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Role of hemoglobin oxygenation in the modulation of red blood cell mechanical properties by nitric oxide

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Abstract

It has been previously demonstrated that both externally generated and internally synthesized nitric oxide (NO) can affect red blood cell (RBC) deformability. Further studies have shown that the RBC has active NO synthesizing mechanisms and that these mechanisms may play role in maintaining normal RBC mechanical properties. However, hemoglobin within the RBC is known to be a potent scavenger of NO; oxy-hemoglobin scavenges NO faster than deoxy-hemoglobin via the dioxygenation reaction to nitrate. The present study aimed at investigating the role of hemoglobin oxygenation in the modulation of RBC rheologic behavior by NO. Human blood was obtained from healthy volunteers, anticoagulated with sodium heparin (15 IU/ml), and the hematocrit was adjusted to 0.4 L/L by adding or removing autologous plasma. Several two ml aliquots of blood were equilibrated at room temperature ($22 \pm 2^\circ\text{C}$) with moisturized air or 100% nitrogen by a membrane gas exchanger. The NO donor sodium nitroprusside (SNP), at a concentration range of 10^{-7} to 10^{-4} M, was added to the equilibrated aliquots which were maintained under the same conditions for an additional 60 minutes. The effect of the non-specific NOS inhibitor L-NAME was also tested at a concentration of 10^{-3} M. RBC deformability was measured using an ektacytometer with an environment corresponding to that used for the prior incubation (i.e., oxygenated or deoxygenated). Our results indicate an improvement of RBC deformability with the NO donor SNP that was much more pronounced in the deoxygenated aliquots. SNP also had a more pronounced effect on RBC aggregation for deoxygenated RBC. Conversely, L-NAME had no effect on deoxygenated blood but resulted in impaired deformability, with no change in aggregation for oxygenated blood. These findings can be explained by a differential behavior of hemoglobin under oxygenated and deoxygenated conditions; the influence of oxygen partial pressure on NOS activity may also play a role. It is therefore critical to consider the oxygenation state of intracellular hemoglobin while studying the role of NO as a regulator of RBC mechanical properties.

Keywords

Erythrocyte deformability; erythrocyte aggregation; nitric oxide; oxygen partial pressure; hemoglobin

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Introduction

In addition to its pivotal role in the regulation of vascular smooth muscle tonus [1], many other regulatory functions are now attributed to nitric oxide (NO) [2–5]. Red blood cell (RBC) properties and function have been demonstrated to be affected by NO [6–12], and NO was proposed to be a regulatory factor of RBC mechanical properties since inhibitors of endogenous NO synthesis induce decreased RBC deformability [8;10;13]. This suggestion is supported by the existence of functional NO synthase (NOS) in mature RBC [10]. In addition to this intrinsic RBC regulatory role, NO is exported from RBC under appropriate conditions and is important for vascular control [14;15].

In general, most prior studies have concluded that, due to effective scavenging of NO by hemoglobin, RBC-NO interaction is an important determinant of local NO availability in the vasculature [16;17]. However, the reaction of NO with hemoglobin encapsulated in RBC is 1,000 times slower than with free hemoglobin [18], with this difference related to the diffusion barrier around the RBC membrane and the various factors which can modulate this barrier [19]. Oxygenated and de-oxygenated RBC have also been demonstrated to have significantly different NO scavenging activities [18] which can be explained by the higher membrane permeability of deoxygenated RBC to NO [20]. Additionally, the dioxygenation reaction of NO with oxyhemoglobin is faster than its binding to deoxyhemoglobin, which may also account for the faster scavenging of NO by oxyhemoglobin [20].

It has been suggested that S-nitrosylation of hemoglobin at the beta cystein 93 position, yielding S-nitrosohemoglobin (SNO-Hb), is a way of conserving NO that is then released from hemoglobin for delivery to vascular beds to serve as vasodilator [15;21]. This concept is supported by the observation that S-nitrosylation is favored in oxygenated RBC and SNO-Hb is destabilized in the deoxygenated state, thereby allowing NO derivatives to be released from hemoglobin [22;23]. However, this hypothesis has recently been seriously challenged in a mouse model expressing human hemoglobin with beta cystein 93 replaced by alanine, thereby precluding S-nitrosylation [24]: the substitution did not cause serious hemodynamic-vascular effects and RBC-dependent hypoxic vasodilation was conserved [24].

Recent reports have confirmed another important source of NO by demonstrating functional NO synthase (NOS) in RBC [8;10]. RBC NOS uses L-arginine as a substrate and has properties similar to endothelial NOS. Although not yet specifically demonstrated for RBC NOS, eNOS activity is known to be dependant on the presence of oxygen [25]. Thus, while hypotheses regarding its control and target mechanisms remain to be proven [26], it has been demonstrated that RBC NOS can play a role in maintaining RBC mechanical properties [8] and that NO generated in RBC can be exported from the cell [10].

The short discussion above indicates that NO availability within RBC may be strongly affected by the oxygenation state of hemoglobin. It should also be noted that the oxygenation status of hemoglobin also affects important RBC properties including interactions between hemoglobin and membrane proteins, enzyme activities and intracellular ion concentrations [27]. Further, it has recently been shown that RBC deformability differs between oxygenated and deoxygenated aliquots of blood from the same donors [28]. Based on these considerations, it has been hypothesized that the modulation of RBC rheological behavior (i.e., deformability and aggregation) by NO donors and by NOS inhibition may depend on their oxygenation state; the present study was designed to evaluate this possibility for normal human blood.

