Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance


1Hematology Division, Albert Einstein College of Medicine, Bronx, New York; 2Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel; and 3Institute of Bioengineering, Department Cell Biophysics, Aachen University of Applied Sciences, Juelich, Germany

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Kaul DK, Koshkaryev A, Artmann G, Barshtein G, Yedgar S. Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance. Am J Physiol Heart Circ Physiol 295: H1788–H1793, 2008. First published August 29, 2008; doi:10.1152/ajpheart.253.2008.—To explore the contribution of red blood cell (RBC) deformability and interaction with endothelial cells (ECs) to circulatory disorders, these RBC properties were modified by treatment with hydrogen peroxide (H2O2), and their effects on vascular resistance were monitored following their infusion into rat mesocoeom vasculature. Treatment with 0.5 mM H2O2 increased RBC/EC adherence without significant alteration of RBC deformability. At 5.0 mM H2O2, RBC deformability was considerably reduced, inducing a threefold increase in the number of undeformable cells, whereas RBC/EC adherence was not further affected by the increased H2O2 concentration. This enabled the selective manipulation of RBC adherence and deformability and the testing of their differential effect on vascular resistance. Perfusion of RBCs with enhanced adherence and unchanged deformability (treatment with 0.5 mM H2O2) increased vascular resistance by about 35% compared with untreated control RBCs. Perfusion of 5.0 mM H2O2-treated RBCs, with reduced deformability (without additional increase of adherence), further increased vascular resistance by about 60% compared with untreated control RBCs. These results demonstrate the specific effects of elevated adherence and reduced deformability of oxidized RBCs on vascular resistance. These effects can be additive, depending on the oxidation conditions. The oxidation-induced changes applied in this study are moderate compared with those observed in RBCs in pathological states. Yet, they caused a considerable increase in vascular resistance, thus demonstrating the potency of RBC/EC adherence and RBC deformability in determining resistance to blood flow in vivo.

Address for reprint requests and other correspondence: G. Barshtein, Dept. of Biochemistry, Hebrew Univ.-Hadassah Medical School, Jerusalem, Israel 91120 (e-mail: gregb@cc.huji.ac.il).

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human RBCs. In a previous study we have found that treatment of RBCs with H$_2$O$_2$ suppressed their aggregability. However, depending on its concentration, H$_2$O$_2$ differentially elevated their adherence to ECs and their rigidity (reduced deformability) (35). This therefore provides a method for the aggregation-free modulation of RBC deformability and/or adherence and was used in the present study to explore the direct specific effect of RBC deformability and adherence on vascular resistance.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, glutamine, and trypsin were purchased from Biological Industries. Poly-L-lysine cell culture glass slides were purchased from Sigma (St. Louis, MO). Polystyrene slides were purchased from Electron Microscopy Science (Washington, PA).

Preparation of RBC samples. Human blood samples were collected from healthy donors (under their consent under Helsinki Committee Permit, Hadassah Hospital 20-30/03/01, Israel). RBCs were isolated from blood by centrifugation, washed (3 times) from their plasma by centrifugation in phosphate-buffered saline (PBS, pH 7.4), and resuspended at a hematocrit of 10% in PBS.

Treatment of RBCs with H$_2$O$_2$. RBC suspension (10% of cells in PBS buffer) was supplemented with H$_2$O$_2$ (0.5 or 5.0 mM final concentration) and 0.4 mM NaN$_3$ and incubated for 1 h at 37°C in a shaking bath. The RBCs were then separated and washed three times with PBS by centrifugation and resuspended at 10% hematocrit in PBS, supplemented with 1 mM CaCl$_2$ and 0.5% albumin. Since H$_2$O$_2$ levels may decrease while mixed in medium, we determined the change in its concentration during treatment of RBCs by monitoring the change in optical density produced by its interaction with potassium thiocyanate as described by Hildebrandt et al. (20). It was found that during 1 h treatment of RBC with 0.5 or 5.0 mM H$_2$O$_2$, its concentration decreased by 11% and 13%, respectively.

EC culture. Transformed human bone marrow ECs (HBMECs) were grown at 37°C in 5% CO$_2$ and 95% humidity in DMEM supplemented with 10% FCS, 25 mM HEPES, 100 μM penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cell cultures were expanded by trypsinization with 0.25% trypsin in PBS containing 0.025% EDTA. HBMECs were used for all experiments until passage 14. HBMECs were seeded onto poly-L-lysine glass culture slides coated with gelatin (1%) and grown to confluence.

