Nitric oxide generated by red blood cells following exposure to shear stress dilates isolated small mesenteric arteries under hypoxic conditions

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Abstract. Red blood cells (RBC) possess a functional nitric oxide synthase (NOS) enzyme located in the cell membrane and cytoplasm. It has previously been observed that shear stress acting on RBC activates NOS and causes enhanced NO export. The aim of the present study was to investigate the physiological importance (e.g., in local blood flow regulation) of RBC-derived NO stimulated by application of shear stress. Blood samples and arterial vessel segments were obtained from Wistar rats; RBC suspensions were adjusted to a hematocrit of 0.1 l/l using Krebs solution. In order to apply shear stress to the RBC suspensions they were continuously flowed through a small-bore glass tube for 20 minutes at a wall shear stress of 2 Pa. The RBC suspensions were then perfused through endothelium denuded small mesenteric arteries having a diameter of $\sim 300\ \mu\text{m}$ under both high oxygen ($\text{PO}_2 \sim 130\ \text{mmHg}$) and hypoxic conditions. Perfusion of vessel segments with sheared RBC suspensions caused a significant dilation response under hypoxic conditions but not at high oxygen levels. Incubation of RBC suspensions with the non-specific NOS inhibitor L-NAME ($10^{-3}\ \text{M}$) prior to shear stress application abolished this dilation response. Our results indicate that NO released from RBC due to shear stress activation of NOS results in vasodilation of vessel segments under hypoxic conditions, and strongly suggest that NO originating from RBC may have a functional role in local blood flow regulation.

Keywords: Erythrocyte NOS, mechanical stimulation, NOS activation, blood flow regulation

1. Introduction

Red blood cells (RBC) are well known as oxygen (O_2) transporters between the respiratory system and metabolizing tissues owing to their high intracellular concentration of hemoglobin as oxygen carrier. However, in recent years additional roles have been attributed to RBC, including regulation of local blood flow by releasing vasoactive agents [16, 19, 49]. The proposed mechanisms attributed to erythrocytes as a flow regulator are: 1) ATP release upon deoxygenation and mechanical deformation, with ATP in

[†]Diseased.

turn stimulating nitric oxide (NO) generation by endothelial cells [26, 48, 50]; 2) reduction of nitrite to NO by deoxyhemoglobin [14, 29]; 3) transport of NO by RBC in the form of S-nitroso-hemoglobin and its release under hypoxic conditions [33]; 4) enzymatic generation of NO by activation of NO synthase (NOS) in RBC [36]. However, the contributions and relative importance of these mechanisms in local blood flow regulation have not yet been fully elucidated [8, 10, 11, 22, 31].

In the vascular system, generation of NO has been traditionally attributed to vascular endothelium containing the eNOS enzyme that produces NO using L-arginine as a substrate [43, 45]. Hemoglobin within RBC has been considered to play an important role in determining the local bioavailability of NO: hemoglobin is a powerful scavenger of nitric oxide and thus a major sink for NO [1, 37]. However, RBC have also been considered as a source for NO, generating NO either by non-enzymatic [33, 41, 51] or enzymatic mechanisms [10, 36, 56]. Kleinbongard, et al. demonstrated that a functional NOS activity exists in RBC, that this activity is located both at the internal surface of the cell membrane and in the cytoplasm, and that RBC NOS has properties similar to endothelial NOS (eNOS) in terms of its phosphorylation sites controlling enzymatic activity and the dependence of its activity on intracellular calcium and L-arginine concentrations [36]. These findings thus suggest that the role of RBC should be reconsidered in order to incorporate the positive contributions of RBC to the local bioavailability of nitric oxide.

