MORPHOLOGY AND PATHOMORPHOLOGY

Effect of Peroxynitrite on Mechanical Properties of Human Erythrocyte Membranes

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Atomic force microscopy showed that treatment of human whole blood with peroxynitrite modified local mechanical and physical characteristics of erythrocyte membranes, specifically, increased their rigidity.

Key Words: *peroxynitrite; human erythrocytes; lipid domains; atomic force microscopy*

Peroxynitrite ($ONOO^-$; PN) belongs to the class of active nitrogen forms and is generated in humans under pathological conditions as a result of diffusion-controlled reaction between superoxide anion radical with nitrogen monoxide. PN not only oxidizes SH groups and aromatic amino acid residues in cell membrane and cytosol, but also induces LPO in biological membranes [2,3,8]. LPO modulates physicochemical parameters, ultrastructural organization, and functional characteristics of biological membranes and underlies the pathogenesis of many human diseases [6,12,13]. It is hypothesized that LPO processes cause cell membrane reorganization due to phase separation of lipids, formation and enlargement of lipid domains with different physical mechanical characteristics [7]. At 4-40°C, the lipid component of biomembranes is presented by a mixture of gel and liquid phases. The presence of cholesterol in biomembranes promotes the formation of one more L_0 phase, a liquid phase of long range ordering, combining the properties of two previous phases [7]. Domains with different lipid phases differ by their thickness and physical mechanical characteristics. Phase L_0 domains, several tens of nanometers in diameter, form so-called rafts, functional units of cell membrane [7,9]. During LPO, unsaturated lipids (liquid phase lipids) are converted into saturated (gel phase lipids). The area occupied by the gel phase increases with increasing the concentration of saturated lipids. These lipid phase separation processes are observed during cell membrane cooling [7]. However, the mechanism of membrane reorganization in LPO processes, for example, induced by active nitrogen forms (PN) in a single cell, is not quite clear. Methods for studies of nanostructures, atomic force microscopy (AFM), can help to clear out the problem.

Here we detected local changes in mechanical and physical characteristics of human erythrocyte membrane by AFM after PN treatment of the whole blood.

MATERIALS AND METHODS

PN was obtained in $NaNO_2$ and H_2O_2 reaction in acid water solutions with their subsequent rapid alkalization [10]. To this end 100 ml 1.2 M $NaNO_2$ was rapidly mixed with 100 ml H_2O_2 (10 ml 30% H_2O_2, 60 ml 1 M HNO_3, 30 ml H_2O) and 100 ml 1.5 M NaOH was added to the mixture. H_2O_2 ex-

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cess was removed by filtration of PN solution through granulated MnO_2. PN solution was frozen and stored at -20°C for 3-7 days, after which its upper (yellow) layer was used as the working solution of PN. The concentration of PN was evaluated spectrophotometrically (ε_{302nm}=1670 $cm^{-1}M^{-1}$). $NaNO_2$, NaH_2PO_4, and Na_2HPO_4 from Reakhim were used.

Blood samples from volunteers was incubated with PN (2.5 mM) for 10 min at ambient temperature (experimental samples; n=16). Blood samples not treated with PN serves as control (n=8).

The degree of oxidative stress in experimental samples was evaluated by hemoglobin oxidation. The content of methemoglobin in control samples did not exceed 4-5%, while in experimental samples its level was ≥96-100%. Erythrocytes were fixed in 1% glutaraldehyde for 1 h, washed in phosphate buffer and 3 portions of distilled water. Erythrocyte suspension was applied onto glass for examination of cell surface by the AFM method in the air.

