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Hemoglobin-Mediated Nitric Oxide Signaling

Christine C. Helms
University of Richmond, chelms@richmond.edu

D. B. Kim-Shapiro

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Christine Helms and Daniel B. Kim-Shapiro

Department of Physics and Translational Science Center, Wake Forest University, Winston-Salem, NC 27109

Address correspondence to Dr. Daniel Kim-Shapiro, Department of Physics, Wake Forest University, Winston-Salem, NC 27109, shapiro@wfu.edu 336-758-4993

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Abstract

The rate that hemoglobin reacts with nitric oxide (NO) is limited by how fast NO can diffuse into the heme pocket. The reaction is as fast as any ligand/protein reaction can be and the result, when hemoglobin is in its oxygenated form, is formation of nitrate in what is known as the dioxygenation reaction. As nitrate, at the concentrations made through the dioxygenation reaction, is biologically inert, the only role hemoglobin was once thought to play in NO signaling was to inhibit it. However, there are now several mechanisms that have been discovered by which hemoglobin may preserve, control, and even create NO activity. These mechanisms involve compartmentalization of reacting species and conversion of NO from or into other species such as nitrosothiols or nitrite which could transport NO activity. Despite the tremendous amount of work devoted to this field, major questions concerning precise mechanisms of NO activity preservation as well as if and how Hb creates NO activity remain unanswered.

Introduction

Nitric oxide, having a free electron, is a free radical, making it an interesting signaling molecule. NO functions as a neurotransmitter, a macrophage-derived host-defense molecule, inhibits platelet aggregation and endothelium adhesion molecule expression, is an antioxidant, and is a potent vasodilator[1,2]. Nitric oxide is an endothelium-derived relaxing factor (EDRF). It is synthesized from arginine by nitric oxide synthase (NOS) in endothelial cells and diffuses rapidly to smooth muscle cells where it can activate soluble guanylate cyclase (sGC), which converts GTP to cyclic GMP, initiating a signaling cascade leading to vasodilation. Nitric oxide can also be converted to other species including nitrosothiols, nitrated lipids, and nitrite that have been proposed to be an important part of signal transduction in many different physiological processes [3-7]. Due to its many roles, loss of NO bioactivity contributes to disease in a variety of conditions including hemolystic anemias like sickle cell disease [8], transfusion of older blood [9], and endothelial dysfunction that occurs in pathological conditions like metabolic syndrome [10] and during aging [11,12]. Restoration of NO bioavailability in these conditions is an attractive therapeutic avenue.

Hemoglobin (Hb) is a tetrameric, 64 Kilodalton protein. It is a dimer of dimers, having two alpha/beta dimers or two alpha and two beta subunits. Each of the four subunits has an iron-containing heme prosethic group that is attached to the protein via a histidine bond. The heme iron is the primary site for ligand binding so that Hb can carry out its main function; transporting oxygen from the lungs to the tissues. About 200 micromolar oxygen could dissolve in plasma while Hb is present at a concentration of about 10 mM in heme (this is 2.5 mM in Hb tetramers or 16 grams per deciliter). Thus, the oxygen carrying capacity of blood is increased fifty-fold by including Hb.

Hemoglobin has evolved to be an efficient oxygen transporter through an allosteric mechanism. This is understood in terms of a two-state model comprised of a high oxygen affinity relaxed or R-state and a low affinity T-state. At low oxygen pressures, when the Hb is fully deoxygenated, it is in the T-state. Each heme can bind one oxygen molecule. As the oxygen pressure increases, when the T-state molecule binds 2-3 oxygen molecules it transitions to the high affinity R-state. In this way, oxygen binding is cooperative so that binding subsequent oxygen molecules becomes easier as more and more bind. Having these two states allows Hb to be fully saturated with oxygen at the lungs where the oxygen pressure is high, while it can easily release oxygen at the tissues where oxygen pressure is low. Hemoglobin only binds oxygen when the iron heme is ferrous (+2), forming oxyhemoglobin

(oxyHb). When the iron is oxidized to the ferric form (+3), oxygen no longer binds. The ferric form of hemoglobin is called methemoglobin (metHb).

Nitric oxide also binds to the ferrous heme forming iron nitrosyl Hb, $Fe^{II}NO$ Hb (in this abbreviation the superscript represents the iron (Fe) oxidation state). Whether NO is bound to the alpha or beta subunits is distinguishable by electron paramagnetic resonance (EPR) spectroscopy and each species is referred to as alphanitrosyl or betanitrosyl hemoglobin. When alphanitrosyl hemoglobin is in the T-state, the bond between the histidine and iron breaks so the heme is pentacoordinate (one bound to the NO and four to the heme porphyrin ring) and this species is distinct when observed by EPR. The affinity of NO for the ferrous heme is enormous, with a dissociation constant (K_d) of 10^{-10} to 10^{-11} M [13]. Nitric Oxide can also bind to the ferric heme, but with a substantially lower affinity ($K_d = 2.5 \times 10^{-4}$ M).

NO can also interact with the accessible cysteine thiol at the beta-93 position to form S-nitroshemoglobin (SNO-Hb). Formation of SNO-Hb formally involves addition of NO^+ to the reduced thiol and is thus chemically described as nitrosation,. However, the nitrosonium ion, NO^+, exists transiently in aqueous environments at pH 7 due to its rapid reaction with H_2O forming nitrous acid so that other oxidized NO species are more likely to be responsible for SNO-Hb formation. In addition , SNO-Hb could form in a direct reaction of NO with a thiyl radical. Formation of SNO-Hb is facilitated when the Hb is in the R-state [14].

