Decreased Fluidity of Isolated Erythrocyte Membranes in Type 1 and Type 2 Diabetes. The Effect of Resorcylidene Aminoguanidine

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Abstract. Changes in the physico-chemical properties of erythrocyte membranes induced by nonenzymatic glycation as well as the possible prevention of their rise were studied. Using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), fluorescence anisotropy values were determined in erythrocyte membranes isolated from type 1 and type 2 diabetic patients with and without complications. The mean anisotropy values for the groups of diabetic patients were significantly higher than those for the control group ($p < 0.01$). This indicated pathologically decreased fluidity in cell membranes in the diabetics regardless of the type of diabetes or the presence of complications. The fluorescence anisotropy positively correlated ($p < 0.01$) with clinical parameters, such as glycohaemoglobin and plasma cholesterol content, which are important for the monitoring of the compensation status of the diabetic patient. Our results support the suggestion that protein crosslinking and oxidative stress induced by nonenzymatic glycation contribute to changes in the physico-chemical properties of erythrocyte membranes.

In vitro testing of a new potential drug resorcylidene aminoguanidine (RAG) showed its ability to increase significantly ($p < 0.001$), to various extent ($p < 0.01$), the fluidity of both diabetic and control erythrocyte membranes. Upon the administration of RAG, reduced fluorescence anisotropy values for the groups of diabetic patients approached the normal values obtained for the controls. This may play an important role in the improvement of impaired cell functions found in diabetes that are controlled by the cell membrane.

Key words: Erythrocyte membranes — Glycation — Resorcylidene aminoguanidine — Membrane fluidity — Fluorescence anisotropy

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Abbreviations: DPH – 1,6-diphenyl-1,3,5-hexatriene; DM – diabetes mellitus; AGEs – advanced glycation end products; RAG – resorcylidene aminoguanidine

Introduction

Diabetes mellitus (DM) is associated with a host of chronic complications, including microvascular disease, premature atherosclerosis, and early mortality. For many years, the hypothesis has been generally accepted that prolonged exposure to hyperglycaemia is the major cause responsible for the onset of the complications (Brownlee et al. 1984; Brownlee 1991). An increased plasma glucose content in diabetics results in non-enzymatic glycation of proteins (the Maillard reaction), including those in the blood. Excessive chemical interactions of reducing saccharides with proteins and other biomolecules initiate a chain of reactions that result in the formation of advanced glycation end products (AGEs). AGEs accumulate in tissue proteins during the disease as well as with age. Their formation requires autooxidative conditions which produce reactive oxygen species and lead to the fragmentation, cross-linking and additional rearrangement of proteins (Baynes 1996; Wolff 1996). This damage may explain the occurrence of an altered structure and function of proteins in diabetics, such as glycated collagen and low-density lipoproteins, leading to arterial rigidity and capillary weakness (Brownlee 1991).

The existing evidence suggests that depression of some blood cell functions evoked by non-enzymatic glycation may contribute to the impaired microcirculation, chronic pseudohypoxia and to a high incidence of diabetic vascular disease (Caimi et al. 1992; Watala 1993). The most commonly used model membrane system to investigate altered cell functions is the one that uses erythrocytes because of their ready availability and relatively uncontaminated plasma membrane preparation (Bernhardt et al. 1999; Ivanov 1999). Abundant information is now available about membrane changes in diabetic condition. The following effects have been reported to occur in the membranes: increased protein glycation (Watala and Winocour 1992), disordered biochemical composition (Bryszewska et al. 1986; Watala et al. 1987), hyperpolarization (Zavodnik et al. 1997), lowered deformability (McMillan 1990) and changed membrane fluidity. Most studies on the membrane fluidity of erythrocytes in human diabetic subjects report decreases in this parameter compared with control subjects (Baba et al. 1979; Bryszewska et al. 1986; Watala and Winocour 1992; Candiloros et al. 1995). However, other researchers suggested that the fluidity is increased (Juhan-Vague et al. 1986) or unchanged in diabetes (Caimi et al. 1992).

