Chapter 17

ROLE OF THE RED BLOOD CELL IN NITRIC OXIDE HOMEOSTASIS AND HYPOXIC VASODILATION

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Abstract: Nitric oxide (NO) regulates normal vasomotor tone and modulates important homeostatic functions such as thrombosis, cellular proliferation, and adhesion molecule expression. Recent data implicate a critical function for hemoglobin and the erythrocyte in regulating the bioavailability of NO in the vascular compartment. Under normoxic conditions the erythrocytic hemoglobin scavenges NO and produces a vasopressor effect that is limited by diffusional barriers along the endothelium and in the unstirred layer around the erythrocyte. In hemolytic diseases, intravascular hemolysis releases hemoglobin from the red blood cell into plasma (decompartmentalizes the hemoglobin), which is then able to scavenge endothelial derived NO 600-fold faster than erythrocytic hemoglobin, thereby dysregulating NO homoestasis. In addition to releasing plasma hemoglobin, the red cell contains arginase which when released into plasma further dysregulates arginine metabolism. These data support the existence of a novel mechanism of human disease, hemolysis associated endothelial dysfunction, that potentially participates in the vasculopathy of iatrogenic and hereditary hemolytic conditions. In addition to providing an NO scavenging role in the physiological regulation of NO-dependent vasodilation, hemoglobin and the erythrocyte may deliver NO as the hemoglobin deoxygenates. Two mechanisms have been proposed to explain this principle: 1) Oxygen linked allosteric delivery of S-nitrosothiols from S-nitrosated hemoglobin (SNO-Hb), and 2) a nitrite reductase activity of deoxygenated hemoglobin that reduces nitrite to NO and vasodilates the human circulation along the physiological oxygen gradient. The later newly described role of hemoglobin as a nitrite reductase is discussed in the context of hypoxic vasodilation, blood flow regulation and oxygen sensing.

Key Words: SNO-Hb, hemoglobin, vasodilation, nitrite, nitric oxide, hemolysis, arginase

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INTRODUCTION

Hypoxic vasodilation is a highly conserved physiological response required to match blood flow and oxygen delivery to tissue metabolic demand. This systemic response has been appreciated for more than 100 years since the initial description by Roy and Brown in 1879 (76). Feedback hypoxic vasodilation requires oxygen or pH sensing to detect an "error signal" in the normal relationship between delivered blood oxygen content and tissue oxygen consumption (90). This error signal leads to the feedback generation of vasodilatory effectors that increase blood flow to maintain the balance of oxygen delivery and oxygen consumption. In mammals such vasodilation occurs as the hemoglobin desaturates from 60 to 40%, around a partial pressure of oxygen ranging from 40-20 mm Hg (75). Recent measurements of microcirculatory oxygen tension and hemoglobin oxygen saturation suggest that this degree of deoxygenation occurs within the resistance arterioles, especially in the case of skeletal muscle (87). In other tissues, such as heart and brain, more oxygen is extracted within the capillary network. Work by Segal and Duling suggests that NO or ATP delivery to the capillary circulation produces retrograde intracellular propagation of a vasodilating signal to the precapillary resistance vessels (80-82). These data in aggregate suggest that the oxygen or pH sensor is responsive to tissue oxygen partial pressures of 20-40 mm Hg and hemoglobin saturations of 40-60% (around the hemoglobin P_{50}).

Despite the physiological appreciation of this conserved blood flow response to hypoxia, the identity of the oxygen sensor mechanism and the specific feedback vasodilators remains uncertain and controversial. Even the site of sensing remains unknown with oxygen sensing and vasodilation either occurring within the A3-A4 arterioles or in the capillary network with retrograde propagation of a vasodilating signal through endothelium to the precapillary resistance arterioles. A number of mediators have been considered, including adenosine, nitric oxide (NO), K_{ATP} channels, endothelium derived hyperpolarizing factor (candidates include CO, H_2O_2 or $ONOO^-$), and prostacyclin, however, blockade of many of these pathways fails to completely inhibit hypoxic vasodilation (90, 91). These studies suggest that the system is either intricate and overbuilt with multiple effectors or that other undiscovered pathways exist.

An alternative paradigm has been advanced since 1995: That hemoglobin per se is the oxygen sensor with the oxygen linked allosteric structural transition of the hemoglobin tetramer from the oxygenated conformation (relaxed or R state) to the deoxygenated conformation (tense or T state) signaling the release or generation of a vasodilating signal from the erythrocyte (12). The first such hypothesis suggested that this R-to-T transition produced a release of ATP from the erythrocyte, which by binding to purinergic receptors in endothelium resulted in vasodilation (8, 12, 24, 37). This mechanism is supported by the observation of increasing concentrations of ATP in venous blood following hypoxia, and the in vitro generation of ATP from hypoxic or acidic erythrocytes. Evidence for retrograde propogation of the ATP/purinergic receptor/eNOS signal from the capillaries to precapillary arterioles further supports this hypothesis (12). The details and experimental evidence supporting this hypothesis will be covered in detail by Dr. Sprague's contribution to this series.

The second two hypotheses suggest that hemoglobin deoxygenation results in NO (equivalent) release from the red blood cell and subsequent NO-dependent vasodilation, however, the proposed mechanisms are fundamentally different. The first prosposed mech-

anism is that S-nitrosated hemoglobin (SNO-Hb) releases S-nitrosothiols during hemoglobin deoxygenation with subsequent generation of NO and vasodilation (25, 26, 38, 86). The second proposed mechanism suggests that hemoglobin is an allosterically regulated nitrite reductase which reduces nitrite to NO by deoxyheme as hemoglobin deoxygenates (5, 56). The present review will briefly review the overall role of the erythrocyte in vascular NO homeostasis, the specific mechanistic challenges facing the SNO-Hb hypothesis and then summarize the mechanism and emerging data supporting the nitrite reductase hypothesis.

NITRIC OXIDE AS A PARACRINE AND ENDOCRINE VASODILATOR (Figure 1)

Nitric oxide is produced from endothelial NO synthase and participates in the regulation of basal blood vessel tone and vascular homeostasis (antiplatelet activity, modulation of oxidant stress, endothelial and smooth muscle proliferation and adhesion molecule expression) (15, 33, 34, 64, 65). NO is a paracrine signaling molecule as it is produced in endothelium and then diffuses to vicinal smooth muscle, binds avidly to the heme of soluble guanylyl cyclase which produces cGMP, activates cGMP dependent protein kinases ultimately leading to smooth muscle relaxation. NO that diffuses into the lumen of the blood vessel is expected to react at a nearly diffusion limited rate (10^7 $M^{-1}sec^{-1}$) with both oxy- and deoxyhemoglobin to form methemoglobin/nitrate and iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO), respectively (62). These reactions limit the half life and diffusional distance of NO in blood (<2 millisecond half time) and maintain NO as a paracrine vasoregulator . The rapid irreversible nature of these NO-hemoglobin reactions and the massive concentration of hemoglobin in blood (10,000 μM heme) also suggest a great paradox in vascular biology: while we know that NO is a paracrine vasodilator, kinetic calculations suggest that all of the NO produced by endothelium should be inactivated by hemoglobin and the sphere of diffusion of NO should be extremely limited, i.e. diffusion to adjacent smooth muscle should be impossible (44).