Experimental procedures

Blood Samples

Venous blood samples, anticoagulated with heparin (15 IU/ml), were obtained from healthy adult male volunteers. The hematocrit of the samples was adjusted to 0.4 L/L by adding or removing autologous plasma based upon hematocrit determined using a microcentrifuge. Each experiment detailed below was conducted on blood samples obtained from a group of 8 individuals, not necessarily from the same group of volunteers for all experiments. All experiments and measurements described below were completed within 4 hours after blood collection.

Oxygenation and Deoxygenation Procedures

Blood samples were either oxygenated or deoxygenated at room temperature (22 ± 2 °C) by equilibration with ambient air or 100% nitrogen using a microfiber array gas exchanger (Model OX, Living Systems, Inc.). Two ml aliquots of blood were prepared and pumped back and forth through the gas exchanger for 15 min at a flow rate of 4.8 ml/min using a syringe pump (Model NE1000, New Era Pump Systems Inc., Wantagh, NY, USA). Ambient air or 100% nitrogen was fed to the gas chamber of the exchanger and both air and nitrogen were moisturized by bubbling through distilled water prior to entering the gas exchanger. Oxygen and carbon dioxide partial pressures (pO_2 and PCO_2) and pH in the blood samples prior and after equilibration were determined using a blood gas analyzer (Stat Profile® Critical Care Xpress, Nova Biomedical, USA).

Effect of NOS inhibition and NO donor

In some experiments, the non-specific NOS inhibitor N-omega-nitro-L-arginine methyl ester (L-NAME, N-5751, Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 10^{-3} M or the NO donor sodium nitroprusside (SNP, S-0501, Sigma) at a concentration range of 10^{-7} to 10^{-4} M were added to the oxygenated or deoxygenated aliquots of RBC suspension. All manipulations were done in the appropriate gas atmosphere (i.e., air or 100% nitrogen) and the aliquots were incubated for 60 min at room temperature (22 ± 2 °C) under the same conditions with gentle shaking. RBC deformability and aggregation were measured immediately after the incubation period as described below.

RBC deformability measurements

RBC deformability was determined at various fluid shear stresses by laser diffraction analysis using an ektacytometer (LORCA, RR Mechatronics, Hoorn, The Netherlands). The system has been described elsewhere in detail [29]. Briefly, a very dilute suspension of RBC (Hct < 0.01 L/L) in an isotonic viscous medium (PVP, 4% polyvinylpyrrolidone 360 solution, MW 360 kDa) is sheared in a Couette system composed of a glass cup and a precisely fitting bob, with a gap of 0.3 mm between the cylinders. A laser beam is directed through the sheared sample and the diffraction pattern produced by the deformed cells is analyzed by a microcomputer. Based upon the geometry of the elliptical diffraction pattern, an elongation index (EI) is calculated as: $EI = (L-W)/(L+W)$, where L and W are the length and width of the diffraction pattern. An increased EI at a given shear stress indicates greater cell deformation and hence greater RBC deformability. All measurements were carried out at 37 °C; for the measurements of deoxygenated samples the PVP was deoxygenated prior to suspending RBC and the measurement chamber of the ektacytometer was filled with 100% nitrogen.

EI values were measured for nine shear stresses between 0.3 – 30 Pascal (Pa). Subsequently, the shear stress for half-maximal deformation ($SS_{1/2}$) was calculated using this nine-point data set for each measurement by employing a Lineweaver-Burk analysis as described elsewhere

[30]. Briefly, the shear stress-EI curve was linearized by plotting the reciprocal of EI as a function of the reciprocal of shear stress: $1/(EI) = [(SS_{1/2}) / (EI_{max})] (1/SS) + 1/ (EI_{max})$ where SS is shear stress and EI_{max} is the theoretical EI at infinite shear stress. The x-intercept of this line corresponds to the negative reciprocal value of shear stress causing half-maximal deformation (i.e., $SS_{1/2}$); increased $SS_{1/2}$ values indicate decreased RBC deformability. Note that this approach for data analysis utilizes the entire range of shear stress and thus provides a comprehensive view of RBC stress-deformation behavior.

Determination of RBC aggregation

RBC aggregation was quantified using the Couette system described above (LORCA, RR Mechatronics, Hoorn, The Netherlands). A one ml blood sample is sheared at a shear rate of 400 sec^{-1} for five sec to disperse pre-existing aggregates, the shearing then abruptly stopped, and laser light reflection from the sample recorded for 120 sec. The recorded light reflection-time curve, termed a sylectogram, is analyzed by a microcomputer and two parameters of RBC aggregation are calculated as described in detail elsewhere [29;31]: 1) an Aggregation Index (AI) reflecting the extent of aggregation at stasis; 2) an Aggregation Half Time ($t_{1/2}$) being the time required to reach 50 percent of light reflectance change during the 120 s period. RBC aggregation measurements were carried out at 37°C and the measurement chamber of system was filled with 100% nitrogen for the measurements on deoxygenated samples.

Red blood cell hematology indices and morphology

MCV, MCH and MCHC values were determined using an electronic hematology analyzer (Cell-Dyn 3500R, Abbott Diagnostic Division, Illinois, USA). RBC shape was examined in dilute, unstained wet-mount preparations under light microscopy.

Statistics

Results are expressed as mean $\pm$ standard error (SE). Statistical comparisons between groups were done by "repeated measures ANOVA" followed by "Newman-Keuls post test", with p values <0.05 accepted as statistically significant.

