Red Blood Cells under Mechanical Stress

M. Kodíček, J. Suttnar, L. Mirčevová, and T. Mařík

Institute of Hematology and Blood Transfusion, U nemocnice 1, 128 20 Praha 2, Czechoslovakia

Abstract. The effect of mechanical stress on erythrocytes suspended in various media was studied. The ability of the cells to increase their glucose consumption was found to be the major criterion allowing to divide the media into two groups. In plasma, serum or in Ringer's solution supplemented with albumin and glucose the energy consumption by mechanically stressed erythrocytes increased 20 to 50 %; no morphological changes of the cells were observed either in suspension or on Giemsa smears. The cells behaved in the same way in Mg^{2+}-free medium. The other group included protein-free medium (Ringer's solution supplemented with glucose) and Ca^{2+}-free Ringer's solution supplemented with albumin and glucose; under these conditions erythrocytes were unable to raise their energy consumption in response to mechanical stress, and after some period structural impairment of the membrane could be observed on Giemsa smears. No differences in metabolism-associated nucleotide concentrations (ATP, ADP, NAD, NADP) were observed between the samples. Resealed red cell ghosts with high concentrations of intracellular components were prepared as a model of cells with damaged membrane. In these ghosts (with low ATP concentration) mechanical stress produced increased proportions of echinocytes, even in the "native" suspension. These results have confirmed the vital role of the energy-consuming contractile apparatus in the erythrocyte membrane, and supplied a clue to the role of Ca^{2+} in its activation and to the influence of extracellular proteins on the maintenance of in red cell shape.

Key words: Erythrocyte membrane — Glucose consumption — Mechanical energy — Divalent ions

Introduction

During recent years a number of experiments have shown that erythrocyte membrane cannot be described as a passive structure with transport as the only active function (Sheetz 1983). Gradually it is realized that the unique shape and the rheological features of red cells are determined by the inherent contractile apparatus present in the membrane (Mirčevová et al. 1983). An impressive

support to this concept was provided by Fowler et al. who demonstrated the presence of tropomyosin (Fowler and Bennet 1984) and myosin (Fowler et al. 1985) in the erythrocyte membrane. Sufficient amounts of ATP for this contractile ATPase is produced by the glycolytic system; its ATP production covers twice the consumption by transport ATPases and kinases (Rapoport et al 1984).

In our previous reports (Kodíček 1986; Kodíček et al. 1987) based on preliminary results we could show that restoration of the natural smooth biconcave shape of the red cell is an active, energy consuming process which is connected with the activity of the conctractile apparatus in the cell membrane. The present paper is based on extended experiments and discusses in more detail the behaviour of erythrocytes under mechanical stress.

Materials and Methods

Fresh heparinized blood from a healthy donor was centrifuged ($5600 \times g$, 20 min) and plasma and buffy coat were removed. The erythrocytes were then washed by 4 volumes of the respective medium (see below); the remaining leucocytes and thrombocytes were removed by another centrifugation ($3000 \times g$, 5 min). The erythrocytes (9 ml) were mixed with the medium (12 ml) to make a suspension with a hematocrit of 40 %. This suspension was "equilibrated" at 37 °C in water bath for 15 min.

Each experiment was performed at least three times with blood (or erythrocytes) from different healthy donors (men and women) of the age from 18 to 55 years. No age or sex differences were observed in our experiments.

A rotational ektacytometer was used to produce reproducible shear stress on erythrocytes. It consists of two concentric cylinders, the gap width between the fixed and rotating ones being 0.5mm. The apparatus was placed in a polystyrene box carefully thermostated at 37 °C. The equilibrated suspension of red cells was divided into two aliquots: 6.5 ml were poured into the apparatus to be mechanically stressed, the rest was left at the same temperature in the box to serve as a control. Every 15 min the control was gently stirred to prevent sedimentation and to allow sufficient supply of nutrients to the cells. During the experiment the pH value was stabilized by inserting a small piece of dry ice into the box. After finishing each experiment, the pH of both experimental and control sample was measured; the nonsystematic differences were below 0.1 pH units. No differences were found in packed cells volumes either. Hemolysis never exceeded 1 %.