However, we know that such diffusion is indeed possible, and the proposed solution to the paradox is illustrated in the central panel of Figure 1: during normal physiology the reaction of NO with hemoglobin is limited by compartmentalization of hemoglobin within the erythrocyte membrane (79). This compartmentalization of hemoglobin from endothelium creates two diffusional barriers: a cell free diffusion barrier along the endothelium in laminar flowing blood (3, 46) and an unstirred bulk diffusional barrier around the erythrocyte membrane (49). Additional studies suggest an intrinsic barrier within the membrane and submembrane protein matrix that further limits NO entry (29). Similar diffusional barriers modulate oxygen diffusion across the erythrocyte (4). Such barriers suggest a potential role for plasma enrichment in the microcirculation in NO homeostasis (the "Fahreus-Lindqvist" effect) and explain the morbidity and mortality of stroma-based blood substitutes and hemolytic disease (62, 73).

Hemolysis associated endothelial dysfunction and vasculopathy

Indeed, hemolytic diseases such as sickle cell disease and paroxysmal nocturnal hemo-

globinuria are associated with relative hypertension (compared to the hypotension of non-hemolytic anemias) (74), pulmonary hypertension (19, 39), esophageal and smooth muscle dystonias during paroxysms of hemolysis (28), and recently, in the case of sickle cell disease, the characterization of a state of NO resistance (73). Vasodilation during infusions of NO donors (nitroprusside, nitroglycerin, NONOates) is blunted in patients with sickle cell disease (10, 73) and in transgenic mouse models of sickle cell disease (41, 42, 60), and this resistance to NO correlates with plasma hemoglobin levels (42, 73). Not only does cell free plasma hemoglobin released during hemolysis disrupt the normal diffusional barriers but may also extravate into the extracellular space and directly intercept NO diffusing between endothelium to smooth muscle (58, 62). In addition to the release of hemoglobin during hemolysis, the red blood cell also contains large quantities of arginase, such that hemolysis increases plasma levels of this enzyme and metabolizes arginine to ornithine, reducing the substrate for endothelial NO synthase (19, 55, 83). Impairment of endothelium-dependent vasodilation leads to a state of hemolysis associated endothelial dysfunction, and with chronic hemolysis, a progressive proliferative vasculopathy. It is increasingly clear that multiple systems have evolved to limit the toxicity of cell free plasma hemoglobin, including the high molecular weight haptoglobin system (prevents extravasation of hemoglobin and limits NO scavenging) (11, 57, 92), hemopexin, CD163 hemoglobin scavenger protein (which not only mediates haptoglobin-hemoglobin clearance but also upregulates IL-10 and hemeoxygenase 1) (69), and the hemeoxygenase 1/biliverdin reductase/p21 pathways which exert anti-inflammatory, anti-oxidant and anti-proliferative effects (1, 13, 59, 63, 77).

Endocrine properties of NO

In addition to a paracrine vasodilator function, there is increasing appreciation that NO may be stabilized by the formation of NO modified proteins, peptides and lipids, as well as by oxidation to the anion nitrite. The principle that NO may be thus stabilized in blood, and the inactivation reactions with hemoglobin thus limited, was first proposed by Loscalzo and Stamler. They hypothesized that NO (abstraction of an electron required) could form a covalent bond with cysteine residues on albumin to form S-nitrosated albumin (SNO-albumin) (78, 85). This paradigm was later extended by the Stamler group to S-nitrosated hemoglobin (SNO-Hb) (38). While this field is extremely controversial, largely secondary to major questions about the concentrations and importance of SNO-albumin and SNO-hemoglobin in the human circulation (reported values range from a few μM to undetectable, with more modern methodologies documenting levels of less than 10 nM) (18, 20, 50, 70, 71, 84, 92), it is likely that there are a number of intravascular species capable of endocrine vasodilation, including S-nitrosothiols (61, 85), nitrite (5), N-nitrosamines (27, 48, 70), iron-nitrosyls (18), and recently identified nitrated lipids (47). This review will briefly review the SNO-Hb hypothesis, the major challenges to this theory, and then focus on the role of nitrite in vasoregulation and hypoxic vasodilation.

