Physiol. Res. 48: 215-219, 1999

Anoxia/induced Membrane Changes in Human Red Blood Cells

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Received June 3, 1998
Accepted January 22, 1999

Summary

The cation-osmotic hemolysis was studied in human red blood cells incubated under anoxic conditions. In relation to the time course of anoxia, two phases of hemolysis were distinguished. A significant decrease of hemolysis was found between 3 and 24 h of incubation. On the other hand, hemolysis was significantly increased after prolonged incubation (48-72 h). Using the method of cation-osmotic hemolysis, the properties of two membrane constituents, spectrine membrane skeleton and membrane bilayer, were studied. The relation between cation-osmotic hemolysis and erythrocyte deformability is being discussed.

Key words

Cation-osmotic hemolysis • Human red blood cells • Anoxia • Metabolic depletion

Introduction

It is known that the hemorheological membrane properties of red blood cells (RBC) may be changed under hypoxic conditions (Backman 1986). Human RBC treated under anoxic conditions for an adequate time period lose their discoid form in favor of spiculated or spherical forms (Reinhart and Chien 1987). Erythrocytes and hemoglobin have been shown to be a source of enhanced superoxide production under hypoxic conditions (Balagopalakrishna *et al.* 1997). It is believed that oxidative stress causes membrane damage associated with membrane protein cross-linking and the loss of deformability can be a consequence of these membrane changes (Rifkind *et al.* 1991). Furthermore, it was found that ATP-depletion under anoxic conditions increases spectrin cross-linking to oligomers and accumulation of intracellular calcium (Kamada *et al.* 1983). An increase in intracellular calcium results in a decrease of RBC deformability (Clark *et al.* 1981, Chien 1987). Increased osmotic fragility in metabolic depleted RBCs has also been observed (Tozzi-Ciancarelli *et al.* 1992).

On the other hand, Kaniewski *et al.* (1994) found that hypoxic conditions did not alter the biophysical properties of human RBC membranes. Using the ectacytometric method, these authors examined human, cat, dog, rabbit, and rat erythrocytes under normoxic and hypoxic conditions. Decreased deformability was observed only in rat erythrocytes.

The aim of our paper was to study the erythrocyte deformability changes in human RBC under anoxic conditions using the method of cation-osmotic hemolysis (COH).

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Method

The blood used for *in vitro* experiments was withdrawn from a group of 35 healthy donors 19 to 46 years. The blood samples in volumes of 2 ml were collected according to the rules of the International Hemorheological Committee (ICSH Expert Panel on Blood Rheology 1986).

The erythrocyte membrane deformability was studied using the method of cation-osmotic hemolysis (Nicák and Mojžiš 1992, Mojžiš and Nicák 1993). Immediately after blood sampling, 15 µl of blood were added to 3 ml of the incubating medium, which contained different concentrations of NaCl and isotonic glucose. The concentrations of NaCl used (ionic strength) were as follows (in mmol.l^{-1}): 15.4, 30.8, 46.2, 61.6, 77.0, 92.4, 107.8, 123.2, 138.6 and 154.0. On the other hand, the concentrations of isotonic glucose were (in mmol.l^{-1}): 258.3; 229.6; 200.7; 172.2; 143.5, 114.8, 86.1, 57.4, 28.7 and 0.0. The hemolysis was induced by $HgCl_2$ (0.15 mmol.l^{-1}) present in the incubating medium. The samples were incubated for 60 min at 37 °C and centrifuged afterwards for 5 min at 700 g. Hemolysis was established spectrophotometrically at 540 nm and expressed as the hemolytic rate in relation to hemolysis in distilled water, which was arbitrarily set as 100 %.

The remaining portion of blood samples were incubated during the period of 72 h at 37 °C under anoxic conditions. The changes of membrane deformability were established 3, 6, 12, 24, 48 and 72 h after the onset of incubation according to the method described above.

