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Commentary

Physiological reactions of nitric oxide and hemoglobin: A radical rethink

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With the recent visit of Drs. Furchgott, Ignarro, and Murad to Nobel-land, the discovery of nitric oxide (NO) as a vascular signaling molecule achieved global appreciation. This elevates NO to the lofty status of hemoglobin (Hb), arguably the best-understood protein from a molecular structure/function viewpoint and one that has been recognized by the well-deserved award of the 1962 Nobel prize in Chemistry to Max Perutz. Nonetheless, there is trouble in paradise. At the intersection of NO and hemoglobin biochemistry lies an abyss in our understanding that is so fundamental and deep as to challenge the biological function of each molecule. This problem has been largely ignored, but it has been visited in a recent report by Gow *et al.* (1).

Simply stated, the problem is that our dogmatic knowledge of chemical interactions of NO and hemoglobin predicts that (*i*) NO should not achieve a concentration in the vascular wall sufficient to elicit vasorelaxation, and (*ii*) nitrosylation of Hb, considered to be essentially irreversible, should result in the build-up of a complex that ultimately compromises life by interfering with tissue oxygenation. The obvious facts that endogenously produced NO is bioactive and Hb can deliver O_2 indicate that the chemistry which actually occurs between NO and Hb needs major revision. Notably, this chemistry had been defined by *in vitro* experimentation using supraphysiological concentrations of NO under nonbiological conditions (2). Although correct, it fails to adequately describe the situation at normal physiological levels of NO (3), where interaction of NO would occur with only a single globin subunit, at most, per predominantly O_2-saturated Hb tetramer. A growing body of evidence directs our attention to Hb allostery as the answer to this dilemma in adequately understanding NO/Hb chemistry.

It is well accepted that reaction with Hb is the major mechanism for disarming NO bioactivity. Moreover, reaction with Hb is certainly the major fate of NO in mammals. Two classical reactions of Hb and NO have been considered to be relevant. These are oxidation, in which NO reacts with oxy-Hb to yield met-Hb and nitrate, and addition, in which NO reacts with Fe(II)-Hb to give NO-Fe(II)-Hb. Only the oxy-Hb reaction was thought to be significant in physiology. Understandably, this view was based on a massive concentration of oxy-Hb resident in the circulation (4–8 mM, depending on location within the circulatory tree) and an *in vitro* reaction rate of NO with oxy-heme that is near diffusion-limited (2, 4, 5). The reaction between NO and oxy-Hb lies at the core of NO biology. The oxy-Hb reaction is assumed to be the fate of NO in vertebrates, explaining the endogenous origin of plasma nitrate (6, 7). NO biologists have capitalized on the perceived efficiency of this reaction for quantification of NO synthesis by enzymes and tissues, using met-Hb accumulation as a measure (8). A hallmark for establishing a role for endothelium-derived relaxing factor (EDRF)/NO in any biological system has been whether oxy-Hb scavenges the bioactivity (9–12).

Unfortunately, the oxy-Hb reaction of NO is so rapid that it should preclude all other reactions of NO, including those with established biological targets (e.g., soluble guanylyl cyclase). Estimates of NO biosynthesis in mammals (13) suggest that the NO/oxy-Hb reaction must be orders of magnitude slower than generally assumed to explain the detected levels of NO in biological systems (3, 14, 15). Thus, our present understanding of the oxy-Hb reaction is incompatible with a possible role for NO in biology. On the presumption that NO is not a practical joke played by tens of thousands of scientists on this year's Nobel Prize Committee, heme-NO chemistry cries out for a revisit.

