9-8-2016

Recent Insights Into Nitrite Signaling Processes in Blood

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

Xiaohua Liu

Daniel B. Kim-Shapiro

Recommended Citation

Christine C. Helms*, Xiaohua Liu and Daniel B. Kim-Shapiro, "Recent insights into nitrite signaling processes in blood," *Biological Chemistry* 398, no. 3 (2016): 319-329, doi:10.1515/hsz-2016-0263.

Review

Christine C. Helms*, Xiaohua Liu and Daniel B. Kim-Shapiro*

Recent insights into nitrite signaling processes in blood

DOI 10.1515/hsz-2016-0263
Received August 4, 2016; accepted August 30, 2016; previously published online September 8, 2016

Abstract: Nitrite was once thought to be inert in human physiology. However, research over the past few decades has established a link between nitrite and the production of nitric oxide (NO) that is potentiated under hypoxic and acidic conditions. Under this new role nitrite acts as a storage pool for bioavailable NO. The NO so produced is likely to play important roles in decreasing platelet activation, contributing to hypoxic vasodilation and minimizing blood-cell adhesion to endothelial cells. Researchers have proposed multiple mechanisms for nitrite reduction in the blood. However, NO production in blood must somehow overcome rapid scavenging by hemoglobin in order to be effective. Here we review the role of red blood cell hemoglobin in the reduction of nitrite and present recent research into mechanisms that may allow nitric oxide and other reactive nitrogen signaling species to escape the red blood cell.

Keywords: erythrocyte; hemoglobin; hypoxia; nitric oxide; vasodilation.

Introduction: nitric oxide in the blood

Endothelium-derived nitric oxide (NO) is an important cardiovascular signaling molecule whose production increases blood flow, reduces blood pressure, decreases blood cell adhesion, and diminishes platelet activation. In normoxic conditions, nitric oxide synthase (NOS) found on endothelial cells and red blood cells catalyzes the production of NO and L-citrulline from molecular oxygen and L-arginine (Andrew and Mayer, 1999; Forstermann and Sessa, 2012). The source of normoxic vasodilation is widely believed to be endothelium derived NO (Moncada and Higgs, 1993). As oxygen is a substrate for NOS, at low oxygen levels NO production by NOS is diminished (Totzeck et al., 2012). Therefore, at low oxygen tension mechanisms of hypoxic NO production, such as nitrite reduction by deoxygenated hemoglobin (Hb) in red blood cells (RBCs) can take over.

Hb is present in the blood at a concentration of about 10 mM and Hb rapidly scavenges NO. NO reacts with oxygenated Hb (oxyHb) at a rate of $5–6\times10^7$ M^{-1} s^{-1} and deoxygenated Hb (deoxyHb) at a rate of $2–6\times10^7$ M^{-1} s^{-1} (Cassoly and Gibson, 1975; Morris and Gibson, 1980; Doyle et al., 1981; Cooper, 1999; Kim-Shapiro et al., 2006). Additionally, NO has a high affinity for Hb, with a dissociation constant of about 10^{-11}–10^{-12} (Cooper, 1999).

Although Hb is abundant in the blood it is compartmentalized to the RBC and the RBC is pushed to the center of the blood vessel in circulation. These mechanisms keep the Hb away from the endothelium, a major source of NO in oxygenated conditions, and minimize NO scavenging (Lancaster, 1994; Butler et al., 1998; Liu et al., 1998; Liao et al., 1999).

However, in deoxygenated and acidic conditions when the NOS no longer efficiently produces NO, the RBC has been shown to play a role in NO production through the use of nitrite. Therefore, it is important to determine how NO can be produced by nitrite and the RBC and how NO or another reactive nitrogen signaling species can escape the Hb rich RBC.

Nitrite acquisition

Nitrite concentration in the blood is derived through two main mechanisms. First, nitrite is formed from the oxidation of nitric oxide (NO) produced by nitric oxide synthase, found in the RBC itself and in endothelial cells (Kleinbongard et al., 2003, 2006; Cortese-Krott et al., 2012; Grau et al., 2013; Wood et al., 2013; Bizjak et al., 2015). The auto-oxidation of NO to nitrite proceeds slowly ($k=2\times10^6$ M^{-2} s^{-1}) compared to oxidation of nitric oxide to nitrate by hemoglobin ($k=8\times10^7$ M^{-1} s^{-1}) (Shiva, 2012).

***Corresponding authors: Christine C. Helms,** Physics Department, University of Richmond, Richmond, VA 23235, USA, e-mail: chelms@richmond.edu; and **Daniel B. Kim-Shapiro,** Physics Department, Wake Forest University, Winston-Salem, NC 27109, USA, e-mail: shapiro@wfu.edu
Xiaohua Liu: Physics Department, Wake Forest University, Winston-Salem, NC 27109, USA

However, ceruloplasmin in the plasma can catalyze the conversion of NO to nitrite (Shiva et al., 2006). The oxidation of endothelial derived NO accounts for roughly 70% of resting plasma nitrite (Kleinbongard et al., 2003). This is an approximation as it is difficult to quantitatively estimate the participation of each source of plasma nitrite as there could be interactions between nitrite sources.

Secondly, nitrite is obtained through dietary consumption. Nitrite, found in cured meats and cereal products, is efficiently absorbed by the intestines (Fritsch et al., 1979; Lundberg et al., 2008). However, the main dietary source of plasma nitrite is reduced dietary nitrate. Nitrate is found at high levels in root and leaf vegetables (Hord et al., 2009; Machha and Schechter, 2011). In the mouth some of the nitrate is converted to nitrite by anaerobic bacteria (Tannenbaum et al., 1976; Duncan et al., 1995). The nitrate and nitrite travel to the intestine where again part of it is absorbed and makes its way into the blood stream (Fritsch et al., 1979) and part passes on to be excreted in the urine (Carlsson et al., 2001). At this point a spiraling process begins where nitrate in the plasma is absorbed, concentrated and excreted in the saliva where it then interacts with bacteria in the mouth and is reduced to nitrite then travels to the intestine and so on (Lundberg et al., 2008). Endothelial and dietary derived nitrite lead to total plasma nitrite levels between 50 and 350 nM (Kelm et al., 1999; Rassaf et al., 2004; Lundberg et al., 2008).

Nitrite interactions

Nitrite was originally thought to be a biochemically inert end product in plasma (Lauer et al., 2001). However, for the past few decades this belief has been upturned and nitrite is now regarded as a storage pool for NO. Gladwin et al. (2000) measured significant arterial-venous plasma nitrite gradients indicating the consumption of nitrite during it transit to a lower PO_2 and studies by Cosby et al. showed nitrite infused into human forearms at physiological levels leads to vasodilation, a response indicative of NO production (Cosby et al., 2003). Multiple studies investigating the effect of nitrate consumption show increases in plasma nitrite are associated with a decrease in blood pressure, antiplatelet properties, a decrease in leukocyte adhesion, and an increase blood flow; all known signaling effects of NO (Jansson et al., 2008; Webb et al., 2008a; Stokes et al., 2009; Presley et al., 2011; Srihirun et al., 2012; Liu et al., 2015; Wightman et al., 2015).