The duration of the experiment itself was 3 hours. Every 30 min the glucose concentration in the medium, deproteinized by uranyl acetate, was determined in the stressed and in the control sample using a Lachema kit (Brno), working on the glucose oxidase — peroxidase — chromophore principle. After completion of each experiment, hematocrit and nucleotide concentrations were determined, and classical Giemsa-stained smears were prepared. Morphology of defective cells was defined according to Bessis classification (Bessis 1973). The time course of glucose concentration (Fig. 1) was analysed using least-square linear regression. Two straight lines were obtained for each experiment; a ratio of their slopes (value H, expressed in %) was used as the characteristic of the increase of glucose consumption in response to stress.

The following media were used to prepare erythrocyte suspensions:

A: Autologous heparinized plasma

B: Serum of the same blood group

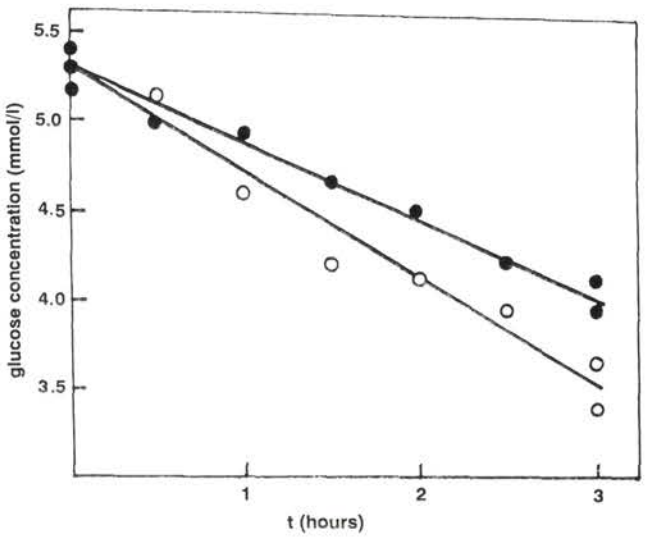

Fig. 1. Time course of glucose consumption by erythrocytes suspended in autologous heparinized plasma (○ — shear rate 2000 s^{-1}, ● — control). The slopes of the straight lines are −0.59 mmol/l/hour and −0.45 mmol/l/hour resp.; their ratio (H value) is 131 %.

C: Ringer's solution (141 mmol/l Na^+, 5.9 mmol/l K^+, 3.5 mmol/l Ca^{2+}, 1.2 mmol/l Mg^{2+}, 128.2 mmol/l Cl^-, 1.2 mmol/l $(H_2PO_4)^-$, 1.2 mmol/l $(SO_4)^{2-}$, CO_2), pH 7.4
D: Ringer's solution (C) supplemented with 5 % human serum albumin
E: Ringer's solution supplemented with albumin (D) but without Ca^{2+}
F: Ringer's solution supplemented with albumin (D) but without Mg^{2+}
To the media C — F glucose was added to final concentration of 5 mmol/l.

Resealed ghosts with high concentrations of hemoglobin and of other intracellular components were prepared. To one volume of fresh erythrocytes washed 3 times in cold Ringer's solution, 1.5 volume of ice-cold water was added dropwise. After 10 min in ice bath saturated NaCl solution was added slowly while stirring to restore isotonicity. After 15 min of incubation at 37 °C the resealed ghosts were collected by centrifugation (5600 × *g*/20 min). The ghosts were washed with Ringer's solution, suspended in their original plasma and used for the experiment.

