Hemoglobin and nitrite export from red cells

Regulation of nitrite transport in red blood cells by hemoglobin oxygen fractional saturation

Dario A. Vitturi¹, Xinjun Teng¹, José C. Toledo², Sadis Matalon³,⁴,⁵, Jack R. Lancaster Jr.²,³,⁴,⁵, Rakesh P. Patel¹,²,³,⁵*

Department of ¹Pathology, ²Anesthesiology, ³Environmental Health Sciences, ⁴Physiology & Biophysics and ⁵Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA.

Running Head: Hemoglobin and nitrite export from red cells

Address correspondence to: Rakesh P Patel, PhD, Department of Pathology, University of Alabama at Birmingham, 901 19th street south, BMR-2, room 302, Birmingham, AL 35294. Tel: 205 975 9225. Fax: 205 934 7447. E-mail: rakeshp@uab.edu
Hemoglobin and nitrite export from red cells

Abstract
Allosteric regulation of nitrite reduction by deoxyhemoglobin is proposed to mediate nitric oxide (NO) formation during hypoxia. Nitrite is predominantly an anion at physiological pH raising questions about the mechanism by which it enters the red blood cell (RBC) and whether this is regulated and coupled to deoxyhemoglobin mediated reduction. We tested the hypothesis that nitrite transport by RBC is regulated by fractional saturation. Using human RBC, nitrite consumption was faster at lower fractional saturations consistent with faster reactions with deoxyheme. A membrane-based regulation was suggested by slower nitrite consumption with intact versus lysed RBC. Interestingly, upon nitrite addition, intracellular nitrite concentrations attained a steady state which despite increased rates of consumption did not change with decreasing oxygen tensions, suggesting a deoxygenation sensitive step that either increases nitrite import or decreases the rate of nitrite export. A role for anion exchanger-1 (AE1) in controlling nitrite export was suggested by increased intracellular nitrite concentrations in RBC treated with DIDS. Moreover, deoxygenation decreased steady state levels of intracellular nitrite in AE1-inhibited RBC. Based on these data we propose a model in which deoxyhemoglobin binding to AE1 inhibits nitrite export under low oxygen tensions allowing for the coupling between deoxygenation and nitrite reduction to nitric oxide along the arterial-to-venous gradient.

Keywords
Oxygen, Anion Exchanger-1, DIDS
Hemoglobin and nitrite export from red cells

**Introduction**

Nitrite (NO$_2^-$) was for a long time considered a relatively inert end product and marker of nitric oxide (NO) metabolism (23). More recently however, this paradigm has been challenged by multiple studies highlighting the potential for nitrite as an endogenous and therapeutic mediator of NO homeostasis during hypoxia (41). This concept is derived from studies using low physiologic concentrations of nitrite (nM-μM) which are also safe from a therapeutic perspective (35). A key element of the proposal that nitrite is mediator of NO-function, is the elucidation of mechanisms that operate under biological conditions to reduce nitrite to NO. These include reactions between nitrite and xanthine oxidoreductase, and/or deoxyheme proteins including neuroglobin, cytoglobin, myoglobin and hemoglobin (2, 5, 11, 13, 18, 26-29, 31, 42, 46, 48, 56, 62, 63, 65). With the latter, the general model proposed links heme deoxygenation with a reactivity that results in the one-electron reduction of nitrite to NO. In the case of hemoglobin, we and others have shown that nitrite reduction occurs by an allosterically-regulated nitrite-reductase activity of deoxyhemoglobin, that is kinetically maximal at oxygen tensions that result in approximately 50% hemoglobin oxygen fractional saturation i.e. the hemoglobin P$_{50}$ (13, 28). Desaturation of hemoglobin close to its P$_{50}$ may occur during transit of red cells through the resistance (arteriolar) vasculature under physiological and hypoxic stress conditions (6, 59) leading to the proposal that NO derived from nitrite reduction by the RBC is a mediator of hypoxic vasodilation (11, 22, 31, 38, 42, 44, 50, 51).
Hemoglobin and nitrite export from red cells

Most of the insights with respect to deoxyhemoglobin-nitrite reactions have been gleaned from cell-free based studies with key observations including maximal kinetics at the hemoglobin P50 being verified with red blood cells (RBC) also (13, 24, 27, 28). Nitrite is predominantly an anion at physiologic pH (pKa = 3.2) raising the question of what role the RBC membrane plays in regulating nitrite movement and subsequent reactions with hemoglobin. Under basal conditions, significant concentrations of nitrite exist in the RBC (~300 nM) (15). Given that both oxy- and deoxyhemoglobin consume nitrite, these data suggest a continual import of nitrite into the RBC. As RBC deoxygenate, the rate of nitrite-heme reactions increases (27, 28, 46). Moreover, transit of RBC across one circuit of the coronary microcirculation results in an increase in intraerythrocytic markers of nitrite-deoxyhemoglobin reactivity (52) further underscoring the requirement for movement of nitrite from the plasma into the RBC compartment during hypoxemia. How nitrite movement across membranes is controlled remains unclear. In this study we hypothesized that during hypoxia, net nitrite transport into RBC also increases to match accelerated nitrite-deoxyheme reactions and moreover that transport of nitrite is also regulated by hemoglobin fractional saturation.

