STRUCTURAL ALTERATION IN THE MEMBRANE OF ERYTHROCYTES FROM RATS WITH STREPTOZOTOCIN-INDUCED DIABETES

Boyana Paarvanova[1], Anna Tolekova[2], Petya Hadzhibozheva[2], Tsvetelin Georgiev[2], Ivan Ivanov1*

1) Department of Physics, Biophysics, Roentgenology and Radiology, Medical Faculty, Thracian University, Stara Zagora 6000, Bulgaria

2) Department of Physiology, Pathophysiology and Pharmacology, Medical Faculty, Thracian University, Stara Zagora 6000, Bulgaria

**) Author for correspondence: ivanov_it@gbg.bg*

ABSTRACT

Diabetes damages tissues mainly through non-enzymatic glycosylation and free radical oxidation of cellular constituents. This study was designed to determine the combined effect of melatonin and streptozotocin (STZ)-induced diabetes in rats on the structural stability of spectrin, the main cytoskeletal protein of erythrocytes. Diabetes was induced by a single injection of STZ (60 mg/kg, i.p.). Melatonin is strong antioxidant that does not prevent glycosylation of proteins. It was administered to the rats (10 mg/kg, i.p.) for 7 consecutive days after the inducement of diabetes. Wistar male rats were divided into three groups: control (n = 6), untreated-diabetic (n = 5), and melatonin- treated diabetic (n = 5). On the 42th day after STZ injection, the animals were anesthetized with Nembutal (50 mg/kg i.p.) and exsanguinated, about 1 ml heparinized blood were taken and washed with 9 ml of 150 mM NaCl. 50 μl of washed erythrocytes were suspended in 150 mM NaCl, hematocrit 0.50, and heated from 20 to 60 °C at 3.0 °C/min heating rate in a conductometric cuvette. Spectrin denaturation was registered as a threshold increase in suspension admittance, Ys (50 kHz), at Td, the spectrin denaturation temperature. The temperature derivative of the change in Ys at Td depicted a peak with a width at half-peak height, ΔT1/2, which is a measure for the cooperativity of spectrin denaturation. Changes in the structural stability of spectrin were quantified by the changes in Td and ΔT1/2. Differences in the mean values of Td ± standard deviation, σ, were low and statistically insignificant: 50.93 ± 0.778 oC for control rats; 51.44 ± 0.994 oC for diabetic rats; and 51.38 ± 0.403 oC for melatonin - treated diabetic rats. By contrast, differences in ΔT1/2 ± σ were great and statistically significant: 2.58 ± 0.420 oC for control rats, 4.31 ± 0.213 oC for diabetic rats and 2.59 ± 0.334 oC in the group of melatonin - treated diabetic rats. The effect of melatonin indicates that the increase in the ΔT1/2 of spectrin of diabetic rats was possibly due to the oxidative damage, not to the accompanying glycolysation of this important protein.

***Key words**: spectrin cytoskeleton, erythrocyte deformability, oxidative stress.*

Introduction. Diabetes mellitus (DM) is a metabolic disorder characterized by varying or persistent hyperglycemia, due to decreased production of insulin or impaired utilization of glucose. DM is a major and increasingly significant health problem worldwide. World Health Organization predicts

5 % of world's population to suffer from diabetes in 2030 (Amos et al., 2010). The risks of blindness, stroke, coronary heart disease or peripheral vascular disease increase strongly in diabetic patients (Giugliano et al., 1995; Gispen and Biessels, 2000; Jeppesen and Bek, 2004).

The biochemical alterations that underlie the pathogenesis of diabetes include oxidation of protein sulfhydryls and non-enzymatic glycosylation of proteins. An enhanced oxidative stress has been observed in diabetics as indicated by increased free radical production, lipid peroxidation and diminished antioxidant defence (Hiramatsu and Arimori, 1988; Baynes, 1991). Oxidative stress is regarded as an important mechanism of delayed complications of DM, moreover, it also plays an important role in the pathogenesis of DM (Lyons, 1991).

Erythrocytes are an important determinant of the rheological properties of blood because of their large number, extreme deformability and elasticity, and aggregation tendency. Major

determinants of erythrocyte deformability are the rheological characteristics of plasma membrane and intracellular fluid as well as the surface to volume ratio (Shin et al., 2007). The major factor of them is the erythrocyte membrane deformability, which depends on the submembraneous cytoskeletal network comprised primarily of spectrin and actin (Picart et al., 2000).

In vitro oxidative stress on erythrocytes and hemolysis has been caused by such concentrations of glucose (10-20 mM) as in diabetic patients (Marar, 2011). In vitro restructuring of erythrocyte membrane was produced by the increased level of glucose which manifest by deterioration of the mechanical properties of membrane as measured by the micropipet aspiration method (Traykov TT, Jain, 1987). DM induces changes in erythrocytes as a consequence of the glycosylation of proteins and oxidation of protein sulfhydryls. Diabetic erythrocytes have diminished (Na+/ K+)ATPase and (Ca2+)ATPase activity and, relatedly, disturbed intracellular ionic balance. Of the erythrocyte membrane proteins spectrin is the most heavily glycosylated and oxidatively damaged and the extent of its oxidation clearly correlates its glycosylation (Schwartz et al., 1991). With the increase in plasma concentration of glucose the erythrocytes tend to transform from discocytes to echinocytes with concomitant decrease in deformability (Babu and Singh, 2004). Besides diabetes, RBCs may also exhibit reduced deformability in other pathological situations, such as heart disease, hypertension, malaria, and sickle cell anemia.