In a manner similar to eNOS in endothelial cells, RBC-NOS has been hypothesized to be activated by shear stress acting on RBC [2, 36, 40]. Fischer, et al. reported that NOS immuno-staining in RBC was increased in a time dependent manner during exposure of RBC to extracorporeal circulation in patients undergoing cardiac surgery, thereby indicating shear stress induced activation of NOS [27]. Ulker, et al. also confirmed this hypothesis in various experimental models by exposing RBC to different types and levels of shear stress including continuous pumping of RBC suspensions through a 1.2 mm diameter glass tube [56], filtration of RBC suspensions through five μm pores [55] and subjecting RBC attached to a glass surface in a rectangular flow channel to fluid flow [57]. The range of applied shear stress in these studies was between 0.1 to ~ 110 Pa and application time was in the range of 1 ms to 30 minutes [55–57]. Shear stress induced NOS activation was confirmed by increased immunostaining for serine-1177 phosphorylated NOS [56, 57] (i.e., the activated form of the NOS enzyme [20, 36]). The results of these studies also showed that NO concentrations in RBC suspensions as measured by electrochemical NO probes were significantly enhanced in response to shear stress, indicating that NO was exported from RBC [55, 56].

The data summarized above confirm that enzymatic NO generation and export by RBC is enhanced under the influence of shear stress. However, it has not been experimentally demonstrated whether this NO originating from RBC significantly contributes to the local NO bioactivity and influences local vasomotor balance. The present study was designed to investigate the following hypothesis: RBC-derived NO produced by activated NOS enzyme in response to shear stress can mediate dilatation of resistance arteries and hence affect the main regulators of local blood flow.

2. Materials and methods

2.1. Study design

Endothelium-denuded resistance arteries ($\sim 300 \mu\text{m}$ diameter) isolated from rat mesentery were perfused *in vitro* with RBC suspensions at a constant flow rate (20 microliters/minute) and constant

intraluminal pressure (40 mmHg) under high oxygen partial pressure (PO_2) and hypoxic conditions: mean PO_2 values were ~ 130 mmHg and ~ 33 mmHg, respectively. Each experiment consisted of three separate phases in which the properties of the RBC suspensions were different:

1. Control (C): Perfusion with normal rat RBC suspensions prepared as described below.
2. Shear stress (SS): Perfusion with RBC suspensions prepared using the same procedure as C but exposed to a nominal SS of 2 Pa for 20 minutes as described below.
3. Shear Stress + NOS inhibitor (SS + L): Perfusion with RBC suspensions exposed to SS in the presence of the NOS inhibitor N-omega-nitro-L-arginine methyl ester (L-NAME, 10^{-3} M).

The arterial segment diameters were monitored and compared during each of the three phases described above.

The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University and was in accordance with the Declaration of Helsinki and IASP guidelines.

2.2. Animals, blood and tissue samples

12 to 14 week-old female Wistar rats weighing 260–290 g were used in the study. Animals were housed in a temperature- and light-controlled room with 12/12 h light/dark cycle and had free access to food and water until the day of the experiment. Rats were anesthetized by an intraperitoneal injection of thiopental sodium (80 mg/kg, ip.) and a mid-line laparotomy was performed. Arterial blood was obtained from the abdominal aorta and anticoagulated with EDTA (1.5 mg/mL). A portion of mesentery and associated intestinal segment was excised and transferred to a Petri dish containing cold Krebs solution (119 mM NaCl, 4.7 mM KCl, 24 mM $NaHCO_3$, 1.18 mM KH_2PO_4 , 1.17 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 11.2 mM glucose). The arterial segments were isolated and cannulated immediately. All experiments were completed within six hours after blood sampling.

2.3. Preparation of red blood cell suspensions

Eight mL of blood was layered on top of 16 mL of a polysucrose (60 g/L) and sodium diatrizoate (167 g/L) solution (Histopaque 1119, Sigma Chemical Co., St. Louis, MO, USA) in a 50 mL polypropylene tube and centrifuged at $700 \times g$ for 30 minutes to remove leukocytes. The RBC pellet was washed three times and re-suspended to a hematocrit of 0.1 L/L with Krebs solution. L-Arginine (A5131, Sigma) was added to the prepared suspensions at a concentration of 1 mM and incubated for 30 minutes at $37^\circ C$ [56].

