Protein disulfide isomerase may facilitate the efflux of nitrite derived S-nitrosothiols from red blood cells

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A B S T R A C T

Protein disulfide isomerase (PDI) is an abundant protein primarily found in the endoplasmic reticulum and also secreted into the blood by a variety of vascular cells. The evidence obtained here, suggests that PDI could directly participate in the efflux of $NO^+$ from red blood cells (RBC). PDI was detected both in RBC membranes and in the cytosol. PDI was S-nitrosylated when RBCs were exposed to nitrite under −50% oxygen saturation but not under ~100% oxygen saturation. Furthermore, it was observed that hemoglobin (Hb) could promote PDI S-nitrosylation in the presence of ~600 nM nitrite. In addition, three lines of evidence were obtained for PDI−Hb interactions: (1) Hb co-immunoprecipitated with PDI; (2) Hb quenched the intrinsic PDI fluorescence in a saturable manner; and (3) Hb−Fe(II)−NO absorption spectrum decreased in a [PDI]−dependent manner. Finally, PDI was detected on the surface RBC under ~100% oxygen saturation and released as soluble under ~50% oxygen saturation. The soluble PDI detected under ~50% oxygen saturation was S-nitrosylated. Based on these data it is proposed that PDI is taken up by RBC and forms a complex with Hb, Hb−Fe(II)−NO that is formed from nitrite reduction under ~50% $O_2$, then transfers NO$^+$ to either Hb−Cy3 or directly to PDI resulting in S-nitroso-PDI which transverses the RBC membrane and attaches to the RBC surface. When RBCs enter tissues the S-nitroso-PDI is released from the RBC-surface into the blood where its $NO^+$ is transferred into the endothelium thereby inducing vasodilation, suggesting local oxygen-dependent dynamic interplays between nitrite, NO and S-nitrosylation.

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Introduction

When RBCs enter a hypoxic region of the vasculature they release effector(s) that induce vasodilation thus ensuring that the oxygen they release is effectively distributed. This phenomenon termed hypoxic vasodilation is highly conserved and although first reported some 90 years ago [1–3], the sensing mechanisms as well as the vasodilatory substances released by RBCs remain to be clearly identified. Current research in this area supports either that nitric oxide (NO) and related compounds (NOx) [4–6] or ATP [7–12] as vasodilator-triggers released from RBCs.

The first hypothesis put forward for RBC-mediated hypoxic vasodilation is through release of ATP upon decrease in $HbO_2$ saturation. The ATP released diffuses to the endothelium and binds to the purinergic receptors leading to increase NO production via eNOS activation [7–13]. Furthermore, recent studies have shown that deoxy−Hb interacts with nitrite and dislodges the membrane bound glycolytic regulatory subunits enhancing intracellular ATP that is released under hypoxic conditions [14–16]. The ATP is released from RBC not only when RBC deoxygenates but also in response to mechanical deformation when RBC travel through narrow vessels [11,12]. Various factors are likely to regulate the role of ATP in vasodilatation such as the activity of transporters that regulate ATP release, enzymes that regulate ATP concentrations and purinergic receptor expression levels [17]. The NO (or NOx) based hypotheses can be further subdivided into those that depend on the scavenging of endothelia-generated or more recently RBC−eNOS−generated NO [18–21] to yield S-nitrosohemoglobin (SNO−Hb) or those that transform nitrite to NO within the RBC by hemoglobin acting as a nitrite reductase. In the S−NHO−Hb hypothesis, deoxygenated Hb in its T-state scavenges
the endothelial NO to yield a mixture of HbFe(II)–NO and HbFe (III)–NO. When the RBCs arrive in the lungs the Hb undergoes a conformational change to the R-state where the O2 displaces the heme-bound NO to Cys β93 of Hb to form SNO-Hb. When the RBC reach the hypoxic tissues, Hb then undergoes conformational changes to the T state which leads to the concomitant release of O2 and transfer the SNO-bound NO bioactivity to the outside of the RBC possibly via transnitrosation reactions to induce endothelial vasodilatation [4,22–24].

