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Thermal sensitivity and haemolysis of erythrocytes with membranopathy

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ABSTRACT

Measuring the impedance of heated suspensions of erythrocytes and erythrocyte ghost membranes, two thermally-induced alterations are registered in the plasma membrane at T_A (denaturation of spectrin with inducing temperature at 49,5 °C) and T_G (hyperthermic activation of basal ion permeability with inducing temperature at 60.7 °C). In this study erythrocytes from 9 healthy patients and 15 patients with hemolytic anemia were studied and divided into four groups depending on their T_A and T_G top temperatures. The T_A and T_G of erythrocytes with hemoglobinopathy were the same as those of control erythrocytes while those of erythrocytes with membranopathy were significantly reduced. In erythrocytes with severe membranopathy, the T_G was decreased by about 5 °C. In latter cells the normal value of T_G was restored and the resistance to thermal haemolysis was increased by 90% after the specific stabilization of band 3 protein by 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). Obtained results indicate the involvement of band 3 in the membrane alteration at T_G and in the heat target responsible for thermal haemolysis.

1. Introduction

Hereditary hemolytic anemias that originate from congenital defects in erythrocyte plasma membrane proteins (membranopathies) include hereditary spherocytosis (De-Franceschi et al., 1997), poikilocytosis and pyropoikilocytosis, stomatocytosis (Vives-Corrons et al., 1995) and hereditary elliptocytosis. Hereditary spherocytosis (HS) is due to decreased surface area of erythrocytes as a result of defect or deficiency of band 3, spectrin and some minor cytoskeletal proteins.

Hereditary hemolytic anemias, due to membranopathy, are relatively rare but acute anemic conditions that demonstrate themselves during the first months and years of childhood (Gallagher and Jarolim, 2005; Perrotta et al., 2008). Their correct therapy relies on how well they can be differentiated from other types of secondary and primary anemia, especially from the hemoglobinopathy. For this aim a multitude of methods has been developed. Frequently used are the osmotic resistance test, incubated osmotic fragility test, level of haemolysis, shape of erythrocytes, dimensions of spleen and thermal sensitivity of erythrocytes (Guetarni et al., 1992; Gallagher, 2005). The autohaemolysis test, hypertonic cryohaemolysis test, ektacytometry and the acidified glycerol test suffer from lack of specificity and are not widely used. Flow cytometric analysis of eosin-5-maleimide binding to erythrocytes (King et al., 2000) has recently been explored as a screening test for HS diagnosis. Specialized testing, such as membrane protein quantitation by gel electrophoresis, and genetic analyses, are performed for studying difficult cases or when additional information is needed. Some of these methods are non-specific, others are time consuming.

Recently, a new method, thermal analysis of erythrocyte suspension impedance, has been described as sensitive to defects in erythrocyte membrane with xerocytosis (Ivanov et al., 2007a). With the erythrocytes of healthy donors the method detected two changes in the impedance, whose inducing temperatures were 49.5 °C and 60.7 °C. These changes, designated as A peak and G peak on impedance thermogram, have been shown to demonstrate thermally-induced events (structural transitions) in erythrocyte membrane (Ivanov, 2007). In contrast to G peak, the A peak was independent of the amplitude and direction of the transmembrane gradient of ion concentration (Ivanov and Benov, 1992; Ivanov, 2007). It corresponded to the drop in electric capacitance of membranes (Ivanov, 1999) associated with the heat denaturation of peripheral protein spectrin (Ivanov, 1997), which takes place at 49.5 °C (Brandts et al., 1977). The G peak corresponded to the collapse of transmembrane gradient of ion concentration due to the hyperthermic activation of passive permeability to ions with inducing temperature of 60.7 °C (Ivanov and Benov, 1992; Ivanov, 1992, 2007). With the erythrocytes of different mammals the inducing temperature of G peak has been shown to vary strongly correlating the

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sphingomyelin content and resistance of erythrocytes against thermal haemolysis (Ivanov, 1993, 2007). The G peak has been also registered in plant cells correlating the thermal stability of their plasma membranes (Hardin et al., 1999). According to studies on healthy erythrocytes (Ivanov et al., 2007b; Ivanov et al., 2011) the membrane event described by the G peak apparently involved a predenaturational, initially reversible alteration of the anion exchanger, the band 3 protein of erythrocyte membrane.

