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Forum Review

Oxygen Sensing in the Circulation: "Cross Talk" Between Red Blood Cells and the Vasculature

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ABSTRACT

Oxygen (O_2) sensing in blood and regulation of microvascular tone appear to involve hemoglobin (Hb) conformational changes resulting from O_2 desaturation. This observation has prompted the thought that Hb functions as both an O_2 sensor and regulator of microvasular blood flow to meet local tissue oxygen demand. The mechanism(s) by which this is accomplished has recently been the subject of increasing debate. Three primary hypotheses are described within the literature and include release of adenosine 5'-triphosphate by red blood cells (RBCs), release of *S*-nitrosylated molecules from RBCs originally bound to β93 cysteine residues of oxyHb, and nitrite conversion and storage of nitric oxide by Hb at the site of ferric (Fe^{3+}) and ferrous (Fe^{2+}) Hb. Within extravascular cells, the global regulator of oxygen homeostasis is hypoxia-inducible factor-1 (HIF-1). This transcriptional factor is tightly regulated by O_2 and cellular redox-sensitive mechanisms. HIF-1 activation is responsible for the up-regulation of proteins, which increase O_2 supply. We believe that there are important and yet unexplored mechanisms by which RBCs can directly or indirectly communicate via redox intermediates with extravascular sites as part of the global O_2 sensing mechanism. *Antioxid. Redox Signal.* 6, 1000–1010.

INTRODUCTION

In 1939, Cannon described a fundamental observation and problem of microvascular physiology still being rigorously studied 65 years later. In *The Wisdom of the Body* (6), Cannon posed the following: "The dilation of the arterioles and capillaries in active muscles is one of the most remarkable emergency adjustments. What causes the capillaries to dilate is not yet understood. However, the capillaries may be opened, the great importance of their being opened should not be overlooked." In this review, we pose the logical question: Do oxygen (O_2) carriers and sensors in the blood and vasculature cross talk? There is a rapidly accumulating body of experimental evidence and recently developing hypotheses that suggest hemoglobin (Hb) functions as an O_2 sensing mechanism in blood and initiates, via conformational and redox transitions, activation of vasodilatory regulators. O_2 sensors (*i.e.*, Hb) and second messengers that function routinely to detect local microvascular changes in O_2 concentration have been proposed to involve adenosine 5'-triphosphate (ATP), nitric oxide (NO), *S*-nitrosylated thiols (RSNO), and nitrite (NO_2^-). Although the precise mechanisms by which these regulators of blood flow and subsequently tissue oxygenation are not entirely well defined, each hypothesis contains a commonality of thought focused on Hb. Additionally, each of these hypotheses proposes NO as a common end-stage regulator of perfusion. It is of interest that vascular O_2 sensing is focused on heme proteins and redox regulation of what has been termed the global or master regulator of O_2 homeostasis, hypoxia-inducible factor-1 (HIF-1). However, it is not well established, and in fact rarely mentioned, that a potential route of communication, other than O_2 itself, exists be-

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tween the O_2 sensing and regulation mechanisms in blood and the vascular environment. We have recently suggested that extracellular Hb influences HIF-1 expression during conditions of hypoxia and that the interaction between extracellular Hb and HIF-1 expression apparently occurs through redox mechanisms (84, 85). We would similarly suggest that mechanisms of O_2 sensing and regulation that occur in blood directly influence O_2 sensing and regulation in vascular cells. It seems likely that the common links between the two redox sites are Hb, NO, and HIF-1. Messengers other than NO such as superoxide anion ($O_2^{\bullet -}$) originating from red blood cells (RBCs) have also been proposed (64, 65, 75).

O_2 SENSING IN BLOOD: MODULATORS OF FLOW AND OXYGENATION

The ability of RBCs to sense local O_2 partial pressure (pO_2) and adjust blood flow to meet O_2 demand has been suggested according to several hypotheses. Additionally, current thinking in this area is the subject of much debate; however, certain basic principles are common across all lines of thought. Hb, for example, is central to these hypotheses, and each relies on either a direct or indirect role of Hb in modulation and release of regulators of vascular tone. Three potential mechanisms by which O_2 sensing occurs in blood with subsequent regulation of flow and O_2 delivery are presented, and general limitations to these hypotheses are discussed.

ATP

Several investigators have demonstrated that RBCs release ATP from a large intracellular pool during conditions of hypoxia (1, 18, 19). Figure 1 summarizes events described in the literature leading up to and following ATP production and release from RBCs. ATP released into the blood vessel lumen binds directly to and activates the type $2Y_1$, $2Y_2$, $2Y_{11}$, and $2X_4$ purinergic receptors on the luminal surface of endothelial cells and appears to result in dilation of microvascular resistance vessels via a NO-mediated mechanism (82). Conversely, vasoconstriction is mediated via type $2Y_2$, $2Y_6$, and $2X_1$ purinergic receptors on the cells of the vascular smooth muscle, indicating that the vasoregulatory action of ATP in response to hypoxia is confined to the vascular lumen. McCollough *et al.* (50) demonstrated that intraluminal addition of 1 μ*M* ATP produced an arteriolar diameter increase of 8–10% and a 17% increase in blood flow in the hamster cheek pouch retractor muscle. This effect was blocked by the NO synthase inhibitor, *N*ω-nitro-L-arginine methyl ester (L-NAME), alone and after addition of L-arginine + L-NAME. Under normal conditions, RBCs produce and retain millimo-