The nitrite/nitrate reductase hypothesis involves transport of nitrite to RBC, reaction of nitrite with deoxy-Hb, transport of NO bioactivity from RBC and finally vasodilatation [5,15,25,26]. The plasma levels of nitrite are reportedly between ∼120 nM and 290 mM [17,27]. Recent studies suggest that plasma nitrite can accumulate to near μM levels in RBC under hypoxia, via the deoxyHb-mediated inhibition of the anion transporter (AE1) which is responsible for nitrite efflux from the RBC [28]. Within RBC the nitrite could be converted to NO by the previously demonstrated nitrite reductase activities of xanthine oxidoreductase, hemoglobin [28] and eNOS [21,29–31].

The next important question concerns the mechanisms by which intracellular NO or NO-equivalents exit the RBC which contains ∼30 mM Hb. The amount of NO produced by the SNO-Hb or the nitrite routes are expected to be in the submicromolar levels. Under these conditions, any NO that is formed can react with deoxyhemoglobin (Fe2+) and yield heme-nitrosylHb (HbNO) which can either react with oxygen to form nitrate plus methemoglobin (Fe3+) or react with RBC-thiols to yield S-nitrosothiols (SNO). The efflux of SNO-bound NO from RBC could be plausible via a series of transnitrosation reactions where the SNO moiety would be transferred/shuttled from Hb to other intracellular proteins then to membrane spanning proteins eventually ending up as cell surface or secreted SNO-proteins which can deliver their NO into endothelia, thus effecting vasodilation. In fact, several studies have implicated the transmembrane anion exchanger 1 (AE1) or band 3, one of the most abundant RBC-proteins in mammalian cells. Although it is largely an endoplasmic reticulum—(ER)—resident enzyme, it is secreted or leaks out of cells where it forms weak associations with the cell surfaces of many cell types including pancreatic cells [34,35], B cells [36,37], hepatocytes [38], platelets [39,40], endothelial cells [41], leukocytes [42,43] and platelet derived microparticles [44]. Several studies in RBCs have identified membrane associated PDI. However, the physiological role of PDI in RBCs is unknown [40,45–47].

Previous studies have shown that in endothelial cell surface PDI facilitates the transfer of extracellular SNO to the cytosol [48] and that PDI catalyze the release of NO from SNO-PDI as well as other S-nitrosothiols [49]. In this study, we report the potential involvement of PDI in a nitrite-dependent and oxygen regulated process for the efflux of NO (or NO-equivalents) from RBCs.

**Materials and methods**

**Materials**

Buffer salts, diethylentriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), penicillamine, sodium pyrophosphate (PMSF), noryln phenoxophenetylethanol (NP-40), sodium dodecyl sulfate (SDS), hemoglobin, sodium dithionite, bovine maleimide and immunoblotting reagents were obtained from Sigma-Aldrich (St. Louis Mo). All antibodies were purchased from AbCam (Cambridge MA).

Bicinchoninic acid assay (BCA assay), Aminolink Plus coupling resin and spin columns were purchased from Thermo Scientific (Rockford, Ill). The nitrite/nitrosylated PDI by immunoblotting

RBCs were prepared for experiments under different oxygen saturations using previously established protocols. Fresh blood was collected from healthy human volunteers by venipuncture into BD tubes containing anticoagulant. Blood was centrifuged at 1000 x g for 10 min to remove plasma and buffy coat. RBCs were washed with buffer (pH 7.4) of following composition 6.9 g/l NaCl, 2.28 g/l NaHCO3, 0.35 g/l KCl, 0.136 g/l KH2PO4, 0.144 g/l MgSO4, 2.0 g/l D-glucose to prevent hemolysis. Experiments with different oxygen saturations were performed in septa sealed vials. The buffer used in the experiments was also pre-equilibrated for 30 min at respective oxygen saturations. Isolated and washed RBCs in buffer (pH 7.4) were held under 16% O2 or hypoxia 4% O2 for 15 min [28,50]. Nitrite stock solution was prepared in phosphate buffered saline (PBS) with DTPA (100 μM) and added to RBC suspension to a final concentration of 600 nM using a syringe and further incubated for 10 min.