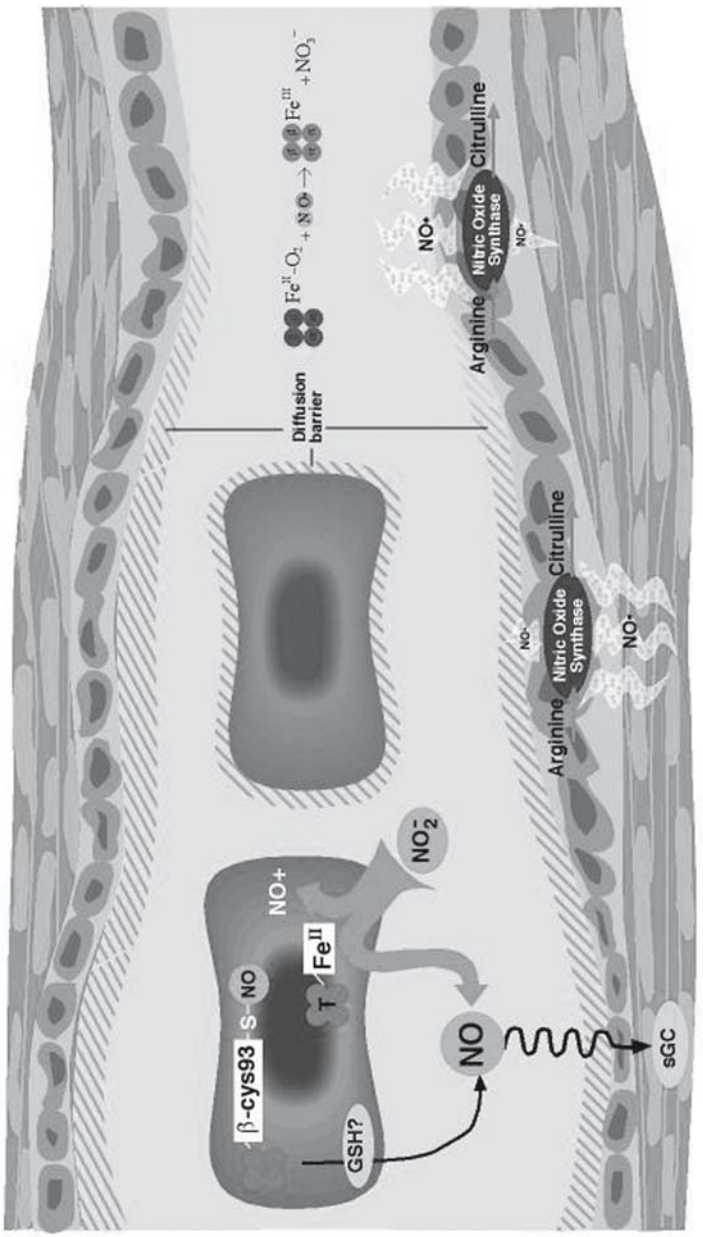

Figure 1. Endocrine, paracrine and autocrine properties of NO. Nitric oxide as a paracrine vasodilator (middle of figure), whose scavenging by hemoglobin is limited by the compartmentalization of hemoglobin within the intact erythrocyte. Nitric oxide as an endocrine vasodilator (left side of figure) being transported in blood as nitrite or an S-nitrosothiol. Nitric oxide as an autocrine vasodilator (right side of figure), whose diffusion is limited by scavenging by cell free plasma hemoglobin released during pathological hemolysis. Adapted with permission from: Schechter AN and Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med* 348: 1483-1485, 2003.

The SNO-hemoglobin hypothesis

Based on observed artery-to-vein gradients in SNO-Hb in the rat and the ability of S-nitrosated hemoglobin to vasodilate aortic ring preparations and the rat circulation it was proposed that NO represented a third gas molecule in the human respiratory cycle (26, 38, 86). A complicated mechanism was proposed suggesting that NO produced by the endothelium would react with a vacant heme on oxygenated hemoglobin (three $HbFe^{II}$-O_2 per tetramer and one $HbFe^{II}$-NO per tetramer) and thus trap and "preserve" the NO on hemoglobin (25, 52). The NO would lose an electron (mechanism not demonstrated) and then migrate to the ß-globin chain cysteine 93 residue to form an S-nitrothiol bond. This SNO-Hb would then transfer the NO+ group by transnitrosation to the erythrocyte membrane anion exchange protein (AE1 or band 3) thiols followed by export of a yet to be identified intermediate species (called X-NO) (68). This would presumably be an S-nitrosothiol which would need to be reduced to NO to activate soluble guanylate cyclase.

While the principle was elegant, the mechanism has been severely challenged (Figure 2) (17). Multiple laboratories have now shown that NO does not bind preferentially to vacant hemes on oxygenated hemoglobin (cooperative NO binding is not observed) (18, 30, 31, 40, 96). The required transfer of NO from the heme to the cysteine has not been observed using electron paramagnetic resonance spectroscopy (unless large concentrations of nitrite contaminate the experiment) (95). Importantly, this transfer requires the abstraction of an electron from the NO and a mechanism for this reduction has not been determined over the last 9 years. Finally, multiple groups have been unable to reproduce the levels of both SNO-albumin or SNO-hemoglobin reported by the Stamler group, and the artery-to-vein gradients have not been detected in the human circulation by other groups (18, 22, 50, 61, 70-72, 95). Finally, SNO-Hb is not stable in the reductive intra-erythrocytic environment at 37°C and is rapidly reduced by intracellular glutathione and ferrous heme (6, 7, 22). Thus the mechanism for formation, levels in the circulation, and oxygen dependent delivery of the S-NO group have all been challenged.

While our work suggests that SNO-Hb does not participate in the process of hypoxic vasodilation in the basal human circulation, we do find support for the principle that the red blood cell and hemoglobin participates in oxygen dependent NO homeostasis. Rather than hemoglobin being a reservoir of S-nitrosated hemoglobin, we find that hemoglobin is an enzymatic nitrite reductase with a deoxyheme-nitrite reaction generating NO as hemoglobin deoxygenates within the circulation (5, 16).

Vasoactivity of nitrite in the human circulation

While large doses of nitrite given as an antidote for cyanide poisoning clearly produces hypotension in humans (94), the large concentrations of nitrite required to vasodilate aortic ring bioassay systems led to a dismissal of nitrite as a vasoactive reservoir of NO in the circulation. Indeed, nitrite at concentrations exceeding 100 μM was shown to vasodilate aortic ring bioassays by Furchgott as far back as 1952, and shown by Murad and Ignarro to activate guanylate cyclase in the mid 1970's and early 1980's (14, 35, 36, 54). However, studies published by Laur and colleagues suggested that nitrite had no intrinsic vasodilator activity and led to a premature dismissal of nitrite as a physiological vasodilator (45, 51, 67).

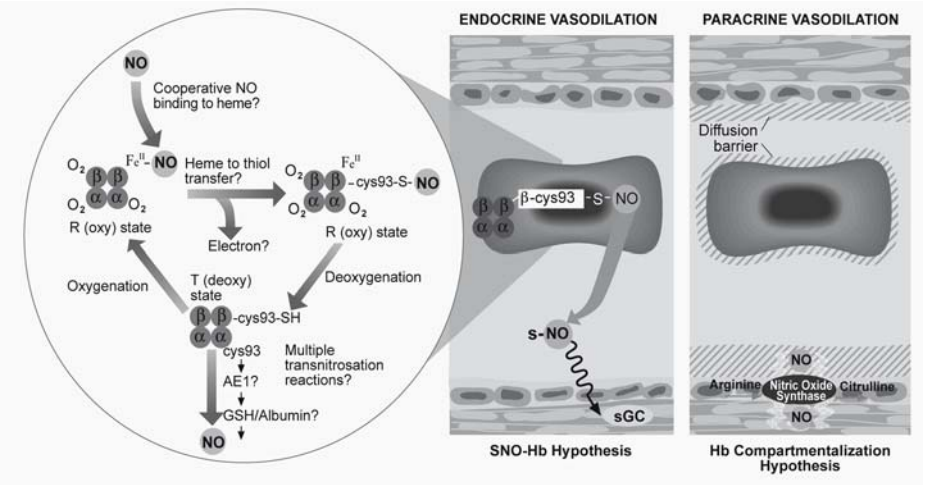

Figure 2. Mechanism proposed for SNO-hemoglobin mediated hypoxic vasodilation (left panel). A complicated mechanism was proposed suggesting that NO produced by the endothelium would react with a vacant heme on oxygenated hemoglobin (three $HbFe^{II}$-O_2 per tetramer and one $HbFe^{II}$-NO per tetramer) and thus trap and "preserve" the NO on hemoglobin (25, 52). The NO would lose an electron (mechanism not demonstrated) and then migrate to the ß-globin chain cysteine 93 residue to form an S-nitrothiol bond. This SNO-Hb would then transfer the NO+ group by transnitrosation to the erythrocyte membrane anion exchange protein (AE1 or band 3) thiols followed by export of a yet to be identified intermediate species (called X-NO) (68). This would presumably be an S-nitrosothiol which would need to be reduced to NO to activate soluble guanylate cyclase. The question marks reflect specific challenges to the mechanism discussed in the text. Figure reproduced with permission from: Gladwin MT, Lancaster JR, Freeman BA, and Schechter AN. Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. *Nat Med* 9: 496-500, 2003.