Dietary studies add additional complexities such as the effect of the acidic reduction of nitrite in the stomach which can be enhanced by the consumption of polyphenol and/or ascorbic acid (Moriya et al., 2002; Takahama et al., 2002; Peri et al., 2005; Gago et al., 2007). For example, Medina-Remón et al. measured the influence of the Mediterranean diet, which is high in polyphenols, on plasma nitrite, plasma nitrate and blood pressure. They found that eating the Mediterranean high in polyphenols decreased blood pressure and increased plasma nitrite and nitrate and they attributed these effects to the catalyzed reduction of nitrite in the gut by polyphenols. However, the study did not measure the total intake of nitrite and nitrate and therefore it is not certain whether the measured effects are due to acidic reduction in the gut or increased nitrite in the blood stream due to consumption of nitrite and nitrate (Medina-Remón et al., 2015).

The above *in vivo* studies clearly point to a mechanism involving nitrite that leads to vasodilation. To identify the mechanism responsible for nitrite reduction, studies have investigated nitrite reduction by deoxygenated hemoglobin, xanthine oxidoreductase, carbonic anhydrase and deoxygenated myoglobin in the blood vessel, acid in the stomach, and bacteria in the oral cavity (Duncan et al., 1995; McKnight et al., 1997; Huang et al., 2005b; Webb et al., 2008b; Aamand et al., 2009; Totzeck et al., 2012; Tiso and Schechter, 2015). Additionally, studies have examined increased ATP production by red blood cells (RBCs) in the presence of nitrite and subsequent ATP release by red blood cells followed by stimulation of endothelial nitric oxide synthase (Dietrich et al., 2000; Cao et al., 2009). Each of these mechanisms are capable of producing NO and may contribute to the overall bioavailability of NO (see Figure 1).

Support for the deoxyRBC and nitrite pathway

The role of deoxygenated RBCs in nitrite bio-activation is evident from *in vitro* and *in vivo* studies where the addition of deoxygenated RBCs and nitrite lead to vasodilation (Cosby et al., 2003; Jensen and Agnisola, 2005; Crawford et al., 2006). In these studies, vasodilation was diminished when nitrite or RBCs were infused alone (Cosby et al., 2003; Jensen and Agnisola, 2005; Crawford et al., 2006). One explanation for increased NO production following RBC and nitrite exposure is the increased synthesis of RBC ATP in the presences of nitrite (Cao et al., 2009) followed by the hypoxic release of ATP from RBCs which then stimulates NOS (McMahon et al., 2002; Crawford et al., 2006). However, a response indicative of NO production is often seen in the presence of NOS inhibitors L-NAME or LMMA,

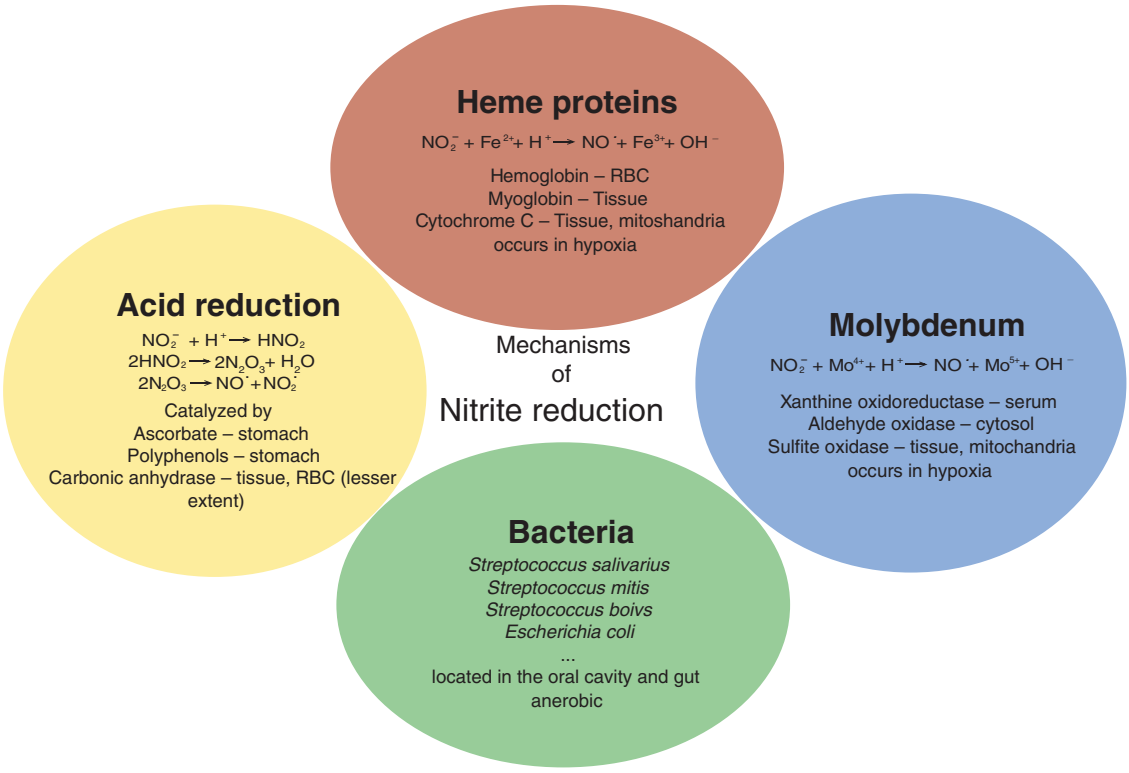


Figure 1: Mechanism of nitrite reduction.
We comprised a non-extensive collection of molecules and bacteria that reduce nitrite to NO and indicate where they are most commonly found.

suggesting ATP release by RBCs cannot be the sole mechanism responsible for vasodilation (Crawford et al., 2006; Liu et al., 2015). Others have shown an intrinsic vasodilation of vessels exposed to nitrite under hypoxic conditions in the absence of RBCs (Dalsgaard et al., 2007; Isbell et al., 2007), however, the timescale of this response does not match the timescale of transit from arteries to veins and the response to nitrite is increased in the presence of RBC (Crawford et al., 2006; Allen et al., 2009). Together these data points to a RBC mechanism that reduces nitrite to NO under deoxygenated conditions.

Very strong support for the role of RBCs in bioactivation of nitrite comes from studies of platelet activation and aggregation (Srihirun et al., 2012; Park et al., 2014; Liu et al., 2015; Wajih et al., 2016). When nitrite is added to platelet rich plasma after platelet activation is induced by ADP or another agent, there is no effect unless deoxyRBCs are present (Srihirun et al., 2012). When deoxyRBCs are present, the nitrite leads to inhibition of platelet activation and aggregation and this inhibition is potentiated by hypoxia but abrogated when a NO scavenger is added (Srihirun et al., 2012; Wajih et al., 2016). These affects are also observed in slowing down clot formation and maximum strength (Park et al., 2014). Importantly, effects are seen at physiological levels of nitrite (Srihirun et al., 2012; Park et al., 2014; Liu et al., 2015; Wajih et al., 2016).

Additional *in vitro* studies exclude other mechanisms from playing a large role in nitrite reduction by deoxygenated red blood cells. We recently showed that xanthine oxidoreductase does not play a large role in RBC mediated bio-activation of nitrite. In fact, the inhibition of xanthine oxidoreductase by allopurinol did not significantly change the production of NO (Liu et al., 2015). Additionally, inhibition of carbonic anhydrase did not affect the production of NO in the presence of nitrite and RBC when measured by EPR and chemiluminescence and only affected platelet activation at nonphysiological carbon dioxide concentration (Liu et al., 2015), suggesting that carbonic anhydrase is not the major source of nitrite reduction by RBCs. These data support the hypothesis that hemoglobin plays a prominent role in nitrite reduction by RBCs.