Immunoprecipitation

RBCs membranes were prepared using standard protocols as described previously [17,27,51]. Briefly, to the RBC pellet (1 mL) 40 volumes of ice-cold 5 mM phosphate buffer containing 0.1 mM PMSF, 20 mM NEM and 100 μM DTPA was added. RBCs were then incubated on ice for 20 min to induce hemolysis. After centrifugation at 12,000 x g for 10 min at 4 °C, RBC membranes were washed twice with the same buffer.

RBC membranes were dissolved as described earlier [51,52]. RBC membranes were solubilized in lysis buffer containing Hepes (50 mM), NaCl (150 mM), EDTA (5 mM), EGTA (5 mM), sodium pyrophosphate (20 mM), NEM (20 mM), orthovanadate (1 mM), NaF (20 mM), K3Fe(CN)6 (10 mM), NP-40 (1%), PMSF (0.1 mM) and protease inhibitor (1:200). The samples (100 μg) were precleared with protein A/G (40 μL) by incubation and mixing for 1 h at 4 °C. Samples were then incubated with anti-PDI antibody (1:50 dilution) or mouse anti-AE1 antibody (1:100 dilution) or rabbit anti-glut 1 antibody (1:100 dilution). After incubation for 2 h at 4 °C, protein A/G beads (50 μL) were added to the samples and further incubated for 3 h. The beads were washed three times with lysis buffer. Proteins were eluted from beads using SDS-PAGE sample buffer devoid of β-mercaptoethanol by incubating at 95 °C for 10 min and analyzed by immunoblotting. For experiments with RBC homogenates, RBC samples (100 μL) were homogenized in PBS with NEM (20 mM), K3Fe(CN)6 (10 mM), DTPA (100 μM), NP-40 (1%), PMSF (0.1 mM) and protease inhibitor (1:200) followed by immunoprecipitation as described above.

**Detection of S-nitrosylated PDI by immunoblotting**

Nitrite supporting PDI-S-nitrosylation in-vitro in presence of oxy-hemoglobin (oxyHb) was determined as follows: Hb(1 mM) was reduced with dithionite (50 mM) under argon in serum sealed vials and transferred to septa sealed vials containing constant PDI (1 μM), Hb (0.6 mM) and varying amounts of nitrite (78 mM –5 μM) in PBS. The headspace of the vial contained 20 ppm O2. After incubation for 5 min at room temperature the samples were subjected to biotin switch assay as previously described [50,53,54]. The supernatant was treated with 100 μM DTPA, 20 mM NEM, 10 mM K3Fe(CN)6 and 1% SDS and incubated at 50 °C for 30 min with frequent vortexing. Two volumes of ice-cold acetone were added to precipitate the proteins. The precipitant was further
RBCs were isolated and adjusted to previously established protocols with some modification. Deoxy-hemoglobin was prepared as previously described. Preparation of Hb samples for UV spectroscopy secondary antibodies and visualized using chemiluminescence anti-Hb (1:1000), mouse anti-PDI (1:1000) and streptavidin-HRP followed by immunoblotting. The membranes were probed with μ protein in samples were determined by BCA assay. Protein chilled acetone and resuspended in PBS. The concentrations of 1 mM biotin-maleimide and incubated in dark at room temperature for 1 h. Biotin-labeled proteins were precipitated using pre-

Detection of soluble PDI

The buffer of the RBC suspension under 16% O₂ and 4% O₂ oxygen with and without nitrite treatment were probed for soluble PDI. The soluble PDI was isolated as described previously. The supernatant of the RBC suspension was subjected to two centrifugation steps first at 1000×g to remove the cells then at 13,000×g to remove cellular debris. The resulting supernatant containing soluble proteins was used for immunoprecipitation of PDI. The supernatant (1 mL) was precleared with protein A/G (40 μg/well) by incubation and mixing for 1 h at 4°C. The suspension was centrifuged at 1000×g for 1 min. The beads were washed three times with wash buffer and incubated with 100 μL of elution buffer for 5 min. Immunoprecipitated proteins were collected by centrifugation at 1000×g for 2 min. Protein concentrations were determined by BCA assay. Protein samples, ~20 μg/well, were resolved on non-reducing SDS-PAGE gel followed by immunoblotting. The membranes were then incubated with rabbit anti-PDI antibody (1:1000) antibody for 2 h followed by HRP conjugated secondary antibodies (1:2500) for 1 h and visualized using chemiluminescence substrate. For determining S-nitrosylation of soluble PDI at various oxygen saturations, 10 μL of immunoprecipitated protein was injected into NO analyzer. The concentration of NO was determined from standard curve.