Despite the apparent lack of bioactivity of nitrite in these more recent studies, we observed arterial-to-vein gradients in nitrite across the human forearm, with increased consumption of nitrite during exercise stress, suggesting that nitrite was metabolized across the peripheral circulation (21). We therefore hypothesized that nitrite might be reduced to NO during hypoxic and acidic stress by the actions of xanthine oxidoreductase (23, 53) or by acidic reduction (disproportionation) (97). To test this we infused nitrite into the forearm brachial artery of 18 healthy volunteers and to our surprise, observed substantial vasodilation, even without exercise stress. Nitrite was remarkable potent, increasing blood flow by 170% at 200 µM and by 22% at 2.5 µM. Even levels of 900 nM vasodilated during exercise stress with concurrent NO synthase inhibition with L-NMMA (Figure 3) (5). A vasodilation at these concentrations under normal physiological non-stress conditions was inconsistent with a mechanism of reduction by xanthine oxidoreductase or disproportionation, as both of these pathways require very low pH and extreme hypoxia, thus suggesting an alternative mechanism of nitrite bioactivation. Additional studies have been published in the last year confirming the vasodilating effects of nitrite (32, 43, 88, 89, 93).

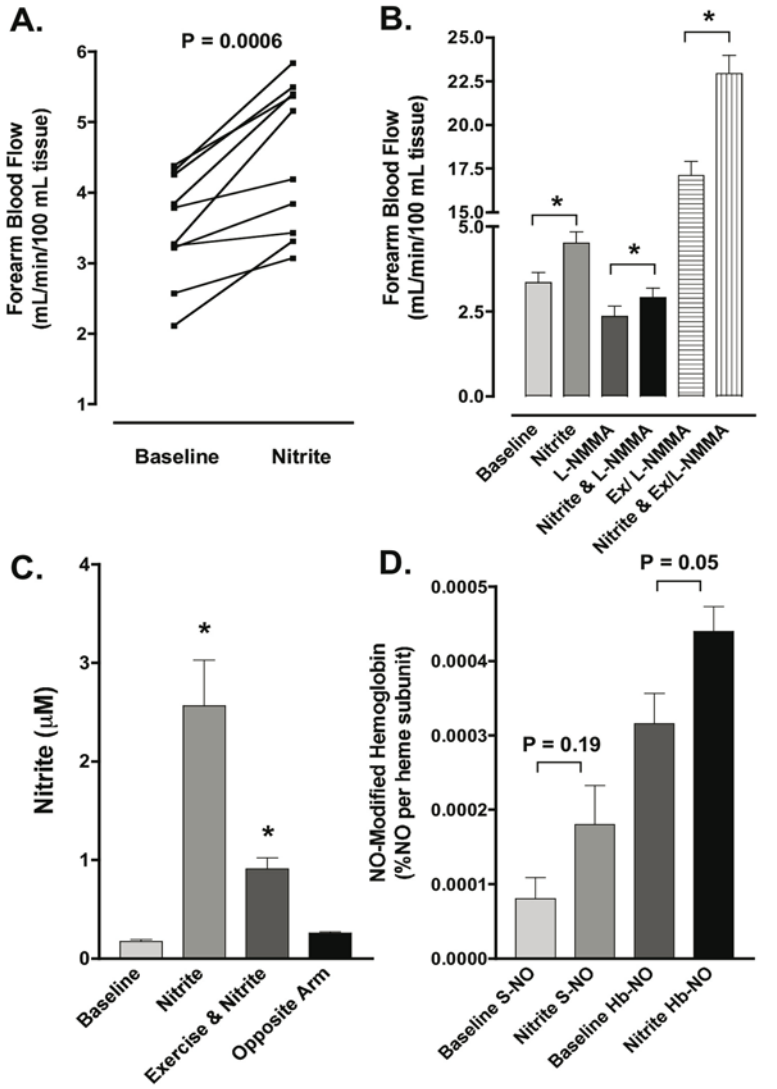

Figure 3. Nitrite vasodilates the human circulation at near physiological concentrations. Panel A: Nitrite infusion increases blood flow 22% in ten normal volunteers. Panel B: Increase in blood flow occurs at rest, during exercise and during exercise with NO synthase inhibition and L-NMMA infusion. Panel C: Blood flow increases with regional nitrite concentrations of 2.5 μM at rest and 900 nM during exercise. Panel D: Blood flow increases during nitrite infusions are associated with the formation of iron-nitrosyl-hemoglobin from artery-to-vein. Figure reproduced with permission from Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, and Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498-1505, 2003.

Hemoglobin as an allosterically and electronically regulated nitrite reductase

During nitrite infusions into the brachial artery we observed the artery-to-venous formation of iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO) suggesting that nitrite was being reduced to NO rapidly within one half-circulatory time (5). An analysis of the iron-nitrosyl-hemoglobin levels during all experimental conditions revealed a striking inverse correlation with oxyhemoglobin saturation, i.e. as hemoglobin deoxygenated more NO formed. These physiological observations were consistent with a reaction between nitrite and deoxyhemoglobin to form NO as described by Doyle and colleagues in 1981 (9):

$NO_2^- + HbFe^{II}$ (deoxyhemoglobin) $+H^+ \rightarrow$ NO (nitric oxide) + $HbFe^{III} + OH^-$

Much of the formed NO is then captured as iron nitrosyl-hemoglobin ($HbFe^{II}$-NO) on viscinal hemes and measured as a "dosimeter" of NO production in venous blood:

NO + $HbFe^{II}$ (deoxyhemoglobin) $\rightarrow$ $HbFe^{II}$-NO (iron-nitrosyl-hemoglobin)

Consider the potential physiological implications of this simple equation. The reaction requires deoxyhemoglobin and a proton, providing oxygen and pH sensor chemistry, and generates NO, a potent vasodilator. Methemoglobin formed during the reaction will not autocapture and inactivate the NO formed. In additional experiments we found that nitrite, red cells (or hemoglobin), and hypoxia were required for in vitro hypoxic vasodilation of rat aortic rings. Indeed, in the presence of hypoxia and erythrocytes (conditions never tested in historical aortic ring bioassay studies) nitrite now vasodilated aortic rings at physiological concentrations of 200-500 nM (Figure 4) (5).

