Effect of high glucose concentrations on human erythrocytes in vitro

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A B S T R A C T
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, and in red blood cells (RBCs) treated with glucose [9], and Raftos et al. found that Vmax of active Ca2+-ATPase 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 glication of membrane enzymes along with oxidative stress leading to decrease in activity of Na+/K+-ATPase and other changes in erythrocyte membranes [1].

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 membrane, altered lipid–protein interactions, enzyme glycation and peroxidation which account for many complications [1]. 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].

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**Abbreviations:** ATPase, adenosinetriphosphatase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetracetic 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 Ca2+-ATPase; PS, phosphatidylserine; RBCs, red blood cells; RQJB, red 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 (2000g, 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 H2O) 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 ethylenediaminetetraacetic acid (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) as proteolytic inhibitors, and centrifuged at 4 °C at 20,000g 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 CaCl2. 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 Ca2+ 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 1000g 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 Ca2+ - 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).

Ca2+–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 MgCl2, 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
the tubes were added with 200 μl of the molybdate reagent (1.75% w/v (NH₄)₂MoO₄·4H₂O in 3.15 M H₂SO₄), incubated for 10 min, added with 200 μl of 0.035% Malachite Green in 0.35% w/v polyvinyl alcohol and incubated at room temperature for another 30 min. Then absorbance of the samples was measured at 620 nm. The Ca²⁺-AT-Pase activity was calculated as the difference between the activity measured with calcium lactate and the basal activity measured without calcium.

Measurement of glycated hemoglobin

Glycated hemoglobin content was measured spectrophotometrically by the method of Standerfer and Eaton [27] and expressed as a percent of total hemoglobin. Glycosylation of hemoglobin was expressed as μmoles of 5-(hydroxymethyl)-2-furaldehyde (HMF)/g hemoglobin.

Measurement of intracellular superoxide

Intracellular superoxide was estimated by labeling cells with dihydroethidine (Karlsruhe, Germany) in the dark at 37 °C for 20 min. The probe was added to a final concentration of 5 μM. A total of 10⁵ cells were counted with an LSRII multicolor flow cytometry (Becton Dickinson).

Lipid peroxidation assay

This assay is dependent upon the sensitivity of the fluorophore BODIPY (581/591) C₁₂ to oxidation by peroxyl/alkoxyl radicals [28]. This probe readily incorporates into biological membranes and responds to free radical attack with a spectral emission shift from red to green, which can be readily monitored and quantified by flow cytometry [29].

Enzymatic activities

Catalase

Catalase activity was determined according to Aebi [30]. To 1 ml of 50 mM phosphate buffer, pH 7.0 and 1.95 ml of 50 mM phosphate buffer, pH 7.0, 50 μl of hemolysate was added and the decrease in hydrogen peroxide concentration was measured spectrophotometrically at 240 nm, at 25 °C during 1 min. The kinetic curves were analyzed using the Cary-WinUV-software. One unit of enzyme activity was defined as the activity required to degrade 1 μmol of hydrogen peroxide in 60 s. Enzyme activity was expressed as units per mg of Hb (U/mg Hb).

Glutathione S-transferase (GST)

GST activity was determined by the method described by Hubig et al. [31], 250 μl of 0.02 M glutathione (GSH) in 0.1 M phosphate buffer, pH 6.5, was added to 750 μl of 0.1 M phosphate buffer, pH 6.5, 10 μl of 0.1 M chloro-2,4-dinitrobenzene (CDNB) in ethanol and 10 μl of hemolysate was added to the cuvette. The increase of absorbance was measured at a wavelength of 340 nm for 3 min against the reagent blank. Enzyme activity was expressed as units per gram of Hb (U/g Hb).

Glutathione reductase (GR)

GR activity was determine using Glutathione Reductase Assay (RANDOX, GR2368).

Hemolysis assay

Following incubation, the samples were centrifuged (5000g, 10 min, 23 °C) and hemoglobin content in the supernatant was read at 540 nm (A). A value of 100% lysis was assigned to the supernatant of the sample with control hemolysate, obtained by freezing and thawing the cell suspensions (A₁). The percentage of hemolysis (H) in each assay suspensions was calculated by the equation:

\[ H [%] = A/A₁ \times 100 \]

Estimation of GSH and GSSG content

Glutathione determination was performed according to Senft et al. [32] as modified by Robaszkiewicz et al. [33]. Suspension of RBCs in PBS (hematocrit of 16%) treated with different concentrations of glucose was centrifuged (5000g, 10 min, 23 °C). The pellet was washed three times with PBS. Proteins of the erythrocyte pellet were precipitated with RQB–TCA buffer (20 mM HCl, 5 mM diethylenetriaminepentaacetic acid, 10 mM ascorbic acid, 5% trichloroacetic acid (TCA)), then centrifuged (5000g, 10 min, 23 °C) and the supernatant was taken for GSH and GSSG assay.

