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Nitric oxide, vasodilation and the red blood cell

Michael J. Simmonds[a], **Jon A. Detterich**[b], and **Philippe Connes**[c,d,e,*]

[a]Heart Foundation Research Centre, Griffith Health Institute, Griffith University, Queensland, Australia

[b]Division of Cardiology, Childrens Hospital Los Angeles, Los Angeles, CA, USA

[c]UMR Inserm 1134, Hôpital Ricou, CHU de Pointe à Pitre, Pointe à Pitre, Guadeloupe

[d]Institut Universitaire de France, Paris, France

[e]Laboratory of Excellence GR-Ex “The red cell: from genesis to death”, PRES Sorbonne Paris Cité, Paris, France

Abstract

Since the identification of the elusive endothelium-derived relaxing factor as nitric oxide (NO), much attention has been devoted to understanding its physiological effects. NO is a free radical with many roles, and owing to its neutral charge and high diffusion capacity, it appears NO is involved in every mammalian biological system. Most attention has been focused on the NO generating pathways within the endothelium; however, the recent discovery of a NO synthase (NOS)-like enzyme residing in red blood cells (RBC) has increased our understanding of the blood flow and oxygen delivery modulation by RBC. In the present review, pathways of NO generation are summarized, with attention to those residing within RBC. While the bioactivity of RBC-derived NO is still debated due to its generation within proximity of NO scavengers, current theories for NO export from RBC are explored, which are supported by recent findings demonstrating an extracellular response to RBC-derived NO. The importance of NO in the active regulation of RBC deformability is discussed in the context of the subsequent effects on blood fluidity, and the complex interplay between blood rheology and NO are summarized. This review provides a summary of recent advances in understanding the role played by RBC in NO equilibrium and vascular regulation.

Keywords

Erythrocyte; nitric oxide synthase; nitrite; vascular function

[*]Address for correspondence: Philippe Connes, UMR Inserm 1134, Hôpital Ricou, CHU de Pointe à Pitre, 97159 Pointe à Pitre, Guadeloupe. Tel.: +590 690 36 76 28; Fax: +590 590 83 05 13; pconnes@yahoo.fr.

In memory of our inspiring mentor and friend, Prof. Oguz K. Baskurt

1. Nitric oxide

1.1. Introduction

The field of nitric oxide (NO) biology has expanded considerably over the past decades. Many works demonstrated the key role of NO in the cardiovascular system, and the importance of this discovery led to the 1998 Nobel Prize Award in Physiology/Medicine to Robert Furchgott, Louis Ignarro and Fred Murad. NO is a free radical, which can diffuse at a rate of approximately 50 μm/s in aqueous solution [76]. *In vivo*, the direct action of NO is regionally limited due to its half-life of ~10 s [60], although several longer-lived NO-related species exist that increase the temporal and spatial bioactivity of NO.

1.2. NO and vasodilation

Although the functions of NO are numerous, including beta-adrenergic dependent modulation of myocardial contractility [1], inhibition of platelet activation and adhesion, action as a neurotransmitter and involvement in host defense reactions, it is best known as a powerful modulator of vasomotor tone and blood flow resistance [44,59]. Generation of NO occurs endogenously by a family of structurally similar enzymes (nitric oxide synthase; NOS), in addition to NOS-independent reactions that involve NO-containing compounds and/or the inorganic anions nitrate and nitrite. The NOS isoforms are classified by the tissue within which they were first identified, and not surprisingly also indicates their principle functions: endothelial NOS (eNOS), for example, is critically involved in maintaining vasomotor tone, and is sensitive to free calcium. Consequently, upon increased intracellular calcium concentration and/or an association with the calcium/calmodulin complex, eNOS is phosphorylated and generates NO from L-arginine. The diffusion of NO into adjacent smooth muscle cells results in its binding of the ferrous (Fe^{2+}) heme group of soluble Guanylyl Cyclase (sGC). This activated form of sGC enzymatically converts guanosine triphosphate (GTP) into the second messenger cyclic guanosine monophosphate (cGMP), which interacts with and activates cGMP-dependent protein kinases (PKGs). Activated PKGs induce smooth muscle relaxation by multiple mechanisms, including the modulation of myosin light chain kinase, decreased intracellular free calcium, as well as hyperpolarization of the cell membrane by regulating the activity of potassium channels or Na^+/K^+ ATPase [15,46,70]. Increased cGMP may also act to regulate intracellular calcium concentration and dephosphorylate myosin light chain, and thus in doing so, reduce its calcium sensitivity [44,89].