Using an in vitro aortic ring bioassay systems designed by the Patel lab to simultaneously measure vessel force tension and oxygen tension, we find that vasodilation is measureably potentiated by as low as 200 nM nitrite under hypoxic conditions (5). Importantly, these studies reveal that nitrite-red blood cell dependent vasodilation is initiated at an oxygen tension around the intrinsic hemoglobin P_{50} (PaO_2 of 40 mm Hg for rat erythrocytes and 30 mm Hg for human erythrocytes). In ongoing unpublished work from four laboratories (the Gladwin, Patel, Kim-Shapiro, and Hogg groups) we have now found that this vasodilation occurs as hemoglobin unloads oxygen to 50% saturation, and that this vasodilation is mediated by a maximal nitrite reductase activity of hemoglobin allosterically linked to its intrinsic P_{50}. This maximal reductase activity is allosterically regulated as oxygen binding to one heme decreases the redox potential of the other hemes in the tetramer, thus increasing the ability of the hemes to donate an electron and reduce nitrite. An ideal balance of available deoxyhemes for nitrite binding and oxyhemes - required to lower redox potential of the vacant hemes - is met at the 50% hemoglobin saturation (the P_{50}). Indeed the measured rate of nitrite reduction by hemoglobin is maximal at a hemoglobin-oxygen saturation between 40-60%. Such a maximal reductase activty at P_{50} is biochemically consistent with a role in hypoxic vasodilation because physiological studies demontrate an onset of hypoxic vasodilation at 40-60% hemoglobin oxygen saturation (75).

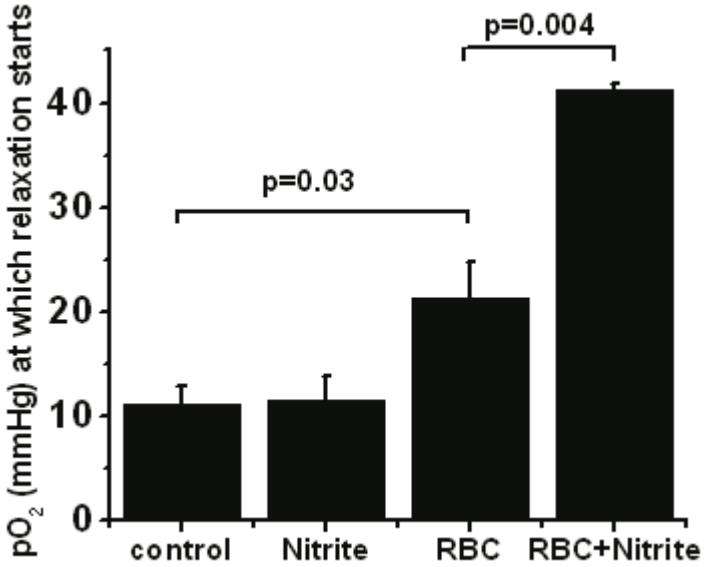

Figure 4. In the presence of hypoxia and erythrocytes nitrite vasodilates aortic rings at a PaO_2 of 30-40 mm Hg. Using an in vitro aortic ring bioassay system designed by the Patel laboratory to simultaneously measure vessel force tension and oxygen tension, we find that vasodilation is measureably potentiated by nitrite under hypoxic conditions. While control rat aortic rings and nitrite alone vasodilate at an oxygen tension of approximately 10 mm Hg, nitrite and red blood cells vasodilate at an oxygen tension around the intrinsic hemoglobin P_{50} (PaO_2 of 40 mm Hg for rat erythrocytes as shown in this figure and 30 mm Hg for human erythrocytes - data not shown). This experiment utilizes 2 μM nitrite and 0.3% red cell hematocrit. Figure based on data from Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, and Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498-1505, 2003.

INTEGRATED BIOCHEMICAL PHYSIOLOGY

Nitrite appears to fit requirements for a physiological mediator of hypoxic vasodilation as it maximally reacts with hemoglobin at 40-60% hemoglobin saturation, an oxygen tension (20-40 mm Hg) significantly higher than that required for SNO-hemoglobin deoxygenation, i.e. cysteine 93 liganded hemoglobins have very high oxygen affinities (2, 66). In the normal skeletal muscle circulation oxygen tension decreases from the A1 caliber arterioles (100 μmeter diameter) to the A4 caliber arterioles (20 μmeter diameter) to values as low as 20 mm Hg prior to the capillary circulation (87). These data suggest that much of the oxygen delivery occurs within the arterioles allowing for spacially linked oxygen delivery and vasomotor control. Additional mechanisms suggest that NO or ATP delivery to the capillary circulation produces retrograde intracellular propagation of vasodilating

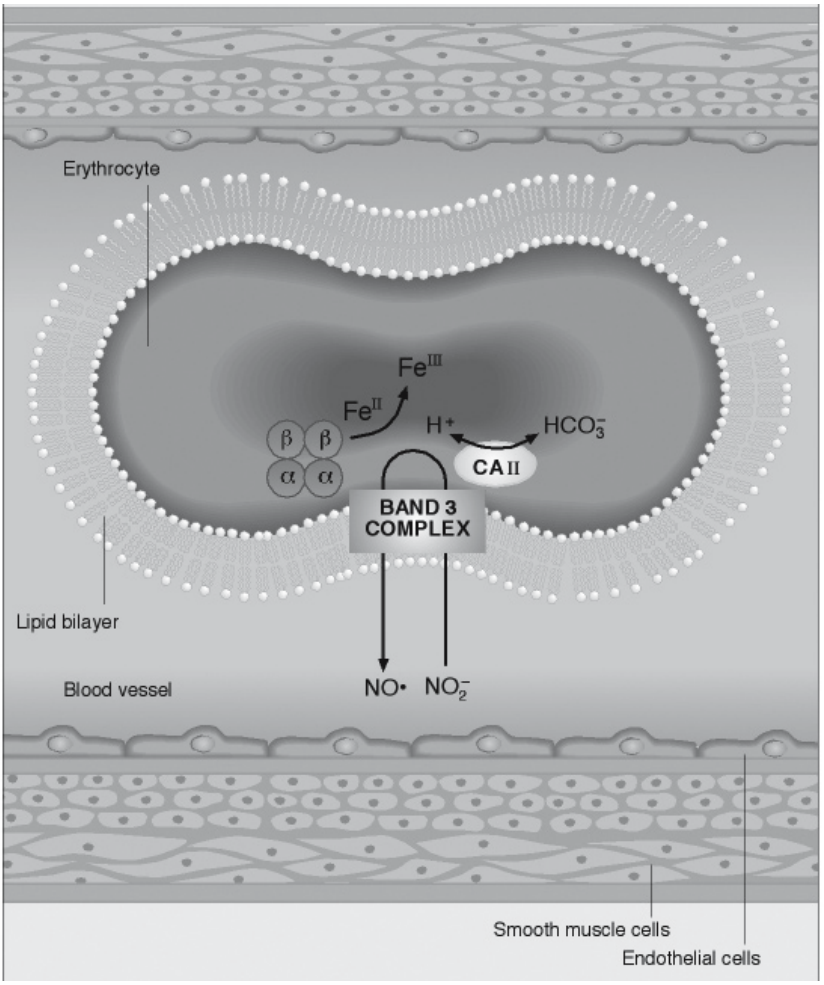

Figure 5. Putative nitrite reductase metabolon. We speculate that the erythrocyte membrane proteins provide a potential nitrite reductase metabolon function composed of deoxyhemoglobin and methemoglobin, anion exchange protein, carbonic anhydrase, aquaporin and Rh channels. Such as system would concentrate nitrite, proton, deoxyheme and highly hydrophobic channels at the membrane complex. Reproduced with permission from: Gladwin MT, Crawford JH, and Patel RP. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radic Biol Med* 36: 707-717, 2004.