The RBC contains multiple membrane proteins that regulate ion movement enabling appropriate transport of metabolic products such as carbon dioxide and protons, regulation of blood pH, adequate oxygen delivery and modulation of erythrocytic volume (4, 20). Key amongst these is anion exchanger-1 (AE1) which represents ~25% of the RBC membrane protein (9). Both C- and N-termini of AE1 are cytoplasmic and have been shown to selectively interact and hence modulate cytosolic protein function (e.g.
Hemoglobin and nitrite export from red cells

glycotlyic enzymes) (9). Interestingly, deoxyhemoglobin has higher affinity than oxyhemoglobin for the N-terminus of AE1 (61). This specific binding has been shown to inhibit movement of sulfate through this channel at low oxygen tensions (19). Thus AE1 represents a possible candidate to link hemoglobin oxygen sensing with nitrite metabolism by the RBC. Previous studies using RBC ghosts suggested a role for AE1 in controlling nitrite efflux (55). However, this and other studies evaluating nitrite–RBC interactions have only been performed under oxygenated conditions (1, 43, 60, 64). Studies evaluating the role of oxygen saturation on RBC nitrite metabolism are largely limited to teleost fish, in which case faster nitrite consumption rates at lower oxygen tensions are observed (33). Interestingly, this effect is not observed with pig RBC (34) nor in rat RBC (18). Herein, we provide evidence that hemoglobin deoxygenation regulates nitrite transport in human RBC and that this effect is mediated by deoxygenation-sensitive inhibition of nitrite export via AE1. These data suggest the hypothesis that nitrite reduction to NO by RBC occurs via a concerted mechanism that matches increased rates of nitrite reduction by deoxyhemoglobin with an inhibition of nitrite export across RBC membranes by AE1.

**Experimental Procedures**

**Materials:** DIDS (4,4’-diisothiocyanostilbene disulphonic acid), was purchased from Sigma-Aldrich Co (St Louis. MO), NEM (N-ethylmeleimide) from Pierce (Rockford, IL), and L-NAME (N\(^G\)-nitro-L-arginine methyl ester) from Alexis Biochemical (Lausen, Switzerland). All other reagents were of analytical grade and purchased from Sigma-Aldrich Co.
Hemoglobin and nitrite export from red cells

Red Blood Cell preparation: Blood was obtained from healthy volunteers according to protocols approved by the University of Alabama at Birmingham Institutional Review Board. Briefly, blood was collected into vacutainers (BD, Franklin Lakes, NJ) containing ACD anticoagulant and centrifuged for 10 minutes at 1000g, 4°C. Both plasma and the leukocyte coat were carefully removed and the remaining RBC pellet washed three times in Krebs-Henseleit buffer pH 7.4 supplemented with 0.5% Bovine Serum Albumin (KH/BSA). Under these conditions, hemolysis was typically <0.5%. For experiments in which nitrite consumption by hemolysates was studied, ice-cold RBC suspensions at ~50% hematocrit in KH/BSA buffer were mechanically lysed using a tissue homogenizer (PowerGen 125, Fischer Scientific) at medium-high speed for up to 20 min. Methemoglobin formation under these conditions did not significantly change compared to intact RBC (not shown). Finally, we note that the absolute rate of nitrite consumption varied up to ~2-3 fold between RBC preparations isolated from different donors (see Fig 4 legend). We have not systematically evaluated the nature of these differences and note that i) times between venipuncture and experiment (<3hr), RBC P50’s and protocols for RBC preparation remained consistent for all presented studies and ii) that the relative effects of hypoxia on increasing rates of nitrite consumption was similar in all RBC preparations. Therefore when presenting data from multiple experiments, nitrite consumption at different oxygen tensions is presented as fold-change as indicated in respective figure legends.

Determination of RBC hemoglobin oxygen affinity: Oxygen binding curves were measured by open-flow respirometry coupled with UV-Vis measurement of intraerythrocytic hemoglobin as previously described(13).
Nitrite consumption by RBC: Experiments under different oxygen tensions were performed in a controlled-atmosphere chamber (Plas Labs, Lansing MI, USA) using gases containing different O₂ tensions balanced with N₂ and 5% CO₂ at either 22°C or 37°C. For these studies Krebs-Henseleit buffer was incubated overnight under a 5% CO₂ atmosphere to ensure full equilibration before the addition of BSA and RBC. RBC suspensions at 5% hematocrit in KH/BSA buffer were equilibrated in six-well tissue culture plates with gentle rocking. We used 15 mins equilibration time for experiments performed at 10% oxygen (75mmHg) and 30 mins for experiments performed at 4% oxygen (30mmHg). Preliminary observations indicated that these times were sufficient for oxygen fractional saturations to equilibrate. For data shown in Fig 2B however, 30 min equilibration time was used for all oxygen tensions tested. The pH of the RBC suspensions was adjusted to 7.4 after equilibration with desired oxygen tension and immediately before the addition of nitrite (100 µM final concentration in the RBC suspension, unless otherwise specified). To ensure pH was not a variable, pH was measured at the end of experiments also and remained constant during the course of the incubations. In some experiments, RBC were pre-equilibrated with L-NAME, or DIDS for 15-30 min prior to equilibration with oxygen tension. Appropriate vehicle control incubations were also included. To initiate experiments, nitrite was added (nitrite stock solutions made in PBS + 100µM DTPA pre-equilibrated also at respective O₂ tension) and aliquots then removed at indicated times. Aliquots were taken out of the chamber and immediately centrifuged 2000g, 30 sec. The extracellular fraction (supernatant) was collected and RBC pellet washed 3 times with ice-cold PBS containing 100µM DTPA at
Hemoglobin and nitrite export from red cells

pH 7.4. After 3-washes (~3-4 min total), pellets were processed for determination of nitrite, S-nitrosothiols or methemoglobin as described below. Alternatively, unwashed pellets were collected for measurement of iron-nitrosyl species. In all experiments, parallel incubations of nitrite alone in KH/BSA were included and aliquots collected. Nitrite in the extracellular fraction was measured by triiodide-mediated reduction followed by chemiluminescent detection using a nitric oxide analyzer (Sievers NOA Model 280, Boulder CO, USA) as described previously (39). Nitrite consumption from the extracellular compartment is defined as the nitrite concentration difference between nitrite alone versus RBC + nitrite conditions.