1.3. Shear stress modulation of endothelial NO production

Endogenous production of NO within endothelial cells may occur both with, and without, the involvement of calcium, and is mainly regulated by shear stress (the product of shear rate times blood viscosity). G protein–phospholipase interactions that involve tyrosine-kinase (TK)-dependent phosphorylation result in transient inositol tri-phosphate (IP3) evoked calcium release from internal stores. The subsequent depletion of these stores promotes influx of calcium via store-operated channels (SOC) that appear to be activated by mechanisms involving TK-dependent phosphorylation and epoxyeicosatrienoic acids derived from arachidonic acid via cytochrome P-450 monooxygenase (CYP450). Shear stress and elevations in internal calcium availability also stimulate K^+ channel activity, and the

associated more negative membrane potential promotes calcium entry by increasing the electrochemical gradient for influx. Elevated intracellular calcium concentration is of significance, given eNOS has a calmodulin binding site on its reductase domain; the binding of the calcium/calmodulin complex to eNOS is believed to initiate electron exchange between the domains of the eNOS dimer, resulting in NO synthesis [28].

eNOS is expressed in several intracellular compartments, and also at the plasma membrane where it co-localizes and is negatively regulated by the structural coat protein caveolin. Moreover, TK-dependent phosphorylation and G proteins may stimulate phosphatidylinositol 3-kinase (PI3K), which is an upstream activator of protein kinase B (Akt/PKB) that in turn phosphorylates eNOS to increase its activity in a calcium-independent manner in response to shear stress. Increased shear stress favors the activation of eNOS via phosphorylation at various sites [23,30,31] although phosphorylation of a serine residue at 1177 (Ser1177) has been accepted as the activated form of the enzyme under various conditions [23,32]. Once activated via this shear stress induced pathway, eNOS consumes L-arginine to produce NO. It may also be noted that shear stress may increase NO production by releasing endothelium-dependent agonists such as ATP, bradykinin or substance P [57]. Moreover, the level of shear stress has been shown to regulate eNOS expression and therefore is critically involved in the production of NO by endothelial cells [65]. The enhancement of eNOS mRNA expression in bovine aortic endothelial cells by increasing shear stress is not inhibited by dexamethasone, inhibitors of tyrosine kinases (TK), or inhibition of G-protein signaling. In contrast, chelation of intracellular calcium in endothelial cells reduced shear stress induction of eNOS mRNA by 70% [100].

Increased shear rate associated with change in local blood flow, is known to increase the production of NO through shear stress-dependent mechanisms in different vascular beds: conduit arteries [75], resistance arteries [39] and arterioles [50]. However, few studies addressed the effects of viscosity (another component of shear stress). Several authors reported that high-molecular-weight dextran solutions increased NO release from isolated vessels, although the mechanism appeared to be independent of flow rate [43]. The group of Intaglietta [88] also found that increased plasma viscosity in anemic animals stimulated the expression of eNOS, increased NO production and resulted in vasodilation. Blood viscosity-related increases in NO production are likely to contribute to the increased organ blood flow found in high viscosity states, such as in polycythemia [98], although it is clear that other disorders associated with hyperviscosity (e.g., hypertension, hypercholesterolemia) result in reduced eNOS activity and decreased NO bioavailability [68]. Finally, the increase in blood flow and blood viscosity during acute exercise are presently thought to result in increased NO production through shear stress-dependent mechanisms [19].

1.4. RBC as a source of NO

The interactions between RBC- and endothelial-derived NO, and the subsequent physiological responses are provided in Fig. 1. Plasma free-hemoglobin scavenges NO by reducing NO to nitrate in the bloodstream and by depositing itself at the basement membrane of endothelial cells [33]. Furthermore, NO entering into RBC is rapidly

inactivated by oxyhemoglobin (HbO_2) via conversion to methemoglobin (MetHb) and nitrate, which cannot be converted back to nitrite or other NO equivalents except in oral/GI bacteria; hence, this outcome is a virtual “dead end” of nitric oxide activity [97]. The production of NO within such close proximity to the scavenging effects of free-hemoglobin at the endothelium level, in the presence of free heme or within RBC which contain a high concentration of hemoglobin, raises the question regarding the associated bioactivity given the inherent challenge for NO escaping this environment [33]. However, the unstirred diffusional barrier that exists around RBC limits the rate of NO–hemoglobin reactions by approximately 600-fold [52]. Moreover, and perhaps of more biological significance, S-nitrosylated hemoglobin or nitrite-treated deoxyhemoglobin may behave as NO donorsinstead of NO scavengers [34]. Consequently, there is increased interest regarding the role that RBC could play in the regulation of NO bioavailability, including:

1. When NO enters into RBC, it can bind to the highly conserved β-globin chain cysteine 93 residue to form bioactive S-nitrosohemoglobin (SNO–Hb) [71]. SNO–Hb associates with the membrane primarily through the interaction with Band 3 (i.e. the transmembrane anion-exchanger protein 1; AE1) via its N-terminal cytoplasmic domain (CDAE1). Upon deoxygenation, transfer of the NO group from β-Cys-93 of SNO–Hb to a cysteine thiol within CDAE1 favors generation and release of significant portion of RBC vasodilator activity; i.e., release of S-nitrosothiols (SNO) [71,72]. The role of circulating SNO–Hb on vasodilation has been challenged with results suggesting a minor role of SNO–Hb and SNO in the regulation of basal vascular tone [35]. Indeed, a knock-in mouse model that contained human hemoglobin modified to express an alanine in place of cysteine 93 residue on the β-chain resulted in no change to the level of RBC-induced hypoxic vasodilation [45]. These results supported a limited bioactive role for SNO–Hb, although this mouse model still expressed typical SNO abundance. Pawloski et al. [72] investigated the possibility of sickle RBC to mediate hypoxic vasodilation through the involvement of SNO-Hemoglobin S (SNO–HbS). The authors demonstrated that both SNO–HbS formation, and transfer of NO groups from SNO–HbS to a cysteine thiol within the CDAE1, are deficient in sickle RBC. The authors concluded that RBC-induced vasodilation is impaired in sickle cell disease.

2. NO may be formed from nitrite entering RBC, mediated by the reductive potential of deoxyhemoglobin [34]. Nitrite reduction by hemoglobin results in a large fraction of the NO generated being retained in the intermediate state where NO is bound to MetHb and is in equilibrium with the nitrosonium bound to hemoglobin [67]. This pool of NO, unlike heme-nitrosylated hemoglobin, is weakly bound and can be released from the heme, particularly during hypoxic conditions to provide compensatory vasodilation (i.e., hypoxic vasodilation) to increase local blood flow and reestablish oxygen supply to metabolically active tissues [21,42,58,62]. Huang et al. [42] demonstrated that the nitrite reduction rate is maximal when hemoglobin is 40–60% saturated with oxygen. Supporting this hypothesis was the *in vivo* observation that there is a significant and positive

arteriovenous nitrite gradient, consistent with the oxygen uptake (and RBC deoxygenation) across active tissue [22].

3. A third method of NO production by RBC has been recently described in humans by Kleinbongard et al. [47]. They demonstrated that RBC express an active and functional eNOS-like enzyme (RBC NOS), which is localized in the RBC membrane and cytoplasm. RBC NOS has similar properties than eNOS in terms of phosphorylation sites controlling enzymatic activity and the dependence of its activity on intracellular calcium and L-arginine concentrations [47]. Extracorporeal circulation has been reported to increase RBC NOS activity [29]. More recently, Ulker et al. demonstrated that RBC exposed to shear stress resulted in RBC NOS activation (increased immunostaining for Ser1177 phosphorylated NOS), and subsequent production and exportation of NO (increased NO levels in RBC suspensions as measured by electrochemical NO probes) [91–93]. Similar to the vasculature, shear stress appears to stimulate mechanosensitive channels in the RBC plasma membrane that facilitate calcium flux into the cell. Ulker et al. [93] demonstrated that extracellular calcium availability (1 mM extracellular [Ca^{2+}]) and importantly, shear stress-mediated influx of calcium, was requisite for RBC NOS activation. Chelation of extracellular calcium inhibited intra-cellular NO formation, and thus emphasized the involvement of calcium in shear stress activation of RBC NOS. Basal free intracellular [Ca^{2+}] in the RBC is between 30–60 nM and total intracellular [Ca^{2+}] (bound +free) may be as high as 5.7 μM, whereas extracellular [Ca^{2+}] may approach 1.8 mM, thus creating a large gradient by which RBC influx via Ca^{2+} channels may increase intracellular [Ca^{2+}] > 10-fold [89]. It must be acknowledged that while it seems shear stress is the main stimulus for RBC NOS activation, the amount of NO exported from RBC is higher in hypoxic than in normoxic conditions [92,93], supporting the complementary roles that SNO–Hb and nitrite reduction may contribute to RBC-derived NO generation.