Results and discussion

PDI is detected in RBC homogenates and membrane fractions

Recent studies indicate that PDI along with several other chaperones are lost in erythroid progenitor cells as they mature to become RBCs. Despite this, PDI was detected on the surface of RBCs as well as in proteomic profiles of RBCs. Here, the various components of human RBCs were probed with the aid of anti-PDI antibodies (RL-90). Western blots indicated PDI was present in RBC homogenates and in the plasma membrane (Fig. 1).

RBC-PDI can be S-nitrosated in the presence of Hb plus nitrite under oxygenated conditions

The nitrite reductase hypothesis requires a significant portion of the RBC Hb to be in the deoxyHb form. In addition, the intra RBC nitrite accumulation proposed by Vitturi et al. also requires large amounts of deoxyHb to inhibit Band3 thereby blocking the efflux of nitrite. In current study, we explored the ability of RBC-PDI to become S-nitrosylated upon incubation of intact RBCs with nitrite under 4% O₂ or 16% O₂ saturation

![Western immunoblot of: RBC membrane fraction-Lane 1; RBC homogenate-Lane 2; and standard human PDI-Lane 3, all probed with anti-PDI primary antibodies.](image)

Fig. 1. Western immunoblot of: RBC membrane fraction-Lane 1; RBC homogenate-Lane 2; and standard human PDI-Lane 3, all probed with anti-PDI primary antibodies.
conditions. These two O₂ levels were chosen because 4% O₂ corresponds to ∼27 Torr or ∼36 μM oxygen which is the P50 for human Hb, representing oxygenation levels in venous blood. Therefore, at this oxygen tension, Hb is half saturated. Previous studies [6] have demonstrated that the highest rate of nitrite reduction takes place at the P50 of Hb which is 4% O₂. The 16% level was chosen as this represents the oxygen tension in the arteries ∼144 μM. At this level Hb is fully saturated with oxygen.

The S-nitrosylation status of PDI was determined by the biotin switch assay. Our data indicate that RBC-PDI is S-nitrosylated under 16% O₂ (Fig. 2, Lane 3), whereas it is not S-nitrosylated at 4% O₂ (Fig. 2, Lane 1).

The next question was to determine whether Hb was responsible for nitrite-mediated PDI-S-nitrosylation under normoxia. To do this, constant amounts of PDI (1 μM), Hb (0.6 mM-dithionite reduced) was incubated with varying amounts of nitrite (78 nM to 5 μM) in PBS, in septa sealed vials equilibrated with 16% O₂.

The PDI-S-nitrosylation was detected by the biotin switch assay visualized with streptavidin-HRP. The results indicate that [nitrite] as low as ∼625 nM can support PDI-S-nitrosylation (Fig. 3, Lane 4). Furthermore, these experiments dramatically demonstrate the requirement of Hb in the S-nitrosylation of PDI: ∼50% of PDI is S-nitrosylated with 5 μM nitrite in the presence of Hb (Fig. 3, Lane 7). However, there is no detectable PDI-S-nitrosylation when Hb is excluded (Fig. 3, SNO-PDI: streptavidin-HRP-Hb or Lane 8).

**PDI co-immunoprecipitates with Hb and Hb quenches the PDI-intrinsic fluorescence**

In order for Hb to be able to S-nitrosylate PDI these two proteins must come in close contact so that the nitrosation ion on moiety on Cys β93-S-NO of Hb can be transferred to the free thiols of PDI. Here we tested the potential interaction of Hb and PDI by two different methods. First RBC homogenates were immunoprecipitated with anti-PDI: protein A/G-sepharose beads and the immunoprecipitation product was immunoprobed with either anti-PDI or anti-Hb primary antibodies. The immunoprecipitation product contained both PDI and Hb and an indication that the two proteins interact in RBC.