Intracellular nitrite and nitrosothiol determination: Washed RBC pellets were immediately lysed in a stabilization solution containing 10 mM K₃Fe(CN)₆, 20 mM N-ethylmaleimide, 1% IGEPAL and 100 µM DTPA in PBS and then frozen at -80°C resulting in stabilization of RBC pellets within 5min of sample collection. Nitrite or S-nitrosothiols in RBC samples were measured within 4 days of collection and freezing was not found to affect measured concentrations of these NO-species (not shown). For S-nitrosothiol determination lysates were treated with acid sulfanilamide (1.5% wt:vol in 2 M HCl, 5 minutes at room temperature) in the presence or absence of mercuric chloride (50 mM) and measured by reductive chemiluminescence as described previously (39). For the measurement of intracellular nitrite levels, samples were deproteinated by the addition of an equal volume of ice-cold methanol followed by centrifugation at 15000g for 2 minutes. Nitrite in the resulting supernatants is measured as described above.
Hemoglobin and nitrite export from red cells

Background nitrite levels in stock solutions were <125 nM and <500 nM for DIDS and L-NAME respectively.

*Methemoglobin determination:* Washed RBC pellets were resuspended in four volumes of KH/BSA buffer, transferred to EPR tubes and frozen in liquid nitrogen. Methemoglobin was measured in an X-band EPR Spectrometer E Lexsys E500 (Bruker Biospin, MA, USA) with the following settings: modulation amplitude = 15 G, microwave power = 100.4 mW, temperature = 120 K, frequency = 9.437 GHz, time constant = 10.24 ms, conversion time = 40.96 ms. Each spectrum was the average of 8 scans and the signal at $g = 6$ quantified by comparison to methemoglobin standards.

*Intracellular HbNO determination:* RBC pellets were lysed in three volumes of high purity water containing 50µM DTPA and measured in the same day or flash-frozen in liquid nitrogen and thawed immediately prior to measurement. HbNO was measured by chemiluminescence using 50 mM K$_3$Fe(CN)$_6$ in PBS supplemented with 1% SE-15 antifoam (Sigma) at 70°C and pH 7.4 as previously described (8, 39).

*Carbon monoxide treatment:* RBC were resuspended at 5%Hct in a CO saturated Tris buffer (NaCl 140.5 mM, Tris 21 mM, KCl 4.7 mM, MgSO$_4$ 1.2 mM, CaCl$_2$ 2 mM, Glucose 0.1% containing DTPA 100µM, BSA 0.5%). The resulting suspension was then exposed to a gentle stream of CO for a further 10-15 minutes prior to removing aliquots for experiments in the controlled atmosphere chamber. This protocol yielded ≥90%
Hemoglobin and nitrite export from red cells
conversion to carboxyhemoglobin as determined by spectral measurement of hemoglobin after RBC lysis in 4 volumes of high purity water.

Data normalization: Extracellular and intracellular nitrite concentrations were normalized to heme concentrations measured by the Drabkin’s assay (17). Briefly, lysed RBC are treated with excess K$_3$Fe(CN)$_6$ and KCN to quantitatively convert all hemoglobin species into cyanomethemoglobin. Cyanomethemoglobin concentration is determined by measuring the resulting absorbance at 540 nm using an extinction coefficient of 11 mM$^{-1}$cm$^{-1}$.

Vessel relaxation studies. Rat aortic rings were equilibrated under either 21% or 0% oxygen in Krebs-Henseleit buffer at 37$^\circ$C in the presence of 5% CO$_2$ as previously described (29). Vessels were pre-treated with indomethacin (5µM), L-NMMA (1mM) and pre-contracted with phenylephrine (200nM) prior to the addition of RBC (0.3% HCT final concentration). Upon reaching a stable tone, vasorelaxation was elicited by the addition of a single dose of either sodium nitrite (10µM final) or the NO donor Maha-NONOate (MNO, 30nM final). Vasorelaxation response was determined in the absence and presence of RBC and percent inhibition of nitrite or MNO dependent vasodilation by RBC calculated.

Statistical Analysis. All experiments were performed >3-times with ≥3 replicates within each experiment per condition unless otherwise specified. Data are expressed as means ± SEM with statistical analysis indicated in respective figure legends. Data was analyzed
Results

Effects of RBC oxygen fractional saturation on nitrite and NO-dependent vasodilation

Previous studies using isolated aortic ring bioassays have shown that the effects of cell-free hemoglobin on nitrite-dependent vasodilation depends on the oxygen fractional saturation(14, 29). Cell-free oxyhemoglobin inhibits nitrite and NO-dependent vasodilation consistent with heme based NO-scavenging. Under deoxygenated conditions a similar inhibition of NO-dependent vasodilation is observed, however nitrite-dependent vasodilation is unaffected consistent with a nitrite-reductase activity of deoxyhemoglobin that generates NO and counters NO-scavenging. Figure 1 extends this concept to intact RBC and shows that under the employed experimental conditions both oxygenated or deoxygenated RBC inhibit NO-dependent vasodilation to the same extent. In contrast, inhibition of nitrite dependent vasodilation by RBC is significantly attenuated at 0% oxygen compared to 21% oxygen.

