Effects of nitric oxide on red blood cell deformability

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Bor-Kucukatay, Melek, Rosalinda B. Wenby, Herbert J. Meiselman, and Oguz K. Baskurt. Effects of nitric oxide on red blood cell deformability. Am J Physiol Heart Circ Physiol 284: H1577–H1584, 2003.—In addition to its known action on vascular smooth muscle, nitric oxide (NO) has been suggested to have cardiovascular effects via regulation of red blood cell (RBC) deformability. The present study was designed to further explore this possibility. Human RBCs in autologous plasma were incubated for 1 h with NO synthase (NOS) inhibitors [Nω-nitro-L-arginine methyl ester (L-NAME) and S-methylisothiourea], NO donors [sodium nitroprusside (SNP) and diethylenetriamine (DETA)-NONOate], an NO precursor (L-arginine), soluble guanylate cyclase inhibitors (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one and methylene blue), and a potassium channel blocker [triethylammonium (TEA)]. After incubation, RBC deformability at various shear stresses was determined by ektacytometry. Both NOS inhibitors significantly reduced RBC deformability above a threshold concentration, whereas the NO donors increased deformability at optimal concentrations. NO donors, as well as the NO precursor L-arginine and the potassium blocker TEA, were able to reverse the effects of NOS inhibitors. Guanylate cyclase inhibition reduced RBC deformation, with both SNP and DETA-NONOate able to reverse this effect. These results thus indicate the importance of NO as a determinant of RBC mechanical behavior and suggest its regulatory role for normal RBC deformability.

Nitric oxide (NO) plays a major role in cardiovascular regulation (5, 27), with its action mainly attributed to the effects on vascular smooth muscle cells (5, 25, 27). However, it has also been shown that NO synthesized in endothelial cells not only diffuses to the adjacent smooth muscle cells but also to the vascular lumen (7, 24). In addition to its effects on leukocytes and platelets, NO interacts with red blood cells (RBCs) via binding to the heme portion of hemoglobin to form S-nitrosohemoglobin (16, 45) and inducing the formation of methemoglobin (23, 43, 45). Human RBCs are positive for both inducible (NOS2 or iNOS) and constitutive (NOS3 or eNOS) forms of NO synthase (NOS), and thus are capable of synthesizing their own NO (17). Petrov et al. (37, 38) have demonstrated the existence of particulate and soluble guanylate cyclase as well as phosphodiesterases in human RBCs. It has therefore been suggested that NO synthesized within RBCs may modulate RBC physiological behavior and thus that both extracellular and intracellular sources of NO can affect the cell (9, 17).

Korbut and co-workers (21, 22, 41) have suggested that NO may have a regulatory effect on RBC deformability and aggregation and have shown that this effect is concentration dependent. The same authors also demonstrated that NOS inhibitors have a protective effect on erythrocyte deformability in septic shock and in the acute phase of endotoxemia in rats (22, 42). Furthermore, Mesquita et al. (29) have shown that both acetylcholine (ACH) and the NO donor spermine NONOate improve RBC deformability and suggest that the effect of ACh may be due to induced NO synthesis mediated by M1-type ACh receptors on the RBC membrane. Chronic inhibition of NOS by Nω-nitro-L-arginine methyl ester (L-NAME) has been found to significantly reduce RBC deformability in rats (6); the mechanical properties of RBC from these animals were normalized in vitro by a low dose (10 μM) of sodium nitroprusside (SNP), whereas higher doses were ineffective (6).

The present study was designed to provide further insight into the effects of NO on the rheological behavior of human RBCs. In particular, the in vitro effects of NOS inhibitors, NO donors, and guanylate cyclase inhibitors on RBC deformation in defined shear fields were evaluated. Our results indicate the marked influence of NO on RBC deformability and thus lend support to the possibility that NO has an important regulatory effect on this cellular mechanical property.

MATERIALS AND METHODS

Blood Sampling

Blood samples, anticoagulated with heparin (15 IU/ml), were obtained from healthy adult male volunteers. RBCs were isolated from whole blood by centrifugation (1,400 g, 6 min) followed by two washing steps in PBS (pH = 7.4, 290 mosmol/kg). The washed RBCs were then resuspended in plasma at a hematocrit of 40% for the experimental studies.

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All RBC preparations, incubations, and measurements were carried out within 4–6 h after blood collection.