The determination of nucleotide concentrations in red cells was based on the method of Crescentinini and Stocchi (1984). Perchloric acid extracts of erythrocytes were neutralized with potassium carbonate, centrifuged and applied on a Separon SGX C18 (TESSEK, Czechoslovakia) 30 × 0.3 cm reversed-phase column. The LKB HPLC system worked with a 254 nm monitor. The mobile phase consisted of 0.1 mol/l KH_2PO_4 solution, pH 5.8, and contained 2.5 % methanol (isocratic arrangement). One analysis took about 15 min at a flow rate of 0.5 ml/min at room temperature.

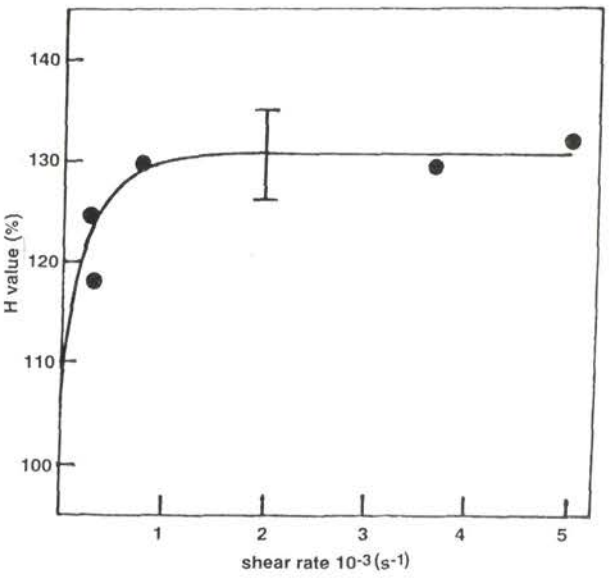

Fig. 2. The dependence of H value on shear rate for erythrocytes in autologous plasma.

Results

Previously (Kodíček et al. 1987) we reported H values (see Methods) for red cells in autologous heparinized plasma to range between 120 and 150 %, with an average value of approx. 130 %. These results were obtained for a shear rate interval of 2000—5000 s^{-1}. The dependence of the H value on the shear rate (Fig. 2) shows that the increase of glucose consumption reaches a plateau at relatively low levels of shear stress. All experiments described below were performed at the shear rate of 2000 s^{-1} (4 revolutions of the rotating cylinder per second) where the H value only weakly depends on shear rate changes.

In attempting to obtain information about the state of metabolism in the stressed erythrocytes we analysed the content of nucleotides in the cells after completion of the experiment (Fig. 3). The method of HPLC, using deproteinization by perchloric acid, allowed to determine concentrations of ATP, ADP, NAD and NADP. No shifts in nucleotide concentrations studied were observed in plasma suspended, stress exposed red cells. The shape of stressed and control cells were compared in native state in suspension (light microscope, phase contrast) and on Giemsa-stained smears after the completion of the experiment. No differences were disclosed by any of these methods. These observations

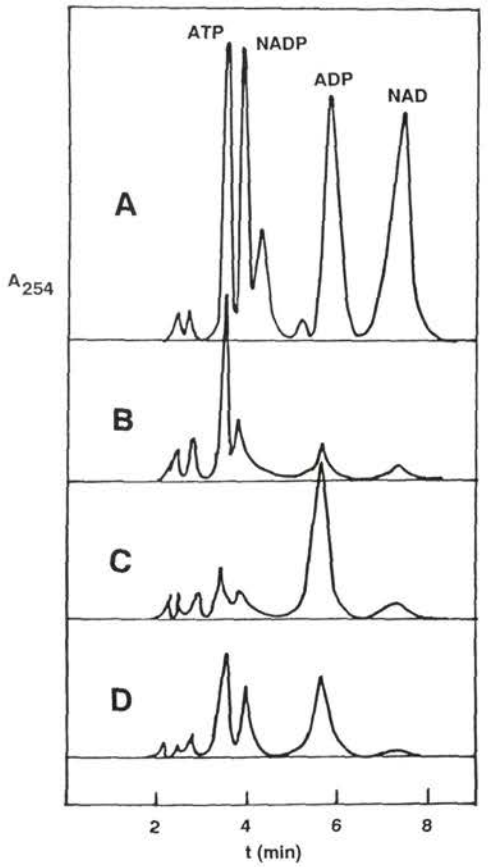

Fig. 3. HPLC profiles of nucleotide concentration analysis. *A*: Mixture of standards; *B*: Fresh erythrocyte extract; *C*: Extract of metabolically depleted erythrocytes (48 hours at 37 °C); *D*: Extract of resealed red ghosts (enlarged 10 ×).