For GSH determination, 2 μl of deproteinized supernatant diluted to 25 μl with RQB–TCA were put on two wells (denoted ‘−’ and ‘+’) of a 96-well black plate. The sample ‘+’ was added with 4 μl of 7.5 mM N-ethylmaleimide in RQB–TCA, both samples added with 40 μl of 1 M potassium phosphate buffer, pH 7.0, mixed for 1 min and incubated at room temperature for 5 min. Then 160 μl of 0.1 M potassium phosphate buffer was added, followed by 25 μl of 0.5% o-phenaldehyde in methanol and the plate was shaken (1 min). After 30-min incubation (room temperature) the fluorescence was read at 355 nm/460 nm. The value obtained for the ‘−’ sample was subtracted from that obtained for the ‘+’ value and GSH concentration was read from a calibration curve obtained with glutathione as a standard.

For determining GSSG concentration, two paired samples, ‘−’ and ‘+’, each containing 25 μl of deproteinized supernatant, were added with 4 μl of 7.5 mM N-ethylmaleimide in RQB–TCA and 40 μl of 1 M potassium phosphate buffer. Then, 5 μl of 100 mM sodium dithionite in RQB–TCA was introduced into the sample ‘+’. The mixture was incubated at room temperature for 60 min. The remaining part of the procedure was the same as for GSH estimation. The calibration curve was prepared with GSSG.

Hemoglobin assay

Hemoglobin (Hb) concentration was estimated according to the Drabkin’s method [34]. 20 μl of the hemolysate was added to 5000 μl of the Drabkin reagent (0.03% K₃[Fe(CN)₆], 0.1% NaHCO₃, 0.005% KCN) and incubated for 15 min; then the absorbance was measured at a wavelength of 540 nm against a reagent blank. 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., Switzerland).

Statistical analysis

Data were expressed as means of the percentage of control ± S. D. Statistical comparison between groups was performed using the one-way ANOVA followed by Bonferroni test for mutual comparison of treated samples with control and by STATISTICS, version 10 (2010; StatSoft Inc., Tulsa, OK, USA). Differences among means were considered significant at *p < 0.05, **p < 0.01 and ***p < 0.001.

Results

The level of glycated hemoglobin in control samples (5 mM glucose) was practically not changed with increasing time of incubation. High concentrations of glucose induced glycation of hemoglobin in 48 h (100 mM glucose) and 72 h of incubation (45 and 100 mM glucose) (Fig. 1).

When erythrocytes were incubated with normal (5 mM), high (45 mM) and extremely high (100 mM) concentrations of glucose
for 24, 48 and 72 h, PMCA activity decreased with increasing incubation time in control sample (5 mM glucose) as well as in samples incubated with 45 mM or 100 mM glucose. There was no significant effect of glucose concentration on the loss of PMCA activity (Fig. 2).

Cytosolic calcium concentration increased in control sample (5 mM glucose) incubated for up to 48 and 72 h. The presence of high glucose concentrations (45 mM and 100 mM) decreased the level of calcium after incubation for up to 48 h when compared to samples with 5 mM glucose (Fig. 3). Eryptosis significantly increased only after incubation of the cells with 5 mM glucose for up to 72 h. High glucose levels attenuated eryptosis in comparison with control samples (5 mM glucose) after 72-h incubation (Fig. 4). Increase of hemolysis was observed with 5 mM glucose and incubation time of up to 48 and 72 h. Under conditions of high concentrations of glucose hemolysis was attenuated and observed only after the longest incubation time (72 h; Table 1).

The intracellular content of superoxide declined with increasing incubation time in control erythrocytes and in cells with both high concentrations of glucose (Fig. 5). No significant differences in the superoxide level were found between samples with high glucose (45 mM and 100 mM) in comparison with the control (5 mM glucose). Lipid peroxidation had a tendency to a time-dependent increase in all samples tested, which, however, did not reach statistical significance except for samples containing 100 mM of glucose where the increase after 72 h was statistically significant (Fig. 6). The activity of catalase decreased drastically in control samples (containing 5 mM glucose) after 48 h and 72 h of incubation (Table 1). 45 mM glucose prevented the decrease of catalase activity observed in control cells; however, a decrease was observed in samples incubated with 100 mM glucose. In control samples (5 mM glucose) significant decreases of both GSH and GSSG were observed within the whole period of time tested (24–72 h). Glucose (45 and 100 mM) attenuated both GSH and GSSG decrease after 48 and 72 h. The activity of GST was decreased by prolonged incubation at high glucose concentrations (48 h for 45 mM glucose, 48 h and 72 h for 100 mM glucose). GR activity was decreased by incubation in high glucose (24–72 h for 45 mM glucose, 48 and 72 h for 100 mM glucose) (Table 1).
Table 1

Effect of incubation in various glucose concentrations on selected erythrocyte parameters; *p < 0.05, **p < 0.001.