Erythrocyte surface was examined by AFM under an NT-206 microscope (Mikrotestmaschiny Company) in the static scanning mode. Standard probes NSC11 (MikroMasch) were used. Topographic images, torsion angle, and deviation of the microscope console were registered in this mode. The torsion angle images (lateral force map) are interpreted as the map of mechanical properties heterogeneity and the object composition at the scanned site (property map). The study was carried out at first on 25×25 μ sites, in which discocytes were selected, and then on 5×5 μ surface sites of these cells. A total of 8-16 lateral force maps were analyzed for samples of each type (16 μ^2). Quantitative evaluation of mechanical properties of membrane sites was carried out by static force spectroscopy. The values of local elasticity modulus (E) in an i point of the examined surface was calculated by the formula:

$$E_i = \frac{3}{4}(1-\omega^2)\frac{k}{R^{1/2}}\frac{Z_{defl}}{(Z_{pos}-Z_{defl})^{3/2}},$$

where ν is Poisson's coefficient for the sample, R radius of the probe tip rounding, k probe console rigidity, Z_{defl} console deflection value, and Z_{pos} the probe position [1]. The elasticity modulus decreases with deeper insertion of the AFM probe tip and deformation of membranes of erythrocytes fixed by glutaraldehyde (Fig. 1). The maximum depth of the probe insertion was no more than 500 nm. Elasticity modulus values at the probe insertion into the depth of 500 nm (18.7±7.5 MPa) was taken for elasticity modulus characteristic of a certain erythrocyte membrane and was used for comparing the mechanical properties of experimental and control samples. Comparison of the selected means was carried out using Student's t test with consideration for Fisher's test.

RESULTS

Physical properties of erythrocyte membrane changed after PN treatment of the whole blood. The distribution of elasticity modulus for membranes of PN-treated erythrocytes was bimodal. In one group of data (n=10) the elasticity modulus was within the confidence interval for the mean Young modulus of control membranes and was 22.0±1.5 MPa; in the other it was significantly (p<0.001) higher in comparison with the values characteristic of control membranes and was 29.2±2.6 MPa (n=6).

PN caused changes in erythrocyte membrane structure. Microareas with different mechanical and physical properties emerged on the membrane of the same erythrocyte. The location of the lipid domains can be examined using maps of lateral forces of different sites of erythrocyte membranes. The lateral force maps for membranes of experimental samples were characterized by higher values of AFM probe tip torsion angle in comparison with the values characteristic of control samples (Fig. 2). Areas of different configuration with different values of torsion angle values were seen on PN-treated erythrocyte membrane lateral forces maps (Fig. 2). Changes in the lateral forces were not paralleled by changes in the membrane topology and erythrocyte shape, as only discoid erythrocytes were selected as control and experimental samples. The changes in the composition and structure of the membrane lipid component could be evaluated by

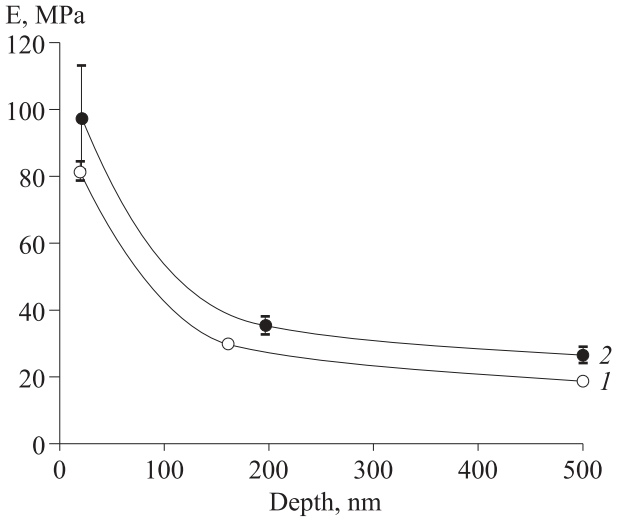

Fig. 1. Relationship between membrane elasticity modulus of erythrocytes fixed by glutaraldehyde and the depth of insertion of atomic force microscope probe tip into the cell. *1*) control (n=8); *2*) experimental samples (n=16).