Since under-membrane spectrin cytoskeleton of erythrocytes is the main factor responsible for the deformability of intact erythrocytes we studied the changes in the structural stability of spectrin in erythrocytes of diabetic rats. These changes were quantified by the variations in the peak temperature, Td, and width at the half-peak height, $\Delta T1/2$, of the denaturation of spectrin by heat. We have probed the dependence of Td and $\Delta T1/2$ on melatonin administration into diabetic rats as melatonin displays high antioxidant activity (Shcherba et al., 2000) and protective effect in diabetic rats (Yavus et al., 2003).

Materials and Methods

Thermal dielectroscopy of erythrocyte samples. Wistar rats were divided into three groups; control (n = 6), untreated-diabetic (n = 5) and melatonin-treated diabetic rats (n = 5). Diabetes was induced by a single intraperitoneal injection of 60 mg Streptozotocin per kg body weight of rats. The formation of diabetes was checked on the third day by measuring fasting plasma glucose levels. Only the rats with blood glucose levels more than 16 mmol/l, measured on the 72nd hour after the STZ application, were considered to be diabetic. Plasma glucose levels in diabetic rats were about 3 times as high as that in control rats. The protective effect of melatonin was studied by administration to the rats (10 mg/kg, i.p.) for 7 consecutive days after STZ application.

On the 42th day after STZ injection, the animals were anesthetized with Nembutal (50mg/kg i.p.) and exsanguinated. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. About 1 ml fresh heparinized blood was obtained from each rat and washed with 9 ml of 150 mM NaCl to eliminate hyperglycemia and blood plasma. The erythrocytes were immediately isolated by centrifugation (3000 x g, 5 min) after careful removing the upper layer of white blood cells. About 50 μl of washed erythrocytes were suspended in 150 mM NaCl, hematocrit 0.50, and heated from 20 to 60°C at 3.0°C/min heating rate in a conductometric cuvette, equipped with two electrodes of platinum wire. During the heating the suspension admittance was continuously measured with Solartron 1260A Impedance/Gain-phase analyzer, England, connected to computer (Ivanov, 2010). To measure the suspension admittance alternating voltage of 100 mV, 50 kHz, was applied to the electrodes.

*Thermal denaturation of protein***.** The thermally induced transition of protein from a native to a denatured conformation represents structural melting or unfolding of the molecule. The process is accompanied by the rupture of inter- and intra-molecular bonds, and occurs in a cooperative manner. We used the method of thermal dielectroscopy to determine two important parameters of

the denaturation of spectrin, major protein of erythrocyte under-membrane cytosketon: the denaturation temperature, *Td*, which is the transition peak temperature, and the width at half-peak height, *ΔT1/2*.

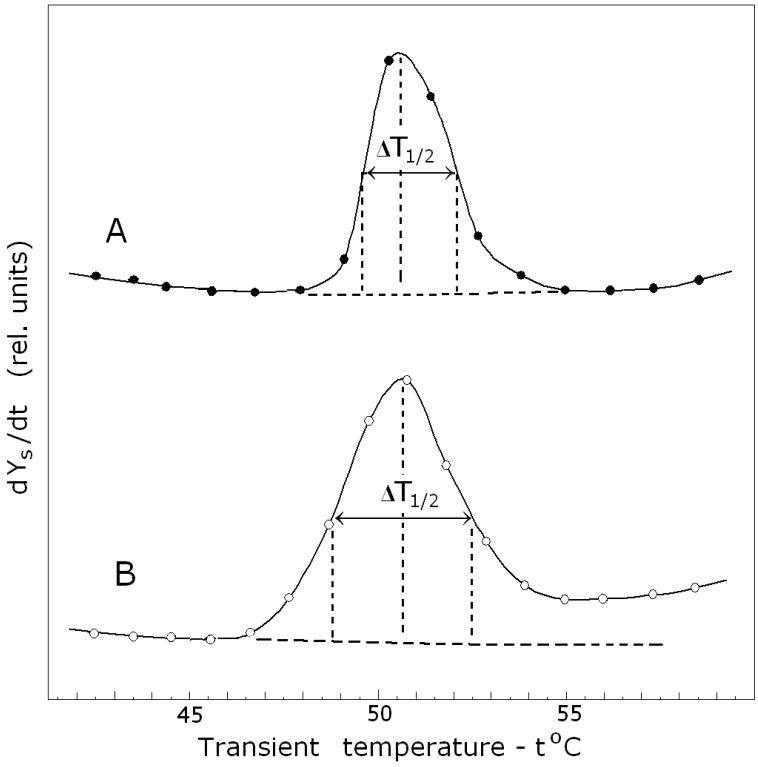

Fig. 1. *Changes in the admittance, Ys,of erythrocyte suspension as a function of temperature, t oC. dYs / dt is the admittance derivative against temperature. Shown is the determination of the width at half-peak height, ΔT1/2, for spectrin denaturation by heat. A – control erythrocytes, B – diabetic erythrocytes.*

In general, the *Td* represents the temperature at which the energy of heating is enough to cause half of the protein to unfold. *Td* is a measure of the thermal stability of protein. For cases where the thermal denaturation is not reversible, the *Td* value will depend on the heating rate; hence the same heating rate was applied for all blood samples.