suggested that mechanical stress, as produced in our experiments, did not induce any irreversible changes in cell metabolism or in membrane structure.

A different situation occurred when the red cells were suspended in some nonphysiological media. In Ringer's solution (medium C) which contained all the inorganic components of plasma (but no proteins), the red cells did not increase their glucose consumption (H = 100 %). Neither their nucleotide concentrations nor native cell shape did change; on Giemsa smears, however, many knizocytes occurred in the stressed sample while the control showed a normal picture of discocytes.

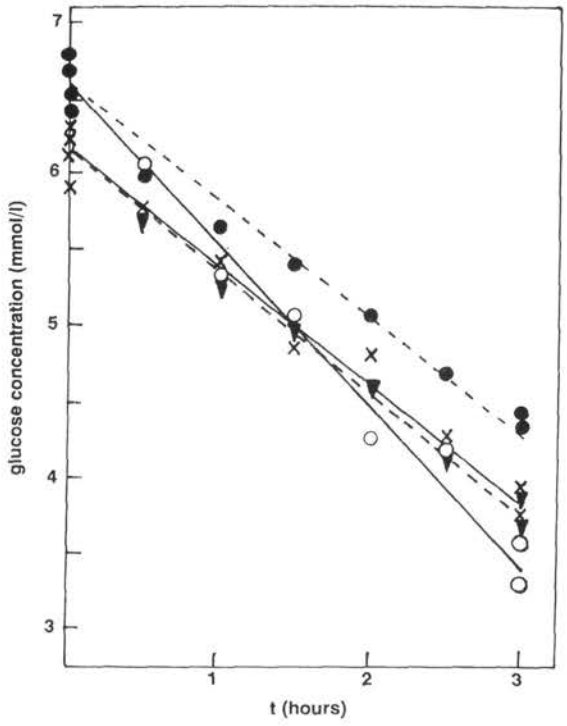

Fig. 4. Time course of glucose concentration in the absence of extracellular Mg^{2+} (○, ● — medium F), and in the absence of Ca^{2+} (×, ▼ — medium E): (full lines, mechanically stressed erythrocytes; dashed lines, controls).

This above observation suggested that the absence of proteins in the medium presents some "difficulties" for mechanically stressed cells. This idea was supported by the experiment in which albumin was added to Ringer's solution (medium D). In this medium the red cells behaved similarly as they did in plasma or serum: the glucose consumption was increased by stress, and no morphological changes were observed either in the suspension or on smears.

In further experiments the effects of divalent ions present in the medium on the behaviour of red cells under stress were studied. The absence of Mg^{2+} (medium F) was not associated with any significant change; stress raised the glucose consumption (Fig. 4), and the cells did not change their morphology. On the other hand, in the absence of Ca^{2+} (medium E) the cells were unable to increase their glucose consumption (Fig. 4); the nucleotide concentrations and

