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AGING OF THE ERYTHROCYTE

IV. SPIN-LABEL STUDIES OF MEMBRANE LIPIDS, PROTEINS AND PERMEABILITY

GREZEGORZ BARTOSZ

Department of Biophysics, Institute of Biochemistry and Biophysics, University of Łódź, Banacha 12/16, 90-237 Łódź (Poland)

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Spin-label studies demonstrated age-related alterations of the erythrocyte membrane concerning both lipid and protein components. Decrease in fluidity of membrane lipids correlated with decreased membrane permeability to a hydrophobic spin label TEMPO, permeability to a more hydrophilic TEMPOL being less affected. The rigidification of membrane lipids was much more pronounced in whole membranes than in liposomes composed of membrane lipids, suggesting changes in lipid-protein interactions as an important factor in the decrease of lipid fluidity in aged red cells. ESR spectra of membrane-bound maleimide spin label evidenced alterations in the state of membrane proteins during cell aging in vivo.

Introduction

Alterations in membrane properties must belong to the main events in the intravascular aging of the red blood cells. The membrane interacts with extracellular environment and changes in the membrane are probably responsible for the selective removal of senescent erythrocytes from circulation. It has been suggested that such membrane alterations as descrease in deformability [1] or exposure of specific antigens [2] permit recognition of aged erythrocytes in situ.

The fluidity of membrane lipids may affect the course of cell immune reactions and, additionally, may influence cellular deformability [3,4]. A recent spin-label study demonstrated a decrease in lipid fluidity of in vivo aged human erythrocytes [5]. This report confirms that finding in bovine red cells, lending further evidence in support of the view that the decreased lipid fluidity of aged erythrocytes is conditioned mainly by altered lipid-protein interactions. Moreover, spin-label data presented here point to functional counterparts of the lipid rigidification and indicate age-related changes in the state of erythrocyte membrane proteins.

Materials and Methods

Bovine blood was obtained in an abbatoir. Erythrocytes were separated according to density (and presumably age) by the metod of Murphy [6]. The stratified cell column was divided into six equally sized fractions. The validity of this procedure for age separation of bovine red cells will be discussed elsewhere (unpublished observations).

Erythrocyte membranes were isolated by the method of Dodge et al. [7]. Membrane lipids were extracted after Folch et al. [8]. Cholesterol was estimated according to Babson et al. [9], lipid phosphorus according to Bartlett [10] and protein by the method of Lowry et al. [11] with the modification of Lees and Paxman [12]. Liposomes were prepared from extracted lipids by vortexing vessels containing deposited lipid films in phosphate-buffered saline (152 mM NaCl in 10 mM sodium phosphate, pH 7.4). Spin labels (Fig. 1): methyl 5-doxylpalmitate (I),

Abbreviations: TEMPO, 1-oxyl-2,2,6,6-tetramethylpiperidine; TEMPOL, 1-oxyl-2,2,6,6-tetramethylpiperidin-4-ol.

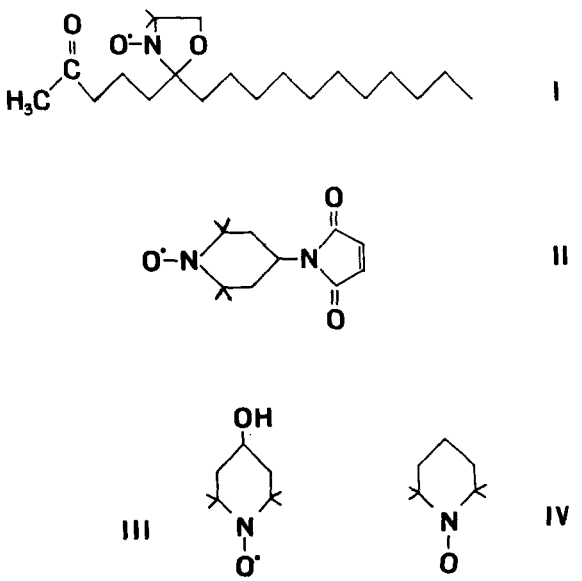

Fig. 1. Spin labels used.