**Incubations**

RBC suspensions were incubated at room temperature for 1 h in the presence of various chemical agents, after which RBCs from these suspensions were used for the determination of RBC size, geometry, and cellular deformability. This 60-min incubation period was selected based on preliminary studies that indicated a gradual increase of effects up to 40 min, with no additional change thereafter. The following agents were used for the incubations:

- **NOS inhibitors and/or NO donors.** Both a nonspecific NOS inhibitor (l-NAME) and a specific iNOS inhibitor [S-methylisothiourea (SMT)] were used. The effects of these NOS inhibitors were first tested at concentrations between 10^{-6} and 10^{-5} M, and the most effective doses were then selected to be used for the rest of the l-NAME and SMT studies. SNP and diethylenetriamine (DETA)-NONOate were used as NO donors at concentrations between 10^{-4} and 10^{-3} M and were employed alone or with NOS inhibitors.

- **NO precursor.** The effect of the NO precursor l-arginine was evaluated at a concentration of 10^{-6} M.

- **Soluble guanylate cyclase inhibitors.** The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) was used with or without NO donors. ODQ was tested at various doses (10^{-6}–10^{-3} M), after which 10^{-3} M was selected to be used throughout the study. Additionally, in a separate series of experiments, methylene blue, an inhibitor of both NOS and soluble guanylate cyclase, was tested at 10^{-5} M.

- **Potassium channel inhibitor.** The effect of the nonselектив potassium channel inhibitor tetraethylammonium chloride (TEA) was first tested at concentrations between 10^{-6} and 10^{-3} M and then used at 10^{-5} M with or without l-NAME.

All chemicals were obtained from Sigma (St. Louis, MO) except for DETA-NONOate, which was obtained from Cayman Chemicals (Ann Arbor, MI). The soluble guanylate cyclase inhibitor ODQ was dissolved in DMSO and later diluted in PBS to the desired final concentration; the other chemicals were directly dissolved in PBS.

**RBC Deformability Measurements**

RBC deformability (i.e., the ability of the entire cell to adopt a new configuration when subjected to applied mechanical forces) 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 (18). Briefly, a low hematocrit suspension of RBC in an isotonic viscous medium (70-kDa dextran) was 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 was directed through the sheared sample, and the diffraction pattern produced by the deformed cells was analyzed by a microcomputer. On the basis of the geometry of the elliptical diffraction pattern, an elongation index (EI) was calculated: 

$$EI = (L - W)/(L + W),$$

where $L$ and $W$ are the length and width of the diffraction pattern, respectively. EI values were calculated for shear rates between 0.5 and 15 Pa; 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.

**Determination of Cytosolic Calcium Concentration**

Cytosolic calcium concentration was determined in a separate series of experiments for RBC incubated with l-NAME (10^{-3} M) or SNP (10^{-6} and 10^{-5} M) using a method modified from David-Dufilho et al. (11). RBCs were separated using a density gradient (Histopaque-1077) to eliminate white blood cell contaminants. The cells were washed twice in PBS and once in HEPES buffer [containing (in mM) 123 NaCl, 5 KCl, 1 MgCl2·6H2O, 1.3 CaCl2, 10 glucose, and 25 HEPES; pH = 7.4] and then resuspended in the HEPES buffer at a hematocrit of 40%. After the addition of the NO donor SNP or the nonspecific NOS inhibitor l-NAME, the suspension hematocrit was immediately reduced to 1%, and RBCs were incubated at room temperature (22 ± 1°C) for 30 min. Fura 2-AM at a concentration of 10 nM was added to this suspension and incubated at 37°C for 30 min (i.e., total incubation time of 60 min). The suspension was then centrifuged at 350 g for 5 min, and the packed RBCs were resuspended in PBS to a hemoglobin concentration of 0.05 g/dl. The fluorescence spectrum was measured using an excitation range of 335–385 nm with emission monitored at 510 nm using a spectrofluorophotometer (Shimadzu RF-5000; Tokyo, Japan); the ratio of fluorescence intensities for the fura 2-Ca2+ complex and the unchanged fura 2 reflects cytosolic calcium concentration.

**Statistics**

Results are expressed as means ± SE. Statistical comparisons between groups were done by repeated-measures ANOVA, followed by Newman-Keuls posttest, with $P$ values <0.05 accepted as statistically significant.

**RESULTS**

**RBC Deformability at Various Shear Stresses**

RBC deformability (i.e., the EI) for control RBCs was measured at various shear stresses and, as expected
(18), was found to increase with increasing applied stress levels (Fig. 1). Figure 1 also presents EI values at various shear stresses for RBCs incubated with L-NAME ($10^{-3}$ M), where it can be seen that L-NAME-treated cells are significantly less deformable at stress levels of 5 Pa or less: the difference in the EI was more prominent at the lower stress levels and disappeared at shear stresses higher than 5 Pa. This pattern of altered RBC deformability (i.e., notable effects at lower stress levels) was consistent for all experimental protocols, and therefore EI values measured at 1.58 Pa are presented throughout as a measure of RBC deformability.