Nitrite interactions with Hb

Nitrite reacts with oxyHb, deoxyHb and methemoglobin (metHb). Nitrite reacts with oxyHb to form metHb and

nitrate. The oxyHb/nitrite reaction is autocatalytic at high ratios of nitrite to hemoglobin and involves H_2O_2 and NO_2. However, the autocatalytic character is lost at lower nitrite:oxyHb ratios and as the oxysaturation of hemoglobin decreases (Grubina et al., 2007; Keszler et al., 2008). Keszler et al. argued that *in vivo* the nitrite oxyHb reaction will never become autocatalytic due to low nitrite:oxyHb ratios and the presences of antioxidants such as catalase and ascorbate (Keszler et al., 2008).

The reaction between nitrite and deoxyHb, given in equation 1, was first studied by Brooks in 1937 and extended by Doyle in 1981 (Brooks, 1937; Doyle et al., 1981). The reaction leads to the formation of metHb and NO.

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow HbFe^{3+} + NO + OH^- \quad (1)$$

The reaction rate of nitrite with deoxyHb is dependent on the conformation state of Hb. Therefore, under partial oxygenation or in the presence of metHb or HbNO the reaction rate increases (Huang et al., 2005a,b). NO produced through the reduction of nitrite by deoxyHb rapidly reacts with any unreacted deoxyHb. Therefore, the reaction of nitrite with deoxyHb lead to a 1:1 production of metHb and HbNO (Brooks, 1937; Huang et al., 2005b).

The reaction between deoxyHb/nitrite and oxyHb/nitrite proceed in parallel with similar maximum reaction rates at 50% saturation (Grubina et al., 2007). The reaction of nitrite with deoxyHb provides a mechanism for production of NO. Data supports the formation and escape of NO from the deoxyRBC. The Gladwin group has shown that NO can be detected by chemiluminescence after reacting Hb and nitrite (Huang et al., 2005b). Crawford et al. measured NO by chemiluminescence in the head space of deoxyRBCs reacted with nitrite (Crawford et al., 2006). Other groups have also observed NO detected by chemiluminescence after reacting NO with RBCs (Ghosh et al., 2013; Liu et al., 2015). Crawford et al. also measured stimulation of NO dependent processes such as cyclic guanosine monophosphate production, inhibition of mitochondrial respiration, and vasodilation which were sensitive to NO scavenger C-PTIO (Crawford et al., 2006). Additionally, work by Wajih et al. showed diminished platelet activation in the presence of nitrite and deoxygenated RBCs that was inhibited by NO scavenger C-PTIO (Wajih et al., 2016).

However, when one considers the end product of the nitrite deoxyHb reaction, NO, and its rapid interaction with deoxyHb, or oxyHb which are abundant in the RBC, the question of how NO escapes the RBC arises. Compartmentalization of NO or reaction intermediates could reduce scavenging as well as the production of an alternative reactive nitrogen species. A metabolon has been suggested where compartmentalization at the RBC membrane serves to concentrate NO in the membrane itself (Gladwin et al., 2004).

N_2O_3

One possible reactive nitrogen signaling species that may escape the RBC is N_2O_3. N_2O_3 does not react with the ferrous heme and is freely diffusible. Basu and colleagues detected the intermediate metHb-nitrite during the reaction of nitrite with deoxyHb and proposed that this intermediate may react rapidly with NO to form N_2O_3, a nitrosylating species (Basu et al., 2007; Roche et al., 2013). One caveat of this reaction is that metHb-nitrite must outcompete any surrounding Hb for NO, therefore, compartmentalization of this reaction may be necessary for it to play a physiological role. Friedman and coworkers have suggested that a N_2O_3 bound Hb species forms during the reaction of metHb and nitrite. They detected the formation of an intermediate capable of S-nitrosation and GSNO formation under multiple conditions when metHb, NO, and nitrite were mixed (Roche et al., 2013). Also, the addition of reducing agent L-cysteine to metHb and nitrite reduced a small population of metHb to deoxyHb. This deoxyHb was then able to react with nitrite to form NO thus enabling the formation of the intermediate species (Roche et al., 2015). However, studies have provided experimental or theoretical arguments against N_2O_3 formation (Tu et al., 2008; Koppenol, 2012). On the other hand, if N_2O_3 does form it could potentially diffuse out of the RBC and then form NO and $NO_2^{\cdot}$.

S-nitrosothiols

Another pool of NO derivatives that may escape or transport NO signaling within the RBC are S-nitrosothiols. S-nitrosothiols react slowly with heme proteins (Spencer et al., 2000), therefore, once an S-nitrosothiol is formed the signal could be passed from S-nitrosothiol to S-nitrosothiol without reacting with deoxyHb.

One example of S-nitrosation (also referred to as S-nitrosylation emphasizing its role in signaling) is the formation of SNO-Hb. Stamler et al. demonstrated the ability of SNO-Hb to relax vessels through the hypoxic release of NO activity from the β-93 cysteine of Hb (Stamler et al., 1997; McMahon et al., 2002) and hypothesized the mechanism for this release was a decreased stability of SNO-Hb in the deoxygenated T-state compared to the oxygenated

R-state. *In vitro* research by Doctor and co-workers supported this theory by demonstrating SNO-Hb content is coupled to Hb oxygen saturation (Doctor et al., 2005) and Diesen et al. used blood vessel assays to show that decreased RBC SNO-Hb content abolished vessel relaxation by hypoxic RBCs (Diesen et al., 2008). However, there are many questions surrounding the SNO-Hb mechanism of hypoxic vasodilation and its physiological relevance. For more information on the hypoxic release of NO from SNO-Hb we refer you to reviews by Allen et al. and Singel et al. (Singel and Stamler, 2005; Allen and Piantadosi, 2006), but also suggest contrary literature (Gladwin et al., 2002, 2003; Xu et al., 2003; Huang et al., 2006).

The above work does not consider the role of nitrite in hypoxic vasodilation and raises the question of how SNO-Hb is formed. In circulation SNO-Hb must be replenished following release in order for it to play a continuous role in hypoxic vasodilation. Experiments into the formation of SNO-Hb have ruled out the transfer of NO from HbNO to the β-93 cysteine (Xu et al., 2003; Huang et al., 2006). Nagababu et al. and Angelo et al. suggest a semi-stable metHb-NO intermediate may form during nitrite reduction by deoxyHb and would be capable of SNO-Hb formation, thereby linking S-nitrosothiol formation with hypoxia and nitrite (Angelo et al., 2006; Nagababu et al., 2006). But, Basu et al. were unable to detect a stable metHb-NO intermediate and demonstrated metHb-nitrite is a major intermediate during nitrite reduction by deoxyHb (Basu et al., 2007).

Additionally, a study by Isbell et al. in mice with a β-93 substitution of alanine for cysteine indicate SNO-Hb is not essential for nitrite associated vasodilation (Isbell et al., 2007). These findings provide strong evidence that militates against the role SNO-Hb in nitrite mediated hypoxic vasodilation. However, a role for S-nitrosothiols is not ruled out. Possible S-nitrosothiol mechanisms are nitrosation of glutathione directly by metHb-NO, expected as a transient intermediate in the reduction of nitrite by deoxyHb, or the formation of SNO via N_2O_3. The formation of S-nitrosothiols that utilizes nitrite, provides a mechanism for hypoxic nitrite reduction that produces NO bio-activity that avoids scavenging by oxyHb o deoxyHb. However, the mechanism of S-nitrosothiol formation requires further research and reproducible detection of GSNO or other S-nitrosothiols exported from the RBC have not been reported.

