Effect of Structural Modification of Membrane Proteins on Lipid-Protein Interactions in the Human Erythrocyte Membranes

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UDC 616.98:578.832.1

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol.116, № 11, pp. 488-491, November, 1993
Original article submitted April 5, 1993

Key Words: *erythrocytes; lipid-protein interaction; molecular probes*

During pathological processes, structural alterations in the erythrocyte membrane play a key role in determining the functional changes taking place in cells and in the recognition and selective removal of senescent erythrocytes. The cytoplasmic surface of the erythrocyte membrane is stratified with a protein skeleton, which contributes to membrane stabilization, regulation of cell shape and deformability, and limitation of the lateral mobility of transmembrane proteins [1-3]. There is evidence that modification of the protein skeleton goes along with alterations in the structure of the erythrocyte membrane, including the changes of membrane protein mobility, exposure to the senescent cell antigen, increased susceptibility to proteolysis, and conformational changes in the membrane proteins [2-4]. This, in turn, affects the dynamics of the lipid matrix, and causes an increase of membrane permeability and erythrocyte hemolysis.

Recently, it has been suggested that the disturbances of membrane function which are caused by active forms of oxygen [2-4] and by nitric oxide represent a certain molecular mechanism of the development of various pathological processes [5]. In order to explore the effect of oxidation stress on the lipid-protein interactions in the erythrocyte membrane, we used a number of protein-modifying agents, such as tert-butylhydroperoxide (TBH) and N-ethylmaleimide [2,3]. The changes in motility of the lipids forming the microenvironment of membrane proteins were assessed as the eximer-monomer ratio during pyrene steady-state fluorescence caused by emission energy transfer from tryptophan residues to nearby pyrene molecules. The effect of reagents on the bulk lipid matrix was monitored by the method of spin probes, using merocyanin 540.

MATERIALS AND METHODS

Blood was drawn from the vein of healthy volunteers into heparinized tubes and handled within 30 min of collection. Erythrocytes were isolated by a 5-min centrifugation and then thrice resuspended and washed with 1 mM Ringer solution buffered with sodium phosphate (pH 7.4).

Pyrene, a fluorescent probe which lodges in the hydrophobic portion of the lipid matrix, was used for monitoring the state of the microenvironment of membrane proteins [6,7]. Ethanol solutions of pyrene were added to 1 ml of a 4% erythrocyte suspension so that the final ethanol concentration in the solution did not exceed 0.1%. Following a 30-min incubation at 37°C, the erythrocytes were centrifuged and twice treated with various concentrations of TBH, with 0.5 mM diamide, or with 0.5 mM N-ethylmaleimide (10-min incubation at 4°C). The accumulation of fluores-

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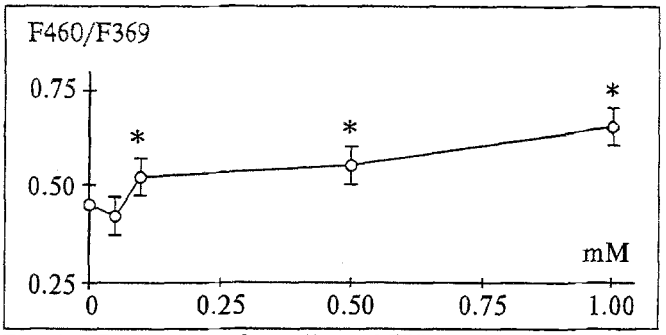

Fig. 1. Dose–dependent effect of TBH on eximer/monomer ratio in microenvironment of membrane proteins. An asterisk indicates reliability of differences vs. the control ($p<0.01$).

cent products of lipid peroxidation was measured in the erythrocyte membranes as described previously [5]. The treated erythrocytes were washed twice with Ringer solution. Formation of pyrene eximers, attended by fluorescence energy transfer from tryptophan residues of the protein, offered a means of monitoring the changes of lipid-protein interaction in the hydrophobic microenvironment of intrinsic proteins. The maximal excitation wavelength of pyrene was 332 nm. The maximum emission spectra of monomers were observed at 375 nm. The fluorescent signal corresponding to eximer formation was determined to appear at 460 nm. The maximum intensity of tryptophan fluorescence for the membrane proteins was detected at 360 nm (the excitation wavelength being 282 nm [8]). This spectrum largely overlaps with the absorption spectrum of pyrene, this providing for effective transfer of energy from tryptophan residues to pyrene molecules.