signal to the precapillary resistance vessels (80-82). Thus a maximal nitrite reductase activity at the hemoglobin P_{50} appears ideal for oxygen sensing and hypoxic vasodilation as this allosteric point is thermally, chemically and electronically responsive to physiologically relevant tissue metabolic stress. We speculate that the erythrocyte membrane proteins provide a potential nitrite reductase metabolon function composed of deoxyhemoglobin and methemoglobin, anion exchange protein, carbonic anhydrase, aquaporin and Rh channels

(16). Such as system would concentrate nitrite, proton, deoxyheme and highly hydrophobic channels at the membrane complex (Figure 5) (16). The lipophilicity and potency of NO (EC_{50} of only 1-5 nM) requires very little NO escape to regulate vasodilation, especially considering that flow is proportional to the radius to the fourth power.

REFERENCES

1. Baranano DE, Rao M, Ferris CD, and Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A* 99: 16093-16098, 2002.
2. Bonaventura C, Ferruzzi G, Tesh S, and Stevens RD. Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. *J Biol Chem* 274: 24742-24748, 1999.
3. Butler AR, Megson IL, and Wright PG. Diffusion of nitric oxide and scavenging by blood in the vasculature. *Biochim Biophys Acta* 1425: 168-176., 1998.
4. Coin JT and Olson JS. The rate of oxygen uptake by human red blood cells. *J Biol Chem* 254: 1178-1190., 1979.
5. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, and Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498-1505, 2003.
6. Deem S, Kim JU, Manjula BN, Acharya AS, Kerr ME, Patel RP, Gladwin MT, and Swenson ER. Effects of S-nitrosation and cross-linking of hemoglobin on hypoxic pulmonary vasoconstriction in isolated rat lungs. *Circ Res* 91: 626-632., 2002.
7. Deem S, Kim SS, Min JH, Eveland R, Moulding J, Martyr S, Wang X, Swenson ER, and Gladwin MT. Pulmonary vascular effects of red blood cells containing S-nitrosated hemoglobin. *Am J Physiol Heart Circ Physiol*, 2004.
8. Dietrich HH, Ellsworth ML, Sprague RS, and Dacey RG, Jr. Red blood cell regulation of microvascular tone through adenosine triphosphate. *Am J Physiol Heart Circ Physiol* 278: H1294-1298, 2000.
9. Doyle MP, Pickering RA, DeWeert TM, Hoekstra JW, and Pater D. Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. *J Biol Chem* 256: 12393-12398, 1981.
10. Eberhardt RT, McMahon L, Duffy SJ, Steinberg MH, Perrine SP, Loscalzo J, Coffman JD, and Vita JA. Sickle cell anemia is associated with reduced nitric oxide bioactivity in peripheral conduit and resistance vessels. *Am J Hematol* 74: 104-111, 2003.
11. Edwards DH, Griffith TM, Ryley HC, and Henderson AH. Haptoglobin-haemoglobin complex in human plasma inhibits endothelium dependent relaxation: evidence that endothelium derived relaxing factor acts as a local autocoid. *Cardiovasc Res* 20: 549-556, 1986.
12. Ellsworth ML, Forrester T, Ellis CG, and Dietrich HH. The erythrocyte as a regulator of vascular tone. *Am J Physiol* 269: H2155-2161, 1995.
13. Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, and Snyder SH. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat Cell Biol* 1: 152-157, 1999.
14. Furchgott RF and Bhadrakom S. Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J Pharmacol Exp Ther* 108: 129-143, 1953.
15. Furchgott RF and Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373-376, 1980.
16. Gladwin MT, Crawford JH, and Patel RP. The biochemistry of nitric oxide, nitrite, and

hemoglobin: role in blood flow regulation. *Free Radic Biol Med* 36: 707-717, 2004.
17. Gladwin MT, Lancaster JR, Freeman BA, and Schechter AN. Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. *Nat Med* 9: 496-500, 2003.
18. Gladwin MT, Ognibene FP, Pannell LK, Nichols JS, Pease-Fye ME, Shelhamer JH, and Schechter AN. Relative role of heme nitrosylation and beta -cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. *Proc Natl Acad Sci U S A* 97: 9943-9948, 2000.
19. Gladwin MT, Sachdev V, Jison ML, Shizukuda Y, Plehn JF, Minter K, Brown B, Coles WA, Nichols JS, Ernst I, Hunter LA, Blackwelder WC, Schechter AN, Rodgers GP, Castro O, and Ognibene FP. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *N Engl J Med* 350: 886-895, 2004.
20. Gladwin MT and Schechter AN. NO contest: nitrite versus S-nitroso-hemoglobin. *Circ Res* 94: 851-855, 2004.
21. Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, Ognibene FP, and Cannon RO, 3rd. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci U S A* 97: 11482-11487, 2000.
22. Gladwin MT, Wang X, Reiter CD, Yang BK, Vivas EX, Bonaventura C, and Schechter AN. S-nitrosohemoglobin is unstable in the reductive red cell environment and lacks O2/NO-linked allosteric function. *J Biol Chem* 21: 21, 2002.
23. Godber BL, Doel JJ, Sapkota GP, Blake DR, Stevens CR, Eisenthal R, and Harrison R. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *J Biol Chem* 275: 7757-7763, 2000.
24. Gonzalez-Alonso J, Olsen DB, and Saltin B. Erythrocyte and the regulation of human skeletal muscle blood flow and oxygen delivery: role of circulating ATP. *Circ Res* 91: 1046-1055, 2002.
25. Gow AJ, Luchsinger BP, Pawloski JR, Singel DJ, and Stamler JS. The oxyhemoglobin reaction of nitric oxide. *Proc Natl Acad Sci U S A* 96: 9027-9032, 1999.
26. Gow AJ and Stamler JS. Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391: 169-173, 1998.
27. Gruetter CA, Barry BK, McNamara DB, Kadowitz PJ, and Ignarro LJ. Coronary arterial relaxation and guanylate cyclase activation by cigarette smoke, N'-nitrosonornicotine and nitric oxide. *J Pharmacol Exp Ther* 214: 9-15, 1980.
28. Hillmen P, Hall C, Marsh JC, Elebute M, Bombara MP, Petro BE, Cullen MJ, Richards SJ, Rollins SA, Mojcik CF, and Rother RP. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. *N Engl J Med* 350: 552-559, 2004.
29. Huang KT, Han TH, Hyduke DR, Vaughn MW, Van Herle H, Hein TW, Zhang C, Kuo L, and Liao JC. Modulation of nitric oxide bioavailability by erythrocytes. *Proc Natl Acad Sci U S A* 98: 11771-11776., 2001.
30. Huang Z, Louderback JG, Goyal M, Azizi F, King SB, and Kim-Shapiro DB. Nitric oxide binding to oxygenated hemoglobin under physiological conditions. *Biochim Biophys Acta* 1568: 252-260., 2001.
31. Huang Z, Ucer KB, Murphy T, Williams RT, King SB, and Kim-Shapiro DB. Kinetics of nitric oxide binding to R-state hemoglobin. *Biochem Biophys Res Commun* 292: 812-818., 2002.
32. Hunter CJ, Dejam A, Blood AB, Shields H, Kim-Shapiro DB, Machado RF, Tarekegn S, Mulla N, Hopper AO, Schechter AN, Power GG, and Gladwin MT. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. *Nat Med* 10: 1122-1127, 2004.
33. Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G. Endothelium-derived

relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84: 9265-9269., 1987.
34. Ignarro LJ, Byrns RE, Buga GM, and Wood KS. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res* 61: 866-879, 1987.
35. Ignarro LJ and Gruetter CA. Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. *Biochim Biophys Acta* 631: 221-231., 1980.
36. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ, and Gruetter CA. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J Pharmacol Exp Ther* 218: 739-749, 1981.
37. Jagger JE, Bateman RM, Ellsworth ML, and Ellis CG. Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation. *Am J Physiol Heart Circ Physiol* 280: H2833-2839, 2001.
38. Jia L, Bonaventura C, Bonaventura J, and Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control (see comments). *Nature* 380: 221-226, 1996.
39. Jison ML and Gladwin MT. Hemolytic anemia-associated pulmonary hypertension of sickle cell disease and the nitric oxide/arginine pathway. *Am J Respir Crit Care Med* 168: 3-4, 2003.
40. Joshi MS, Ferguson TB, Jr., Han TH, Hyduke DR, Liao JC, Rassaf T, Bryan N, Feelisch M, and Lancaster JR, Jr. Nitric oxide is consumed, rather than conserved, by reaction with oxyhemoglobin under physiological conditions. *Proc Natl Acad Sci U S A* 17: 17, 2002.
41. Kaul DK, Liu XD, Chang HY, Nagel RL, and Fabry ME. Effect of fetal hemoglobin on microvascular regulation in sickle transgenic-knockout mice. *J Clin Invest* 114: 1136-1145, 2004.
42. Kaul DK, Liu XD, Fabry ME, and Nagel RL. Impaired nitric oxide-mediated vasodilation in transgenic sickle mouse. *Am J Physiol Heart Circ Physiol* 278: H1799-1806., 2000.
43. Kozlov AV, Costantino G, Sobhian B, Szalay L, Umar F, Nohl H, Bahrami S, and RedlH. Mechanisms of vasodilatation induced by nitrite instillation in intestinal lumen: possible role of hemoglobin. *Antioxid Redox Signal* 7: 515-521, 2005.
44. Lancaster JR, Jr. A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* 1: 18-30., 1997.
45. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, and Kelm M. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci U S A* 98: 12814-12819., 2001.
46. Liao JC, Hein TW, Vaughn MW, Huang KT, and Kuo L. Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc Natl Acad Sci U S A* 96: 8757-8761., 1999.
47. Lim DG, Sweeney S, Bloodsworth A, White CR, Chumley PH, Krishna NR, Schopfer F, O'Donnell VB, Eiserich JP, and Freeman BA. Nitrolinoleate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity. *Proc Natl Acad Sci U S A* 99: 15941-15946, 2002.
48. Lippton HL, Gruetter CA, Ignarro LJ, Meyer RL, and Kadowitz PJ. Vasodilator actions of several N-nitroso compounds. *Can J Physiol Pharmacol* 60: 68-75, 1982.
49. Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, and Lancaster JR, Jr. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem* 273: 18709-18713, 1998.
50. Marley R, Feelisch M, Holt S, and Moore K. A chemiluminescense-based assay for S-nitrosoalbumin and other plasma S- nitrosothiols. *Free Radic Res* 32: 1-9, 2000.

51. McMahon TJ. Hemoglobin and nitric oxide. *N Engl J Med* 349: 402-405; author reply 402-405, 2003.
52. McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, Stolp BW, Gow AJ, Pawloski JR, Watke P, Singel DJ, Piantadosi CA, and Stamler JS. Nitric oxide in the human respiratory cycle. *Nat Med* 3: 3, 2002.
53. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, and Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* 427: 225-228, 1998.
54. Mittal CK, Arnold WP, and Murad F. Characterization of protein inhibitors of guanylate cyclase activation from rat heart and bovine lung. *J Biol Chem* 253: 1266-1271, 1978.
55. Morris CR, Morris SM, Hagar W, Van Warmerdam J, Claster S, Kepka-Lenhart D, Machado L, Kuypers FA, and Vichinsky EP. Arginine Therapy: A New Treatment for Pulmonary Hypertension in Sickle Cell Disease? *Am J Respir Crit Care Med*, 2003.
56. Nagababu E, Ramasamy S, Abernethy DR, and Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin mediated nitrite reduction. *J Biol Chem*, 2003.
57. Nakai K, Ohta T, Sakuma I, Akama K, Kobayashi Y, Tokuyama S, Kitabatake A, Nakazato Y, Takahashi TA, and Sadayoshi S. Inhibition of endothelium-dependent relaxation by hemoglobin in rabbit aortic strips: comparison between acellular hemoglobin derivatives and cellular hemoglobins. *J Cardiovasc Pharmacol* 28: 115-123, 1996.
58. Nakai K, Sakuma I, Ohta T, Ando J, Kitabatake A, Nakazato Y, and Takahashi TA. Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. *J Lab Clin Med* 132: 313-319, 1998.
59. Nath KA, Katusic ZS, and Gladwin MT. The perfusion paradox and vascular instability in sickle cell disease. *Microcirculation* 11: 179-193, 2004.
60. Nath KA, Shah V, Haggard JJ, Croatt AJ, Smith LA, Hebbel RP, and Katusic ZS. Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease. *Am J Physiol Regul Integr Comp Physiol* 279: R1949-1955., 2000.
61. Ng ES, Jourd'heuil D, McCord JM, Hernandez D, Yasui M, Knight D, and Kubes P. Enhanced S-nitroso-albumin formation from inhaled NO during ischemia/reperfusion. *Circ Res* 94: 559-565, 2004.
62. Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, and Lemon DD. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med* 36: 685-697, 2004.
63. Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, and Choi AM. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 6: 422-428, 2000.
64. Palmer RM, Ashton DS, and Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664-666, 1988.
65. Palmer RM, Ferrige AG, and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524-526, 1987.
66. Patel RP, Hogg N, Spencer NY, Kalyanaraman B, Matalon S, and Darley-Usmar VM. Biochemical Characterization of Human S-Nitrosohemoglobin. Effects on oxygen binding and transnitrosation. *J Biol Chem* 274: 15487-15492, 1999.
67. Pawloski JR. Hemoglobin and nitric oxide. *N Engl J Med* 349: 402-405; author reply 402-405, 2003.
68. Pawloski JR, Hess DT, and Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature* 409: 622-626., 2001.
69. Philippidis P, Mason JC, Evans BJ, Nadra I, Taylor KM, Haskard DO, and Landis RC. Hemoglobin Scavenger Receptor CD163 Mediates Interleukin-10 Release and Heme Oxygenase-1 Synthesis. Antiinflammatory Monocyte-Macrophage Responses In Vitro, in

