THE EFFECT OF GLUCOSE AND INSULIN UPON HUMAN ERYTHROCYTE MEMBRANE ATPases

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Abstract. Elevated level of glucose in the blood is known to cause membrane damage. By using human erythrocytes the present study has examined the changes that are induced by hyperglycemia in the absence or presence of a different amount of insulin on the activity of membrane ATPases. Normal human erythrocytes were incubated with varying concentrations of glucose at 37 °C for 72 h, in the presence or absence of 10 mIU/l or 200 mIU/l insulin. Erythrocytes incubated with an elevated level of glucose showed a modified electrophoretic profile of membrane proteins, a significantly increased protein glycation and reduced ATPases activity. A significantly positive correlation was observed between the extent of protein glycation and the activity of membrane ATPases. 200 mIU/l insulin restores the electrophoretic profile of membrane protein, reduces the level of protein glycation and increases the enzymatic activity to 90% of control value.

Key words: human erythrocyte membrane, hyperglycemia, diabetes mellitus, Na^+, K^+-ATPase, Ca^{2+}, Mg^{2+}-ATPase.

INTRODUCTION

Diabetes mellitus is a disease associated with a confusing array of metabolic abnormalities, which can also concern erythrocyte function. The changes that affect the respiratory function of the erythrocyte are reflected upon every tissue of the body. Erythrocytes of diabetic patients have reduced life span [22], altered membrane dynamic properties [27] and increased membrane thermostability [19]. It has also been reported that diabetic patients with poor metabolic control have lower erythrocyte membrane enzymes activity as compared to healthy control subjects [8, 9]. Diabetes has been correlated with an increase in glycation of erythrocyte membrane proteins [4]. The role of protein glycation in the long-term

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pathogenesis of diabetes has been extensively investigated [16, 28]. The modified proteins have altered functions such as modified enzymatic activities [1, 10], lower affinities for their receptors [21], etc.

The aim of the present work was to obtain some information about the changes of the membrane enzymes from erythrocytes incubated for 72 h in hyperglycemic medium culture, in the presence and absence of variable amounts of insulin. Our data show a decrease in the membrane Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities. In all the cases, the ATPases activity was negatively correlated with the incubation time, glucose concentration and protein glycation. 200 mIU/l insulin reduces the level of protein glycation and partially restores the enzymatic activity.

MATERIALS AND METHODS

CHEMICALS

Chemicals of analytical grade were obtained from Sigma. Insulin was produced by NovoNordisk.

SEPARATION AND INCUBATION OF ERYTHROCYTES

Normal erythrocytes were isolated from blood samples (20 – 25 ml) drawn by venous puncture, with informed consent of the subject, into tubes containing 1 mmol/l EDTA as anticoagulant. Venous blood samples were centrifuged at 1000 × g for 15 min at 4 °C and the plasma and buffy white layer of cells were removed. The packed erythrocyte pellet was washed three times with an equal volume of sterile 150 mM NaCl, pH = 7.4. Healthy human erythrocyte suspensions containing 4×10^6 cells/ml, prepared in one of the following media, were incubated for 72 h at 37 °C:

a. 1 g/l glucose DMEM
b. 3.6 g/l glucose DMEM
c. 3.6 g/l glucose DMEM + 10 mIU/l insulin
d. 3.6 g/l glucose DMEM + 200 mIU/l insulin

We carried out the studies from the cells harvested after 24, 48 and 72 h.

MEMBRANE PREPARATION

Erythrocyte membrane isolation was done using the procedure of Hanahan and Ekholm [11]. The erythrocytes were isolated by centrifugation and washed three times in 170 mM Tris-HCl, pH = 7.4. The washed cells were hemolysed in 20

vol. of 10 mmol/l Tris-HCl buffer, pH = 7.4 and the membranes ("ghosts") were collected by centrifugation at 10,000 × g for 60 min at 4 °C. The membranes were washed free of hemoglobin by repeated centrifugation through 10 mmol/l Tris-HCl buffer, pH = 7.4 until the pellet was white or pale pink in color. The pellet was suspended in 10 mmol/l Tris-HCl buffer, pH = 7.4 at a protein concentration of 1 mg/ml. The protein amount was determined by the procedure of Bradford [5].

