Research Paper

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EPR Study of Erythrocyte Properties After in Vitro Treatment With Urea and Hydrogen Peroxide

Medical Science

KEYWORDS : erythrocytes, urea, electron paramagnetic resonance (EPR)

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ABSTRACT

The aim of this study was to evaluate erythrocyte properties after in vitro treatment with urea and hydrogen peroxide. Changes in erythrocyte membrane fluidity, conformational state of membrane proteins and hemoglobin were investigated using EPR spectroscopy.

The urea treatment resulted in increase of lipid membrane fluidity in polar region and hydrophobic core indicated by 5- and 12-doxylstearic acids. The combination of urea and H2O2 lead to deeper changes in lipid microviscosity. The conformational state of membrane proteins was measured using maleimide (MSL) and iodoacetamide (ISL) spin labels. ISL spectra showed a significant decrease in the membrane proteins motion. However, in the combination of urea and H2O2 the spin labeled proteins mobility was similar to control value. Analysis of EPR spectra of MSL attached to hemoglobin showed significant increase in the motion of spin label residues after urea and H2O2 treatment. Similar results were observed for ISL but differences were insignificant.

INTRODUCTION

Chronic renal failure (CRF) is a debilitating condition which is responsible for high morbidity and mortality of suffering patients. The bad condition of the patient may be exacerbated due to the constant presence of high concentration of urea and oxidative stress. As a consequence of chronic renal failure (uremia), the plasma levels of urea are greatly increased (30-50 mM) above normal (3-7 mM) [1]. In vivo and in vitro urea is spontaneously decomposed to cyanate and ammonia [2]. Cyanate is rapidly converted into its active form, isocyanic acid, which can react with amino groups of proteins causing their carbamylation [2,3]. Carbamylation alters the structural properties of proteins by destabilization of their secondary and tertiary structures [1,2,3].

In CRF hemodialysed patients oxidative stress plays also an important role. Reactive oxygen species react with other molecules (e.g., lipids, proteins, DNA, etc.) that come in contact with them [4,5]. In uremia structural changes in albumin and increase in lipid peroxidation in plasma was observed [6]. Simultaneously numerous changes in erythrocyte properties such as the shape of cells and alterations in the cell membrane of CRF ppatients with chronic renal failure were shown [6,7,8].

Structural changes in the cell membrane may result from carbamylation or/and oxidation of its components by higher level of urea and other uremic toxins and also reactive oxygen species including free radicals [9,10]. These alterations in physical properties of the plasma membrane may lead to accelerated aging process of erythrocytes and premature removal from the circulation. Both high levels of uremic toxins and oxidative stress may influence on higher incidence of complications such as anemia, atherosclerosis, cardiovascular disease, accelerated aging processes [7].

The aim of this study was to evaluate properties of red blood cells after treatment with urea and combination of urea and hydrogen peroxide. The changes in erythrocyte plasma membrane fluidity, conformational state of membrane proteins as well as conformational state of hemoglobin were investigated using spin labeling technique in electron paramagnetic resonance (EPR).

MATERIAL AND METHODS

Chemicals

5-, 12-, 16- doxylstearic acids (5-DS, 12-DS and 16-DS), 4-maleimido-2,2,6,6,-tetramethylpiperidine-1-oxyl (MSL), 4-iodoacetamide-2,2,6,6,-tetramethylpiperidine-1-oxyl (ISL) obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were analytical grade products from POCh (Gliwice, Poland).

Experimental procedures

Human whole blood from healthy volunteers (under sterile conditions) was incubated with urea to a final concentration of 35 mM in phosphate buffer saline (PBS, pH 7.4) at 37°C for 24h. After that hydrogen peroxide (H_2O_2) was added (final concentration 50 mM) and incubated in the same conditions for 1h. For experiments the blood was centrifuged and isolated erythrocytes (RBC) were washed three times with phosphate buffered saline (PBS, pH 7.4).

