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Red blood cell aggregability is enhanced by physiological levels of hydrostatic pressure

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Abstract

The effect of hydrostatic pressure of up to 15 bars on the aggregability of rat and human red blood cells (RBC), i.e., their capability to form aggregates, was studied using computerized image analysis. The aggregate size distribution was determined under ambient pressure, following application of hydrostatic pressure for various durations up to 2 h. It was found that RBC aggregability markedly increases, up to three-fold, as the pressure which had been applied was increased. Accordingly, higher shear stress is required for dispersing the aggregates of pressure-treated RBC than those of untreated cells. The median size of human RBC aggregates was about three times higher than that of rat RBC, and this ratio was maintained following pressure treatment. RBC aggregability is a major determinant in blood flow, especially in the microcirculation. Pressure at the levels used in this study occurs in physiological states such as hyperbaric treatment or diving. The enhanced aggregability induced by application of such pressure implies that blood flow in microvessels might be altered under conditions associated with elevated hydrostatic pressure.

Key words: Hydrostatic pressure; Aggregation; Shear stress; (Erythrocyte)

1. Introduction

The study of the effect of hydrostatic pressure on cell function has shown that different activities may be sensitive to different levels of pressure. High hydrostatic pressure in the range of hundreds of bars, has been shown to alter cell functions and biochemical processes, such as membrane lipid molecular order [1] and phase transition [2], release or crosslinking of erythrocyte membrane proteins [3,4], protein-receptor dissociation [5], microtubule assembly [6], and histone mRNA level [7]. Other studies have shown that lower pressure levels, in the range of tens of bars may affect cell functions such as platelet aggregation [8,9], release of neurotransmitters [10,11], and cellular distribution of cytoskeletal and adhesion proteins [12].

Of special interest to this study is the pressure effect on intact red blood cell (RBC) functions, as they have been shown to be influenced by a wide range of hydrostatic pressures. For example, ionic regulation in deep sea fish RBC is modulated by hundreds of bars [13], while ion transport and ATP metabolism in human RBC (Goldinger, Hall), are effected at tens of bars [14,15]. Further to that, in a previous study [16] we have shown that hydrostatic pressure in the range of several bars induces changes in the membrane composition of red blood cells (RBC), leading to resistance of the cells to hemolysis by phospholipase A_2. It thus seems that RBC functions may be selectively modulated by a wide range of hydrostatic pressures.

Red blood cells (RBC) in the presence of plasma proteins or other macromolecules form aggregates, called rouleaux [17–19]. The aggregability of RBC is the result of opposing forces; the repulsive force between the negatively charged cells, the cell-cell attraction induced in the presence of macromolecules [17,18], and the disaggregating shear force generated by the flow [19]. Thus, the aggregates formed in stasis or low-flow states are dispersed with increasing flow rate [19].

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The aggregability of RBC is a major determinant of blood flow, especially in the microcirculation [20]. Increased aggregability contributes to the pathophysiology of various diseases associated with impaired microcirculation [21]. Of special interest to the present study is the observation that long-term diving is associated with retinal microcirculatory disorders [22].

RBC aggregability is susceptible to the physical and chemical properties of the cell membrane, and is altered by changes in the membrane composition [23,24]. It is therefore likely that the changes in RBC membrane composition induced by the application of pressure are associated with altered aggregability.

This study was undertaken to examine the effect of hydrostatic pressure of several bars, such as that applied in diving or hyperbaric treatment, on RBC aggregability. For this purpose we have employed a computerized image analysis system for monitoring blood cells in a narrow flow-chamber constructed in our laboratory. This system provides quantitative measures of RBC aggregate size distribution [25].

It was found that application of such pressure results in the enhancement of RBC aggregability, which is associated with the removal of membrane constituent(s) to the extracellular fluid.

2. Experimental

2.1. Preparation of RBC suspension

Blood was drawn from rats or from human volunteers in EDTA and centrifuged at low speed and aqueous column, which were not sufficient to induce pressure effect. The RBC pellet was washed in Tris buffer (140 mM NaCl, 5 mM KCl, and 5 Mm Tris-HCl at pH = 7.4) supplemented with 1% albumin and 10% plasma, and resuspended in the same buffer at the desired concentrations.

