Erythrocyte Membrane Fluidity in Ageing, Type 2 Diabetes and Stroke Patients

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This study was conducted to investigate the values of the order parameter (S) and correlation time (τc) in electron spin resonance spectra for 5-DS(doxyl-stearic acid) and 16-DS (doxyl-stearic acid) in old normal controls and in patients with type 2 diabetes and stroke in order to assess any association between age-related disorders and membrane fluidity.

<u>Materials and Methods:</u> We measured the membrane fluidity (a reciprocal value of membrane microviscosity) of erythrocytes in old healthy controls and in individuals with age-related disorders by using an electron paramagnetic resonance and spin-labeling method.

The subjects were eleven type II diabetic patients (5 males and 6 females) with poor blood glucose (HbA1c 8.48±1.05%), eight old healthy volunteers (4 males and 4 females) with (HbA1c 5.3±1.03%), and ten diabetic stroke patients (5 males and 5 females) with (HbA1c 7.65±1.3).

Erythrocyte membrane fluidity was determined by probes, 5- and 16- doxyl-stearic acid methyl ester (SALM) inserted into the erythrocyte membrane bilayer, after which electron spin resonance (ESR) spectra were obtained. Fasting plasma glucose and other blood tests, were measured by standard methods.

<u>Results:</u> The order parameter (S) for the spin label agent (5-DS) and the correlation time for 16-DS in the electron spin resonance spectra of erythrocytes were significantly higher in stroke and diabetic patients than in old healthy controls (OH), indicating that membrane fluidity of erythrocytes was decreased in age-related disorders, compared with old healthy controls. The results indicated the lower levels of erythrocyte membrane fluidity using 16-DS with a motion parameter (p<0.05) but not using 5-DS (p<0.05) in diabetic and stroke patients compared with OH individuals.

<u>Conclusion:</u> Use of the 16-DS probe showed a significant decrease in membrane fluidity in patients with Type 2 diabetes and cerebrovascular diseases.

Introduction

Diabetes is a multifactorial disease that affects cardiovascular regulation via metabolic dysfunctions. The cellular bases of these processes resides in the inability of pancreatic cells to produce insulin and/or by defects in insulin action. Recent evidence suggests that increased superoxide formation after high glucose–induced throughout in the mitochondrial electron-transport chain generates reactive oxygen species, which are

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involved in the development of diabetic complications.[1,2]

Various factors, including hyperglycemia, glycation of proteins, and accumulation of sorbitol, have been proposed to contribute to the pathogenesis of cellular dysfunction leading to the vascular complications of diabetes.[3-5] Red blood cells (RBCs) and other cells of diabetic animals and patients have elevated levels of lipid peroxidation products.[3-5]

In diabetes mellitus, the high incidence of microvascular and atherosclerotic disease has been associated with abnormalities of erythrocyte composition and rheological function and with increased oxidative stress, among many other factors. The increased blood viscosity seen in diabetes[6] and more so in patients with established complications[7] has been ascribed to a decrease in erythrocyte deformability[8] and to changes in erythrocyte membrane fluidity.[9,10] The extent to which these changes are due solely to alterations in the lipid composition of the erythrocyte membrane is still controversial. Increased cholesterol content and cholesterol/phospholipid ratio, which correlated with the decrease in membrane fluidity in type 1 diabetes mellitus (T1DM), were identified as contributing factors[11] in addition to glycation of membrane proteins.[12] In contrast, other authors have found no alterations in erythrocyte membrane lipids of type 2 diabetes mellitus (T2DM) patients[13] or even the opposite, changes such as decreased cholesterol content[14] or increased phospholipid content in normolipemic T1DM and T2DM patients.[15] Concerning membrane fatty acid composition, again there is no consensus on the direction of changes in diabetes. The relative increase of polyunsaturated acids (PUFA; mainly 20:4 and/or 22:6) found by the three groups mentioned seems in contradiction to others.[16,17] In T1DM, the unifying mechanism behind all of these changes has been postulated to be the deficiency in insulin because it has been observed that insulin treatment, which is known to improve membrane fluidity[9,10] and blood viscosity,[18] also increases conversion of dietary (n-6) fatty acids (mainly linoleic acid, 18:2) to PUFA (mainly arachidonic acid, 20:4).[19] Another possible mechanism, independent of the effect of insulin, is suggested by the higher levels of lipid peroxidation products such as lipofuchsin[20] and malondialdehyde (MDA) found in erythrocytes from diabetic patients.[21-23]

A human erythrocyte survives in circulation for nearly 120 days, after which it is removed by the narrow splenic sinusoides or the reticuloendothelial system. During the life span of the erythrocyte, its membrane undergoes changes in lipid and protein content.