Lipid membrane fluidity

Isolated erythrocytes were investigated to determine lipid membrane fluidity at three different depths of membrane bilayers, using 5-DS, 12-DS and 16-DS. The erythrocytes were labeled with 0.1 M spin labels in ethanol solution (50:1) and incubated for 30 minutes at room temperature. Based on EPR spectra of 5-DS the order parameter was calculated from equation [11].

$$S = \frac{A_{II} - A_{\perp}}{A_{zz} - A_{xx}} \times \frac{a_N}{a'_N}$$

Where: the distance between outer $2A_{II}$ and inner $2A_{I}$ (The same as in equation below) hyperfine spectral splitting, A_{zz} and A_{xx} are hyperfine splitting parameters for nitroxide derivative in host crystal, a_N and a'_N are the isotropic hyperfine coupling constants of nitroxide in membrane and cristal state.

$$a'_N = \frac{1}{3}\left(A_{II} + 2A_{\perp}\right)$$

In the case of 12-DS and 16-DS the ratio of the amplitude of low field line and middle field line (h_{+1}/h_0) was determined as a semi-empirical parameter of hydrocarbon chain mobility [12].

Hemolysate preparation

Erythrocytes were hemolysed with water in the following relation (1:1.5), then vortexed for 10 min, and centrifuged at 16 000 rpm for separation of erythrocyte membranes. The crude hemoglobin was labeled with covalently binding spin labels.

Erythrocyte ghost preparation

Erythrocyte ghosts were prepared using a modified method of Dodge et al. [13]. The ghosts were successively washed with 20, 10, and 5 mM phosphate buffer (pH 7.4) at 4°C.

Spin labeling of erythrocyte ghost and hemoglobin

For investigation of conformational changes of membrane proteins and hemoglobin two spin labels, 0.1 M ethanol solutions, were used (MSL and ISL). Samples were incubated with labels (50:1) for 1h at 4°C. The unbound spin labels were removed from erythrocyte ghosts by several washings with 5 mM phosphate buffer (pH 7.4). The unbound spin label was removed from hemolysate through the dialysis using 20 mM phosphate buffer, pH = 7.4.

Based on EPR spectra the following parameters were calculated, for MSL the ratio h_w/h_s, where h_w is weakly immobilized and h_s strongly immobilized spin label residues [14], while for ISL the relative rotational correlation time according to Kivelson equation [15]:

$$\tau_C = kw_0\left(\sqrt{\frac{h_0}{h_{-1}}}-1\right)$$

where:

tau_c - time when the spin label undergoes full rotation

k - constant equal to 6.5×10^{-10} s

w_0 - width of the mid-line of spectrum

h_0 - height of the mid-line of spectrum

h_{-1} - height of the high-field line of spectrum.

EPR (electron paramagnetic resonance) measurements

EPR (electron paramagnetic resonance) spectra were measured on the Bruker ESP 300 E spectrometer at room temperature, operating at a microwave frequency of 9.73GHz. The instrumental settings were as follows: the microwave power was 10 mW, the centre field was set at 3480 G, with a range of 80 G, and a 100 kHz modulation frequency, a modulation amplitude of 1.01 G and a time scan of 256 s.

Statistical analysis

Statistical analysis included the calculation of means and S.D. The significance of differences for multiple comparisons was estimated using Tukey's test. Statistical significance was accepted at $p < 0.05$ in comparison to control RBC.

RESULTS

The changes in erythrocyte membrane fluidity after treatment with urea and hydrogen peroxide were estimated using three spin labels 5-DS, 12-DS and 16-DS. In the case of 5-DS the order parameter (S) was calculated, whiles for 12-DS and 16-DS the ratio of h_{+1}/h_0 was received. The obtained results indicate significant increase of lipid membrane fluidity (decrease of S) at the polar region of lipid bilayer in erythrocytes after treatment with urea ($p<0.05$) and in combination of urea with H_2O_2 ($p<0.005$) (Fig.1A) determined by 5-DS. The changes in lipid fluidity were higher when the combination of both substances was applied. In the deeper region of lipid layer e.g. 12th carbon atom of hydrocarbon fatty acid chains the significant increase of lipid fluidity only after combination of urea with H_2O_2 was observed ($p<0.05$) (Fig.1B). Generally hydrogen peroxide alone induced lower changes in lipid layer than urea. However, at the depth of 16th carbon atom of hydrocarbon chain of fatty acids no differences in lipid membrane fluidity were observed (data not shown).