The second method used for assessing PDI-Hb interactions was to monitor the intrinsic fluorescence change of PDI as a function of Hb-dosage. PDI has five tryptophan residues in its sequence and its fluorescence was assumed to be similarly sensitive to the detection of protein:protein interactions as thioredoxin [64,65]. Here, the intrinsic fluorescence of PDI (0.75 μM) (λex 278 nm, λem 339 nm) decreased in a [Hb]-dependent manner (Fig. 5) with a half-maximal decrease occurring at ∼1 μM Hb, suggesting a dissociation constant between these two proteins of ∼1 μM or less.

**PDI can denitrosate Hb-NO**

Having obtained evidence for PDI-Hb interactions the next question was, can PDI denitrosate Hb-NO under normoxic conditions? To test this Hb, under Ar, was reduced with dithionite, exposed to nitrite. The formation of Hb-NO was identified via its characteristic absorption spectrum with a maximum at 418 nm (Fig. 6A).

As previously reported, the conversion Hb-NO to SNO-Hb (Cys β93) requires the presence of oxygen [58,66]. To this end, 500 μL of air was added to septa-sealed (Fig. 6B) samples containing Hb-NO. The Hb-NO was stable in the presence of Ar. Upon introduction of air, the Hb-NO peak decreased at a rate of 2.72 nM/min.

However, the rate of the decrease of the Hb-NO peak increased ∼6.5-fold to 17.7 nM/min in the presence of PDI, suggesting that PDI can denitrosate Hb-NO. Next Hb-NO was titrated with PDI in an attempt to determine the stoichiometry of the interaction (Fig. 6C and D). The Hb-NO peak decreased with increasing amounts of PDI and saturated at a ratio of 1:1 [Hb-NO] to [PDI].

![Figure 2](image2.png)

**Fig. 2.** Nitrite promotes RBC-PDI S-nitrosylation under normoxia but not under hypoxia: (A) Freshly isolated RBCs were equilibrated with either 4% O₂ (Lane 1) or 16% O₂ (Lane 3) in septa sealed vials nitrite (600 nM) was introduced and incubated for 10 min. The RBCs were lysed and the S-nitrosylation status of RBC-PDI was determined by the biotin switch assay visualized with streptavidin-HRP. The band corresponding to PDI was identified from the electrophoretic mobility of standard human PDI subjected to SDS-PAGE under identical conditions (Lane 2). (B) Digitized blot densities (ImageJ) of the bands obtained from 3 different experiments with conditions identical to A in each lane. Error bars represent standard deviation (n=3).
PDI associates with the RBC-surface in an \( \text{O}_2 \) and nitrite-dependent manner

As outlined in the introduction, PDI is secreted from many cells comprising the vascular system. Fully active PDI has been detected in the blood, in its soluble form as well as in its microparticle-associated and cell surface-associated forms [33–42].

We wanted to determine if PDI associated with the RBC surface and whether the surface association was dependent on \( \text{O}_2 \) and nitrite levels. To do this, RBCs were treated with and without nitrite (50 \( \mu \text{M} \)) under 16% \( \text{O}_2 \) and 4% \( \text{O}_2 \) saturation. The PDI on the RBC-surface was detected using flow cytometry (Cytomix FC500, Beckman Coulter, USA) using monoclonal mouse anti-PDI antibody (primary Ab) and sheep-antimouse IgG-FITC (secondary Ab).