The manner of NO export from RBC is controversial and depends on which species is being discussed – SNO–Hb, nitrite or NO – and each pathway must overcome barriers to successfully contribute to bioactivity. Transfer of NO from SNO–Hb to membrane-bound AE1 in order to transfer NO out of the RBC is dependent on SNO–Hb state (T or R) and SNO–Hb concentration; therefore, SNO–Hb ability to transfer NO to AE1 or other proteins (e.g., glutathione) appear to be crucial limiting factors. The kinetics and allosteric regulation of hemoglobin nitrosylation by oxygen and pH are consistent with physiologic mechanisms to modulate tissue blood flow whereby acidosis, hypoxemia and tissue hypoxia lead to NO generation by the RBC via SNO–protein transfer of NO activity [24,83]. A barrier to NO export specific to the reduction of nitrite is the potential for irreversible oxidization of nitrite to nitrate [33]. Moreover, when nitrite is converted to NO by deoxyhemoglobin, diffusion of NO out of RBC without being reduced by the now relatively abundant unoccupied Fe^{2+} within heme, or glutathione, poses a significant barrier to bioactivity. RBC NOS production of NO would also be prone to the same diffusion barriers as NO produced from nitrite reduction. While membranes and hydrophobic structures do not present diffusion barriers *per se* due to NO's high solubility [52], it is suggested that NO auto-oxidation may be

accelerated up to 300-times in the presence of a hydrophobic phase [53] – consequently, the lipid bilayer membrane of RBC may also represent a significant barrier to bioactivity. Despite these findings, however, recent observations support that RBC suspensions show a rise in extracellular NO concentration following exposure to shear stress and deoxygenation [91].

The efficiency of NO produced by RBC NOS to promote vasodilation was unknown and debated until the recent work of Ulker et al. [90]. The authors observed that perfusion of vessel segments with pre-sheared RBC suspensions caused a significant dilation response under hypoxic conditions, but not at high oxygen levels (a small but not-significant vasodilation was observed under this condition). Incubation of RBC suspension with L-NAME prior to shear stress application abolished vasodilation under both conditions. These findings indicate that shear stress activation of RBC NOS, leading to NO release, results in vasodilation of vessel segments under hypoxic conditions, and supports that RBC-derived NO has a functional role in local blood flow regulation [90]. When these findings are viewed alongside other studies which demonstrated that shear stress was able to induce ATP release from hypoxic RBC (a potent vasodilator also capable of stimulating vascular eNOS), it is well-founded that RBC are now considered O_2 sensors [25]. This hypothesis is built upon the pivotal role that RBC play in vasoregulation when O_2 tension is low, as is the case within metabolically active tissues.

Polymorphisms and allele number variation of the eNOS gene have been implicated in abnormal responses to exercise, impaired RBC rheology, and elevated disease risk. Kojda [48] demonstrated that mice heterozygous for eNOS (eNOS+/eNOS−) have normal endothelial dependent vasodilation; however, in response to exercise, the increase in eNOS expression and activation was absent [48]. Fatini demonstrated that T-786C and G894T polymorphisms in the eNOS gene independently affect RBC deformability [26] and increase the risk for acute coronary syndromes [27], while others reported that G894T is associated with idiopathic sudden sensorineural hearing loss after adjusting for other vascular risk factors [54]. T-786C polymorphism is associated with RBC membrane fluidic changes [63], coronary artery disease in some Caucasians groups [79] but not those in Australia [37], vasoocclusive events and acute chest syndrome in sickle cell disease [13,80]. Therefore, the mechanism underlying decreased RBC-deformability and vascular disease in healthy as well as chronically ill patients may depend on eNOS polymorphisms, which affect both endothelial cells and RBC.

2. NO and blood rheology

2.1. Short introduction on RBC rheology

RBC deformability and aggregation are known to affect blood viscosity in a shear-dependent fashion with increased RBC aggregation increasing blood viscosity at low shear rate, and elongation and alignment of RBC decreasing blood viscosity at high shear rate [3]. In addition, these two RBC rheological properties may affect blood flow independent of their effects on blood viscosity. Decreased RBC deformability may cause mechanical obstruction at the entry of capillaries and decrease tissue perfusion. Interventions to decrease RBC deformability have demonstrated significant decrements in blood flow for a given perfusion

pressure indicating increased vascular resistance [69]. Moreover, increased RBC rigidity has been demonstrated to cause increased mechanical stress applied on endothelial cells promoting dysfunction and over-expression of vascular cell adhesion molecules, even in the absence of ischemia and oxidative stress [55]. Increased RBC aggregation may increase vascular resistance, particularly at the microcirculation level where RBC aggregates need to be dispersed to facilitate blood flow through narrow apertures [101,103]. Several studies have investigated the RBC rheological responses to metabolic factors such as lactate [17,18,81], oxidative stress [4,82], hydrogen ions [51], etc.; however, evidence is also accumulating that NO is an important modulator of RBC rheology.

