Editorials

See related article, pages 976–983

NO Contest
Nitrite Versus S-Nitroso-Hemoglobin

Mark T. Gladwin, Alan N. Schechter

A recent flurry of research papers and commentaries in this journal\(^1\)–\(^4\) has highlighted a current major controversy in cardiovascular biochemistry and physiology: how is nitric oxide (NO) transported in the bloodstream. Two views have arisen. First, that S-nitrosated hemoglobin and albumin serve as stable storage forms of intravascular NO, and, in the case of S-nitrosated hemoglobin, as an allosterically regulated delivery vehicle for NO.\(^5\),\(^6\) The second is that the anion nitrite, which is present in relative abundance in both blood and tissue, subserves this function.\(^7\)

The controversy is relevant to the study by James et al in this issue of Circulation Research that examined the ability of red blood cells treated with NO, from healthy subjects and patients with diabetes, to vasodilate rabbit aortic rings.\(^4\) The investigators report that exposure of oxygenated red blood cells to NO in vitro results in increased levels of NO-modified hemoglobin, specifically iron-nitrosyl-hemoglobin (NO bound to the heme group) and S-nitroso-hemoglobin (NO bound to the cysteine 93 residue of the \(\beta\)-globin chain), and that these cells vasodilate rabbit aortic rings. The ability of the NO-treated red blood cells to vasodilate increases with greater NO exposure, raising intracellular S-nitrosohemoglobin levels and with progressive hypoxia. Additionally, they find that diabetic red blood cells form less S-nitroso-hemoglobin with NO exposure than normal red blood cells, dilate more at 1% oxygen concentration, and dilate less at 2% oxygen. They ascribe these observations to an impairment in NO delivery from glycohemoglobin (at 2% oxygen) and suggest that the reduced NO bioavailability in diabetes can be attributed to the red blood cells, in addition to the generally accepted mechanism of reduced endothelial NO production and NO inactivation by reactive oxygen species. Indeed, the authors suggest that elevated levels of iron-nitrosyl-hemoglobin may be a marker for poor glycemic control.

This commentary will not specifically focus on this newly proposed mechanism for microvascular pathology based on impaired NO delivery from blood, but on the two competing mechanisms for that delivery in the context of normal physiology—a topic far from resolved in the work of James et al.\(^4\) We begin by reviewing what we believe are the currently accepted principles, supported by both laboratory and clinical studies, for the interaction between NO and hemoglobin in blood.

Common Ground

NO Production From the Endothelium Accounts for Basal Physiological NO-Dependent Vascular Tone

It has been appreciated for almost two decades that endothelial NO production by NO synthases produces tonic vasodilation (approximately 25% of basal blood flow in humans) and accounts for the majority of both flow (shear stress)-mediated vasodilation and acetylcholine-mediated vasodilation. Thus, any putative intravascular NO-donating species cannot compensate for the loss of regional endothelial NO synthesis. The dominance of endothelial-derived NO versus blood-transported NO in basal blood flow regulation is illustrated by the fact that NO gas inhalation in humans,\(^8\) and recently demonstrated in cats,\(^3\) does not increase peripheral blood flow under normal physiological conditions. It is only with local inhibition of NO synthase activity (by infusion of L-NMMA or L-NAME) that subtle peripheral blood flow effects of blood-transported NO can be appreciated.\(^8\),\(^9\) Thus, the physiological role for blood-transported NO is now generally ascribed to that of hypoxic vasodilation.\(^2\),\(^7\)

Hemoglobin, per se, Is an NO Destroyer

Nitric oxide reacts in a nearly diffusion-limited reaction with oxyhemoglobin and deoxyhemoglobin to form methemoglobin and iron-nitrosyl-hemoglobin.\(^10\) These reactions inactivate NO and form part of the experimental basis supporting the identity of endothelium-derived relaxing factor as NO: investigators could show that addition of hemoglobin to blood vessels inhibited acetylcholine-mediated vasodilation. The NO scavenger and vasopressor effects of hemoglobin are limited by compartmentalization of hemoglobin within the erythrocyte. An unstirred diffusional barrier around the red blood cell and perhaps unique submembrane properties limit the rate of NO hemoglobin reactions by approximately 600-fold.\(^11\)–\(^14\) Thus, erythrocyte-free or plasma hemoglobin inactivates NO and produces vasoconstriction, an effect consistently described in basic animal and clinical studies of stroma-free hemoglobin blood substitutes and more recently in patients with hemolytic disease.\(^10\),\(^15\)–\(^17\) However, a paradoxical effect is observed with chemically S-nitrosated he-
moglobin or with nitrite-treated deoxyhemoglobin, the NO scavenger becomes an NO donor.