The membrane fluidity of cells plays a role in the control of a number of physiological processes, though the mechanism on molecular scale is not exactly known (Gennis 1989). The fluidity of erythrocyte membranes may be a component of the system that controls blood microcirculation, oxygen transport activity, as well as cell interactions (Brewer 1975). If abnormal, it may be involved in some pathological states. The membrane structure and fluidity can be investigated using fluorescence polarization techniques which have been recently developed. The techniques are

based on employing appropriate fluorophores. The methods can be instrumental in the study of protein interactions (Fedunová and Antalík 1998), of the origin of the pathological states (Watala et al. 1996) as well as their treatment (Ziegelhöffer et al. 1997). The techniques are based on the fact that the fluorescence anisotropy of a probe that is inserted in the membrane of a cell investigated is inversely proportional to the mobility of the probe. Hence, from increased fluorescence anisotropy values it can be inferred that the membrane exhibits decreased fluidity (Shinitzky and Barenholz 1978).

Since the biological consequences of non-enzymatic glycation appear to explain some features of diabetic complications, pharmacological agents have been sought to inhibit these processes. They are expected to accomplish it both by selectively blocking the reactive carbonyls on early glycation products as well as by their antioxidant activity. We studied the Schiff base resorcylidene aminoguanidine (RAG), synthesized by J. Čársky, as a potential antiglycation and antioxidation agent. Our earlier studies revealed that RAG possesses inhibitory properties in the processes of nonenzymatic glycation, oxidation of proteins, and membrane lipoperoxidation (Ziegelhöffer et al. 1997; Hrnčiarová et al. 1998; Jakuš et al. 1999). Moreover, RAG is able to form a quinoid structure as well as a chelate complex, which is important for its antioxidative capacity (Čársky et al. 1980; Onuska et al. 1996). In this paper, we present a study of changes in erythrocyte membrane fluidity that occur in diabetic type 1 and type 2 patients with and without complications. Correlations between the fluorescence anisotropy data and the clinical parameters were also investigated. As RAG acts as inhibitor in the processes, which could prevent or delay diabetic complications, we tested the *in vitro* effect of RAG on the fluidity of erythrocyte membranes isolated from diabetic patients.

Materials and Methods

Experimental groups

Adult diabetic patients (28) were divided into four groups: Group A included type 1 DM without complications ($n = 5$); Group B included type 1 DM with complications ($n = 5$); Group C were patients with type 2 DM without complications ($n = 7$); Group D comprised type 2 DM with complications ($n = 11$). The groups were compared with a control group ($n = 10$). The control group was recruited from age-matched healthy subjects giving their consent to participate in the study.

Incubation and preparation of erythrocyte membranes

Blood samples were obtained from the Department of Medicine II, University Hospital in Bratislava. Blood from healthy donors (controls, $n = 10$) was provided by the Clinic of Haematology and Transfusiology, University Hospital.

Blood was taken into heparinized tubes and incubated immediately with RAG (molecular weight of RAG.HCl: 230.65) at a concentration of 2.5×10^{-4} mol/l for 30 minutes at 37 ℃. After the incubation, the blood was centrifuged to remove the plasma and buffy coat. The erythrocytes were washed three times in cold

phosphate-buffered saline (PBS: 0.15 mol/l NaCl, 1.9 mmol/l NaH_2PO_4, 8.1 mol/l Na_2HPO_4; pH 7.4).

Erythrocyte membranes were isolated according to the standard method of Hanahan and Ekholm (1974). Lysis was induced by adding a cold hypotonic buffer (10 mmol/l Tris.HCl; pH 7.4) at a 1 : 14 volume ratio. Haemoglobin was removed by four to five washing steps in the buffer. The erythrocyte membranes were resuspended in 10 mmol/l Tris.HCl (pH 7.4). Membrane protein concentration was determined by the Lowry method (Lowry et al. 1951). Prior to anisotropy measurements, the erythrocyte membranes were diluted to the appropriate concentration of membrane proteins to avoid depolarization effects due to light scattering.