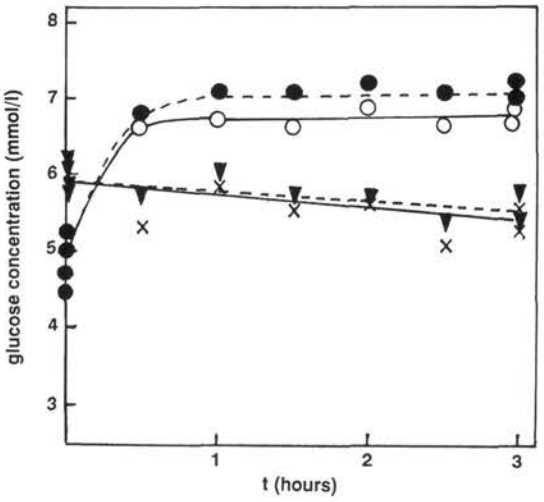

Fig. 5. Time course of glucose concentration; resealed red cell ghosts suspended in plasma (full lines, mechanically stressed ghosts; dashed lines, controls). In the first experiment (×, ▼) the samples were deproteinized with perchloric acid, and the obtained values represent the overall amounts of glucose in the samples. In the second experiment (○, ●) uranyl acetate was used, which induces no red cell hemolysis.

native morphology (in suspension) were normal, whereas many dacryocytes and acanthocytes were found on the Giemsa smears.

To study the effect of membrane perturbations on cell resistance to stress, resealed red cell ghosts were prepared (see Methods) with reduced ATP/ADP ratio (to 1 as compared with the normal value of approx. 10, Fig. 3). The time course of glucose concentration was completely different from that in intact cells. When perchloric acid was used as the deproteinizing agent, the glucose concentration in the extracts had a slightly decreasing tendency (Fig. 5), and glucose consumption by the stressed sample was not enhanced but insignificantly. In uranyl acetate extracts the glucose concentration increased and rapidly reached a limit value; this time course suggested that the membrane had been irreversibly damaged during the ghosts preparation, and that transport, after some time at physiological temperature, differs from that in intact cells, the membranes being unable to prevent diffusion of glucose out of the ghosts. Under these experimental conditions mechanical stress caused irreversible shape changes. In the "native" suspensions of the stressed cells there were many echinocytic ghosts (up to 50 %) as compared to the control value of approx.

15 %; the unstressed ghosts tended to form rouleaux. The differences between stressed cells and controls were less pronounced on smears; in both cases various deviated shapes could be seen: knizocytes, echinocytes, dacryocytes and target cells. These experiments with resealed ghosts confirmed that the methods used were sufficiently sensitive to reflect deviations in cell metabolism, glucose transport and membrane structure that mechanical stress might produce on red cells.

Discussion

The present experiments confirmed our previous observations that mechanical stress strongly enhances the consumption of glucose by red cells under "physiological" conditions, i.e. in plasma, serum or in Ringer's solution supplemented with albumin. In these media mechanical stress does not produce any changes in morphology; an analysis of nucleotide concentrations suggested that the content of the intracellular chemical energy remains unaffected. Similarly, no changes were found when the cells were suspended in Mg^{2+}-free protein-containing Ringer's solution; the intracellular Mg^{2+} concentration was not affected by mechanical stress, and sufficient amount of Mg-ATP complex, the substrate of intracellular ATPases, was formed.

On the other hand, Ca^{2+} ions play an important role in the restoration of erythrocyte shape after deformation. It has been known since the beginning of this decade that Ca^{2+} permeability of human erythrocytes is enhanced by shear stress (Larsen et al. 1981), and that these ions activate the contractile membrane ATPase (Mirčevová et al. 1979). This activation of the supramolecular contractile complex seems to be vital for the proper function of the membrane. If Ca^{2+} ions are not present in medium, this mechanism does not work, and after some time the structure of the membrane is broken. Even if native cells in suspension gave a normal picture, stress during preparation of the smears produced a number of abnormal shapes in the mechanically stressed samples.

It is still not entirely clear why the cells need an extracellular protein to be able to function properly when exposed to mechanical stress. Proteins affect cell properties in a very complex manner: they change the surface tension of the solution and interact in many ways with the cell membrane affecting its deformability (Leonhardt and Reinhardt 1977) as well as cell shape (Mehta 1983). The decreased membrane stability leads to some structural changes under stress: on smears, they are reflected as morphological deviations resembling those occurring in the absence of Ca^{2+}.

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