2]'_x}\beta\text{93-cys]} + \mathrm{RSNO} + \mathrm{(x\text{-}x')O_2}. \quad 13.$$

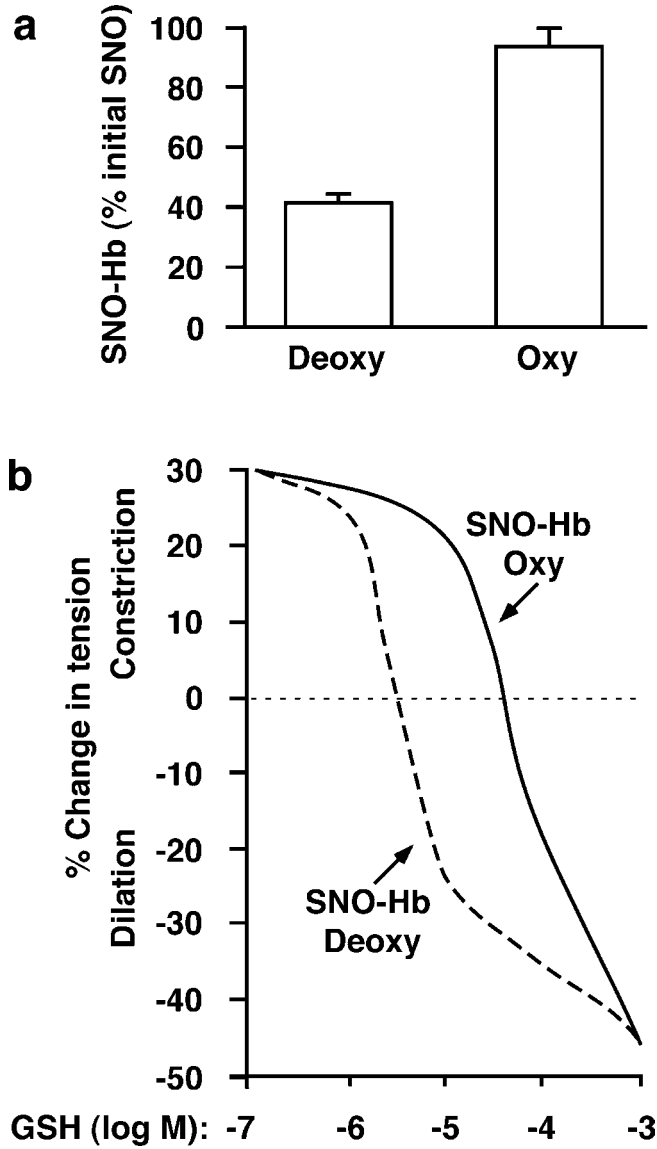

Figure 10 Allostery-regulated generation of GSNO by SNO-Hb and induced vasoactivity. (*a*) SNO-Hb (50 μM) is stable in room air in the presence of glutathione (1 mM) (Oxy) but decays immediately upon deoxygenation (Deoxy) with production of GSNO (not shown). (*b*) Vasorelaxation by SNO-Hb (1 μM) is potentiated by hypoxia (1% O_2; Deoxy versus 21% O_2; Oxy) and glutathione, which favors production of the vasodilatory mediator GSNO (representing a shift in the position of the equilibrium in Equation 13 to the right). Data are derived from McMahon et al. (41).

Under certain experimental conditions, relaxations by SNO-Hb have been observed at both high and low pO_2 (and conversely SNO-Hb vasoconstriction has been seen at both high and low pO_2) (54). Some argue that these data rule out allosteric control of SNO-Hb vasoactivity (54). However, when data are considered over an appropriately broad range of O_2 and thiol concentrations, as illustrated in Figure 10, the flaw in this argument is apparent. The vasoactivity of SNO-Hb at both high and low thiol concentrations is independent of pO_2, as would be expected by a process governed by Equation 13. At intermediate thiol concentrations, however, the O_2 dependence is unmistakenly revealed. In vivo concentrations of SNO-Hb and allosteric effectors, nature of thiol, and rates of reactions will modify the quantitative behavior illustrated in Figure 10. Nevertheless, the data underscore both the operation of allostery and the importance of experimental conditions in probing for it. Crystal structures again corroborate the importance of allostery: Exposure of R-state carbonmonoxyHb crystals to NO leads to iron- and S-nitrosylation, whereas exposure of T-state crystals to NO leads only to iron nitrosylation (30, 31) (Figure 2).

