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Research Paper

Effect of high glucose concentrations on human erythrocytes *in vitro*

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ABSTRACT

Exposure to high glucose concentrations *in vitro* is often employed as a model for understanding erythrocyte modifications in diabetes. However, effects of such experiments may be affected by glucose consumption during prolonged incubation and changes of cellular parameters conditioned by impaired energy balance. The aim of this study was to compare alterations in various red cell parameters in this type of experiment to differentiate between those affected by glycoxidation and those affected by energy imbalance. Erythrocytes were incubated with 5, 45 or 100 mM glucose for up to 72 h. High glucose concentrations intensified lipid peroxidation and loss of activities of erythrocyte enzymes (glutathione *S*-transferase and glutathione reductase). On the other hand, hemolysis, eryptosis, calcium accumulation, loss of glutathione and increase in the GSSG/GSH ratio were attenuated by high glucose apparently due to maintenance of energy supply to the cells. Loss of plasma membrane Ca^{2+}-ATPase activity and decrease in superoxide production were not affected by glucose concentration, being seemingly determined by processes independent of both glycoxidation and energy depletion. These results point to the necessity of careful interpretation of data obtained in experiments, in which erythrocytes are subject to treatment with high glucose concentrations *in vitro*.

Introduction

Hyperglycemia is the most important factor in the onset and progress of diabetic complications. Diabetes mellitus is a metabolic disorder resulting in decreased number of pump units on the erythrocyte membrane, altered lipid–protein interactions, enzyme glycation and peroxidation which account for many complications [1]. Human erythrocytes are continuously exposed to glucose in plasma during their circulatory life span of 120 days. Passive transport through insulin-independent glucose transporter, GLUT1, ensures that the glucose concentration in the erythrocyte cytosol is close to that in the plasma, normally about 5 mM, and increased under conditions of hyperglycemia [2]. The erythrocytes and their membranes have always been objects of studies as they play an important role in various physiological and metabolic events. Human erythrocyte properties such as deformability and elasticity are affected by Ca^{2+} ions [3,4] whose level is regulated by plasma membrane Ca^{2+}-ATPase (PMCA). A rise in internal Ca^{2+} concentration leads to changes in cell shape and volume, increased cellular rigidity, and hemolysis [5,6].

Ca^{2+}-ATPase activity of erythrocyte membranes is known to be decreased in diabetic patients with elevated blood glucose [7,8] and in red blood cells (RBCs) treated *ex vivo* with glucose [9], and there is evidence to suggest that such effects are related to glycation of plasma membrane Ca^{2+}-ATPase (PMCA) [10,11]. However, Raftos et al. found that V_{max} of active Ca^{2+} extrusion from intact RBCs is unaffected by their exposure *in vivo* or *in vitro* to high concentration of glucose for up to 6 h [12].

The red blood cell membrane contains approximately equal weight amounts of lipids and proteins [1]. Lipid peroxidation alters the microenvironment of membrane bound enzymes by changing phospholipids and fatty acid composition [13]. In addition, membrane proteins are also glycated, which may lead to decrease in their activities [14]. Hyperglycemia in diabetes mellitus causes glycation of membrane enzymes along with oxidative stress leading to decrease in activity of Na^+/K^+-ATPase and other changes in erythrocyte membranes [1].

Abbreviations: ATPase, adenosinetriphosphatase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FITC, fluorescein isothiocyanate; GLUT1, glucose transporter 1; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; Hb, hemoglobin; HMF, 5-(hydroxymethyl)-2-furaldehyde; PBS, phosphate-buffered saline; PMCA, plasma membrane Ca^{2+}-ATPase; PS, phosphatidylserine; RBCs, red blood cells; RQB, redox quenching buffer; TCA, trichloroacetic acid

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Asymmetry of transmembrane phospholipids is the characteristic feature of biological membranes. Phospholipids containing amino groups such as phosphatidylserine (PS) are localized mainly or exclusively in the inner leaflet of the plasma membrane [15]. Loss of membrane lipid asymmetry is associated with a number of physiological and pathological events [16]. Under hyperglycemic conditions, the concentration of glucose metabolite methylglyoxal is dramatically elevated in plasma as well as in erythrocytes. Increased concentration of methylglyoxal induces exposure of PS on the outer surface of erythrocytes, disrupting the asymmetry of membrane phospholipids [17]. As erythrocytes lack nucleus and mitochondria, death of erythrocytes has been called eryptosis [18] to distinguish it from apoptosis of nucleated cells. Increase of both, eryptosis and advanced glycation products, was reported in high glucose (40 and 100 mM) treated erythrocytes [19].