In this study above method was used to investigate the thermal sensitivity and haemolysis of erythrocytes from patients with membranopathy. Depending on the values of T_A (top temperature of A peak) and T_G (top temperature of the G peak), the patients (n = 24) were divided into four groups; healthy (n = 9), patients with haemoglobinopathy (n = 5), patients with membranopathy on band 3 (n = 2), and patient with HS (n = 8). DIDS is a membrane impermeable amino reagent which specifically binds the band 3 integral protein of erythrocyte membrane, the anion exchanger (Cabantchik, Rothstein, 1974), and strongly stabilizes its structure (Snow et al., 1978). In all patients having erythrocytes with inherited strongly reduced value of T_G, DIDS-treatment of erythrocytes restored the normal value of T_G and increased the resistance against thermal haemolysis by 90%.

2. Materials and methods

2.1. Materials

4,4′-Diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS), ethylene diamine tetraacetic acid (EDTA), NaCl and sucrose were purchased from Sigma Chemicals Co, St. Louis, MO, USA.

2.2. Preparation of erythrocytes

0.3 ml citrated blood was taken through venipuncture from healthy donors (control) and anemic patients in the pediatric hospital of the Medical faculty of Thracian University, Stara Zagora, Bulgaria. Each anemic disorder was established by classical procedure. In several hours erythrocytes were isolated, twice washed by centrifugation in excess volume of cold NaCl saline and used.

2.3. DIDS treatment of erythrocytes

Washed erythrocytes were suspended at hematocrit of 0.10 in 150 mM NaCl saline, containing 5 mM phosphate buffer, pH 7.8, and 50 μM DIDS at dark and at room temperature for 15 mins. The DIDS - treated cells were isolated and washed in excess volume of cold 150 mM NaCl saline to remove non-reacted DIDS. Under these conditions, more than 95% of the DIDS resides on band 3 protein (Jennings and Passow, 1979) resulting in strong inhibition and thermal stabilization of band 3 protein (Snow et al., 1978).

2.4. Derivative thermogram of suspension conductance

Deviations in the structure of the main proteins of erythrocyte plasma membrane, spectrin and band 3, were revealed determining the denaturation temperatures of these proteins as explained earlier (Ivanov, 1997; Ivanov et al., 2011). Due to their immense number and random orientation the suspended erythrocytes were regarded as an ensemble of spherical particles. The dielectric interaction between erythrocytes was reduced using sufficiently low hematocrit value (0.17). To impose strong transmembrane gradient of ion concentration, 50 μl of packed washed erythrocytes from healthy and anemic patients were suspended in 230 μl isotonic solution, containing 30 mM NaCl and sucrose, pH 7.4. Prior to testing, the suspension was introduced with a syringe into a vertical U-tube glass conductometric cuvette (total length 240 mm, outside diameter 4 mm, wall thickness 0.5 mm). Two platinum electrodes, spaced at about 7 mm, were attached at the common bottom