Biologic membranes have a lipid bilayer which proteins are inserted into the bilayer.[24] The diffusion of protein and lipid molecules within the membrane is known as membrane fluidity (MF) that is dependent on the presence of saturated and polyunsaturated fatty acid.[24,25]

MF is essential for the functioning of biological membranes, and any changes in the composition of the membrane in T2DM patients may have an effect on these dynamic properties.

Lipid protein interaction is a determinant factor regulating MF; to investigate its membrane properties either in normal or pathologic conditions, the Electron Spin Resonance spectroscopy (ESR), a powerful technique, is used for evaluating the motional properties of MF in ageing processes.[26]

The plasma membrane is the only organelle in the erythrocyte and is an interesting model for studies of age related changes in structure and function.

The aim of this study was to explore the dynamic properties of intact erythrocyte membrane in erythrocyte membrane fluidity in ageing, type 2 diabetic and stroke patients by means of electron spin labels of 5-DS and 16-DS.

Materials and Methods

Study design: Eight old healthy volunteers (4 males and 4 females), aged 66±5.7 yr, eleven type II diabetic patients (5 males and 6

females) aged 80.63±5.10 yr and ten stroke patients (5 males and 5 females) aged 81.5±6.13 yr were studied. The diagnosis of DM was based on the American Diabetes Association criteria for type 2 DM (fasting plasma glucose level higher than 126 mg/dL and/or glucose level exceeding 200 mg/dL at 2 hours in the 75 g oral glucose tolerance test). Stroke was defined as sudden onset of a neurological deficit occurring 48 hours prior to admission, and persisting for more than 24 hours.

Venous blood samples were obtained early in the morning after subjects had fasted overnight. We used heparin as the anticoagulant (5 U heparin/5 mL blood; 5 ml heparinizend venous blood was obtained from healthy volunteers and patients. Erythrocytes were separated by centrifugation at 3000 rpm for 10 min to remove the plasma. Erythrocytes were washed three times with Phosphate-Buffered Saline (PBS), PH=7.4, 310 mosmol at 4oc, and the washed erythrocytes were then added to the same volume of PBS and mixed thoroughly.[27]

Erythrocyte membrane fluidity: Erythrocyte membrane fluidity was studied by the Kamada and Otsuji method,[28] which involves the incorporation of spin labels using different probes 5 and 16- doxyl-stearic acid methyl ester (SALM) into the erythrocyte membrane bilayer, after which electron spin resonance (ESR) spectra were obtained.

The MF of the environment surrounding (S) and deeper (tc), as order parameter and correlation time, were measured, respectively.[28]

The erythrocyte suspensions were immediately subjected to spin labeling, and electron spin resonance (ESR) measurement.

Two stearic acid spin labels (SAL): 5-SAL and 16-SAL were purchased from Syva co. (Palo Alto, California); these are stearic acid analogues and each has a nitroxide radical ring at the 5th, and 16th carbon position counted from the carboxyl group of the acyl-respectively the spin label technique has been established as a valuable tool to obtain conformational and dynamic data concerning the physical state of the biologic membrane. These spin labels embedded in the biologic membrane exhibit their freedom of anisotropic motion in conformity with the position of the nitroxide ring on the alkyl fatty acid chain. This anisotropic motion reflects the molecular motion of the lipid bilayer, the so-called membranc fluidity. We measured parameters from the ESR spectra of spin labels embedded in intact erythrocyte membrane to estimate the dynamic states of the lipid bilayer of the membrane.

The incorporation of these spin labels into the erythrocyte membrane bilayer was readily accomplished by the following procuedure. Four microliters of 5 µg/mL spin label solutions in 100% ethanol were diluted with 500 ml of PBS and the resultant label solution was added to 500 ml of the erythrocyte suspension in PBS. After incubation for 20 min at 37°C by gentle shaking, erythrocytes were washed three times with 20 vol of PBS to eliminate free spin labels. The labeled erythrocytes were packed by centrifugation at 3200 rpm for 20 min and used immediately for ESR measurements. About 50 µL was transferred to a capillary quartz cell and ESR spectra were obtained at 37°C controlled by a variable temperature controller (JES-VT-3A2) on a JEOL X-band spectrometer Model JES FEIX (JEOL Ltd., Tokyo, Japan). There were no morphologic changes in the labeled erythrocytes when the analysis was performed.