Results

Red blood cell indices and morphology

MCV values ranged between 88 and 89 fL with a mean value of 88.6 ± 0.2 fL and there were no significant effects related to oxygenation, deoxygenation or incubation with the NO donor or the NOS inhibitor; MCH and MCHC values were also not altered. RBC morphology was observed to be the normal biconcave shape in all blood aliquots.

pO₂, pCO₂ and pH values

As sampled from the donor (i.e., Control samples), blood pO₂ was in the range of 41.5 – 58.9 mmHg, pCO₂ ranged between 34.6 – 75.8 mmHg, and pH was between 7.22 – 7.49. Mean values for pO₂, pCO₂ and pH for Control, Oxygenated and Deoxygenated aliquots are presented in Table 1. The greatest difference was observed in pO₂, being 3-fold higher than Control for blood samples equilibrated with air and about 30% lower for nitrogen-equilibrated samples; the pO₂ of the Oxygenated samples was almost 5-fold greater than Deoxygenated. Compared to Control, pCO₂ was found to be decreased and pH was increased following both oxygenation and deoxygenation; however, neither pCO₂ nor pH values differed significantly between the oxygenated and deoxygenated samples.

Effect of NO donor on red blood cell deformability

The effects of the NO donor SNP at 10^{-6} M on RBC deformability for oxygenated aliquots are shown in Table 2. EI values measured at shear stresses between 0.53 – 1.69 Pa were found to be significantly increased by an average of 7% after incubation with SNP. The effect of SNP on deoxygenated RBC was also characterized by an enhancement of EI (Table 3), with the alteration significant over a wider range of shear stresses (0.30 – 16.89 Pa) and more prominent at most shear stresses. It should be noted from Table 2 and Table 3 that EI measured in the aliquots which were not exposed to SNP was higher in oxygenated blood aliquots at shear stresses between 0.53 to 5.34 Pa ($p < 0.01$): the magnitude of the increase was shear-rate dependent (i.e., 18% at 0.53 Pa, 3% at 5.34 Pa). In addition, comparisons between the oxygenated and deoxygenated aliquots exposed to 10^{-6} M SNP also revealed highly significant differences in EI, being higher in deoxygenated samples at shear stresses between 0.53 – 3 Pa ($p < 0.001$).

The effects of SNP on RBC deformability at concentrations between 10^{-4} to 10^{-7} M are shown in Figure 1 in terms of $SS_{1/2}$ in order to simplify comparisons of EI-shear stress data (see Experimental procedures). Note that $SS_{1/2}$ and RBC deformability are inversely related so that a decrease of $SS_{1/2}$ indicates less rigid, more deformable cells. The values in Figure 1 are normalized by the control values of $SS_{1/2}$ measured in the absence of SNP in order to account for the $SS_{1/2}$ difference between the Oxygenated and Deoxygenated aliquots (2.78 ± 0.06 and 3.40 ± 0.13 Pa, respectively) due to differences in EI (Table 2 and Table 3). Comparison of Figures 1a and 1b indicates that SNP was more effective in decreasing $SS_{1/2}$ if the RBC were deoxygenated during the incubation period. Although the SNP effects at all concentrations were greater in the deoxygenated aliquots compared to oxygenated counterparts, the most effective dose for both conditions was 10^{-6} M.

Effect of NOS inhibition on red blood cell deformability

Table 4 and Table 5 indicate the effects of the non-specific NOS inhibitor L-NAME at 10^{-3} M on EI - shear stresses relations for oxygenated and deoxygenated blood aliquots. For oxygenated samples, L-NAME significantly decreased EI measured at shear stresses higher than 0.95 Pa (Table 4), resulting in an 11.5% increment in $SS_{1/2}$ compared to oxygenated RBC not incubated with L-NAME (Figure 2). Conversely, L-NAME had *no* significant effect on the deformability of deoxygenated RBC (Table 5 and Figure 2).

Effect of NO donor on red blood cell aggregation

Aggregation Indexes (AI) for Oxygenated and Deoxygenated aliquots without incubation with SNP differed by about 3% (63.48 ± 3.61 and 61.68 ± 4.17 , respectively, $p < 0.05$). The aggregation half time ($t_{1/2}$) was slightly but not significantly affected by oxygenation (2.32 ± 0.47 sec oxygenated, 2.62 ± 0.61 sec deoxygenated). There were significant individual variations of aggregation parameters, especially the aggregation index, as indicated by the 16% coefficient of variation in oxygenated samples and 19% in deoxygenated samples; data related to the effects of SNP on aggregation parameters are thus presented as percent of Control.

The NO donor SNP significantly decreased the aggregation index for both Oxygenated and Deoxygenated blood aliquots (Figure 3). However, the effect with deoxygenated blood was more prominent: the difference from Control for the oxygenated RBC was only significant at 10^{-7} M (Figure 3a) whereas significant decreases were found for deoxygenated cells at all four SNP levels (Figure 3b). SNP increased the aggregation half time ($t_{1/2}$) with a pattern similar to that seen for AI (Figure 4): for oxygenated RBC the change was only significant at 10^{-7} M (Figure 4a) whereas significant increases were found for deoxygenated cells at all four SNP levels (Figure 4b).

Effect of NOS inhibition on red blood cell aggregation

The non-specific NOS inhibitor L-NAME did not significantly affect the RBC aggregation parameters AI and $t_{1/2}$ for either oxygenated or deoxygenated RBC suspensions incubated with 10^{-3} M L-NAME (Figure 5).