Hemoglobin oxygen fractional saturation regulates nitrite consumption by RBC

Previous studies have shown that the nitrite-deoxyhemoglobin (nitrite-reduction) reaction is faster compared to the nitrite-oxyhemoglobin (nitrite oxidation) reaction (27, 28). To test if this occurs with intact RBC and if this effect is mediated by lower dissolved oxygen concentrations or by different hemoglobin oxygen saturations, RBC-dependent
Hemoglobin and nitrite export from red cells

Nitrite consumption was evaluated at 22°C and 37°C. These two temperatures were used to modulate oxygen affinity (being higher at lower temperatures (Fig 2A)), and hence hemoglobin fractional saturation at a given oxygen tension. Figure 2B shows that, regardless of the temperature used in the assay, nitrite consumption increases as hemoglobin fractional saturation decreases reaching maximum at saturation values close to the hemoglobin P$_{50}$ and then decreasing as hemoglobin oxygen saturation decreases beyond the P$_{50}$. Note that maximal nitrite consumption over 15 min is different at the two temperatures studied, being 106.1 nmoles/µmole heme at 28.8 mmHg O$_2$ and 28 nmoles/µmole heme at 7.8 mmHg O$_2$ at 37°C and 22°C respectively. Taken together these data demonstrate that increased nitrite consumption is related to the oxygen fractional saturation of hemoglobin in a bimodal fashion. This conclusion is verified by almost complete inhibition of hypoxemia-dependent nitrite consumption by RBC that had been pre-equilibrated with carbon monoxide (CO) (Fig 2C).

**Role of the RBC membrane in controlling nitrite-RBC reactions**

In order to study the role of the membrane in controlling nitrite reaction with hemoglobin, nitrite consumption was measured with intact (<0.5% hemolysis) or lysed (>70%) RBC. Since hemoglobin fractional saturation affects the kinetics of nitrite-heme reactions, and RBC P$_{50}$ is regulated by effectors (e.g. 2,3-biphosphoglycerate), RBC were lysed mechanically to avoid dilution and loss of allosteric effectors. P$_{50}$ of intact or hemolyzed RBC were not different (27.6 mmHg and 25 mmHg respectively, data not shown) ensuring heme-reaction rates were not a variable in these experiments. Figure 3 shows that hemolysis of RBC significantly increases nitrite consumption at half fractional
Hemoglobin and nitrite export from red cells

saturation. Similar results showing faster rates of nitrite consumption by lysed RBC were observed at high oxygen fractional saturations also (Figure 3), consistent with previous reports using oxygenated RBC (64). These data imply that compartmentalization by the membrane slows the overall consumption of nitrite by erythrocytic hemoglobin and suggest that modulating how nitrite crosses the membrane will impact on nitrite metabolism by RBC.

Evidence for an oxygen-sensitive step in RBC nitrite transport

We next determined the time course for nitrite consumption from the extracellular compartment and the parallel changes that occur in intracellular nitrite levels. Fig 4A presents data from one representative experiment (i.e. from one RBC donor) showing that nitrite consumption occurs in a time-dependent manner which is faster at lower hemoglobin fractional saturations. Interestingly, intracellular nitrite levels increase upon nitrite addition and immediately reach a steady state that lasts throughout the course of the observation period. Furthermore, unlike nitrite consumption, the steady state observed for intracellular nitrite is not altered by lowering the oxygen tension. The nitrite concentrations inside the RBC were ~1.5 µM which is significantly lower than the amount of nitrite consumed from the extracellular compartment. Due to variation between RBC preparations from different donors, Figures 4B and 4C show normalized data from 3-4 independent experiments in which nitrite consumption and intracellular accumulation were followed over 30 minutes further confirming the results obtained in Fig 4A. Figures 4D and 4E show respectively the nitrite concentration dependence on nitrite consumption from the extracellular compartment and intracellular nitrite steady
state levels as calculated by the nitrite:heme ratio. Nitrite consumption was higher at lower oxygen tension at all tested nitrite concentrations. However, whereas intracellular nitrite increased in proportion to nitrite dose added, this was not altered by lowering oxygen tension. These data are consistent with intracellular nitrite levels representing a balance between nitrite entry and heme-based consumption, a result further confirmed by time-dependent increases in both methemoglobin and nitrosylhemoglobin (HbNO) (Figure 4F). MetHb is a product of both nitrite-oxyHb and nitrite-deoxyHb reactions, whereas HbNO is an ultimate product of nitrite-deoxyHb reactions. MetHb formation was similar at both fractional saturations tested whereas HbNO formation was significantly higher at the lower fractional saturation. These data suggest that the lack of effect of fractional saturation on intracellular nitrite levels is not due to lack of reactions with heme but instead it represents a steady state comprising a balance between nitrite entry and heme-based consumption.

To ensure that measured intracellular nitrite levels were not affected by nitrite remaining from extracellular fractions, RBC were washed either two, three or four times. Intracellular nitrite levels remained the same with this procedure (not shown) suggesting contamination from extracellular nitrite is unlikely. Other possible sources of intracellular nitrite are decomposition of HbNO (and subsequent NO-autooxidation) upon sample processing and activity of RBC eNOS (37, 63). We note that HbNO levels were 4 to 5-fold higher at lower oxygen tensions suggesting nitrite-derived from this pool is unlikely to contribute to measured intracellular nitrite levels which do not change with varying oxygen tensions. eNOS-dependent nitrite generation was excluded since all studies were
Hemoglobin and nitrite export from red cells

performed in the absence of exogenous L-Arginine and moreover addition of the NOS inhibitor L-NAME (3 mM) did not affect either extracellular consumption rates (40.7 vs. 37.8 nmoles/µmole heme / 15 min in the absence and presence of 3mM L-NAME respectively p = 0.41) or intracellular nitrite levels (129.1 vs. 128.9 pmoles/µmole heme /15min) in the absence and presence of 3mM L-NAME respectively p = 0.99 NS; PO$_2$ 30.5 mmHg, 15 min incubation, 37° C, 100µM nitrite, significance determined by t-test).

