ERYTHROCYTE MEMBRANE FLUIDITY IN DIABETICS: FLUORESCENCE STUDY

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Erythrocyte membrane fluidity is changed in diabetic subjects with long-term complications. As membrane fluidity indicator, the mean steady-state fluorescence anisotropy was measured in 1,6-diphenylhexa-1,3,5-triene labelled erythrocyte membranes prepared from six control healthy donors and six poorly controlled diabetic subjects. Fluorescence anisotropy values of membranes prepared from erythrocytes of diabetic subjects were significantly higher than in control subjects. This indicates a decreased fluidity of membranes prepared from diabetic subjects. The decreased fluidity of diabetic membranes was raised by glycation inhibitors – penicillamine, captopril, and lipoic acid.

Key words: Erythrocytes, human; Glycation; Membrane fluidity; Penicillamine; Captopril; Lipoic acid; Antiglycation therapy; Diabetes.

Red blood cells and platelet membranes, like other proteins, can be nonenzymatically glycated. This process is enhanced in diabetic patients with macrovascular complications[1–5]. Several abnormalities have been identified in the erythrocytes of diabetics: reduced life span, excessive aggregation, altered membrane phospholipid asymmetry and membrane fluidity[6]. It is likely that membrane glycation (nonenzymatic glycosylation) and also oxidative processes[7] alter membrane fluidity and thus may contribute to development of diabetic long-term complications[8,9]. However, a correction of abnormal membrane fluidity of diabetic erythrocytes is possible by insulin[10,11], metformin[12,13], and selenium[14] added *in vitro* and *in vivo*.

In this study there were investigated:

– Membrane fluidity and extent of glycation in erythrocyte membranes of healthy donors and diabetic subjects.

– Influence of glycation inhibitors[15–18] – penicillamine, captopril, and lipoic acid on fluidity of erythrocyte membranes from diabetic subjects.

EXPERIMENTAL

Chemicals

1,6-Diphenylhexa-1,3,5-triene (DPH, Sigma), glucose-oxidase E.C. 1.1.3.4 (from *Aspergillus niger*, Sigma), captopril (Sigma), penicillamine (Sigma), and lipoic acid (Asta Medica).

Preparation of Isolated Human Erythrocyte Membranes

Isolated erythrocyte membranes (red blood cell (RBC) ghosts) were prepared from blood of six healthy donors and six poorly controlled diabetic subjects. Samples were stored in heparinized plastic syringes at 4 °C. The blood was hemolyzed with 15 mM sodium phosphate buffer[19], pH 7.4 (NaH_2PO_4/Na_2HPO_4/0.9% NaCl). Thereafter, the samples were centrifuged with a Sorvall RC 5-B (GSA) at 14 000 *g* for 10 min. Washing was done with the 15 mM and subsequently with the 10 mM sodium phosphate buffer for at least ten times. At the end of the washing procedure (the membranes are not red), distilled water was used once. Thereafter, the membranes were resuspended with 10 mM sodium phosphate buffer, lyophilized, and kept at –80 °C.

Incubation of Isolated Diabetic Erythrocyte Membranes with Glycation Inhibitors

Isolated diabetic erythrocyte membranes were suspended in a solution of NaCl (0.9%) and sodium phosphate (10 mmol/l) to a protein concentration of 0.1 mg/ml (determined by Lowry method[20]). Membranes were homogenized with a 5-ml potter and then sonicated five times for 10 s by means of a Branson Sonificator W-250. The temperature during sonication was kept at 5 °C. Subsequently, the membrane suspension was incubated with glycation inhibitors – penicillamine, captopril, and lipoic acid (20 mmol/l) for 30 min at 37 °C. After incubation the membrane fluidity was measured.

Fluidity Measurements

Membrane fluidity was determined by measuring fluorescence anisotropy with a hydrophobic probe, 1,6-diphenylhexa-1,3,5-triene (DPH) in tetrahydrofurane (final concentration of DPH was 1 nmol/l). DPH was used to assess anisotropy in the deep hydrophobic regions of the lipid bilayer. The steady-state fluorescence anisotropy was measured at room temperature after adding DPH with a Perkin–Elmer LS-3 spectrofluorometer equipped with polarizers in the excitation and emission beams. The excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence anisotropy ⟨r⟩ was calculated according to Shinitzky and Barenholz[21] using the equation: $\langle r \rangle = (I_0 - I_{90})/(I_0 + 2I_{90})$, where I_0 and I_{90} are the intensities of the emitted light, the polarization plane of which is oriented parallel and perpendicular to the polarization plane of the excitation beam, respectively. Fluorescence anisotropy values mainly reflect lipid structural order and membrane fluidity was defined as the reciprocal of the lipid structural order parameter.