The rotational mobility of monomer and eximer molecules of pyrene was monitored by assessing parameter P, characterizing the steady-state fluorescence depolarization [9]. The membrane protein-induced depolarization of excitation light of pyrene was monitored with the use of erythrocyte ghosts; the value obtained constituted 0.42±0.03.

Merocyanin 540 was used for assessing the effect of reagents on the lipid packing in the bulk lipid matrix [10]. The maximum fluorescence spectrum of the dye incorporated in the membranes of intact erythrocytes was detected at 594 nm (excitation wavelength 360 nm). The fluorescence spectra were recorded on a Perkin-Elmer LS-SB luminescence spectrophotometer equipped with thermostatically controlled compartments for the cells. The spectra were corrected with respect to the background intensity of fluorescence (Raman spectrum peak) (slit width 5 nm). Fluorescent-labeled cells were examined with a Polivar Reicher-Jung epifluorescence microscope. Monitoring of the lipid bilayer in erythrocytes was performed by the method of spin probes. The final concentration of 16-doxylstearic acid (16-DS) in a 50% erythrocyte suspension in buffered Ringer solution was 10^{-5} M. Spin-labeled erythrocytes (50 μl) were aspirated in flat quartz cells thermostated at a temperature within the limits of the structural transition of human erythrocyte membranes (21±0.5°C). The spectra were obtained on a spin-resonance spectrometer (Svetlana, St. Petersburg) at a central field strength of 0.328 Tl, scanning time 8 min per 0.01 Tl, time constant 0.25 sec, modulation amplitude 2.0 G, and microwave power 20 mW. The motility of lipid methyl groups in the erythrocyte membranes was assessed after Henry and Keith [11] by calculating the correlation time (τ) of rotation of spin-labeled fatty acid.

Pyrene, merocyanin 540, diamide, N-ethylmaleimide, TBH, and 16-DS were supplied by Sigma Chemicals (USA).

The results were expressed as $M\pm m$ of six experiments. The ratio between the fluorescence intensities of pyrene monomers and eximers is

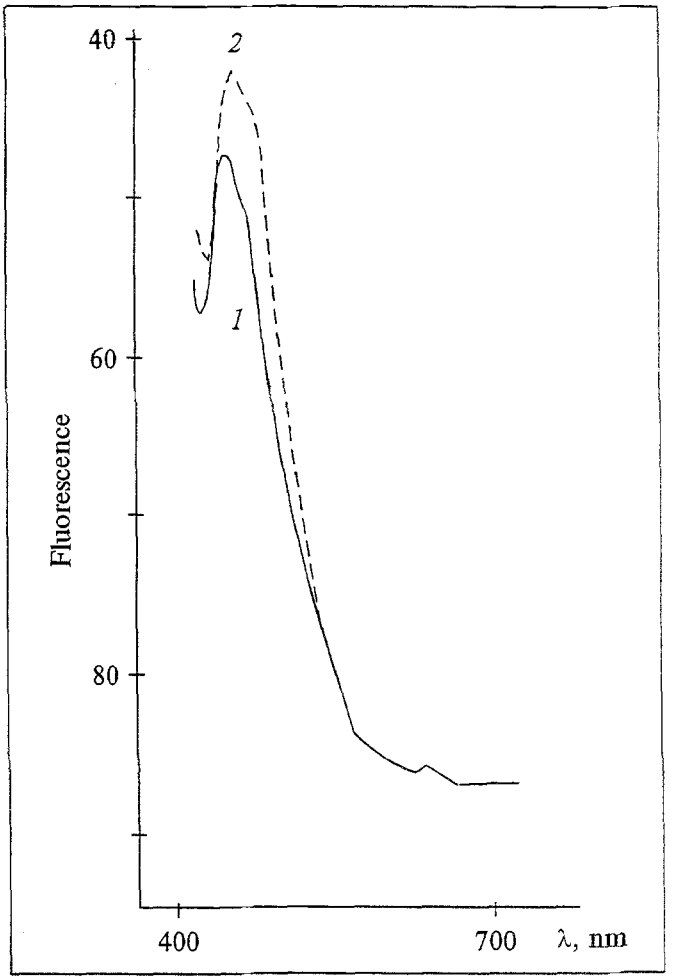

Fig. 2. Accumulation of fluorescent products of lipid peroxiadation caused by 0.1 mM TBH in erythrocyte membranes. *1*) control; *2*) effect of TBH.