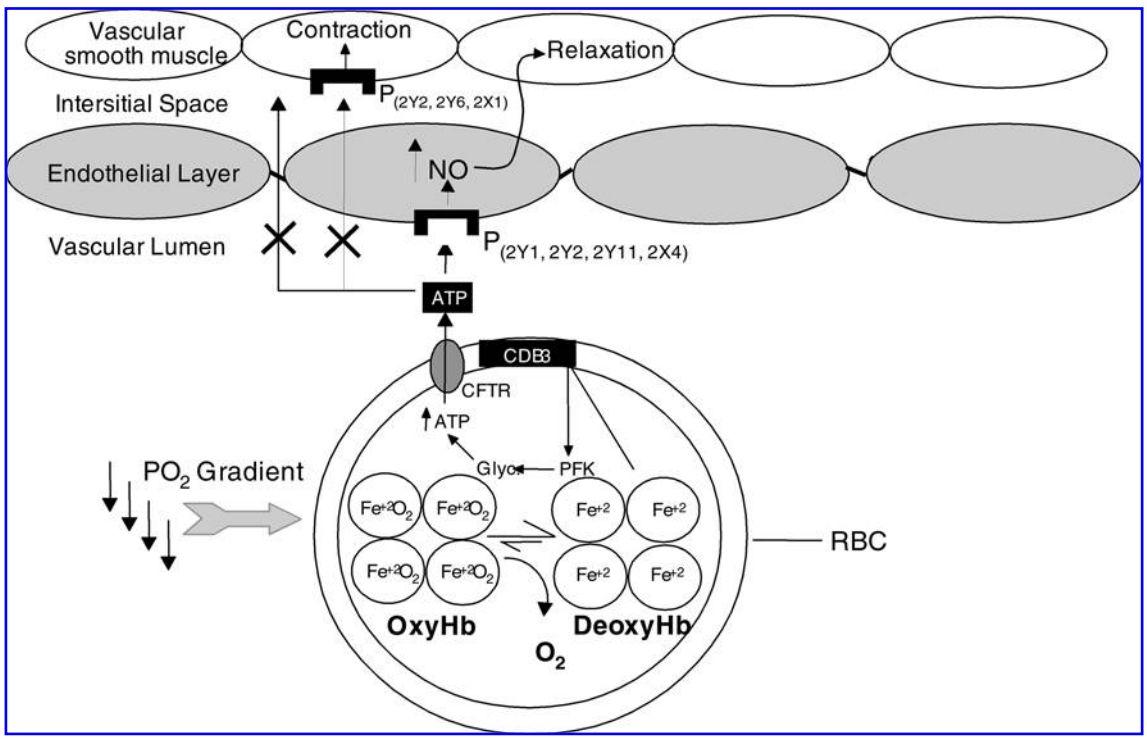

FIG. 1. A representation of ATP in the regulation of blood flow and tissue oxygenation under conditions of decreasing O_2 availability. Within the vascular lumen, oxyHb undergoes conformational transitioning to deoxyHb with release of O_2 as a decreasing gradient of pO_2 is experienced from arterioles to capillaries to venules by RBCs. Subsequently, deoxyHb may displace phosphofructokinase (PFK) from cytoplasmic domain of band 3 (CDB3), creating increased glycolysis (Glyc.) and ATP accumulation within the RBC **(bottom)**. ATP efflux from the RBC is believed to occur via cystic fibrosis transmembrane receptor (CFTR), allowing ATP to activate endothelial cell purinergic-2 receptor (P_2) subtypes, which increases endothelial cell production of NO **(middle)**. The end result is vascular smooth muscle (VSM) relaxation as opposed to contraction, which is associated with ATP activation of VSM localized P_2 subtypes **(top)**.

lar quantities of ATP (54); thus, it is conceivable that quantities of ATP released under conditions of hypoxia could reach the micromolar concentrations required for dilation of microvascular resistance vessels. The mechanism(s) by which ATP is released from RBCs in hypoxic environments is not completely defined; however, Jagger *et al*. (37) suggest that reducing the O_2 saturation of Hb is likely the initial step. Experiments performed by this group demonstrate that decreasing oxyhemoglobin (oxyHb) (R state) and subsequently increasing deoxyhemoglobin (deoxyHb) (T state) is correlative with increasing plasma ATP concentrations. By locking Hb in its R state via carbon monoxide (CO) saturation, the same group demonstrated no change in plasma ATP concentrations compared with normal conditions, furthering the idea that Hb's conformation plays an important role in the process (37). Moreover, excess deoxyHb binds to the N-terminus of the cytoplasmic domain of band 3 (CDB3) in RBCs, displacing the key regulator of glycolysis, phosphofructokinase (PFK), resulting in increased glycolysis and ATP production (46). Thus, a potential mechanism for increasing intracellular levels of ATP during periods of Hb desaturation seems to support this hypothesis. ATP appears to be released from RBCs, after the following sequence of events: Hb desaturation, deoxyHb binding to CDB3, increased ATP production, and RBC deformation. Experimental evidence is reported by Sprague *et al*. (72) to support a cyclic AMP-mediated activation of the cystic fibrosis transmembrane conductance regulator (CFTR), one of several ion channels making up the ATP binding cassette in the regulation of intracellular ATP efflux (63). Clinical support for this overall hypothesis of RBC O_2 sensing is demonstrated in vascular airway diseases such as cystic fibrosis, where CFTR is known to be nonfunctional (11), and in primary pulmonary hypertension (73). In both conditions, decreased ATP release and RBC deformation are reported in response to hypoxia (70, 71).