**Blood, Red Blood Cells, and Hemoglobin Treated With NO or Nitrite Can Mediate Vasodilation**

Inhalation of NO gas\(^8\) can produce distal vasodilation in the human forearm if local NO synthesis is inhibited. Infusions of NO-equilibrated buffer solutions have been shown to elicit vasodilation distal to the site of infusion.\(^1\) Similar effects have been observed in the feline intestine pretreated with NO synthase inhibitors\(^9\) or after ischemia-reperfusion injury.\(^3\) Consistent with this observation, numerous laboratories have used the experimental model of James et al\(^4\) and demonstrated that red blood cells treated with high concentrations of NO,\(^19\) S-nitrosocysteine,\(^20\) or nitrite\(^7\) can export an NO-derived species and produce vasodilation of rat or rabbit aortic rings.

The principle that blood may carry an NO derivative in an endocrine fashion to mediate distal vasodilation was first proposed by Stamler and Loscalzo and ascribed to S-nitrosated albumin.\(^21\) This paradigm was then extended to S-nitrosated hemoglobin, and a role for the red blood cell in oxygen-dependent NO delivery was further championed by Stamler’s group.\(^6\) These groups deserve full credit for advancing the then novel principle of NO storage and transport in blood. The relative balance of NO scavenging, limited by compartmentalization of hemoglobin within the erythrocyte membrane, and NO delivery, by S-nitroso-hemoglobin or the nitrite reductase activity of deoxyhemoglobin, would determine whether the red blood cell can transport/deliver or scavenge NO for a given physiological condition or vascular bed.

**The Heart of the Controversy**

With a clear understanding that we recognize that red blood cells can export NO activity under certain conditions and may contribute to oxygen-dependent NO homeostasis, studies of the mechanism for this effect have led to two alternative models. In particular, is the storage form of NO in blood an S-nitrosated protein or is it the anion nitrite? Is NO delivered from the hemoglobin cysteine residue by an oxygen-linked allosteric mechanism, or is the NO formed by the nitrite reductase activity of deoxyhemoglobin? Distinguishing between these two mechanisms is an absolute requirement for translational application to human disease and therapeutics. For example, will nitrite or S-nitroso-hemoglobin serve as sensitive and specific biomarkers of vascular dysfunction and NO deficiency? Which molecule will provide a therapeutic target for ischemic tissue or limit ischemia-reperfusion injury? Beyond a technical debate about methodologies, hemoglobin allostery, and chemical reactions lies the answer to a question with potentially broad therapeutic implications.

**The Devil in the Details**

**The Problem With S-Nitroso-Proteins as a Biological Storage Form for NO**

Two issues pose significant problems for the S-nitroso-hemoglobin paradigm: (1) It is not clear if significant amounts of S-nitrosated proteins exist in the basal human circulation. (2) There are no detectable arterial-to-venous gradients of S-nitrosated hemoglobin in the human circulation.

Prior studies by Stamler and colleagues first reported the level of S-nitrosated albumin in human blood to be 7 \(\mu\)mol/L\(^21\) and later lower at 700 nmol/L.\(^22\) However, multiple groups using validated techniques with high sensitivity and specificity, including the recent study by Ng et al in this journal,\(^3\) found plasma levels less than 40 nmol/L. NO-modified protein and this NO-protein complex is largely mercury stable (consistent with an N-nitrosamine or iron-nitrosyl complex and not an S-nitrosothiol).\(^8,18,21-33\) In our hands, the levels of plasma S-nitroso-albumin are less than 5 nmol/L, and we have been unable to detect higher levels of S-nitrosated albumin at rest or during NO gas inhalation in humans using Cu/cysteine- and tri-iodide-based reductive chemiluminescence, biotinylation assays, or the classic Griess-Saville assays (even with collection of samples in NEM and DTPA—required for stabilization of added standards). These nonphotolysis-based chemiluminescent methodologies used by multiple laboratories have been extensively validated. A recent editorial in this journal suggested that S-nitrosated hemoglobin or albumin standards cannot be used to validate analytic methods;\(^2\) however, by all conventions, an oxyhemoglobin molecule with a 29 mass unit NO group (subtracted proton in S-NO linkage) on the cysteine 93 residue of the \(\beta\)-chain (synthesized by a variety of methods) must be considered a viable standard or the science cannot be tested.