Role of the RBC membrane

Band 3, also known as AE1, is an abundant ion transporter in the RBC membrane and has also been proposed as a mechanism for SNO transport. Pawloski et al. proposed that SNO-Hb formed in the RBC would S-nitrosate a cysteine on the cytosolic domain of Band 3 forming SNO-AE1 (Pawloski et al., 2001). However, the subsequent export of NO bio-activity is still unclear but could involve low molecular weight thiols. In the nitrite mediated vasodilation work of Isbell et al., using mice with β-93 cysteine substituted with alanine, argued against SNO-Hb playing an important role in nitrite related vasodilation, however, they did not rule out the role of S-nitrosothiol formation all together (Isbell et al., 2007). Therefore, low molecular weight SNO formed via nitrite reduction could transnitrosylate AE1 and eventually escape the RBC through and AE1 mechanism as proposed by Pawloski and coworkers (Pawloski et al., 2001), although lack of S-nitrosothiol detection outside of the RBCs argues against this hypothesis.

Kallakunta et al. investigated a role for protein disulfide isomerase (PDI) in nitrite derived S-nitrosothiol export from the RBC (Kallakunta et al., 2013). Their work supports a process where under oxygenated conditions in the presence of nitrite, PDI forms a complex with Hb, is S-nitrosylated and then attaches to the surface of the RBC. Upon deoxygenation SNO-PDI is released from the membrane (Sliskovic et al., 2005). Past research has shown a role for PDI in the import of NO derivatives into various cell types (Zai et al., 1998; Ramachandran et al., 2001; Bell et al., 2006). This new proposal puts forward its importance as an exporter of vasodilatory activity. One challenge to this hypothesis is that RBCs are not likely to contain much PDI, so that only a small fraction of Hb would be associated with PDI and nitrite-mediated PDI S-nitrosylation would be inefficient.

In addition to its role in SNO export, the RBC membrane, specifically AE1, plays a role in nitrite transport. For nitrite to react with hemoglobin and lead to NO bioactivation it must first enter the RBC. The transport of nitrite across the RBC membrane is affected by the oxygen saturation of hemoglobin (Vitturi et al., 2009). Early studies showed that hemoglobin binds to the RBC membrane through an interaction with AE1 (Shaklai et al., 1997; Galtieri et al., 2002; Chu et al., 2008). The binding of hemoglobin to AE1controls its transport activity (Galtieri et al., 2002; Drew et al., 2004). In the case of nitrite transport, oxygen saturation has no effect on the import of nitrite by AE1 however, deoxygenation of the RBC reduces nitrite export by AE-1 (Vitturi et al., 2009). This change in nitrite export leads to a steady state level of nitrite in the RBC. This steady state is maintained through the combination of faster scavenging of nitrite by deoxyHb balanced with the decreased export of nitrite by deoxyHb bound

AE1 (Vitturi et al., 2009). It should be noted however, that substantial nitrite traverses the membrane directly as nitrous acid.

The affinity of hemoglobin for the RBC membrane is dependent upon its adduct. It has been suggested that nitrite reacted Hb, which may lead to metHb-NO or Hb-NO^+, has a greater affinity for the RBC membrane than deoxyHb and deoxyHb has a greater affinity for the RBC membrane than oxyHb (Salgado et al., 2015). Salgado argues that this greater affinity allows the lower concentrations of nitrite reacted Hb to compete with deoxyHb and oxyHb for the AE1 binding sites. Additionally, the binding of the nitrite reacted Hb to AE1 when nitrite is in its reduced form [Hb(II)NO^+ or Hb(III)NO] facilitates the release of NO (Salgado et al., 2015); however, the lifetime of this intermediate is debated (Nagababu et al., 2003; Angelo et al., 2006; Basu et al., 2007). When nitrite reacted Hb is bound to AE1, NO release occurs near the RBC membrane decreasing the distance NO would need to diffuse to escape scavenging by Hb in the RBC, but escape is still a great challenge. Therefore, Hb binding to the RBC membrane leads to yet another potential mechanism through which the RBC membrane may facilitate NO bio-activity from hypoxic nitrite reduction.

Protein AE1 was also suggested to be part of a metabolon involved in NO export from the RBC (Gladwin et al., 2004). In this hypothesis, the metabolon (possibly on a RBC lipid raft) includes carbonic anhydrase which provides protons for the nitrite reaction (see equation 1) and AE1 which binds to Hb that reduces nitrite to NO. NO itself is them exported (Gladwin et al., 2004).

Lastly, recent work by our lab has shown a potential role for RBC membrane S-nitrosylation in nitrite bioactivation (Wajih et al., 2016). We showed nitrite inhibits platelet activation when in the presence of deoxyRBCs. The addition of SNAP, a membrane impermeable nitrosating agent, to RBCs had a similar effect on platelet activation, suggesting a role for RBC membrane S-nitrosylation. The addition of N-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), thiol blocking agents, eliminated the abrogation of platelet activation by nitrite and deoxyRBCs (Wajih et al., 2016). These data suggest a role for S-nitrosylation of the external RBC membrane in the pathway of nitrite bioactivation.

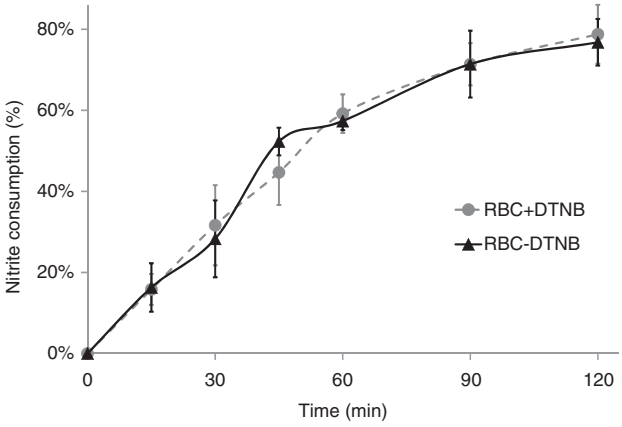


Figure 2: Nitrite consumption by RBCs treated or not treated with DTNB.
For RBC treated with DTNB, we incubated RBCs at 50% Hct with 2.5 mM DTNB in the dark for 1 h at room temperature. Next, we washed the RBCs three times by centrifugation to remove extra DTNB and deoxygenated them with nitrogen. Nitrite (0.1 mM) was added to deoxyRBCs at 15% Hct and nitrite remaining in the solution was determined at 15 and 30 min intervals by ozone chemiluminescence. There was no difference in the consumption of nitrite by RBCs exposed or not exposed to DTNB.

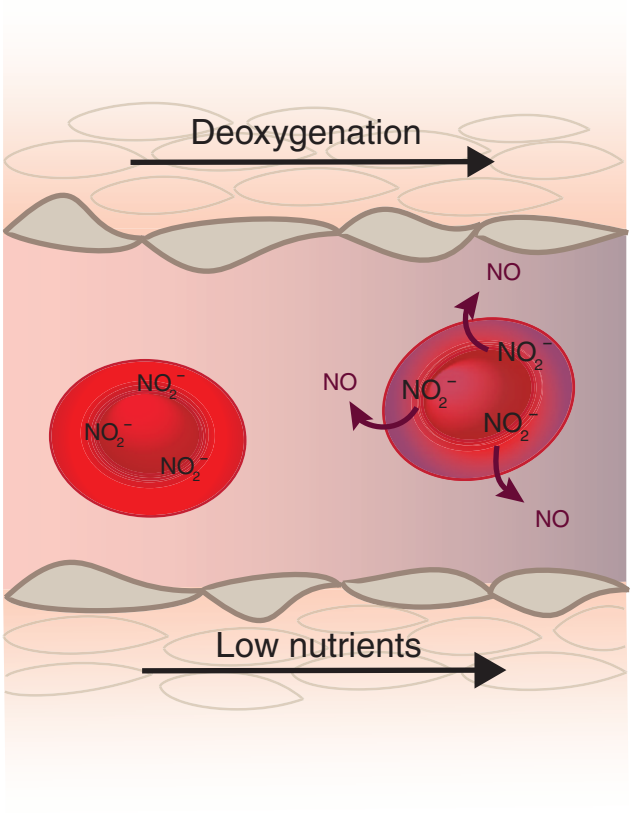


Figure 3: Physiological application of nitrite reduction by the RBC. Nitrite and the deoxygenated RBC produce NO and other reactive nitrogen signaling species in deoxygenated conditions as shown through vessel assays and platelet activation studies, tending to increase blood flow into areas of low oxygen. Recent data using platelet activation to measure NO bioavailability show that NO bioavailability is greatest when nutrients such as leucine and glucose are low, suggesting that blood flow may also be directed to areas of low nutrients.