**Effects of NOS Inhibitors on RBC Deformability**

Both the nonspecific NOS inhibitor L-NAME and the specific iNOS inhibitor SMT affected RBC EI in a biphasic manner (Fig. 2). No significant decreases of the EI were observed at concentrations below $10^{-3}$ M for L-NAME and $10^{-4}$ M for SMT, whereas at these levels RBC deformability was significantly impaired. Increasing the L-NAME concentration above $10^{-3}$ M and the SMT concentration above $10^{-4}$ M did not result in a further decrease of EI for either agent, but rather the absence of significant differences and a trend toward control EI levels. Therefore, fixed concentrations of L-NAME ($10^{-3}$ M) and SMT ($10^{-4}$ M) were chosen and used throughout the study.

**Effects of NO Donors on RBC Deformability**

RBC deformability was significantly enhanced by SNP at $10^{-6}$ M, with EI values not different from control values at lower or higher concentrations (Fig. 3A). DETA-NONOate yielded a similar trend (i.e., maximal effect at $10^{-6}$ M), although the increase of deformability did not reach significance (Fig. 3B). Note that at higher concentrations the effects of NO donors were not present and RBC deformability tended to decrease.

**Reversal of NOS Inhibitor Effects by NO Donors**

The impairment of RBC deformability by $10^{-3}$ M L-NAME shown in Figs. 1 and 2 was also confirmed in a separate series of experiments designed to determine the combined effects of an NOS inhibitor plus an NO donor. As shown in Fig. 4, both NO donors (SNP and DETA-NONOate) reversed the reduced deformability caused by $10^{-3}$ M L-NAME in a dose-dependent manner, with the maximum effective dose greater for SNP (i.e., $10^{-5}$ M for SNP vs. $10^{-6}$ M for DETA-NONOate). Note that even in the presence of $10^{-3}$ M L-NAME, RBC deformability for cells incubated with $10^{-6}$ M DETA-NONOate was significantly enhanced compared with control (Fig. 4B).

The decreased RBC deformability due to the specific iNOS inhibitor SMT ($10^{-4}$ M) was also reversed by the
NO donors SNP and DETA-NONOate (Fig. 5). SNP at 10^{-6} and 10^{-5}M significantly reversed the decrease due to SMT; DETA-NONOate at 10^{-6}M yielded the same result.

Effects of Soluble Guanylate Cyclase Inhibitors on RBC Deformability

ODQ concentrations of 10^{-4}–10^{-6} M were employed to determine the effects of this soluble guanylate cyclase inhibitor on RBC deformability. RBC deformability was reduced to the same extent at all three concentrations (data not shown), and thus 10^{-5} M was used for subsequent studies. DMSO at a final concentration identical to that employed as the solvent for ODQ did not significantly affect RBC deformability.

The decrease of RBC deformability due to 10^{-5} M ODQ was reversed, in a dose-dependent manner, by the NO donors SNP and DETA-NONOate (Fig. 6), with the results indicating a pattern similar to the effects of SNP and DETA-NONOate shown in Fig. 6. Thus the reversal of the ODQ effects at both 10^{-5} and 10^{-4} M ODQ essentially eliminates the possibility that this reversal pattern results from an inadequate inhibition of guanylate cyclase.

In a separate set of experiments (n = 5) conducted using 10^{-5} M methylene blue, an inhibitor of both NOS and soluble guanylate cyclase, a significant impairment in the RBC EI was observed. This decrease of the EI was prevented by SNP, with a dose-dependent pattern similar to that observed with ODQ (data not shown).

Effects of the NO Precursor L-Arginine

The decreases of RBC deformability due to the NOS inhibitors L-NAME (10^{-3} M) and SMT (10^{-4} M) and the soluble guanylate cyclase inhibitor ODQ (10^{-5} M) were prevented by the NO precursor l-arginine at 10^{-5} M (Fig. 7). As shown in Fig. 7, l-arginine alone had no effect on RBC deformability.