2.2. NO and RBC deformability

One of the first works suspecting an effect of vascular-derived NO (extracellular NO) on RBC deformability was the study of Starzyk et al. [84] who demonstrated that intravenous infusion of L-NAME (an eNOS inhibitor) in rats caused a reduction of RBC elongation index. Bor-Kucukatay et al. [10] independently reported several eNOS inhibitors (L-NAME and S-methylisothiourea) that reduced NO availability also decreased RBC elongation index, suggesting that basal release of NO actively maintains RBC deformability. Korbut et al. [49] demonstrated that, in the presence of a small number of polymorphonuclear leukocytes, RBC filterability was increased by NO donors, such as sydnonimine or sodium nitroprusside, but was reversed by NOS inhibition. The positive effects of NO donors, including sodium nitroprusside and diethylenetriamine, for increased RBC deformability were also reported [10]. Horn et al. [41], using intra-vital microscopy, tested the effects of NO on RBC velocity in the microcirculation of the chorioallantoic membrane of the avian embryo at day 7 post-fertilization, when all vessels lack smooth muscle cells and therefore blood velocity is dependent more on blood rheology since the vessels do not change diameter. The authors confirmed that inhibition of enzymatic NO synthesis and NO scavenging decreased intracellular RBC NO concentration and avian RBC velocity *in vitro*. In addition, these authors demonstrated that injection of an eNOS-inhibitor or NO-scavenger into the previously defined microcirculation *in situ* decreased capillary RBC velocity and deformation, while the diameter of the vessels remained constant. These findings indicate that NO scavenging and inhibition of NO synthesis decrease RBC velocity (i.e., increases RBC transit time) by the active modulation of RBC deformability [41]. Nevertheless, it is important to note that very high NO concentrations, as can be the case in various inflammatory states, may be deleterious for RBC deformability [49,56,84]. Thus, in summary, it appears that extracellular NO influences RBC structural and functional properties in a non-linear, dose-dependent manner [56].

While NO from extracellular sources may have an impact on the deformability of RBC, several works strongly suggest that endogenous RBC NO synthesis may also modulate RBC deformability [47]. Suhr and colleagues [86] demonstrated that exercise-induced increase in shear stress activated RBC NOS (increased RBC NOS phosphorylation at Ser1177) via the PI3-kinase/Akt kinase pathway and this enhanced activity caused a greater production of NO, which was critical to maintain ability of RBC to deform in flow (i.e., RBC elongation index) during exercise. This exercise bout did not change the total RBC NOS content or RBC NOS phosphorylation at threonine 495 – a residue associated with inhibition of NO

production. Grau et al. [38] further extend these findings by showing that RBC NOS activation by pharmacological treatment (insulin) lead to increased RBC NO content and improved RBC elongation index through direct S-nitrosylation of cytoskeleton proteins, most likely α- and β-spectrins. In contrast, the use of RBC NOS inhibitors (wortmannin or L-N5-(1-Iminoethyl)-ornithin) resulted in a decrease of RBC NOS Ser1177 phosphorylation, NO content, cytoskeleton proteins S-nitrosylation and RBC elongation index.

There are currently several explanations for the NO-mediated alteration in RBC deformability. As previously outlined, the effects of NO on the vascular smooth muscle are known to be mediated by pathways involving sGC – an enzyme also expressed in RBC [73,74]. It is not surprising to observe that guanylyl cyclase inhibition, thereby limiting the production of the second messenger cGMP, results in decreased RBC elongation index [10]. However, both sodium nitroprusside and diethylenetriamine reversed the effect of guanylyl cyclase inhibition in a dose-dependent manner [10] indicating that the effect of NO on RBC deformability is partially mediated by sGC. NO has also been shown to affect potassium transport across RBC membrane [12]. Bor-Kucukatay et al. [10] have demonstrated that the use of a potassium channel blocker (triethylammonium chloride) reversed the deleterious effects of L-NAME on RBC elongation index [10] suggesting that the inhibition of NO synthesis by this nonspecific NOS inhibitor may lead to deterioration of RBC mechanical properties by increasing the potassium permeability of the RBC membrane. Baskurt et al. [5] also demonstrated that sodium nitroprusside prevented the deterioration of RBC (i.e. sub-hemolytic damage) exposed to high shear stress for 15–120 s. The authors observed a similar protective effect of the triethylammonium chloride suggesting that the beneficial effect of NO on both RBC deformability and fragility is mediated by the inhibition of potassium leakage from RBC [5].

