Nitric oxide generation by endothelial cells exposed to shear stress in glass tubes perfused with red blood cell suspensions: role of aggregation

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Yalcin O, Ulker P, Yavuzer U, Meiselman HJ, Baskurt OK. Nitric oxide generation by endothelial cells exposed to shear stress in glass tubes perfused with red blood cell suspensions: role of aggregation. Am J Physiol Heart Circ Physiol 294: H2098–H2105, 2008. First published March 7, 2008; doi:10.1152/ajpheart.00015.2008.—Endothelial function is modulated by wall shear stress acting on the vessel wall, which is determined by fluid velocity and the local viscosity near the vessel wall. Red blood cell (RBC) aggregation may affect the local viscosity by favoring axial migration. The aim of this study was to investigate the role of RBC aggregation, with or without altered plasma viscosity, in the mechanically induced nitric oxide (NO)-related mechanisms of endothelial cells. Human umbilical vein endothelial cells (HUVEC) were cultured on the inner surface of cylindrical glass capillaries that were perfused with RBC suspensions having normal and increased aggregation at a nominal shear stress of 15 dyn/cm2. RBC aggregation was enhanced by two different approaches: 1) poloxamer-coated RBC suspended in normal, autologous plasma, resulting in enhanced aggregation but unchanged plasma viscosity and 2) normal RBC suspended in autologous plasma containing 0.5% dextran (mol mass 500 kDa), with a similar level of RBC aggregation but higher plasma viscosity. Compared with normal cells in unmodified plasma, perfusion with suspensions of poloxamer-coated RBC in normal plasma resulted in decreased levels of NO metabolites and serine 1177 phosphorylation of endothelial nitric oxide synthase (eNOS). Perfusion with normal RBC in plasma containing dextran resulted in a NO level that remained elevated, whereas only a modest decrease of phosphorylated eNOS level was observed. The results of this study suggest that increases of RBC aggregation tendency affect endothelial cell functions by altering local blood composition, especially if the alterations of RBC aggregation are due to modified cellular properties and not to plasma composition changes.

Wall shear stress, as the hydrodynamic factor affecting the endothelium, is a function of fluid velocity and viscosity (28, 40). However, it should be noted that both velocity and viscosity are not homogeneous over the cross section of a blood vessel, with velocity being maximum at the central flow zone (24). Furthermore, there is a tendency for red blood cells (RBC) to accumulate in the central region of the vessels during flow because of the process known as axial migration (24). Axial migration of RBC results in a phase separation, leading to the formation of a cell-poor layer adjacent to the vessel wall. Since blood viscosity increases with hematocrit (Hct), the viscosity of blood flowing in this marginal zone can be expected to be lower than that of the bulk phase. RBC aggregation tends to promote axial accumulation, resulting in a less viscous, plasma-rich region near vessel walls (1, 13). It has been demonstrated that flow resistance in tubes is decreased with the use of RBC suspensions having enhanced RBC aggregation (13).

Diminished wall shear stress, resulting from greater axial migration promoted by enhanced RBC aggregation, could be expected to influence mechanically induced effects in blood vessels. Decreased systemic blood flow (e.g., left ventricular failure) in the vasculature is well known to downregulate endothelial NO-related mechanisms (45), whereas intermittently increased blood flow (e.g., exercise training) upregulates these mechanisms (36, 45). The effect of enhanced RBC aggregation on endothelial NO-synthesizing mechanisms has also been demonstrated in an animal model (5). RBC aggregation was chronically enhanced in rats after exchange transusions with suspensions of coated RBC with greater aggregation tendency. Both flow-mediated dilution in blood vessels isolated from skeletal muscle and eNOS expression in the same muscle tissue were found to be suppressed after 4 days. Additionally, arterial blood pressure increased gradually over the 4 days following the exchange transusions (5). These findings suggest that this interaction between RBC aggregation and NO-related vascular mechanisms may have significant clinical consequences, inasmuch as enhanced RBC aggregation is a common consequence of acute-phase reactions (3).