Key evidence in support of the hypoxia-dependent release of NO-bioactivity from RBCs comes from the work of McMahon et al. (22) and, more recently, of James et al. (33). RBCs were shown to actuate a unique, rapid, and graded, vasodilator and vasoconstrictor response across a physiological range of pO_2 (Figure 3). Thus, RBCs were shown not only to dilate blood vessels, but in a manner that recapitulates the autoregulation of vessel tone by the physiological O_2 gradient. This response is uniquely characteristic of SNO-Hb (Figure 10*b*). Thus, the inference that SNO-Hb relaxations are not allosterically regulated by O_2, drawn from the observation that relaxations by NO itself are also potentiated by hypoxia (120), is ill-conceived: The pO_2 versus activity relationship for NO and SNO-Hb shows little, if any, resemblance (22, 33)—in particular, GSNO relaxations fail to show the same graded responsiveness over the physiological oxygen gradient as does SNO-Hb. The enhanced activity of NO itself under hypoxia does not have an allosteric basis; SNO-Hb relaxations are not mediated by free NO (120, 121).

Linkage

S-nitrosylation of Hb results in enhanced oxygen affinity, as indicated in Figure 5 (41, 122). These measurements were conducted on Hb-$(SNO)_2$, whereas Hb-(SNO) is more likely to predominate in the physiological situation. Enhanced oxygen affinity was seen by some to be inconsistent with the idea that hypoxic vasodilation is regulated by the coupling of NO-bioactivity release to deoxygenation. The argument was, "NO transfer would be limited to regions of extremely low oxygen tension, if this were to occur from deoxygenated hemoglobin" (122). This perspective is fundamentally flawed. At any finite oxygen saturation there is a population of deoxy-Hb subunits. Not only does the number of these molecules increase monotonically with decreasing pO_2, but in view of the cooperativity of oxygen binding, that increase is nearly linear in oxygen saturation. Thus a lowering

of oxygen tension that causes a drop in oxygen saturation of the S-nitrosylated Hbs labilizes a proportionate number of S-nitrosothiols. While extremely low oxygen tensions would be required to denitroslyate all nitrosothiols, physiological levels reached in the microcirculation are sufficient for dispensing enough activity through transnitrosylation to modulate vessel tone, particularly in view of the great vasodilatory efficacy of SNO-Hb, in the presence of thiols (Figures 3 and 10). Moreover, the fact that decreased oxygen tension results in destabilization of the SNO-moiety actually implies that S-nitrosylation stabilizes oxygen ligation: The observed behavior, rather than representing a paradox, should be expected. In principle, the thermodynamics could be trumped by kinetic bottlenecks, but such bottlenecks seem not to be significant, provided species such as thiols are present to accept the released NO group (41).

SNO-Hb Stability in RBCs

Gladwin and coworkers (74) published the results of experiments ostensibly demonstrating that SNO-Hb was too unstable in the RBC to exert biological activity, and that the decay of SNO-Hb in the cells was not accelerated by deoxygenation, ruling out allosterically controlled release. However, there is no foundation for asserting that their measured half-life for SNO-Hb (~10 min) is too short for physiological efficacy inasmuch as A-V transit times are measured in ~seconds. There is also little foundation for asserting that the measured half-life for SNO-Hb under the in vitro conditions employed is relevant to the physiological situation. The cells these authors examined are raised to extreme NO levels by incubation with high millimolar S-nitrosocysteine. The kinetic data presented in the paper are too limited to precisely determine the kinetic order, information required for extrapolation to physiological concentrations (zero to third-order kinetics fit the sparse data equally well). The results of Gladwin et al. are directly refuted by their own subsequent measurements in which no such instability in SNO-Hb content was observed (75). Most significant, untreated RBCs withdrawn from blood into room air likewise show no such instability in their SNO-Hb levels (22).