Labeling

The suspension of the erythrocyte membranes was stained with a DPH probe (1,6-diphenyl-1,3,5-hexatriene; Serva, Heidelberg, Germany). A stock solution of DPH was prepared in acetone at a concentration of 5×10^{-4} mol/l (Plášek and Jarolím 1987). For labeling, the stock solution was diluted 1 : 250 with 10 mmol/l Tris.HCl (pH 7.4) and vigorously stirred for 30 minutes to remove acetone. This solution was then mixed 1 : 1 with the diluted erythrocyte membrane suspension. The final concentration of DPH in the samples was 1 μmol/l, and the final concentration of the membrane proteins was 15 μg/ml. A fresh labeling solution was prepared for each measurement.

Fluorescence anisotropy measurement

Fluorescence anisotropy measurements were made with a Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany). The samples were excited at 360 nm, and the emission was recorded through a standard fluorescence holder (Carl Zeiss Jena, Germany). Dichroic filters were used as the polarizers and analyzers in the excitation and emission light paths, respectively. The changes in DPH anisotropy were checked at room temperature (22 ℃) every 5 minutes during an hour. Within the first 40 minutes, the DPH molecules were incorporated into the erythrocyte membrane suspensions from both untreated blood and blood treated with RAG. Over the next 20 minutes complete DPH incorporation persisted, and the anisotropy fluorescence did not further change (Šikurová et al. 1998). After recording the 60-minute values, the anisotropy values were calculated according to equation (1) to assess membrane ordering.

$$r = (I_{vv} - I_{vh} \times G)/(I_{vv} - 2I_{vh} \times G) \quad (1)$$

where I_{vv} and I_{vh} represent the components of emitted light intensity which are detected through the polarizers oriented parallel and perpendicularly to the direction of vertically polarized excitation light. The factor $G = I_{hv}/I_{hh}$ is a correction factor for the inequality of the detection system sensitivity to horizontally and vertically polarized emission (Shinitzky and Barenholz 1978).

Measurements of biochemical parameters

All the desired biochemical tests were performed in the clinical setting. Fasting blood glucose was measured using an electrochemical method by means of glucose oxidase. The glycation of haemoglobin was assayed using an immunoturbidimetric method. Plasma cholesterol and triglycerides were determined enzymatically with a colorimetric method (CHOD-PAP and GPO-PAP, respectively).

Data analysis

Mean steady-state anisotropy values and standard deviations were calculated for each group. Statistically significant differences among the diabetic and normal groups were tested using the non-parametric Kruskal-Wallis test. Wilcoxon test and Student's paired t-test were applied to test the significance of anisotropy changes induced by RAG treatment. Pearson correlation coefficients were used to determine whether membrane fluidity correlated with clinical descriptives.

Results

Fluidity in the hydrocarbon regions of erythrocyte membranes isolated from type 1 and type 2 diabetic patients was quantitatively assessed in terms of DPH fluorescence anisotropy. The fluorescence anisotropy values were significantly higher ($p < 0.01$) than those in the healthy donors (Table 1), which corresponded to decreased membrane fluidity (Shinitzky and Barenholz 1978). As suggested by the data shown in Table 1, the phenomenon of decreased fluidity of erythrocyte membranes was present regardless of the type of diabetes, and it was insignificantly pronounced in the diabetics with complications. Glycaemia, plasma cholesterol, and glycohaemoglobin content were significantly increased ($p < 0.01$) in the diabetic patients. The decreased fluidity in the whole group of diabetics ($n = 28$) correlated with the glycohaemoglobin content ($p < 0.01$; Fig. 1), with the total plasma cholesterol content ($p < 0.01$; Fig. 2), as well as with the age of the diabetic subjects ($p < 0.05$; not shown). A poor correlation was found for glycaemia ($r = 0.307$; $p = 0.11$). A smaller group ($n = 12$) was set up from the diabetics with

Table 1. Comparison of the fluorescence anisotropy of DPH between labeled erythrocyte membranes isolated from untreated and RAG-treated blood

	A	B	C	D	Control
r_0	0.288±0.012	0.300±0.010	0.288±0.015	0.295±0.010	0.259±0.012
r_{RAG}	0.262±0.011	0.269±0.010	0.265±0.015	0.270±0.012	0.247±0.012

A – type 1 DM without complications, B – type 1 DM with complications, C – type 2 DM without complications, D – type 2 DM with complications, r_0 – anisotropy of DPH at 60 minutes in untreated sample, r_{RAG} – anisotropy of DPH at 60 minutes in RAG-treated sample.