The representative spectrum of SAL spin labels embedded in the erythrocyte membrane is shown in Fig. 1. The observed values of the outer ($2T\parallel$) and inner ($2T\perp$) hyperfine splitting (in Gauss) were used to calculate the order parameter (S) in 5 and 12 SAL, according to the Gaffney formula1[28] However in the case of 12 SAL, the outer splitting value could not be measured since the low–field peak was not resolved. The order parameter was determined from the observed inner splitting value and the calculated outer splitting value using the

relation: $T_\parallel = 3a - 2T_\perp$ where a is the isotropic hyperfine splitting; 3a was taken to be 44.5G.

$$S = \frac{T_\parallel - T_\perp + -C}{T_\parallel + 2T_\perp + 2C} \times 1.723 \quad (1)$$

Where $C = 1.4G - 0.053(T_\parallel - T_\perp)$.

The motion parameter (τc) was calculated from formula (2) of Henry and keith, where w0 is the line width h0 is the mid-field height and h-1 is the high – field height.

$$\tau c = K.W0[(h0/h\text{-}1)1/2\text{-}1] \quad (2)$$

The constant $K = 6.5.10\text{-}10$ (seconds) is dependent on the anisotropic hyperfine coupling values and the g-tensor terms when the correlation time $\tau c > 10\text{-}9$. Although equation 2 was derived for the analysis of isotropic motion, the peak height ratio (h0/h-1) and motion parameter could be used as parameters of 16 SAL anisotropic mobility for the purpose of comparison. Motion parameter was used for comparison of membrane fluidities throughout the present study.

The fatty acid spin label agents are believed to be anchored at the lipid aqueous interface of the cell membranes by their carboxyl ends, whereas the nitroxide group moves rapidly through a restricted angle around the point of attachment. Therefore, the ESR spectra of the fatty acid spin label agents are used to detect an alteration in the freedom of motion in biological membranes and to provide an indication of membrane fluidity. In addition, 5-NS could be an example of the properties of superficial membrane layers, whereas 16-NS could be an indicator referring to the more hydrophobic core of the lipid membranes.[27,28] For indices of membrane fluidity of erythrocytes, we have evaluated the values of outer and inner hyperfine splitting.[27,28]

Statistical Analysis: The data are expressed as means±SD. All statistical analyses were performed using the Statistical Package for Social Sciences program (SPSS for Windows, version 11.5). Variables were compared using unpaired t test for normally distributed variables. ANOVA followed by Scheffe's test was used to compare the group means. P values less than 0.05 were considered significant.

Results

Table 1 shows the order parameters(S) and correlation times (tc) using 5, 16 deoxyl and 5-stearic acid methyl ester (5- SALM) and age in each group of subjects.

Table 1. Comparison of membrane fluidity of erythrocytes using different probes in old normal controls, type 2 diabetes and diabetic stroke patients

Subjects	Age (yr)	HbA1c (%)	Sex M/F	(5 probes)	Tc (10^{-10} 16 probes	P value	
						OH† controls vs Type 2 DM	OH controls vs Stroke patients
OH (n=8)	66±5.7*	5.3±1.03	4/4	0.653±0.04	17.19±0.74	-	-
T2DM (n=11)	80.6±5.1	8.48±1.1	5/6	0.673±0.66	19.64±0.97	<0.05	-
Stroke (n=10)	81.5±6.1	7.65±1.3	5/5	0.685±0.57	19.22±0.69	-	<0.05

* Data are presented as mean±SD; Statistically significant; † OH, old healthy controls; Tc, Correlation times.

The values of the S for 5-DS in the ESR spectra of erythrocytes were not significantly higher in O.H individuals than in aged – related disorders (S for O.H: mean ± standard deviation, 0.653±0.04, n=8; S for stroke: 0.685±0.57, n=10, S for T2DM: 0.673±0.66, n=11 p> 0.05. tc for OH: 17.19±0.74, n=8; tc for stroke: 19.22±0.69, n=10, tc for T2DM: 19.64±0.97, n=11 p<0.05). Findings indicated that membrane fluidity of erythrocytes was decreased in age–related disorders compared with OH individuals.