S-Nitrosohemoglobin (SNO-Hb)

Another proposed mechanism for O_2 sensing, regulation of blood flow and oxygen delivery by RBCs relies on the hypothesis that Hb transitioning between R state and T state modulates binding and release of NO from the 93 position β_1 and β_2 globin chain cysteine residues (Cysβ93) (38). It proposes that deoxyHb initially binds NO at the site of β globin chain hemes (β$HbFe^{2+}$NO) in the venous circulation. During oxygenation in the lungs, transitioning from T to R state conformation facilitates the release and transfer of NO to the thiols of the two Cysβ93 residues forming SNO-Hb (47). As SNO-Hb enters the microvascular circulation and traverses the arterioles and capillaries, a decreasing gradient of pO_2 is experienced, causing release of O_2 from oxyHb and transition of Hb conformation from the R to the T state. As pO_2 becomes exceedingly low in the resistance vessels (<6 mmHg), NO is then released from Cysβ93 residues (74) as summarized in Fig. 2A. However, factors influencing the fate of NO within the RBC, transport of NO out of the RBC, and the mechanism of vasorelaxation caused by NO originating from SNO-Hb are all unclear. The authors of the SNO-Hb hypothesis suggest that NO released from SNO-Hb is transferred to or undergoes a transnitrosylation with RBC glutathione (GSH) or thiols (RSH) on the band 3 protein forming *S*-nitrosoglutathione (GSNO) and RSNO, respectively (51). These nitrosylated thiols (GSNO and RSNO) are speculated to be transported from the RBC by a yet to be defined mechanism and dilate the vasculature. Additional controversy associated with this hypothesis of O_2 sensing in blood is that it does not directly account for the high rates of NO reactivity with oxyHb at the heme sites, which forms methemoglobin (metHb; $HbFe^{3+}$) and nitrate (NO_3^-) (17) or the high rate of NO binding to deoxyHb at α globin chain heme sites (7). Thus, NO entering the erythrocyte should readily be consumed at sites other than the two Cysβ93 residues. Moreover, several factors restrict the free access of NO to Hb within RBCs as demonstrated by a diffusion barrier created by the vascular endothelium, a RBC free zone close to the luminal boundary of the blood vessel, varying distances through the luminal space, an unstirred layer surrounding the RBC, flow rate of RBCs, and finally the RBC membrane (43, 76–78). Taken together, these physical barriers to NO diffusion and consumption by Hb within the RBC are limited by 500–1,000 times that of acellular Hb circulating in the vascular space (77). Therefore, the idea of SNO-Hb raises several questions, which challenge the *in vivo* relevance of the hypothesis as it pertains to O_2 sensing and blood flow regulation. Yet another challenge to this hypothesis comes from the well documented hypertensive side effect associated with all cell-free Hb-based blood substitutes due to their ability to avidly scavenge NO at the heme sites. This effect is caused in part by the absence of physical barriers to NO, but in some cases by a lack of T to R state transition created by chemical and/or recombinant modification to Hb that limits β globin chain thiol accessibility (unpublished observations).

NO_2^- reduction

An alternative hypothesis to that of SNO-Hb, which also depends on NO as a regulator of blood flow and tissue oxygenation, suggests that nitrite (NO_2^-) originating from extracellular sites and deoxyHb within RBCs may play an important role in O_2 sensing and subsequent modulation of blood flow and O_2 delivery. The basis of this hypothesis relies on the observation that a storage pool of a stabilized NO species, potentially NO_2^-, is available for conversion to active NO during periods of decreased O_2 availability (24). Thus, significant reserves of an inactive stored form of NO (*i.e.*, NO_2^-) should be present *in vivo*, which on its own exerts no biological action under normal conditions. Rassaf *et al*. (61) reported plasma NO_2^- to be ~200 n*M* in both rats and humans, whereas Rodriguez *et al*. (66) demonstrated that the major NO-related storage products in rat thoracic aorta are NO_2^- (~10 μ*M*) and NO_3^- (~ 40 μ*M*). *S*- and *N*-nitroso compounds were found to be present at ~30–40 n*M* concentrations in the thoracic aorta of rats, indicating a role for NO_2^-, which may be more relevant than SNO-Hb (66). These data suggest that NO_2^- may in part be a relevant storage product of NO. Cosby *et al*. (13) evaluated the physiologic role of NO_2^- during conditions of increased metabolic activity by measuring the blood flow response to NO_2^- infusion at rest and during exercise in the human forearm. The investigators found that NO_2^-