Discussion

The results of this study confirm previously reported findings indicating improvement of RBC deformability by NO donors [7;8;10–12], and provide new results demonstrating that this effect is modulated by the oxygenation status of RBC: the effect of the NO donor SNP was significantly more prominent for deoxygenated RBC. This effect of deoxygenation is indicated by the wider range of SS, including higher stress values, where differences were observed (Table 2 and Table 3), the larger effects for deoxygenated conditions, and the more pronounced decrement in $SS_{1/2}$ at SNP concentrations between 10^{-7} to 10^{-4} (Figure 1). The dose dependence of the SNP is thus consistent with earlier findings [8] indicating that there is a biphasic effect, with 10^{-6} M being the most effective dose of SNP for both oxygenated and deoxygenated RBC.

The mechanisms by which NO affects RBC deformability are not yet clearly understood. NO may interact with cytoskeletal proteins directly or affect RBC mechanics indirectly by intermediates (e.g., peroxynitrate) oxidizing cellular proteins [32;33]. RBC have intracellular signaling elements that may mediate the effects of NO, including functional guanylate cyclase and cyclic GMP-dependent protein kinase [27]; the effect on NO on RBC deformability has been demonstrated to be partly mediated by soluble guanylate cyclase [8]. NO may also affect intracellular ion homeostasis, including calcium [34]: 1) intracellular RBC calcium concentrations of rats treated with the non-specific NOS inhibitor L-NAME are significantly increased [13]; 2) Ismail, et al. report that NO prevents endotoxin-induced increases of RBC intracellular calcium concentration [35]; 3) NO prevents the effect of increased calcium concentration on annexin binding in RBC treated with a calcium ionophore and has been suggested to be involved in the regulation of RBC survival [36]. There is also evidence indicating that the RBC membrane potassium conductance may be modulated by NO [9;37], and that this modulation may play role in the maintenance of normal RBC deformability [6; 8]. Interestingly, it has been demonstrated that density fractionated RBC are affected differently by the NO donor SNP: the deformability of older RBC (i.e., the denser fraction) was not affected by 10^{-6} M SNP whereas the deformability of younger RBC (i.e., the less dense fraction) were significantly improved by the same treatment [38]. These findings suggest that some yet unknown targets of NO might be at a lower level or lower activity in senescent RBC.

The modulation of NO effects on RBC deformability by oxygenation status may have two separate mechanisms: 1) Oxygenation status may interfere with NO availability within RBC. 2) Oxygenation status may interfere with the target mechanisms of NO. It has been reported that deoxygenated RBC have significantly higher NO permeability than oxygenated RBC [20], thus accounting for the relatively faster scavenging of external NO by deoxygenated RBC [18]. Furthermore, oxyhemoglobin has a more powerful scavenging effect for NO compared to deoxyhemoglobin [18;39]. Therefore, with an external source of NO (e.g., SNP), intracellular NO concentrations would be expected to be higher in deoxygenated RBC, thereby helping to explain the findings of the present study. That is, at equal concentrations, SNP had a greater effect on RBC deformability for deoxygenated RBC. However, this mechanism cannot easily explain why SNP concentrations higher than 10^{-6} M improved deformability in deoxygenated RBC, while for oxygenated RBC these concentrations were found to be on the rising phase of the biphasic effect (Figure 1a). If a given concentration of SNP results in higher concentrations of NO in deoxygenated RBC, this “rising phase” should have been shifted to lower SNP concentrations and levels greater than 10^{-6} M should correspond to ineffective

doses for oxygenated RBC (e.g., 10^{-4} M, Figure 1a). Obviously, this observation suggests the need to consider other factors (e.g., modifications of target mechanisms), in addition to increased NO concentrations, in order to explain the increased impact of NO in deoxygenated RBC.

As discussed above, the target mechanisms of NO related to the modulation of RBC deformability are not well defined. However, oxygenation and deoxygenation cause important alterations of RBC as reviewed by Barvitenko, et al. [27]. These effects include: 1) Alterations in hemoglobin binding of membrane proteins (e.g., band III, spectrin); 2) Increased association of band III with membrane skeletal proteins; 3) Modulation of intracellular magnesium concentration; 4) Altered ion transport, including stimulation of anion exchange by oxygenation and increased potassium-chloride co-transport by deoxygenation; 5) Acceleration of glycolysis by deoxygenation. Further, RBC deformability has been shown to be affected by the degree of hemoglobin oxygenation in both this study (Table 2 and Table 3) and a prior report [28]. Unfortunately, it is not yet possible to formulate reliable conclusions based on the observed relations between oxygenation status and NO effects. It is also not yet possible to fully reconcile our deformability-oxygenation findings for control RBC (Table 2–Table 5) with prior reports indicating no measurable effects of oxygen tension [40]. One tentative explanation may reside in the sensitivity and reproducibility of the ektacytometer used in this study and its ability to measure RBC deformation at low levels of SS [41]: differences between control and experimental RBC are more evident at low forces.

The experiments with the non-specific NOS inhibitor L-NAME provide insight into another aspect of NO in RBC function. L-NAME is a competitive inhibitor of NOS [42], and previous experiments using L-NAME and other NOS inhibitors suggest that NO synthesized enzymatically in RBC plays a role in maintaining normal deformability [8;10]. Experiments with oxygenated RBC in the present study provided a similar result: significantly impaired deformability under the influence of 10^{-3} M L-NAME for oxygenated RBC with the effect not seen for deoxygenated cells (Table 4 and Table 5). This difference between oxygenated and deoxygenated RBC most likely relates to various mechanisms of active NO generation. It has been reported that oxygen is an important determinant of NOS activity in endothelial cells, with activity suppressed at lower levels of pO_2 [25;43]. Furthermore, NOS activity is not the only source of NO generation in RBC. In addition to enzymatic generation of NO, deoxyhemoglobin acts as a reducing agent for nitrite to generate NO [44;45]. NO generated by nitrite reduction can be released from hemoglobin and may be an important source of the RBC NO pool, especially in the deoxygenated state [45]. The results of this study thus support the suggestion of Nagababu, et al. [45] in that non-enzymatically generated NO accounts for the majority of the intracellular pool in deoxygenated RBC, thereby explaining the lack of effect of NOS inhibition on RBC deformability under deoxygenated conditions. Conversely, NOS makes a significant contribution to the NO pool in oxygenated RBC in which hemoglobin acts as an effective sink for NO [46].