ELECTROPHORESIS

Membrane peptides were separated using sodium dodecylsulfate (SDS) polyacrylamide system described by Laemmli (15). The slab gel consisted of a running gel of 6 – 12% acrylamide and 5% staking gel. Samples of 100 μg protein were applied and the electrophoresis was carried out at 15 mA (40V) for 1.5 h and at 20 mA (100V) until the dye reached the bottom of the running gel (about 15 hours) in the running buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS). The gels were fixed for 1 h in 45% (vol./vol.) methanol/10% (vol./vol.) acetic acid and then stained 15 min in the solution containing 0.07% (wt./vol.) Coomassie brilliant blue R-250. Destaining was performed with 10% (vol./vol.) acetic acid.

ASSAY OF Na^+, K^+-ATPase AND Mg^{2+}-ATPase

ATP-hydrolysis by erythrocyte ghosts was measured at 37 °C in 50 mmol/l Tris-HCl buffer, pH = 7.4, containing 130 mmol/l NaCl, 20 mmol/l KCl, 7 mmol/l $MgCl_2$ and 4 mmol/l ATP in the presence and absence of 10^{-3} mol/l ouabain following the method of Garner [8]. The ghosts were tested with ATP solution ± ouabain in order to discriminate the purely ouabain-independent Mg^{2+}-dependent ATPase activity. Na^+, K^+-ATPase-dependent ATP hydrolysis was computed as the difference between the rates determined in the absence and presence of 10^{-3} mol/l ouabain, and is reported as nmoles Pi released/h /mg protein.

ASSAY OF Ca^{2+}, Mg^{2+}-ATPase

Membrane bound Ca^{2+}, Mg^{2+}-ATPase activity was determined at 37°C in a medium containing 120 mmol/l KCl, 30 mmol/l Tris-HCl (pH = 7.4), 4 mmol/l $MgCl_2$, 0.1 mmol/l ouabain, 1.1 mmol/l $CaCl_2$ and 2 mmol/l ATP in the presence and absence of 1 μg/ml calmodulin [9].

PROTEIN GLYCATION

Glycation of red blood cell membrane proteins was evaluated by the method of Murtiashaw et al [18]. Results are expressed as nmoles of 5-hydroxymethyl-2-furaldehyde (HMF) released from glycated proteins submitted to hydrolysis with oxalic acid.

STATISTICAL ANALYSIS

All data are presented as means ± SEM. The Student's *t*-test performed with Microsoft Excel was used to determine two-tailed *P* values. Differences were considered significant if $P < 0.05$.

RESULTS

ELECTROPHORESIS

The electrophoretic profile of erythrocyte membrane proteins after 72 h incubation in DMEM containing 1 g/l glucose (a), 3.6 g/l glucose (b), 3.6 g/l glucose and 10 mIU/l insulin (c) and 3.6 g/l glucose and 200 mIU/l insulin (d) was analyzed using the Scion Image program and the results are presented in Fig. 1. In lane e are shown the molecular weight markers. There is a marked difference between the gel profile of membrane proteins from erythrocytes incubated in medium containing 1 g/l glucose (Fig. 1a) and the gel profile of membrane proteins from erythrocytes incubated with hyperglycemic medium (Fig. 1b). 10 mIU/l insulin in hyperglycemic medium does not affect the electrophoretic profile (Fig. 1c), while 200 mIU/l insulin in hyperglycemic medium partially restores the electrophoretic profile of membrane proteins back to normal (Fig. 1d).

MEMBRANE ATP-ases ACTIVITY

Our results show that erythrocyte membrane ATPases activity was significantly lower ($P < 0.01$) after 72 h incubation in hyperglycemic medium as compared to those incubated in culture medium with 1 g/l glucose (Table 1). 10 mIU/l insulin in hyperglycemic medium does not enhance the enzymes activity, but the presence of insulin in a concentration of 200 mIU/l in hyperglycemic medium leads to an increase of enzymes activity.

The sodium pump activity in erythrocytes incubated in hyperglycemic medium was reduced with 60% after 72 h of incubation as compared to control. 200 mIU/l insulin restores pump activity to 80% as compared to control (Fig. 2).