To further support this hypothesis and exclude an effect of DTNB on nitrite uptake we measured nitrite uptake by the RBC in the presence of DTNB. Nitrite uptake by RBCs was not altered by the addition of DTNB (Figure 2). Thus, RBC surface nitrosation appears to play a part in nitrite bioactivation by the RBC as measured by inhibition of platelet activation.

Further complexities and physiological action

As stated above, a new paradigm has been proposed whereby RBCs, upon sensing hypoxia and acidosis, reduce nitrite to NO thereby increasing blood flow to where it is needed.

Recent research by our lab found that physiological levels of leucine and glucose inhibit nitrite bioactivation by RBCs through a mechanism that is not related to SNO export by L-type amino acid transporter 1 (Wajih et al., 2016). This adds further complexity to the mechanism of nitrite bioactivation. Although this novel work needs further exploration, it suggests that nitrite bioactivation by RBCs not only increases blood flow to areas of low oxygen, but also to areas of low nutrients (Figure 3).

Conclusions

The reaction between nitrite and deoxygenated hemoglobin in the RBCs leads to the production of NO or another reactive nitrogen signaling species capable of dilating blood vessels and inhibiting platelet activation. The *in vitro* reaction between deoxyHb and nitrite produces metHb and NO. However, in the RBC NO would be quickly scavenged by the surrounding Hb. Therefore, researchers have proposed and tested many hypotheses involving NO or related reactive nitrogen signaling species that avoid scavenging (Figure 4). A dominant element in many hypotheses is the formation of S-nitrosothiols however, the mechanism for their formation and transport remains elusive.

Currently, research has suggested (1) the infusion of nitrite and deoxyRBCs produces vasodilation, (2) there is an arterial to venous nitrite gradient, (3) nitrite transport through the RBC membrane is partially controlled by AE-1, (4) Hb interacts preferentially with AE1 on the RBC membrane according to its adduct, (5) PDI, nitrite and hemoglobin lead to PDI-S-nitrosylation, (6) leucine and glucose diminish the production of a species that abrogates platelet activation and (6) S-nitrosylation of the RBC membrane produces a species capable of decreasing platelet activation.

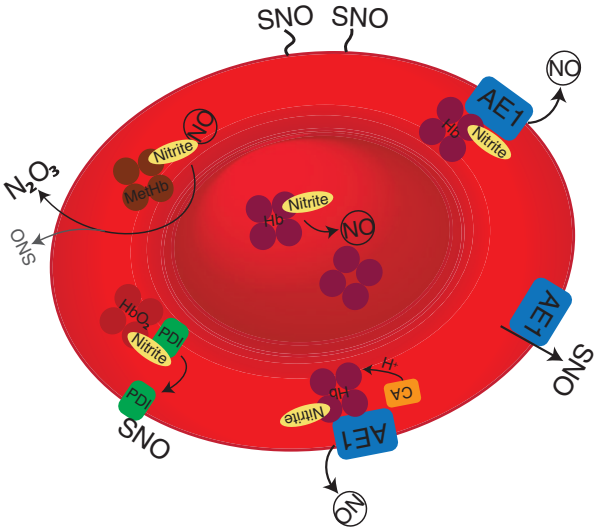


Figure 4: Potential players in the escape of NO and other reactive nitrogen signaling species from the RBC.
In the center of the RBC is the reaction between nitrite and deoxyHb which leads to NO production. However, NO produced inside the RBC is quickly scavenged by Hb leading to the question of how NO escapes the RBC. Starting at the top and moving clockwise: We showed S-nitrosylation of the RBC membrane is necessary for platelet inhibition. Salgado et al. showed preferential binding of nitrite reacted Hb to AE1 and suggested this binding would place NO production at the membrane making it more likely to escape scavenging. Stamler et al. suggested AE1 may become S-nitrosylated through transnitrosation reactions and facilitate transport of S-nitrosothiols. Gladwin et al. hypothesized a membrane metabolon consisting of AE1, carbonic anhydrase, deoxyHb and nitrite that would compartmentalize NO production by the metabolon to the membrane thereby facilitating NO escape. Kallakunta et al. showed a reaction between oxyHb, nitrite and PDI leading to PDI S-nitrosylation and argued *in vivo* SNO-PDI may embed in the membrane and be release during deoxygenation of the RBC. Lastly, we showed the reaction between metHb, nitrite and NO can produce N_2O_3 which could escape through the RBC membrane or form S-nitrosothiols which could escape through transnitrosylation, as previously proposed.

Acknowledgments: This work was supported by NIH grant HL058091.

References

Aamand, R., Dalsgaard, T., Jensen, F.B., Simonsen, U., Roepstorff, A., and Fago, A. (2009). Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. Am. J. Physiol. Heart Circ. Physiol. *297*, H2068–H2074.

Allen, B.W. and Piantadosi, C.A. (2006). How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. Am. J. Physiol. Heart Circ. Physiol. *291*, H1507–H1512.

Allen, B.W., Stamler, J.S., and Piantadosi, C.A. (2009). Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation. Trends Mol. Med. *15*, 452–460.

Andrew, P.J. and Mayer, B. (1999). Enzymatic function of nitric oxide synthases. Cardiovasc. Res. *43*, 521–531.

Angelo, M., Singel, D.J., and Stamler, J.S. (2006). An S-nitrosothiol (SNO) synthase function of hemoglobin that utilizes nitrite as a substrate. Proc Natl. Acad. Sci. USA *103*, 8366–8371.

Basu, S., Grubina, R., Huang, J., Conradie, J., Huang, Z., Jeffers, A., Jiang, A., He, X., Azarov, I., Seibert, R., et al. (2007). Catalytic generation of N_2O_3 by the concerted nitrite reductase and anhydrase activity of hemoglobin. Nat. Chem. Biol. *3*, 785–794.

Bell, S., Shah, C., and Gordge, M. (2006). Protein disulfide-isomerase mediates delivery of nitric oxide redox derivatives into platelets. Biochem. J. *403*, 283–288.

Bizjak, D.A., Brinkmann, C., Bloch, W., and Grau, M. (2015). Increase in red blood cell-nitric oxide synthase dependent nitric oxide production during red blood cell aging in health and disease: a study on age dependent changes of rheologic and enzymatic properties in red blood cells. PLoS One *10*, e0125206.

Brooks, J. (1937). The action of nitrite on haemoglobin in the absence of oxygen. Proc. R. Soc. Lond. B Biol. Sci. *123*, 368–382.