Alterations of RBC aggregation due to pathophysiological processes are, in part, related to modified plasma composition (i.e., increased acute-phase reactants including fibrinogen) and viscosity (3). Thus, the viscosity of plasma should also be taken into account when evaluating wall shear stress (40): plasma is

IT IS NOW WELL ESTABLISHED that shear forces acting on the vascular wall are one of the main factors regulating endothelial function (11). Endothelial cells regulate their nitric oxide (NO) synthesis in response to altered wall shear stress, with the synthesis playing a key role in vasomotor control (21, 39, 40, 43). The details of mechanotransduction and cellular control mechanisms, including the activation of NO synthesis mechanisms, have been investigated widely and are of current interest (16, 17, 21). The enzyme endothelial nitric oxide synthase (eNOS) is expressed at a basal level in endothelial cells, with the level of expression modulated depending on the magnitude of local shear forces (21, 39). Additionally, the activity of expressed eNOS is modulated by calcium-dependent and -independent mechanisms through phosphorylation of various domains of the eNOS protein (20, 37).

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the interface between endothelium and blood, and its significance is amplified with enhanced axial RBC migration resulting in a plasma-rich fluid in contact with the vessel wall. Increased plasma viscosity has been demonstrated to enhance endothelial NO synthesis and decrease vascular resistance (9, 44). Therefore, increased plasma viscosity and enhanced RBC aggregation may result from the same compositional changes in plasma, yet have opposing effects on endothelial function. Finally, it should be noted that RBC aggregation may be increased because of modification of cellular properties (i.e., altered aggregability of RBC) without any change of plasma properties (3).

On the basis of the above considerations, it can be hypothesized that the impact of enhanced RBC aggregation may depend on the mechanisms responsible for this alteration. More specifically, the response of endothelial cells may reflect whether plasma viscosity also changes with altered RBC aggregation. The present study thus explored NO-related responses of human umbilical vein endothelial cells (HUVEC) coated on the inner surface of 0.1-cm-internal diameter (ID) glass capillaries when perfused with RBC suspensions with normal or modified aggregation properties. RBC aggregation was modified by two separate approaches, one of which increased aggregation without affecting plasma properties. It was thus possible to examine the effects of elevated aggregation with and without elevated plasma viscosity.

MATERIALS AND METHODS

Preparation of HUVEC Culture

Primary HUVEC were isolated and grown to confluence as previously described (27). Briefly, umbilical veins were cannulated and washed thoroughly with sterile phosphate-buffered saline (PBS, pH 7.4; Gibco, Invitrogen, Carlsbad, CA). The vessel was filled with 1 mg/ml collagenase (Sigma Chemical, St. Louis, MO) in PBS, clamped on both ends, and incubated at 37°C for 15 min. Detached HUVEC were flushed from the vein with a bolus of PBS, washed, and resuspended in medium 199 (M199; Gibco) containing 20% fetal bovine serum, penicillin (10,000 U/ml) and streptomycin (10 mg/ml), hydrocortisone (1 mg/ml), epidermal growth factor (1 μg/ml), and amphotericin (2.5 μg/ml) from Sigma. The cells were cultured until confluent (∼5–6 days) in 25-cm² culture flasks (BD Falcon) pretreated with 1% gelatin solution (Sigma). HUVEC were transferred to cylindrical glass capillaries after the proliferation of primary endothelial cells was sufficient to coat 70–80% of the tissue culture flasks. The endothelial identity of the cultured cells was confirmed by von Willebrand factor (27). Each experiment described below utilized first-passage HUVEC cultures from a different donor.

HUVEC Coating of Cylindrical Glass Capillaries

HUVEC were cultured onto the inner surface of 7.5-cm long, 0.1-cm-ID cylindrical glass capillaries previously coated with (3-aminopropyl)triethoxysilane (APES; Sigma) as described previously (14). APES-coated and autoclaved capillaries were filled with a 1:1 mixture of gelatin (10 mg/ml type B; Sigma) and PBS and incubated for 30 min at 37°C immediately before use to improve the efficiency of seeding with HUVEC (42).