RBC aggregation was also affected by the NO donor SNP (Figure 3 and Figure 4). The effect was more prominent for deoxygenated RBC, with significantly decreased aggregation indexes at all four concentrations of SNP, while only 10^{-7} M was effective for oxygenated RBC. The aggregation half time was increased by SNP in a manner supporting the aggregation index results; both aggregation index and aggregation half time findings of the present study are consistent with those reported by Starzyk, et al. [12]. Note, however, that the pattern for the effect of SNP on aggregation was different from that on RBC deformability (Figure 1, Figure 3 and Figure 4). Alternatively in the present study, non-specific NOS inhibition did not induce any significant alteration in RBC aggregation. RBC aggregation in a defined medium (e.g., plasma or polymer solution), termed RBC “aggregability”, is a cellular property primarily determined by membrane surface properties [47]. It seems possible that these surface properties

may not be acutely altered by the changes of the intracellular NO pool which were suggested to affect deformability. NO as a highly reactive radical has been reported to induce modifications in a wide variety proteins (e.g., S-nitrosation, glutathiolation, arginylation) and also in lipid structures (e.g., lipid nitration) [32]. Understanding the complex nature of such NO-induced modifications is far from being comprehensive, and therefore does not yet provide a mechanism for the observed alterations of RBC aggregation.

Both deformability and aggregation are important rheological properties of RBC, significantly contributing to the flow properties of blood. RBC deformability affects blood viscosity and flow resistance in larger blood vessels as well as contributing significantly to flow resistance in microcirculation [48;49]. RBC aggregation is also accepted as the major determinant of blood fluidity [50], especially when flowing in low-shear zones of the circulation (i.e., venous vessels), although the hemodynamic effects of RBC aggregation is still controversial [51].

The improvement of RBC deformability by NO is a phenomenon which may have significant hemodynamic consequences. It is notable that this effect is greater under low oxygen tension, implying that NO plays a more important role in hypoxic conditions. While there was a significant difference in RBC deformability between oxygenated and deoxygenated states (i.e., deoxygenated less deformable, Table 2 and Table 3), increased NO exposure reversed this difference making deoxygenated RBC more deformable. This effect should help to improve blood flow in the microcirculation under hypoxic conditions, thereby providing another mechanism by which NO reduces vascular resistance and improves blood flow.

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References

1. Ignarro LJ, Cirino G, Casini A, Napoli C. Nitric oxide as a signaling molecule in the vascular system: An overview. *J. Cardiovasc. Pharmacol* 1999;34:879–886. [PubMed: 10598133]
2. Prast H, Philippu A. Nitric oxide as modulator of neuronal function. *Prog. Neurobiol* 2001;64:51–68. [PubMed: 11250062]
3. Sethi S, Dikshit M. Modulation of polymorphonuclear leukocytes function by nitric oxide. *Thromb. Res* 2000;100:223–247. [PubMed: 11108909]
4. Brune B, von Knethen A, Sandau KB. Nitric oxide (NO): an effector of apoptosis. *Cell Death Diff* 1999;6:969–975.
5. Erusalimsky JD, Moncada S. Nitric oxide and mitochondrial signaling from physiology to pathophysiology. *Arteriosclerosis Thromb. Vasc. Biol* 2007;27:2524–2531.
6. Baskurt OK, Uyuklu M, Meiselman HJ. Protection of erythrocytes from sub-hemolytic mechanical damage by nitric oxide mediated inhibition of potassium leakage. *Biorheology* 2004;41:79–89. [PubMed: 15090678]
7. Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am. J. Physiol. Heart. Circ. Physiol* 2001;280:H2848–H2856. [PubMed: 11356644]
8. Bor-Kucukatay M, Wenby RB, Meiselman HJ, Baskurt OK. Effects of nitric oxide on red blood cell deformability. *Am. J. Physiol Heart Circ. Physiol* 2003;284:H1577–H1584. [PubMed: 12521942]
9. Caramelo C, Riesco J, Outeirino I, Milas G, Blum B, Monzu V, Casado L, Sanchez JR, Mosquera S, Casado S, Fare AL. Effects of nitric oxide on red blood cell: changes in erythrocyte resistance to hypotonic hemolysis and potassium efflux by experimental maneuvers that decrease nitric oxide. *Biochem. Biophys. Res. Com* 1994;199:447–454. [PubMed: 8135785]
10. Kleinbongard P, Schutz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch H-G, Godecke A, Weber A-A, Robenek M, Robenek H, Bloch W, Rosen P,