There was essentially no PDI detected on the surface of RBCs under 4% \( \text{O}_2 \). However, at 16% \( \text{O}_2 \), in the absence of added nitrite there was a low amount of PDI (corresponding to \( \sim 0.65\% \pm 0.2\% \) of total fluorescence). Upon exposure to nitrite, the population distribution increased to \( \sim 8.6\% \pm 3.8\% \) (Fig. 7A). These results suggest that PDI associates with the RBC surface at 16% and is released at low (4%) \( \text{O}_2 \). If this hypothesis is correct, then there should be more soluble PDI in the suspension buffer of RBCs exposed to 4% \( \text{O}_2 \) than 16% \( \text{O}_2 \). To test this, we removed the RBCs from the buffer by gentle centrifugation and probed the RBC suspension buffer for PDI by immunoprecipitation. Furthermore, the immunoprecipitation products, isolated from the suspension buffer, were probed for the degree of S-nitrosylation by directly injecting them into an NO-analyzer (acetic acid-iiodine).

Interestingly, the amount of soluble PDI found in RBC suspensions was \( \sim 11\times \) larger under 4% \( \text{O}_2 \) plus nitrite in comparison to 16% \( \text{O}_2 \) plus or minus nitrite, (Fig. 7B and C) this was the exact opposite of the RBC surface associated PDI levels determined by flow cytometry (Fig. 7A). This supports our hypothesis that PDI is strongly associated with the RBC surface under normoxia and weakly associated under conditions of hypoxia.

Next, we determined the S-nitrosoation status of soluble PDI with the chemiluminescent NO analyzer (NOA). In these experiments, immunoprecipitation product was injected into the NOA containing iodine in acetic acid. Under these conditions, PDI-SNO would be reduced to NO by \( \text{I}_3^- \) and detected by the NOA. To test this, the amount of NO in the immunoprecipitated samples exposed to 16% \( \text{O}_2 \) plus or minus nitrite or those exposed to 4% \( \text{O}_2 \) – nitrite. In contrast, RBC exposed to nitrite under 4% \( \text{O}_2 \) yielded \( \sim 274 \text{ pmol} \pm 74 \text{ pmol of NO per mg protein} \) (\( n = 4 \)). This corresponds to \( \sim 2\% \) SNO/mol of Hb, not surprising in view of the lability of SNO functionality in proteins and the \( \sim 2\text{h} \) workup required for the isolation and analysis of the soluble PDI fraction.

We believe the data presented in this in vitro study makes a compelling case for PDI to have a role in the efflux of NO equivalents from RBCs. First, RBCs reportedly become devoid of PDI as they mature from erythroid progenitor cells [64] yet this protein is detected on the RBC surface [38,39] and in total cellular proteomic profiles [45,46]. In addition, in the present study, we detected PDI in membrane fractions and total cell homogenates (Fig. 1). In addition, the fact that PDI was found to co-immunoprecipitate with Hb (Fig. 4) indicates that PDI is also in the cytosol of RBCs.

We can therefore speculate that RBCs pick up soluble PDI from blood as PDI is readily secreted into the vasculature from many cell types [33–43]. PDI is also known to readily effluxed from cells. The current observation that it is detected intracellularly in RBC suggests that PDI can freely cross plasma membranes in both directions.

Our results also show that RBC-PDI is S-nitrosylated upon exposure of RBCs to nitrite, that nitrite levels as low as 0.6 \( \mu \text{M} \) could support significant SNO–PDI formation and there was an absolute requirement for Hb in this process (Fig. 3). In order for PDI to get transnitrosylated from SNO-Hb (Cys \( \approx \text{NO} \)) or directly denitrosate Hb–Fe(II)–NO these two proteins must interact. Here, we presented multiple lines of evidence for Hb:PDI interactions: (1) these two proteins co-immunoprecipitate (Fig. 4); (2) the intrinsic PDI-fluorescence is perturbed in a saturable manner with Hb (Fig. 5); and (3) we have clear UV/vis spectral evidence that PDI can denitrosate Hb–Fe(II)–NO with a \( \sim 1:1 \) stoichiometry (Fig. 6).

In order for PDI to "carry" NO-equivalents out of RBCs it must be secreted. Here we were able to use flow cytometry to show that the PDI associates with the RBC surface in the presence of nitrite under normoxia but not under hypoxia. And that under hypoxia most of the PDI is secreted as soluble PDI. Furthermore, the secreted PDI was S-Nitrosated (Fig. 7). These potentially redox...