It is of great biological significance that the Stamler laboratory has demonstrated a third relevant reaction of NO with Hb: S-nitrosylation of a cysteine residue that is conserved in β-globins of all birds and mammals (βCys^{93} of human Hb), yielding SNO-Hb (3, 16, 17). Importantly, SNO-Hb retains EDRF/NO-like bioactivity and is capable of transferring NO to low molecular weight thiol-containing molecules. Since *S*-nitrosothiols do not react with Fe(II)-Hb, they provide a protected route for delivery of bioactive NO equivalents from the erythrocyte to targets that affect smooth muscle relaxation (e.g., guanylyl cyclase). Notably, allosteric structural transitions of Hb, triggered by changes in oxygen tension *in vivo*, contribute to the molecular gymnastics that dictate NO addition and release from Hb-βCys^{93} (3, 17). In essence, S-nitrosylation of Hb occurs preferably in the R structure of Hb, which dominates at high oxygen tension (i.e., in lung), and release of NO from the resultant SNO-Hb occurs with transition to T structure at low oxygen tension (i.e., in capillaries) (3, 17). The net effect is that when O_2 is released from Hb in regions of low pO_2, the shift in Hb to T structure triggers NO release. By means of transnitrosation, this system allows NO bioactivity to be delivered to vascular smooth muscle as an X-SNO, a form that is protected from heme scavenging. Moreover, it enables NO equivalents to be released preferentially where pO_2 is lowest, dilating vessels and directing blood flow to the most ischemic tissues. Despite the elegance of this collaboration between NO and Hb in a system for optimized oxygen delivery, the *in vivo* formation of SNO-Hb was viewed with initial skepticism, given the perception of a voracious appetite for oxy-Hb to consume NO.

Gow *et al.* (1) report that the oxy-Hb reaction may in fact be an unimportant reaction of NO *in vivo*. Indeed, they argue that at physiological concentrations of NO, the oxy-Hb reaction is dominated by the addition reaction of NO to Fe(II)-Hb. Because Hb is typically 70–99% O_2-saturated under physiological conditions (18), the predominant Hb species for NO addition will be in the predominantly oxygenated (3 or more bound oxygen molecules) or R structure. Despite the extremely rapid rate constant for the NO/oxy-heme reaction of 3.7×10^7 $M^{-1} \cdot sec^{-1}$ (2), Gow *et al.* indicate that the NO/Fe(II)-Hb addition reaction to the R structure of Hb may be 100-fold faster, approaching the diffusion limit of 10^9 to 10^{10} $M^{-1} \cdot sec^{-1}$. Notably, this ultrarapid rate constant was unanticipated from earlier investigations of the

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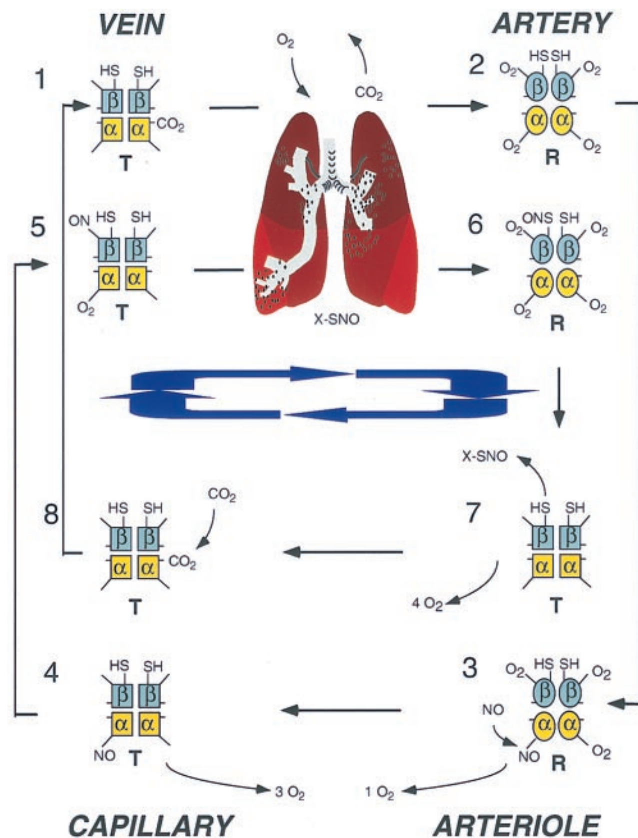