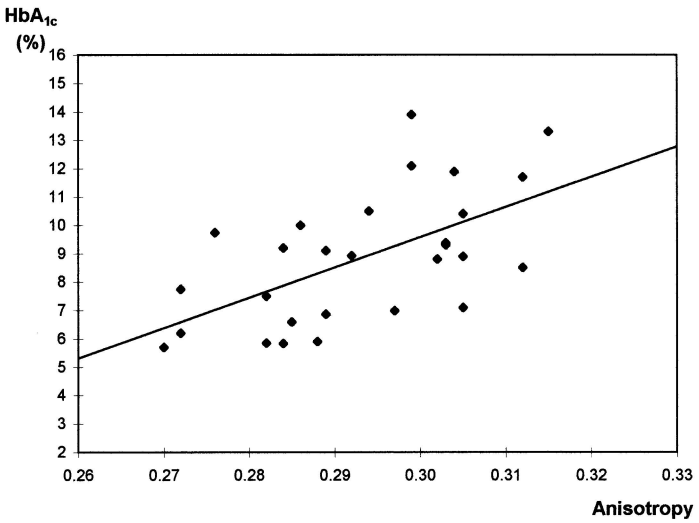

Figure 1. Relationship between fluorescence anisotropy of DPH-labeled erythrocyte membranes and glycohaemoglobin content: $r = 0.567$; $p < 0.01$; $n = 28$.

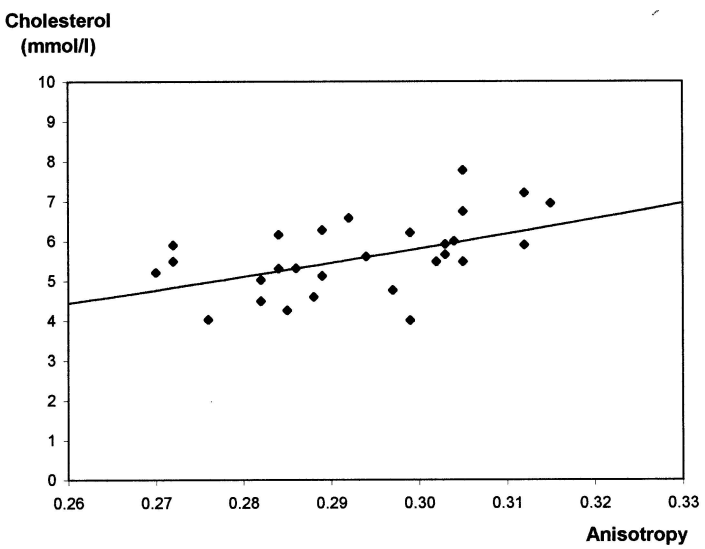

Figure 2. Relationship between fluorescence anisotropy of DPH-labeled erythrocyte membranes and plasma cholesterol content: $r = 0.514$; $p < 0.01$; $n = 28$.

Table 2. Pearson correlation coefficients of the parameters investigated in the group of 12 diabetics

	r_0	Age	D DM	Com.	BMI	Glyc.	GHb	PCh	TG	HDL	VLDL	LDL
r_0	*	0	1	0		0	1	2^{S}	2^{S}	0	1	1^{s}
Age	0.339	*			0		0		0		0	0
D DM	0.565	0.204	*	1^{S}				1^{s}				0
Com.	0.414	0.230	0.711	*		0						0
BMI	−0.13	0.307	−0.18	−0.18	*	0	1^{s}			0		
Glyc.	0.445	−0.26	0.008	0.302	−0.36	*	1^{s}		0	1^{s}		
GHb	0.519	−0.43	0.223	0.240	−0.63	0.567	*			1		
PCh	0.778	0.284	0.507	0.290	0.030	0.282	0.242	*	2^{SS}		1^{S}	2^{SS}
TG	0.753	0.404	0.272	0.207	0.192	0.317	0.117	0.811	*		2^{SS}	2^{S}
HDL	0.372	−0.10	0.271	0.281	−0.42	0.594	0.506	0.083	−0.09	*	0	
VLDL	0.564	0.357	0.212	0.202	0.192	0.128	−0.02	0.719	0.915	−0.42	*	1^{S}
LDL	0.602	0.306	0.387	0.090	0.236	0.028	0.055	0.937	0.754	−0.19	0.719	*