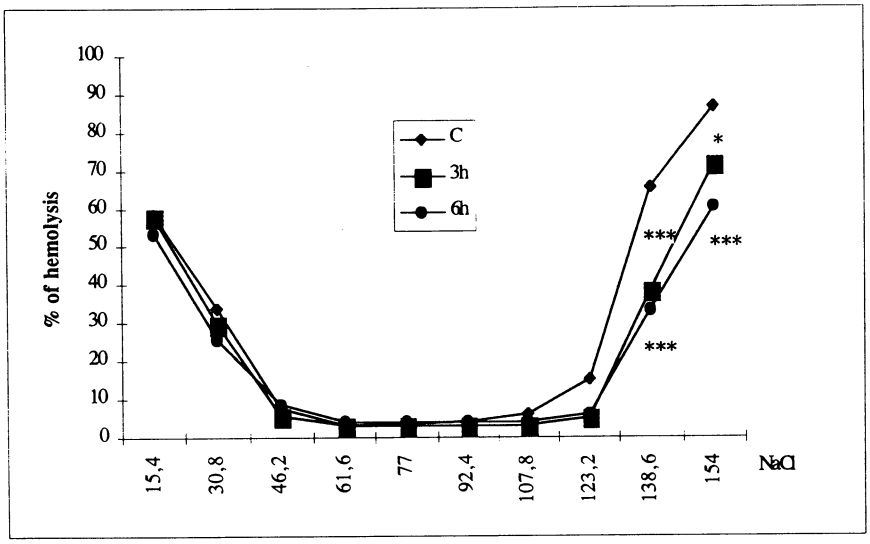

Fig. 1. *Cation-osmotic hemolysis of human erythrocytes after 3 h and 6 h of incubation under anoxic conditions. Ordinate: % of hemolysis. Abscissa: concentration of NaCl in mmol.l^{-1}. Statistically significant differences for * $p<0.05$, *** $p<0.001$*

Results

The results of cation-osmotic hemolysis after three and six hours of incubation are shown in Figure 1. No significant differences were observed in solutions with ionic strength from 15.4 to 123.2 mmol.l^{-1} of NaCl. However, in the solutions with higher ionic strength (138.6-154.0 mmol.l^{-1} of NaCl) hemolysis was significantly lower ($p<0.001$ and $p<0.05$, respectively). Figure 2 demonstrates cation-osmotic hemolysis after 12 and 24 h of incubation. After 12 h of incubation, hemolysis was significantly lower in the medium containing 30.8 mmol.l^{-1} of NaCl. However, hemolysis after 24 h of incubation in the solutions with 15.4 and 30.8 mmol.l-1 of NaCl was significantly lower ($p<0.001$). In the solutions with high ionic strength (138.6-154.0 mmol.l^{-1} of NaCl) significant decrease of hemolysis was observed only after 12 hours of incubation ($p<0.001$).

Dramatic changes of cation-osmotic hemolysis occurred after 48 and 72 h of incubation. After 48 h of incubation in solutions ranging from 46.2 to 154.0 mmol.l^{-1} of NaCl hemolysis significantly increased ($p<0.01$ and $p<0.001$, respectively) (Fig. 3). More intensive increase of hemolysis was observed after 72 h of incubation. Significant enhancement of hemolysis was found in all incubating solutions except those containing 15.4 mmol.l^{-1} of NaCl (30.8-154.0 mmol.l^{-1} of NaCl) ($p<0.001$).

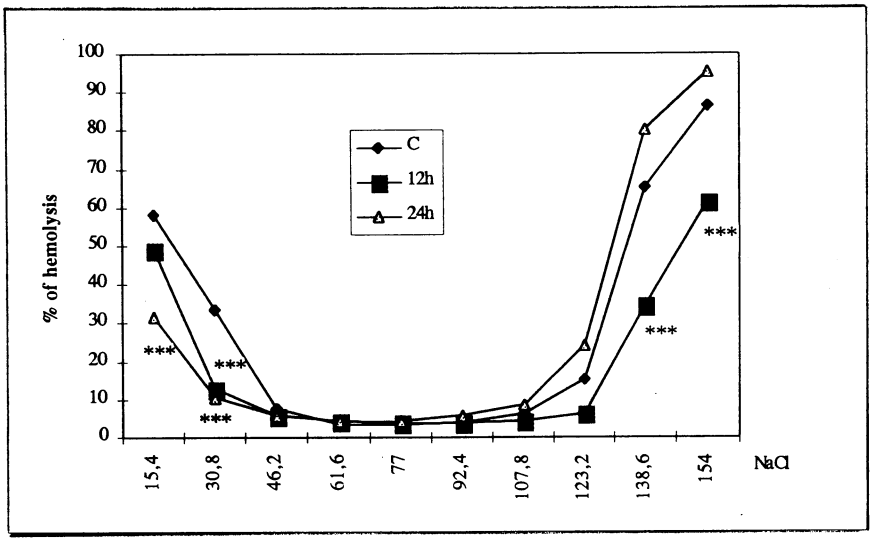

Fig. 2. *Cation-osmotic hemolysis of human erythrocytes after 12 h and 24 h of incubation under anoxic conditions. Ordinate: % of hemolysis. Abscissa: concentration of NaCl in mmol.l^{-1}. Statistically significant differences for *** $p<0.001$*

