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Short Communication

## CARBAMYLATION OF PROTEINS LEADS TO ALTERATIONS IN THE MEMBRANE STRUCTURE OF ERYTHROCYTES

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**Abstract:** The effect of the sodium cyanate-induced carbamylation (carbamylation) of proteins in erythrocytes was studied using spin labelling and spectrophotometric methods. The experiments were conducted in whole blood and in erythrocytes in phosphate buffer using 25 mmol/L of sodium cyanate. Lipid membrane fluidity was determined using three spin-labelled fatty acids: 5-, 12- and 16-doxyloleic acids (5-DS, 12-DS, 16-DS). Internal viscosity was measured with Tempamine, using also EPR spectroscopy. Osmotic fragility was determined spectrophotometrically. Incubation of whole blood with sodium cyanate led to an increase in lipid membrane fluidity in the deeper region of the lipid layer, indicated by 12- and 16-doxyloleic acid, and a decrease near the surface (5-DS). Statistically significant results were obtained for the internal viscosity and osmotic fragility of erythrocytes. An increase in internal viscosity and increase in osmotic fragility were found in erythrocytes after incubation of whole blood, as well as in erythrocytes incubated with sodium cyanate in buffer. Alterations in internal viscosity were stronger in erythrocytes incubated with sodium cyanate in blood than in erythrocytes in the buffer. On the other hand, higher osmotic fragility was observed for erythrocytes in the buffer.

**Key words:** Carbamylation, Erythrocyte, Spin Labelling, Internal Viscosity, Membrane Fluidity, Osmotic Fragility

### INTRODUCTION

Carbamylation (carbamylation) of peptides and proteins and oxidative stress occurs in erythrocytes in uremia where the concentration of urea is approximately 7-fold higher than in healthy controls. Cyanate is formed by the transformation of urea. The reactive form of cyanate is isocyanic acid, which can

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Abbreviations used: 5-DS - 5-doxyloleic acid; 12-DS - 12-doxyloleic acid; 16-DS - 16-doxyloleic acid; CRF - chronic renal failure; PBS - phosphate buffer saline; Tempamine - 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl.

react with amino groups of peptides and proteins. Generally, carbamylation leads to changes in protein properties, enzyme activities and lipoprotein modifications [1, 2]. In chronic renal failure (CRF), carbamylation and oxidative stress can modify erythrocyte components.

In the erythrocyte membrane, the level of carbamylated proteins was higher than in healthy donors [3]. Erythrocytes from CRF patients have an impaired antioxidant system, e.g. a decrease in enzymes activities and a decrease in the level of low molecular weight antioxidants [4, 5]. Thus, the erythrocytes are exposed to internal and external sources of toxic oxygen intermediates. Dialysing membranes can activate neutrophils, which release oxygen free radicals and other toxic oxygen species during hemodialysis sessions [6, 7]. Free radicals and other toxic oxygen forms, such as hydrogen peroxide, hypochlorous acid and singlet oxygen, lead to red blood cell damage [8, 9]. In our previous study, the modification of erythrocyte membrane proteins upon treatment with sodium cyanate was shown [10].

The effect of carbamylation on erythrocyte membrane properties was examined in an *in vitro* study. The experiments were conducted with sodium cyanate in whole blood and in erythrocytes in a phosphate buffer. Lipid membrane fluidity, internal viscosity of erythrocytes and osmotic fragility were determined using the spin-labelling method.

## MATERIALS AND METHODS

Human blood (anticoagulated with sodium citrate) from healthy volunteers was centrifuged and washed three times with phosphate buffer saline (PBS, pH 7.4). Whole blood or isolated erythrocytes in PBS were incubated for 24 h at 37°C with 25mmol/l (final concentration) sodium cyanate in sterile conditions.