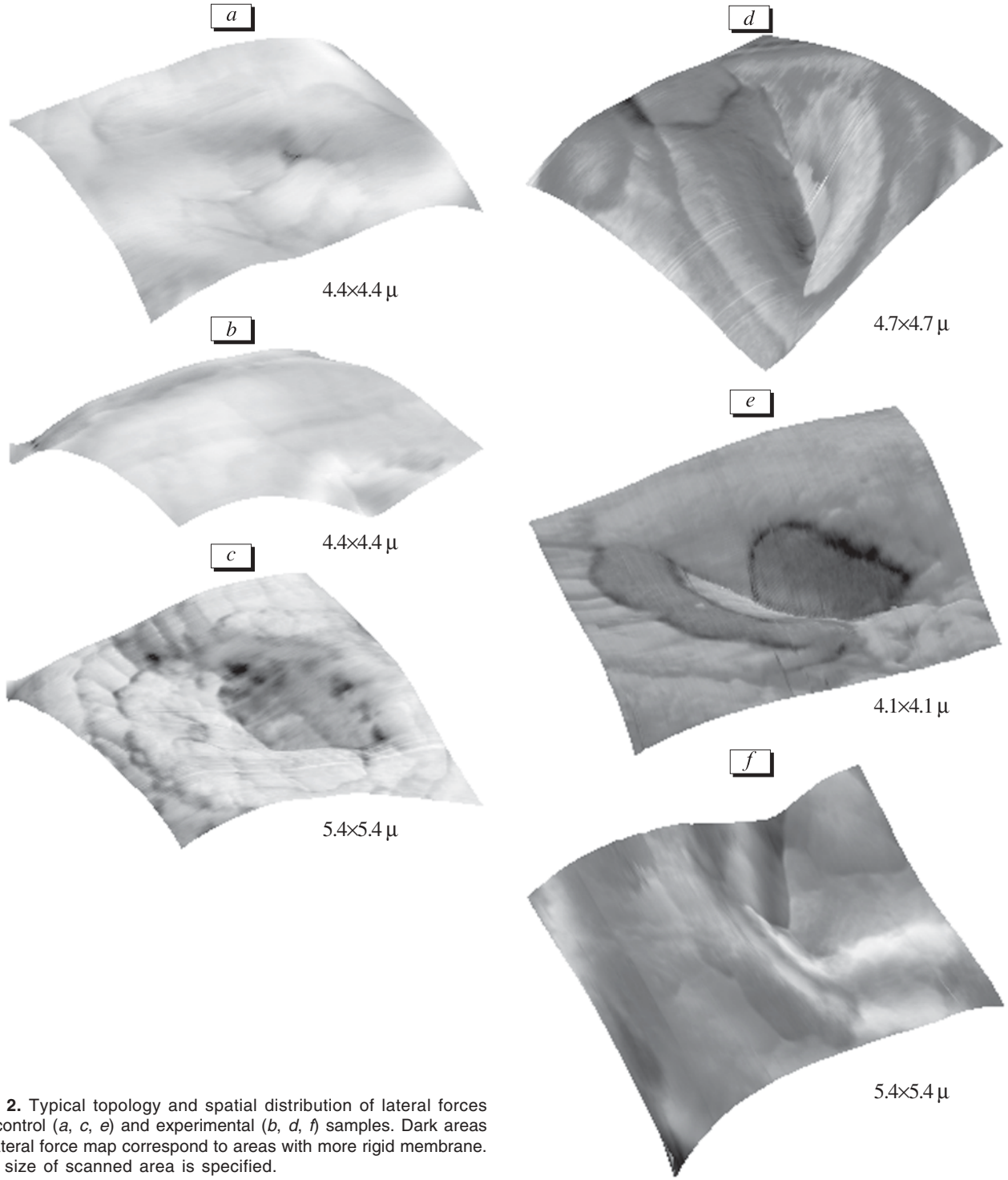

Fig. 2. Typical topology and spatial distribution of lateral forces for control (*a*, *c*, *e*) and experimental (*b*, *d*, *f*) samples. Dark areas of lateral force map correspond to areas with more rigid membrane. The size of scanned area is specified.

the lateral force maps due to equivalence of membrane shape in control and experimental samples.

