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**MEMBRANE FLUIDITY AND SHAPE OF HUMAN RED BLOOD CELLS
ARE ALTERED BY PHYSIOLOGICAL LEVELS OF HYDROSTATIC PRESSURE**

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Abbreviations used:

RBC = Red blood cells

DPH = 1,6 diphenyl-1,3,5-hexatriene

TMA-DPH = 1,4(trimethylamino)-phenyl-1,6-diphenyl-1,3,5-triene

ANS = anilinonaphthalene-8-sulfonic acid

AA = 12-(9-anthryl)-11-trans-dodecenoic acid

ASM = N-[12-(9-anthryl)-11-trans-dodecenoyl]-sphingosine-1-phosphocholine

APC = 1-acyl-2-[12-(9-anthryl)-11-trans-dodecenoyl]-sn-glycero-3-phosphocholine

NAPE = N-acyl-phosphatidylethanolamine

RET = Resonance energy transfer.

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ABSTRACT

The effect of hydrostatic pressure at physiological levels, such as that applied to humans in diving or hyperbaric chamber, on human red blood cell (RBC) membrane fluidity and morphology was studied. Membrane fluidity was determined by fluorescence anisotropy (FA) of lipid probes (mainly diphenyl hexatriene (DPH), and of tryptophan), as well as by energy transfer from the tryptophan to the lipid probes, in ghosts prepared prior to or after application of pressure to intact RBC. The morphology of intact RBC, prior to or after application of pressure, was evaluated by scanning electron microscopy. It was found that 1. The FA of DPH, which resides in different apolar membrane regions, was increased as a function of the pressure applied and the duration of the treatment. At 15 atm the FA increased by 50%, reaching a plateau after 60 min of application of pressure. 2. Increased FA, to various extents, was observed also with the lipid probes which reside in the membrane lipid core, but not with probes which monitor the polar/apolar phospholipid interface, or the cell surface. 3. The same treatment increased tryptophan anisotropy by about 20%. 4. Tryptophan energy transfer to the lipid probes which resides in the lipid core was increased to various degrees which were related to the increase of the FA of these probes. 5. Following application of 15 atm for 1 hour, at least 60% of the RBC change their shape from discocytes to stomatocytes. These results demonstrate that hydrostatic pressure at physiological levels, might induce reduction of the membrane fluidity of RBC, which affects the physical state of protein environment, and might alter RBC morphology. Since the membrane fluidity and shape of RBC play an important role in cellular and rheological functions, the pressure-induced alterations in RBC properties might be pertinent to the microcirculatory disorders observed in humans subjected to elevated pressure.

INTRODUCTION

Pressure of several or even tens of atmospheres is applied in hyperbaric treatment, or in diving (in commercial and experimental diving the diver may reach the depth of 300 meter, i.e. 30 atm). This range of pressures is therefore considered "physiological" [Haskin & Cameron, 1993]. In the clinic, blood cells are routinely separated by centrifugation, and in research, during experimental procedures cells and membranes are often subjected to high speed centrifugation. As the hydrostatic pressure induced in spinning is a function of the angular velocity [Halle et al., 1988 ; Wu et al., 1985] these procedures may exert a pressure of several or tens of atmospheres (in the separation of blood cells) or hundreds or thousands of atmospheres (in experimental procedures).

Many studies have demonstrated that hydrostatic pressure in the range of hundreds of atmospheres alters cell functions and biochemical processes, such as membrane lipid molecular order [Macdonald, 1984] and phase transition [Wu et al., 1985], release or crosslinking of erythrocyte membrane proteins [Deckman et al., 1985; Kitajima et al., 1990], protein - receptor dissociation [Royer & Weber, 1986], microtubule assembly [Bourns et al., 1988], and histone mRNA level [Symington et al., 1991]. Other studies have shown that lower pressure levels, in the range of tens of atmospheres may affect cell functions such as platelet aggregation [Pickles et al., 1990; Philp, 1990], release of neurotransmitters [Ashford et al., 1982; Gilman et al., 1991], and cellular distribution of cytoskeletal and adhesion proteins [Haskin & Cameron, 1993].