Incubation of erythrocytes under conditions of high glucose concentrations is usually used as a model to mimic the *in vivo* situation of hyperglycemia in diabetes. However, another aspect of this type of experiments should be taken into account: if no glucose is added during the incubation, cells incubated with initial physiological concentration of glucose utilize it during the incubation while those incubated with high glucose have longer supply of energy source. As a result, some their parameters, not so strongly affected by glycation, change less than in control erythrocytes incubated with initially physiological levels of glucose. We noted such a phenomenon in preliminary experiments; this study was aimed at comparison of behavior of several parameters critical for the function of erythrocytes during incubation with initially physiological glucose concentration (5 mM) and with high glucose (45 and 100 mM). We expected to identify by this approach those parameters whose changes are mainly dependent on glycation and those whose changes depend critically on maintenance of the energy balance of erythrocytes.

Material and methods

Ethical approval

The study was approved by the Research Bioethics Committee of the University of Łódź (Poland).

Chemicals and equipment

All basic reagents were from Sigma-Aldrich Company (Poznan, Poland). All reagents used were of analytical reagent grade. All the spectrophotometric analyses were done using a Varian Cary 50 UV–vis spectrophotometer (Varian Inc., Cary, NC, USA). Fluorescence was read using an Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland).

Glucose incubation routine

Freshly drawn human whole blood was obtained from the Blood Bank in Łódź. Blood was centrifuged (2000*g*, 10 min, 4 °C), plasma and leukocyte buffy coat were aspirated and the erythrocyte pellet was washed three times with 4 volumes of phosphate buffered saline (PBS; 1 tablet of PBS/100 ml H_2O) per 1 volume of a suspension. Residual leukocytes were removed by passing erythrocyte suspensions through a 1:1 (w/w) mixture of a cellulose and microcrystalline cellulose [20].

Washed red blood cells (RBCs) were suspended to a final hematocrit of 10% in PBS containing varying concentrations of glucose (5, 45 or 100 mM) and 10 μM ampicillin, and incubated at 37 °C for 24, 48 and 72 h with continuous mixing. The moderately high (45 mM) and very high (100 mM) glucose concentrations employed had been applied in earlier studies by other authors [9,12,21–23]. Erythrocytes suspended in PBS-glucose (5 mM) were used as a control, because of normal blood glucose level in non-diabetic humans before meal is about 4–5.9 mM.

Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared from washed erythrocytes according to the method of Dodge et al. with some modifications [24]. Briefly, after incubation, erythrocytes were hemolyzed on ice with 20 volumes of 20 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetatic acid (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) as proteolytic inhibitors, and centrifuged at 4 °C at 20,000*g* for 20 min. The ghosts were resuspended in ice-cold 10 mM and then 5 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA, centrifuged and this process was continued until the ghosts were free from residual hemoglobin. Finally, the erythrocyte ghosts were resuspended in 20 mM phosphate buffer, pH 7.4. The protein concentration was estimated by the method of Lowry et al. [25].

Flow cytometry analysis

For evaluation of eryptosis, erythrocytes (6% hematocrit) were washed in annexin-V-binding buffer containing: 125 mM NaCl, 10 mM HEPES (pH 7.4) and 5 mM $CaCl_2$. Erythrocytes were then stained with FITC-Annexin V at a 1:20 dilution and mixed gently on a vortex mixer. After 20 min incubation in the dark at room temperature, samples were diluted 1:5, thoroughly mixed to obtain single cell suspensions, and analyzed by flow cytometry in a Becton Dickinson LSR II cytometer. Cell volume differences were estimated by forward scatter (FSC), and FITC-annexin-fluorescence intensity was measured in the FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm as previously described [17].

Measurement of intracellular Ca^{2+} in erythrocytes was performed according to Nicolay et al. [17]. Briefly, erythrocytes were loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) by addition of 10 μl of a Fluo-3/AM stock solution (2.0 mM in dimethylsulfoxide) to 10 ml erythrocyte suspension (0.16% hematocrit) in Ringer solution. The cells were incubated for 15 min under vigorous shaking. An additional 10 μl of a Fluo-3/AM was added and the cells were incubated 25 min. Fluo-3/AM-loaded erythrocytes were centrifuged at 1000*g* at 22 °C and washed twice with Ringer solution containing 0.5% bovine serum albumin (BSA) and once with albumin-free Ringer buffer. Fluo-3/AM-loaded erythrocytes were analyzed by forward and side scatter and Ca^{2+}-dependent fluorescence intensity was measured in the FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm using Becton Dickinson LSR II cytometer (BD Biosciences Franklin Lakes, New Jersey, USA).

Ca^{2+}-ATPase activity

After the treatment, ATPase activity was estimated as described previously [26]. Briefly, the membranes obtained by lysis and washing with 20 mM borate buffer instead of phosphate to avoid high phosphate background were diluted with the assay medium (final concentrations: 10 mM $MgCl_2$, 1 mM ATP, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM ouabain in 100 mM Tris–HCl buffer) and incubated at 37 °C in the presence or absence of 1 mM calcium lactate, at a protein concentration of 1.5 mg/ml, for 30 min. Samples incubated on ice served as blanks. Incubation was stopped by addition of an equal volume (70 μl) of 10% trichloroacetic acid, the samples were centrifuged and 100 μl aliquots of the supernatants were added to eppendorf tubes containing 1 ml of deionized water. Then