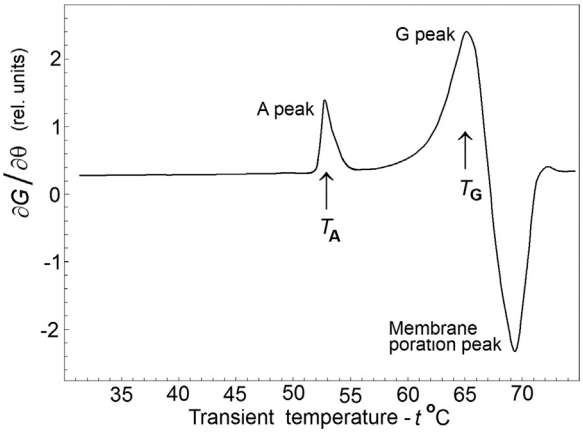

Fig. 1. Temperature profile of d$G/$dθ. The d$G/$dθ is the first derivative of suspension electrical conductance, G, against time, θ, and is shown as dependent on the temperature, t. The suspension contained control erythrocytes, suspended in isotonic medium of 30 mM NaCl and sucrose, and was heated. The hematocrit value, frequency and heating rate were 0.17, 7 kHz and 1.8 °C/min.

of the left and right tubes. About two-third of the cuvette was submerged into the water bath of a thermostate whose temperature, t, was measured by a thermocouple. The water bath was heated with a constant heating rate, d$t/$dθ = 1.8 °C/min, where θ is time. During heating an alternating electric voltage of 150 mv was applied between electrodes and the admittance, $Y^* = G + j.B$, of tested suspension was continuously measured at 7 kHz and separated into its real (G) and imaginary (B) parts using Solartron 1260 Impedance Frequency Analyzer (Schlumberger Instruments, Hampshire, England), controlled by a computer.

The conductance, G, of erythrocyte suspension increased linearly with the temperature, t, unless a denaturation (structural alteration) took place in erythrocyte membrane causing sharp sigmoid change in G at the denaturation temperature. In order to better express the temperature profile of each change we calculated the quantity d$G/$dθ, which is the first derivative of suspension conductance, G, against time, θ, and determined its dependence on the temperature, t. The obtained derivative conductance thermogram, i.e., the temperature profile of d$G/$dθ, appeared as a horizontal line with sharp peak centered at each denaturation temperature (Fig. 1). There were two denaturation temperatures, respectively two peaks, further designated as A peak and G peak (Fig. 1).

2.5. Resistance of erythrocytes against thermal haemolysis

It was assessed by two protocols as previously described (Ivanov, 1993). The first protocol consisted in determination of the time rate of thermal haemolysis. Briefly: 0.1 ml packed cells were suspended in 5 ml isotonic NaCl saline, containing 3 mM EDTA. The EDTA was obligatory in order to inhibit the proteolytic activity of residual leucocytes and to bind the egressed free iron ions capable of precipitating oxidative attack on membranes. The thermohaemolysis was assayed at 53.5 °C by measuring the optical density at 700 nm, E_{700}, of 0.2 ml aliquots periodically withdrawn and diluted to 1.5 ml by NaCl saline. The thermal resistance was defined by the time of exposure, resulting in 50% haemolysis. With the second protocol, the resistance of erythrocytes to thermal haemolysis was represented by the top temperature of the G peak, T_G, on derivative conductance thermogram as the membrane G transition is relevant to thermal haemolysis (Ivanov, 1993; Ivanov et al., 2011). On the other hand, the top temperature of the A peak, T_A, corresponds to the thermal sensitivity of erythrocytes (Vives-Corrons et al., 1995).

Table 1
Values of T_A (denaturation temperature of spectrin) and T_G (denaturation temperature of band 3) in the erythrocyte membrane of patients with hereditary anemia compared to healthy patients. The T_A and T_G top temperatures were determined at 1.8 °C/min heating rate.

Number of group	Type of disorder	Number of patients in group	T_A °C	T_G °C
I	Control erythrocytes	9	52,080 ± 0334	65,11 ± 0226
II	Hemoglobinopathy	5	52,10 ± 1,01	63,9 ± 0,34
III	Membranopathy on band 3	2	52,65 ± 0,35	61 ± 0,50
IVa	Severe HS	2	49,0 ± 0,50	60,6 ± 0,40
IVb	HS	6	50,75 ± 0,29	63,08 ± 0,47