- Kelm M. Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 2006;107:2943–2951. [PubMed: 16368881]
11. Mesquita R, Pires I, Saldanha C, Martins e Silva. Effects of acetylcholine and spermineNONOate on erythrocyte hemorheologic and oxygen carrying properties. *Clin. Hemorheol. Microcirc* 2001;25:153–163. [PubMed: 11847418]
 12. Starzyk D, Korbut R, Gryglewski RJ. Effects of nitric oxide and prostacyclin on deformability and aggregability of red blood cells of rats ex vivo and in vitro. *J. Physiol. Pharmacol* 1999;40:629–637. [PubMed: 10639013]
 13. Bor-Kucukatay M, Yalcin O, Gokalp O, Kipmen-Korgun D, Yesilkaya A, Baykal A, Ispir M, Senturk UK, Kaputlu I, Baskurt OK. Red blood cell rheological alterations in hypertension induced by chronic inhibition of nitric oxide synthesis in rats. *Clin. Hemorheol. Microcirc* 2000;22:267–275. [PubMed: 11081464]
 14. Allen BW, Piantadosi CA. How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. *Am. J. Physiol. Heart. Circ. Physiol* 2006;291:H1507–H1512. [PubMed: 16751292]
 15. Pawloski JR, Hess DT, Stamler JS. Export by red blood cells of nitric oxide bioactivity. *Nature* 2001;409:622–626. [PubMed: 11214321]
 16. Imig JD, Gebremedhin D, Harder DR, Roman RJ. Modulation of Vascular Tone in Renal Microcirculation by Erythrocytes - Role of Edrf. *Am. J. Physiol. Heart. Circ. Physiol* 1993;264:H190–H195.
 17. Liao JC, Hein TW, Vaughn MW, Huang KT, Kuo L. Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc. Nat. Acad Sci. USA* 1999;96:8757–8761. [PubMed: 10411948]
 18. Azarov I, Huang KT, Basu S, Gladwin MT, Hogg N, Kim-Shapiro DB. Nitric Oxide Scavenging by Red Blood Cells as a Function of Hematocrit and Oxygenation. *J. Biol. Chem* 2005;280:39024–39032. [PubMed: 16186121]
 19. Han TH, Liao JC. Erythrocyte nitric oxide transport reduced by a submembrane cytoskeletal barrier. *Biochim. Biophys. Acta-Gen. Subj* 2005;1723:135–142.
 20. Huang KT, Huang Z, Kim-Shapiro DB. Nitric oxide red blood cell membrane permeability at high and low oxygen tension. *Nitric Oxide-Biol. Chem* 2007;16:209–216.
 21. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 1996;380:221–226. [PubMed: 8637569]
 22. Doctor A, Platt R, Sheram ML, Eischeid A, McMahon T, Maxey T, Doherty J, Axelrod M, Kline J, Gurka M, Gow A, Gaston B. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O₂ gradients. *Proc. Nat. Acad Sci. USA* 2005;102:5709–5714. [PubMed: 15824313]
 23. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 1997;276:2034–2037. [PubMed: 9197264]
 24. Isbell TS, Sun CW, Wu LC, Teng XJ, Vitturi DA, Branch BG, Kevil CG, Peng N, Wyss JM, Ambalavanan N, Schwiebert L, Ren JX, Pawlik KM, Renfrow MB, Patel RP, Townes TM. SNO-hemoglobin is not essential for red blood cell-dependent hypoxic vasodilation. *Nature Medicine* 2008;14:773–777.
 25. Whorton AR, Simonds DB, Piantadosi CA. Regulation of nitric oxide synthesis by oxygen in vascular endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol* 1997;16:L1161–L1166.
 26. Ozuyaman B, Grau M, Kelm M, Merx MW, Kleinbongard P. RBC NOS: regulatory mechanisms and therapeutic aspects. *Trends Mol. Med* 2008;14:314–322. [PubMed: 18539530]
 27. Barvitenko NN, Adragna NC, Weber RE. Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell. Physiol. Biochem* 2005;15:1–18. [PubMed: 15665511]
 28. Uyuklu M, Meiselman HJ, Baskurt OK. Effect of hemoglobin oxygenation and deoxygenation on red blood cell deformability and aggregation parameters. *Clin. Hemorheol. Microcirc.* 2008In press
 29. Hardeman MR, Goedhart PT, Dobbe JGG, Lettinga KP. Laser-assisted optical rotational cell analyzer (lorca). 1. A new instrument for measurement of various structural hemorheological parameters. *Clin. Hemorheol* 1994;14:605–618.
 30. Baskurt OK, Meiselman HJ. Analyzing shear stress-elongation index curves: comparison of two approaches to simplify data presentation. *Clin. Hemorheol. Microcirc* 2004;31:23–30. [PubMed: 15272150]