Given the abundance of Hb in the body and its complexity, and the importance of NO signaling in many pathways, one can imagine many ways that Hb mediates NO signaling. Indeed, as more questions in the complex field are answered more arise. In this review we will explore the different ways that hemoglobin can influence NO signaling, discussing how Hb impedes NO signaling, preserves it, or creates it.

Hemoglobin, destroyer of NO bioactivity

One of the clues that led to the identification of NO as the EDRF was that its activity is inhibited in the presence of hemoglobin [15]. This inhibition of NO signaling is mainly due to its reaction with oxyHb whereby oxyHb reacts with NO to form metHb and nitrate,

$$Fe^{II}O_2\ Hb + NO \rightarrow Fe^{III}\ Hb + NO_3^- \quad . \qquad (1)$$

This reaction, known as the dioxygenation reaction, occurs at a rate of 6-8 × 10^7 $M^{-1}s^{-1}$ [16-18]. It is rate-limited by diffusion of NO to the heme pocket; once at the heme pocket NO reacts [19,20]. Thus, the dioxygenation reaction is as fast as any ligand/heme reaction can possibly be.

With 10 mM Hb in the blood, the half-life of NO under oxygenated conditions would be predicted to be about 1 μs. During this time, NO could only diffuse about 0.1 μm (assuming a diffusion constant of 3000 $\mu m^2\ s^{-1}$). One might think that the microanatomy of the blood vessels and surrounding tissue (Figure 1) make it so that NO produced in the endothelium is not greatly affected by Hb since Hb is on the opposite side of the endothelium from the smooth muscle. However, as Lancaster pointed out in 1994 [21], if it were not for compartmentalization of Hb in red blood cells (as discussed in the next section), Hb scavenging of NO would make it so that NO produced in the endothelium could not function as the EDRF. Basically any NO that diffuses into the lumen and encounters Hb would be immediately converted to nitrate, so most NO that randomly walks from the endothelium on its way to the smooth muscle would be destroyed.

Based on recent work showing that nitrate can actually be converted back to NO, secondary to first being converted to nitrite by oral bacteria [6], one might argue that conversion of NO to nitrate in the dioxygenation reaction does not stop NO signaling. However, the amount of nitrate in plasma is typically tens of micromolar [6] while NO is present in the nanomolar

range or less [22]. Thus, conversion of NO to nitrate does not significantly increase the concentration of plasma nitrate which can later be taken up by salivary glands and converted to nitrite. The formation of nitrate via the dioxygenation is a dead end in NO signaling.

In addition to reacting with oxyHb, NO also binds to the deoxygenated ferrous Hb and this reaction can be substantial in partially oxygenated blood,

$$Fe^{II}\ Hb + NO \leftrightarrow Fe^{II}NO\ Hb \quad . \qquad (2)$$

Iron nitrosylation does not permanently eliminate NO activity as in the dioxygenation reaction. In principle, NO can dissociate from the heme so that, in a sense, iron nitrosyaltion preserves NO activity. However, the dissociation rate constant of NO from the ferrous heme ranges from $1 \times 10^{-3}\ s^{-1}$ for T-state (beta hemes), $4 \times 10^{-4}\ s^{-1}$ for T-state alpha hemes (pentacoordinate), and $1 \times 10^{-5}\ s^{-1}$ for R-state [23-25]; all quite slow. Thus, when NO binds to ferrous hemes it is not permanently removed from the biological NO signaling pool, but barring other mechanisms discussed below (including intramolecular transfer to SNO-Hb or oxidative denitrosylation), it is held by the Hb for a long time. Moreover, once it does come off, it is susceptible to dioxygenation via oxyHb which is always abundant in the red blood cell.

Based on the relative yield of nitrosyl Hb vs metHb when NO was added to partially oxygenated Hb, it was proposed that R-state deoxygenated hemes bind NO faster than T-state hemes and that this is one method for reducing dioxygenation and preserving NO activity [26]. This proposal is counter to earlier work by Gibson and colleagues that found no cooperativity in the rate of NO binding to Hb [27,28], although the rate of dissociation is dependent on quaternary state [23,24]. In subsequent experiments examining nitrosyl Hb yield vs metHb yield, no evidence for an increased rate of NO binding to R-state hemes compared to T-state ones was found [29]. In addition, experiments that directly measured the rate that NO binds R-state Hb found that the rate constant was the same as for T-state Hb [30]. Other studies suggested that apparent increased binding to R-state Hb may have been due to NO reactions that occur upon bolus addition of high concentrations of NO [31-33]. Overall, the majority of evidence suggests that R-state Hb binds NO with the same rate constant as T-state Hb, so that NO activity is not preserved through faster R-state binding [34].

Given the extremely fast rate constants for the reactions described in Equations (1) and (2), and the abundance of oxyHb and deoxyHb (ferrous Hb with no ligands bound), one might expect that Hb only acts to destroy NO activity. However, as discussed below, hemoglobin's role in NO signaling is far more complex.