The lack of effect of lowering fractional saturation on intracellular nitrite levels despite decreasing extracellular concentrations of nitrite at each time point provides evidence for regulation of nitrite transport by Hb deoxygenation. This conclusion was derived by consideration of the scheme shown in Fig 5 in which nitrite was assumed to enter RBC in a reversible manner and independent of oxygen tension (i.e. HNO$_2$/NO$_2^-$ passive diffusion). In this model, the following criteria were applied i) deoxyHb reacts with nitrite faster compared to oxyHb, therefore in this model the only role of oxygen on nitrite consumption will be by regulating Hb fractional saturation, ii) nitrite transport is independent of oxygen tension and iii) intracellular nitrite reaches steady state. These criteria were developed based on existing (36) and presented data (Fig 4) and assuming the Null hypothesis that nitrite transport was independent of oxygen tension. In this model (Fig 5) the concentration of intracellular nitrite can be solved by applying the steady state approximation resulting in equation 1:

\[
[N O_2^-_{IN}] = \frac{k_1[N O_2^-_{OUT}]}{[k_{-1} + k_{ox} [oxyHb] + k_{red} [dHb] + k_2]} \]
Hemoglobin and nitrite export from red cells

Since $[NO_2^-]_{IN}$ does not change with oxygen tension (Fig 4C), four possibilities can be proposed; i) hemoglobin is not important for intracellular metabolism of nitrite, i.e. $k_1$, $k_2$ >>> $(k_{ox}[oxyHb] + k_{red}[dHb])$ and $k_1$, $k_2$ are unaffected by oxygen, ii) deoxygenation increases $(k_{ox}[oxyHb] + k_{red}[dHb])$ and decreases $k_2$, iii) deoxygenation increases $(k_{ox}[oxyHb] + k_{red}[dHb])$ and increases nitrite import ($k_1$), iv) deoxygenation increases $(k_{ox}[oxyHb] + k_{red}[dHb])$ and decreases nitrite export ($k_{-1}$). Since nitrite-metabolism by RBC was dependent on fractional saturation and inhibited by CO (suggesting hemoglobin as the primary reaction target for nitrite, i.e. $(k_{ox}[oxyHb] + k_{red}[dHb])$ >>> $k_2$), options i) and ii) can be excluded leaving the possibility that in order to explain the lack of effect of deoxygenation on intracellular nitrite despite increased rates of consumption, deoxygenation also modulates nitrite transport by increasing import ($k_1$) and/or decreasing export ($k_{-1}$).

AE1 controls deoxygenation-dependent nitrite export.

We next tested the potential for AE1 in mediating increased nitrite-uptake by hypoxemic RBC. The AE1 inhibitor DIDS was added to RBC and hypoxia-dependent nitrite consumption and intracellular accumulation measured. In the presence or absence of DIDS, lowering fractional saturation increased nitrite consumption to the same extent (Fig 6A) suggesting AE1 is not involved in how nitrite enters the RBC. Interestingly, intracellular nitrite increased in DIDS-treated RBC with the steady state level being ~3-4 fold higher compared to control RBC (Fig 6B). These data suggest that AE1 controls nitrite export from RBC. In order to assess if an inhibition of export pathway is responsible for maintaining intracellular nitrite levels in hypoxic RBC (Fig 4C), we
Hemoglobin and nitrite export from red cells

hypothesized that intracellular nitrite levels will decrease when oxygen tension is lowered in DIDS-treated RBC. In other words, if oxygen-dependent modulation of nitrite export through AE1 is the mechanism responsible for maintaining constant intracellular nitrite levels, then in the presence of DIDS (where AE1 is already inhibited), the only oxygen-sensitive step in RBC-nitrite interactions would be control over heme reactivity and not hemoglobin dependent effects on AE1. Figure 6C shows that in control cells, deoxygenation does not change the intracellular steady-state nitrite concentrations compared to oxyRBC consistent with data shown in Figure 4C. However, in DIDS-treated cells deoxygenation is associated with significant decrease in the intracellular steady-state nitrite levels suggesting AE1 is responsible for maintaining intracellular nitrite levels upon RBC deoxygenation. As a control we investigated whether DIDS would affect cell-free deoxyhemoglobin reactivity with nitrite and observed no effect (not shown).

Finally, to demonstrate that inhibition of export results in a greater nitrite-reductase activity due to higher intracellular nitrite levels, RBC S-nitrosothiols (SNO) were measured. These species are relatively stable by-products of nitrite-reduction by deoxyhemoglobin (3, 11, 47) and evidence the intermediate formation of reactive nitrogen species as recently demonstrated(5). Consistent with this prediction, Figure 6D shows that RBC SNO levels are increased ~2-fold in DIDS treated RBC.

Discussion
Hemoglobin and nitrite export from red cells

How nitrite crosses biological membranes, whether this is an active or passive transport process, and subject to regulation by electrochemical and/or concentration gradients remain unclear. Nitrite transporters have been identified in plants (58), but limited data exists in mammals. Interestingly, intraperitoneal administration of nitrite results in a rapid (within 5 min.) redistribution through all major organs/tissues in the rat and results in nitrite concentrations that vary significantly from tissue to tissue (8) suggesting i) rapidity in nitrite movement and ii) either regulation of nitrite transport and/or tissue-specific rates of nitrite metabolism. Recent studies using a liposomal model (in the absence of protein channels) suggest that despite being predominantly (>99.9%) in the anionic form at pH 7.4, addition of sodium nitrite to the extracellular compartment results in acidification of the liposomal interior, consistent with the generation of nitrous acid (HNO₂) which can freely diffuse across the bilayer (54).