The sharpness of the transition peak can be measured as width at half-peak height, *ΔT1/2*, and is an index of the cooperative nature (domino-like effect) of the transition from native to denatured state. If denaturation occurs within a narrow temperature range (a low *ΔT1/2* value), the transition is considered highly cooperative.

Results and discussion

The filaments of spectrin, the major peripheral protein of human erythrocyte membrane unfolds at 49.5oC (Td) (Brandts et al., 1977). On the other hand threshold decrease in the electric capacity and increase in the admittance of the plasma membrane were detected at the spectrin denaturation temperature (Ivanov, 2010; Ivanov et al., 2012). Fig. 1 shows this

temperature – induced increase in the admittance of suspensions containing control and diabetic erythrocytes. Several lines of evidence have indicated that this change is due to the thermal denaturation of spectrin (Ivanov, 1997).

The thermally induced transition of spectrin from a native to a denatured conformation is outlined by the derivative peak on Fig. 1. The peaks of different samples control erythrocytes have horizontal base line and asymmetric shape with its right half side broader than the left one (Fig. 1). By contrast, the peaks of diabetic erythrocytes have symmetric shape and base line with raised right part.

The top peak temperature corresponds to the spectrin denaturation temperature, Td. The determination of the width at half-peak height, *ΔT1/2* is explained in the Fig. 1. The obtained data for Td and ΔT1/2 for the different blood samples is shown in Table 1.

The mean values of Td ± its standard deviation, σ, in the indicated groups of animals were as follows: 50.93 ± 0.778 oC for control rats; 51.44 ± 0.994 oC for diabetic rats; and 51.38 ± 0.403 oC for melatonin - treated diabetic rats. Thus, differences in Td were not statistically significant. By contrast, differences in ΔT1/2 ± σ were great and statistically significant (P<0.05): 2.58 ± 0.420 oC

for control rats, 4.31 ± 0.213 oC for diabetic rats and 2.59 ± 0.334 oC in the group of melatonin - treated diabetic rats. In conclusion, using diabetic erythrocytes we have measured a 65 % decrease in the cooperativity of the denaturation of spectrin. This is far more than the corresponding 20-25 % decrease in the deformability index of diabetic erythrocytes, determined by ektacytometry.

***Table 1**. Data for the denaturation temperature, T_d, and width at half-peak height, $\Delta T_{1/2}$ for the thermal denaturation of spectrin in different blood samples.*

№	Control rats		Diabetic rats		Melatonin – treated diabetic rats	
	T_d (^oC)	$\Delta T_{1/2}$ (^oC)	T_d (^oC)	$\Delta T_{1/2}$ (^oC)	T_d (^oC)	$\Delta T_{1/2}$ (^oC)
1	50.1	3.22	50.5	4.25	51.6	2.37
2	50.5	2.81	50.6	4.21	51.5	2.78
3	51.3	2.81	52.5	4.69	50.8	2.24
4	52.2	2.11	52.5	4.19	51.7	2.50
5	51.2	2.68		-	51.4	3.07
6	51.2	2.05		-		-
Mean	50.93	2.58	51.44	4.31	51.38	2.59
σ	± 0.778	± 0.420	± 0.994	± 0.213	± 0.403	± 0.334

Recent study (Gumustekin et al., 2007) evidenced that administration of melatonin to rats with STZ – induced diabetes improved renal and kidney injuries in diabetic rats, probably by decreasing oxidative stress, but did not affect the decreased erythrocyte deformability. Other authors (Brown et al., 1993) claimed that long-term treatment of rabbits with aloxane-induced diabetes using aminoguanidine, a drug known to prevent cross-linking between glycosylated proteins, normalized the impaired erythrocyte deformability. Moreover, the discontinuing aminoguanidine in a subset of diabetic rabbits restored the deteriorated erythrocyte deformability. Using rats with streptozotocin-induced diabetes another authors reported that treatment of animals with carnosine, a dipeptide with strong antioxidant potency (50 mg/kg/day during 7 days, per os), prevents the decrease in the hemolytic stability of diabetic erythrocytes (Korobov et al., 2000).

Based on the obtained in this study effect of melatonin and above mentioned reports we assume that the decrease in the intramolecular cooperativity of spectrin from diabetic rats was possibly due to oxidative damage, not to the accompanying glycosylation of this important membrane protein. The findings suggest usefulness of melatonin as a possible treatment of diabetes patients.

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