The treatment of RBCs used by Gladwin and collaborators (74, 75) was originally tried in the Stamler laboratory but abandoned when it was found not only to obscure the relevant chemistry but also to dramatically alter the cellular contents through extensive nitrosylation and oxidation of Hb and all of the protein and peptide contents, including membrane-bound proteins. To the extent that SNO-Hb is activated by deoxygenation for release to nitrosylate band 3 or glutathione then, under Gladwin and colleagues' conditions, this oxygen dependence would be short-circuited owing to the artifactual nitrosylation of band 3 and glutathione. (The fact that Gladwin and colleagues' deoxygenate on a timescale similar to their half-life further masks allosteric release.) Because of these problems, a new method of RBC treatment with NO was introduced that affords NO mass balance among Fe(II)NO and SNO-Hb and maintains the NO concentrations in the physiological realm (40). This method should be the operating standard for NO/RBC studies.

PHYSIOLOGICAL ISSUES: NO DELIVERY WITH NO GRADIENTS

Observations

In our first report we observed that the amount of SNO-Hb was apparently greater in oxygenated left-ventricular blood than in partially deoxygenated right-ventricular blood, whereas the opposite was true of iron nitrosyl Hb (8). The total amount of NO bound to Hb (i.e., SNO plus FeNO), however, was the same on either side of the heart (within error of the measurement) (8, 37). A correlation between oxygen saturation of Hb (but not pO_2) and amount of SNO-Hb was demonstrated in situ across 80 measurements in human patients (22) (Figure 1). In vitro, oxygenation of the partially deoxygenated human blood samples displaced the NO from the hemes, with accompanying formation of SNO-Hb (22, 40, 64). Much the same chemical behavior, i.e., the displacement of NO from the hemes of venous blood Hb by oxygenation, has recently been reproduced in experiments of Herold & Röck (65), Rifkind et al. (85), and Datta et al. (32).

We interpreted these data as evidence for the oxygenation-induced transfer of some NO from hemes to thiols of Hb within the lung and for some migration of NO back to hemes upon deoxygenation of blood in the microcirculation and veins; the amounts of NO that apparently transferred ranged widely from low nanomolar to micromolar in our studies (22). The adult human placenta serves the function of the fetal lung in utero. Funai et al. have reported analogous gradients in SNO-Hb across the umbilical vein and artery of 19 babies (36). Arterial-venous differences in SNO-Hb/FeNO-Hb have now been measured in humans by five groups (22, 32, 36, 85; A. Doctor & B. Gaston, personal communication), and recently correlated directly with O_2 extraction in both health (22, 32) and disease (32).

Simulation of the oxygen-mediated transfer of NO from heme to thiol of Hb in vitro requires accurate recapitulation of the physiological conditions. In particular, we reported that the efficiency of NO exchange between heme and thiol is an inverse function of both the NO/Hb(FeII) ratio and the amounts of NO present (64), and that the NO-group transfer from heme to thiol shows saturation behavior with a plateau at $\sim$1 μM (64). Thus, as heme-bound NO increases, the efficiency of transfer that is coupled to oxygenation decreases such that the transferred NO groups do not exceed micromolar levels. As noted above, these core observations have recently been confirmed in other laboratories (65), and indicate that the regulation of the disposition of the NO group incorporates a brake to limit formation of SNO-Hb when NO exceeds physiological levels. Under physiological conditions, where oxygen delivery is principally regulated by variation in blood flow, the formation of SNO-Hb, which regulates hypoxic vasodilation and hyperoxic vasoconstriction, is enabled (22, 32, 36). In septic shock, however, vasodilation by SNO-RBCs would aggravate hypotension and thus tissue hypoxemia. Hb then effectively circumvents the potential problem of excess SNO-Hb formation by trapping the excess NO on the α hemes (thus lowering the affinity of the other

hemes for O_2). This chemistry accomplishes NO "detoxification" while possibly enhancing O_2 delivery, and is exemplified in the ~0.3–1 μM A-V Hb heme-NO gradients observed by Mason and coworkers (62), under conditions ranging from basal NO levels to those characteristic of septic shock. It also consistent with the excess heme-Fe(II)NO over SNO-Hb reported recently in a murine model of sepsis (54) and with inhalation of supraphysiological NO (87).