One milliliter of ethylene diaminetetraacetic acid (EDTA) solution (0.02%) was added for 1 min to the HUVEC culture flask in order to remove excess calcium from the environment. Subsequently, 3 ml of trypsin-EDTA solution (2.5 g/ml trypsin, 0.5 mM EDTA; Sigma) was added to the culture flask and kept at 37°C for 1 min. The enzyme activity was terminated by adding 6 ml of M199, and the cells were transferred to a sterile 5-ml tube and centrifuged at 1,500 rpm for 5 min at room temperature. The supernatant was aspirated, and the HUVEC were resuspended in 300 μl of M199 to a cell concentration of ~2.5 × 10⁶/ml.

HUVEC suspensions harvested from two separate culture flasks (300 μl each) were used to coat each glass capillary. The capillary was positioned horizontally, 100 μl of cell suspension was used to fill the tube and allowed to settle for 15 min, and then the tube was emptied with gentle suction. This protocol was repeated with a new aliquot of cell suspension for every 90° rotation of the tube along its axis to coat the entire inner wall. The capillaries were placed in sterile glass dishes having internal ports for direct connection to the capillary (School of Engineering, University of Birmingham, Birmingham, UK). The dish was filled with M199, the capillary was attached to the ports, and the external ports were attached to a perfusion system providing a M199 flow rate of 0.4 ml/min for 30 s every hour. Thus medium contained in the capillary tubes was changed regularly in order to maintain the growth of the HUVEC; the capillaries were used in the experiments after 24 h of incubation. All culturing and coating procedures were carried out at 37°C, with the 24-h incubation of capillaries in atmospheric air containing 5% CO₂.

Experimental Groups

The HUVEC-coated glass capillaries were randomly assigned to one of the following groups. Control (zero flow) group. Capillary tubes were filled with normal RBC suspensions in autologous plasma and kept at 37°C for 30 min with no flow through the capillary.

Normal group. Capillary tubes were perfused with normal RBC suspensions in autologous plasma for 30 min at a flow rate calculated to maintain a wall shear stress of 15 dyn/cm² as described below.

F98 group. Capillary tubes were perfused with suspensions of poloxamer F98-coated RBC in autologous plasma for 30 min as described for the Normal group.

Dextran group. Capillary tubes were perfused with normal RBC suspensions in autologous plasma containing 0.5% of 500-kDa dextran for 30 min as described for the Normal group.

It should be noted that the RBC suspensions used in the F98 and Dextran groups had an enhanced degree of RBC aggregation (2, 47) but differed in plasma viscosity (i.e., higher for Dextran group).

Preparation of RBC Suspensions

Blood samples were obtained from healthy adult volunteers and anticoagulated with EDTA (1.5 mg/ml; Sigma). RBC were separated from whole blood by centrifugation (2,700 rpm, 10 min) followed by three washings in PBS. The washed RBC were then resuspended in autologous plasma for the Control and Normal groups and were resuspended in autologous plasma containing 0.5% of 500-kDa dextran for 30 min as described for the Normal group.

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solution (in mM: 100 NaF, 50 HEPES, 150 NaCl, 1 MgCl₂, 1 EGTA, 2.5 g/ml trypsin, 0.5 mM EDTA) for 1 min and then washed to detach the cells. The cell lysate described above was centrifuged at 10,000 rpm for 10 min, and the protein content of the supernatant was determined with Bradford reagent (Bio-Rad Laboratories). The protein content of each sample was adjusted to 50 μg, and the sample was loaded onto 7.5% SDS-PAGE and separated by electrophoresis (80 V, 2 h). This was followed by blotting onto nitrocellulose membranes. Immunoblotting was performed with anti-eNOS, anti-phospho(Ser 1177) eNOS, and anti-phospho(Thr 495) eNOS antibodies (Sigma) at a dilution of 1:1,000. Immunoreactive bands were visualized with ECL detection reagent (Amersham Biosciences, Little Chalfont, UK). To detect the β-actin levels to serve as the loading control, the membranes were reprobed with an anti-β-actin antibody at 1:1,000 dilution (Sigma). The intensities of the bands were quantified bydensitometric analysis using a gel scanner, and the results were expressed as the ratio of densities of eNOS-specific bands to β-actin bands.