Preservation of NO bioactivity by compartmentalization of Hb

In 1998, Anthony Butler conducted some simulations that suggested that the encapsulation of hemoglobin in red cells, together with a red cell-free zone that develops between the endothelium and the red blood cell (Figure 1), results in much reduced NO scavenging by hemoglobin [35]. Bernoulli's law states that as velocity increases, pressure decreases. As the velocity near the endothelium is lowest (due to friction) and the velocity in the center of a blood vessel is highest, there is a pressure gradient that tends to push red cells away from the NO producing endothelium. This creates a cell-free zone so that NO scavenging is much reduced. The presence of a cell-free zone and its ability to reduce NO scavenging was demonstrated by Liao and coworkers using single arteriole myography and examining effects on vasodilation of red cells and cell-free Hb in the presence and absence of flow [36]. Assuming a cell-free zone of 5 μm (a reasonable estimate for many arterioles [35,37]), the lifetime of NO would be about 10 milliseconds, before it would reach the red-cell rich zone and be rapidly scavenged (ignoring scavenging by other species within the cell-free zone, calculation based on diffusion, not

including any effect convection may have on NO lifetime [38]). This is about a 10,000 fold increase in the lifetime of NO compared to if it were simply exposed to 10 mM Hb.

Even in experiments where there is no cell-free zone, such as where NO is mixed with red blood cells in suspension or after rapid mixing in a stopped-flow apparatus NO was observed to react up to 1000 times slower with red cell encapsulated Hb than with cell-free Hb [39]. This phenomenon had been described previously for oxygen binding to hemoglobin [40-43]. Slower NO uptake by red cells in these experiments has generally been ascribed to three different mechanisms (Figure 1): (1) NO uptake being rate-limited by diffusion of NO to the RBC, which contributes to the phenomenon of an unstirred layer around the RBC [39,40,44-46], (2) an intrinsic, physical RBC membrane barrier to NO diffusion [47-51], and (3) NO uptake being rate-limited by diffusion of NO within the RBC (intracellular diffusion) [47,52]. The relative contribution of these three different factors is a matter of continuing research and debate. Strong arguments for a major role of external diffusion come from experiments that demonstrate that the bimolecular rate constant for NO uptake by red cells increases as the distance between the cells decreases (accomplished by increasing the hematocrit) [51] and by experiments that show that the rate constant for NO uptake by red blood cells also depends on the viscosity of the buffer suspending the cells [53]. Strong evidence for a role of a physical membrane barrier comes from experiments where the red cell membrane was altered, either chemically or mechanically, and this increased the rate of NO uptake by red blood cells [49,54]. For physiological conditions where the concentration of NO is much less than that of Hb, rate limitations due to intracellular diffusion are suggested to be due to the fact that NO would only interact with a thin shell of Hb just inside the RBC and therefore the rate of reaction of Hb with NO would be different than the reaction rate of a homogenously mixed solution of the same concentrations of Hb and NO. This notion is inconsistent with observed independence of NO uptake by red cells on internal Hb concentration. [39]. Thus, one might argue that some consensus is being achieved in recent work where external diffusion is the main limiting factor while membrane permeability also plays some role [39,51,53,55].

Regardless of which of the above described mechanisms is mainly responsible for limiting NO uptake by red blood cells, they all break down upon red cell hemolysis. (Figure 1) Cell-free Hb does not remain in the cell-free zone and can even extravasate into the endothelium, exacerbating destruction of NO activity. Loss of NO has been identified as a major factor in pathology associated with the use of hemoglobin-based blood substitutes [56]. In addition, NO scavenging by cell-free hemoglobin is also implicated in pathology in hemolytic anemias like sickle cell disease [8,57,58], malaria [59-61], and transfusion of older stored blood [9]. The extent of contribution of NO scavenging upon hemolysis in sickle cell disease has been debated [62,63], but experimental studies show correlations between NO reactivity and cell-free Hb levels [8,61,64] and computational studies support the notion that even a few micromolar of cell-free Hb can substantially reduce NO bioavailability even in the background of ten millimolar red cell encapsulated Hb [65,66].

In addition to hemolysis and release of cell-free hemoglobin, red cell breakdown also can result in the formation of hemoglobin-containing red cell microparticles. These particles have a large range in size reported from 50 nm to 2 μm in diameter [67]. However, much research has focused on red cell microparticles that are on the order of 100 to 200 nm. It was shown that red cell microparticles that form as blood ages during storage scavenge NO only 2.5 to 3 times slower than cell-free Hb, which is practically 1000 times faster than NO reacting with red blood cell-encapsulated Hb [9]. These results are consistent with previous ones that also showed that the rate of oxygen uptake by red cells or other hemoglobin-containing vesicles increases as the diameter of the particles decreases and the internal Hb concentrations increases [68].

Preservation of NO bioactivity by formation of SNO-Hb

Although elucidation of precise mechanisms for their formation, degradation, signaling pathways continue to emerge, S-nitrosothiols are potentially an effective transporter of NO bioactivity either through conversion to NO, or through pathways that mimic NO signaling in some ways [69,70]. Although they do react with both oxygenated and deoxygenated Hb, they do so at a much slower rate than NO dioxygenation or NO binding to the heme [71,72]. Importantly, the NO^+ moiety can be transferred from one thiol to another in what is known as trans-nitrosation so that formation of SNO-Hb represents a potentially very effective way of preserving and subsequently transducing NO bioactivity [73].

An intriguing mechanism of NO activity preservation developed by Stamler and colleagues has become known as the SNO-Hb hypothesis [14,74-78]. A central component of the hypothesis is that NO bound to the heme of T-state Hb is transferred to the β93 cysteine when the Hb undergoes a T to R transition, forming SNO-Hb from iron nitrosyl Hb. Iron nitrosyl Hb is formed in the tissues and, upon oxygenation at the lungs, this becomes SNO-Hb which can deliver NO activity via nitrosothiols when the Hb approaches the tissues again [77]. This elegant model thus suggests that Hb is a carrier of NO activity that harnesses Hb allostery for delivery.