RBC suspensions were then divided into three aliquots with one used as the Control suspension (C). The second aliquot (SS) was exposed to 2 Pa SS as described below. The third aliquot (SS + L) was exposed to 2 Pa SS after pre-incubation with L-NAME (N5751, Sigma) at 10^{-3} M concentration for 30 minutes [56].

2.4. Shear stress application to red blood cell suspensions

RBC suspensions were continuously exposed to shear stress at a nominal level of 2 Pa for 20 minutes by flow through a 0.06 cm radius, 33 cm long cylindrical glass tube. The volumetric flow rate through the tube was maintained at a level calculated as follows [44].

$$Q = \delta \cdot \pi \cdot r^3 / 4 \cdot \eta \quad (1)$$

where δ is wall shear stress (2 Pa), r is the radius of the cylindrical glass tube (0.06 cm), and η is the viscosity of the RBC suspensions. The viscosity of RBC suspensions at 37°C was measured at a shear rate of 750 s⁻¹ using a cone-plate viscometer (DV II+ Pro, Brookfield Engineering Labs, Middleboro, MA). Since the viscosities of all 0.1 L/L hematocrit suspensions were found to be very similar, the flow rate was calculated using a value of 1.8 mPa.s for all experiments. A pair of synchronized syringe pumps (Model NE1000, New Era Pump Systems Inc., Wantagh, NY) operating in a reciprocal manner were used to maintain the calculated flow rate. A more detailed description of the components of the system and a schematic drawing of the instrumentation are provided elsewhere [56]. The 20-minute application of 2 Pa shear stress did not result in detectable levels of hemolysis as assessed by hemoglobin determinations of the supernatant after centrifugation of RBC suspensions at 1,500 × g for five minutes. After sampling the C, SS and SS + L suspensions for determination of eNOS activation by immunohistochemistry and for nitrite/nitrate measurements, the RBC suspensions were used immediately for perfusing the vessel segments.

2.5. Isolation, cannulation and perfusion of vessel segments

A third-order branch of the mesenteric artery was isolated from the excised mesentery under a dissecting microscope, and arterial segments having a 260 to 356 μ m external diameter were cleared of fat and connective tissue. Endothelial denuding was performed by rubbing the inner surface of the vessel with a 40 μ m diameter tungsten wire. The endothelium-denuded arterial segments were then transferred to a special vessel chamber (CH/1, Living Systems Instrumentation, Burlington, VT) containing two horizontal, opposing, 150 μ m diameter glass micro-cannulas filled with Krebs solution; the chamber, segment and micro-cannulas were maintained at 37°C. The isolated arterial segments were fitted to these cannulas at both ends and tied with 11-0 nylon surgical sutures.

The vessel chamber containing the isolated and cannulated vessel segment was placed on the stage of an inverted microscope (Eclipse TS100, Nikon, Japan) equipped with a charge-coupled device camera (XC73CE, Sony, Japan). The camera was connected to a video dimension analysis system (Model V94, Living Systems Instrumentation, Burlington, VT) to monitor the outer diameter of the arterial segment.

The intra-luminal pressure of the arterial segment was maintained at 40 mmHg using a pressure servo-controlled perfusion system composed of two roller pumps, two pressure transducers monitoring inflow and outflow pressures, and a control unit (PS/200/Q, Living Systems Burlington, VT). The segment was allowed to equilibrate without flow for 45 minutes at 40 mmHg intraluminal pressure while filled with Krebs solution.