Nitric Oxide Gradients Contrasted with O_2 Gradients

Notwithstanding these observed A-V NO differences in situ, gradients of NO compounds have been mistakenly analogized to O_2 gradients (52, 88). In the realm of the oxygen-delivery function of Hb, A-V gradients have a particular significance: They measure O_2 delivery to tissues. A-V gradients in vasodilatory NO compounds, however, have no such obvious significance (123). If we assume that NO gradients reflect NO delivery, then the measured gradients, typically in the range of ~0.1–1 μM, are highly problematic because they imply a utilization of NO that vastly exceeds endogenous production (~1 mmol/70 kg/24 h), and a dose that would lead to lethal hypotension [SNO at 1–10 nM is adequate for regulation of blood flow (22, 41)]. Thus, the argument against SNO delivery by RBCs (88), which is based on measurement of a gradient, is not constrained by any quantitative standards of efficacy. In their study, Gladwin et al. (88) detected A-V gradients of up to 60 nM SNO-Hb, yet, without any rationale, viewed these as insignificant. Datta et al. (32) found micromolar A-V Hb NO gradients that correlate directly with A-V Hb O_2 gradients, both in normal subjects and in patients with heart failure.

Why are NO gradients different from O_2 gradients? Whereas O_2 uptake in the lung and release in tissue are compatible with the simple accounting scheme that balances the gradients and the blood flow with the amount delivered, NO and bioactive products (particularly SNOs) are produced throughout the circulatory system. Salient properties, such as locations, fluxes, etc., of the sources of NO/SNO relevant to their Hb chemistry as well as the sinks of Hb-NO compounds are not fully understood. However, the gradient-determining properties are very different for the O_2 versus the NO systems; the former is designed for wholesale delivery of an electronic sink; the latter, in contrast, is for the regulated dispensing, as a signal, of tiny doses of a high-potency messenger (41). Likewise, the gradient of NO compounds cannot be identified with NO delivery, as is the case for O_2. Total amounts of NO bound to Hb do not change, within the limits of sensitivity of assays, during the A-V cycle (8, 22, 37), a finding in keeping with predictions regarding NO mass balance (41, 55). Thus, the notion that the relative sizes of the A-V gradients, exhibited by various NO-compounds, provides a basis for identifying endogenous vasodilators (52, 53, 88, 123) betrays a misunderstanding of NO biological chemistry in general and the SNO-Hb mechanism in particular.

Dispensing NO

It is well documented (22, 32, 36, 37, 65, 85, 90, 92) that pO_2 regulates the disposition of NO bound in Hb, i.e., the SNO content of blood is proportional to O_2 saturation, whereas heme-NO varies inversely with O_2 saturations. A decline in SNO-Hb is generally accompanied by the capture of NO on β hemes (41, 90), thus creating the appearance of an A-V gradient in SNO-Hb and FeNO [total Hb-NO does not change within limits of detection (8, 22, 37)]. These gradients can be revealed ex vivo provided A-V O_2 differences are carefully maintained during sampling (22, 32). [Gladwin et al. (74) do not detect these gradients, perhaps because they expose both venous and arterial samples to room air.] These gradients may be viewed as informative of steady state situations in vivo. They have no real bearing, however, on the amounts of NO/SNO that transfer per A-V cycle or that are exported from RBCs. Indeed, amounts of NO/SNO delivered by RBCs in vivo should not be expected to be any more accessible to measurement than are the amounts of NO/SNO (EDRF) delivered by endothelial cells in vivo (in the presence of RBCs). Neither should be detectable. Moreover, the gradient-forming transfer of NO from β-cys93 to β hemes (see Thiol-to-Heme Transfer, above) is inevitably accompanied by a second reaction in which NO groups are exchanged with receptor thiols in the RBC membrane (band 3) (40, 121) and in the cytosol (glutathione) (8, 41) to provide (and conserve) NO bioactivity in proportion to degree of hypoxia (22, 41). The pO_2 does not strictly regulate the amount of SNO exported from RBCs (as recently misconstrued) (74), but rather exerts an effect on the position of the equilibrium between SNO-Hb and acceptor thiols (e.g., band 3) that provides bioactivity (Equation 13).