The validity of the SNO-Hb hypothesis has been a subject of much debate on issues ranging from concentrations of SNO-Hb that are present in vivo, stability of SNO-Hb in the red blood cell, evidence for NO delivery in vivo, and (the focus that this discussion will now take) the allosterically-controlled intramolecular transfer itself [78-88]. Firstly, it should be noted that NO itself does not bind to a reduced thiol, it must first be oxidized to NO^+ (a transient species in aqueous environments) or a thiol radical must be present. This requirement does not prove that intramolecular allosteric transfer cannot occur, it just suggests that the chemistry is more complicated and an electron acceptor would have to be present. However, experiments designed to test transfer from the heme to thiol are relatively straight-forward. One need only make iron-nitrosyl Hb, effect a T to R quaternary change in the Hb, easily accomplished by oxygenation, and measure changes in heme-nitrosyl and SNO-Hb. These types of studies have not demonstrated the expected heme to thiol transfers [82,88]. It was suggested that transfer is most efficient from betanitrosyl Hb [78], but experiments specifically designed to test transfer from the beta hemes also did not show evidence for transfer [88].

The lack of an allosterically controlled intramolecular transfer from heme to thiol was attributed by some to poor methodology [84]. In fact much of the controversial issues surrounding the SNO-Hb hypothesis have been attributed to variations in chemiluminescence assays used to measure nitrosothiols [89,90]. We hold that any of these methods are capable of accurately measuring S-nitrosothiols under a variety of conditions as long as proper controls are included. The tri-iodide chemiluminescent method has been largely criticized [84,89], but this is likely due to the method not having been properly implemented. A recent study validated the tri-iodide method in comparison to a novel mass-spectrometry-based method [91]. In any case, use of electron paramagnetic resonance spectroscopy as a gold standard to measure nitrosyl Hb content has not, to our knowledge, been contested. Thus, studies using this technique should have, but did not find evidence for allosterically-controlled intermolecular transfer from the heme iron to the β93 cysteine [82,88].

These studies using electron paramagnetic resonance strongly militate against the hypothesis that when iron nitrosyl Hb undergoes a T to R-state transition, the heme NO transfers to the β93 cysteine to form SNO-Hb. However, this does not mean that NO bound to the heme cannot, with some additional chemistry, form an S-nitrososthiol in the form of SNO-Hb or otherwise. An allosteric transition is simply not sufficient to cause the formation of SNO-Hb from iron nitrosyl Hb. In fact, other mechanisms of SNO-Hb formation are discussed below. In addition the electron paramagnetic resonance studies do not argue against proposed therapeutic uses of SNO-Hb. Those remain to be tested and while the phenotype (including

survival) of a β93 cysteine knockout mouse suggests that SNO-Hb is not essential for normal physiological function [85], the role of SNO-Hb in various conditions continues to be worthy of experimentation.

Preservation by mechanisms involving Hb oxidation

Whereas NO dioxygenation through reaction with oxyHb (Equation 1) destroys NO activity and NO binding to the ferrous heme (Equation 2) sequesters the NO due to the slow dissociation rate, NO binding to the ferric heme does not substantially impede NO signaling. In fact, Liu et al showed that oxidation of appreciable amounts of intracellular Hb had no effect on NO uptake by RBCs [39]. Although the rate constant for NO binding to metHb is pretty large (on the order of 10^3 $M^{-1}s^{-1}$) the dissociation rate constant is also quite fast (on the order of 1 s^{-1}) [92]. Thus, NO binding to metHb is quite reversible,

$$Fe^{III}\ Hb + NO \leftrightarrow Fe^{III}NO\ Hb \quad . \qquad (3)$$

The latter species, which is designated $\{FeNO\}^6$ according to the Enemark-Feltham notation [93] and which has also been written as MetHb-NO and HbFe(II)-NO^+ is actually a distinct species where the electron is neither bound to the iron or to the NO but rather is delocalized.

Oxidation of cell-free Hb (which remains mainly in the oxygenated form in plasma) to metHb has been recognized as a potential treatment strategy to counter NO scavenging by plasma cell-free Hb [8,94-97]. This strategy takes advantage of the fact that cell-free Hb reacts with NO on the order of 1000 times faster than red cell encapsulated Hb, so that the cell-free Hb is preferentially oxidized while red cell metHb levels do not rise too high.

Recent work by Isakson and colleagues have proposed that interconversion of hemoglobin alpha subunits between the ferrous and ferric state acts as a sort of traffic light for NO between endothelial cells and smooth muscle (Figure 2) [98,99]. Alphahemoglobin is proposed to line myoendothelial junctions that extend from endothelial cells through internal elastic lamina to smooth muscle cells. When the Hb is in the reduced, ferrous state it limits NO signaling through dioxygenation, preventing it from reaching smooth muscle cells. When the Hb is oxidized, NO diffusion to smooth muscle cells is not impeded. In addition, cytochrome b5 reductase 3 (also known as metHb reductase) acts to reduce metHb back to its reduced form (as it does in the red blood cell). In this way, the oxidation state of Hb is harnessed to control how much NO reaches the smooth muscle.

Given that the dissociation rate constant for MetHb-NO is at least 1000 times faster than that for ferrous iron nitrosyl Hb, oxidation of ferrous iron nitrosyl Hb would be expected to be an efficient way to release NO [79,80,100]. Indeed, a process named oxidative denitrosylation involves oxidation of a ferrous nitrosyl species to rapidly release NO [101]. Oxidative denitrosylation was discovered in the context of reactions of partially oxygenated Hb with nitrite. In this reaction deoxyHb makes NO from nitrite (the subject of the next section in this review) that can form $Fe^{II}NO$ and nitrite also undergoes a complex reaction with oxyHb that makes several intermediate species including NO_2 radical that can oxidize the heme iron of the $Fe^{II}NO$ [101,102]. Through this mechanism, NO can be released from ferrous iron nitrosyl Hb and may be available for use in signaling pathways secondary to heme oxidation, barring dioxygenation via oxyHb.