Determination of RBC flow properties in vitro. In the present research we employed the computerized cell flow properties analyzer (CFA) design, developed in our laboratory (7, 16, 24, 30) for monitoring RBC aggregability, deformability, and adherence as a function of shear stress by direct visualization of their dynamic organization in a narrow-gap flow chamber under controllable flow conditions, resembling those in a microvessel. In this setup, the CFA consists of a thermostated flow chamber with a 200-μm gap, designed by G. M. Artmann (2), and placed under a microscope. RBC suspension (50 μl) was inserted into the flow chamber and subjected to flow induced by a syringe pump (model PHD 2000P, Harvard Apparatus, South Natick, MA). The wall shear stress (τ) is calculated from pressure (P) at the entrance to the flow chamber using a pressure-measuring station (PowerLab 2/20, ADInstruments) according to the following equation: τ = 0.5P × b/t, where b and t are the chamber length and gap, respectively. The RBC images are transmitted by a microscope connected to a camera (model Coolpix995, Nikon) and to a computer equipped with homemade software that provides comprehensive and integrative parameters (some newly defined) for characterization of RBC adherence to ECs (24, 30) and deformability (6, 30).

Determination of RBC deformability. RBC suspension (50 μl; 5% of cells in PBS-albumin buffer) were inserted into the flow chamber. The flow chamber consisted of a polystyrene slide that was placed in the base. After a 15-min incubation of RBC suspension over the slide, the flow of buffer was applied, forming a stress of 3.0 Pa, and the deformation of adherent RBCs were monitored at constant shear stress (3.0 Pa). During measurements, 15–20 randomly chosen fields (width area, 0.1 mm$^2$) were collected. The image analysis of the cell shape provides the elongation index (EI) of individual cells and their distribution in the RBC population (6, 30). The average cell count was 1,200 ± 180. For all cells, we estimated the major (a) and minor (b) axes, and EI was calculated by the formula EI = ab/h, ranging from 1 to 3 (30). EI = 1 reflects round RBCs that are not deformed at the shear stress applied in this study (up to 3.0 Pa). In the imaging system used here, the resolution (in pixels per square inch) resulted in a variance of 10% in determining EI. Therefore, we defined undeformable cells as those having EI ≤ 1.1.

Determination of RBC adhesion to cultured ECs. A slide of confluent cultured ECs was placed in the flow chamber and covered with RBCs for 20 min at 37°C. The nonadherent RBCs were then washed with 5 ml of Ca$^{2+}$-PBS-albumin solution under controllable increasing τ. According to the shear stress applied, the washing time ranges from 5 min at 0.1 Pa to 10 s at 3.0 Pa. The number of adherent RBCs was continuously counted from images by using original software. To each shear stress on 10 randomly chosen fields (with area 0.1 mm$^2$), the number of adherent RBCs remaining was plotted as a function of 1/τ, respective with the equation N = N$_0$ + α/τ, where N is the number of adherent RBCs at a specific τ (N$_0$ is the number of adherent RBC at extrapolated τ → ∞) and α is the adherence coefficient, expressing the strength of intercellular interaction (30).

Determination of vascular resistance in the ex vivo rat mesocecum preparations. Blood samples (1 ml) were drawn from healthy volunteers (no patients) upon their consent under a protocol approved by the Institutional Review Board (Albert Einstein College of Medicine, Bronx, NY). All experimental protocols for the use of animals were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine. Perfusion studies were performed in the isolated, acutely denervated, and artificially perfused ex vivo rat mesocecum vasculature (n = 8) according to the method of Baek et al. (3) as modified by Kaul et al. (22) for the infusion of erythrocytes. Briefly, the perfusion pressure in the mesocecum was maintained at 60 mmHg, and venous outflow pressure was kept at 3.8 mmHg. During perfusion with Ringer-albumin solution containing 3% bovine albumin, a 0.2-ml bolus of a given red blood cell suspension (Hct, 40%) was infused via the arterial injection port over ~5 s. Peripheral resistance units (PRU) were determined as described (2) (expressed in mmHg⋅ml$^{-1}$⋅min$^{-1}$⋅g$^{-1}$). PRU = ΔP/Q, where ΔP is the arteriovenous pressure difference and Q is the rate of venous outflow (in ml/min) per gram of tissue weight. Pressure flow recovery time ($T_{pr}$), defined as the time (in s) required for the arterial pressure and the venous outflow to return to their baseline levels, was determined after the red blood cell infusion. $T_{pr}$ is an estimate of total transit time throughout the isolated vasculature and is influenced by red blood cell deformability and adherence. Direct intravital microscopy and video recording of the microcirculatory events were carried out using a Nikon microscope (model E400; Nikon, Melville, NY), equipped with Dage-MTI-CCD television camera (model CCD-300T-RC; Dage-MTI, Michigan City, IN) and a Sony U-matic video recorder (model VOS5800; Sony, Teaneck, NJ).