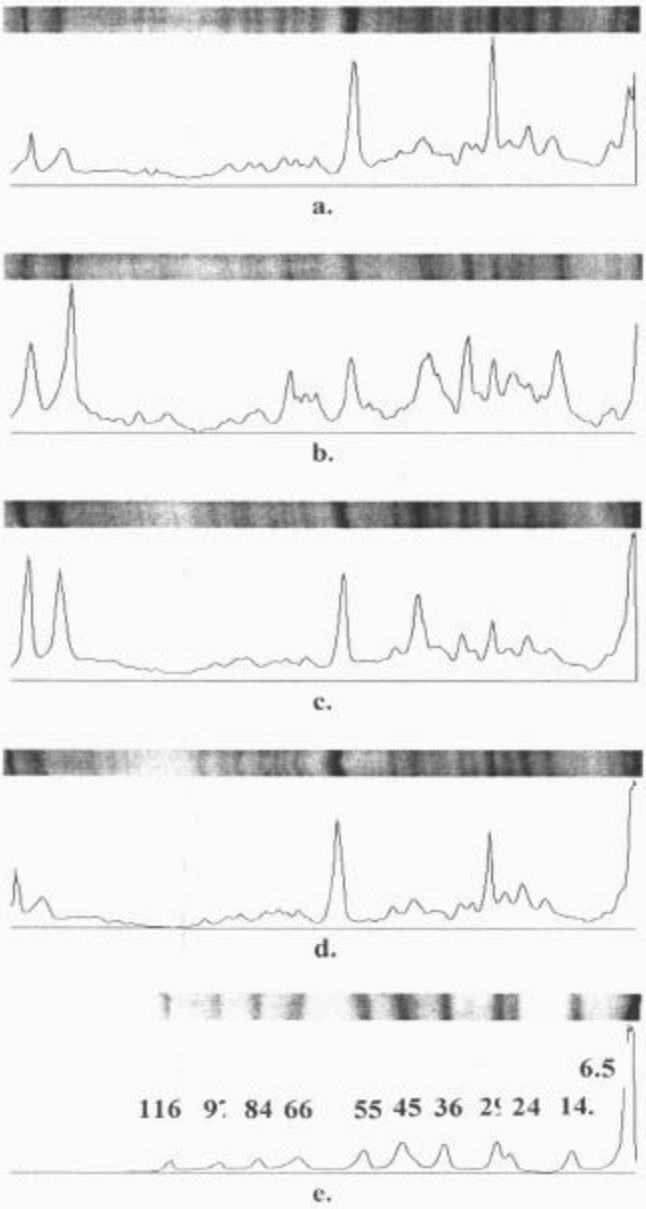

Fig. 1. – Gel profile of erythrocyte membrane proteins after 72 h incubation in DMEM containing 1 g/l glucose (a.), 3.6 g/l glucose (b.), 3.6 g/l glucose and 10 mIU/l insulin (c.) and 3.6 g/l glucose and 200 mIU/l insulin (d.); molecular weight markers (e.) (kD).

Mg^{2+}-ATPase retained 73% of the activity after 72 h incubation in culture medium with 3.6 g/l glucose, as compared to control. 200 mIU/l insulin restores the pump activity to 78% of control value (Fig. 3).

Ca^{2+}, Mg^{2+}-ATPase was inhibited by 50% after 72h incubation in hyperglycemic DMEM, as compared to control. 200 mIU/l insulin restores pump activity to 84% as compared to control (Fig. 4).

Table 1

Erythrocyte membrane Na^+, K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase activities and protein glycation after 72 h erythrocyte incubation, at 37 °C, in hyperglycemic DMEM with or without different concentrations of insulin (* $P < 0.01$).

Incubation medium	Na^+, K^+-ATPase activity (nmol P_i/h/mg protein)	Mg^{2+}-ATPase activity (nmol P_i/h/mg protein)	Ca^{2+}, Mg^{2+}-ATPase activity (nmol P_i/h/mg protein)	Protein glycation (nmol HMF/mg protein)
1 g/l glucose	504.0 ± 17.50	245.0 ± 3.00	1177.0 ± 24.10	11.0 ± 0.06
3.6 g/l glucose	196.3 ± 47.60*	179.0 ± 2.00*	589.0 ± 34.70*	37.4 ± 0.32*
3.6 g/l glucose + 10 mIU/l insulin	131.7 ± 14.40*	167.3 ± 1.73*	644.0 ± 14.60*	36.7 ± 0.17*
3.6 g/l glucose + 200 mIU/l insulin	400.0 ± 8.08	190.3 ± 1.00	993.0 ± 6.67	14.2 ± 0.14