N-1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl-*N*-maleimide (II), *N*-1-oxyl-2,2,6,6-tetramethylpiperidin-4-ol (III; TEMPOL) and *N*-1-oxyl-2,2,6,6-tetramethylpiperidine (IV; TEMPO) were synthesized and kindly provided by Dr. K. Gwoździński from this Department.

Spin-labeling of erythrocyte membranes was performed as described elsewhere [13]. Labeled liposomes were prepared by adding the spin label to the chloroform solution of lipids evaporated for film formation. Transport of compounds III and IV across the erythrocyte membrane was measured by monitoring the rate of disappearance of the electron spin resonance (ESR) signal of these labels from erythrocyte suspensions [14].

Results

(i) Membrane lipid fluidity

The ESR spectrum of bovine erythrocyte membranes labeled with compound I is shown in Fig. 2. From spectra of this type, the order parameter, S, of membrane lipids was calculated [13]. This parameter increased with increasing density (mean age) of erythrocyte fractions of which the membranes were prepared (Table I). Liposomes prepared from these membranes showed a much smaller increase in rigidity.

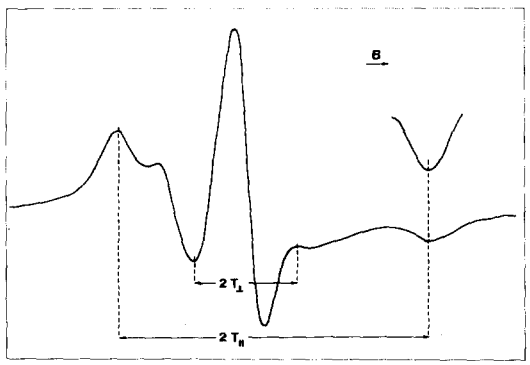

Fig. 2. ESR spectrum of spin label I embedded in bovine erythrocyte membranes prepared from the lighest cell fraction (fraction 1).

The content of both cholesterol and lipid phosphorus decreased with increasing cell density when expressed per g of membrane protein. The magnitude of this decrease was practically the same for both these compounds so the elevation of lipid rigidity cannot be due to an elevation of the cholesterol/phospholipid ratio (Table II).

(ii) Membrane permeability to non-electrolytes

In view of the relation between membrane lipid fluidity and permeability to substances diffusion through the lipid phase of the membrane [15], it seemed worthwhile to examine whether the lipid rigidification in aged erythrocytes involves alterations in membrane permeability. Therefore transport of two non-electrolyte spin labels (compounds III and IV) of different hydrophobicity into erythrocytes of different age fractions was studied. The method used is based on a rapid reduction to non-paramagnetic derivatives inside the cell. As the membrane transport is the rate-limiting event in this process, the rate of permeation of nitroxide compounds can be followed by monitoring the rate of decrease of ESR signal of the permeant introduced into erythrocyte suspension [14].

Kinetics of permeation of both labels obeyed a simple exponential relationship within the incubation period employed (up to 1 h) at ambient temperature:

$$c = c(0)\exp(-kt)$$

where: c is concentration of a label, t is time, k is the permeation constant and $h \propto c$ (h is the height of the

TABLE I

ORDER PARAMETERS OF LIPIDS

Order parameter,

$$S = \frac{T_{\parallel} - T_{\perp}}{2.63\ \text{mT}} \cdot \frac{1.487\ \text{mT}}{1/3(T_{\parallel} + 2T_{\perp})}$$

of lipids of different density (age) fractions of bovine eythrocytes measured in membranes and in liposomes prepared of membrane lipids (mean ± S.D., $n = 6$)