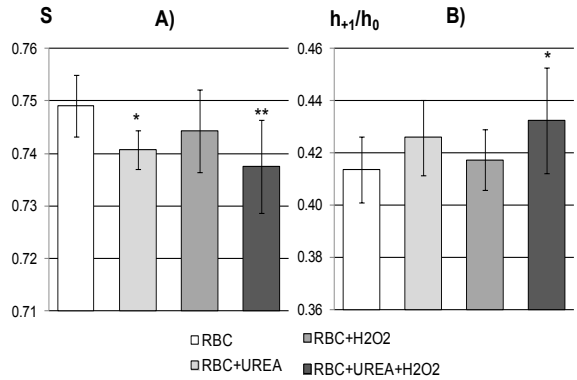

Fig.1. Erythrocyte lipid membrane fluidity after treatment with urea and hydrogen peroxide at the A) 5th and B) 12th carbon atom of fatty acid chain, estimated by order parameter (S) and h_{+1}/h_0 ratio, respectively. *, p<0.05; **, p<0.005.

Physical state of membrane proteins was estimated using two covalently binding spin labels: MSL and ISL. Both spin labels react with –SH groups but with different proteins [16,17]. Using the data from the EPR spectra of MSL the h_w/h_s ratio (h_w height of the amplitude of weakly immobilized and h_s height of the amplitude of strongly immobilized spin label residue, respectively) was calculated [14]. The obtained results showed decrease (not significant) in h_w/h_s ratio of maleimide attached to membrane proteins after whole blood treatment with urea or hydrogen peroxide alone. Similar result was obtained for the combination of urea with H_2O_2 (Fig.2A). On the other hand significant decrease of relative rotational correlation time of ISL spin label, attached to membrane proteins, after treatment with urea, H_2O_2 ($p<0.0002$) and their combination ($p<0.005$) has been observed (Fig.2B).

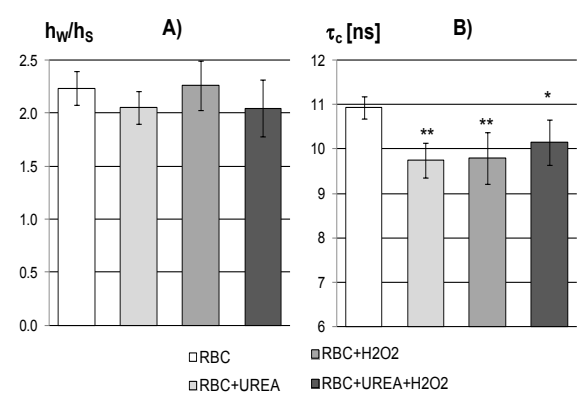

Fig.2. Conformational state of erythrocyte membrane proteins in whole blood treated with urea, H_2O_2 and combination of both substances measured using A) MSL and B) ISL. *, p<0.005; **, p<0.0002.

Similarly to membrane proteins, the conformational state of hemoglobin was measured using the MSL and ISL. Urea and hydrogen peroxide applied alone did not induce changes in hemoglobin. However, incubation of whole blood with combination of urea and H_2O_2 resulted in significant increase of h_w/h_s ratio for MSL attached to hemoglobin ($p<0.01$) (Fig.3A). However, for ISL attached to hemoglobin no statistically sig-

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nificant differences after blood incubation either with urea or H_2O_2 separately or with their combination (Fig3B) were detected.

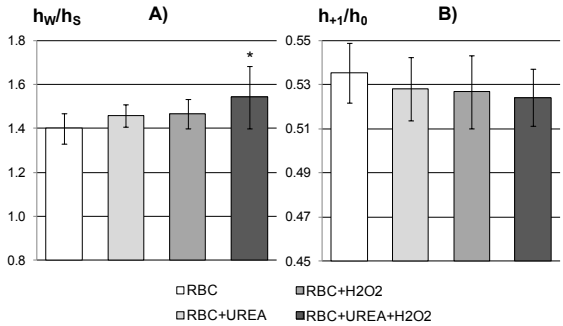

Fig.3. Conformational state of hemoglobin after whole blood treatment with urea, H_2O_2 and their combination measured using A) MSL and B) ISL *, p<0.01.

Fig 3 shows the increase in h_w/h_s ratio which reflects the increase of the motion of MSL labeled hemoglobin.