31. Hardeman MR, Dobbe JGG, Ince C. The laser-assisted optical rotational cell analyzer (LORCA) as red blood cell aggregometer. *Clin. Hemorheol. Microcirc* 2001;25:1–11. [PubMed: 11790865]
32. Godecke A, Schrader J, Reinartz M. Nitric oxide-mediated protein modification in cardiovascular physiology and pathology. *Proteomics Clin. Applic* 2008;2:811–822.
33. Piagnerelli M, Zouaoui Boudjeltia K, Vanhaeverbeek M, Vincent JL. Red blood cell rheology in sepsis. *Int. Care Med* 2003;29:1052–1061.
34. Clementi E. Role of nitric oxide and its intracellular signalling pathways in the control of Ca²⁺ homeostasis. *Biochem. Pharmacol* 1998;55:713–718. [PubMed: 9586942]
35. Ismail NH, Cohn EJ, Mollitt DL. Nitric oxide synthase inhibition negates septic-induced alterations in cytoplasmic calcium homeostasis and membrane dynamics. *American Surgeon* 1997;63:20–23. [PubMed: 8985066]
36. Nicolay JP, Liebig G, Niemoeller OM, Koka S, Ghashghaieina M, Wieder T, Haendeler J, Busse R, Lang F. Inhibition of suicidal erythrocyte death by nitric oxide. *Pflugers Archiv-Eur. J. Physiol* 2008;456:293–305. [PubMed: 18058124]
37. Adragna NC, Lauf PK. Role of nitrite, a nitric oxide derivative, in K-Cl cotransport activation of low-potassium sheep red blood cells. *Memb. Biol* 1998;166:157–167.
38. Bor-Kucukatay M, Meiselman HJ, Baskurt OK. Modulation of density-fractionated RBC deformability by nitric oxide. *Clin. Hemorheol. Microcirc* 2005;33:363–367. [PubMed: 16317245]
39. Gow AJ, Luchsinger BP, Pawloski JR, Singel DJ, Stamler JS. The oxyhemoglobin reaction of nitric oxide. *Proc. Nat. Acad. Sci. USA* 1999;96:9027–9032. [PubMed: 10430889]
40. Mohandas, N.; Ballas, SK. Red cell deformation. In: Embury, SH.; Hebbel, RP.; Mohandas, N.; Steinberg, MH., editors. *Sickle cell disease*. New York: Raven Press; 1994. p. 195-214.
41. Hardeman MR, Goedhart PT, Schut NH. Laser-assisted optical rotational cell analyzer (LORCA); II Red blood cell deformability: elongation index versus cell transit time. *Clin. Hemorheol* 1994;14:619–630.
42. Zatz R, Baylis C. Chronic nitric oxide inhibition model six years on. *Hypertension* 1998;32:958–964. [PubMed: 9856957]
43. Kantrow SP, Huang YC, Whorton AR, Grayck EN, Knight JM, Millington DS, Piantadosi CA. Hypoxia inhibits nitric oxide synthesis in isolated rabbit lung. *Am. J. Physiol. Lung Cell. Mol. Physiol* 1997;272:L1167–L1173.
44. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, Ringwood LA, Jiang A, Hogg N, Kim-Shapiro DB, Gladwin MT. Concerted Nitric Oxide Formation and Release from the Simultaneous Reactions of Nitrite with Deoxy- and Oxyhemoglobin. *J. Biol. Chem* 2007;282:12916–12927. [PubMed: 17322300]
45. Nagababu E, Ramasamy S, Abernethy DR, Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J. Biol. Chem* 2003;278:46349–46356. [PubMed: 12952953]
46. Wennmalm A, Benthin G, Petersson AS. Dependence of the Metabolism of Nitric-Oxide (No) in Healthy-Human Whole-Blood on the Oxygenation of Its Red-Cell Hemoglobin. *Br. J. Pharmacol* 1992;106:507–508. [PubMed: 1504736]
47. Rampling MW, Meiselman HJ, Neu B, Baskurt OK. Influence of cell-specific factors on red blood cell aggregation. *Biorheology* 2004;41:91–112. [PubMed: 15090679]
48. Baskurt OK, Meiselman HJ. Blood rheology and hemodynamics. *Semin. Thromb. Hemost* 2003;29:435–450. [PubMed: 14631543]
49. Chien S. Red cell deformability and its relevance to blood flow. *Ann. Rev. Physiol* 1987;49:177–192. [PubMed: 3551796]
50. Cokelet, GR.; Meiselman, HJ. Macro- and micro-rheological properties of blood. In: Baskurt, OK.; Hardeman, MR.; Rampling, MW.; Meiselman, HJ., editors. *Handbook of Hemorheology and Hemodynamics*. Amsterdam, Berlin, Oxford, Tokyo, Washington, DC: IOS Press; 2007. p. 45-71.
51. Baskurt OK, Meiselman HJ. Hemodynamic effects of red blood cell aggregation. *Ind. J. Exp. Biol* 2007;45:18–24.

A: Oxygenated **B: Deoxygenated** **Figure 1.**

Effect of sodium nitroprusside (SNP) on shear stress at half maximum deformation ($SS_{1/2}$): A) In oxygenated aliquots; B) In deoxygenated aliquots. Data are expressed as percentage of Control incubated without SNP and presented as mean $\pm$ standard error; n=8. Difference from Control *: $p < 0.05$, **: $p < 0.01$ tested by one-way ANOVA.

Figure 2.

Effect of N-omega-nitro-L-arginine methyl ester (L-NAME) on shear stress at half maximum deformation ($SS_{1/2}$) values for red blood cells in oxygenated and deoxygenated aliquots. Data are expressed as percentage of Control cells incubated without L-NAME and are mean $\pm$ standard error, n=8. Difference from Control; **: p<0.01.

A: Oxygenated **B: De-oxygenated**

Figure 3. Effect of sodium nitroprussid (SNP) on red blood cell aggregation index (AI). A) In Oxygenated aliquots. B) In Deoxygenated aliquots. Data are expressed as percentage of Control incubated without SNP and are mean $\pm$ standard error; n=8. Difference from Control; **: p<0.01; tested by one-way ANOVA.

A: Oxygenated **B: De-oxygenated**

Figure 4. Effect of sodium nitroprussid (SNP) on red blood cell aggregation half time ($t_{1/2}$). A) In Oxygenated aliquots. B) In Deoxygenated aliquots. Data are expressed as percentage of Control incubated without SNP and are mean $\pm$ standard error; n=8. Difference from Control; **: p<0.01; tested by one-way ANOVA.