RESULTS

Effect of H$_2$O$_2$ on RBC adherence and deformability. In search for a method for differentially testing the effect of RBC/EC and RBC rigidity on vascular flow, we have found that RBC oxidation by H$_2$O$_2$ exerts differential effects on RBC adherence and deformability, depending on the H$_2$O$_2$ concentration. Figure 1 shows that treatment with 0.5 mM H$_2$O$_2$ enhanced RBC/EC adherence by ~3.5-fold, and this
was not further increased by a higher H2O2 concentration. Using the same methodology, we have found that RBC/EC adherence might reach a much higher level under different treatments, e.g., a 115-fold elevation by Ca2+/H11001 ionophore (unpublished data). It thus appears that the H2O2 effect on RBC/EC adherence reaches saturation already at 0.5 mM.

Since cultured cells do not have the surface glycolcalyx that vascular ECs in vivo have, the projection from ECs in vitro behavior to in vivo conditions has been questioned. Therefore, subsequent to determining the adhesion of H2O2-treated RBCs to cultured ECs, we examined their adhesion to vessel wall following their perfusion into rat mesocecum as described in MATERIALS AND METHODS. Figure 2 demonstrates that H2O2-treated RBCs exhibit considerable adhesion to the vessel wall of postcapillary venules in the rat mesocecum preparation.

Effect of H2O2-treated RBCs on vascular resistance. The results (in Fig. 4) provide a method for examining the flow considering in vivo, circulatory disorders, the increase in the rigid cell portion might be more important than the average change in EI. Figure 3B indeed shows that the H2O2 effect is expressed prominently in the number of rigid undeformable RBCs (cells with EI < 1.1), exhibiting a threefold increase.

Fig. 2. Videomicrographs of artificially perfused mesocecum infused with a bolus of H2O2-treated human RBCs. A: a bolus infused of H2O2-treated RBCs during artificial perfusion with Ringer-albumin solution results in adherence of RBCs to vessel wall following their perfusion into rat mesocecum (arrowheads). Arrows indicate the flow direction from a small-diameter venules to a large-diameter venules. B: scanning of the microvascular revealed adhesion of the treated RBCs in several postcapillary venules. C: higher magnification revealed significant adhesion in many venules.

Fig. 1. Red blood cell (RBC) adhesion to endothelial cells (ECs) after treatment with H2O2 (0.5 and 5.0 mM). A: adherence of RBCs as a function of shear stress (τ). The number of RBCs that remained adherent to the cultured ECs under the indicated τ is determined by cell flow properties analyzer and are plotted vs. 1/τ, yielding the function \( N = N_0 + \alpha/\tau \), where \( N \) is the number of adherent RBCs at a specific τ and \( \alpha \) is the adhesiveness coefficient. Data are means ± SE for \( n \) blood samples (15 for the control and 9 for the H2O2-treated RBCs). B: relative \( \alpha \) for H2O2-treated RBCs. \( P < 0.005 \) for difference between treated and control, untreated RBCs.
functions under the perfusion of RBCs with elevated adhesion to ECs with normal deformability (treatment with 0.5 mM), whereas the effect of RBCs with both elevated adherence and rigidity is determined by the perfusion of RBCs treated with 5 mM H2O2. A subtraction of the first from the latter provided the net effect of RBCs with reduced deformability, all these under aggregation-free conditions.

Figure 4 depicts the changes in vascular resistance, expressed by PRU (Fig. 4A) and $T_{pf}$ (Fig. 4B), occurring following the perfusion of rat mesocecum with H2O2-treated RBCs. As shown in Fig. 4, a considerable elevation of vascular resistance occurs already at 0.5 mM H2O2, and this was further increased at 5 mM H2O2. Analyzing these data of Fig. 4 in light of Figs. 1 and 3 suggests that RBC/EC adherence is responsible for the increased vascular resistance at 0.5 mM H2O2. At 5 mM H2O2, which reduced RBC deformability without a further increase in RBC/EC adherence, the increase in the percentage of undeformable RBCs was associated with an additional increase in vascular resistance (Fig. 4).

DISCUSSION

Experimental data and theoretical considerations have suggested that rigid RBCs may attenuate perfusion in peripheral tissues since they can block microvessels and capillaries in particular (27). Adherent RBCs at the vessel wall may impair the flow pattern in large vessels and directly occlude microvessels and capillaries in particular (5, 25).