Changes in the probability of lateral force distribution density on erythrocyte membrane surface sites after PN treatment reflected the physical mechanical properties of erythrocyte membranes (Fig. 3). The mode of lateral force distribution for experimental samples was shifted towards higher values in comparison with the distribution mode for control samples. The peak of lateral force distribution for experimental samples was wider than for control samples.

Changes in the parameters of PN-treated erythrocyte membranes, fixed by AFM methods, indicate changes in their composition, mechanical characteristics, and structure. Lipid fractions with

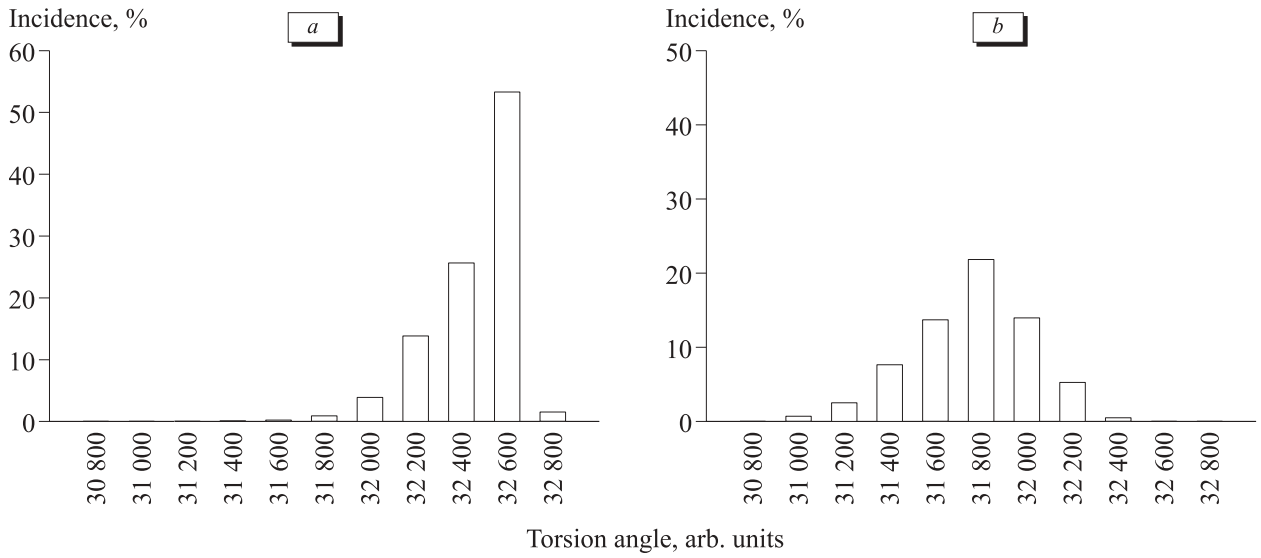

Fig. 3. Lateral force distribution for membranes of intact (*a*) and PN-treated erythrocytes (*b*). Medians of samplings of 6 cells are presented. Torsion angle distribution mode for control erythrocyte membranes: 32,600 arb. units; for experimental samples: 31,800 arb. units.

unsaturated and saturated (oxidized) fatty acids, occupying separate areas on the membrane, were detected on membranes of PN-treated erythrocytes. The formation of lipid domains with different composition leads to a sharp increase in the heterogeneity of erythrocyte membrane structure and properties in the same cell. This can serve as a mechanism stimulating various cell processes with participation of membrane enzymes and as a mechanism of barrier function loss by the cell [4,5]. It is known that some membrane proteins are included in domains with a certain lipid phase, while others are excluded from them because of specific structure and physical properties of these lipid domains and proteins [7]. Redistribution of membrane proteins, changes in their environment, and formation of LPO products modulate the membrane protein functions [7,11]. As a result, the cell exposed to an oxidizing agent, can react to an external stimulus by different modes.

Hence, due to AFM methods we detected changes in the local mechanical and physical properties of human erythrocyte membranes after treatment of whole blood by PN, as well as increase of these cells' membrane rigidity in comparison with the membranes of erythrocytes not exposed to an oxidizing agent.

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