Miscellaneous Methods

The extent of RBC aggregation was estimated by measuring RBC sedimentation rates. The Hct of RBC suspensions was adjusted to 0.1 l/l, and the time course of RBC sedimentation in Westergren tubes was recorded for 60 min. The viscosity of plasma and RBC suspensions was measured at 37°C with a Wells-Brookfield cone-plate viscometer (DV II + Pro, Brookfield Engineering Labs, Middleboro, MA). Plasma viscosity was measured at 1,500 s⁻¹.

Statistics

Individual data points for each experiment are presented in Figs. 2–6, with median values also shown except for Fig. 2A. Statistical comparisons between groups were done by Kruskal-Wallis variance analysis, followed by Dunn’s posttest, with P values <0.05 accepted as statistically significant.

RESULTS

RBC Sedimentation Rate, Plasma and RBC Suspension Viscosities, and Calculated Flow

RBC sedimentation behavior for suspensions with Hct adjusted to 0.1 l/l is presented in Fig. 2A, where it can be seen that sedimentation was markedly accelerated in the F98 and Dextran suspensions. RBC sedimentation reached a maximum at ~40 min and then leveled off in these two groups, whereas it continued to increase in the Normal group until achieving the maximum value for F98 and Dextran groups at 60 min. Figure 2B shows individual values and medians for sedimentation at 30 min, indicating a significance difference of both F98 and Dextran values versus Normal (P < 0.05) but no difference between Dextran and F98 values.

Plasma viscosity was significantly increased in the Dextran group versus both Normal and F98 groups (P < 0.05), but there was no significant difference between Normal and F98 groups (Fig. 3A). RBC suspension viscosities measured at 750 s⁻¹ are presented in Fig. 3B. Suspension viscosities were found to be higher in F98 and Dextran groups compared with the
Normal group, although the difference was significant only for the Dextran group (P < 0.05).

RBC suspension viscosity measured at 750 s⁻¹ was used to calculate the volumetric flow rate required for that suspension to maintain a wall shear stress of 15 dyn/cm² (Eq. 1). These calculated values are presented in Fig. 4. As anticipated on the basis of its lower viscosity (Fig. 3B), the calculated flow was highest in the Normal group; flow values for the Dextran and F98 groups were less, with the difference from the Normal group statistically significant for the Dextran group (P < 0.05).

Nitrite/Nitrate Concentrations of HUVEC

Nitrite/nitrate contents of HUVEC harvested from capillaries after perfusion with RBC suspensions are presented in Fig. 5. Perfusion using normal RBC suspensions in autologous plasma (Normal group) resulted in significantly increased nitrite/nitrate content of HUVEC (P < 0.05) compared with the no-flow Control group. If the suspension flowing in the capillary contained RBC with enhanced aggregability but normal plasma properties (i.e., the F98 group), there was no significant increment of nitrite/nitrate content compared with the Control group. In contrast, nitrite/nitrate content was significantly increased in the Dextran group compared with the Control group, with the increase slightly less than for the Normal group. It should be noted that the Dextran group had a degree of aggregation similar to that of the F98 group but a higher plasma viscosity (Figs. 2 and 3A).

**eNOS Expression and Phosphorylation**

Compared with the no-flow Control group, the expression of total eNOS was not affected by 30 min of 15 dyn/cm² shear stress in the Normal, F98, or Dextran group (Fig. 6A). However, serine 1177 phosphorylated eNOS protein was significantly increased in the Normal group compared with the Control group, with this increment of phosphorylation greatly reduced in both the F98 and Dextran groups (Fig. 6B). Note that although the serine 1177 phosphorylated eNOS level was higher in the Dextran versus the F98 group the difference was not statistically significant. Threonine 495 phosphorylated eNOS protein levels were also slightly, but nonsignificantly, enhanced in HUVEC exposed to flow for 30 min regardless of the nature of the perfusion suspension (Fig. 6C).
DISCUSSION

Previous studies have demonstrated that alterations of endothelial cell NO-synthesizing mechanisms closely reflect the applied shear stress on these cells (15, 39, 49). This observation has also been confirmed in the present study, with significantly increased nitrite/nitrate content of endothelial cell lysates harvested after the application of shear stress of 15 dyn/cm² for 30 min (Fig. 5). In addition, increased serine 1177 phosphorylation of eNOS protein, known to be related to the activation of this enzyme, was found to accompany this increment (Fig. 6B).