We anticipate therefore that RBCs will have a propensity to deliver NO bioactivity (involving only a small fraction of the total Hb-NO) throughout the low pO_2 circuit, which includes arterioles, capillaries, veins, and pulmonary artery, and thus influence both blood flow systemically, as well as pulmonary arterial oxygenation and pressure (Figure 4*a*). Conversely, RBC-NO will have a lesser tendency to deliver NO bioactivity in the high pO_2 circuit, including pulmonary veins and large systemic arteries.

PHYSIOLOGICAL VERSUS PATHOPHYSIOLOGICAL NO/HB BIOCHEMISTRY

Principles

The importance of adhering to physiological ratios of the reagents in in vitro studies aimed at elucidating the fundamental chemistry of NO Hb interactions has been chronically underestimated. As noted above, the disposition of NO bound to Hb and reactivity of the molecule is a function of many variables, including the amount of NO, the NO/heme ratio, the details of reagent mixing, and the lag time after NO infusion (55, 57, 60–65). These principles dictate exercise of caution in drawing physiologically relevant inferences from studies conducted with extreme

NO concentrations. Such experiments include the work of Mason and colleagues, who infuse DEA-NO to give concentrations of NO characteristic of septic shock and other pathological conditions (57, 124, 125); Gladwin et al. (74, 75); Xu et al. (75); Huang et al. (73); and Han et al. (70, 72), who add more NO to RBCs (500–10,000 μM) than is found in any pathophysiological situation (74, 75); and Gladwin et al., who introduce inhaled levels of NO at 80 ppm ($\sim$4 μM) in a flow of 40 liters/min—a suprapathological dose (87).

Hemoglobin Structural Effects

As previously shown by Kosaka et al. (57), such high concentrations of NO, in concert with the low pH that accompanies hypoxia at the tissue level, favor production of Hb molecules with 5-coordinate α-subunit heme Fe(II)NO moieties, which are viewed as a signature of the T-state conformation. These T-state molecules switch to R structure upon oxygenation without immediately dislodging NO from the α-chain, but exhibit a low affinity for O_2 on their vacant hemes (61). The lower affinity for O_2 has been suggested as providing a mechanism to improve O_2 delivery (126). This relative lack of affinity for O_2 clearly affects the propensity of bound-NO toward displacement, including β heme-to-thiol NO-group transfer, as $[O_2]$ varies in a physiological cycle. This effect can be understood by recognizing that SNO-Hb is disfavored in T-state molecules (22, 30, 37, 90, 92). Moreover, NO-transfer between 6-coordinate α and β hemes (in R structure), which facilitates production of significant SNO-Hb, is inhibited by the very O_2 molecules that must first bind to β hemes in order to induce the 6-coordinate R structure. Thus Hb may switch the control mechanism by which O_2 delivery is regulated in pathophysiological states. Instead of relying on SNO-Hb vasodilation, which would be counterproductive in shock, nature might exploit NO as an allosteric effector of oxygen delivery (58, 64).