In addition, NO could decrease the risk for hemolysis and increase RBC survival rate through its effects on eryptosis since NO is able to down-regulate caspase 3 activity through S-nitrosylation [14]. More recently, another group demonstrated that the NO donor sodium nitroprusside inhibited the decrease of RBC deformability induced by ionophore A23187-mediated calcium influx in RBC [2]. Increased intra-cellular calcium concentration activates calcium-sensitive K^+ (gardos) channels, resulting in potassium-efflux and decreased cell-volume, which in turn increases the stiffness of RBC; however, the presence of sodium nitroprusside abolished this calcium-induced impairment in RBC deformability [2]. Barodka et al. [2] suggested that sodium nitroprusside may have limited calcium influx, thereby inhibiting the activation of gardos channels, and thus, maintaining cell volume and RBC deformability. However, this interpretation is in contrast to the findings discussed previously regarding the importance of calcium influx for RBC NOS activation and intracellular NO accumulation, which positively improves RBC deformability [93]. Nicolay et al. [64] demonstrated that sodium nitroprusside, as well as the NO-donor papanonoate, was able to reverse the phosphatidylserine exposure and cell shrinkage induced by the use of Ca^{2+} ionophore ionomycin, but not the increase of cytosolic Ca^{2+} activity. Moreover, papanonoate was able to reverse the decrease of protein nitrosylation and thioredoxin activity induced by ionomycin [64]. These findings collectively suggest a protective role of NO on RBC

rheological properties [2] and eryptosis [9] through its effects on cGMP, protein nitrosylation and thioredoxin activity [64].

2.3. NO and RBC aggregation

Few works have tested the effects of NO on RBC aggregation properties [11,85]. Bor-Kucukatay et al. [11] demonstrated that incubation of RBC with sodium nitroprusside decreased RBC aggregation while giving L-NAME to rats resulted in a rise of their RBC aggregation. The underlying mechanisms at the origin of these findings are unclear but might involve membrane/cytoskeletal protein nitrosylation or oxidative stress modulation [11].

To our knowledge, there have been no studies designed to investigate the effects of RBC aggregation on endogenous NO production by RBC. One could theorize the effects of RBC aggregation on available surface area for NO diffusion, changes to the unstirred layer surrounding the RBC and most importantly, the effect of the depletion layer on NO scavenging and NO release. This would be particularly important in the venous system and in conditions that would create passive blood flow to an arterial system, i.e., pulmonary arteries receiving passive blood flow in the Fontan type circulation for single ventricle palliation.

Few works have investigated the effects of RBC aggregation on NO production by endothelial cells. It has been established that blood flow and wall shear stress in the vascular system are important determinants of NO synthesis by endothelial cells [20,61,77], whereas decreased blood flow suppressed eNOS expression and flow-mediated dilation responses in small arteries [95]. An elegant study performed by Baskurt et al. [6] on rats submitted to exchange transfusion with aggregating RBC suspensions demonstrated that enhanced RBC aggregation resulted in suppressed expression of endothelial NO synthesizing mechanisms, thereby leading to altered vasomotor tonus. The authors interpreted these findings by proposing that the decreased blood flow associated with increased RBC aggregation ultimately results in reduced wall shear stresses [6]. It should be noted, however, that the effects of RBC aggregation on hemodynamic resistance are not a straightforward relationship, but rather depends on the perfusion pressure and the ability of the vascular system to dilate [101]. Moreover, enhanced RBC aggregation would also tend to promote axial migration of RBC away from blood vessels towards central flow streamlines, resulting in a less-viscous, plasma-rich region near vessel walls [16]. Decreased wall shear stress resulting from this non-uniform radial composition of blood should be expected to influence the NO-related mechanisms in the vascular system [16,36]. In that case, the viscosity of plasma near the vessel wall could be the main modulator of shear stress, eNOS activation and NO production. Yalcin et al. [102] tested the effect of enhanced RBC aggregation, with (use of 500 kDa dextran) or without (use of poloxamer-coated RBC) altering plasma viscosity, on NO production and eNOS activation in perfused human umbilical vein endothelial cells. Perfusion with poloxamer-coated RBC resulted in decreased NO production and reduced phosphorylation of eNOS Ser1177. In contrast, perfusion with RBC suspended in plasma containing dextran resulted in a NO concentration that remained elevated, highlighting the role of plasma viscosity on NO production. Tsai et al. [88] found