At the end of the no flow equilibration period, the arterial segment was constricted by 10⁻⁶ M phenylephrine HCl (Phe, P-6126, Sigma) added to the vessel chamber. The Phe caused vasoconstriction to about 60–65% of the starting diameter, with the specific response dependent on the initial, spontaneous tonus of the vessel: the constriction response thus confirmed the viability of the vascular smooth muscle. The absence of endothelium functionality was confirmed by the lack of a dilation response of the Phe pre-constricted vessels to 10⁻⁶ M acetylcholine (ACh, A6625, Sigma); ACh was applied immediately after the vessel diameter reached a steady level, usually within 60 seconds. As expected, control experiments using a separate series of vessel segments with intact endothelium indicated a significant increase in vessel diameter after the addition of ACh.

The purinergic receptor blocker pyridoxal-phosphate-6-azophenyl-2,4-disulfonic acid (PPADS, P178, Sigma) at a concentration of 10⁻³ M was added to the vessel chamber and incubated for 30 min to preclude

possible effects of any ATP, released from erythrocytes in response to mechanical stress and hypoxia, that are mediated via P2X purinoceptors on vascular smooth muscle [58].

After the PPADS incubation period, the vessel was constricted again with 10^{-6} M Phe to 60–65% of the initial diameter, and after waiting for five minutes, sequentially perfused at a flow rate of 20 μ L/min with the three RBC suspensions (C, SS, SS + L; see Section 2.3) in that order. Each perfusion period with one type of RBC suspension lasted for 10 minutes and was divided into two five-minute sub-intervals for perfusion under high PO_2 and under hypoxic conditions. During the first five minute period, the vessel chamber was filled with Krebs solution that was equilibrated with room air ($PO_2 \sim 130$ mmHg). For the second five minute period, the fluid surrounding the arterial segment was replaced with Krebs solution equilibrated with 100% nitrogen in an external reservoir by bubbling for 20 minutes ($PO_2 \sim 33$ mmHg) and continued to be bubbled with 100% nitrogen during the hypoxic perfusion period. There was no delay between the two phases of the experiment and the vessel did not return to the control conditions during this transition from high to low PO_2 . The sequence of the treatment was the same for all experiments. The PO_2 levels of the RBC suspensions after perfusing the vessel segment were determined using a blood gas analyzer (Stat Profile[®] Critical Care Xpress, Nova Biomedical, USA): PO_2 values were 138 ± 15.6 mmHg and 48 ± 7.1 mmHg for the high PO_2 and hypoxic phases, respectively.

The vessel outer diameter was monitored throughout the perfusion periods and the values at the start and end of each five-minute perfusion period (D_{0m} and D_{5m} respectively) were recorded to calculate the change in diameter as the percentage of the maximal possible dilation (see below). Following the three consecutive perfusion periods with RBC suspensions (i.e., C, SS, SS + L), the external fluid in the vessel chamber was replaced with Ca^{+2} -free Krebs solution and the vessel segment filled with the same solution. The vessel was then incubated for 30 minutes, at an intra-luminal pressure of 40 mmHg with no flow, to determine its maximal passive diameter (D_p).

2.6. Nitrite/nitrate measurement

Nitrite/nitrate concentrations were measured in the supernatant of RBC suspensions. Three hundred μ L samples from each RBC suspension, obtained immediately after shear stress application for the SS and SS + L samples, were centrifuged at 2,700 RPM. The supernatants were stored at -20°C until making nitrite/nitrate concentration measurements; deproteinization of the supernatant was achieved by passage through a filter with a 10 kDa cut-off by centrifugation at 14,000 RPM for 120 min. A commercial kit was used for nitrite/nitrate determination (780001, Cayman Chemical Co., Ann Arbor, MI). The filtrate was incubated with nitrate reductase and the cofactor (NADPH) for three hours at room temperature to convert nitrate in the sample to nitrite, and the total nitrite concentration calculated using absorbance measured at 540 nm. Note that the calculated amount corresponds to the total nitrite + nitrate concentration in the samples.