The reactivity of NO and Hb at physiological levels (nanomolar NO, millimolar Hb) is very different. At low NO/heme ratios, selective interactions of NO with β-subunit heme have been observed under a variety of conditions (22, 55, 63, 90, 92), notwithstanding the tendency of the NO groups to reach the α-subunit heme Hb at higher NO levels and/or longer NO incubations (55, 57, 60, 61). In the R state, a partitioning is also established between the 6-coordinate β-subunit heme and thiol (22, 32, 33, 55, 63, 90). The more NO that binds within the T state (and the lower the pH), the greater the tendency to form the relatively inert 5-coordinate α-nitrosyl Hb (more so in rat than in human blood), and β-subunit heme-to-thiol transfer is suppressed. Thus the formation of 5-coordinate α-nitrosyl Hb may be nature's way of limiting SNO-Hb formation (and consequent hypotension) in situations of hypotension characterized by NO plus SNO-Hb excess (54, 58, 64). In the interactions of NO with Hb, the amount of a given product does not respond linearly to the NO concentration; the relative product distribution is not constant. Rather, Hb is a model of complexity whose subunit inequivalences and allosteric states enable a wide-ranging responsiveness to ambient conditions.

OUTLOOK: DISORDERS OF RBC-NO PROCESSING

The integrated vascular response that mediates autoregulation of blood flow in pulmonary and systemic vessels to support O_2 uptake and delivery on physiological timescales has a counterpart in SNO-Hb RBC vasodilation, distinguished from EDRF (shear-induced vasodilation) (35, 49, 58) and pharmacological nitrite vasodilation (53). Inasmuch as this physiological response is paramount to the respiratory cycle, we anticipate that defects in RBC vasodilation might underlie diseases of heart, lung, blood, and potentially other organs.

Impaired release of NO by RBCs (8, 22, 32, 42) may have direct implications for hematological abnormalities such as the vaso-occlusive crises of sickle cell disease, the hypertension of thalessemia, and the thrombotic diathesis of paroxysmal nocturnal hemoglobinuria. More broadly, it may shed light on unexplained and underappreciated medical problems identified with RBCs. Thus, RBC counts, even within the normal range, are directly correlated with blood pressure (127), and RBCs may actively contribute to clot formation (128). These observations may bear directly on the findings that RBC transfusions (128–131, 144), erythropoietin (132) (which raises Hb levels), and Hb-based blood substitutes (133) that are devoid of NO bioactivity have each been associated with increased cardiovascular morbidity and mortality. Indeed, stored blood, which over typical storage intervals likely loses vasodilator SNO, has been shown to raise pulmonary and systemic pressures, induce ventilation-perfusion mismatching, and create mesenteric ischemia (134, 134a). Both erythropoietin (135, 136) and Hb-based blood substitutes (137, 138) induce similar cardiovascular dysfunction, evidently by creating an NO/Hb imbalance. The new understanding that RBCs precisely regulate their function through a controlled NO/Hb balance, thus raises the idea that dysfunction of RBCs (through NO/Hb imbalance or compromised NO processing) may contribute broadly to the pathogenesis of thrombotic, hematologic, and ischemic disorders and perhaps other organ dysfunctions (100). Consistent with this possibility are the initial reports of altered RBC-SNO processing in subjects with diabetes (84), heart failure (32), sickle cell disease (J.R. Pawloski, D.T. Hess & J.S. Stamler, unpublished results), pulmonary hypertension (18), and sepsis (21). For example, patients with pulmonary arterial hypertension (18) and diabetes show impaired RBC relaxations (18), the former associated with SNO-Hb deficiency and the latter with deficient SNO release, as glycosylation of Hb, presumably by promoting the R structure, shifts the equilibrium in Equation 13 to favor reactants (18, 84, 139). Both diabetic (84, 139) and septic (21) patients exhibit excessive SNO-Hb levels, but the latter show increased relaxations (18, 54), whereas the former, because of this shift, show decreased relaxations (18). More generally, impaired vasodilation by RBCs is suggested to originate at multiple levels, ranging from impairments in oxygenation, to Hb itself (allostery), to alterations in interactions of Hb with the membrane band 3 and the RBC NO export function. If true, a new perspective on the alterations in blood flow and tissue oxygenation that characterize many vascular, respiratory, hematologic, and ischemic diseases may emerge from attention to RBC

dysfunctions. Reconstitution of RBC-NO activity may also provide a novel therapeutic strategy to treat NO deficiency and/or hypoxemia-associated states (100).