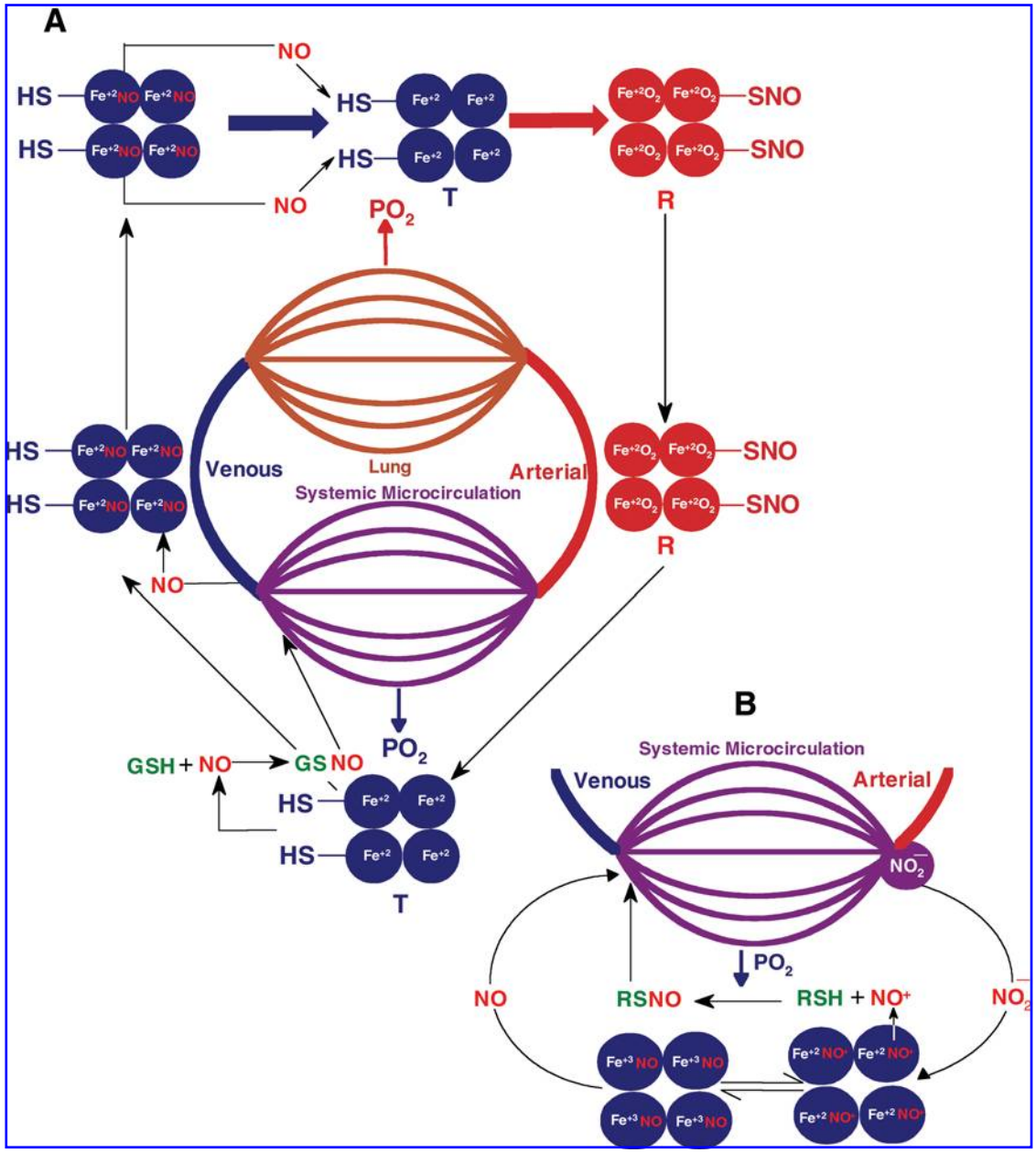

FIG. 2. (A) A representation of NO binding and release from Hb (RBC not depicted) in the circulation based on the SNO-Hb hypothesis. OxyHb or R-state conformation Hb with NO bound to thiol groups (SH) of cysteine residues (Cysβ93) is shown leaving the lung or pulmonary circulation **(top right)**. Thus, in the arterial circulation, SNO-Hb predominates until the decreasing pO_2 is experienced in the systemic microcirculation **(center right)**. Under decreasing pO_2, oxyHb (R state) changes to deoxyHb (T state), favoring the release of NO from SNO-Hb's Cysβ93 residues. NO then complexes with RBC thiols such as GSH forming GSNO, which is thought to be transported out of the RBC and exert a vasodilatory effect on vascular smooth muscle to increase blood flow and tissue oxygenation **(center bottom)**. As deoxyHb leaves the systemic microcirculation and enters the venous circulation, NO is scavenged by heme iron **(center left)**. Iron nitrosylated Hb enters the pulmonary circulation **(top left)** and is speculated to release NO to the Cysβ93 residues **(top center)** upon transitioning from deoxyHb (T state) back to Oxy-SNO-Hb (R state), completing the cycle **(top right)**. **(B)** A representation of the NO_2^- hypothesis. Circulating NO is converted to NO_2^- in the plasma (reaction shown in text) **(center right)**, NO_2^- reacts with Hb and exists in the RBC (not depicted) as $HbFe^{3+}NO$ **(bottom left)** and in equilibrium with $HbFe^{2+}NO^+$ **(bottom right)**. Under conditions of reduced O_2 availability, such as in the systemic microcirculation, NO may be released from readily dissociable intermediate storage sites ($HbFe^{3+}NO$ and $HbFe^{2+}NO^+$) and complex with GSH in the RBC **(center middle)** or may potentially be released from $HbFe^{3+}NO$ and subsequently the RBC **(center left)**. The result is vasodilation of the microcirculation following release of NO or GSNO in an effort to increase blood flow at sites of low local pO_2 **(top left)**.

increased blood flow at rest and to a greater extent during exercise with and without infusion of N^{ω}-monomethyl-L-arginine. The investigators also suggested that deoxyHb catalyzes the reduction of NO_2^- to NO and metHb as evidenced by increased blood concentrations of iron nitrosylated Hb ($HbFe^{2+}NO$). Doyle *et al*. (15) used a spectrophotometric technique to demonstrate that nitrous acid, or protonated NO_2^- (HONO), reacts with deoxyHb to form metHb and NO, which then rapidly binds to another deoxyHb forming $HbFe^{2+}NO$. Using both chemiluminescence and electron paramagnetic resonance spectrometry, Nagababu *et al*. (55) showed that, under hypoxic conditions, deoxyHb reacts with NO_2^- to form heme-NO. Significant arterial/venous heme-NO concentration differences were detected, with greater overall heme-NO detected in venous blood. However, ~75% of the heme-bound NO existed as $HbFe^{3+}NO$, an unstable complex that readily releases NO. The authors point out that the release of NO is only partially bioavailable to regulate vascular tone, and the remainder exists in equilibrium between $HbFe^{2+}NO^+$ and $HbFe^{3+}NO$. However, most important is that NO_2^- reduction occurs in the microcirculation, making small amounts of NO available where it is needed, as summarized in Fig. 2B. Additional i*n vitro* data presented by Cosby *et al*. (13) were obtained by mixing NO_2^- with oxygenated and deoxygenated human RBCs. These data suggested that NO production was significantly enhanced after concentrations of both NO_2^- and deoxygenated RBCs were increased. Two additional *in vitro* studies performed by the same investigators evaluated vasoactivity of deoxyHb and NO_2^- using rat aortic ring preparations. Reduced aortic ring tension in the NO_2^- and deoxygenated RBC group at lower pO_2 values (10–40 mm Hg) was shown. Additionally, comparing cell-free Hb with and without inositol hexaphosphate and varying concentrations of NO_2^- demonstrated that a reduction in rat aortic ring tension could be achieved at lower NO_2^- concentrations (100 n*M*) with Hb locked in the T state compared with nearly 100 μ*M* concentrations of NO_2^- required to reduce tension when Hb was evaluated in the R state (13). Gladwin *et al*. (24) suggest several limitations of this overall hypothesis and point to areas where clarity is lacking, particularly, mechanism for NO_2^- uptake by RBCs, the function of additional components in the process (*e.g.*, band 3 and carbonic anhydrase), and mechanisms of release of NO from the RBC.