FIG. 1. This model depicts the physiologically relevant reactions of NO with Hb that have been proposed to occur during the respiratory cycle in erythrocytes (1, 3, 16, 17). Notably, these are the reactions that are considered to be most relevant for controlling NO bioactivity in blood vessels. An important aspect is that these reactions are modulated by R to T state structural transitions of Hb, assuming the simple two-state paradigm (49). Stages of addition and release of NO, O_2, and CO_2 by a single molecule of Hb are shown as Hb makes two complete cycles through the circulation. The first cycle is represented by the series of Hb molecules labeled **1–4**, and the second cycle by molecules labeled **5–8**. Molecule **8** is ready to begin the cycle anew as molecule **1**. Essential features are the efficient capture of NO by Fe(II)-Hb in T structure (molecule **3**), formation of SNO-Hb by NO transfer from Fe(II) to βCys^{93} (molecule **6**), and facilitated transnitrosation to produce X-SNO upon transition of Hb from R to T structure (molecule **8**). Over the two cycles, there is a net scavenging of one molecule of NO that is subsequently released as a bioactive NO equivalent (X-SNO, which can depart from the erythrocyte and elicit vascular responses). The Hb molecule shown represents approximately 1 in 1,000 that may carry NO at any given moment (3).

NO addition reaction that had been conducted under anaerobic conditions where NO was the sole ligand (i.e., Hb in T structure) (2, 5, 19). Thus, earlier studies failed to investigate the addition of NO to the biologically relevant form of Hb in which one unliganded Fe(II)-Hb subunit is typically available per otherwise oxygen-clad Hb tetramer.

If NO-Fe(II)-Hb possessed the stability ascribed to it in the literature (20, 21), this adduct would be expected to accumulate in the circulation. By analogy to the marked increase in O_2-binding affinity to Hb when CO is bound, it might have been expected that NO would similarly increase O_2-binding affinity (22) and accordingly, diminish the capacity for O_2 release. Fortunately for our survival as mammals, NO-Fe(II)-Hb is not the rock-stable molecule it was thought to be, and in fact it accumulates in the circulation to a significant extent only during pathological conditions of NO excess, such as endotoxic shock (23–25). Moreover, even in the case of endotoxic shock, there are indications that NO addition to Fe(II)-Hb does not mimic CO in its ability to increase oxygen-binding affinity (26).

The revision by Gow *et al.* (1) in our appreciation of the relative extent of NO reaction with oxy-heme vs. unliganded Fe(II)-Hb provides far more than a quantitative refinement of known chemical rates: from a biological viewpoint it harks a major paradigm shift. Inasmuch as NO-Fe(II)-Hb can transfer NO groups to thiols, via Hb-βCys^{93} (3), the NO addition reaction allows preservation of NO bioactivity, rather than eradication. Fig. 1 provides an integrated model of the respiratory cycle, highlighting recently proposed physiological reactions of Hb (1, 3, 16, 17) that may shape NO biology. Whilst the diminished capacity for the oxy-Hb reaction resolves a major paradox in NO biology, it also raises several new questions.

If the oxy-Hb reaction is not the major pathway of NO inactivation, it remains to be determined what is. Similarly, the source of NO-derived plasma nitrate demands redefinition. Possibilities for either of the above include reaction of NO with non-blood-borne globins (e.g., myoglobin) or perhaps other specialized NO-metabolizing enzymes that await discovery. Consideration of Fig. 1 draws attention to intracellular thiols as a key element for salvaging NO bioactivity from Hb. Can disease-associated or drug-induced oxidative stress diminish thiol levels to a point where NO delivery to physiological targets becomes compromised and vascular dysfunction results? If so, can we effectively increase thiol levels to enhance NO bioactivity in patients, thereby reconstituting physiological NO/Hb chemistry for clinical benefit? Such an elevation in thiols may contribute to apparent beneficial actions of antioxidants in conditions associated with deficient NO-mediated vasodilatation (e.g., hypertension, atherosclerosis, diabetes) (27, 28). In patients where NO replacement therapy may become a clinical goal, reactions shown in Fig. 1 would predict that a nitrosothiol could provide a more direct route for NO delivery to the vessel wall than a donor of NO itself.

Pulmonologists in intensive care units have been administering inhaled NO for treatment of patients suffering from acute respiratory distress syndrome (ARDS), assuming that scavenging of NO by oxy-Hb would prevent any systemic toxicity (29–31). The findings of Gow *et al.* (1) challenge this view and explain the otherwise problematic reports that inhaled NO can indeed elicit systemic actions, manifest as increased blood flow to ischemic tissues (32), enhanced glomerular filtration rate (33), lowered systemic blood pressure (30), and elevated cGMP accumulation in the aorta (34). Systemic NO actions may indeed contribute to the reported failure of inhaled NO to improve mortality in ARDS patients (35, 36) and accordingly, warrant judicious consideration.