MetHb-NO, sometimes referred to as $Fe^{III}NO\ Hb$ (but see discussion of the delocalized electronic structure above), has ferrous nitrosonium, $Fe^{II}NO^+\ Hb$ character. It is perhaps more accurate to describe $Fe^{III}NO\ Hb$/ $Fe^{II}NO^+\ Hb$ as a single species it is truly one that has character of both. The nitrosonium can react with a thiol to form a nitrosothiol, thereby preserving NO activity. MetHb-NO was thus proposed as a species lending itself to SNO-Hb formation that

was demonstrated when NO was added to metHb or ferrous nitrosyl Hb was oxidized [103]. This intramolecular transfer of the nitrosonium was found to be most efficient when concentrations of NO were low compared to Hb (as would be the case under physiological conditions) [103,104]. S-nitrosation of low molecular weight thiols could also occur from MetHb-NO either directly or through trans-nitrosation from SNO-Hb. The extent to which nitrosation occurs through these mechanisms under various conditions including in vivo needs further exploration.

In addition to forming metHb and NO as NO dissociates as in Equation 3, Ferric iron nitrosyl Hb can also react with hydroxide or water to form nitrite [105-108],

$$Fe^{III}NO\,Hb + OH^- \rightarrow Fe^{II}Hb + HNO_2 \quad . \qquad (4)$$

The reaction described by Equation 4 is referred to as reductive nitrosylation, when NO is in excess, ferrous heme nitrosylation can occur at the reduced heme. As described below, nitrite (or nitrous acid as in Equation 4) can be viewed as a molecule that stores nitric oxide activity so that formation of nitrite by reductive nitrosylation preserves NO activity. The reaction of NO with metHb is found to be faster at the beta subunits [103], and is also, surprisingly, faster when the concentration of NO is decreased [109].

The above discussion shows that there are three possible fates for MetHb-NO: (1) the NO dissociates and leave a ferric heme, (2) an NO^+ moiety can be transferred to a low molecular weight thiol or to the β93 cysteine to form SNO-Hb, or (3) it can react with OH^- or water to leave a ferrous heme and make nitrite. The probability of each pathway remains to be completely elucidated for various conditions including physiological ones. In addition, given that the rate constant for NO binding to metHb is at least 10,000 times slower than dioxygenation or binding to a ferrous heme, and the fact that metHb is usually less than 1% of Hb present in the red cell, one wonders if some kind of compartmentalization within the red blood cell is necessary for these reactions to be of importance apart from separate mechanisms that may form MetHb-NO.

Production of NO bioactivity via Hb reaction with nitrite

The reaction of nitrite with reduced, deoxygenated Hb was first described by Brooks in 1937 and further investigated by Doyle and colleagues in the 1980s [110,111]. Many aspects of the reaction and its role in physiology and therapeutics have been reviewed recently [112,113]. The reaction produces metHb and NO [114,115],

$$NO_2^- + Fe^{2+}Hb + H^+ \rightarrow NO + Fe^{3+}Hb + OH^- \, . \qquad (5)$$

When carried out under anoxic conditions, the NO that is produced binds to deoxyHb so that equimolar concentrations of nitrosyl and metHb are produced. Interestingly, when nitrite is reacted with the deoxyHb with nitrite in excess, the kinetics of the reaction resemble zero order rather than the expected pseudofirst order [114,115]. This phenomenon is attributed to R-state hemes reacting with nitrite faster than T-state hemes so that as the reaction progresses the loss in vacant ferrous heme available to react with nitrite is balanced by an increase in the Hb tetramers that are in the R-state (as nitrosyl and metHb formation leads to R-state Hb) [114-116]. The rate constant for the reaction of T-state Hb has to be estimated to be 0.2 $M^{-1}s^{-1}$ and to increase 100-fold for R-state Hb [112]. Thus, Hb has been referred to as an allosterically-controlled nitrite reductase [115], although to consider this process to be enzymatic one would need to include red cell metHb reductase as part of the system.

Although the reaction of nitrite with deoxyHb to form NO has been known for a long time, that it could have physiological relevance was not suggested until 2003 along with the discovery that just slightly supraphysiological levels of nitrite can cause increased blood flow in humans [5]. The notion that nitrite acts as a physiological vasodilator is strongly supported by several reports that show that nitrite derived from dietary nitrate decreases blood pressure [117-119]. However, the convincing evidence that nitrite acts as a vasodilator does not mean that Hb is necessarily involved; many other mechanisms for nitrite reduction to NO have been proposed [120-135]. It is likely that several different mechanisms of nitrite reduction are active in different tissues and different conditions.