Determination of Extent of Glycation by Hydrazine Method

Determination was based on the hydrazine method of Kobayashi *et al.*[22,23]. In our modification of his procedure, the sample and the equivalent of glucose-oxidase were incubated at 37 °C (pH 5.0). Hydrazine monohydrate was added and the mixture heated for 30 min at 100 °C (pH 9.4). Then phenylhydrazine hydrochloride was added followed by an incubation for 1 h at 60 °C. The absorbance of the supernatant obtained after centrifugation at 1 000 *g* was measured at 390 nm. The extent of glycation of membranes was calculated from the net absorbances of sample and standard solution (1 mM *N-p*-tolyl-1-amino-1-deoxy-D-glucose) and expressed as concentration of protein-bound glucose (μmol/l).

Statistical Evaluations

Statistical significance was calculated using Student's *t* test.

RESULTS AND DISCUSSION

The present study shows that the mean-steady-state fluorescence anisotropy value in DPH labelled isolated membranes from diabetics was significantly higher than that from control subjects. Thus, fluidity in membranes of diabetic patients is reduced (Table I).

Proteins were glycated significantly more extensively in membranes from diabetics than from in those control subjects (Table II).

In membranes from six diabetic subjects, there was a significant correlation between the steady-state fluorescence anisotropy values and the extent of glycation of proteins ($r = 0.78$, $p < 0.05$) (Fig. 1).

TABLE I
Steady-state anisotropy of DPH in erythrocyte membranes

Membrane	Fluorescence anisotropy	s.d.[a]
Control[b]	0.212	0.005
Diabetic membranes[b]	0.250	0.005
Diabetic membranes with PNCA (20 mmol/l)	0.238	0.005
Diabetic membranes with lipoic acid (20 mmol/l)	0.233	0.005
Diabetic membranes with captopril (20 mmol/l	0.221	0.005

[a] Standard deviation for fluorescence measurement. [b] Average value for 6 donors.

The finding that the erythrocyte membrane proteins from diabetic subjects were more extensively glycated than those from control subjects is compatible with previous reports of increased glycation of proteins from isolated erythrocyte[24] and platelet[25] membranes from diabetic subjects.

Changes in fluidity in diabetic erythrocyte membranes are probably caused by protein–protein interactions which lower protein rotational and diffusional motion in the membrane, by changes in the composition of lipid classes and in the level of total lipids.

The decreased fluidity of diabetic membranes was significantly raised by penicillamine, captopril and lipoic acid in 20 mM concentration. This could occur due to interactions of the thiol-containing drugs with erythrocyte membrane. This opens a possibility that antiglycation therapy with these drugs could influence positively the membrane state. However, additional investigations with intact erythrocytes are essential to confirm these data and their potential importance for therapy of diabetic subjects.

TABLE II
Extent of glycation of erythrocyte membranes

Membrane	Glycation extent[a]	s.d.
Control[b]	41.5 μmol/l	7.9
Diabetic membranes[b]	92.9 μmol/l	10.8

[a] Expressed as concentration of protein-bound glucose, determined by hydrazine method. [b] Average value for 6 donors.

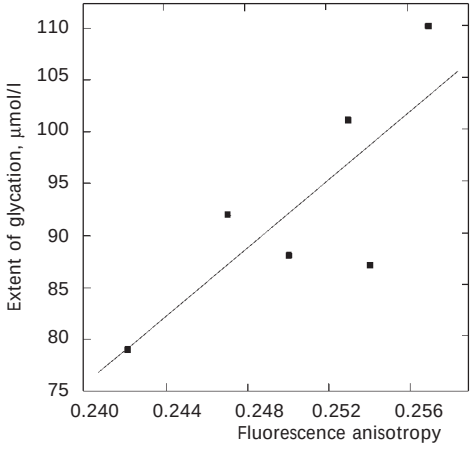

FIG. 1
Correlation between steady-state fluorescence anisotropy and extent of glycation (μmol/l of protein-bound glucose) in erythrocyte membranes from diabetic subjects

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