2.2. Application of pressure

As previously discussed [16] cells at the bottom of a spinning tube are subjected to hydrostatic pressure P, which is a function of the angular velocity and the height of the aqueous column on the cells, according to the equation:

$$P = P_0 + \tfrac{1}{2}\rho\omega^2\left(R^2 - R_0^2\right) \quad (1)$$

where P_0 is the atmospheric pressure, ρ is the aqueous phase density, ω is the angular velocity of the spinning rotor, R and R_0 are the distances from the center of rotation to the bottom of the tube and to the air-water meniscus, respectively ($R - R_0$ is the height of the aqueous column on the cells). Accordingly, in this study pressure was applied to RBC by centrifugation at angular velocity and buffer column corresponding to the desired pressure (for example a pressure of 10.1 bar (10 atm) was obtained by spinning at 3000 rpm under 5.5 cm buffer in a centrifuge having a radius of 19.5 cm). After application of pressure the cells were returned to ambient pressure, their supernatant was collected, and the cells were suspended in new buffer.

To rule out possible drag or shear effect of the spinning, the RBC to be pressurized were placed at the bottom of the tube and the buffer was layered on top before spinning. As shown in Eq. (1), the pressure applied by spinning depends on the height of the aqueous column on top of the spinning cells. Thus, in the control experiment the RBC underwent the same procedure, but were centrifuged under an aqueous column of 0.5 cm, which was not sufficient to induce a significant pressure effect even at the highest speed used for pressure treatment. This rules out the possibility that the effect is due to centrifugation-induced cell-cell contact. To further exclude effects of drag, shear or cell-cell contact, in a few experiments the cells were subjected to hydraulic pressure by applying force directly on a syringe filled with RBC suspension (free of bubbles), and connected to a manometer. (The latter system, assembled in our laboratory, was limited to a narrow pressure range and much less practical than spinning, and therefore the routine method used here for application of pressure was centrifugation). Following the application of hydraulic pressure the cells were separated from the extracellular fluid by centrifugation at low speed and water column (50 g under 2.5 cm height of buffer column) which were not sufficient to induce significant pressure effect. As in our previous study [16], we have found also here that application of hydraulic pressure induced the same effect as that of centrifugation-induced pressure (see Fig. 2).

In all experiments following application of pressure the cells were suspended in buffer at ambient pressure by gentle tilting of the suspension tube.

2.3. Determination of RBC aggregability

The aggregability of the cells before and after application of pressure was studied using a computerized image analysis system developed and constructed in our laboratory [25]. This system includes a transparent flow-chamber having a gap of 30 μm, providing a single layer of RBC aggregates. The flow-chamber, thermostated and connected to a pump and a pressure transducer to control the shear stress, is placed under a microscope equipped with a CCD camera. The RBC images are video recorded and transmitted to a computer equipped with software (developed in our laboratory) for image analysis. As shown in Fig. 1, this system provides the distribution of RBC aggregate size under

various shear stresses. From the distribution curve, depending on its shape (e.g., symmetric or asymmetric), one can derive different parameters of aggregation, such as median aggregate size, corresponding to the aggregate size under which 50% of the cell population is found, as well as the size corresponding to any selected percentile of the cell population, as well as average or peak size.

RBC aggregation was induced by the addition of concentrated solution of dextran-500 to obtain final concentration of 0.5% dextran and 10% RBC in the Tris buffer, supplemented with 0.5% albumin and 10% plasma. All aggregation measurements were carried at 37°C in the thermostated flow-chamber.

3. Results

Fig. 1 depicts representative images of untreated and pressurized RBC at three values of shear stress, and the corresponding aggregate size distribution curves. It should be noted that similar to a previous report [26], application of flow to cells in stasis first induced increased aggregation at low shear stress, reaching a peak at 0.125 dyne/cm^2. Beyond that, disaggregation is induced and increases with increasing shear stress. Therefore, the reference shear stress in this study was 0.125 dyne/cm^2 (Fig. 1AI). As demonstrated by this figure, application of pressure clearly enhances the aggregability, i.e., a marked shift to higher aggregate size is obtained. This figure also indicates that higher shear stress is required for dispersing pressurized RBC aggregates.

The dependence of the aggregation on the pressure is shown in Fig. 2, which depicts the median aggregate size as a function of the pressure applied prior to the determination of the aggregability. As shown in this figure the aggregation increases with increasing pressure in a sigmoidal manner, and an appreciable effect is observed above 4 bar.

As indicated in Fig. 1, higher shear stress was required for disaggregation of pressure-treated RBC compared to the untreated cells. This is further elaborated in Table 1, presenting the median aggregate size at different shear stresses.