The study of the effect on pressure on red blood cells (RBC), which is the subject of the

present study, has shown that different RBC functions may be sensitive to different levels of hydrostatic pressure. For example, ionic regulation in deep sea fish RBC is modulated by hundreds of atmospheres [Shelton et al., 1985], while ion transport and ATP metabolism in human RBC are effected at tens of bars [Goldinger et al., 1980; Hall et al., 1982]. Further to that, in previous studies we have observed that application of hydrostatic pressure in the range of several atm to RBC induces changes in the membrane composition, leading to the resistance of the cells to hemolysis by phospholipase A2 [Halle & Yedgar, 1988], and to enhancement of their aggregability [Chen et al., 1994].

These pressure-induced changes in RBC membrane indicate that such pressure may alter physical properties and morphology of the cells, which are closely related. This study was undertaken to examine the effect of hydrostatic pressure at physiological levels on the membrane fluidity and shape of human RBC. It was found that application of pressure of up to 15 atm reduces the membrane fluidity, as measured by fluorescence anisotropy of lipid probes, and changes the cell morphology from discoid to stomatocytic shape.

EXPERIMENTAL

Preparation of RBC suspension: Blood was drawn from human volunteers in EDTA and centrifuged at low speed (gravity of 300 g) under aqueous column of 0.5 cm., i.e. under conditions which were not sufficient to induce significant pressure (less 0.5 atm). The RBC pellet was washed in Tris buffer (140 mM NaCl, 5 mM KCl, and 5 mM Tris-HCl) at pH = 7.4, and separated by centrifugation as indicated above. This procedure was repeated 3 times.

Application of pressure: As previously discussed [Halle et al., 1988 ; Wu et al., 1985], cells at the bottom of a spinning tube are subjected to hydrostatic pressure which is a function of the angular velocity and the height of the aqueous column in the spinning tube, as expressed by the equation:

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

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 the present study pressure was applied to RBC by centrifugation, in a swinging bucket rotor, at angular velocity and buffer column corresponding to the desired pressure (for example, a pressure of 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 and their supernatant was collected. The cells were washed twice by isotonic Tris buffer at pH = 7.4, by centrifugation (10 min) at 300 x g under aqueous column of 0.5 cm, which, as noted above, does not produce significant pressure effect (additional 0.5 atm), and suspended in fresh Tris buffer.

As shown in Equation 1, the pressure applied by spinning depends on the height of the aqueous column on top of the spinning cells. Therefore, to obtain equal pressure on all the cells, the amount of RBC used in these experiments was small enough (0.2 ml of packed RBC) to form thin layer at the bottom of the tube, with no significant height difference within the RBC layer. 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 to the desired height before spinning. As in the previous studies [Halle et al 1988, Chen et al., 1994], 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.

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

Preparation of RBC ghosts: RBC were subjected to lysis by osmotic shock according to the common procedure [Schachter et al., 1982]. In this procedure the membranes are separated from the intracellular content by high speed centrifugation, that already exerts a pressure of at least hundred of atm, which is obviously undesirable in the present study. To circumvent this drawback, in the present study the membranes were separated by column chromatography. RBC, prior to or following application of pressure, were lysed in Tris buffer (0.5 mM) containing 5 mM NaCl and 0.15 mM KCl, and the membranes were separated from hemoglobin on a Sephadex G-100 column [Aevi et al., 1964].

Determination of membrane fluidity: Membrane fluidity was determined by the fluorescence anisotropy of lipid probes interacted with the RBC membrane. To avoid the interference of hemoglobin with the fluorescence measurements, the fluorescence anisotropy was measured in ghosts prepared from RBC prior to or following application of pressure (see

preparation of ghosts below).