Taken together, these hypotheses for O_2 sensing in blood are, in general, not well defined, but nevertheless they focus our attention on the central role played by Hb/RBCs in O_2 balance within the circulatory system. Along this line of thinking, the cardiovascular and respiratory systems each play key roles in O_2 homeostasis. It is becoming evident that physiological responses to hypoxia involve rapid changes in gene expression leading to expression of proteins responsible for hypoxic adaptation mediated by transcriptional activation of HIF-1. RBCs in circulation may communicate with the vascular and extravascular sites of O_2 sensing and regulation to maintain normal O_2 balance, which raises the provocative question: do O_2 carriers and sensors in blood cross talk with O_2 sensors at vascular and extravascular sites to regulate HIF-1 expression?

OXYGEN SENSING IN THE VASCULATURE

HIF-1

HIF-1 is the primary transcriptional factor responsible for downstream target gene activation of numerous protein and enzymatic pathways, which function to increase cellular O_2 delivery and promote physiologic adaptive responses to low O_2. Transcription of target gene pathways activated by HIF-1 can loosely be divided into those responsible for regulating delivery of O_2 (*e.g.*, tyrosine hydrolase, inducible NO synthase (iNOS), heme oxygenase, erythropoietin (EPO), vascular endothelial growth factor (VEGF), and angiopoietins) and those responsible for survival during long-term O_2 deprivation (*e.g.*, adrenomedullin, heat shock proteins, insulin growth factor, glucose transporter, glycolytic enzymes, EPO, and VEGF). Given the complex matrix of regulatory HIF-1 functions and its ubiquitous influence in states of low O_2 tension, we will attempt to focus primarily on hypoxia, O_2 sensing, and HIF-1 as it relates to circulatory function. Moreover, we propose a potential link between O_2 carrying/sensing mechanisms in blood with vascular and extravascular O_2 sensing and regulatory mechanisms, which we believe warrants further investigation.

STRUCTURE AND CHARACTERISTICS

The discovery of HIF-1 by Wang *et al*. (80, 81) revealed a heterodimeric protein complex. The complex consists of a cytosol-originating HIF-1α (120 kDa) and an O_2-insensitive nuclear HIF-1β (~93 kDa) alternatively known as the arylhydrocarbon receptor nuclear translocator (ARNT). Under conditions of normal O_2 tension (normoxia), HIF-1α is rapidly modified by an O_2-dependent pathway and degraded (33), thus preventing translocation to the nucleus, binding with HIF-1β to form the HIF-1 complex, and subsequent activation of hypoxia response elements (HREs) (32, 80). Conversely, under conditions of reduced O_2 tension (hypoxia), HIF-1α accumulates in the cytoplasm. Accumulation allows for nuclear translocation of the HIF-1α subunit and facilitates dimerization with HIF-1β and formation of the heterdimeric complex HIF-1. The result is activation of HREs and adaptation to acute and chronic hypoxia via activation of proteins responsible for maintaining O_2 homeostasis. Acute changes resulting from exposure to hypoxia occur rapidly (within seconds to minutes) and result in increased glycolytic enzyme functioning, increased peripheral vasculature relaxation, increased cardiac function (heart rate and cardiac output), and subsequently increased blood flow (67). Meanwhile, long-term responses to hypoxia occur by altering gene expression within a time frame of minutes to hours (31) and result in increased RBC production, angiogenesis, vascular remodeling, and increases in mRNA associated with the numerous proteins responsible for increasing perfusion, oxygenation, and oxygen utilization (67). For example, peripheral chemoreceptors are highly sensitive to pH, pCO_2, and pO_2 changes in blood and respond immediately via increasing respiration according to demand. Regulation of tyrosine

hydroxylase gene expression in the peripheral chemoreceptors (*e.g.*, carotid and aortic bodies) is an important function of HIF-1 in the acute and chronic response to hypoxia (58). During chronic hypoxia (*e.g.*, decreased pO_2 following high-altitude exposure), changes in ventilation are modulated in response to acute modification of respiration initially determined by changes in pO_2 made by the carotid bodies (41). Numerous genes are regulated by HIF-1 during chronic hypoxia, perhaps most recognized of which is HIF-1-induced up-regulation of EPO and a fairly rapid rise in plasma EPO and induction of RBC production in response to chronic hypoxia (41).

Previous work by Goldberg *et al*. (28) demonstrated that regulation of the EPO gene was via an oxygen-sensitive heme protein. Thereafter, Goldberg and Schneider (26) demonstrated that expression of both VEGF and EPO was similarly regulated by O_2 sensing mechanisms. These discoveries have implicated a heme protein within the plasma membrane as the O_2 sensing mechanism associated with HIF-1 activation and downstream gene regulation during hypoxia (86).