As noted in the Introduction, we have previously shown that application of pressure of several bars induces the release of a membrane component to the extracellular fluid, and subsequently reduces the susceptibility of RBC to hemolysis. Incubation of pres-

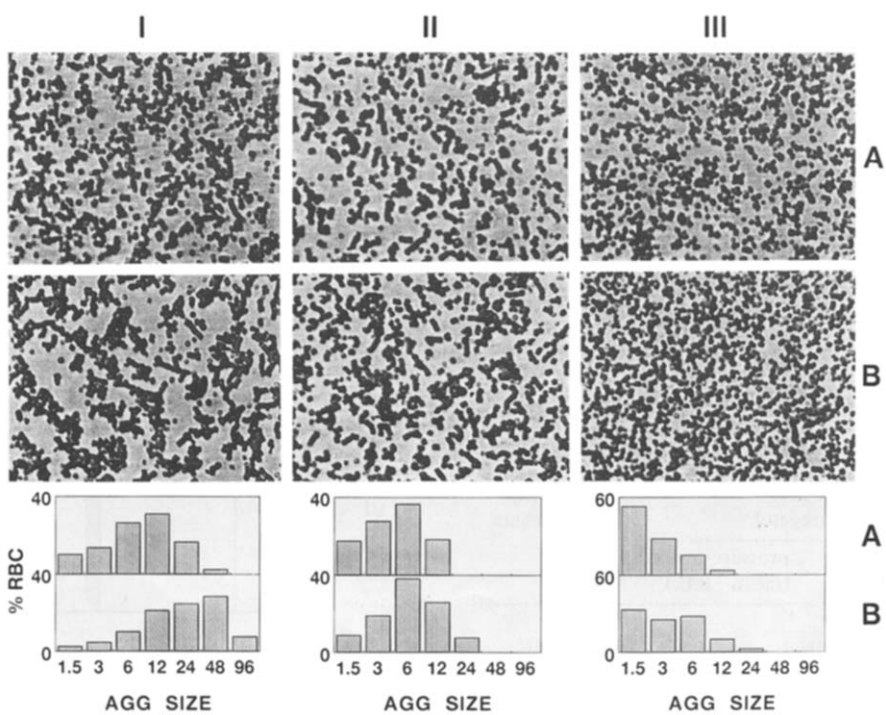

Fig. 1. Images of control and pressure-treated rat RBC aggregates and corresponding aggregate size distribution diagrams at three levels of shear stress. Rat RBC were subjected to a pressure of 15.2 bar (15 atm) for 1 h and returned to ambience. The pressure treated cells, and equivalent amount of control, untreated cells were separated from the extracellular medium and suspended at concentration of 10% in fresh buffer (see Experimental for further details). The figure depicts the images of untreated (A) or pressure-treated (B) RBC aggregates at 0.125 (I), 0.5 (II) and 2.0 (III) dyne/cm^2, analyzed, and the corresponding aggregate size distribution diagrams provided by the computerized image analysis system. The aggregate size at the abscissa of the distribution diagram corresponds to the number of cells per aggregate. This value indicates the mean of a range of aggregate sizes, and varies on a logarithmic scale. The ordinate of the distribution diagrams depicts the percent of the RBC population in aggregates of the size indicated in the abscissa.

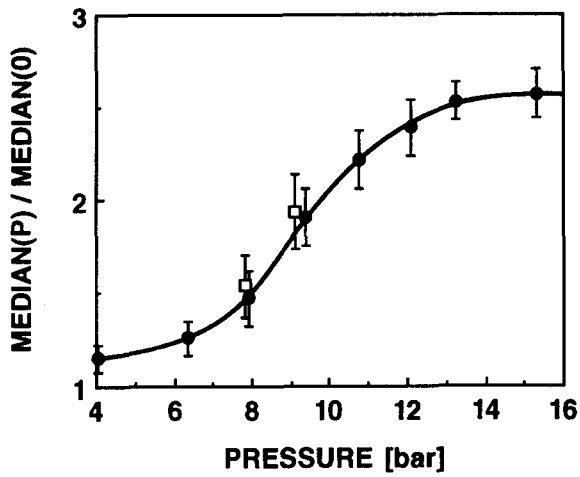

Fig. 2. Median aggregate size as a function of applied pressure. Rat RBC were subjected to centrifugation-derived (•) or hydraulic (□) pressure for 1 h and further treated as in the experiment of Fig. 1. The median aggregate size, at shear stress of 0.125 dyne/cm^2, of pressure-treated cells, Median(P), is expressed by its ratio to the median aggregate size of untreated cells, Median(0). Each datum is mean ± S.E. for four replications.