2.7. Determination of Serine 1177-phosphorylated-NOS

Serine 1177 phosphorylated NOS (ser1177P-NOS) levels were estimated by immunohistochemistry [36] in samples obtained from RBC suspensions C and SS. These samples were obtained immediately after the exposure to SS and before being used in the perfusion studies. RBC suspensions were layered onto the cover-slips pre-coated with poly-l-lysine and allowed to settle and attach to the surface at room temperature. Cover slips with attached RBC were then air-dried for 30 minutes followed by fixing in 4%

paraformaldehyde in 0.1 M PBS for an additional 30 minutes. After three washing steps with PBS, the slides were incubated for 1.5 hours in PBS containing 3% bovine serum albumin (BSA) to block non-specific binding and 0.05% Tween 20 for RBC membrane permeabilization. RBC were immuno-labeled with primary polyclonal rabbit antibodies against serine-1177 phosphorylated eNOS (Cell Signaling, Boston, USA) at a 1 : 10 dilution, by incubating for three hours. The cover slips were then washed with PBS and incubated with an anti-rabbit FITC-conjugated secondary antibody (Invitrogen, Carlsbad CA, USA) for one hour. The slides were mounted in fluorescence mounting medium (Vector Laboratories, Peterborough, Cambridgeshire, UK) and examined with a fluorescence microscope (Olympus AX70; Japan); pictures were taken with a CCD camera and processed by an Olympus DP controller program version 3.2.1.276. In order to determine Ser1177P-NOS cellular distribution on each slide, the intensity of immunoreactivity was evaluated semi-quantitatively: RBC were classified as no staining, moderate staining or intense staining.

2.8. Calculations and statistics

All data are presented as mean $\pm$ standard error of mean (SEM). Changes in diameter in response to perfusion with RBC suspensions were normalized according to the corresponding passive diameter and expressed as change in diameter as percentage of maximal dilation (Δd) using following equation [21]:

$$\Delta d = (D_{5m} - D_{0m}) / (D_p - D_{0m}) \times 100$$

where D_{5m} is the measured diameter of the vessel at the end of a 5 minute perfusion period, D_{0m} is the baseline diameter of the vessel at the start of the perfusion period, and D_p is the maximal passive diameter of the vessel. It should be noted that the outer diameters of vessel segments were used in these calculations in as much as technical difficulties prevented detecting the inner diameter. Therefore, the calculated values do not reflect alterations of vessel wall thickness in response to vasomotor influences.

Statistical comparisons were made using one-way ANOVA for repeated measures followed by the Bonferroni post test. Percentage change of vessel diameter (i.e., Δd , % maximum dilation) was tested against zero change using one-way ANOVA. P values < 0.05 were considered significant.

3. Results

3.1. Characteristics of RBC suspensions used for perfusion studies

Immuno-fluorescence staining specific to the ser1177P-NOS protein in RBC was significantly enhanced in response to exposure to 2 Pa SS for 20 minutes. Figure 1a and 1b present typical examples of ser1177P-NOS expression in C and SS samples. Semi-quantitative analysis of immuno-fluorescent staining of ser1177P-NOS is presented in Fig. 1c.

Figure 2 presents nitrite/nitrate concentrations measured in the suspending medium under control and shear stress conditions. Shear stress induced a significant, $\sim 60\%$ increase of nitrite/nitrate concentration compared to Control ($p < 0.05$). Inclusion of L-NAME in RBC suspensions during shear stress exposure resulted in decreased nitrite/nitrate concentrations compared to the SS group ($p < 0.05$).

Fig. 1. Immuno-fluorescent staining of Serine 1177 phosphorylated NOS protein in control RBC suspensions (C) and suspensions exposed to shear stress (SS). C suspensions were not exposed to shear stress (Panel a), while SS suspensions were exposed to 2 Pa shear stress for 20 min (Panel b). Semi-quantitative analysis of Ser1177P-eNOS immuno-fluorescent staining is shown in Panel c as the percent of unstained, moderately stained or intensely stained RBC. Data are mean $\pm$ SE; $n = 3$ for both groups. Difference of staining category from that for C: * $p < 0.05$.