Using similar assays, our group and the Feelisch group have reported that S-nitroso-hemoglobin levels are less than 40 nmol/L or undetectable in human red blood cells.\(^20,31\) No arterial-to-venous gradients of S-nitroso-hemoglobin are observed by either group. Higher published values for S-nitroso-albumin and hemoglobin under normal physiological conditions have largely been measured using high-energy UV photolysis, a methodology that itself triggers conversion of blood nitrate to NO.\(^22\) Thus, methods based on high-energy UV photolysis of NO ligands from albumin or hemoglobin yield measurements 100- to 1000-fold higher than other high-sensitivity assays but are likely not specific for S-nitrosated proteins.

Of particular concern, the levels of S-nitroso-hemoglobin (0.0031 NO/Hb or 7.7 \(\mu\)mol/L in whole blood) and iron-nitrosyl-hemoglobin (0.0088 NO/Hb or 22 \(\mu\)mol/L in whole blood) reported in the present study by James and colleagues are orders of magnitude higher than any values currently measured in the field. While the chemiluminescent measurement of NO-modified proteins is the subject of considerable controversy, the measurement of NO-heme by electron paramagnetic resonance spectroscopy (EPR) is an accepted, highly specific gold standard with a sensitivity approaching 250 to 500 nmol/L in whole blood. Using this technique, iron-nitrosyl-hemoglobin is not detectable in the human circulation.\(^40\) The levels of iron-nitrosyl-hemoglobin of 22 000 nmol/L reported by James et al would provide a massive signal by EPR that has quite simply never been observed in human blood. We suggest that these authors
Is NO Delivery From S-Nitrosated Hemoglobin Oxygen-Dependent and Under Hemoglobin Allosteric (Oxygen-Linked) Control?

The S-nitroso-hemoglobin model has been described as requiring three steps:

1. NO binds cooperatively to a deoxygenated heme group of oxyhemoglobin (faster on R-state or oxyhemoglobin) to preserve the NO molecule and protect it from reaction with oxyhemoglobin. This step has been refuted by six independent groups

2. As hemoglobin reoxygenates (presumably after deoxygenation in the peripheral circulation) the NO group “transfers” to the cysteine. The problem is that this transfer requires a one-electron oxidation to an NO equivalent and has only been demonstrated in the presence of excess nitrite. This observation is in fact an effect of nitrite, not an oxygen-linked heme to thiol NO transfer. Without added nitrite, NO migration from heme to thiol is not observed, and with added nitrite, isotopic labeling has proven that the source of iron-nitrosyl-hemoglobin is from nitrite and not S-nitroso-hemoglobin.

3. Deoxygenated S-nitrosated hemoglobin releases the NO group by transnitrosation to the anion exchange protein on the red blood cell membrane for export, as a yet unidentified NO species. This last step is invoked in the present study by James et al to explain their observation that oxygenated red heme. It is therefore critically important to carefully compare the oxygen-dependent vasodilatory effects of NO-treated red blood cells with other nonallosteric NO donors.

Furthermore, an allosteric function of S-nitrosated hemoglobin is limited by reversible second-order transnitrosation reactions with intracellular glutathione. In the glutathione-rich reductive red blood cell, S-nitrosated hemoglobin rapidly decomposes independent of oxygen tension. Again, multiple groups have shown that S-nitrosated hemoglobin will vasodilate under both normoxia and hypoxia in the presence of greater than 100 μmol/L glutathione, i.e., under normal physiological glutathione concentrations. In this sense, S-nitrosated red blood cells can be considered “leaky bags” of NO, leaking NO out by transnitrosation, independent of oxygen tension; the apparent hypoxic effect appears to be secondary to the well-described hypoxic potentiation of all NO donors and nitrite, not an intrinsic allosteric property of S-nitroso-hemoglobin.