The experiments reported here were conducted with an in vitro perfusion system whose design differed from those widely used to expose endothelial cells to shear stress. Prior reports have used rectangular flow chambers with various dimensions (32, 41, 42) and with various fluids, usually cell-free culture media, flowed over cultured endothelial cells to generate shear stress (7, 20, 29, 31, 49). However, in the present study, HUVEC were cultured on the inside of cylindrical glass capillaries of 0.1-cm diameter with the use of a slight modification of a method for culturing HUVEC in rectangular glass capillaries (41), after which the capillary was perfused with various RBC suspensions. This model is thought to be a better reflection of in vivo conditions, enabling investigation of the role of hemodynamic mechanisms, such as axial migration of RBC, that can be relevant to blood flow in vessels of this size (13). The HUVEC-coated tubes thus seem more suitable for the purpose of this study, since both the anticipated effect of RBC aggregation behavior on the distribution of RBC and the local properties of the fluid near the vessel wall strongly depend on the geometry of the flow system.

The specific aim of this study was to explore the role of RBC aggregation in shear stress-mediated responses of HUVEC-coated capillaries after perfusion with RBC suspensions, with experimental parameters relevant to published information. Details of shear stress effects (e.g., temporal studies of eNOS phosphorylation, activation or inactivation of the enzyme, altered eNOS protein expression) and of the molecular mechanisms of mechanotransduction have been reported (16, 19, 21). It is well known that activation of eNOS by phosphorylation processes starts in minutes and saturates within an hour, with no further increase up to 4 h (46). Therefore, a 30-min period of exposure to fluid stress was used, and the effects of applied shear stress on eNOS activation were indexed by NO.
synthesis (30) and eNOS phosphorylation (7, 48). It should be noted that total eNOS protein expression was not altered because of shear stress regardless of which RBC suspension was used during the 30 min of perfusion (Fig. 6A). This finding confirms previously published observations indicating that longer periods of shear stress are required for alterations of protein expression (39).

Phosphorylation of eNOS protein at different amino acid locations is known to control enzyme activation (8), and it has been demonstrated that eNOS is phosphorylated at serine, threonine, and tyrosine residues (15, 21, 23, 35). Phosphorylation of serine at position 1177 of eNOS is associated with increased enzyme activity (17, 22), whereas eNOS threonine 495 phosphorylation is an indicator of enzyme inactivation (10). Application of 15 dyn/cm² shear stress to endothelial cells isolated from ovine fetoplacental arteries induced phosphorylation of eNOS serine 1177 in 20 min (31). However, phosphorylation at other positions of eNOS was demonstrated to be slower (7). Thus serine 1177 and threonine 495 phosphorylation of eNOS were selected in this study as parameters to reflect the activation of the enzyme by shear stress. Compared with Control group data for HUVEC not exposed to shear, serine 1177 phosphorylated eNOS levels were found to be significantly increased by shear stress in the group perfused with normal RBC in autologous plasma (i.e., the Normal group; see Fig. 6B). No significant alterations were detected in threonine 495 phosphorylated eNOS protein levels regardless of the perfusion suspension (Fig. 6C).

Two of the RBC suspensions used to perfuse the coated tubes had increased RBC aggregation, but only one of them (i.e., Dextran suspension) had elevated plasma viscosity. In the F98 group, RBC coated with poloxamer F98 were resuspended in their unmodified, autologous plasma. These suspensions had increased aggregation due to enhanced RBC aggregability (i.e., intrinsic tendency of RBC to aggregate), but the suspending phase viscosity was identical to that for the Normal group. The RBC suspensions used in the Dextran group also had increased aggregation at a level similar to that of the F98 group (Fig. 2), but their suspending phase viscosity was significantly increased by ~20% compared with both Normal and F98 groups (Fig. 3A). An important finding of this study was the different responses of HUVEC perfused at similar nominal wall shear stresses with these two RBC suspensions. That is, compared with elevated levels for the Normal group 1) nitrite/nitrate content was markedly reduced for the F98 group with increased aggregation and normal plasma viscosity but not for the Dextran group with similar aggregation and increased plasma viscosity (Fig. 5) and 2) serine 1177 phosphorylation of eNOS for the F98 group was significantly reduced to essentially the Control group level, whereas the reduction for the Dextran group was more modest (Fig. 6B).

