Redox Biology 1 (2013) 373–380

Contents lists available at SciVerse ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

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

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ARTICLE INFO

Article history:
Received 20 June 2013
Received in revised form
8 July 2013
Accepted 9 July 2013

Keywords:
Nitrite reductase
S-nitrosohemoglobin
Hypoxic vasodilation
Protein disulfide isomerase
Red blood cells
S-nitroso-protein disulfide isomerase

ABSTRACT

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–Cys β93 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.

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 vasodilatation 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 SNO-Hb hypothesis, deoxygenated Hb in its T-state scavenges

Abbreviations: BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; NOx, nitric oxide related species; NP-40, nonyl phenoxypolyethoxylethanol; PDI, protein disulfide isomerase; PMSF, penylmethylsulfenyl-fluoride; RBC, red blood cells; SNO-Hb, S-nitrosohemoglobin; SNO, *S*-nitrosothiol; SDS-PAGE, sodium dodecyl sulfate, poly acrylamide gel electrophoresis

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http://dx.doi.org/10.1016/j.redox.2013.07.002

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 O_2 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 O_2 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/nitrite reductase hypothesis involves transport of nitrite to RBC, reaction of nitrite with deoxy-Hb, transport of NO bioactivity from RBC and finally vasodilation [5,15,25,26]. The plasma levels of nitrite are reportedly between ~120 nM and 290 nM [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 (Fe^{2+}) and yield heme-nitrosylHb (HbNO) which can either react with oxygen to form nitrate plus methemoglobin (Fe^{3+}) 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, of accepting SNO-Hb-bound NO via transnitrosation [23,24,28,32,33].

Protein disulfide isomerase (PDI) is another enzyme that could potentially play a role in the efflux of NO equivalents from RBCs for the following reasons: PDI accounts for ~1% of total cellular 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, diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), penylmethylsulfenylfluoride (PMSF), nonyl phenoxypolyethoxylethanol (NP-40), sodium dodecyl sulfate (SDS), hemoglobin, sodium dithionite, biotin-maleimide and immunoblotting reagents were obtained from Sigma-Aldrich (St. Loius Mo). All antibodies were purchased from AbCam (Cambridge MA). The bicinchoninic acid assay (BCA assay), Aminolink Plus coupling resin and spin columns were purchased from Thermo Scientific (Rockford, Ill).

RBC preparation

RBCs were prepared for experiments under different oxygen saturations using previously established protocols. Fresh blood was collected from healthy human volunteers by venipunture into BD tubes containing anticoagulant. Blood was centrifuged at $1000 \times g$ 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 $NaHCO_3$, 0.35 g/L KCl, 0.136 g/L KH_2PO4, 0.144 g/L $MgSO_4$, 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% O_2 or hypoxia 4% O_2 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 $12{,}000 \times g$ 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), $K_3Fe(CN)_6$ (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° for 10 min and analyzed by immunoblotting. For experiments with RBC homogenates, RBC samples (100 μL) were homogenized in PBS with NEM (20 mM), $K_3Fe(CN)_6$ (10 mM), DTPA (100 μM), NP-40 (1%), PMSF (0.1 mM) and protease inhibitor (1:200) [53] 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 septa sealed vials and transferred to septa sealed vials containing constant PDI (1 μM), Hb (0.6 mM) and varying amounts of nitrite (78 nM–5 μM) in PBS. The headspace of the vial contained 20 ppm O_2. After incubation for 5 min at room temperature the samples were are subjected to biotin switch assay as previously described [50,53,54]. The supernatant was treated with 100 μM DTPA, 20 mM NEM, 10 mM $K_3Fe(CN)_6$ 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