Regulation of O_2 sensing and cellular signaling. A single mechanism of cellular O_2 sensing in the regulation of HIF-1 function is not well defined. Several HIF-1-regulating mechanisms are reported in the literature and supported by experimental evidence; therefore, the possibility exists that multiple (rather than a single) O_2 sensing mechanisms regulate HIF-1 and that several mechanisms of O_2 sensing are potentially at work in different cells or tissues. Three hypotheses for vascular and O_2 sensing and subsequent regulation of HIF-1 will be discussed here (see Fig. 3).

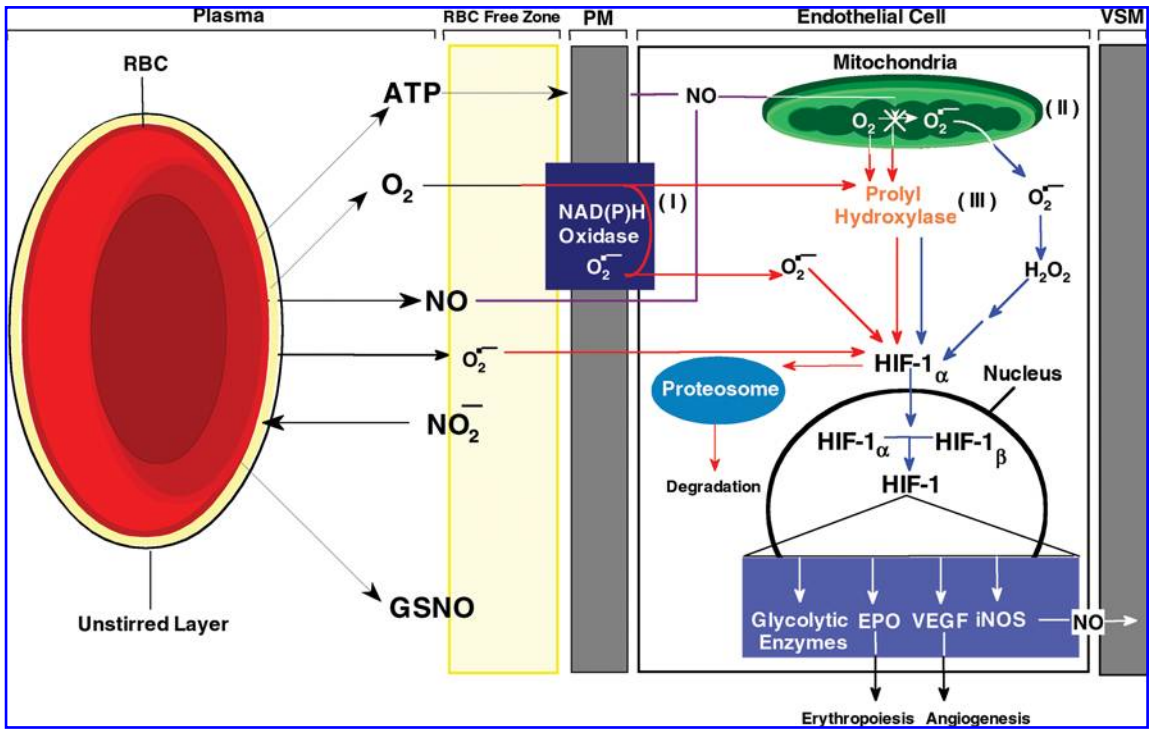

FIG. 3. Representation of a hypothesis that demonstrates how O_2 carriers may communicate with extravascular O_2 sensing mechanisms to increase or decrease HIF-1 expression. The RBC is depicted in the plasma with physical barriers to diffusion shown as the unstirred layer surrounding the RBC, the plasma itself, and the RBC free zone. Therefore, ATP, NO_2^-, NO, GSNO, $O_2^{\bullet -}$, and O_2 are subjected to diffusion barriers in route to the endothelial layer **(left to right)**. The plasma membrane (PM) **(center)** is depicted with **(I)** NAD(P)H oxidase, a potential sensor of O_2 released from RBCs, which converts O_2 to $O_2^{\bullet -}$ under normoxia, inactivating HIF-1α and targeting it for proteosomal degradation. **(II)** The mitochondrial cytochrome oxidases represent a second potential O_2 sensing mechanism by which decreased O_2 availability increases $O_2^{\bullet -}$ with subsequent cytosolic H_2O_2 formation and steps leading to the activation of phosphatidylinositol 3-kinase and HIF-1α stabilization. **(III)** The cytoplasmic prolyl hydroxylase represents a third potential O_2 sensing mechanism by which decreased O_2 availability prevents hydroxylation of specific HIF-1α proline residues; the opposite occurs during normoxia. Thus, hydroxylation targets HIF-1α for proteosomal degradation whereas the absence of O_2 prevents hydroxylation and allows for accumulation of HIF-1α. Dotted red arrows indicate HIF-1α destabilization and degradation; solid blue arrows indicate HIF-1α cytosolic accumulation, nuclear translocation, complexation with HIF-1β to form HIF-1, and activation of downstream gene targets such as glycolytic enzymes, EPO, VEGF, and iNOS **(bottom right)**. The effect of iNOS-generated NO is depicted on vascular smooth muscle. Under conditions of decreasing pO_2 in the microcirculation, RBC modulation of NO (dotted magenta lines) and O_2 (solid black line) likely limits endothelial O_2 sensing mechanisms from allowing HIF-1α accumulation and initiation of global regulators of O_2 homeostasis.