Fluorescence probes: The following fluorescent lipid probes, which incorporate into and monitor different regions of the membrane, were used: diphenylhexatriene (DPH), which incorporates into different apolar regions of the membrane [Lenz et al., 1976]; the cationic probe 1,4-(trimethylamino)-phenyl-DPH (TMA-DPH), where the fluorophore TMA is located between the upper parts of the fatty acyl chains [Kuhry et al., 1983]; aminonaphthalene-8-sulfonic acid (ANS), which monitors the interface between the apolar tail and the polar head of phospholipids and may be an indicator of changes in surface charge [Radda & Vanderkooi, 1972]; N-[12-(9-anthryl)-11-trans-dodecenoyl]-phosphatidylcholine (APC), the fluorophore of which is located in the center of the bilayer perpendicular to the fatty acyl chains [Molotkovski et al., 1982]; N-[12-(9-anthryl)-11-trans-dodecenoyl]-sphingomyelin (ASM) which is similar to APC, but prefers the proximity of membrane proteins [Molotkovsky et al., 1982], and the fatty acid analog 12-(9-anthryl)-11-trans-dodecenoic acid (AA). Taking into account the extremely slow phospholipid flip-flop in the erythrocyte membrane it may be expected that TMA-DPH, APC and ASM label predominantly the outer half of the RBC membrane, whereas AA distributes between both halves of the bilayer.

Incorporation of fluorescent probes: DPH and TMA-DPH were dissolved in tetrahydrofuran to a final concentration of 2 mM. All other probes were dissolved in ethanol to a final concentration of 6 mM. The probe solutions were added to vigorously stirred 5 mM NaCl solution. One volume of the ghost suspension (in 5 mM NaCl) was incubated with 0.1 volume

of the fluorescent probe solution at 37°C. The incubation time was 0.5 h for DPH and TMA-DPH and 3h for AA, ASM and APC .

Fluorescence anisotropy measurements: Excitation wavelengths were: 365 nm for DPH and TMA-DPH, 370 nm for APC, ASM and AA, and 390 nm for ANS. Emission was measured at 445 nm for DPH and TMA-DPH, 430 nm for APC, ASM and AA nm, and at 490 nm for ANS.

Fluorescence anisotropy was calculated according to the equation

$$r = (I_{vv} - GI_{vh}) / (I_{vv} + 2GI_{vh})$$

where I_{vv} and I_{vh} are the fluorescence intensities measured with a vertical polarizer and an analyzer mounted vertically and horizontally, and $G = I_{hv} / I_{vh}$ is the correction factor (the first character of the indices denotes the position of the polarizer and the second that of the analyzer). For each sample, fluorescence was corrected for the scattering of unlabeled ghost membranes.

Resonance energy transfer (RET) from tryptophan to lipid probes in RBC membranes was determined by the decrease in tryptophan fluorescence intensity induced by the presence of the lipid probes.

RBC morphology, prior to or following application of pressure, was examined by scanning electron microscopy as previously described [Rahamim et al., 1990].

RESULTS

Fig. 1 demonstrates the effect of hydrostatic pressure on the fluidity of RBC membranes,

as measured by the fluorescence anisotropy of DPH in RBC membranes isolated following application of pressure to intact RBC. As shown in this figure, the anisotropy is increased substantially as function of both the pressure applied (Fig. 1A) and the duration of treatment (Fig. 1B), and reaches a plateau following application of 15 atm for about 1.5 hour. This clearly demonstrates that application of a pressure of several atmospheres already induces considerable reduction of RBC membrane fluidity.

To examine a possible effect of pressure on RBC membrane proteins, we determined the anisotropy of tryptophan in RBC membranes following application of pressure up to 15 atm to the intact RBC. As shown in Fig. 1B, the tryptophan fluorescence anisotropy is also increased, although to a lesser extent than that of the DPH, suggesting that following application of pressure, membrane proteins may be in a more rigid environment.