**Fig. 4.** Hb co-immunoprecipitates with PDI: RBC were immunoprecipitated with anti-PDI-Protein A/G-Agarose beads and immunoprecipitated proteins and various controls were immunoblotted with either anti-PDI (A) or anti-Hb (B) primary antibodies: (A) Lane 1: PDI control; Lane 2: anti-PDI:protein A/G agarose; Lane 3: protein A/G agarose; Lane 4: immunoprecipitation product; (B) Lane 1: Hb control; Lane 2: anti-PDI:protein A/G agarose; Lane 3: protein A/G agarose; Lane 4: immunoprecipitation product.

**Fig. 5.** PDI intrinsic fluorescence is quenched by Hb: The intrinsic Trp fluorescence (\( \lambda_{ex} \approx 278 \text{ nm}, \lambda_{em} \approx 339 \text{ nm} \)) of PDI (0.75 \( \mu \text{M} \)) was monitored as a function of [Hb(III)]. The fluorescence was corrected for inner-filter effects by measuring the absorbance of the solution after each addition of Hb and using the equation. 

\[
F_{\text{corrected}} = \frac{F}{1 - 10^{-\varepsilon(c0)}}.
\]

The maximal decrease in the Hb-NO peak was \( \sim 30\% \). Potential explanations for this include that the amount of \( \text{O}_2 \) introduced into the cuvette was not sufficient to displace all of the Hb-NO. Another possibility is that only some of the Hb-NO sites are accessible to PDI at this oxygen tension.

\[
\text{Hb-NO} + \text{PDI} \rightarrow \text{Hb} + \text{PDI-NO}
\]
regulated mechanisms for PDI–RBC surface associations are currently under investigation.

On the basis of these observations, we propose the following mechanism for PDI mediated NO-equivalent efflux from RBC (Scheme 1).

Under hypoxic conditions nitrite reacts with Hb to form Fe(II)–NO (1). PDI from the blood can equilibrate across the RBC plasma membrane (PM) and interacts with Hb, to form a complex (2). When the RBCs arrive at the lungs, the O₂ displaces the NO from the heme to either PDI-thiols or Hb(Cys β93) yielding SNO–PDI.
or Hb-SNO. We can also speculate that under normoxia, PDI interacts with HbSNO to yield SNO–PDI (3). Under normoxia the SNO–PDI is attached to the RBC extracellular surface (4). Upon entering the tissues the PDI–SNO is released from the RBC surface (5). PDI–SNO then interacts with the endothelia releasing its NO+ or as previously demonstrated [48,49] its NO, triggering hypoxic vasodilation (6) (Scheme 1).

In summary, the role of PDI in the export of NOx from the RBC is supported by: (1) an oxygen-dependent mechanism of PDI binding to the RBC membrane, PDI being bound to the membrane in a PDI–SNO form under normoxia while being in a soluble form readily able to cross the membrane and deliver NOx under hypoxia; (2) this mechanism is coupled with a hypoxic nitrite reductase activity: because we could evidence a direct interaction between PDI and Hb, we propose an Hb-dependent redox process. We cannot rule out the involvement of the nitrate reductase activity of eNOS as this enzyme was reported in RBC [19,29,31].

Nitrite reduction requires nitrite influx within RBC, either in the form of HNO2 or via active transport possibly through AE-1 as suggested [28]. This mechanism amounted in vitro 2% of SNO efflux per mg Hb, a quantity that would be likely to be much smaller in vivo, given the mM concentration of Hb in RBC. Importantly, our work clearly suggests a dynamic equilibrium between “reservoirs of NO” as nitrite, RSNO and NOx species, governed by oxygen levels and mediated by PDI, leading to multiple ways for efficient hypoxic vasodilation to take place when necessary. Given the interactions of PDI with many cell surfaces including platelets, platelet derived-microparticles and endothelial cells [33–43], this efflux mechanism may take place at additional interfaces besides RBC/blood.

Acknowledgments

This work was supported by a NSERC Discovery Grant to B.M. and by a Grant of the French Agence Nationale de la Recherche to A.S. V.M.K. was supported by a University of Windsor Post Graduate Tuition Scholarship.

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