Plasma membrane cytochrome b *NAD(P)H oxidase.* HIF-1 regulation has been proposed to involve a low O_2 affinity heme protein as the primary O_2 sensor. Experimental evidence to support this hypothesis was previously demonstrated by Liu *et al*. (45) in studies suggesting that CO and NO could effectively block the activation of VEGF during hypoxia. Additionally, studies conducted by Huang *et al*. (34) demonstrated that the same two diatomic gases could directly inhibit HIF-1 activation. The high affinity of heme proteins, particularly Hb for both CO and NO, that bind to heme Fe^{2+} may suggest the role for a heme protein as the O_2 sensor. Data indicate that the O_2 sensor affinity for both CO and NO is considerably lower than that observed for Hb. Zhu and Bunn (86) point out that a reduced affinity for O_2, CO, and NO would be a strict requirement for a functional O_2 sensing mechanism to be effective under conditions of hypoxia. Studies performed by Palmer *et al*. (57) complicate this otherwise straightforward hypothesis by suggesting that, under conditions of normoxia, excess NO facilitates HIF-1 binding to HRE and downstream gene expression. This effect was not blocked by the addition of oxyHb, which would likely consume NO and produce NO_3^-.

The mechanism(s) by which HIF-1α degradation is inhibited and promotion of nuclear binding to HIF-1β is facilitated is also proposed to involve redox regulation by reactive oxygen species (ROS) such as $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2). Several hypotheses exist, and each provides evidence for different O_2 sensor localization and differing roles for ROS during conditions of hypoxia. One hypothesis suggests that the O_2 sensor is a cytochrome *b* (flavo-heme protein) located on the plasma membrane that functions as reduced NAD(P)H oxidase [Fig. 3 (I)]. This hypothesis suggests that, under conditions of normoxia, O_2 is converted to $O_2^{\bullet -}$ by the cytochrome *b* NAD(P)H oxidase system. Subsequently, $O_2^{\bullet -}$ is converted to H_2O_2 and other ROS such as hydroxyl radical ($^{\bullet}OH$), which favors oxidation of HIF-1α and proteosome degradation. Conversely, under conditions of hypoxia, accumulation of ROS would be limited, favoring HIF-1 formation and up-regulation of hypoxia response gene expression (16, 20).

Mitochondrial cytochrome oxidases. Another mechanism suggests that the heme-protein O_2 sensor is a cytochrome oxidase located within the mitochondrion and that increased rather than decreased ROS generation mediates HIF-1 dimerization (8, 9) [Fig. 3 (II)]. This hypothesis indicates that $O_2^{\bullet -}$ is generated at the electron complex III and IV under conditions of hypoxia. $O_2^{\bullet -}$ then enters the cytosol, where it is converted to H_2O_2 with subsequent activation of phosphatidylinositol 3-kinase, HIF-1α stabilization and downstream hypoxia response gene expression (10). At low O_2 concentrations (~3%), this mechanism appears to be directly affected by NO (<400 n*M*) such that HIF-1α is destabilized. It appears from the work of Mateo *et al*. (48) that at low O_2 concentrations (~3%), NO (<400 n*M*) inhibits mitochondrial respiration, creating an increased local O_2 concentration and stabilization of HIF-1α, whereas higher, potentially pathological concentrations of NO stabilized HIF-1α independent of mitochondrial inhibition. This observation provides an interesting basis for further discussion, as it relates to low-level NO exposure in tissues experiencing brief periods of hypoxia and reduced O_2 tension.

Cytoplasmic prolyl hydroxylase (2-oxoglutarate-dependent dioxygenases). Under conditions of normoxia, the destabilization and degradation of HIF-1α by a cytoplasmic O_2-dependent prolyl hydroxlase result in hydroxylation of either proline 402 or proline 564 located on the O_2-dependent degradation domain of HIF-1α (35) [Fig. 3 (III)]. Following hydroxylation, the destabilized HIF-1α is recognized by the von Hippel–Lindau tumor suppressor protein and targeted for ubiquitylation and degradation in the proteosome (36). The process is dependent on Fe^{2+}-containing oxoglutarate-dependent oxygenases and ascorbic acid and proceeds through a ferryl iron intermediate (49).

It seems apparent that vascular O_2 sensing mechanisms responsible for regulating HIF-1 activation are as complex and unclear as O_2 sensing mechanisms in blood. As described by Semenza, "The regulation of HIF-1 may therefore best be viewed as a web—a structure that is poorly defined by reductionist (*i.e.*, linear) experimental approaches" (68).

DOWNSTREAM REGULATORS OF O_2 HOMEOSTASIS

Numerous genes encoding for proteins responsible for maintaining O_2 homeostasis are influenced by HIF-1 (69). For example, EPO produced under hypoxic conditions, primarily in the kidneys but also in the liver, regulates bone marrow erythroid cell production and function (22). The critical influence of hypoxia on EPO has provided insight into its regulation by HIF-1 (5, 26–28, 30, 79). Investigators have demonstrated the role of HIF-1 in the expression of iNOS in the pulmonary circulation (56), cardiac tissue (39), and the carotid body (14), and inhibition of endothelial NO synthase expression (52). Glycolytic enzymes such as PFK and its associated allosteric activators (53), as well as pyruvate kinase, hexokinase, and the glucose transporters (GLUT1 and GLUT3) (62), are all expressed by HIF-1 under conditions of decreased O_2 availability. Additionally, several mediators of angiogenesis are expressed or suppressed in response to hypoxia. VEGF and platelet-derived growth factor-B (PDGFB) are expressed following cellular exposure to hypoxic conditions, whereas angiopoietin-1 and -2 expression and activation are blocked in response to hypoxia (21). Expressed VEGF and PDGFB bind to VEGF receptors and the PDGF receptor, eliciting endothelial cell proliferation and regulation of endothelial cell/smooth muscle cell interaction, respectively, with the end result being assembly of new blood vessels (40, 44). The work of several investigators has established a critical role for HIF-1 in angiogenic growth factor regulation and expression in normal development and pathologic conditions (23, 25, 42, 60, 83).