To learn about a possible differential pressure effect on the fluidity of different lipid domains, we measured the anisotropy of lipid probes which reside in different lipid regions of the RBC membrane, as well as the energy transfer from tryptophan to these probes. The results, presented in Table I, show a selective effect. Application of pressure increased the fluorescence anisotropy of AA, which incorporates into the membrane and distributes between both halves of the bilayer, and ASM, which incorporates predominantly into the outer half of the RBC membrane [Molotkovsky et al., 1982], in addition to the DPH, which incorporates into different apolar regions of the membrane [Lenz et al., 1976]. Similarly, this treatment increased the energy transfer from tryptophan to these three lipid probes. This may suggest that application

of pressure alters preferentially the physical state of the lipids in center of the bilayer, rather than all the lipid regions, and subsequently affects adjacent proteins.

As noted in the Introduction, the pressure effect on the susceptibility of RBC to hemolysis [Halle et al., 1988], and on RBC aggregation [Chen et al., 1994] is associated with the release of a lipid and a protein from the cell membrane to the extracellular medium, and can be reversed by reincubation of pressure-treated cells with the extracellular fluid collected after application of pressure by centrifugation ("conditioned medium"). In accord with this, Table II shows that incubation of pressure-treated RBC with conditioned medium reduced the anisotropy of DPH considerably (although not completely). While the protein shed into the extracellular fluid has not been characterized as yet, the lipid was identified as N-acyl phosphatidylethanolamine (NAPE), which is found in small amounts in RBC [Matsumoto & Miwa, 1973]. As shown in Table II, the addition of exogenous N-palmitoyl-PE to RBCs to pressure-treated RBC reduced the fluorescence anisotropy of DPH to the level obtained by incubation with the conditioned medium, while PE and PC did not affect it.

The morphology of RBC is closely related to the physical state and composition of the membrane [Daleke & Huestis, 1989], and deviation from the normal discocyte shape has been observed with RBC with altered membrane phospholipid composition [Shiga et al., 1990]. Thus, it is expected that changes in membrane fluidity and compositions of RBC induced by application of pressure are associated with morphological changes. Subsequently, we examined this possibility by scanning electron microscopy of RBC prior to and following application of

pressure, as well as after reincubation of pressure-treated cells with conditioned medium. As demonstrated in **Fig. 2**, application of pressure changes the RBC shape from biconcave to stomatocytic ("cup-shape"). Cell counts in large field photographs, as in Fig 2B, showed that after pressure treatment the stomatocytes accounted for at least 60 % of the cells. Here again this effect was reversed and the majority of the cells resumed the discocyte shape, by incubation of the pressure-treated cells with the conditioned medium, as demonstrated in Fig. 3C.

DISCUSSION

The present study demonstrates that application of hydrostatic pressure of several atmospheres is sufficient to induce considerable changes in the membrane fluidity and morphology of RBC. It should be emphasized that these properties were determined after the cells were returned to ambience following application of pressure. The pressure induces constitutive changes in the membrane, which may be reversed by interaction of the pressure-treated cells with extracellular fluid collected after application of pressure (the conditioned medium).

In the present study we used six different fluorescent lipid molecules, which probe differentially various areas of the membrane. Accordingly, as shown in Table II, some of these probes produce quite different RET values in RBC membranes. Of special interest are the differences in the fluorescence anisotropy between different lipid probes carrying the same fluorophore, i.e. between DPH and TMA-DPH (with a rod like fluorophore), and between AA, APC and ASM, which have a discoid fluorophore. The latter three probes are oriented in the

membrane with their long axis parallel to that of the surrounding fatty acyl chains. Moreover, the anthrylvinyl fluorphore was shown to induce only minor disturbance of the surrounding lipids when attached to the end of the fatty acyl chain [Bredlow et al., 1993]. In a homogenous environment, anthrylvinyl-labeled phospholipids with different polar headgroups show very close or even identical fluorescence parameters [Gromova et al., 1992]. Therefore, the relatively large differences in the RET-values of lipids with different head groups (APS and ASM) suggest that they distribute differently in the RBC membrane, and thus confirm indirectly the existence of lipid domains or microdomains in the RBC membrane.