Role of Potassium Channel Blockade

In a preliminary series of experiments, the nonspecific potassium channel blocker TEA was found to modestly enhance RBC deformability at concentrations between 10^{-4} and 10^{-7} M, presumably due to slight cell swelling and the consequent reduction of cytoplasmic viscosity (data not shown). The highest concentration of TEA that did not affect the RBC EI after 1-h incubation was found to be 10^{-3} M. Notably, however, TEA at this concentration prevented the impairment of RBC deformability induced by 10^{-3} M L-NAME (Fig. 8).
Neither SNP (10^{-6} or 10^{-5} M) nor L-NAME (10^{-3} M) affected intracellular calcium concentration (P > 0.1). At the concentrations used herein, neither RBC volume nor shape were affected by the NO donors, NOS inhibitors, or soluble guanylate cyclase inhibitors.

**Intracellular Calcium Concentration, RBC Shape, and RBC Volume**

Neither SNP (10^{-6} or 10^{-5} M) nor L-NAME (10^{-3} M) affected intracellular calcium concentration (P > 0.1). At the concentrations used herein, neither RBC volume nor shape were affected by the NO donors, NOS inhibitors, or soluble guanylate cyclase inhibitors.

**DISCUSSION**

The ability of the entire RBC to deform (i.e., RBC deformability) is of crucial importance for the maintenance of normal circulation: it allows the passage of erythrocytes through narrow capillaries in the microcirculation and reduces blood viscosity at high shear rates in large blood vessels (10, 40). With the use of a rat cremaster muscle preparation, Lipowsky and co-workers (26, 35) demonstrated that decreased RBC deformability results in impaired microcirculatory perfusion. The major determinants of RBC deformability are cell geometry (i.e., membrane surface area-to-volume ratio), cell shape, cytoplasmic viscosity, and membrane mechanical properties. Although both the lipid bilayer and the RBC cytoskeleton may affect RBC membrane mechanical behavior, the physical properties of the membrane skeleton are the main determinants of the viscous and elastic characteristics of the membrane (30, 31, 40). Maintenance of normal RBC deformability depends critically on the metabolic state of the cell: 1) metabolic energy is necessary for the proper operation of ion pumps (e.g., Na^+–K^+–ATPase and Ca^{2+}–ATPase); 2) Na^+–K^+–ATPase is the primary regulator of RBC volume and hence cytoplasmic viscosity via maintaining the osmotic balance across the cell membrane (32); and 3) Ca^{2+}–ATPase maintains the low intracellular Ca^{2+} concentration essential for normal RBC deformability (33, 34).

The results of the current study confirm and extend prior reports (3, 6, 21, 22, 29, 41, 42) indicating that NO plays a role in maintaining RBC deformability. NO, at some critical concentration, therefore appears necessary for preserving this cellular mechanical property, and thus its role can be postulated as “regulatory” vis-a-vis normal RBC deformability. In support of this suggestion are our observations that inhibition of NOS, by both nonspecific (l-NAME; Figs. 1 and 2) and iNOS-specific (SMT; Fig. 2) inhibitors, resulted in significant impairment of RBC deformability, which could be restored by external NO donors (SNP and DETA-NONOate; Figs. 4 and 5).
The effects of both specific iNOS and nonspecific NOS inhibitors could also be reversed by the NOS substrate L-arginine (Fig. 7), indicating the role of internally synthesized NO in the regulation of RBC mechanical properties. RBCs are exposed to NO synthesized by other cells in their natural environment, such as endothelial cells and granulocytes (5, 27), but also contain systems to synthesize NO internally (9, 17). Jubelin et al. (17) demonstrated that RBCs are positive for iNOS (NOS2) and eNOS (NOS2), whereas Chen and Mehta (9) reported that erythrocytes exhibit eNOS but not iNOS activity. Kang et al. (19) indicated that human RBCs collected from normal adults possess both inducible and endothelial isoforms of NOS but that these proteins are without catalytic activity. In contrast with Kang et al.’s report (19), the results of this study indicated that iNOS may play a role in maintaining normal RBC deformability.

In the present study, impaired RBC deformability due to L-NAME was restored by two NO donors (i.e., SNP and DETA-NONOate) having different chemical structures, thus minimizing the possibility of an effect unrelated to NO release. Additional support for a specific NO effect emerges from the results shown in Figs. 4–6, where it can be seen that the optimal concentration of SNP was higher than that for DETA-NONOate. This difference of optimal concentration most likely relates to the release of two NO molecules for each DETA-NONOate (20) versus only one for SNP, thereby yielding twice the NO level at a given molar concentration. It is interesting to note that NO has biphasic effects on RBC mechanical properties, with this effect especially obvious in the experiments where endogenous NO synthesis was inhibited (Figs. 4 and 5). RBC deformability was found to be maximal at a given concentration that depended on the NO-releasing capacity of the specific NO donor; above this concentration red cell deformability was impaired. The biphasic nature of this effect again suggests that the presence of NO at a critical concentration is crucial for the maintenance of normal RBC deformability and that at higher concentrations NO adversely affects cellular mechanical behavior (6, 21). The reason for the biphasic effect is not yet certain but may relate to the known pathophysiological effects induced by higher concentrations of NO or its metabolites, such as increased oxidant stress (14, 28, 44).