Statistical significance: ^{s} $p < 0.05$; ^{S} $p < 0.01$; ^{SS} $p < 0.001$.
Symbols used: 2 for $r \geq 0.75$; 1 for $0.75 > r > 0.5$; 0 for $0.5 > r > 0.3$ and without sign for $r \leq 0.3$, where r is the relevant correlation coefficient.
Abbreviations used: r_0: anisotropy of DPH at 60 minutes in untreated sample, D DM: duration of diabetes, Com.: complications, BMI: body mass index, Glyc.: glycaemia, GHb: glycohaemoglobin, PCh: total plasma cholesterol, TG: triglycerides, HDL, VLDL, LDL: cholesterol of high-density lipoprotein, very low-density lipoprotein and low-density lipoprotein fractions, respectively.

complete clinical analyses including lipid metabolism, examined by Department of Medicine II, University Hospital. The Pearson correlation coeficients of fluorescence anisotropy and clinical parameters are summarized in Table 2.

Treatment with RAG of the blood withdrawn from the diabetics and controls induced a reduction in the values of DPH fluorescence anisotropy ($p < 0.001$). This reduction was more pronounced in the diabetic groups (Table 1). In the diabetic groups, RAG reduced the values of DPH anisotropy by 9.03 % (A), 10.33 % (B), 7.99 % (C) and 8.48 % (D), respectively. These reduced values approached the normal values obtained for untreated controls. In the control group, the reduction of anisotropy values was only 4.63 % . The calculated differences in the anisotropy values between treated and untreated samples in the diabetic groups were significantly higher than those in the control group ($p < 0.01$). In the diabetic groups there were insignificant intergroup differences in the treated and untreated samples, respectively.

Discussion

In healthy tissue, cell membrane fluidity is at an optimum state which is necessary for the right function of the membrane. Membrane fluidity is influenced in particular by the cholesterol content, the lipid composition, and protein-lipid interactions

(Shinitzky 1984). An increase in the cholesterol-to-phospholipids ratio reflects a decrease in fluidity (Shinitzky and Barenholz 1978). In general, the cholesterol-to-phospholipid molar ratio in DM is increased especially due to an enhanced plasma cholesterol content. The ratio rarely remains unchanged or it is decreased (Hill and Court 1983; Watala 1993). It is known that, in the erythrocyte membrane of diabetic patients, the elevation of the plasma cholesterol content positively correlates with both the cholesterol content and the cholesterol-to-phospholipid molar ratio (Watala et al. 1987). Significant correlations between plasma cholesterol, the membrane cholesterol-to-phospholipid molar ratio and membrane fluidity were found by Bryszewska et al. (1986) and Watala et al. (1987). However, several studies in diabetic patients have reported unchanged or increased membrane fluidity, which was not accompanied by a relevant decrease in the cholesterol-to-phospholipid molar ratio, and *vice versa* (Baba et al. 1979; Juhan-Vague et al. 1986).