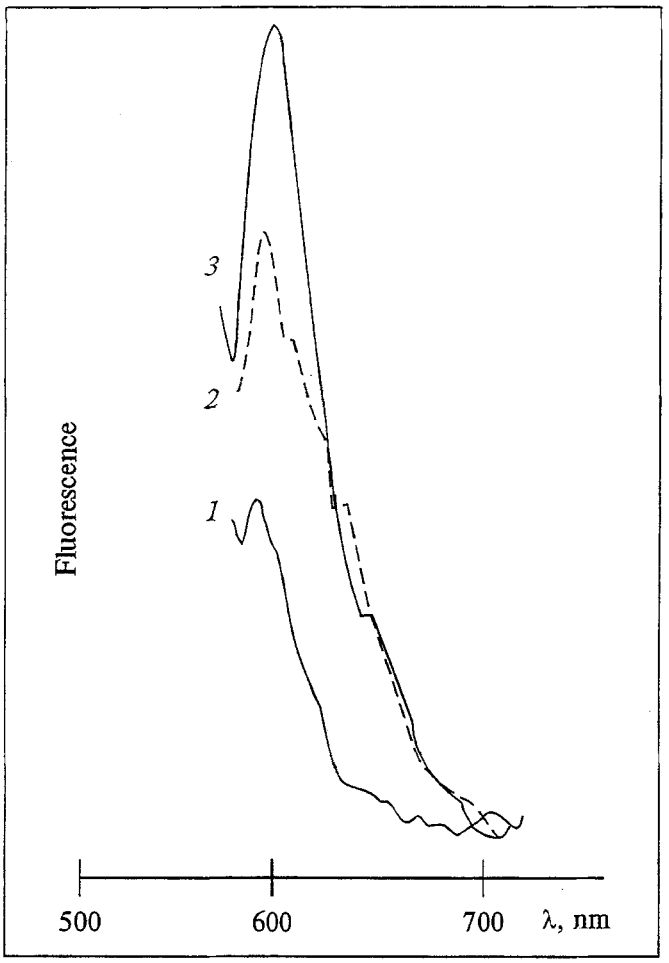

Fig. 3. Effect of modification of membrane proteins on merocyanin 540 binding in erythrocytes. *1*) control; *2*) 0.5 mM N – ethylmaleimide; *3*) 0.1 mM TBH.

presented in relative units. The data were compared using the paired Student *t* test.

RESULTS

In the preliminary experiments the eximer/monomer ratio of pyrene was determined depending on the concentration of pyrene added to the erythrocyte suspension. These values for the membranes of intact erythrocytes were constant over a wide range of pyrene concentrations (10^{-8}-10^{-6} M) and constituted 0.47±0.02 (excitation wavelength 282 nm). We found no effect of erythrocyte hemoglobin on the fluorescence response of pyrene incorporated in the erythrocytes. On the whole, the eximer/monomer ratio in the bulk lipid matrix constituted 0.28±0.02 (excitation wavelength 332 nm). The pyrene depolarization parameter (P) in the bulk lipid matrix was 0.53±0.02 and 0.70±0.04 for monomeric and eximeric forms, respectively. The values of P for pyrene localized in the microenvironment of membrane proteins were 0.64±0.02 and 0.82±0.05, respectively. Analysis of the data obtained suggests that a high level of eximer formation in the microenvironment of the proteins results from the nonspecific binding of pyrene by the hydrophobic moiety of intrinsic proteins. On the basis of this hypothesis we speculated that the eximer/monomer ratio of pyrene depends on diamide- and N-ethylmaleimide-induced alterations in the architecture of the membrane proteins.

Figure 1 shows a reduction of eximer formation caused by polymerization of membrane proteins following treatment with diamide (which cross-links the SH groups). On the other hand, we observed no effect of diamide upon the bulk lipid bilayer monitored by the fluorescence and spin-probe methods. No differences between the intact and diamide-treated erythrocyte membranes were revealed wiht regard to the level of pyrene eximer formation in the bulk lipid matrix (excitation wavelength 332 nm). It was found that the value of τ for 16-DS in the diamide-treated erythrocyte