Although both our findings and literature reports (e.g., 3, 6, 21, 22, 29, 41, 42) demonstrate the role of NO in regulating RBC mechanical properties, the mechanism(s) responsible for the effects of NO on RBC deformability has yet to be fully defined. Classically, the effects of NO are known to be mediated by cGMP produced by soluble guanylate cyclase, and Petrov et al. (37, 38) demonstrated the existence of soluble guanylate cyclase in RBCs. In support of a role for guanylate cyclase in the regulatory pathway of RBC deformability by NO, it is notable that soluble guanylate cyclase inhibitors (i.e., ODQ and methylene blue) impaired RBC deformability (Fig. 6). However, this effect could be reserved by the NO donors SNP or DETA-NONOate in a dose-dependent manner (Fig. 6). If cGMP was the only mediator of the NO effect, the decrease of RBC deformability by guanylate cyclase inhibitors would not have been reversed by these NO donors (13). Furthermore, the dose-dependent reversal of RBC deformability with these NO donors in the presence of 10^{-5} M ODQ was also evident at a higher concentration (10^{-4} M), thereby essentially excluding the possibility of only partial inhibition of guanylate cyclase.

These above-mentioned findings suggest that the regulatory effect of NO on erythrocyte deformability is only partially mediated by soluble guanylate cyclase and that other mechanisms need to be considered. For example, there are several reports indicating the cGMP-independent effects of NO in various tissues. Ahmmed et al. (2) demonstrated that NO modulates Na^{+}K^{+} current in guinea pig and mouse ventricular myocytes via both cGMP- and cAMP-dependent pathways (2), and Pinilla et al. (39) suggested that NO stimulates growth hormone secretion through a specific calcium-cGMP-independent mechanism. It is known that NO has two different effects on megakaryocytes: one of them, the induction of platelet formation, is cGMP independent, whereas the second one, the stimulation of apoptosis, is cGMP dependent (4). Other signal transduction pathways may also be involved, with Oonishi et al. (34) suggesting an important role for cAMP in the maintenance of normal RBC deformability.

Ion transport across the RBC membrane is influenced by NO (36–38), and it has been demonstrated that both the Na^{+}-K^{+}-ATPase and Ca^{2+}-ATPase activities of RBCs are stimulated by NO donors (12, 36). However, in the present study, intracellular calcium concentration was not altered by either the NOS inhibitor L-NAME or the NO donor SNP. NO is also known to affect potassium transport across the membrane, either directly or indirectly (e.g., mediated by nitrite or peroxynitrite) (1, 8, 15). Although potassium currents were not measured in this study, it was observed that blockade of potassium transport through the RBC membrane prevented the adverse effect of L-NAME on RBC deformability (Fig. 8). This finding suggests that the inhibition of NO synthesis by the nonspecific NOS inhibitor L-NAME may lead to deterioration of RBC mechanical properties by increasing the potassium permeability of the RBC membrane. L-Arginine competitive analogs have been shown to increase RBC potassium efflux and to decrease intracellular NO generation (8), thus supporting this suggestion. However, increased potassium release from RBCs also occurs under the influence of NO metabolites (e.g., nitrite and peroxynitrite) (1, 15). Altered potassium transport might be expected to influence RBC shape and volume due to osmotic gradients; however, in our study, neither RBC shape nor volume were altered. Our morphological results are thus consistent with those of Walter et al. (43), who indicated that SNP induced reversible echinocytic shape transformation only at very high levels (10^{-2} M), whereas at lower concentrations SNP failed to alter RBC shape.
In summary, the results of this study clearly indicate that NO can markedly affect RBC deformability and therefore suggest the regulatory role of NO in maintaining normal RBC deformability. Under in vivo conditions, RBC are exposed to two sources of NO: one synthesized by endothelial cells, which diffuses into the bloodstream, and one synthesized within the RBC itself. Thus, in addition to the well-known effect of NO on vascular hindrance, the effect of NO on RBC deformability appears to have a direct role in the control of blood flow. Unfortunately, the target structure or function for the effects of NO on RBC mechanical behavior have not yet been established; although the most probable site for this effect is altered phosphorylation of the RBC cytoskeleton (34), further studies are required to clarify this aspect.

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