A: Aggregation index (AI) **B: Aggregation half time ($t_{1/2}$)**

Figure 5. Effect of 10^{-3} M N-omega-nitro-L-arginine methyl ester (L-NAME) on red blood cell. A) Aggregation index (AI) and B) aggregation half time ($t_{1/2}$) in Oxygenated and deoxygenated aliquots. Data are expressed as percentage of Control incubated without L-NAME and are mean $\pm$ standard error; n=8.

Table 1

pO₂, pCO₂ and pH values for Control and after equilibration with air (Oxygenated) or with 100% nitrogen (Deoxygenated).

	Control	Oxygenated	De-oxygenated
pO ₂ (mmHg)	47.7 ± 2.1	150.7 ± 2.0 ^{***}	31.9 ± 1.9 ^{***,†††}
pCO ₂ (mmHg)	46.5 ± 4.5	6.9 ± 0.9 ^{***}	9.2 ± 1.5 ^{***}
pH	7.39 ± 0.03	7.80 ± 0.06 ^{***}	7.75 ± 0.04 ^{***}

Data are presented as mean ± standard error; n=8.

Difference from Control

^{***}
p<0.001

Difference from Oxygenated

^{†††}
p<0.001.

Table 2

Red blood cell elongation indexes (EI) for blood samples equilibrated with air (Oxygenated) after incubation without (Oxygenated- No SNP) or with 10^{-6} M sodium nitroprusside (Oxygenated-SNP).

Shear stress (Pa)	Oxygenated Without SNP (EI)	Oxygenated With SNP (10^{-6} M) (EI)
0.30	0.051 ± 0.001	0.055 ± 0.003
0.53	0.088 ± 0.001	$0.098 \pm 0.004^{**}$
0.95	0.168 ± 0.001	$0.180 \pm 0.003^{***}$
1.69	0.267 ± 0.001	$0.277 \pm 0.002^{**}$
3.00	0.365 ± 0.001	0.371 ± 0.003
5.34	0.445 ± 0.003	0.445 ± 0.004
9.50	0.507 ± 0.003	0.510 ± 0.005
16.89	0.554 ± 0.002	0.552 ± 0.004
30.04	0.588 ± 0.002	0.586 ± 0.004

Data are mean $\pm$ standard error; n=8.

Difference from Control tested by two-way ANOVA

**
p<0.01

p<0.001.

Table 3

Red blood cell elongation indexes (EI) measured for blood samples equilibrated with nitrogen (Deoxygenated), after incubation without (Deoxygenated-No SNP) or with 10^{-6} M sodium nitroprusside (Deoxygenated-SNP).

Shear stress (Pa)	Deoxygenated Without SNP (EI)	Deoxygenated With SNP (10^{-6} M) (EI)
0.30	0.042 ± 0.004	$0.055 \pm 0.003^{**}$
0.53	0.074 ± 0.003	$0.106 \pm 0.005^{***}$
0.95	0.150 ± 0.003	$0.192 \pm 0.005^{***}$
1.69	0.247 ± 0.004	$0.292 \pm 0.005^{***}$
3.00	0.346 ± 0.003	$0.385 \pm 0.004^{***}$
5.34	0.431 ± 0.004	$0.457 \pm 0.004^{***}$
9.50	0.502 ± 0.004	$0.521 \pm 0.004^{***}$
16.89	0.551 ± 0.005	$0.563 \pm 0.003^{*}$
30.04	0.585 ± 0.004	0.594 ± 0.003

Data are mean $\pm$ standard error; n=8.

Difference from Control-No SNP tested by two-way ANOVA

*
p<0.05

**
p<0.01

p<0.001.

Table 4

Red blood cell elongation indexes (EI) measured for blood samples equilibrated with air after incubation without (Oxygenated-No L-NAME) or with 10^{-3} M N-omega-nitro-L-arginine methyl ester (Oxygenated-L-NAME).

Shear stress (Pa)	Oxygenated Without L-NAME (EI)	Oxygenated With L-NAME (EI)
0.30	0.051 ± 0.004	0.041 ± 0.003
0.53	0.096 ± 0.004	0.086 ± 0.001
0.95	0.180 ± 0.003	0.161 ± 0.002 **
1.69	0.277 ± 0.003	0.253 ± 0.002 ***
3.00	0.371 ± 0.003	0.343 ± 0.003 ***
5.34	0.448 ± 0.004	0.420 ± 0.004 ***
9.50	0.511 ± 0.003	0.486 ± 0.004 ***
16.89	0.557 ± 0.004	0.534 ± 0.003 ***
30.04	0.592 ± 0.004	0.571 ± 0.003 ***

Data are mean ± standard error; n=8.

Difference from "Control" tested by two-way ANOVA

**
p<0.01

p<0.001.

Table 5

Red blood cell elongation indexes (EI) measured for blood samples equilibrated with nitrogen after incubation without (Deoxygenated-No L-NAME) or with 10^{-3} M N-omega-nitro-L-arginine methyl ester (Deoxygenated-L-NAME).

Shear stress (Pa)	Deoxygenated Without L-NAME (EI)	Deoxygenated With L-NAME (EI)
0.30	0.052 ± 0.003	0.046 ± 0.004
0.53	0.090 ± 0.003	0.091 ± 0.003
0.95	0.168 ± 0.003	0.170 ± 0.002
1.69	0.261 ± 0.001	0.267 ± 0.002
3.00	0.354 ± 0.003	0.356 ± 0.002
5.34	0.429 ± 0.006	0.428 ± 0.003
9.50	0.496 ± 0.008	0.490 ± 0.003
16.89	0.541 ± 0.006	0.527 ± 0.013
30.04	0.576 ± 0.006	0.575 ± 0.004

Data are mean ± standard error; n=8.