Noteworthy is the finding that application of pressure affected the fluorescence parameters of different probes to a different degree. As shown in Table II, this treatment resulted in a significant increase in both the tryptophan energy transfer to, and the fluorescence anisotropy of DPH, AA and ASM, whereas with TMA-DPH, ANS and APC these parameters remained unchanged. It follows that application of pressure at this level may have a different effect on each area of the RBC membrane.

High speed centrifugation, at a wide range of angular velocity and durations, is vastly used in biological research, particularly in isolation of cell membranes for characterization of their properties. In these procedures high pressure, which might reach hundreds or even thousands of atmospheres, is applied for various durations, and is capable of altering the membrane composition. An example which is of special interest to the present study, are studies relating to RBC density or age. It has been reported that "old" RBC, which are assumed to be

heavier, and fall to the bottom layer of the packed cell phase in the spinning tube, have reduced membrane fluidity [Gareou et al., 1991]. Since in that procedure high speed centrifugation is used, the cells at the bottom layer are subjected to significantly higher pressure, which might be sufficient to induce changes in the membrane fluidity, as observed in the present study. It is plausible to suggest that when a biological system is subjected to centrifugation, the possible effects of hydrostatic pressure should not be ignored.

Impairment of physiological functions, particularly microcirculatory functions [Polkinghorne et al., 1989] have been observed in humans subjected to elevated pressure, such as in diving or hyperbaric chamber. Membrane fluidity is a regulatory factor in the activity of membrane components, such as phospholipid metabolism [Ko et al., 1994], and transmembrane activities, such as cation [Fu et al., 1992], glucose [Whitesell et al., 1989] and oxygen [Anezaki, 1988] transport. In addition, membrane fluidity is an important determinant in RBC rheological properties, particularly their deformability [Shiraishi et al., 1994]. The reduced fluidity of RBC membrane induced by physiological levels of hydrostatic pressure as observed here, implies that RBC function might be impaired by such pressures, and this might be pertinent to the physiological disorders observed among humans subjected to elevated hydrostatic pressure.

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Table I

Fluorescence anisotropy of lipid probes and energy transfer to tryptophan in pressure-treated human RBC membranes.*

PROBE	RET		Anisotropy	
	Control	After Pressure	Control	After Pressure
DPH	0.20 ± 0.02	0.29 ± 0.02	0.195 ± 0.003	0.268 ± 0.003*
AA	0.45 ± 0.03	0.51 ± 0.03	0.100 ± 0.002	0.125 ± 0.003*
ASM	0.20 ± 0.02	0.29 ± 0.01	0.123 ± 0.002	0.129 ± 0.001*
TMA-DPH	0.26 ± 0.02	0.26 ± 0.01	0.248 ± 0.002	0.250 ± 0.003
ANS	0.30 ± 0.03	0.30 ± 0.03	0.215 ± 0.001	0.215 ± 0.001
APC	0.12 ± 0.02	0.15 ± 0.02	0.119 ± 0.001	0.122 ± 0.002
Tryptophan	—	—	0.138 ± 0.003	0.170 ± 0.002*

*RBC ghosts were prepared from RBC which were subjected to a hydrostatic pressure of 15 atm, or from control RBC, which were subjected to the same procedure without significant pressure, and incubated the indicated lipid probe, as described in Experimental.

Each datum is Mean ± S.D. for three replications.

*Significant at $p < 0.01$.

Table II

Fluorescence anisotropy of DPH in ghosts of pressure-treated human RBC membranes.*

1.	Control RBC	0.184 ± 0.002
2.	Pressure- treated RBC	0.248 + 0.002
3.	Pressure-treated RBC incubated with	
	a. Fresh buffer	0.225 + 0.003
	b. Pressure-conditioned medium	0.210 + 0.005
	c. NAPE	0.204 + 0.004
	d. PC	0.225 + 0.003
	e. PE	0.222 + 0.005