DISCUSSION

The high concentration of urea and other toxins and the presence of oxidative stress are associated with hemodialysis of patients. Urea derivatives e.g. cyanate and its higher reactive form isocyanic acid can cause carbamylation of proteins and other molecules and macromolecules [2,3]. The presence of reactive oxygen species contributes to the oxidation of all of the macromolecules [4,5].

In this work the spin labeling method in EPR spectroscopy for determination of the effects of urea and hydrogen peroxide on erythrocyte membrane properties and conformational state of hemoglobin was applied. This method allows to examine changes in membrane dynamic components e.g. lipids and proteins and also to determine specific interactions between them. Moreover, spin labels attached to macromolecules play a role as reporting groups, providing information about changes in the microenvironment in the nearest region of binding label.

The three doxyl derivatives of stearic acids located at different depths of lipid monolayer allowed to determine membrane fluidity in these regions.

Incubation of blood with urea resulted in an increase of lipid fluidity of the cell membrane at a depth of 5th and 12th carbon atom of hydrocarbon fatty acid chain. The addition of H_2O_2 enhanced the effect of urea. So the changes in membrane fluidity may be a result of oxidation of lipids and/or proteins and changes in lipid-protein interactions as well as lipid and proteins carbamylation. All these processes additionally explain the cause of increased lipid fluidity. In our previous paper we showed that increase of lipid membrane fluidity in CRF patients is correlated with lipid membrane peroxidation [7,17].

Extended analysis of membrane dynamics of the conformational state of membrane proteins was performed, Using covalently bound spin labels MSL and ISL two kinds of membrane proteins were examined. MSL reacts with thiol groups mainly of the spectrin-actin complex of cytoskeletal proteins while ISL reacts with –SH groups of peripheral proteins [14,17]. It has been shown that 75-90% of the total amount of attached spin labels is bound to this complex [14]. Using MSL spin label and calculating the value of h_w/h_s, the slight decrease of this parameter was observed after urea blood treatment. This ratio reflects conformational state of membrane cytoskeletal proteins. Changes in the MSL spectra can be induced by even small alterations in the nearest proximity of the label bound to labeled proteins [14]. In our studies we showed changes in conformational spectrin-actin complex in CRF erythrocytes in comparison to healthy donors [7]. Generally the h_w/h_s ratio increases during protein degradation and oxidation [18,7], which is associated with the increase of segmental motion of label attached to the proteins. The decrease of this ratio can be interpreted as the carbamylation of peripheral –SH groups in spectrin-actin complex. It was a surprise that hydrogen peroxide did not have an influence on this complex, because in isolated RBC hydrogen peroxide significantly increased the ratio h_w/h_s, indicating an increase of spin label mobility.

Membrane proteins were also examined by ISL, which was attached to –SH groups of peripheral membrane proteins [17]. Using this label the significant increase in the motion after urea and hydrogen peroxide treatment as well as a combination of these substances was observed. The conformational state of membrane proteins measured using this label showed a statistically significant change in the rotational correlation time for all investigated points.

MSL and ISL were applied in hemoglobin conformational state investigation. It has been shown that both of these labels bind with the same cysteine residue in the chain of globin giving different spectra, because they vary in the arrangement spatial coupling in this site [19,20].

MSL attached to hemoglobin showed insignificant changes for urea and hydrogen peroxide separately treated whole blood. However, in the case of combined substances significant rise in the ratio h_w/h_s was observed, which reflect conformational changes in the globin chain. It has been shown that oxidation of hemoglobin by hydrogen peroxide lead to cysteine 93 and 112 oxidation to cysteic acid [21]. Additionally at the same time oxidation of methionine and tryptophane was observed [21]. These modifications led to loss of helical structure of globin chain. Our results show that treatment of whole blood with urea facilitates the oxidative modification in globin chain of hemoglobin molecule.

Erythrocytes and plasma components in CRF patients can be damaged by higher concentrations of urea in the blood, which can lead to carbamylation of lipids, proteins, nucleic acid etc. [6]. Our results showed rather slight changes in plasma membrane fluidity and in membrane protein conformation as well as indicated changes in hemoglobin structure. These observations can be a results of short time of urea treatment but also might be caused by an absence of other uremic toxins, which may also play important role in chronic renal failure. Despite of this the obtained data provide the evidence about mechanisms of red blood cell damage in vivo by both higher level of urea and oxidative stress in chronic renal failure of hemodialysed patients.

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