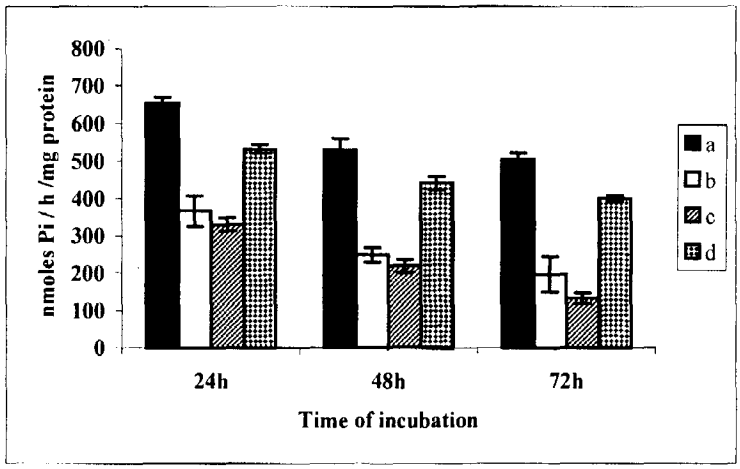

Fig. 2. – Na^+, K^+-ATPase activity after 24, 48 and 72 h incubation in 1 g/l glucose DMEM (a), 3.6 g/l glucose DMEM (b), 3.6 g/l glucose DMEM + 10 mIU/l insulin (c), and 3.6 g/l glucose DMEM + 200 mIU/l insulin (d).

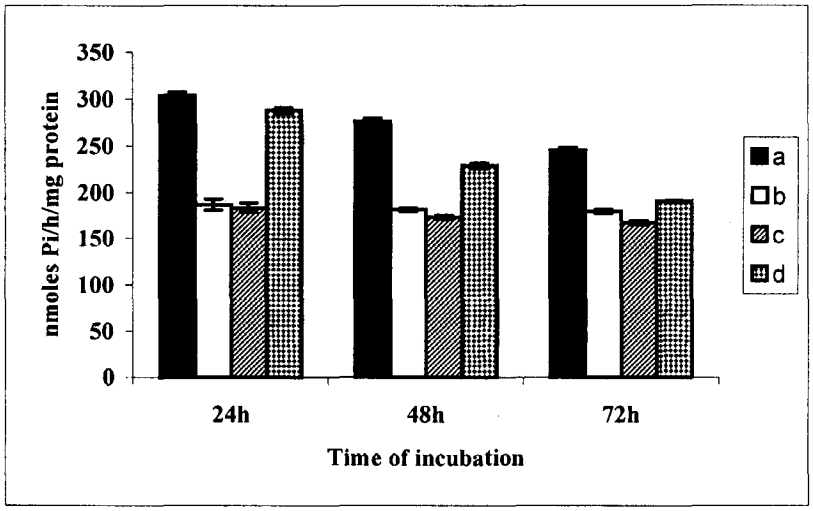

Fig. 3. – Mg^{2+}-ATPase activity after 24, 48 and 72 h incubation in 1 g/l glucose DMEM (a), 3.6 g/l glucose DMEM (b), 3.6 g/l glucose DMEM + 10 mIU/l insulin (c), and 3.6 g/l glucose DMEM + 200 mIU/l insulin (d).