that increasing plasma viscosity in anemic hamster increased wall shear stress, stimulated eNOS and NO production, and thus promoted vasodilation. These two scenarios (decreased blood flow and a less-viscous region near the vessel wall) are not mutually exclusive and most likely are synergistic in decreasing mechanical forces acting on endothelial cells [6–8]. However, while increased RBC aggregation could decrease eNOS activation and NO production, several findings suggest that enhanced RBC aggregation (for a given hematocrit) promote NO bioavailability because the increased plasma cell-poor layer width may actually provide a spatial buffer against the scavenging effects of hemoglobin-containing RBC [66]. Decreased RBC aggregation and RBC deformability (a situation encountered in sickle cell disease [87]) have been shown to interfere with axial migration and to decrease the plasma cell-poor layer width [104], a scenario expected to decrease NO bioavailability because RBC near the vessel wall would consume NO. Finally, the width of the glycocalyx maintains physical separation of RBC and the vessel wall [96]; it follows then that the glycocalyx also represents a spatial–diffusional barrier for endothelium-derived NO to be scavenged by RBC. Indeed, the decreased glycocalyx volume found in sickle cell patients [94] may contribute to the decreased NO bioavailability previously reported in this population.

3. Future considerations and clinical targets

Nitric oxide is a ubiquitous signaling molecule that is present in multiple tissues. The role of RBC involvement in NO bioactivity is still being determined, but the evidence thus far supports that RBC are integral in contributing to vascular regulation through several mechanisms. SNO–Hb is an exciting mechanism to improve tissue oxygenation, thereby mitigating the RBC storage lesion that has been shown to associate increased blood storage age with adverse clinical vascular outcomes [78]. RBC NOS contributes approximately 50% of the circulating nitrite and regulates systemic blood pressure [99]. Nitrite also decreases platelet aggregation in the circulation and is dependent on the presence of RBC [40]. The ongoing discussion regarding the role of specific nitric oxide species in hypoxic vasodilation is not currently settled, and the relative contribution of the RBC in producing these NO species is still being determined; however, our understanding of the role RBC play in NO equilibrium and vascular regulation has improved significantly in the last decade. Figure 2 demonstrates the potential interaction that is at work in the RBC to generate NO. The individual species contribute to NO in the RBC and may each represent one limb of the NO pool, represented as legs on a stool. Whether these mechanisms act in concert to support the NO pool in the circulation or operate independently without regard for the others remains to be seen; however, the interdependence of each would weave an interesting fabric by which the RBC may act as an NO “recharger” in some situations or an NO “sink” in others. Further studies in the setting of different disease states that include abnormal rheology, hemoglobin subtypes, redox disturbances and blood flow conditions will provide insight into the importance for RBC-derived NO for contributing to systemic NO bioavailability.

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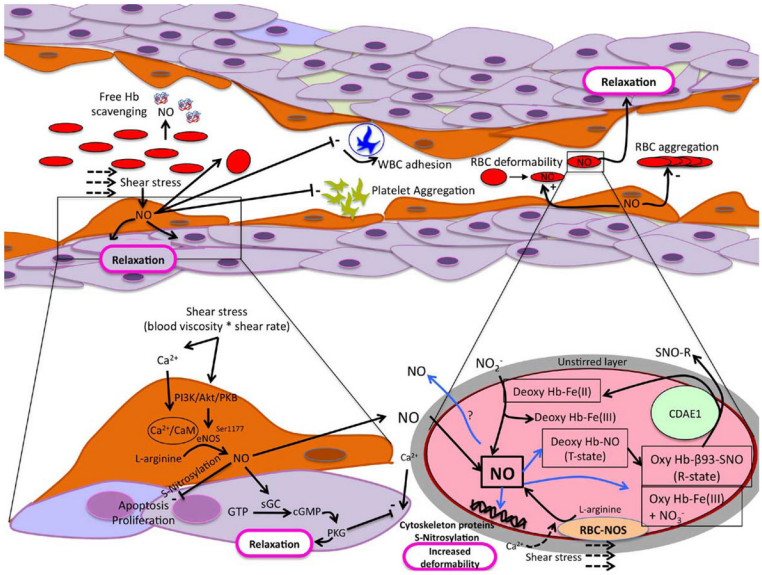

Fig. 1.