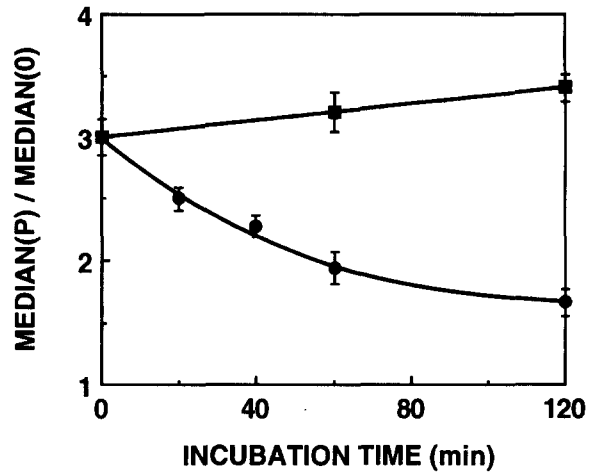

Fig. 3. Reversibility of the pressure effect on RBC aggregation. Rat RBC suspension was divided into two equal portions and both were subjected to pressure of 15.2 bar (15 atm) for 1 h by centrifugation, and returned to ambient pressure. In one portion (•) the cells were resuspended in their own supernatant, while the cells of the other portion (■) were separated from the supernatant and suspended in new buffer. Both portions were incubated at room temperature for the indicated time, and their aggregation was determined at 0.125 dyne/cm^2. Each datum is mean ± S.E. for three replications.

sure-treated cells with this fluid for reincorporation of this factor reverses the pressure effect [16]. Fig. 3 demonstrates that a similar phenomenon was observed with aggregation in the present study; the enhanced aggregability of pressurized RBC is maintained as long as the cell supernatant was removed after treatment and the cells suspended in fresh buffer. On the contrary, when the pressurized cells were resuspended, after treatment, in their own supernatant, and incubated at ambient pressure, the pressure effect was reversed with time, and the aggregation returned almost to that of untreated cells after about 2 h of incubation.

Table 1
Aggregation of human and rat RBC as a function of shear stress

Shear stress (dyne/cm^2)	Median aggregate size (No. of RBC/aggregate)		Statistical significance P
	control (mean ± S.E.)	pressure-treated (mean ± S.E.)	
A. Rat RBC (n = 5)			
0.125	6.97 ± 1.01	18.09 ± 2.01	< 0.003
0.50	4.33 ± 0.23	6.71 ± 0.61	< 0.005
2.00	2.70 ± 0.32	3.10 ± 0.34	n.s.
B. Human RBC (n = 4)			
0.125	19.87 ± 3.21	52.07 ± 4.39	< 0.004
0.50	5.86 ± 0.59	11.94 ± 1.23	< 0.007
2.00	2.21 ± 0.24	3.35 ± 0.68	n.s.

The same procedure as in the experiment of Fig. 1 was performed in these experiments, and median aggregate size at the indicated shear stress was derived from the aggregate size distribution provided by the image analysis.

Furthermore, as shown in Fig. 4, interaction of untreated RBC with pressure-conditioned medium, i.e., the supernatant of pressure-treated cells, reduces their aggregability.

This further supports the conclusion that the application of pressure of several bars induces the release of a membrane component(s) which alters cell function.

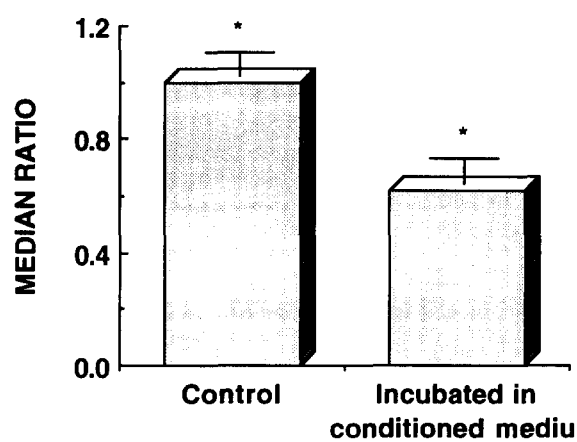

Fig. 4. Effect of pressure-conditioned medium on RBC aggregability. Rat RBC were subjected to pressure of 15.1 bar (15 atm) by centrifugation for 1 h, and their supernatant was collected (pressure-conditioned medium). Equal amounts of untreated RBC were suspended in the conditioned medium (right column) or in new buffer (left column), and incubated at room temperature for the 2 h, prior to determination of the aggregation as in the experiment of Fig. 3. Each column represents mean ± S.E. for four replications. * Significant at $P < 0.01$.