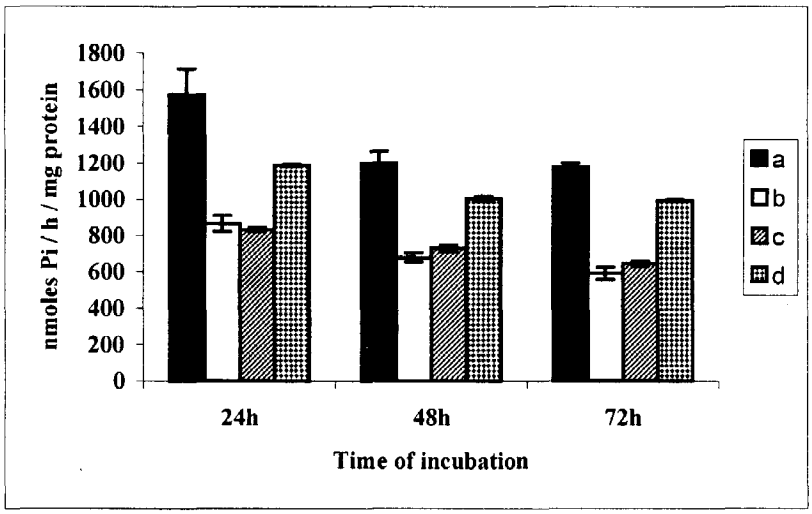

Fig. 4. – Ca^{2+}, Mg^{2+}-ATPase activity after 24, 48 and 72 h incubation in 1 g/l glucose DMEM (a), 3.6 g/l glucose DMEM (b), 3.6 g/l glucose DMEM + 10 mIU/l insulin (c), and 3.6 g/l glucose DMEM + 200 mIU/l insulin (d).

Calmodulin activated Ca^{2+}, Mg^{2+}-ATPase activity is also strongly inhibited during erythrocyte incubation in DMEM with 3.6 g/l glucose for 72 h (Fig. 5).

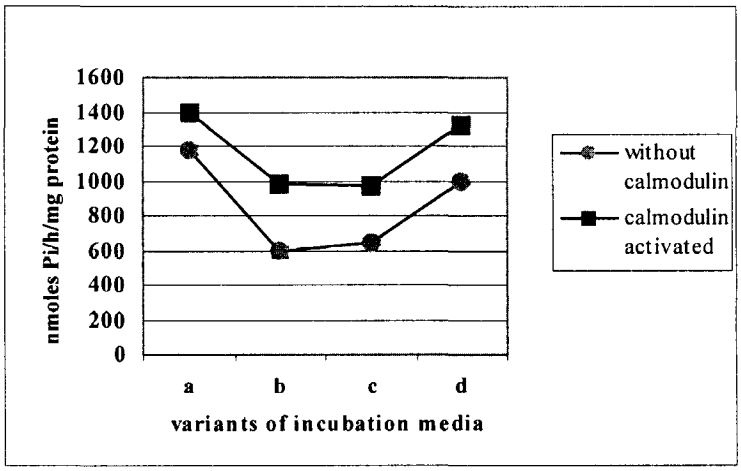

Fig. 5. – Calmodulin activated Ca^{2+}, Mg^{2+}-ATPase activity after 72 h incubation in 1 g/l glucose DMEM (a), 3.6 g/l glucose DMEM (b), 3.6 g/l glucose DMEM + 10 mIU/l insulin (c), and 3.6 g/l glucose DMEM + 200 mIU/l insulin (d).

PROTEIN GLYCATION

Erythrocyte incubation in hyperglycemic DMEM for 72 h at 37 °C leads to a 3.5-fold increase in membrane protein glycation. 200 mIU/l insulin restores the degree of protein glycation to a value comparable with control (Table 1).

DISCUSSION

Diabetes mellitus is a disease associated with abnormal carbohydrate metabolism, arising from insulin deficiency and/or malfunction of insulin receptors. The consequent elevation of glucose in the blood plasma affects primarily the tissues with insulin-independent uptake of glucose like lens fiber cells, the vascular endothelial cells and red blood cells [24]. The hyperglycemia is considered the major causal factor in the development of chronic clinical complications: cataract, retinopathy, nephropathy, neuropathy, generalized microangiopathy and arteriosclerosis [13]. There are some characteristic features of the erythrocyte biology in diabetes mellitus which are thought to contribute to clinical abnormalities of microcirculation: increased red blood cell aggregation, decreased deformability, life-span and membrane ATPases activity [26].

In the present study, isolated normal human erythrocytes were incubated for 72 h at 37 °C in DMEM containing 5 mM and 20 mM glucose in the presence or absence of 10 mIU/l and 200 mIU/l insulin, in order to model normal and

hyperglycemic status, respectively, and to observe the capability of insulin treatment to reverse some of the cellular effects of hyperglycemia. Hyperglycemic treatment of normal erythrocytes produces some of the alterations observed in diabetic cells. In our hyperglycemic model, the activity of membrane ATPases was decreased. The decrease in enzymes activity was accompanied by changes in the electrophoretic pattern of erythrocyte membrane proteins and by an increased glycation of membrane proteins.