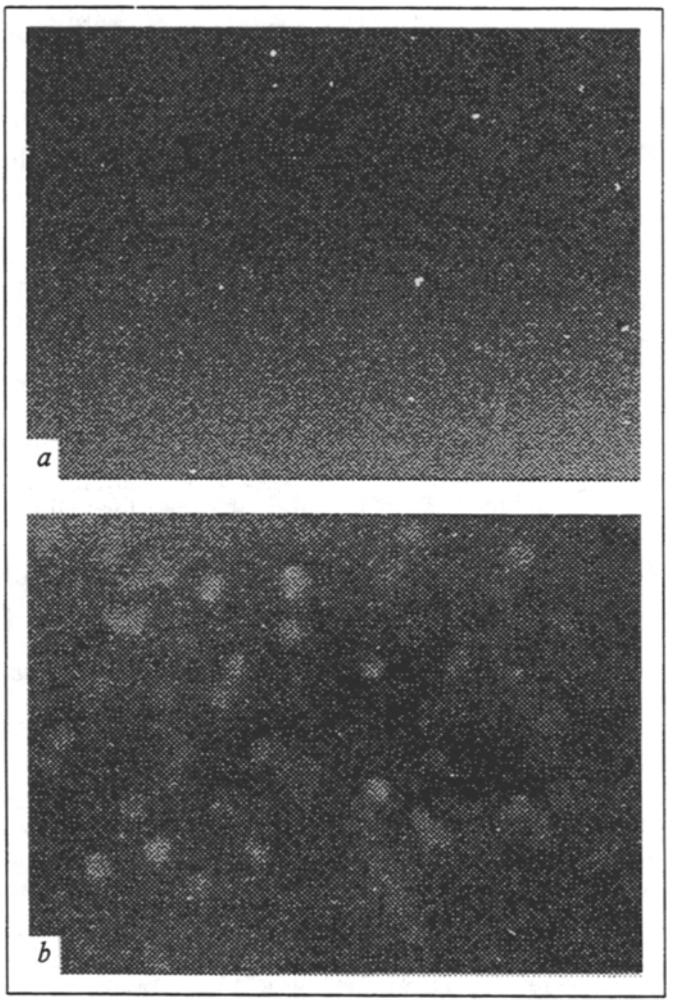

Fig. 4. Staining of erythrocytes with merocyanin 540. *a*) control; *b*) human erythrocytes stained with merocyanin 540 after treatment with 0.1 mM TBH.

TABLE 1. Ratio between Intensities of Fluorescence of Pyrene Eximers and Monomers ($M \pm m$, 6 experiments)

Reagent (0.5 mM)	Fluorescence at 460 nm	Fluorescence at 369 nm	(excitation wavelength 280 nm)
Control		0.44±0.01	
TBH		0.48±0.02*	
N-ethylmaleimide		0.50±0.02*	
Diamide		0.38±0.03*	

Note. Reliability of differences shown by asterisks: one asterisk: $p<0.001$; two asterisks: $p<0.01$.

membranes corresponds to the same parameter in intact membranes ($\tau=1.10\pm0.05$ nsec). The distortions of membrane protein architecture resulting from breakage of disulfide bonds by N-ethylmaleimide were attended by an effect opposite to the effect of diamide. This led to an increase of the eximer-monomer ratio in the microenvironment of membrane proteins in the N-ethylmaleimide-treated erythrocytes (Table 1). In the bulk lipid matrix we observed no changes caused by the molecular probes (data not presented).

In another series of experiments we used TBH, which caused oxidation stress. The treatment of erythrocytes with this substance led to an increase of the eximer/monomer ratio (Table 1) in the microenvironment of intrinsic proteins. The degree of this increase depended on the TBH concentration used (Fig. 1). This effect was not a result of accumulation of lipid peroxidation products (Fig. 2), which are known to reduce membrane rigidity, i.e., to lower the level of pyrene eximer formation. This chaotropic effect may be caused by TBH. In fact, the mobility of intrinsic proteins anchored by cytoskeletal proteins is known to affect the structural transitions in the lipid matrix [3,12-14]. Thus, TBH-induced protein destruction can lead to destruction of the lipid microenvironment of intrinsic proteins.

The data in Figs. 2, 3, and 4 show that TBH-induced oxidation stress goes along with increased merocyanin 540 incorporation in the membranes. This provides evidence that the action of TBH on the lipid matrix of erythrocyte membranes is accompanied by a chaotropic effect. In addition, taking into account that the effect of N-ethylmaleimide is also attended by increased merocyanin 540 incorporation, it may be assumed that TBH- and N-ethylmaleimide-induced modification of membrane proteins results in the destruction of the lipid microenvironment of intrinsic proteins.

Thus, the rearrangement of the membrane protein network which is caused by the oxidation stress is attended by an increase in the mobility of the molecules forming the microenvironment of membrane proteins.

The author is grateful to Prof. A. Arduini (Instituto di Scienze Biochimiche, Universita' degli Studi G. D. Annunzio, Chieti, Italy) for his support of this study and for helpful criticism and revision of the manuscript.

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