Nitric oxide (NO) generation pathways in endothelial cells and red blood cells (RBC). Similarities exist between activation of NO synthase (NOS) in endothelial cells and RBC, including sensitivity to shear stress and the involvement of Ca^{2+} (see in-text for more detail). With respect to blood flow modulation, NO-bioactivity includes decreasing aggregation of platelets, inhibiting cell adhesion, increasing RBC deformability, and vascular smooth muscle relaxation. Whereas endothelium-generated NO can diffuse into adjacent smooth muscle cells and induce vasodilation, for RBC-derived NO to induce vasodilation several NO “sinks” (e.g., hemoglobin in RBC and plasma) must be avoided due to its short half-life and reactivity. While some RBC-NOS generated NO appears to directly modulate blood flow (e.g., increased RBC deformability; vasodilation), it appears that this NO may also substantially contribute to RBC-pools of S-nitrosylated proteins and nitrite, and therefore may provide an increased spatial and temporal effect beyond the RBC membrane. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BIR-140653.)

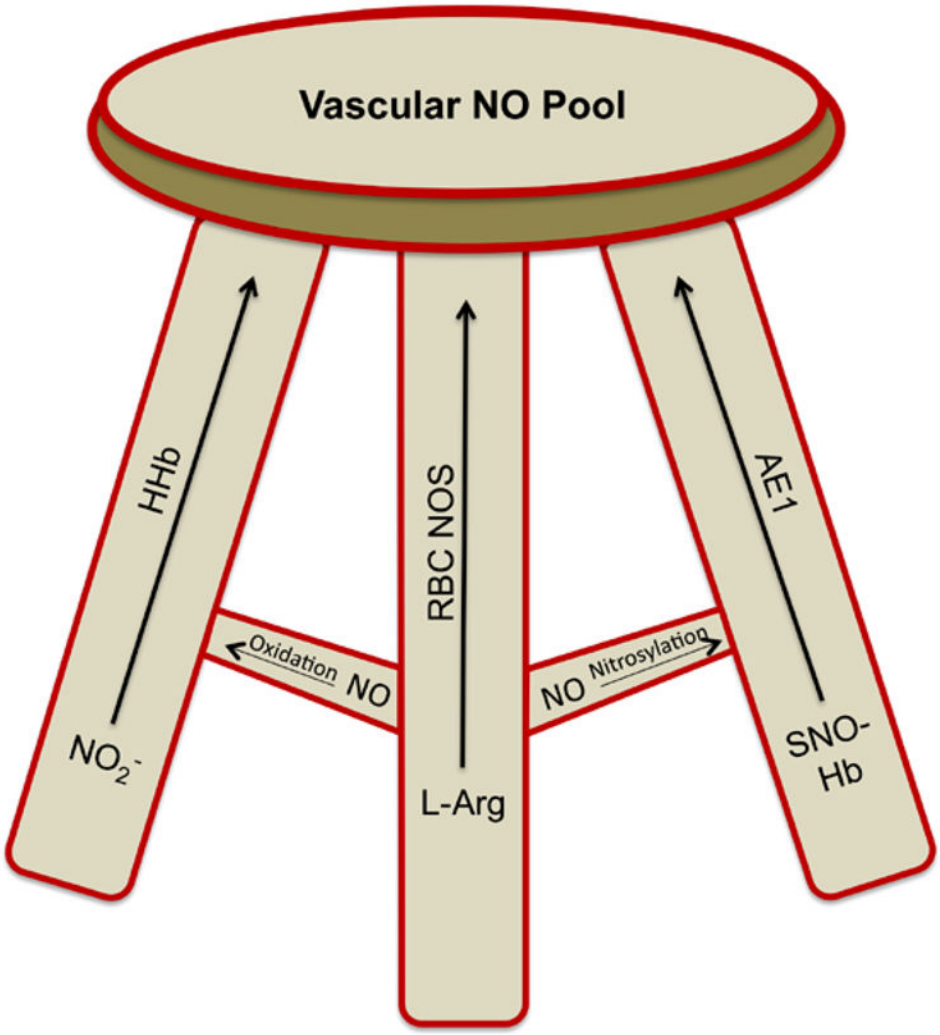

Fig. 2.

A model that represents the interrelationships between the various nitric oxide (NO) sources in red blood cells (RBC), and their collective contributions to NO bioavailability. Recent findings support that there is a certain amount of redundancy in this system, when one 'leg' is impaired (e.g., inhibition of S-nitrosohemoglobin – SNO–Hb – formation in mutant mice), NO bioavailability may be preserved. However, a central role of RBC NO-synthase in providing a basal load of NO that in somehow 'recharges' the nitrite/SNO–Hb pool, in addition to a potential extracellular export of NO, is hypothesized. Further explanations are provided in-text. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BIR-140653.)