We indicated the lower levels of erythrocyte membrane fluidity using 16-DS with a motion parameter but not using 5-DS in diabetic and stroke patients compared with OH individuals.

There was a negative significant correlation between MF of erythrocyte membrane and the motion parameter of 5-DS in erythrocytes from aged–related disorders compared with OH individuals, while we observed significant correlations between the MF and the order parameter of 16-SAL in erythrocytes from age–related disorders compared with OH individuals.

Discussion

The present study was performed to evaluate the possible link between membrane fluidity (a reciprocal value of membrane microviscosity) of erythrocytes in age–related disorders and OH individuals by using the ESR method.

Membrane fluidity is involved in various functions, such as deformability, aggregability, permeability, transport of ions, glucose, or oxygen, and membrane-associated enzymes.[29,30,36] The aim of this study was to investigate the fluidity of erythrocyte membrane in ageing, type 2 diabetes and stroke. In this study, the dynamic physical properties of the erythrocyte membrane were investigated by means of a spin label method and lower levels of membrane fluidity (MF). By using the probe in 16 carbon positions, a significant difference was seen between old

healthy controls compared with T2DM and stroke patients (p<0.05).

Based on previous studies, this suggests that membrane lipid peroxidation and diabetic complications can affect cell deformability and red blood cell survival.[37-42]

The occurrence of changes in the erythrocytes of diabetic patients are evident from several aspects; excessive aggregation, reduced deformability, decreased membrane surface electric charge, elevated glycosylated hemoglobin (HbA1c) are seen in the erythrocytes of diabetic and stroke patients.[30,31]

The values of tc obtained from the ESR spectra of erythrocyte membranes were significantly greater in age–related disorders than in OH individuals. These results suggest that the membrane fluidity of erythrocytes was decreased in age–related disorders compared with OH controls and confirm our previous reports showing that the cell membranes were stiffer and less fluid in age–related disorders.[31-33]

Tetsuro et al also have indicated that the differences for the motion parameter of 16-SALM between normal and diabetic subjects[37] in erythrocyte membrane fluidity were only about 2-3%.

Nevertheless, these changes are worth considering, since there are reports that even slight alterations in membrane fluidity lead to definite changes in membrane enzyme activity.[38]

Investigators agree that with diabetic disease, there are decrease in the total lipid and cholesterol levels and phospholipids of erythrocytes.[39,40]

Lower levels of polyunsaturated fatty acids of erythrocyte membrane in diabetic patients may be associated with a decreased desaturation of fatty acids, which is reportedly caused by an insulin deficiency.[31-34]

Dietary fatty acids (FA) may modulate various important membrane parameters such as membrane associated receptors, tumor antigens, prostaglandin synthesis membrane potential and membrane fluidity. The membrane lipids in erythrocytes are renewed by an exchange with plasma lipids.[28] Therefore,

erythrocyte membrane lipid composition is affected by changes in dietary lipids and subsequently in plasma lipids.[35,36]

Our data results by using 5-DS probe did not show a statistically significant difference in MF, between the old healthy controls and patients with T2DM and stroke. However, using 16-DS as a probe with the outer hyperfine splitting, the membrane fluidity of the diabetic and stroke erythrocytes were significantly decreased in the measurement.

There was no significant difference with the fluidity values measured with 5-DS, which represents the fluidity of rather shallow sites of the erythrocyte membrane.

The rather paradoxical finding of a decrease in MF with the 16-DS probe and the absence of a change in MF with the 5-DS probe may be partially explained by the likely site of insertion of both probes in the biological red cell membrane.[38,39] The discrepancy of the results between the motion parameter of 5-SAL and the order parameter of 16-SAL may be due to the following reasons; variation in the degree of saturation of the acyl-chain is another important factor regulating membrane fluidity; phospholipids with saturated acyl chains form highly ordered membranes in which fluidity is low. Conversely, phospholipids with unsaturated acyl-chains form membranes in which fluidity is high. It has been demonstrated that decreases unsaturation of free fatty acids and phospholipids acyl-chains occurs in diabetes.[40-43] It is very likely that the decreased fluidity in the deeper sites (hydrophobic region) of diabetic erythrocyte membrane demonstrated with 16-SAL in the present study was due to decreased unsaturation.[40-43]

In conclusion, significantly lower levels of erythrocyte membrane fluidity were revealed with 16-SAL as a probe in diabetic and stroke patients compared with old healthy subjects.

Acknowledgments

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