Three spin-labeled fatty acids – 5-, 12- and 16-doxylosteaic acid (5-DS, 12-DS, 16-DS) – were applied to determine lipid membrane fluidity. The erythrocytes were labelled via the introduction of spin label in ethanol solution and incubation for 30 minutes at room temperature. The final ethanol concentration did not exceed 0.05%. For 5-DS, the order parameter was determined thus:

$$S = [(T_{\parallel} - T_{\perp}) a_N] / [(T_{zz} - T_{xx}) a'_N]$$

$T_{\parallel}$  i  $T_{\perp}$  are hyperfine splitting constants for the magnetic field parallel and perpendicular to the bilayer normal, respectively;  $T_{zz}$  i  $T_{xx}$  are hyperfine splitting constants for nitroxide in the host crystal [ $T_{zz} = 32,4$  G,  $T_{xx} = 6,1$  G [11]]; while  $a_N$  i  $a'_N$  are the isotropic hyperfine coupling constants for nitroxide in the membrane and crystal state, respectively, i.e. [ $a_N = (T_{zz} + 2T_{xx})/3$ ], [ $a'_N = (T_{\parallel} + 2T_{\perp})/3$ ].

For 12-DS and 16-DS, the  $h_{+1}/h_0$  ratio and relative correlation time ( $\tau_c$ ) for determination of motion in the membrane were calculated, respectively [12] thus:

$$\tau_c = k w_0 [(h_0/h_{-1})^{1/2} - 1]$$

$k=6.5 \times 10^{-10}$ s,  $w_0$  - width of the spectrum middle line;  $h_0$  - height of the spectrum middle line;  $h_{-1}$  - height of the spectrum low-field line.

The internal viscosity of the erythrocytes was measured with Tempamine (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) in 80 mmol/L of potassium ferricyanide as a broadening agent and calculated from the equation [12]:

$$\eta_{\text{eryth.}} = (\tau_{\text{c eryth.}} / \tau_{\text{cH}_2\text{O}}) \eta_{\text{H}_2\text{O}}$$

where  $\tau_{\text{c eryth.}}$  and  $\tau_{\text{cH}_2\text{O}}$  are the relative correlation times of the spin label inside the erythrocyte and in water, respectively; and  $\eta_{\text{eryth.}}$ ,  $\eta_{\text{H}_2\text{O}}$  are viscosity inside the erythrocyte and in water ( $\eta_{\text{H}_2\text{O}} = 1$  cP).

EPR spectra were recorded on a Bruker ESP 300E (X-band) spectrometer. All the EPR spectra were measured at room temperature.

Osmotic fragility was determined in samples of different concentration of sodium chloride (in the range of 55-155 mmol/l) at 540 nm from the equation:

$$H (\%) = [(A_x - A_0) / (A_{\text{water}} - A_0)] 100 \%$$

$A_x$  - absorbance of the supernatant of the sample;  $A_0$  - absorbance of the supernatant in physiological salt concentration (155 mmol/l);  $A_{\text{water}}$  - absorbance of the supernatant after complete hemolysis in water; Statistical analysis included the calculation of means and S.D. The significance of differences for multiple comparison was estimated using Tukey's test.

## RESULTS AND DISCUSSION

Carbamylation of peptides and proteins in erythrocytes was performed in whole blood and in erythrocytes in phosphate buffer using sodium cyanate. Fig. 1 shows the effect of carbamylation on lipid membrane fluidity. Carbamylation of erythrocyte components led to an increase in lipid membrane fluidity in the deeper region of the membrane layer, determined using 12-, and 16-doxy stearic acid, while near the surface, on the 5-atom of the hydrocarbon of the fatty acid chain, a decrease in fluidity was observed (Fig. 1).

An increase in internal viscosity and an increase in osmotic fragility were found in the erythrocytes of whole blood incubated with sodium cyanate, as well as in erythrocytes incubated with this compound in buffer. The changes in the internal viscosity of the erythrocytes were stronger in the case of carbamylation performed in whole blood than for erythrocytes in the buffer (Fig. 2).

Fig. 3 presents the osmotic fragility of erythrocytes after carbamylation in whole blood and in buffer. For erythrocytes in buffer, osmotic fragility was higher than in whole blood treated with sodium cyanate. Statistically significant results were obtained for the internal viscosity and osmotic fragility of erythrocytes. The presented results clearly show that carbamylation led to the modification of the plasma membrane and the components in intracellular fluid. It seems that changes in lipid membrane fluidity result from carbamylation of membrane proteins, which in consequence leads to changes in lipid-protein interactions.