Butler, A., Megson, I., and Wright, P. (1998). Diffusion of nitric oxide and scavenging by blood in the vasculature. Biochim. Biophys. Acta Gen. Subj. *1425*, 168.

Cao, Z., Bell, J.B., Mohanty, J.G., Nagababu, E., and Rifkind, J.M. (2009). Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation. Am. J. Physiol. Heart Circ. Physiol. *297*, H1494–H1503.

Carlsson, S., Wiklund, N.P., Engstrand, L., Weitzberg, E., and Lundberg, J.O.N. (2001). Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. Nitric Oxide *5*, 580–586.

Cassoly, R. and Gibson, Q.H. (1975). Conformation, co-operativity and ligand binding in human hemoglobin. J. Mol. Biol. *91*, 301–313.

Chu, H., Breite, A., Ciraolo, P., Franco, R.S., and Low, P.S. (2008). Characterization of the deoxyhemoglobin binding site on human erythrocyte band 3: implications for O_2 regulation of erythrocyte properties. Blood *111*, 932–938.

Cooper, C.E. (1999). Nitric oxide and iron proteins. Biochim. Biophys. Acta Bioenerget. *1411*, 290–309.

Cortese-Krott, M., Rodriguez-Mateos, A., Sansone, R., Kuhnle, G.G.C., Thasian-Sivarajah, S., Krenz, T., Horn, P., Krisp, C., Wolters, D., Heib, C., et al. (2012). Human red blood cells at work: identification and visualization of erythrocytic eNOS activity in health and disease. Blood *120*, 4229–4237.

Cosby, K., Partovi, K., Crawford, J., Patel, R., Reiter, C., Martyr, S., Yang, B., Waclawiw, M., Zalos, G., Xu, X., et al. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat. Med. *9*, 1498.

Crawford, J.H., Isbell, T.S., Huang, Z., Shiva, S., Chacko, B.K., Schechter, A.N., Darley-Usmar, V., Kerby, J.D., Lang, J.D., Kraus, D., et al. (2006). Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. Blood *107*, 566–574.

Dalsgaard, T., Simonsen, U., and Fago, A. (2007). Nitrite-dependent vasodilation is facilitated by hypoxia and is independent of known NO-generating nitrite reductase activities. Am. J. Physiol. Heart Circ. Physiol. *292*, H3072–H3078.

Diesen, D.L., Hess, D.T., and Stamler, J.S. (2008). Hypoxic vasodilation by red blood cells: evidence for an S-Nitrosothiol based signal. Circ. Res. *103*, 545–553.

Dietrich, H.H., Ellsworth, M.L., Sprague, R.S., and Dacey, R.G. (2000). Red blood cell regulation of microvascular tone through adenosine triphosphate. Am. J. Physiol. Heart Circ. Physiol. *278*, H1294–H1298.

Doctor, A., Platt, R., Sheram, M.L., Eischeid, A., McMahon, T., Maxey, T., Doherty, J., Axelrod, M., Kline, J., Gurka, M., et al. (2005). Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O_2 gradients. Proc. Natl. Acad. Sci. USA *102*, 5709–5714.

Doyle, M.P., Pickering, R.A., DeWeert, T.M., Hoekstra, J.W., and Pater, D. (1981). Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. J. Bio. Chem. *256*, 12393–12398.

Drew, C., Ball, V., Robinson, H., Clive Ellory, J., and Gibson, J.S. (2004). Oxygen sensitivity of red cell membrane transporters revisited. Bioelectrochemistry *62*, 153–158.

Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., Smith, L., Golden, M., and Benjamin, N. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. Nat. Med. *1*, 546–551.

Forstermann, U. and Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. Eur. Heart J. *33*, 829–837.

Fritsch, P., de Saint Blanquat, G., and Derache, R. (1979). Absorption kinetics of nitrate and nitrite in the intestine of rats. Toxicol. Eur. Res. *2*, 141–147.

Gago, B., Lundberg, J.O., Barbosa, R.M., and Laranjinha, J. (2007). Red wine-dependent reduction of nitrite to nitric oxide in the stomach. Free Radic. Biol. Med. *43*, 1233–1242.

Galtieri, A., Tellone, E., Romano, L., Misiti, F., Bellocco, E., Ficarra, S., Russo, A., Di Rosa, D., Castagnola, M., Giardina, B., et al. (2002). Band-3 protein function in human erythrocytes: effect of oxygenation–deoxygenation. Biochim. Biophys. Acta Biomembr. *1564*, 214–218.

Ghosh, S.M., Kapil, V., Fuentes-Calvo, I., Bubb, K.J., Pearl, V., Milsom, A.B., Khambata, R., Maleki-Toyserkani, S., Yousuf, M., Benjamin, N., et al. (2013). Enhanced Vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. Hypertension *61*, 1091–1102.

Gladwin, M.T., Shelhamer, J.H., Schechter, A.N., Pease-Fye, M.E., Waclawiw, M.A., Panza, J.A., Ognibene, F.P., and Cannon, R.O. (2000). Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc. Natl. Acad. Sci. USA *97*, 11482–11487.

Gladwin, M.T., Wang, X., Reiter, C.D., Yang, B.K., Vivas, E.X., Bonaventura, C., and Schechter, A.N. (2002). S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O_2/NO-linked allosteric function. J. Biol. Chem. *277*, 27818–27828.

Gladwin, M.T., Lancaster, J.R., Freeman, B.A., and Schechter, A.N. (2003). Nitric oxide's reactions with hemoglobin: a view through the SNO-storm. Nat. Med. *9*, 496–500.

Gladwin, M.T., Crawford, J.H., and Patel, R.P. (2004). The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. Free Radic. Biol. Med. *36*, 707–717.

Grau, M., Pauly, S., Ali, J., Walpurgis, K., Thevis, M., Bloch, W., and Suhr, F. (2013). RBC-NOS-Dependent S-Nitrosylation of Cytoskeletal Proteins Improves RBC Deformability. PLoS One *8*, e56759.

Grubina, R., Huang, Z., Shiva, S., Joshi, M.S., Azarov, I., Basu, S., Ringwood, L.A., Jiang, A., Hogg, N., Kim-Shapiro, D.B., et al. (2007). Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. J. Biol. Chem. *282*, 12916–12927.

Hord, N.G., Tang, Y., and Bryan, N.S. (2009). Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am. J. Clin. Nutr. *90*, 1–10.

Huang, K.T., Keszler, A., Patel, N., Patel, R.P., Gladwin, M.T., Kim-Shapiro, D.B., and Hogg, N. (2005a). The reaction between nitrite and deoxyhemoglobin: reassessment of reaction kinetics and stoichiometry. J. Biol Chem. *280*, 31126–31131.

Huang, Z., Shiva, S., Kim-Shapiro, D., Patel, R.P., Ringwood, L.A., Irby, C.E., Huang, K.T., Ho, C., Hogg, N., Schechter, A.N., et al. (2005b). Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. J. Clin. Invest. *115*, 2099–2107.

Huang, K.T., Azarov, I., Basu, S., Huang, J., and Kim-Shapiro, D. (2006). Lack of allosterically controlled intramolecular transfer of nitric oxide from the heme to cysteine in the Î² subunit of hemoglobin. Blood *107*, 2602–2604.

Isbell, T.S., Gladwin, M.T., and Patel, R.P. (2007). Hemoglobin oxygen fractional saturation regulates nitrite-dependent vasodilation of aortic ring bioassays. Am. J. Physiol. Heart Circ. Physiol. *293*, H2565–H2572.