In this study we explored nitrite-transport in RBC and the role of oxygen as a modulator of these processes. We chose this model since i) RBC are endowed with multiple oxygen-sensitive ion channels/transporters and ii) RBC-nitrite interactions have been implicated in modulating hypoxic blood flow. We used RBC in which sufficient time has elapsed post-collection to deplete endogenous (basal) nitrite, precluding this as a variable in our studies. Unlike other ions, the study of nitrite transport in RBC is complicated by the existence of a significant intracellular sink for nitrite i.e. hemoglobin whose reactions with nitrite change with fractional saturation and hence oxygen tension. Therefore any mechanism addressing regulation of transport must integrate differential reactivities of nitrite with oxy- and deoxyHb. Moreover, this reactivity indicates that measurement of
Hemoglobin and nitrite export from red cells

Nitrite consumption from the extracellular compartment alone is not sufficient if transport mechanisms are being assessed.

Consistent with increased rates of nitrite-reaction with deoxyHb, nitrite loss from the extracellular compartment increased with RBC deoxygenation and was inhibited by CO supporting an important role for intracellular reactions with deoxyHb. Moreover, nitrite consumption was faster in lysed RBC suggesting that compartmentalization by the membrane is a critical mechanism in controlling nitrite reactions with erythrocytic hemoglobin. We note that recent studies using sheep RBC (7) did not observe an effect of the RBC membrane and may reflect differences in experimental conditions and/or species-based differences in how RBC metabolize nitrite. In the context of the latter possibility, RBC from different species have been shown to either consume nitrite faster or at the same rate when comparing oxygenated with deoxygenated conditions underlying potential species to species variations in mechanisms that regulate RBC-nitrite reactions (18, 33, 34).

Intracellular nitrite levels increased and immediately (<5 min) attained a steady state. Moreover, this effect was oxygen independent being observed at all hemoglobin fractional saturations tested. This suggests that nitrite can enter RBC in a facile and presumably reversible process, consistent with previous studies showing rapid equilibration of nitrite across oxygenated RBC membrane (43). Whether this is mediated by a specific channel or occurs via diffusion of nitrous acid with RBC remains to be elucidated. Surprising was the fact that intracellular steady-state nitrite level did not
Hemoglobin and nitrite export from red cells

change with decreasing fractional saturation despite increased rates of nitrite consumption and reactions with deoxyheme. The fact that intracellular nitrite-levels remain constant supports a mechanism in which hemoglobin fractional saturation regulates nitrite transport and, moreover, the extent to which deoxygenation affects these transport processes reflects quantitatively the extent to which it increases the rate of hemoglobin-nitrite reactions.

Previous data suggest a role for AE1 in controlling peroxynitrite permeation of the RBC membrane (16). With respect to nitrite specifically, previous work using human, pig or carp RBC showed no effect of DIDS on nitrite consumption by oxygenated or deoxygenated RBC (32, 34, 43, 64) consistent with data presented in Fig 6A of the current study. These previous studies did not directly measure the effects of AE1 inhibition on intracellular nitrite levels however, which we show do increase significantly (Fig 6C). A role for AE1 in mediating nitrite export has also been suggested by Shingles et al using hemoglobin-free RBC ghosts under oxygenated conditions (55). Our data extend this concept to intact RBC and also across oxygen fractional saturations. How AE1 mediated nitrite export occurs and is regulated is not known. Importantly, inhibition of export by DIDS increases intracellular nitrite levels which are reflected in higher concentrations of RBC SNO, consistent with faster velocities of nitrite-deoxyhemoglobin reactions. These data also highlight the potential role of AE1 activity and circulating nitrite levels in determining RBC SNO concentrations, an area of considerable recent interest (12, 21, 49, 57).
Hemoglobin and nitrite export from red cells

We propose that maintaining intracellular nitrite levels during deoxygenation is achieved by deoxyhemoglobin-dependent inhibition of nitrite export via AE-1. Precedence for such a mechanism is provided first by studies showing that deoxyhemoglobin (T-state conformation) has a higher affinity for binding to the N-terminus of AE1 compared to oxyhemoglobin (R-state conformation) and that this phenomenon is responsible for the displacement and activation of AE1-bound glycolytic enzymes in RBC under low fractional saturations (9, 45). Moreover, deoxyhemoglobin binding to AE1 has been shown to inhibit AE1 activity in the context of sulfate transport (19). To investigate a nitrite export based mechanism experimentally, we employed the AE1 inhibitor DIDS. We reasoned that in the presence of DIDS, AE1 will be already inhibited and therefore no longer subject to deoxyhemoglobin mediated inhibition. In this scenario, we predicted that intracellular levels of nitrite would decrease upon deoxygenation due to faster reactions with heme. This result was observed experimentally supporting this model.