Na^+, K^+-ATPase activity was decreased to 40% of control activity after 72 h incubation in hyperglycemic DMEM. 200 mIU/l insulin restores pump activity to 80% as compared to control (Fig. 3). A change in the activity of membrane-bound Na^+, K^+-ATPase can have negative consequences in tissues. For example, changes in cell Na^+ concentration might be expected to result in changes in cell Ca^{2+}, due to alteration of Na^+/Ca^{2+} exchange [8]. Na^+, K^+-ATPase activity dysfunction could be implicated in the pathogenesis of human diabetic neuropathy [20].

Our study also demonstrates a decrease in erythrocyte Mg^{2+}-ATPase activity to 73% of control due to hyperglycemic conditions. 200 mIU/l insulin restores pump activity to 78% as compared to control (Fig. 4). The Mg^{2+}-dependent ATPase activity located in the erythrocyte membranes appears to be responsible for controlling the smoothing of echinocytic erythrocytes to discocytes and stomatocytes. The activity may be associated with a phospholipid translocase which uses the energy released from the hydrolysis of ATP to translocation the aminophospholipids, phophatidylserine and phosphatidylethanolamine from the outer leaflet to the inner leaflet of the lipid bilayer [17]. Phosphatidylserine transport is reduced in a number of normal and pathologic conditions that result in a redistribution of phospholipids, such as platelet activation [3], sickle cell anemia [7] and Ca^{2+}-loading. The newly exposed phosphatidylserine provides a catalytic surface for the activation of the enzymes of the coagulation cascade [2]. Similar alterations in phosphatidylserine asymmetry are associated with several pathologic conditions and may contribute to unwanted thromboses. Diabetic blood cells show an increase in passive transmembrane lipid movement and phosphatidylserine appears in the plasma membrane outer monolayer [28]. The loss of transmembrane phosphatidylserine asymmetry may increase procoagulant activity and contribute significantly to vascular occlusion [26]. Externalized erythrocyte phosphatidylserine may trigger the removal of erythrocytes by macrophages, being similar to the process involved in the programmed cell death [6, 12, 29].

The results obtained in our study showed that hyperglycemia leads to 50% decrease in Ca^{2+}, Mg^{2+}-ATPase activity, too. Calmodulin activated Ca^{2+}, Mg^{2+}-ATPase activity was also decreased in erythrocytes incubated in hyperglycemic DMEM. 200 mIU/l insulin restores pump activity to 84% as compared to control (Fig. 5). Decreased Ca^{2+}, Mg^{2+}-ATPase activity may produce an imbalance in the erythrocyte transmembrane Ca^{2+} flux leading to an increase of erythrocyte Ca^{2+} content. This could be associated with a greater membrane rigidity [23] and phosphatidylserine exposure in the external membrane leaflet. Membrane changes

identified in erythrocyte during Ca^{2+} loading, such as phosphatidylserine exposure, microvesicle release and diminished membrane fluidity may relate to alterations present in several erythrocyte pathologies: cell aging, secondary effects of hypertension, spherocytosis, thalassemia and sickle cell disease. Recent data have revealed that the alterations of membrane properties are responsible for the susceptibility of erythrocytes to secretory phospholipase A_2 and hence to the lysis of intact cells mediated by this enzyme [25].

The presence of insulin in a concentration of 200 mIU/l in hyperglycemic incubation medium reduces the degree of erythrocyte membrane protein glycation to a value comparable with control. This change is positively correlated with a normal electrophoretic pattern of erythrocyte membrane proteins and with an increase in the enzymes activity between 77% and 84% of their control value. Our results are in agreement with the observations made on the biochemical and rheological parameters in erythrocytes from diabetic patients subjected to insulin treatment. The erythrocyte abnormalities observed were corrected after 24 h of normoglycemia obtained by insulin treatment [14].

In conclusion, the incubation of normal red blood cells in hyperglycemic DMEM for 72 h at 37°C leads to a decreased activity of membrane ATPases, correlated with the time of incubation and glucose concentration in the incubation medium. 200 mIU/l insulin in the incubation medium restores the enzymatic activity to a value closer to control.

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