Jansson, E.Å., Huang, L., Malkey, R., Govoni, M., Nihlén, C., Olsson, A., Stensdotter, M., Petersson, J., Holm, L., Weitzberg, E., et al. (2008). A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. Nat. Chem. Biol. *4*, 411–417.

Jensen, F.B. and Agnisola, C. (2005). Perfusion of the isolated trout heart coronary circulation with red blood cells: effects of oxygen supply and nitrite on coronary flow and myocardial oxygen consumption. J. Exp. Biol. *208*, 3665–3674.

Kallakunta, V.M., Slama-Schwok, A., and Mutus, B. (2013). Protein disulfide isomerase may facilitate the efflux of nitrite derived S-nitrosothiols from red blood cells. Redox Biol. *1*, 373–380.

Kelm, M., Preik-Steinhoff, H., Preik, M., and Strauer, B.E. (1999). Serum nitrite sensitively reflects endothelial NO formation in human forearm vasculature: evidence for biochemical assessment of the endothelial L-arginine-NO pathway. Cardiovasc. Res. *41*, 765–772.

Keszler, A., Piknova, B., Schechter, A.N., and Hogg, N. (2008). The reaction between nitrite and oxyhemoglobin: a mechanistic study. J. Biol. Chem. *283*, 9615–9622.

Kim-Shapiro, D.B., Schechter, A.N., and Gladwin, M.T. (2006). Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. Arterioscler. Thromb. Vasc. Biol. *26*, 697–705.

Kleinbongard, P., Dejam, A., Lauer, T., Rassaf, T., Schindler, A., Picker, O., Scheeren, T., Gödecke, A., Schrader, J., Schulz, R., et al. (2003). Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic. Biol. Med. *35*, 790–796.

Kleinbongard, P., Schulz, R., Rassaf, T., Lauer, T., Dejam, A., Jax, T., Kumara, I., Gharini, P., Kabanova, S., Ozuyaman, B., et al. (2006). Red blood cells express a functional endothelial nitric oxide synthase. Blood *107*, 2943–2951.

Koppenol, W.H. (2012). Nitrosation, thiols and hemoglobin: energetics and kinetics. Inorg. Chem. *51*, 5637–5641.

Lancaster, J.R. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. Proc. Natl. Acad. Sci. USA *91*, 8137–8141.

Lauer, T., Preik, M., Rassaf, T., Strauer, B.E., Deussen, A., Feelisch, M., and Kelm, M. (2001). Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc. Natl. Acad. Sci. USA *98*, 12814–12819.

Liao, J.C., Hein, T.W., Vaughn, M.W., Huang, K., and Kuo, L. (1999). Intravascular flow decreases erythrocyte consumption of nitric oxide. Proc. Natl. Acad. Sci. USA *96*, 8757–8761.

Liu, X., Miller, M.J.S., Joshi, M.S., Sadowska-Krowicka, H., Clark, D.A., and Lancaster, J.R. (1998). Diffusion-limited reaction of free nitric oxide with erythrocytes. J. Biol. Chem. *273*, 18709–18713.

Liu, C., Wajih, N., Liu, X., Basu, S., Janes, J., Marvel, M., Keggi, C., Helms, C.C., Lee, A.N., Belanger, A.M., et al. (2015). Mechanisms of human erythrocytic bioactivation of nitrite. J. Biol. Chem. *290*, 1281–1294.

Lundberg, J., Weitzberg, E., and Gladwin, M. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat. Rev. Drug Discov. *7*, 156.

Machha, A. and Schechter, A.N. (2011). Dietary nitrite and nitrate: a review of potential mechanisms of cardiovascular benefits. Eur. J. Nutr. *50*, 293–303.

McKnight, G.M., Smith, L.M., Drummond, R.S., Duncan, C.W., Golden, M., and Benjamin, N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. Gut *40*, 211–214.

McMahon, T.J., Moon, R.E., Luschinger, B.P., Carraway, M.S., Stone, A.E., Stolp, B.W., Gow, A.J., Pawloski, J.R., Watke, P., Singel, D.J., et al. (2002). Nitric oxide in the human respiratory cycle. Nat. Med. *8*, 711–717.

Medina-Remón, A., Tresserra-Rimbau, A., Pons, A., Tur, J.A., Martorell, M., Ros, E., Buil-Cosiales, P., Sacanella, E., Covas, M.I., Corella, D., et al. (2015). Effects of total dietary polyphenols on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial. Nutr. Metabol Cardiovasc. Dis. *25*, 60–67.

Moncada, S. and Higgs, A. (1993). The L-arginine-nitric oxide pathway. N. Engl. J. Med. *329*, 2002–2012.

Moriya, A., Grant, J., Mowat, C., Williams, C., Carswell, A., Preston, T., Anderson, S., Irjima, K., and McColl, K.E.L. (2002). *In vitro* studies indicate that acid catalysed generation of n-nitroso-compounds from dietary nitrate will be maximal at the gastro-oesophageal junction and cardia. Scand. J. Gastroenterol. *37*, 253–261.

Morris, R.J. and Gibson, Q.H. (1980). The role of diffusion in limiting the rate of ligand binding to hemoglobin. J. Biol Chem. *255*, 8050–8053.

Nagababu, E., Ramasamy, S., Abernethy, D.R., and Rifkind, J.M. (2003). Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. J. Biol Chem. *278*, 46349–46356.

Nagababu, E., Ramasamy, S., and Rifkind, J.M. (2006). S-nitrosohemoglobin: a mechanism for its formation in conjunction with nitrite reduction by deoxyhemoglobin. Nitric Oxide *15*, 20–29.

Park, J.W., Piknova, B., Nghiem, K., Lozier, J.N., and Schechter, A.N. (2014). Inhibitory effect of nitrite on coagulation processes demonstrated by thrombelastography. Nitric Oxide *40*, 45–51.

Pawloski, J.R., Hess, D.T., and Stamler, J.S. (2001). Export by red blood cells of nitric oxide bioactivity. Nature *409*, 622–626.

Peri, L., Pietraforte, D., Scorza, G., Napolitano, A., Fogliano, V., and Minetti, M. (2005). Apples increase nitric oxide production by human saliva at the acidic pH of the stomach: a new biological function for polyphenols with a catechol group? Free Radic. Biol. Med. *39*, 668–681.

Presley, T.D., Morgan, A.R., Bechtold, E., Clodfelter, W., Dove, R.W., Jennings, J.M., Kraft, R.A., Bruce King, S., Laurienti, P.J., Jack Rejeski, W., et al. (2011). Acute effect of a high nitrate diet on brain perfusion in older adults. Nitric Oxide *24*, 34–42.

Ramachandran, N., Root, P., Jiang, X., Hogg, P.J., and Mutus, B. (2001). Mechanism of transfer of NO from extracellular S-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. Proc. Natl. Acad. Sci. USA *98*, 9539–9544.

Rassaf, T., Feelisch, M., and Kelm, M. (2004). Circulating no pool: assessment of nitrite and nitroso species in blood and tissues. Free Radic. Biol. Med. *36*, 413–422.

Roche, C.J., Cassera, M.B., Dantsker, D., Hirsch, R.E., and Friedman, J.M. (2013). Generating S-nitrosothiols from hemoglobin: mechanisms, conformational dependence, and physiological relevance. J. Biol Chem. *288*, 22408–22425.

Roche, C.J., Talwar, A., Palmer, A.F., Cabrales, P., Gerfen, G., and Friedman, J.M. (2015). Evaluating the capacity to generate and preserve nitric oxide bioactivity in highly purified earthworm erythrocruorin: a giant polymeric hemoglobin with potential blood substitute properties. J. Biol. Chem. *290*, 99–117.