Fig. 2. Nitrite/nitrate concentrations in the suspending medium of control (C), sheared (SS) and sheared plus L-Name (SS + L) groups. Data are mean $\pm$ SE; $n = 10$ for both groups. Difference from C: * $p < 0.05$, Difference from SS: # $p < 0.05$.

3.2. Change in vessel diameter in response to perfusion with RBC suspensions

Vessel diameters measured for 10 separate arterial segments before and after perfusion with various RBC suspensions under high PO_2 and hypoxic conditions are presented in Table 1 and percent changes of vessel diameter (Δd , see *Calculations and Statistics in Materials and Methods*) are summarized in Fig. 3a and 3b. The passive diameter (D_p) of the isolated vessel segments was $343.7 \pm 11.9 \mu\text{m}$. Perfusion of arterial segments with control (C) RBC suspensions not previously exposed to shear stress resulted

Fig. 3. Changes in vessel diameter expressed as percentage of maximal dilation (Δd ; % maximum dilation) in response to perfusion with RBC suspensions under high PO_2 and hypoxic conditions. Panel a: Perfusion with RBC suspensions under high PO_2 conditions. Panel b: Perfusion with RBC suspensions under hypoxic conditions. Data are mean $\pm$ SE; $n = 10$ for all groups. Significant difference from zero percent change: ** $p < 0.01$.

in significant ($p < 0.01$) decreases of diameter at higher PO_2 levels while this response was not observed under hypoxic conditions. Perfusion of vessel segments with RBC exposed to 2 Pa shear stress resulted in significantly increased arterial diameters under hypoxic conditions ($p < 0.01$) while no significant change was observed under high PO_2 . Perfusion of RBC suspensions incubated with L-NAME prior to shear stress application did not cause any significant changes in vessel diameters under either high PO_2 or hypoxic conditions.

4. Discussion

The results of the present study demonstrate that the diameter of endothelium-denuded mesenteric arterial segments increase in response to perfusion with RBC suspensions pre-exposed to shear stress. This dilation response was only found to be statistically significant under hypoxic conditions. The response was inhibited during perfusion of RBC suspensions pre-exposed to shear stress in the presence of 1 mM L-NAME, a non-specific competitive inhibitor of NOS, thereby strongly suggesting that the response was related to the enzymatic generation of NO. The response of arterial segments perfused with control RBC suspensions not exposed to shear stress was different: with high PO_2 the diameter of the arterial segment decreased in response to perfusion. Therefore, the data obtained in the experiments described herein underline that the response of arterial segments to the flow of RBC suspensions is determined

Table 1
Vessel diameters (μm) measured before and after RBC perfusion under high PO_2 and low PO_2 conditions

	High PO_2		Low PO_2	
	($D_{0\text{m}}$)	After ($D_{5\text{m}}$)	Before ($D_{0\text{m}}$)	After ($D_{5\text{m}}$)
Control	239.3 ± 14.8	220.4 ± 12.1	212.1 ± 10.2	223.3 ± 12.5
SS	231.9 ± 13.2	237.5 ± 14.7	221.7 ± 13.2	246.4 ± 14.2
SS + L	246.9 ± 13.8	237.4 ± 15.3	226.5 ± 17.4	223.7 ± 16.3

Mean passive diameter (D_p) was $343.7 \pm 11.9 \mu\text{m}$. Data are mean $\pm$ SE; $n = 10$ for each group. $D_{0\text{m}}$: outer diameter at time zero; $D_{5\text{m}}$: outer diameter after 5 min.

by two factors: 1) Pre-exposure of RBC to shear stress; 2) The PO_2 level during perfusion of the vessel segment.