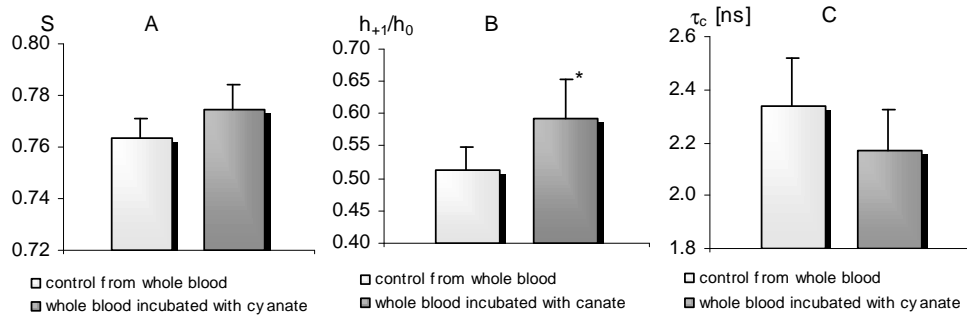


Fig. 1 Changes in (A) the order parameter of 5-DS (not significant); (B) the  $h_{+1}/h_0$  ratio of 12-DS ( $p < 0.01$ ); and (C) the relative correlation time of 16-DS (not significant) in erythrocyte after incubation with sodium cyanate (for each label  $n=11$ ).

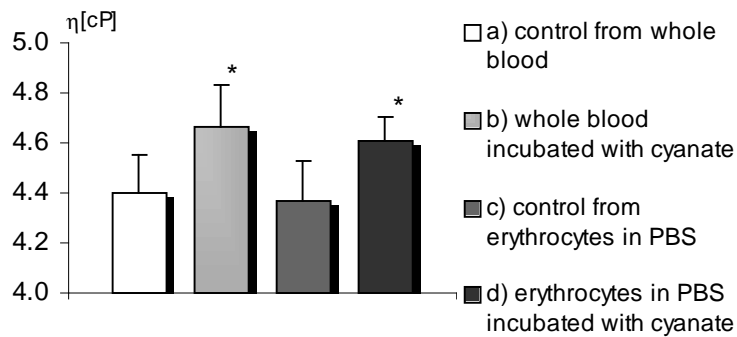


Fig. 2. Internal viscosity of erythrocytes after treatment with sodium cyanate in whole blood and in PBS ( $p < 0.005 \mu_a \neq \mu_b$ ;  $p < 0.0005 \mu_c \neq \mu_d$ ) ( $n=11$ ).

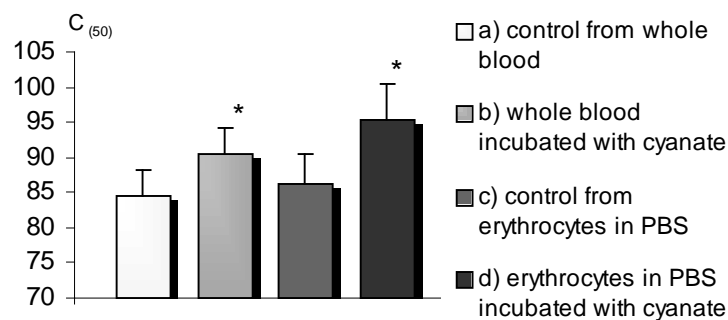


Fig. 3. Osmotic fragility of erythrocytes after treatment with sodium cyanate in whole blood and in PBS ( $p < 0.002 \mu_a \neq \mu_b$ ;  $p < 0.0005 \mu_c \neq \mu_d$ ) ( $n=11$ ).

Interestingly, carbamylation caused an increase in fluidity in the hydrophobic core, but near the surface it caused an increase in rigidity. In our previous study,

we showed that carbamylation produced an immobilisation of the spin label residue in proteins in the membrane [10].

The carbamylation process leads to membrane modification (changes in the fluidity and conformation of membrane proteins) and changes in internal viscosity which reflect alterations in intracellular peptides and proteins. These changes can influence the life span of erythrocytes in chronic renal failure patients.

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