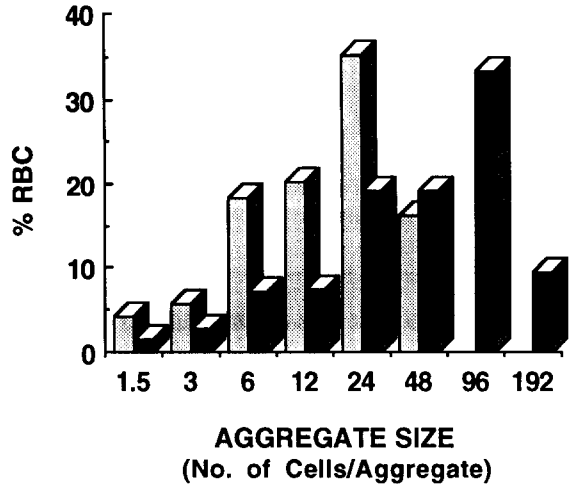

Fig. 5. Aggregate size distribution diagram of human RBC. Human RBC were subjected to the same experimental procedure and analysis as in the experiment of Fig. 1. This figure depicts the aggregate size distribution of untreated (□) and pressure-treated RBC (■) at shear stress of 0.125 dyne/cm^2.

We further studied the effect of application of hydrostatic pressure on human RBC and compared it to that on rat RBC. As shown in Fig. 5 and Table 1B, the same phenomenon was observed with human RBC, but the aggregate size of human RBC was found to be considerably larger then that of rat RBC. The median size of pressurized and untreated human RBC aggregates was about 50 and 20, respectively, while that of rat RBC was about 18 and 7.

4. Discussion

The present study demonstrates that application of hydrostatic pressure of several bars is sufficient to induce enhancement of RBC aggregability, and higher shear stress is required to disperse the aggregates of pressure-treated cells. It should be noted that the aggregability was measured after the cells were returned to ambience following application of pressure. The pressure induces the shedding of a membrane component(s) to the extracellular medium, and normal aggregability can be restored by reincubating the treated cells with the conditioned extracellular medium. Currently we have evidence that application of pressure induced the release of more than one substance, including a phospholipid which contains a free amino group, and migrates close to phosphatidylethanolamine when chromatographed on silica thin layer, as well as a protein of about 30–40 kDa. However, these substances are not yet fully characterized and are the subject of on-going research.

Pressure of several or even tens of bars is applied in hyperbaric treatment, or in commercial diving, and therefore has been considered physiological [12]. High hydrostatic pressure is also applied to blood cells during routine separation by centrifugation for clinical and research purposes. The finding that a pressure of several bars is sufficient to alter RBC function may have implications for organisms and cells subjected to such pressures.

As discussed in the Introduction, previous studies have demonstrated that pressure of tens of bars might affect biochemical and cellular processes. Similarly, pressure effects on physiological function have been observed under hyperbaric treatment. However, in hyperbaric chamber, as well as in experiments where pressure is applied by exposing the reaction mixture to inert gases, the effect of partial gas pressure can not be differentiated from that of hydrostatic pressure as a mechanical force. It has been previously demonstrated that gas pressure and hydrostatic pressure as such may exert differential metabolic effects, and that cells exhibit selective sensitivity to these two parameters [8,27]. This is further supported by the present study which demonstrates that hydrostatic pressure as a mechanical force, in the range applied in a hyperbaric chamber, affects RBC function. This implies that in hyperbaric treatment possible effects of the pressure per se, distinct from partial gas pressure, should be taken into consideration.

It has been reported that professional divers suffer from impaired microcirculation, particularly in the eye [22]. As noted above, RBC aggregability plays a major role in blood flow, especially in the microcirculation. Enhanced aggregation may initiate a self-accelerating process, ending with the formation of 'sludged blood' which leads to reduced tissue perfusion and ischemia [28]. It is thus possible that enhanced RBC aggregability induced by pressures such as those applied in diving, may contribute to the development of diving-associated microcirculatory disorders.

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