Fraction No.	S (Relative) (%)	
	Membranes	Liposomes
1 *	100 **	100 ***
2	100.7 ± 0.7	100.0 ± 0.6
3	101.8 ± 0.5	100.4 ± 0.7
4	101.0 ± 1.0	100.3 ± 0.8
5	102.4 ± 0.8	100.9 ± 0.7
6	103.0 ± 0.8	101.1 ± 0.7

* Lightest.
** $S = 0.701 \pm 0.008$.
*** $S = 0.673 \pm 0.006$.

midfield line of the ESR spectrum) (Fig. 3). Data presented in Table III demonstrate a considerable decrease in the rate of permeation of TEMPO (a moderately hydrophobic compound) and a slight change in that of TEMPOL (a more hydrophilic compound).

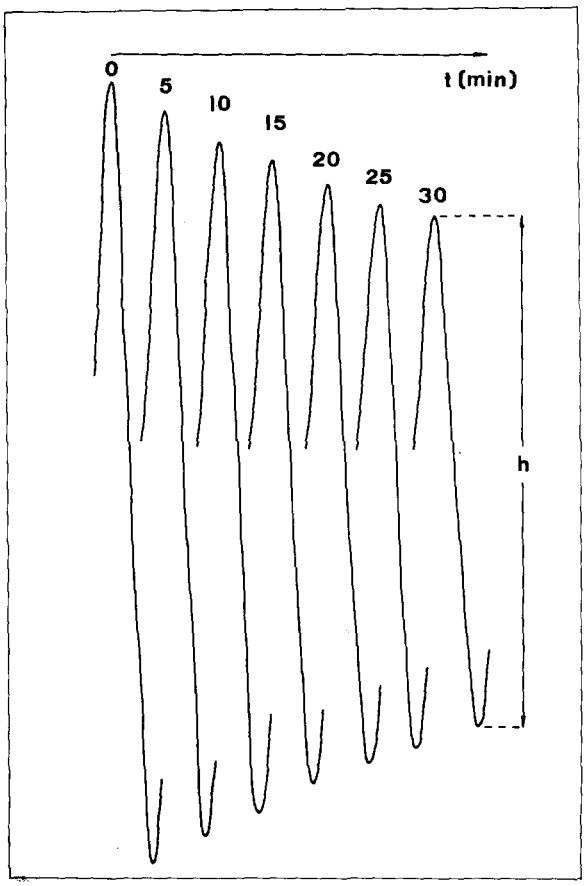

Fig. 3. Permeation of spin label IV into bovine erythrocytes (raction 1). Changes in intensity of the midfield line of a triplet spectrum. Temperature, (20 ± 1)°C; Cell volume fraction, 0.5; $c(0)$, 100 μM.

TABLE II

CHOLESTEROL AND LIPID PHOSPHORUS CONTENT OF MEMBRANES ISOLATED FROM DIFFERENT DENSITY FRACTIONS OF BOVINE ERYTHROCYTES

Contents are given as percentages relative to the fraction 1 values

Fraction No.	Cholesterol (Chol.)	Lipid phosphorus (P_i)	Chol./P_i
1	100 *	100 **	0.86
2	98.6 ± 5.3	96.6 ± 2.4	0.88
3	98.4 ± 2.9	95.1 ± 7.0	0.89
4	95.2 ± 6.9	91.5 ± 5.8	0.90
5	92.4 ± 6.6	88.3 ± 5.2	0.90
6	89.9 ± 7.8	87.2 ± 5.9	0.89

* Content: 703 ± 113 μmol/g membrane protein.
** Content: 815 ± 98 μmol/g membrane protein.