Possible mechanisms of dilation induced by the perfusion of pre-stressed RBC suspensions may include the previously demonstrated RBC NOS activity induced by shear stress [55–57]. It is notable that the existence of enzymatically active NOS in RBC was controversial for decades [34] until Kleinbongard, et al. published convincing data demonstrating the activity of the RBC eNOS protein as shown by various methods [36]. A recent report has questioned the activity level of RBC NOS based on the appearance of [^{15}N] labeled nitrite and nitrate in RBC lysates loaded with L-arginine labeled with the same isotope [5]. However, it should be noted that this recent report does not describe the previous shearing history of RBC that were studied [5]. In contrast however, our previous data [55–57] and the inhibition of vasodilation induced by pre-stressed RBC suspensions containing the competitive inhibitor of eNOS enzyme (L-NAME; Fig. 3b), strongly support the contribution of functional NOS to this response. The inhibition of the response of mesenteric artery segments by L-NAME also rules out the contribution of non-enzymatic mechanisms of NO generation [14, 19, 33] under the experimental conditions used herein, although both enzymatic and non-enzymatic NO generation in RBC have the potential to influence local vasomotor tonus [10, 11].

Whether NO generated by RBC can alter the local NO bioavailability and induce a change in vasomotor balance has been controversial. There have been several mathematical model studies published recently [8–10], yet experimental data to support their conclusions were lacking. Major concerns regarding contributions of NO originating from RBC seem to be: 1) the very short half-life of NO in the biological environment [7, 53]; 2) the possibility of an intrinsic barrier to NO diffusion related to RBC membrane structure [35]; 3) the existence of the potent NO scavenger hemoglobin in RBC [1]. These factors would be expected to make NO escape from RBC difficult and hence only minimally contribute to the local NO pool at a level sufficient to affect smooth muscle tonus [11]. Chen, et al. concluded from their model study that NO originating from RBC only significantly contributes to the local NO pool if there are specific transport mechanisms across the RBC membrane [8]. It has been argued that free diffusion of NO through membrane lipids may not be sufficient for export in meaningful amounts [9, 32, 38, 46], and there is considerable evidence suggesting that certain integral proteins of the RBC membrane may be involved [11]. The anion exchanger band 3 has been considered to be one of the mediators of NO export from RBC [2, 40, 41]. In addition to providing a rapid route for traversing the membrane, band 3 may serve to keep generated NO localized near the membrane in a manner similar to the localization of NOS [40], thereby protecting nascent NO from being scavenged by hemoglobin. The level of hemoglobin oxygenation may also play a role in the regulation of band 3 involvement for NO export from RBC [2, 40].

Ellsworth, et al. demonstrated that ATP is released from RBC in response to hypoxia and mechanical forces, suggesting an alternative mechanism for RBC-induced alterations in vasomotor tonus [23]: ATP from RBC stimulates endothelial cells to release NO which, in turn, results in relaxation of vascular smooth muscle [23–25, 50]. However, in the current study, endothelium-denuded vessel segments were used, thereby precluding the endothelium-mediated mechanism induced by ATP: the observed dilation response of vessel segments to perfusion of RBC suspensions (Fig. 3) is not mediated by ATP. Additionally, in order to block any direct effects of ATP on smooth muscle cells (i.e., contraction), vessel segments were incubated with the purinergic receptor blocker PPADS that inhibits the P2X purinoceptor-mediated contraction in vascular smooth muscle [6, 42]. PPADS has been demonstrated to be a selective inhibitor of this response, specifically in rat mesenteric arteries [58]. Hence, any vasoconstriction effect of ATP on vascular smooth muscle in endothelial-denuded vessels would have opposed the vasodilation influence of NO; blockage by PPADS allows observing the full extent of the NO response without artifacts due to ATP from RBC [23, 50].