Taken together with intracellular nitrite levels increasing in the presence of DIDS, we propose the following scheme (Figure 7) in which nitrite moves into the RBC down an electrochemical / concentration gradient through either a channel and / or as nitrous acid (step 1). Under oxygenated conditions the nitrite concentration gradient is regulated by intracellular reactions with oxyhemoglobin resulting in nitrite oxidation (step 2) and export via AE1 (step 3). As RBC desaturate, nitrite consumption is accelerated due to deoxyhemoglobin reactions resulting in a species (NOx, recently proposed to be N₂O₃ (5) that can ultimately produce NO outside the RBC (step 4) reaching maximal rates at the hemoglobin P₅₀. Concomitantly, deoxyhemoglobin binds to and inhibits AE1, thereby preventing export and maintaining intracellular nitrite levels (step 5). We propose that
Hemoglobin and nitrite export from red cells

such a mechanism allows more nitrite to react with deoxyheme relative to being exported as RBC transit from arterial to venous circulation with the concomitant decrease in fractional saturation and thereby allowing for a more efficient coupling between hypoxia and vasodilation. Put another way, if nitrite transport (modulation of export) was not an oxygen sensitive process, increased hemoglobin reactivity at lower fractional saturations could result in intracellular nitrite levels being lowered to levels that may preclude significant rates of NO formation. Moreover, recent studies indicate that deoxyHb bound to AE1 is a faster nitrite reductase compared to hemoglobin in bulk solution (53). Taken together this suggests a central role for hemoglobin-AE1 interactions in regulating nitrite reduction to NO by RBC.

Limitations: We note that our proposed model is based on results obtained by inhibiting AE1 using DIDS, which despite being a relatively specific inhibitor for AE1 in isolated RBC (30, 40) and being used widely to demonstrate a role for AE1 in controlling transport of species that also react with hemoglobin (e.g. peroxynitrite and nitric oxide) (16, 25), does not provide molecular based evidence for a role of AE1-deoxyHb interactions in regulating nitrite metabolism. We did test the effect DIDS on the ability of RBC to modulate nitrite-dependent vasodilation at different oxygen tensions as described in Figure 1, however AE1 inhibition also decreased NO-scavenging (not shown) consistent with previous reports (25). This observation precludes the use of aortic ring vasodilation as a functional read-out to assess the role of nitrite transport regulation on RBC nitrite-reductase activity. Moreover, recent studies have shown that incorporating an antibody into permeabilized RBC that specifically binds to the cytoplasmic N-terminal
domain of AE1 prevents deoxyHb binding to AE1 (9, 10). We attempted to utilize this approach but observed significant lysis of resealed RBC at low oxygen tensions (not shown) precluding this approach to assess nitrite transport mechanisms by RBC. Further studies utilizing molecular targeted approaches to modulate hemoglobin-AE1 interactions are required to definitely demonstrate a role for deoxyHb-AE1 binding in inhibiting nitrite export.

In summary, the data presented here extend the paradigm of allosteric regulation of RBC-dependent nitrite reduction to include inhibition of nitrite export. We propose that inhibition of nitrite export during hypoxia is crucial in ensuring increased rates of NO production from intra-erythrocytic nitrite as the oxygen tension is lowered and speculate that this may be a general biological mechanism regulating nitrite transport. Finally, these data suggest that inhibition of nitrite export is integrated with diverse RBC functions that include allosteric regulation of glycolytic flux, ion homeostasis and oxygen / NO-delivery.
Hemoglobin and nitrite export from red cells

Acknowledgements

JCT is currently at: Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, CEP 09210-170, Santo André, SP, Brazil.

Grants

We acknowledge funds from the American Heart Association (Southeast Affiliate, AHA 0655312B to RPP and a Pre-doctoral fellowship (0815248E) to DAV) and National Institutes of Health grants HL71189 and HL074391 to JL, U01ES015676 and RO1HL075540 to SM and U54ES017218 to SM and RPP.
Hemoglobin and nitrite export from red cells

References


Hemoglobin and nitrite export from red cells


Hemoglobin and nitrite export from red cells


Hemoglobin and nitrite export from red cells

Hemoglobin and nitrite export from red cells

Figure Legends

Figure 1: Effects of RBC on nitrite and nitric oxide dependent vasodilation at high and low oxygen tensions. Relaxation of aortic rings by bolus additions of either MNO (30nM) or nitrite (10µM) in the absence or presence of RBC (0.3% Hct) was determined at 21% and 0% oxygen. Shown is the inhibition of MNO or nitrite-dependent vasodilation by RBC at 21% and 0% oxygen. Data shown are mean ± SEM (n = 4-6). Indicated P-values calculated by unpaired t-test.

Figure 2: RBC hemoglobin oxygen fractional saturation regulates nitrite-consumption

Panel A) Oxygen binding curves for RBC (0.3-0.5% Hct) in Krebs-Henseleit buffer pH 7.4 determined at 22°C or 37°C in the presence of 5% CO₂. Calculated P₅₀’s and Hill constants were 7.1 mmHg and 1.6 respectively at 22°C and 32.6 mmHg, and 2.9 at 37°C.

Panel B) Oxygen-equilibrated RBC suspensions (5% Hct) were incubated in the presence of 100 µM nitrite at the indicated temperatures and pH 7.4. Remaining nitrite in the media was measured after 15 minutes. Since rates of consumption are faster at higher temperatures, data are presented as relative consumption normalized to maximum at each respective temperature. Data are mean ± SEM (n=3) analyzed by one-way ANOVA and Bonferroni post-test. *P <0.05, ** P <0.01 vs. highest fractional saturation at each respective temperature. Panel C) Control or CO gas pre-equilibrated RBC (5% Hct) in Tris buffer were incubated with nitrite (100µM) at 28mmHg pO₂, 37°C, pH 7.4. for 15 min and nitrite consumption measured. Data are mean ± SEM (n=3) analyzed by two-tailed student’s t test. *P <0.001.
**Figure 3: RBC membrane regulates nitrite-heme reactivity.** Nitrite (100µM) was added to intact (hemolysis < 0.5%) or mechanically lysed (hemolysis > 70%) RBC (5%Hct) at 75mm Hg or 30 mmHg pO₂, 37°C, pH 7.4 and nitrite consumption measured after 10 min. Data are mean ± SEM (n=3) analyzed by unpaired t-test versus intact cells at the respective oxygen tension, *P <0.001.