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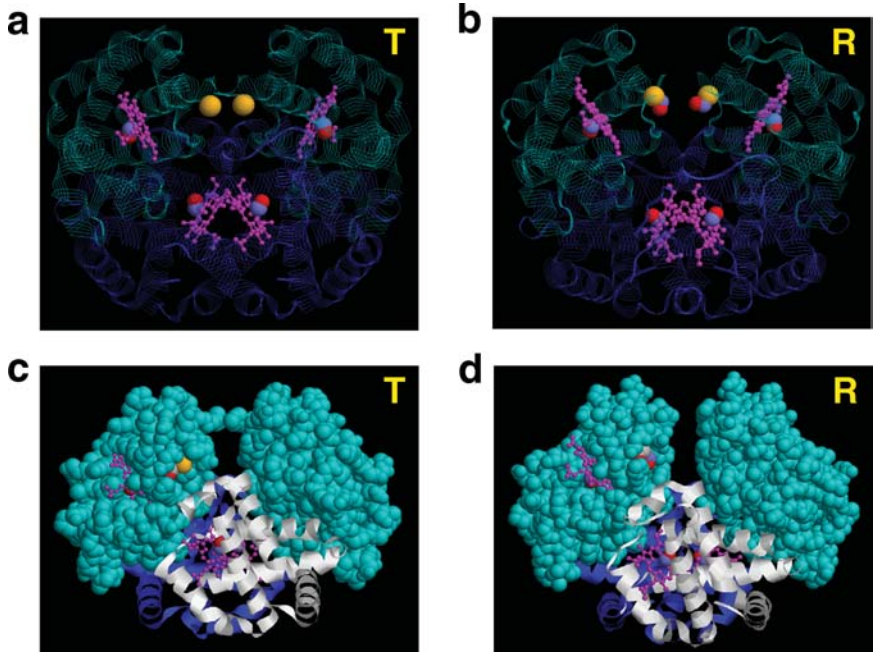

Figure 2 Structures of nitrosylated human hemoglobin in T and R quaternary forms from (30, 31). (*a*, *b*) Strand representations of the globin backbone with β-subunits shown in teal and α-subunits in blue. The hemes are displayed in magenta (ball-and-stick). NO ligands on the hemes, and the β-cys93 sulfur and attached NO group are displayed in space-filling representations with CPK coloration. The T-state nitrosylated protein (*a*) has NO only on the hemes (β-subunit 6-coordinate and α-subunit 5-coordinate), whereas the R-state protein has NO both on heme (all 6-coordinate) and β-cys93. (*c*, *d*). Space-filling representation of the protein β-subunits (*teal*) with ribbon displays of the α-subunits (*blue* and *white*). The heme, β-cys93, and NO groups are represented as in *a* and *b*. In the T structure the β-cys93 sulfur is exposed, whereas in the R structure it is tucked behind its adjoining carbon. In energy-minimized solvated structures (37), the entire SNO-moiety is folded back into the globin and is not solvent accessible.