One feature of the deoxyHb/nitrite reaction (Equation 5) that suggests it is at least partially responsible for nitrite-dependent vasodilation is that it is facilitated under deoxygenated and acidic conditions and so is nitrite-dependent vasodilation [5,136,137]. The allosteric nature of the reaction makes it so that, overall, it is most efficient near the Hb P50 (oxygen tension at which hemoglobin is 50% oxygenated) and is thus modulated at physiological oxygen concentrations [115,136,138,139]. Several studies using aortic ring bioassays show that red blood cells and cell-free Hb act to effect nitrite-dependent vessel dilation upon deoxygenation [5,136,137], although other studies contest this result [140,141]. The apparent discrepancy may be resolved when accounting for the fact that both while Hb produces NO from nitrite, it will also scavenge it via dioxygenation and binding [113,137]. Thus, there is a balance between these two effects that can tip from vasoconstricting to vasodilatory action depending on pH and oxygen tension. Further evidence for a potential role of Hb in nitrite mediated vasodilation comes from experiments with hemoglobin-based blood substitutes that showed that co-administration with nitrite resulted in reduced hypertension that usually occurs with hemoglobin infusion [142]. NO acting through soluble guanylate cyclase is responsible for reduced platelet aggregation and very strong recent evidence for Hb mediated export of NO activity from red blood cells due to the deoxyHb/nitrite reaction comes from studies showing inhibited platelet aggregation due to NO produced by nitrite and red blood cells and facilitated upon deoxygenation. [143,144]. Just 100 nM nitrite (a physiologically-relevant level) was able to significantly reduce platelet aggregation and this effect was abolished when an NO scavenger was introduced [144].

Assuming a bimolecular rate constant for nitrite reduction of 10 $M^{-1}s^{-1}$ when the Hb is 50% oxygen saturated, nitrite would be reduced at a rate of 1 nM/s by Hb in whole blood. However, NO produced in the red blood cell would be rapidly destroyed by dioxygenation. The lifetime of NO is a red blood cell would be about a microsecond. Thus, some alternate or intermediate species must form to transport NO activity out of the red blood cell [145]. Two possible species that could potentially transport NO activity are N_2O_3 and S-nitrosothiols (which could be formed from the strong nitrosating agent N_2O_3) [145-150]. The molecule N_2O_3 can potentially diffuse out of the red blood cell and subsequently form NO or it can nitrosate. Low molecular weight nitrosothiols carry NO or NO-like activity and can be formed from SNO-Hb. SNO-Hb was indeed measured during nitrite infusions [5].

Formation of N_2O_3 has been proposed via a reaction of NO with nitrite bound metHb [147] or by nitrite reacting with a MetHb-NO ($Fe^{III}NO$ Hb) intermediate [146,151]. The MetHb-NO species is likely to form during the reaction of nitrite and deoxyHb. The transient formation of such a species would be similar to that proposed to form during nitrite reduction by certain nitrite reductases [152]. With its fast dissociation rate

constant (1 s^{-1} [13]) this species would not be expected to accumulate to detectable levels, contrary to what has been suggested by [148,153,154]. A more stable form of MetHb-NO has been proposed [148,153,154], but definitive evidence for its existence is lacking. However, formation of N_2O_3 via a nitrite/MetHb-NO intermediate (even if the intermediate is short-lived) is a viable pathway which eliminates the prevention of N_2O_3 formation due to NO scavenging [146]. Moreover, arguments based on kinetics that favor the reaction of nitrite bound metHb with NO [147]have been countered by new data that suggest the kinetics are independent of nitrite [109]. Currently, it is not clear which pathway for N_2O_3 is favored (metHb bound nitrite + NO vs the MetHb-NO + nitrite). Moreover, experimental and theoretical arguments have been made that suggest N_2O_3 is not made in the nitrite/metHb reaction [155,156] whereas others suggest that it is [150].

Since MetHb-NO is a likely intermediate and this species is really the same as $Fe^{II}NO^{+}$ Hb, it makes sense that S-nitrosothiols (including SNO-Hb) could form directly from this species, as suggested by several studies [103,148,149]. In order to investigate whether SNO-Hb or other S-nitrosothiols are formed directly from MetHb-NO, or if the S-nitrosothiols that are formed require the presence of nitrite (so that N_2O_3 can form), a nitrite oxidase system was used to completely eliminate both contaminating nitrite and any nitrite formed when metHb is mixed with NO [109]. The S-nitrosothiol yield was dramatically reduced in the presence of the nitrite oxidase system when NO was added to metHb [109], suggesting that most S-nitrosothiol formation that is made when nitrite is added to deoxyHb (or NO is added to metHb) is due to a nitrite-dependent pathway (such as one involving N_2O_3) formation. One potential problem of this study is that the nitrite oxidase system includes both hydrogen peroxide production and catalase so that the possibility for interfering chemistry is high. Further experiments are required to firmly establish the mechanism of S-nitrosothiol formation in the Hb/nitrite reaction and if the formation of these species accounts for Hb/nitrite mediated vasodilaton or if nitrite-mediated vasodilation (or other effects of nitrite) are actually due to reactions with Hb.

Conclusions

Research has shown the importance of compartmentalization of Hb in the red cell in limiting the destruction of NO by Hb. In addition oxidation of Hb to form metHb reduces the ability of Hb to sequester or destroy NO. A Hb oxidation-reduction mechanism was recently proposed to control NO signaling in myoendothelial junctions. This function of alphahemoglobin in NO signaling may be present in other cell types as alphaHb is found in many cells and its function is not fully understood.

The role of Hb in the vasodilatory role of nitrite is also not fully understood. DeoxyHb can act as a nitrite reductase to produce NO, however, NO produced by Hb in the RBC would then be subject to interactions with other deoxygenated and oxygenated hemoglobins. Formation of N_2O_3 or S-nitrosthiolsfrom the Hb/nitrite reaction may be the key to nitrite mediated NO activity export from red blood cells, but the mechanism remains a matter of debate. It is clear that Hb plays a significant role in NO signaling however many questions remain surrounding the precise mechanisms of NO activity

preservation as well as if and how Hb creates NO activity, making Hb NO research an exciting area for future research.