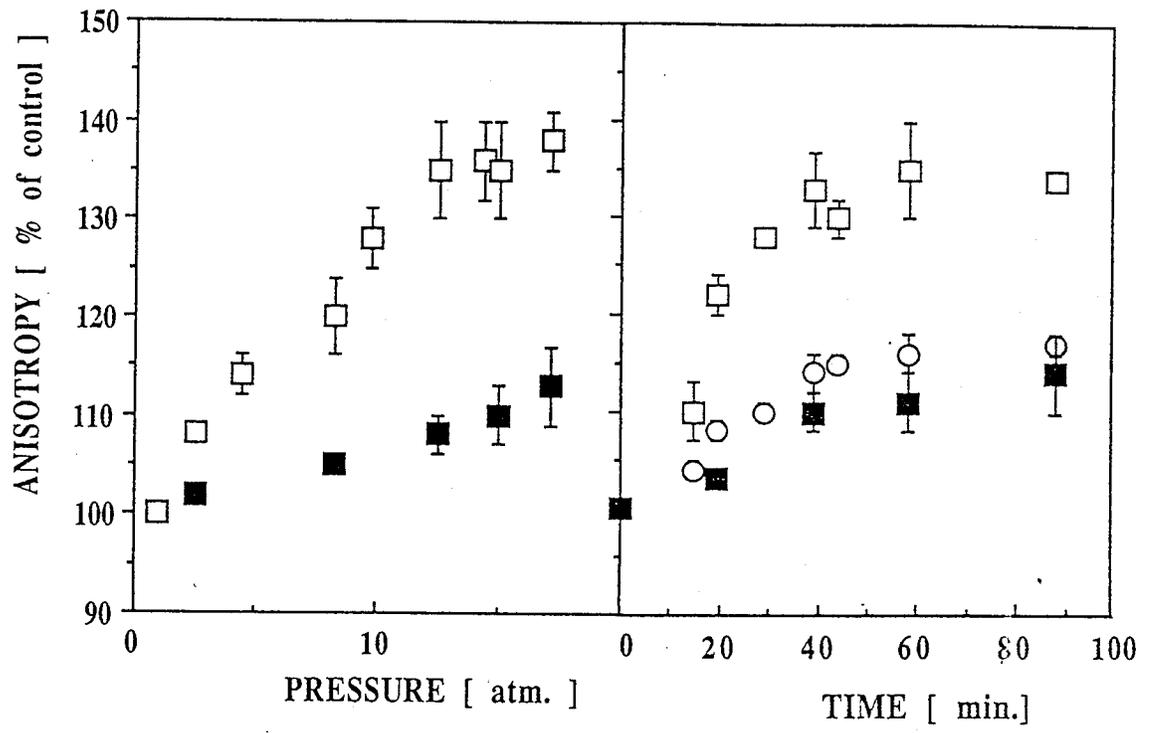
* The same experimental procedure as in the experiment of Table I was performed. In experiments 3a-3e the pressure-treated intact erythrocytes were incubated for 2.0 h at room temperature with either fresh buffer (a), pressure-conditioned medium (b), NAPE (c), PC (d), or PE (e), 10 $\mu\text{g}/\text{ml}$ each. The cells were then lysed and labeled with DPH as indicated above. Each datum is Mean \pm S.D. of triplicates.

LEGENDS TO FIGURES

Figure 1: Effect of application of hydrostatic pressure (1A) and of the duration of application on the fluorescence anisotropy of DPH and tryptophan in RBC ghosts: The symbols in 1A corresponds to DPH after 15 min (■) or 60 min (□) of application of pressure. The symbols in 1B corresponds to DPH after application of 15 atm (□) or 9 atm (■), and to tryptophan after application of 15 atm (○). See Experimental for details.

Figure 2: Effect of hydrostatic pressure on RBC morphology: The morphology of RBC was examined, by scanning electron microscopy, prior to (A) or after (B) application of 15 atm for 1 hour, and following reincubation in conditioned medium (C).

Barshtein et al. Fig. 1



Barshstein et al. Fig.2



A



B



C