The findings of Gow *et al.* (1) also invite caution on the use of the oxy-Hb reaction as the basis for a commonly used assay of NO synthesis. Contrary to popular belief, the efficiency of oxy-Hb oxidation by NO may not be uniform under all *in vitro* conditions where it has been used to measure NO synthesis rate. Nonetheless, it can clearly be used under specific conditions to accurately quantitate NO synthesis (e.g., 100 mM phosphate, 5–10 μM Hb). In any event, as we suggested earlier (37), uncertainty with the oxy-Hb capture assay can be obviated by substituting oxy-myoglobin, which, being an obligate monomer, precludes allosteric-induced changes in NO-binding affinity.

Therapeutic opportunities may be expected to arise from a molecular understanding of the *true* physiological reactions of NO and Hb in areas as diverse as sickle cell anemia, blood substitutes, and septic shock. Binding of NO has been shown to increase oxygen affinity of some sickle cell Hb (HbS) molecules, preventing polymerization of deoxy forms (38). This may contribute to the apparent therapeutic benefit of NO inhalation (38) and hydroxyurea therapy (39) in reducing sickle cell crisis, although NO-mediated vasodilation may be key to the observed clinical benefit. Inasmuch as HbS is typified by poor solubility and abnormal interactions with O_2, it is envisioned that NO chemistry with HbS will also be perturbed. Thus, one would anticipate sickle cell patients to exhibit global dysfunctions in NO-mediated va-

soactivity. Since altered NO/HbS chemistry may contribute to disease etiology, a molecular understanding of the specific dysregulation offers the potential for important new therapeutic insights.

In the field of blood substitutes, development of a useful agent has been thwarted to date by the problem that genetically engineered and chemically modified products invariably suffer from their ability to scavenge NO, thereby eliciting systemic hypertension (40, 41). Although polymerized forms of Hb that minimize extravasation may be beneficial (41), rational design of an optimal agent might incorporate the NO-donating thiol reaction pathway of Hb, while minimizing the capacity for NO oxidation.

Septic shock is a life-threatening vascular dysfunction arising from NO overproduction (42–44). To the extent that NO scavenging proves to be a useful therapeutic approach (compared with selective inhibition of NO synthase isoforms), an agent may be developed which favors the NO oxidation reaction, yet eliminates the ability to donate NO or oxidize O_2 (i.e., produce superoxide anion).

Over the past several years, hemoglobin has revealed itself to be a much smarter and more versatile molecule than even its most zealous aficionados had envisioned. The R to T structural transition of Hb is well established as the molecular switch triggering O_2 delivery from oxy-hemes to oxygen-starved tissues. We now understand that these transitions also modulate reactions of Hb involved in transfer reactions of NO within Hb (from iron to thiol) and finally to a cell-permeant thiol that delivers NO equivalents to biological targets (1, 3, 16, 17). Accordingly, Hb deserves to be recognized as an enzyme that coverts NO to X-SNO. We now appreciate that Hb has evolved to shuttle a triad of gases that are key to life: NO, CO_2, and O_2. While the listing of NO as first among these gases would appear to reflect the authors' bias, it may also reflect the evolutionary origin of Hb. Ancestral hemoglobins appear in bacteria and other microbes where their function cannot be attributed to O_2 delivery (45), but may instead serve a role in the detoxification of NO. This view would be consistent with the evolutionary appearance of simple bacterial hemoglobins at a time when the earth's early atmosphere was anoxic, but perhaps life-threatening in its NO content. Thus, ancestral hemoglobins may have initially functioned to detoxify NO and subsequently evolved toward a molecule that is optimized for oxygen delivery, permitting the evolution of large multicellular life forms. If so, we may owe our very existence to evolutionary pressure imposed by an NO-rich environment. It is notable that some present-day bacteria possess flavohemoglobins that do indeed metabolize NO, protecting them against the toxicity mediated by NO and NO-derived species (46–48).

Whatever their evolutionary origin, it is clear that the chemical interactions of Hb and NO are intricate and mold the biology of these two molecules. While Hb may be the best-understood of all proteins and NO among the simplest of all known molecules, it is remarkable that they continue to surprise us with unforeseen complexities.

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