The present study shows that DM has a pronounced effect on the physicochemical state of erythrocyte membranes. The membrane fluidity is regularly decreased in both type 1 and type 2 diabetics even in uncomplicated DM. The observed anisotropy increase (r_0, Table 1) can be attributed to a higher ordering of phospholipid molecules in the hydrocarbon region of the membranes, i.e. to lowered membrane lipid packing. Our observations are in agreement with previous studies by Baba et al. (1979), Bryszewska et al. (1986), Watala and Winocour (1992), Candiloros et al. (1995), who also observed lowered fluidity in erythrocytes of diabetic patients. The results obtained with different techniques and different probes appear to confirm the finding that fluidity is decreased in diabetic patients (for a review, see Watala 1993). Further, the fluorescence anisotropy values correlated positively with both the glycohaemoglobin and plasma cholesterol contents (Figs. 1, 2), which were significantly increased in diabetic patients. The correlations observed support the suggestion that, besides the cholesterol-to-phospholipid molar ratio, the deteriorative long-term effect of an elevated plasma glucose level plays a role in membrane fluidity alterations. There are reasons to suppose that the processes of nonenzymatic glycation and the impairment of membrane fluidity may be linked with one another at least in some cells (Watala et al. 1996; Ziegelhöffer et al. 1997; Waczulíková and Čársky 1997). The cell membrane of diabetic patients is susceptible to oxidative damage due to the enhanced nonenzymatic glycation and glycoxidation (Bryszewska et al. 1995; Wolff 1996). It is well known that lipid oxidation processes evoked by glycation and glycoxidation lead to linkage between fatty acids, reducing the mobility of the chains (Sills et al. 1994). Further, nonenzymatic glycation may induce structural alterations originating from the crosslinking of AGEs and from oxidative damage (Baynes 1996). Thus, the increased rigidity of erythrocyte membranes of diabetic patients seems to result from either increased glycation and glycoxidation processes, or from an elevated cholesterol-to-phospholipid ratio or from both. It is therefore very likely that, in addition to other factors, alterations in physico-chemical properties of erythrocyte membranes evoked by nonenzymatic glycation and permanent oxidative stress may contribute to the development of long-term complications of diabetes.

In connection to this, aminoguanidine has been studied as an inhibitor of the glycation and glycoxidation processes (Brownlee et al. 1986). It has been suggested that aminoguanidine prevents the formation of AGEs and free radicals partly by blocking reactive oxo-groups of products arising from the glycoxidation processes and partly by its antioxidant activity. The disadvantages of this drug have been its toxicity (Sugiyama et al. 1986) and the pro-oxidant effect (Skamarauskas et al. 1996). Prompted by these findings, we focused on the Schiff base RAG (Čársky et al. 1978, 1980; Onuska et al. 1996). RAG induced a significant diminution of glycated proteins (fructosamine) and decreased the rigidity of cardiac sarcolemma, and normalized the increased malondialdehyde levels in kidney tissue of rats with streptozotocin-induced DM (Ziegelhöffer et al. 1997).

Our results from fluorescence anisotropy measurements in erythrocyte membranes of diabetic patients isolated from RAG pre-treated blood showed that the membrane became more fluid compared with that in untreated blood (Table 1). A significant reduction of fluorescence anisotropy values at the completion of DPH incorporation r_{RAG} (Table 1) was induced by RAG treatment prior to membrane isolation. A similar effect was observed in erythrocyte membranes treated with RAG after the isolation of the membranes measured at 22 ℃ and 37 ℃, respectively (Šikurová et al. 2000). This drug has different effects on erythrocyte membrane fluidity in control and diabetics, which may suggest its inhibitory effect on products originating from glycoxidation processes. Although RAG fails to influence directly the formation of AGEs, it may prevent the formation of reactive oxygen species induced by nonenzymatic glycation. We assume that RAG, acting on some components of the membrane surface, affects interactions contributing to the stabilization of the membrane structure, thus disturbing its structural ordering even in the hydrophobic interior of the membranes.

In summary, data from the DPH fluorescence anisotropy measurements correlate well with the clinical parameters, which are important for the monitoring of the compensation status of the diabetic patients. However, causative details of these correlations at the molecular level have not yet been clarified. Though we have to be satisfied with mediated information provided by fluorescence probes, the correct interpretation of spectroscopical parameters supported by biochemical ones provides valuable information about the structural and functional changes of membranes, about the history of these changes as well as their further possible development.

The *in vitro* testing of RAG, a new potential drug, showed its ability to positively influence pathologically reduced membrane fluidity in diabetes. This may be beneficial in the improvement in diabetics of impaired cell functions controlled by the cell membrane.

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