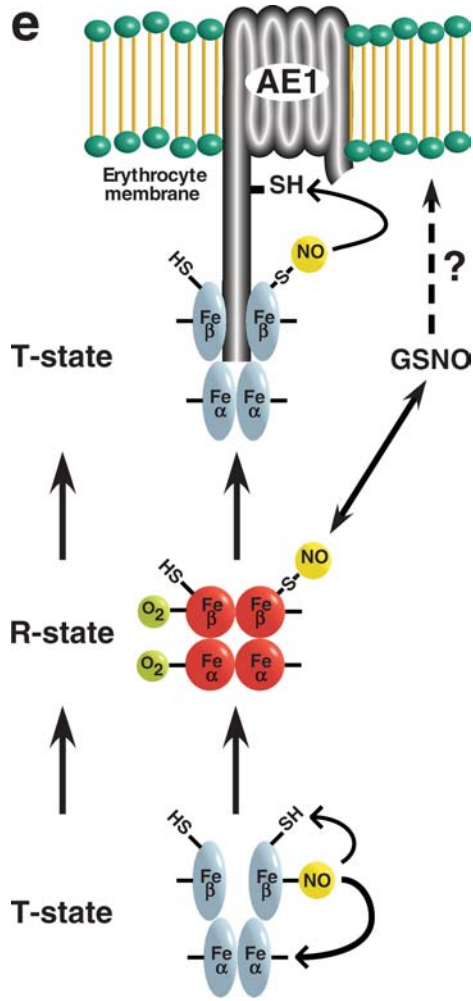

Figure 3*e* Generation of NO bioactivity in RBCs occurs preferentially at the membrane. As illustrated, oxygenation (in the lungs) promotes the transition of hemoglobin from T to R state and the transfer of heme-liganded NO to β-cys93; a significant proportion of NO is retained as an iron nitrosyl species. In the vascular periphery, deoxygenation is associated with the transition from R to T state, which is facilitated in a juxta-membrane population of hemoglobin by interaction with the cytoplasmic domain of AE1 (band 3 protein). Concomitantly, NO is transferred from β-cys93 to a cys thiol within AE1 (and the R to T transition may also facilitate a shift in the intraerythrocytic equilibrium between HbSNO and GSNO); a significant proportion of NO is autocaptured to form Fe-NO. Vasodilatory NO bioactivity is conveyed from this membrane-associated compartment. Both intracellular and extracellular GSNO may provide a source of NO groups that is in equilibrium with SNO-Hb and thereby contributes to RBC vasoactivity.

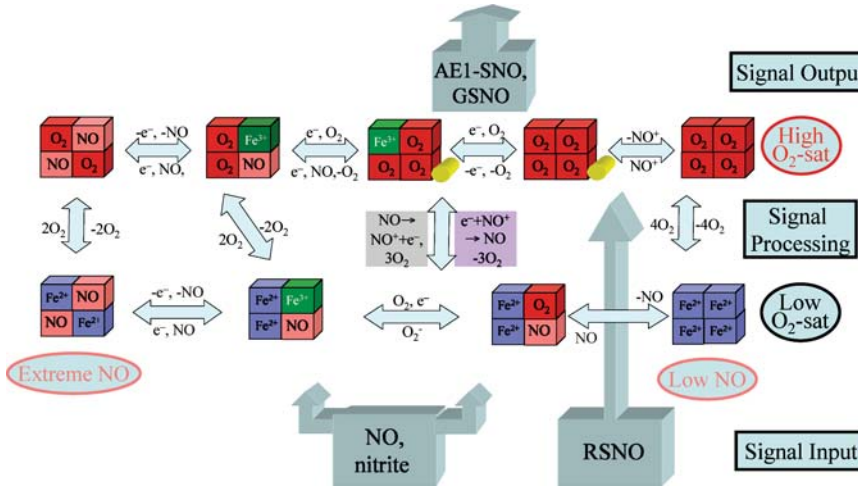

Figure 6 Chemical dynamics of NO interactions with hemoglobin. This perspective envisions Hb as a programmable chemical reactor in which NO chemistry is modulated, as illustrated, by ambient conditions of NO, oxygen, and redox potentials. Allosteric effectors would likewise modulate the chemistry through effects on oxygen saturation, oxidation, and spin states. NO signal input, entailing NO, nitrite, and thionitrites, is processed as directed by ambient conditions to provide appropriate output signals. For hypoxic vasodilation (*low NO*, *right*), output involves dispensing of vasodilatory activity through formation of S-nitrosylated AE-1 and perhaps GSNO (see Figure 3*e*). In the case of high levels of NO (*left*), for example in sepsis, the adaptive chemistry works to brake NO release (5-coordinate a nitrosyl Hb). The species indicated should be taken as exemplary, but underscore the role of minority species in this chemistry. In the tetrameric hemoglobins, shown as four squares, α chains are upper right and lower left and β chains are lower right and upper left.

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