What Is Right About Nitrite

We recently proposed an alternative hypothesis that invokes a simple reaction of nitrite with the heme group of deoxyhemo-
globin, as well as other heme proteins, to generate NO and produce vasodilation.

Nitrite as the Ideal Vascular Storage Pool for NO

Nitrite is present in blood in concentrations of 500 to 1000 nmol/L and in 10 000 nmol/L in tissues. Nitrite is stable in blood compared with NO and S-nitrosothiol, as it is not as readily oxidized or reduced, respectively. Further, nitrite is selectively taken up by deoxygenated red blood cells and relatively stable with oxygenated red blood cells. Finally, nitrite is reduced to NO by deoxygenated hemoglobin. As such, nitrite is an ideal storage form for NO that is conserved under normoxic conditions and utilized under hypoxic conditions.

Nitrite Is a Vasodilator

Although some have argued that nitrite is not a vasodila-
tor, in recent studies, we found that near-physiological levels of nitrite vasodilate the human circulation, and levels of nitrite as low as 500 nmol/L vasodilate rat aortic rings in the presence of deoxygenating red blood cells. Whereas the vasodilatory effects of nitrite are potentiated by hypoxia per se by one order of magnitude, the vasodilatation is potentiated by four orders of magnitude in the presence of deoxygenated hemoglobin. We and others have therefore proposed a function for hemoglobin as a hypoxic-regulated nitrite reductase and suggest that this role might contribute to hypoxic vasodilation. Indeed, a recent study has demonstrated that tissue stores of nitrite are rapidly converted to NO with deoxygenation, suggesting a global role for this storage pool in hypoxia-dependent NO generation.

In the study by James et al, NO donors were added to oxygenated red blood cells. Such additions produce predominantly nitrate and methemoglobin and to a lesser extent nitrite and S-nitroso-hemoglobin. In fact, nitrite levels are anticipated to be 97% higher than S-nitrosothiols for NO additions to blood. Considering the fact that we have found that 500 nmol/L nitrite vasodilates aortic ring bioassays in the presence of deoxygenated erythrocytes, it would be of interest to compare the results observed by James and colleagues with NO donor additions versus equimolar additions of nitrite.

Are the Observed Vasodilatory Effects of Nitrite Secondary to the Formation of S-Nitrosated Hemoglobin?

Nitrite reactions with blood produce NO, iron-nitrosyl-hemoglobin, N-nitrosamines, nitrated tyrosine residues on proteins, and S-nitroso-hemoglobin. In this context, one can consider nitrite as the fundamental storage form for NO in the vasculature capable of forming a vast range of reaction products. This leads to the critical question as to whether S-nitroso-hemoglobin actually mediates the nitrite-dependent...
vasodilation. We believe the answer is that it does not. Definitive experiments using alkylated-blocked cysteine 93 or recombinant hemoglobin with cysteine to alanine, glycine, or leucine substitutions (mutants courtesy of Chien Ho, Carnegie Mellon University; Pittsburgh, Pa) reveal that the rate of nitrite reduction, NO generation, and vasodilation is far from reduced and actually greatly increased (Z. Huang, J. Crawford, M. Gladwin, and R. Patel, unpublished observations, 2004).

In conclusion, we have presented a critical and alternative perspective on how erythrocytes modulate vascular responses to hypoxia. While this function was initially ascribed to S-nitrosated hemoglobin, we find that this function is subserved by nitrite and the nitrite-reductase activity of deoxyhemoglobin. Further studies by multiple research groups evaluating the biochemistry, physiology, and the relative therapeutic activities of nitrite versus S-nitroso-hemoglobin will ultimately provide resolution to this debate. We can all agree that a deeper understanding of the multiple facets of red blood cell and NO biology will provide important insights into the mechanisms of vascular homeostasis and will offer novel therapeutic strategies for the treatment of vascular-related pathologies.

References


KEY WORDS: nitric oxide • hemoglobin • S-nitroso-hemoglobin • iron-nitrosyl-hemoglobin • nitrite
NO Contest: Nitrite Versus S-Nitroso-Hemoglobin
Mark T. Gladwin and Alan N. Schechter

Circ Res. 2004;94:851-855
doi: 10.1161/01.RES.0000126697.64381.37
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/94/7/851

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/