Decreased nitrite/nitrate content and eNOS phosphorylation indicate a blunted shear stress response of HUVEC (Figs. 5 and 6B). Since the calculated shear stress for each group was the same, the differing HUVEC response, as reflected by NO-synthesizing mechanisms, most probably is due to local fluid rheological properties diverging from those based on measurements with the cone-plate viscometer. The mechanical impact of the fluid flowing in a blood vessel is determined by wall shear stress, which in turn is a function of fluid velocity adjacent to the vessel wall and the viscosity of the fluid in that particular region (28, 40). It was shown previously that RBC distribution over the cross section of a tube or vessel is affected by the axial migration process, leaving a cell-poor region with lower Hct and lower viscosity near the vessel wall (13, 24). Axial migration has been demonstrated to be enhanced by RBC aggregation, resulting in decreased frictional resistance at the vessel wall, lower hydraulic resistance (1, 13), and altered tissue Hct (47). However, if the suspending phase viscosity is increased together with enhanced RBC aggregation, the resulting endothelial response could be blunted since the increased suspending phase viscosity and the decreased local Hct due to RBC axial migration would tend to have opposing effects. The results of this study support this suggestion (Figs. 4 and 6B).

Endothelial cells are known to respond to the level of shear stress, although the relationship between the magnitude of shear stress and the response (e.g., activity of NO-synthesizing mechanisms) is not linear (30). Kuchan and Frangos (29) applied various levels of shear stress (2–25 dyn/cm²) to HUVEC cultures and reported that during the first 30 min the maximum change in NO output was observed between 1.8 and 6 dyn/cm²; NO output then decreased with further increases of shear stress.

NO availability in the blood vessel wall is determined by the balance between its rate of synthesis and its rate of degradation. Scavenging by hemoglobin is an important mechanism for NO degradation and is affected by the axial distribution of RBC and flow velocity (26, 44); in turn, these factors are determined by RBC aggregation. With enhanced RBC aggregation, the scavenging effect of hemoglobin would be expected to be reduced because of the greater diffusion distance for NO as a result of an increased thickness of the marginal cell-poor layer. Therefore, measured concentrations of nitrite/nitrate may not appropriately reflect NO synthesis by endothelial cells. However, eNOS phosphorylation results do closely mimic nitrite/nitrate concentrations and thus more clearly reflect the effects on NO-synthesizing mechanisms. Thus enhanced RBC aggregation results in two counteracting effects: reduced NO synthesis due to lower wall shear stress and reduced scavenging due to the greater diffusion distance from wall to flowing RBC.

The method used here to modify RBC aggregability, based on covalent binding of a poloxamer to the RBC surface (2), provided the opportunity to compare different modes for alterations of RBC aggregation. Most previous studies on the effects of RBC aggregation on in vivo blood flow dynamics have utilized models based on including or infusing high-molecular-weight material such as dextrans in the suspending medium (6, 18, 33). This approach results in increased suspending phase viscosity in addition to enhanced RBC aggregation (e.g., the Dextran group). This current study is thus the first in assessing the difference in effects of increased RBC aggregation with or without increased plasma viscosity.

It should be noted that the difference in the methods used to induce alterations in RBC aggregation may have a clinical significance. From a pathophysiologial point of view, RBC aggregation is now generally accepted to be determined by two separate sets of factors (3, 34, 38): 1) suspending (plasma) factors and 2) cellular factors mainly related to the cell’s glycolalx. Acute-phase reactions tend to increase the concentration of certain macromolecules in plasma, such as fibrinogen, increasing both plasma viscosity and RBC aggre-
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REFERENCES