Resolving Skin Blisters In Vivo, and After Cardiopulmonary Bypass Surgery. *Circ Res*, 2003.
70. Rassaf T, Bryan NS, Kelm M, and Feelisch M. Concomitant presence of N-nitroso and S-nitroso proteins in human plasma. *Free Radic Biol Med* 33: 1590-1596., 2002.
71. Rassaf T, Bryan NS, Maloney RE, Specian V, Kelm M, Kalyanaraman B, Rodriguez J, and Feelisch M. NO adducts in mammalian red blood cells: too much or too little? *Nat Med* 9: 481-483, 2003.
72. Rassaf T, Feelisch M, and Kelm M. Circulating no pool: assessment of nitrite and nitroso species in blood and tissues. *Free Radic Biol Med* 36: 413-422, 2004.
73. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, Schechter AN, and Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 8: 1383-1389., 2002.
74. Rodgers GP, Walker EC, and Podgor MJ. Is "relative" hypertension a risk factor for vaso-occlusive complications in sickle cell disease? *Am J Med Sci* 305: 150-156, 1993.
75. Ross JM, Fairchild HM, Weldy J, and Guyton AC. Autoregulation of blood flow by oxygen lack. *Am J Physiol* 202: 21-24, 1962.
76. Roy CS, and Brown, J.G. *Journal of Physiology, London* 2: 323, 1879.
77. Ryter SW, Otterbein LE, Morse D, and Choi AM. Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. *Mol Cell Biochem* 234-235: 249-263, 2002.
78. Scharfstein JS, Keaney JF, Jr., Slivka A, Welch GN, Vita JA, Stamler JS, and Loscalzo J. In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. *J Clin Invest* 94: 1432-1439, 1994.
79. Schechter AN and Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med* 348: 1483-1485, 2003.
80. Segal SS and Duling BR. Communication between feed arteries and microvessels in hamster striated muscle: segmental vascular responses are functionally coordinated. *Circ Res* 59: 283-290, 1986.
81. Segal SS and Duling BR. Conduction of vasomotor responses in arterioles: a role for cell-to-cell coupling? *Am J Physiol* 256: H838-845, 1989.
82. Segal SS and Duling BR. Flow control among microvessels coordinated by intercellular conduction. *Science* 234: 868-870, 1986.
83. Spector EB, Rice SC, Kern RM, Hendrickson R, and Cederbaum SD. Comparison of arginase activity in red blood cells of lower mammals, primates, and man: evolution to high activity in primates. *Am J Hum Genet* 37: 1138-1145, 1985.
84. Stamler JS. S-nitrosothiols in the blood: roles, amounts, and methods of analysis. *Circ Res* 94: 414-417, 2004.
85. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, and Loscalzo J. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci U S A* 89: 7674-7677, 1992.
86. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, and Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 276: 2034-2037, 1997.
87. Tsai AG, Johnson PC, and Intaglietta M. Oxygen gradients in the microcirculation. *Physiol Rev* 83: 933-963, 2003.
88. Tsuchiya K, Kanematsu Y, Yoshizumi M, Ohnishi H, Kirima K, Izawa Y, Shikishima M, Ishida T, Kondo S, Kagami S, Takiguchi Y, and Tamaki T. Nitrite is an alternative source of NO in vivo. *Am J Physiol Heart Circ Physiol*, 2004.
89. Tsuchiya K, Takiguchi Y, Okamoto M, Izawa Y, Kanematsu Y, Yoshizumi M, and Tamaki T. Malfunction of vascular control in lifestyle-related diseases: formation of systemic hemoglobin-nitric oxide complex (HbNO) from dietary nitrite. *J Pharmacol Sci* 96: 395-

400, 2004.
90. Tune JD, Gorman MW, and Feigl EO. Matching coronary blood flow to myocardial oxygen consumption. *J Appl Physiol* 97: 404-415, 2004.
91. Tune JD, Richmond KN, Gorman MW, and Feigl EO. K(ATP)(+) channels, nitric oxide, and adenosine are not required for local metabolic coronary vasodilation. *Am J Physiol Heart Circ Physiol* 280: H868-875, 2001.
92. Wang X, Tanus-Santos JE, Reiter CD, Dejam A, Shiva S, Smith RD, Hogg N, and Gladwin MT. Biological activity of nitric oxide in the plasmatic compartment. *Proc Natl Acad Sci U S A* 101: 11477-11482, 2004.
93. Webb A, Bond R, McLean P, Uppal R, Benjamin N, and Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci U S A* 101: 13683-13688, 2004.
94. Weiss S, Wilkins, R.W., and Haynes F.W. The nature of circulatory collapse induced by sodium nitrite. *Journal of Clinical Investigation* 16: 73-84, 1937.
95. Xu X, Cho M, Spencer NY, Patel N, Huang Z, Shields H, King SB, Gladwin MT, Hogg N, and Kim-Shapiro DB. Measurements of nitric oxide on the heme iron and beta-93 thiol of human hemoglobin during cycles of oxygenation and deoxygenation. *Proc Natl Acad Sci U S A* 100: 11303-11308, 2003.
96. Zhang Y and Hogg N. Mixing artifacts from the bolus addition of nitric oxide to oxymyoglobin: implications for S-nitrosothiol formation. *Free Radic Biol Med* 32: 1212-1219., 2002.
97. Zweier JL, Wang P, Samouilov A, and Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues (see comments). *Nat Med* 1: 804-809, 1995.