Salgado, M.T., Cao, Z., Nagababu, E., Mohanty, J.G., and Rifkind, J.M. (2015). Red blood cell membrane-facilitated release of nitrite-derived nitric oxide bioactivity. Biochemistry *54*, 6712–6723.

Shaklai, N., Yguerabide, J., and Ranney, H.M. (1997). Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. Biochemistry *13*, 5585–5592.

Shiva, S. (2012). Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function. Redox Biol. *1*, 40–44.

Shiva, S., Wang, X., Ringwood, L.A., Xu, X., Yuditskaya, S., Annavajjhala, V., Miyajima, H., Hogg, N., Harris, Z.L., and Gladwin, M.T. (2006). Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. Nat. Chem. Biol. *2*, 486–493.

Singel, D.J. and Stamler, J.S. (2005). Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu. Rev. Physiol. *67*, 99–145.

Sliskovic, I., Raturi, A., and Mutus, B. (2005). Characterization of the S-denitrosation activity of protein disulfide isomerase. J. Biol. Chem. *280*, 8733–8741.

Spencer, N.Y., Zeng, H., Patel, R.P., and Hogg, N. (2000). Reaction of S-nitrosoglutathione with the heme group of deoxyhemoglobin. J. Biol. Chem. *275*, 36562–36567.

Srihirun, S., Sriwantana, T., Unchern, S., Kittikool, D., Noulsri, E., Pattanapanyasat, K., Fucharoen, S., Piknova, B., Schechter, A.N., and Sibmooh, N. (2012). Platelet Inhibition by nitrite is dependent on erythrocytes and deoxygenation. PLoS One *7*, e30380.

Stamler, J.S., Jia, L., Eu, J.P., McMahon, T.J., Demchenko, I.T., Bonaventura, J., Gernert, K., and Piantadosi, C.A. (1997). Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science *276*, 2034–2037.

Stokes, K.Y., Dugas, T.R., Tang, Y., Garg, H., Guidry, E., and Bryan, N.S. (2009). Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. Am. J. Physiol. Heart Circ. Physiol. *296*, H1281–H1288.

Takahama, U., Oniki, T., and Hirota, S. (2002). Oxidation of quercetin by salivary components. Quercetin-dependent reduction of salivary nitrite under acidic conditions producing nitric oxide. J. Agric. Food Chem. *50*, 4317–4322.

Tannenbaum, S.R., Weisman, M., and Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. Food Cosmet. Toxicol. *14*, 549–552.

Tiso, M. and Schechter, A.N. (2015). Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions. PLoS One *10*, e0119712.

Totzeck, M., Hendgen-Cotta, U., Luedike, P., Berenbrink, M., Klare, J.P., Steinhoff, H., Semmler, D., Shiva, S., Williams, D., Kipar, A., et al. (2012). Nitrite regulates hypoxic vasodilation via myoglobin-dependent nitric oxide generation. Circulation *126*, 325–334.

Tu, C., Mikulski, R., Swenson, E.R., and Silverman, D.N. (2008). Reactions of nitrite with hemoglobin measured by membrane inlet mass spectrometry. Free Radic. Biol. Med. *46*, 14–19.

Vitturi, D.A., Teng, X., Toledo, J.C., Matalon, S., Lancaster, J.R., and Patel, R.P. (2009). Regulation of nitrite transport in red blood cells by hemoglobin oxygen fractional saturation. Am. J. Physiol. Heart Circ. Physiol. *296*, H1398–H1407.

Wajih, N., Liu, X., Shetty, P., Basu, S., Wu, H., Hogg, N., Patel, R.P., Furdui, C.M., and Kim-Shapiro, D.B. (2016). The role of red blood cell S-nitrosation in nitrite bioactivation and its modulation by leucine and glucose. Redox Biol. *8*, 415–421.

Webb, A.J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., Rashid, R., Miall, P., Deanfield, J., Benjamin, N., et al. (2008a). Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension *51*, 784–790.

Webb, A.J., Milsom, A.B., Rathod, K.S., Chu, W.L., Qureshi, S., Lovell, M.J., Lecomte, F.M.J., Perrett, D., Raimondo, C., Khoshbin, E., et al. (2008b). Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. Circ. Res. *103*, 957–964.

Wightman, E.L., Haskell-Ramsay, C.F., Thompson, K.G., Blackwell, J.R., Winyard, P.G., Forster, J., Jones, A.M., and Kennedy, D.O. (2015). Dietary nitrate modulates cerebral blood flow parameters and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. Physiol. Behav. *149*, 149–158.

Wood, K.C., Cortese-Krott, M.M., Kovacic, J.C., Noguchi, A., Liu, V.B., Wang, X., Raghavachari, N., Boehm, M., Kato, G.J., Kelm, M., et al. (2013). Circulating blood endothelial nitric oxide synthase contributes to the regulation of systemic blood pressure and nitrite homeostasis. Arterioscler. Thromb. Vasc. Biol. *33*, 1861–1871.

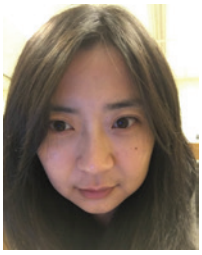
Xu, X., Cho, M., Spencer, N.Y., Patel, N., Huang, Z., Shields, H., King, S.B., Gladwin, M.T., Hogg, N., and Kim-Shapiro, D.B. (2003). Measurements of nitric oxide on the heme iron and ß-93 thiol of human hemoglobin during cycles of oxygenation and deoxygenation. Proc. Natl. Acad. Sci. USA *100*, 11303–11308.

Zai, A., Rudd, M.A., Scribner, A.W., and Loscalzo, J. (1998). Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. J. Clin. Invest. *103*, 393–399.

Bionotes

Christine C. Helms
Physics Department, University of Richmond, Richmond, VA 23235, USA,
chelms@richmond.edu

Christine C. Helms received her PhD in Physics from Wake Forest University in 2010. She then worked as a postdoctoral researcher at Wake Forest University and is now an Associate Professor of Physics at the University of Richmond. Dr. Helms's research focuses on the mechanics of biological nanofibers. Specifically, she studies factors affecting fiber formation and mechanics such as the effect of environmental condition like nitric oxide exposure on fibrin fiber formation and the effect of spinning conditions on electrospun fiber properties.



Xiaohua Liu
Physics Department, Wake Forest University, Winston-Salem, NC 27109, USA

Xiaohua Liu received a Master degree in Applied Physics from Chalmers University of Technology at Gothenburg, Sweden in 2012. Since then, she has been continuing her education towards a PhD in the Department of Physics at Wake Forest University. She joined Dr. Kim-Shapiro's lab and focuses on research on NO production or consumption in red blood cells.

Daniel B. Kim-Shapiro
Physics Department, Wake Forest University, Winston-Salem, NC 27109, USA,
shapiro@wfu.edu

Daniel B. Kim-Shapiro received a PhD in Biophysics from the University of California at Berkeley in 1993. He is now a Professor and the Associate Chair of Physics and holds the Harbert Family Distinguished Chair for Excellence in Teaching and Scholarship at Wake Forest University. He also is the director of the Wake Forest Translational Science Center (TSC); Fostering Independence in Aging. He employs physical tools and theory to solve problems in biology, focusing generally on reactive oxygen species, heme proteins and blood flow. He is currently studying the effects of nitrite and nitric oxide in biological systems, especially as it relates to sickle cell and other blood or cardiovascular diseases. As director of the TSC, he also has a growing interest in aging research.