TABLE III

PERMEATION CONSTANTS OF SPIN LABELS III AND IV INTO BOVINE ERYTHROCYTES OF DIFFERENT DENSITY FRACTIONS

Constants (k) are expressed as percentages relative to the fraction 1 values

Fraction No.	TEMPOL	TEMPO
1	100 *	100 **
2	98.1 ± 7.7	89.1 ± 10.2
3	96.2 ± 5.4	84.3 ± 10.7
4	93.0 ± 5.3	76.3 ± 9.6
5	94.0 ± 6.3	70.1 ± 7.7
6	91.1 ± 5.6	64.1 ± 7.2

* $k = (1.05 \pm 0.13) \cdot 10^{-4}\ s^{-1}$.
** $k = (2.97 \pm 0.24) \cdot 10^{-4}\ s^{-1}$.

TABLE IV

THE h_{s+1}/h_{w+1} RATIO FOR MEMBRANES PREPARED OF DIFFERENT DENSITY FRACTIONS OF BOVINE ERYTHROCYTES, LABELED WITH COMPOUND II

Ratios are expressed as percentages relative to the fraction 1 value.

Fraction No.	h_{s+1}/h_{w+1}
1	100 *
2	99.1 ± 1.3
3	96.7 ± 1.7
4	95.4 ± 2.7
5	91.6 ± 3.3
6	88.9 ± 5.4

* h_{s+1}/h_{w+1} = 0.626 ± 0.016.

(iii) Changes in membrane proteins

In evaluation of ESR spectra of the maleimide spin label (II) bound to proteins, the h_{s+1}/h_{w+1} ratio is usually employed as a relative measure of the sizes of the weakly and strongly immobilized populations, respectively, of the label residues [13]. This ratio showed a regular decrease with increasing erythrocyte density (Table IV), indicating an increase in the contribution of weakly immobilized residues of II.

Discussion

The present results demonstrate that also in the cow, erythrocyte aging in vivo involves an increase in membrane lipid rigidity. In conjunction with the previous study [5], these data evidence that this rigidification is not confined to the hydrophobic zone of the membrane (fatty acid analogs with the nitroxide group bound to the 12th and 16th carbon atoms, respectively) [5] but spreads over the region close to the hydrophilic surface of the bilayer (spin label I with the nitroxide group coupled to the fifth carbon atom). The decrease in membrane lipid fluidity brings about a decrease in the rate of permeation of TEMPO, likely to diffuse through the lipid phase of the membrane. Inhibition of the transport of a more hydrophilic TEMPOL, probably able to make use of hydrophilic channels in the membrane, is definitely less (Table III).

There can be several factors responsible for changes in membrane lipid fluidity, the most important including: (i) the cholesterol/phospholipid ratio; (ii) the degree of unsaturation and length of phospholipid acyl chains; (iii) the lecithin/sphingomyelin ratio and (iV0 the lipid/protein ratio in the membrane [16]. In the present case, the first factor is apparently not relevant (Table II). No data are available on the alterations in phospholipid and fatty-acid composition of bovine red cells of various ages. In other species (human, rabbit) no or only small changes in distribution of phospholipid classes and only small alterations in the content of polyunsaturated fatty acids were found to accompany the red cell aging [17–19]. On the other hand, the lipid/protein ratio decreased in older erythrocytes (Table II), possibly due to a release of protein-poor vesicles during the aging [20,21], and liposomes prepared from lipids of erythrocytes of different age classes showed much smaller changes in rigidity than lipids in intact membranes (Table I). This suggests that alterations in membrane proteins and lipid-protein interactions may be the main cause of cell age-related rigidification of membrane lipids.

ESR spectra of spin label II bound to erythrocyte membrane protein -SH groups [22] evidence changes in the state of membrane proteins during erythrocyte aging (Table IV). These changes can result from alterations in the conformation or composition of membrane proteins. However, the lack of significant changes in the composition of membrane proteins during erythrocyte aging in the cow (unpublished results) as well as in other species [19] speak in favor of progressive conformational transitions of membrane proteins in this process. Whatever the mechanism of these transitions, they may in turn affect lipid-protein interactions and contribute to alterations in the state of membrane lipids.

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