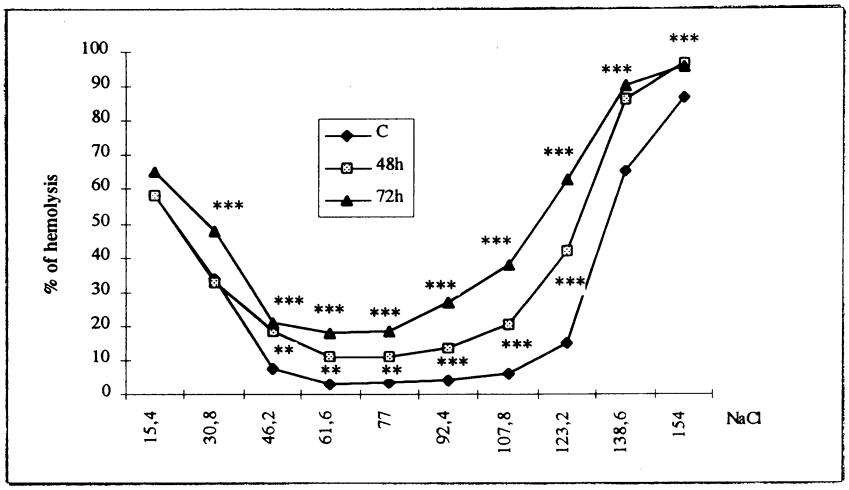

Fig. 3. *Cation-osmotic hemolysis of human erythrocytes after 48 h and 72 h of incubation under anoxic conditions. Ordinate: % of hemolysis. Abscissa: concentration of NaCl in mmol.l^{-1}. Statistically significant differences for * $p<0.01$, *** $p<0.001$*

Discussion

Few years ago we developed the method of cation-osmotic hemolysis (Nicák and Mojžiš 1992). Subsequently, we proved that membrane deformability and cation-osmotic hemolysis are closely related. Hemolysis of the rigid erythrocyte membrane (treated by glutaraldehyde) was significantly lower when compared with non-treated erythrocytes and the filtration time of glutaraldehyde-treated erythrocytes was also much longer as compared with non-treated RBCs (Mirossay *et al.* 1997). On the basis of these results and our previous experience, we suggest that cation-osmotic hemolysis reflects the basic information about erythrocyte deformability.

The method of cation-osmotic hemolysis makes it possible to determine the biophysical state of the spectrin-actin complex (first maximum of hemolysis) and simultaneously changes of the lipid bilayer (second maximum of hemolysis) in relation to the ionic strength of the incubation medium (Nicák and Mojžiš 1993). Both maxima can be considered as a Hg^{2+} blockade of cation and water membrane channels (band 3 and 4.5) (Benga 1988). Thus the lipid bilayer appear to be the only pathway for water permeation (Macey and Farmer 1970).

The influence of metabolic depletion on RBC deformability is a crucial problem. Various intracellular changes of RBC were found, including a decrease of ATP and an increase in the calcium content (Kamada *et al.* 1983). The decrease of erythrocyte deformability (ED) in

metabolically depleted RBC was different according to the methods employed (Weed *et al.* 1969). Oxidative stress was found to be a destructive process connected with membrane peroxidation only after hypoxia associated with hemolysis. However, under conditions of effective anoxia, the intensity of hemolysis is substantially lower (Rifkind *et al.* 1991). These authors later found that oxidative stress at intermediate oxygen pressure induces membrane damage associated with enhanced lysis, membrane protein cross-linking and a decrease of deformability. In our experiments the decrease of COH was observed until 24 h of anoxia. A significant decrease of hemolysis after 3 h of anoxia was only found in solutions of high ionic strength. Later, a significant decrease was observed after 12 h of incubation in media with low and high ionic strength. However, after 24 h incubation a significantly diminished hemolysis was only found in solutions of low ionic strength. These results are closely related to those of Clark *et al.* (1981) who found a decrease of ATP and reduced erythrocyte deformability 18 h after the beginning of incubation. The reduced deformability under hypoxic conditions can also be attributed to enhanced autooxidation (Levy *et al.* 1988) leading to cross-linking between hemoglobin and membrane proteins (Fortier *et al.* 1988)

After 48 and 72 h of anoxia, hemolysis was significantly enhanced. We suggest that this increase is a consequence of resulting ATP depletion. As was previously reported, the metabolic depletion is characterized by increased membrane fluidity (Kamada *et al.* 1983) also accompanied by a loss of membrane stability (Clark *et al.* 1981). Our results are comparable to those of Rifkind and Abugo (1994) who found that during *in vitro* incubation under hypoxic conditions for extended periods of time erythrocyte lysis increase as a consequence of oxidative processes.

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