<table>
<thead>
<tr>
<th>Concentration of glucose (mM)</th>
<th>Incubation time (h)</th>
<th>GST (U/g Hb)</th>
<th>Catalase (U/mg Hb)</th>
<th>Glutatione reductase (U/g Hb)</th>
<th>GSH (μmol/g Hb)</th>
<th>GSSG (μmol/g Hb)</th>
<th>GSSG/GSH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.1 ± 0.11</td>
<td>397 ± 25.39</td>
<td>9.2 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>0.07 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
<td>1.8 ± 0.12</td>
<td>385 ± 16.57</td>
<td>8.5 ± 0.17</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>2.0 ± 0.05</td>
<td>397 ± 25.39</td>
<td>9.2 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>0.07 ± 0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of incubation in various glucose concentrations on superoxide production in erythrocytes.

Dihydroethidine (DHE) was used to measure superoxide level. All glucose concentrations induced significant decrease of superoxide level in comparison with starting values (0 h) after incubation of erythrocytes for 24, 48 and 72 h (p < 0.05).

Fig. 6. Lipid peroxidation in erythrocytes after incubation in various glucose concentrations. All glucose concentrations induced significant increase of lipid peroxidation in comparison with starting values (0 h) after incubation of erythrocytes for 24, 48 and 72 h (p < 0.001). Significant increase (p < 0.01) of lipid peroxidation induced by 100 mM glucose in comparison with 5 mM glucose after 72 h of incubation was observed.

Discussion

Glycated hemoglobin is a minor fraction of hemoglobin in human erythrocytes, generated in vivo by nonenzymatic binding of glucose to N-terminal amino acids of beta chains of hemoglobin A [35,36]. The extent of hemoglobin glycation depends on the age of erythrocytes and glucose concentration in blood. Amount of glycated hemoglobin is higher in older as well as in diabetic erythrocytes. Both intracellular and membrane proteins of erythrocytes are glycated under hyperglycemic conditions [36]. We observed time-dependent increase in hemoglobin glycation under high glucose conditions.

Energy production in the form of ATP is necessary for maintaining erythrocyte function: keeping ion gradients across the membrane via ATP-dependent membrane pumps, maintenance of asymmetry of the membrane phospholipids, synthesis of glutathione and other metabolites, and protection of hemoglobin, enzymes and membrane proteins against oxidative impairment [37].

In the course of incubation within 0, 24, 48 and 72 h of control human erythrocytes with 5 mM glucose in PBS, degradative processes were observed in this study: decrease of Ca$^{2+}$-ATPase activity, increase of cytosolic calcium level, increase of eryptosis, hemolysis, lipid peroxidation and decrease of antioxidant potential as indicated by increasing GSSG/GSH ratio. This may be caused mainly by consumption of glucose as a main substrate for energetic metabolism of erythrocytes [38], associated with decrease of ATP concentration. Energy in erythrocytes is generated only by glycolysis and the pentose phosphate shuttle as these cells lack...
mitochondria. The presence of high levels of glucose (45 and 100 mM) did not affect the decrease in PMCA activity.

Intracellular calcium content in control samples, as well as in erythrocytes treated with high concentrations of glucose increased after 48 and 72 h, correlating with reduction of PMCA activity. Calcium content in erythrocytes treated with 45 mM glucose for up to 24, 48 and 72 h and with 100 mM glucose for up to 24 h was practically equal to that of control erythrocytes. Raftos et al. [12] reported normal (not changed) calcium extrusion in erythrocytes after incubation of intact RBCs in the presence of 30–100 mM glucose for up to 6 h. However, after incubation of erythrocytes with 100 mM glucose for up to 48 and 72 h, decrease in calcium content was observed in comparison with samples incubated with 5 mM glucose. The decrease of calcium under conditions of high glucose may be due to an adaptive mechanisms operating by alternative calcium transport out of cell via NCX transporter. Increased calcium concentration induces eryptosis in erythrocytes [18]. To assess the extent of eryptosis, we tested level the phosphatidylserine (PS) exposure, which is a marker of eryptosis. Eryptosis increased in control samples (5 mM glucose) incubated for up to 72 h and was lower in RBCs incubated with 45 and 100 mM glucose, apparently due to maintenance of energy supply to the cells. Catalase activity decreased in control erythrocytes, most probably due to depletion of NADPH necessary for maintaining the enzyme activity [49] and in erythrocytes incubated with 100 mM but not 45 mM glucose, apparently due to glycation. 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.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References