**Figure 4: Effects of RBC deoxygenation on intracellular nitrite concentration.** Panel A: Nitrite (100µM) was added to RBC (5% Hct) pre-equilibrated at 75 mmHg (---•---) or 30 mmHg (—○—) oxygen as indicated, at 37°C and time dependent loss of nitrite from the extracellular and accumulation in the intracellular compartment determined. Data show changes in absolute nitrite concentrations and are mean ± SEM (n=3) from a single experiment analyzed by two-way ANOVA and Bonferroni post-test ***P <0.001 relative to corresponding time at higher oxygen tension. Intracellular nitrite concentrations were calculated by assuming a RBC volume of 100fl and an intraerythrocytic heme concentration of 20 mM. Panel B and C show normalized data for extracellular nitrite consumption and intracellular nitrite accumulation respectively from 3-4 independent experiments (and RBC preparations). The absolute amounts of nitrite consumed and intracellular levels were found to vary between RBC preparations (from 52 to 139 nmoles/µmole heme, and from 46 to 121 pmoles/µmole heme for maximum consumption and maximal intracellular nitrite levels respectively). To compare results from different RBC preparations therefore, data in panels B and C are plotted as relative to maximal in each preparation. Shown are means ± SEM (n=4 panel B, n=3 panel C) analyzed by two-
Hemoglobin and nitrite export from red cells

way ANOVA and Bonferroni post-test. *P <0.05 and **P <0.001 vs. corresponding time point at 75 mmHg oxygen. Panel D and E Nitrite at indicated doses was added to RBC (5%Hct) pre-equilibrated at either 73.5 mmHg or 21.4 mmHg oxygen at 37°C, pH 7.4 and both nitrite consumption from extracellular compartment (panel D) and intracellular nitrite levels (panel E) were measured after 15 min. Data show mean ± SEM (n=3). P-value indicates two-way ANOVA analysis. Lines in panel D show best fit determined by linear regression. Panel F shows changes in methemoglobin (metHb) and ferrous-nitrosyl hemoglobin (HbNO) after the addition of 100µM nitrite to RBC 5% hct at 37°C, pH 7.4. - -◆-- and --○-- represent metHb at 75 and 30 mmHg oxygen respectively. —◆— and —○— represent HbNO at 75 and 30 mmHg oxygen respectively. Data are normalized to maximal metHb (99.2 nmol / µmol heme) and HbNO (1.81 nmol / µmol heme) respectively and represent mean ± SEM. Data analyzed by two-way ANOVA and Bonferroni post-test. * P <0.05, ** P <0.001 vs corresponding time point at 75 mmHg for HbNO formation.

Figure 5: Model illustrating a proposed reaction scheme assuming fractional saturation does not regulate nitrite transport. \( k_1 \) and \( k_{-1} \) represent the rate constants for reversible nitrite transport across the membrane, \( k_{ox} \) and \( k_{red} \) are the rate constants for the reaction between nitrite and oxy- or deoxyhemoglobin respectively where \( k_{red} > k_{ox} \). \( k_2 \) represents other (non-hemoglobin) sinks for nitrite consumption in the cell. \([NO_2^-]_{IN}\) is intracellular nitrite; \([NO_2^-]_{OUT}\) is extracellular nitrite

Figure 6: Effect of DIDS on nitrite-RBC interactions
Hemoglobin and nitrite export from red cells

Panel A) Control and DIDS-treated RBC (100 µM DIDS) (5%Hct) were equilibrated at either ~75 or ~30 mmHg oxygen at 37°C for 15 or 30 min respectively resulting in fractional saturations of ~0.95 or ~0.5 respectively. Nitrite (100 µM) was then added and consumption from extracellular compartment after 15 mins measured. Data show mean ± SEM (n = 8). *P <0.01 by paired t-test relative to corresponding lower fractional saturation. NS = not significant by t-test. Panel B) shows time dependent changes in intracellular nitrite in control and DIDS-treated RBC at fractional saturation of 0.5. Data are mean ± SEM (n=3). *P < 0.001 relative to corresponding time point in control by two-way ANOVA and Bonferroni post-test. Panel C) shows intracellular steady state levels in control and DIDS treated RBC at oxygenated (0.95) and deoxygenated (0.5) fractional saturations. Data shown mean ± SEM (n=5). *P < 0.01 by paired t-test relative to corresponding high fractional saturation condition. Panel D) Nitrite (100µM) was added to control or DIDS pre-treated RBC (5%Hct) at 27 mmHg oxygen, 37°C and pH 7.4 and RBC S-nitrosothiol (SNO) concentrations measured at 60 min. Data are mean ± SEM (n=3) analyzed by two-tailed student’s t test. *P <0.05.

Figure 7: Scheme illustrating proposed mechanism by which hemoglobin deoxygenation regulates nitrite metabolism by RBC.
$[\text{NO}_2^-]_{\text{out}} \xrightarrow{k_i} \frac{[\text{NO}_2^-]_{\text{in}}}{k_f}$

$\xrightarrow{k_{\text{oxyHb}}} \xrightarrow{k_{\text{rHb}}} \xrightarrow{k_{\text{dHb}}} \xrightarrow{k_i}$

Vitturi et al. Figure 5