The strong dependence of the dilation response on local PO_2 levels also strongly supports the suggestion that this response may be related to enhanced NO generation by RBC. It has been previously demonstrated that the influence of NO on vascular tonus is determined by the oxygenation level: Datta, et al. investigated the ability of RBC to dilate pre-constricted aortic rings under various O_2 tensions and found that RBC induce vasodilation only under hypoxic conditions [16]. It is well known that hemoglobin scavenges NO and that this effect is more prominent if the hemoglobin is oxygenated [1, 28]. Additionally, it has been clearly shown that hemoglobin oxygenation status plays an important role in NO output from RBC; this effect is related to the role of band 3 protein in NO export from RBC [2, 18, 40, 41]. An oxygenation dependence of NO export was also observed in previous studies that examined shear stress effects: higher NO output from RBC was found under hypoxic conditions [55, 56]. It should also be noted that RBC were suspended in a protein-free medium in the current study, therefore the results may differ for RBC suspended in plasma. Interestingly, it has been reported that fibrinogen concentration in the suspending medium may influence NO export from RBC, with the effect being dependent on the degree of band 3 phosphorylation [17].

Sensitization of smooth muscle cells to NO under hypoxic conditions may also contribute to the observed oxygenation dependence of the response. Prior studies have shown that smooth muscle relaxation in response to vasodilators such as GSNO and other NO donors is increased under hypoxic conditions [15]. The classical pathway for NO signaling involves cyclic GMP formation [4, 30, 54]. However, it has been demonstrated that NO may induce an enhanced activation of the sarcoplasmic reticulum calcium pump (SERCA), thereby providing an alternative mechanism contributing to smooth muscle relaxation under hypoxic conditions [39].

The constriction response of endothelium-denuded arterial segments to perfusion with non-stressed RBC suspensions under high PO_2 (Table 1, Fig. 3a) is consistent with widely reported data under high oxygen partial pressures. It has been previously observed in several studies that fluid flow may induce contraction of vessel segments [3, 47, 59]. These reports have shown that smooth muscle cells can sense shear stress via their surface glycocalyx components and can convert this signal into a constriction response in a Ca^{+2} independent manner [13, 52]. Since RBC suspensions were perfused through endothelium-denuded vessel segments in our study, the observed responses are most likely due to the direct influence of hypoxia and/or fluid flow on vascular smooth muscle.

The shear stress level that was applied to RBC in this study (i.e., 2 Pa) was based on previously published data [56]. In prior studies using the shear stress exposure model employed herein, it was observed that 2 Pa was the minimum level of shear stress required for inducing an increase of NO output from RBC,

with NO export becoming maximal within 20 minutes [56]. The effectiveness of this shear stress pattern was confirmed in the present study by the increased immunostaining for serine 1177 phosphorylated NOS in RBC (Fig. 1) and the enhanced nitrite and nitrate levels in suspending media (Fig. 2). These findings confirm our previous reports indicating export of generated NO from RBC [55, 56] and suggest the possible influence of this NO on the tonus of surrounding vascular smooth muscle cells [8, 9]. Therefore, the results of the current study should be interpreted to indicate the contribution of NO generated by mechanically-stressed RBC to local vasomotor control.

The physiological relevance of shear stress-induced NO generation from RBC to vasculature with intact endothelial mechanisms remains to be determined by further studies. The 2 Pa shear stress employed herein seems relevant to an estimate by Chien [12] who indicates pseudo shear rates of 5×10^2 to $2 \times 10^3 \text{ s}^{-1}$ for arterioles in the human circulation: using an apparent blood viscosity of 4 mPa.s yields calculated stresses of 2–8 Pa. However, the constant exposure time of 20 minutes in the present study most likely does not have direct physiological relevance to the human situation. Hence, the contribution of NO generated from RBC by physiologically-relevant shear stress regimes, in terms of magnitude and exposure time, needs to be determined. While exposure of RBC to higher shear forces (e.g., $\sim 120 \text{ Pa}$) for very short duration has been demonstrated to increase NO export from RBC [55], this method for exposure to shear forces was not applicable in the present study due to experimental limitations.

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