Mechanisms for O_2 sensing in blood must alert vascular and extravascular O_2 sensing mechanisms of their immediate control over the local hypoxic environment, or global regulation of oxygenation will take over. Therefore, it is logical to speculate that O_2 sensing mechanisms in blood may be linked

to vascular and extravascular sites of O_2 sensing prior to their global influence.

DO OXYGEN CARRIERS CROSS TALK WITH OXYGEN SENSORS?

Recent work in our laboratory has established a relationship between cell-free Hb and HIF-1 such that HIF-1 expression in bovine aortic endothelial cells is dependent on cell-free Hb's redox status and O_2 carrying capability (84, 85). The question of whether intraerythrocytic Hb can also communicate with regulators of HIF-1 expression, either directly or indirectly, remains to be elucidated. However, likely candidates that may function as messengers between RBCs and sites of HIF-1 regulation (other than O_2) may include small diffusible molecules such as $O_2^{\bullet -}$, NO, H_2O_2, and peroxynitrite ($ONOO^-$).

For example, under conditions of reduced pO_2, intraerythrocytic Hb generates $O_2^{\bullet -}$, which may be produced in sufficient quantities to escape the RBC and act either to damage tissue or alternatively as a signaling molecule (64, 65). In effect, $O_2^{\bullet -}$ generated and released from RBCs under conditions of low pO_2 could conceivably function to inhibit HIF-1α expression similar to $O_2^{\bullet -}$ generated from O_2 at endothelial cell membrane NAD(P)H oxidase. Additionally, NO effectively inhibits mitochondrial cytochrome oxidase complexes I and IV at low concentrations (nanomolar) by replacing O_2 at the oxidase's binding site and subsequently increases local intracellular O_2 concentrations (2–4, 12). As discussed previously, circulating NO_2^- represents a mechanism by which RBCs can convert and release NO under decreasing O_2 tension (*i.e.*, within the microcirculatory resistance vessels). Steady-state plasma levels of NO_2^- range between 200 n*M* and 500 n*M* as reported by Rassaf *et al*. (61) and Preik-Steinhoff and Kelm (59), respectively. Under normal physiological conditions, Nagababu *et al*. (55) suggest that NO_2^- is generated from NO released into the circulation by the following reaction:

$$\text{(Plasma)} \qquad 4NO + O_2 + 2H_2O \rightarrow 4HNO_2 \rightleftarrows 4H^+ + 4NO_2^-$$

NO_2^- is then taken up by RBCs and converted to NO via reaction with $HbFe^{2+}O_2$ or $HbFe^{2+}$ and stored in a dynamic equilibrium (see equilibrium below) prior to release:

$$\text{(RBC)} \qquad HbFe^{2+}NO^+ \rightleftarrows HbFe^{3+}NO$$

Past and more recent work published from the laboratory of Salvador Moncada strongly suggests that low NO concentrations (up to 400 n*M*) function to inhibit mitochondrial cytochrome oxidases, increase cellular O_2 concentrations, facilitate O_2-dependent prolyl hydroxylation of HIF-1α, and increase HIF-1α degradation (29, 48). These data suggest a key role for NO in the regulation of HIF-1α destabilization. It is likely that in an uncontrolled *in vivo* environment (*i.e.*, the microvascular circulation) where a decreasing O_2 gradient is experienced by RBCs, the concept posed by Nagababu *et al*. (55) could account for RBC generation and delivery of sufficient NO to mediate HIF-1α destabilization. Therefore, during conditions of intermittent O_2 reduction that routinely occur in the microvascular resistance vessels, RBCs may communicate with vascular and extravascular O_2 sensing sites via NO originating from NO_2^-, ATP, or SNO-Hb (see Fig. 3). Moreover, it is likely that mechanisms of communication between RBCs and sites regulating HIF-1α stabilization/destabilization depend on the degree and duration of reduced O_2 availability. Thus, under conditions of briefly reduced O_2 availability experienced by RBCs in the microcirculation, control of local O_2 demand is adjusted by RBCs and communication with extracellular sites to destabilize HIF-1α may be facilitated by NO. Conversely, during more extensive reduction and duration of decreased O_2 availability, RBCs may lose control over the local environment and facilitate HIF-1α stabilization by an inability to adjust for and meet local O_2 demand. To this end, the degree and duration of decreased O_2 availability experienced by RBCs and the subsequent influence on the vasculature sites of O_2 sensing/regulation warrant further investigation.

ABBREVIATIONS

ATP, adenosine 5'-triphosphate; CDB3, cytoplasmic domain of band 3; CFTR, cystic fibrosis transmembrane conductance regulator; CO, carbon monoxide; Cysβ93, 93 position β_1 and β_2 globin chain cysteine residues; deoxyHb, deoxyhemoglobin; EPO, erythropoietin; GSH, glutathione; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; HIF, hypoxia-inducible factor; H_2O_2, hydrogen peroxide; HRE, hypoxia response element; iNOS, inducible nitric oxide synthase; metHb or $HbFe^{3+}$, methemoglobin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NO_2^- nitrite; NO_3^-, nitrate; $O_2^{\bullet -}$, superoxide ; oxyHb, oxyhemoglobin; PDGF, platelet-derived growth factor; PFK, phosphofructokinase; pO_2, partial pressure of oxygen; RBC, red blood cell; ROS, reactive oxygen species; RSH, thiols; RSNO, *S*-nitrosoglutathione; SNO-Hb, *S*-nitrosohemoglobin; VEGF, vascular endothelial growth factor.

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