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Conflict of Interest

Dr. Kim-Shapiro is listed as a co-inventor on several patent applications involving the use of nitrite in therapeutics, treating hemolysis through means including oxidation of cell-free Hb, and methods to improve use of blood substitutes.

Figure 1. Schematic of blood flow through a vessel. **A)** Mechanisms to preserve NO bioactivity in typical blood flow. A pressure gradient created by blood flow pushes RBCs into the center of the vessel creating a cell free zone which separates endothelial nitric oxide synthase (eNOS) from Hb inside the RBC. The rapid reaction of NO with Hb creates a NO concentration gradient surrounding the RBC known as the unstirred layer. The RBC membrane encapsulates Hb inside the RBC and contributes as a membrane barrier to the NO Hb reaction. Intercellular diffusion of NO past previously reacted Hb (brown circles) to an unreacted Hb (red circles) limits the reaction of NO (blue circle) after it enters the RBC. **B)** The breakdown of mechanisms preserving NO bioactivity during blood flow. As RBCs age or become damaged they produce microparticles and/or hemolyze. During hemolysis RBCs break open releasing Hb into the blood stream eliminating the unstirred layer, intracellular diffusion and membrane barrier. Microparticles, which bud off from RBCs, and hemoglobin released during hemolysis are not affected by the pressure gradient due to their size and therefore enter the cell free zone. Furthermore Hb can extravasate between cells and out of the blood stream.

Figure 2. Schematic of the traffic light mechanism alphahemoglobin plays in myoendothelial junctions. Alphahemoglobin in the myoendothelial junctions, which stretch across the internal elastic lamina and connect endothelial cells to smooth muscle cells, can be reduced from iron III, metHb (brown circles), to iron II, Hb (red circles). The interaction between MetHb and NO allows NO to continue on as a signaling molecule downstream. However, the interaction between oxygenatedHb and NO produces metHb and nitrate (white circles) blocking downstream signaling of NO. When cytochrome b5 reductase 3, also known as metHb reductase and refered to above as reductase (green), is activated and bound to alpha Hb the Hb is reduced to iron II and is able to block NO signaling.

Figure 3. Possible interactions of nitrite and Hb inside the RBC resulting in NO bioactivity. Hb is represented by colored circles and the redox state of the iron (bright red – oxygenated ferrous Hb, dark red – deoxygenated ferrous Hb, light brown – ferric Hb). Equations are not all balanced as it would be difficult to include all elements and water and protons are explicit. Products which may escape the RBC are located outside of the RBC in the diagram. Reaction 1 depicts the reaction between nitrite and oxygenated Hb producing metHb and nitrate. Reaction 2 indicates the reaction between metHb and nitrite producing nitrite bound met. With the addition of NO, nitrite bound metHb may produce deoxygenated Hb and N_2O_3 as shown in reaction 3. N_2O_3 could itself be exported from the RBC and converted into NO and nitrite,

reaction 4, or it could be used to form S-nitrosothiols, reaction 5, which could be exported from the RBC and lead to NO bioactivity. Reaction 6 indicates the nitrite reductase reaction between nitrite and deoxyHb to produce NO bound metHb. NO bound metHb could dissociate producing MetHb and NO, reaction 7. The NO bound to the metHb could be transferred to a thiol forming SNO-Hb, reaction 8, which could then transfer the nitrosating group to another thiol for export from the RBC. In reaction 9 the NO bound metHb could react with nitrite and hydroxyl to produce deoxyHb and N_2O_3, similar to reaction 3, leading to extracellular NO bioactivity.

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Figure 1

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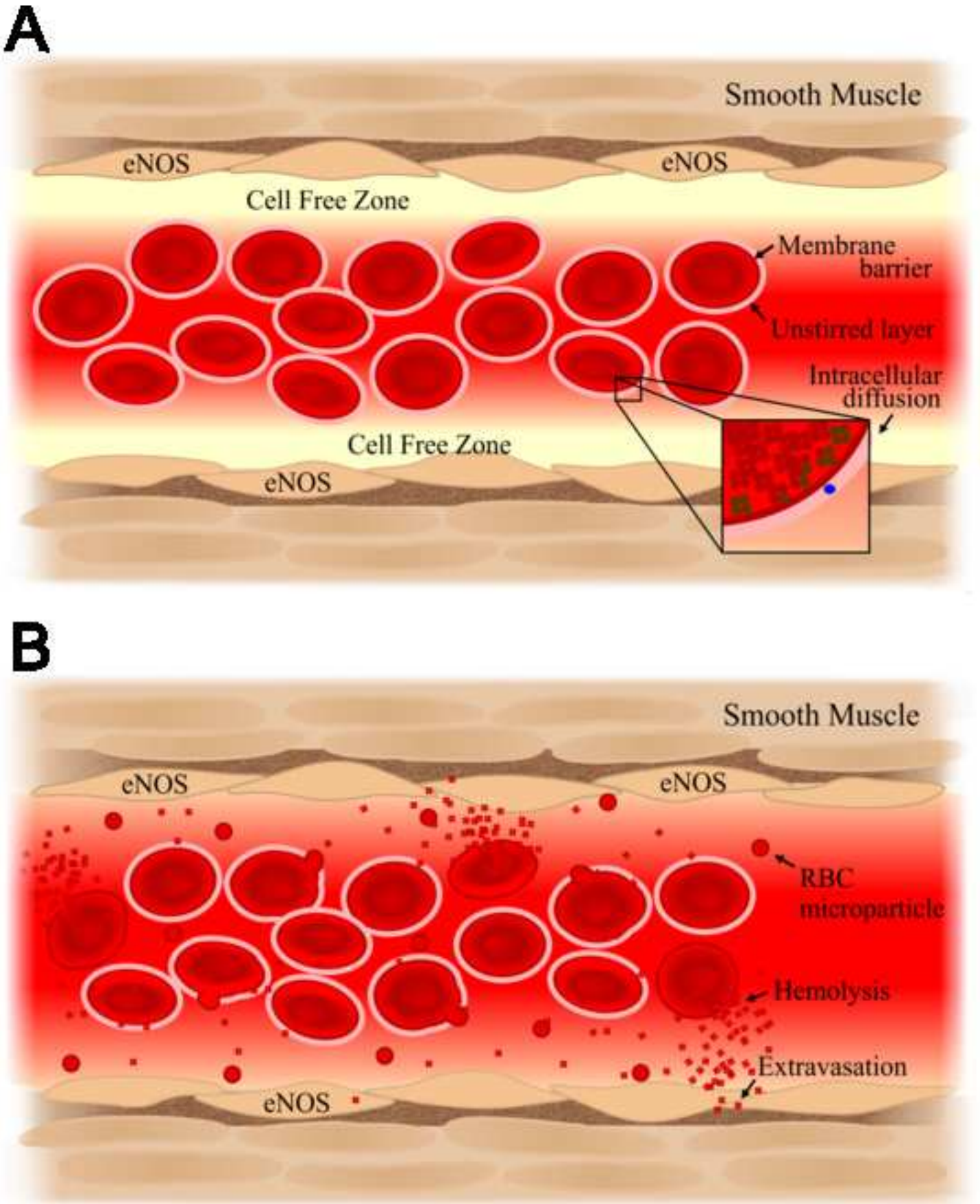