The results presented above show that the perfusion of the mesocoeum vasculature with RBCs with enhanced RBC/EC adherence elevates vascular resistance, as expressed by both PRU and $T_{pf}$. Since this effect is obtained with normal RBC deformability and under aggregation-free conditions, it demonstrates a specific direct effect of RBC/EC adherence on hemodynamics. As shown above, increasing H2O2 concentration (from 0.5 to 5.0 mM) did not further increase RBC/EC adherence but induced a considerable decrease in RBC deformability. The subsequent perfusion of these RBCs further increased vascular resistance in the mesocoeum, showing that the additional increase in vascular resistance is a direct consequence of reduced RBC deformability.

Although, as stated in the Introduction, the objectives of the present study were to demonstrate the in vivo effect of RBC/EC adherence and RBC deformability; the mechanism of the H2O2 effect is obviously of great interest. To gain an insight into this, we have first considered the possibility that H2O2 induces the phosphatidylserine (PS) translocation to the RBC surface, well known to mediate their adherence to ECs. PS externalization has been shown for some OS states (26, 29, ...
examined the H2O2 effect of PS externalization and found that, unlike other OS states (26, 29, 30), the treatment of RBCs with H2O2, at any concentration, did not induce PS translocation, suggesting that H2O2-induced RBC/EC adhesion does not involve RBC surface PS. Another candidate proposed as a mediator of RBC/EC interaction is clustered band-3, which has been shown to occur under OS states (5). In the present study we also observed a slight clustering of band-3 after H2O2 treatment (not shown), but the involvement of band-3 in H2O2-induced RBC/EC adhesion (e.g., testing the adherence in the presence of a blocker of band-3 clusters, not yet available), as well as other possible RBC factors (e.g., CD47), is yet to be explored.

The mechanism of RBC interaction with cultured ECs might be different than with vascular ECs in blood vessels since cultured ECs do not exhibit the same glyocalyx structure and level as in vivo (33). It has been further argued that the glyocalyx hinders RBC accessibility to vessel-wall ECs and thus prevents their interaction (33). On the other hand, in a study by Weinbaum et al. (34), it has been concluded that under low-flow rate (velocity < 20 μm/s), the RBCs can penetrate through the endothelial glyocalyx and interact with the ECs. In support of that, Kaul et al. (23) have shown, using ex vivio rat mesoeceum preparation, that the adhesion of sickle RBCs to vessel walls increases as the vessel diameter decreases and is highest in postcapillary venules where the flow velocity is the lowest, thus providing “the maximal opportunity for circulating erythrocytes to interact with the endothelium.” In addition, it has been shown that malaria-infected RBCs strongly interact with glyocalyx components, specifically hyaluronic acid and chondroitin sulfate (11, 12). In accordance with these findings, the present study demonstrates that RBCs with elevated adherence to cultured ECs (Fig. 1) also adhere to vessel walls (Fig. 2) and elevate vascular resistance (Fig. 4). It may thus be assumed that EC glyocalyx does not necessarily interfere with RBC/EC interaction and might possibly facilitate it in certain conditions.

When compared with those of pathological RBCs, in the present study we induced a relatively moderate elevation of RBC adherence (up to 3- to 4-fold) and reduction of deformability (up to 2.5% of undeformable cells). For example, using the same system applied here, we have found that the adherence of thalassemic or cold-stored RBCs is enhanced by 10- and 8-fold, respectively (21, 30). Similarly, RBC deformability is markedly reduced during blood banking procedures, since the percentage of undeformable RBCs was increased to 4% during cold storage and up to 40% by γ-irradiation (30). Yet, the moderate changes in RBC/EC adherence and deformability applied in the present study still exerted a considerable increase in vascular resistance.

Of particular interest in this regard is our previous finding that the shear stress required to detach adherent RBCs from ECs is at least an order of magnitude higher than that required to disperse RBC aggregates; whereas RBC aggregates are dispersed at about 0.1–0.2 Pa, some oxidized RBCs remain adherent even at 3 Pa (5). This demonstrates the strength of RBC adhesion to the endothelium, which has been considered in recent years to be a potent catalyst of blood vessel occlusion.

In summary, this study presents specific individual effects of elevated RBC/EC adhesion and reduced RBC deformability on the circulation. Even moderate alterations in these properties are sufficient to induce a considerable increase in vascular resistance, thereby demonstrating the potency of RBC/EC adhesion and RBC rigidity to contribute to circulatory disorders.

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REFERENCES