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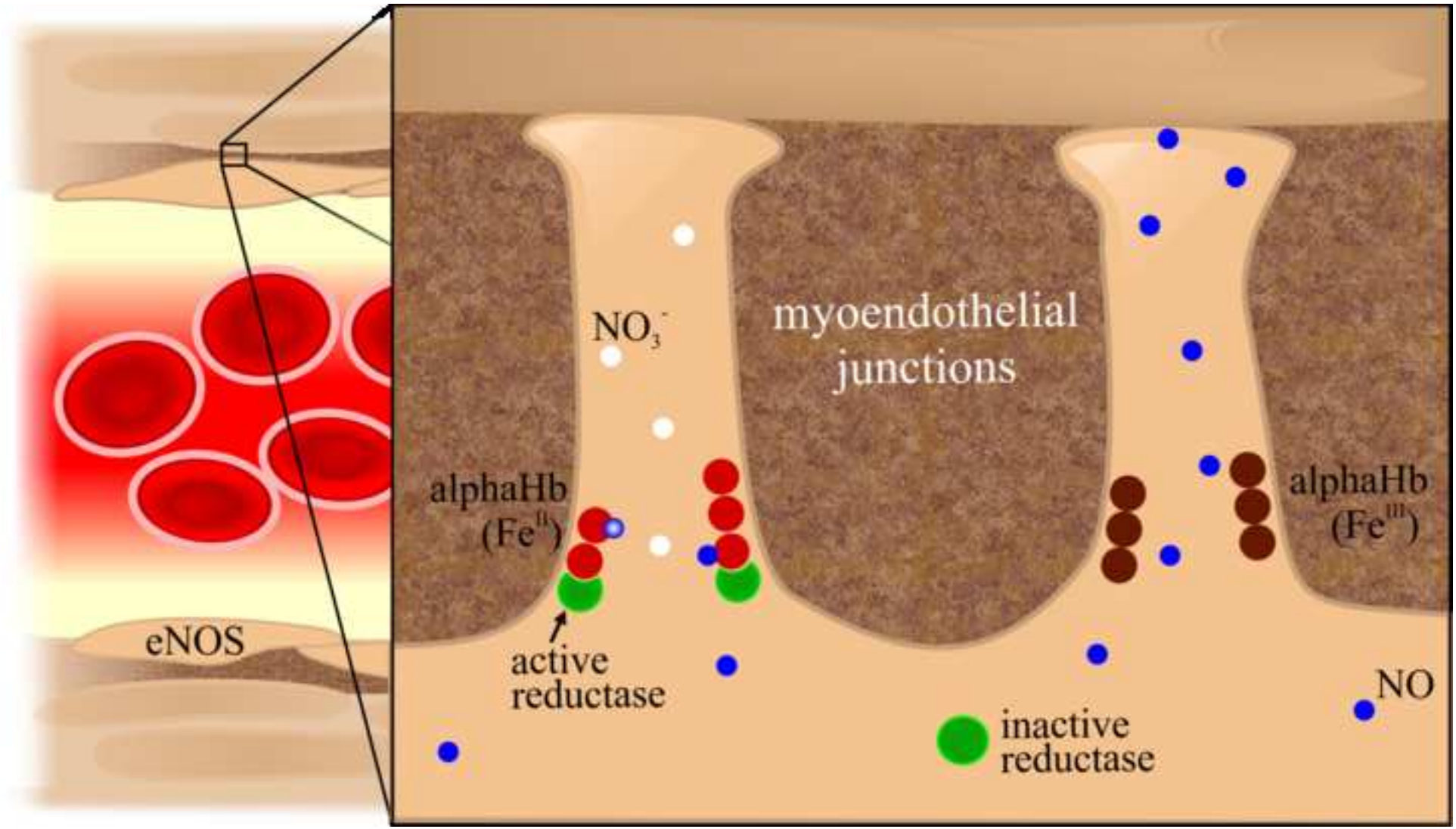

Figure 3

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