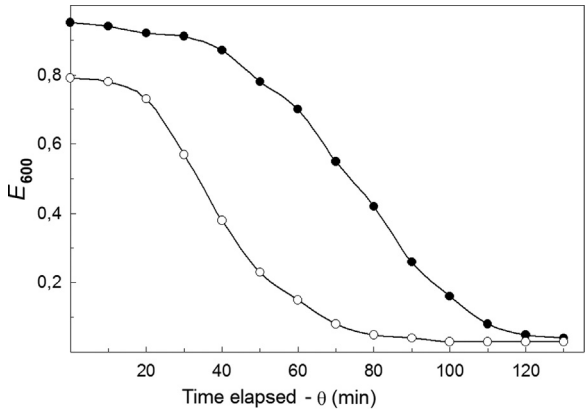

Fig. 2. Time course of the thermal haemolysis of erythrocytes with HS, as affected by the stabilization of band 3. Intact erythrocytes (○) and DIDS-treated erythrocytes (•) were incubated in isotonic medium containing 150 mM NaCl and 3 mM EDTA, hematocrit 0.02. The thermohaemolysis was assayed at 53.5 °C by measuring the optical density E_{700} of 0.2 ml aliquots periodically withdrawn and diluted to 1.5 ml by 150 mM NaCl saline.

3. Results

Fig. 1 shows the derivative thermogram of the conductance, *G*, of a suspension containing control erythrocytes from healthy patients, aged from 10 months to 40 years. Two positive peaks, designated as A and G, were obtained on the thermogram at 52,080 ± 0334 °C and 65,11 ± 0226 °C, correspondingly, at the heating rate of 1,8 °C/min (Table 1). The biophysical significance of each peak is indicated in the Introduction section. The negative peak after the G peak has been explained by the appearance of barrier defects (membrane pores) initially permeable to sucrose and latter to larger molecules (Ivanov and Benov, 1992; Ivanov et al., 2011). Due to the heating rate applied, the top temperatures of A peak, T_A, and of G peak, T_G, were shifted rightwards compared to their inducing temperatures, 49.5 °C and 60.7 °C, respectively. At heating rates tending to zero, these shifts tended to nil.

The T_A and T_G top temperatures demonstrated striking invariability within the large group of healthy individuals (Table 1). The same invariability was obtained repeating many times the determination of these temperatures with the erythrocytes of the same individual (not shown). However, it must be indicated that, in contrast to the T_A temperature, the T_G was reduced by one to two °C in washed erythrocytes subjected to prolonged metabolic starvation (5 h at 23 °C or 24 h at 4 °C). Above results allow usage of T_A and T_G top temperatures as markers for the structural stability of erythrocyte membrane proteins in norm and pathology. Next, these temperatures were determined at patients with different types of hereditary anemia, compared to the group of control patients (Table 1). The reproducibility of T_A and T_G values at anemic erythrocytes was similar to that at control erythrocytes even though the number of replicates was smaller.

The second group contained patients with hemoglobinopathy. The T_A top temperature of their erythrocytes coincided by value and variability with that of control group, while the T_G top temperature was slightly decreased (Table 1). We do not believe that this decrease indicates a deviation in the structure of band 3, rather it could be a secondary effect due to the increased level of free radical production and damage, commonly encountered at the erythrocytes with hemoglobinopathy (Amer et al., 2005).

The third group included two patients with possible deviation in the structure of band 3 protein. Their spectrin was structurally as stable as that of the healthy individuals, however the denaturation temperature of their band 3 was markedly reduced (Table 1). Apparently, these erythrocytes had normal spectrin and genetically altered anion exchanger. Both patients suffered from chronic stomach and lung disorders. These conditions could be related to the inherited deviation in band 3 structure, as this protein is expressed in other cells including the cells of lung alveoli, blood circulation system and kidney (Rysava et al., 1997).

The IVa group included two children with all clinic signs of severe HS. The T_A and T_G denaturation temperatures of their erythrocyte membranes were strikingly reduced (Table 1) indicating severe structural deviations in spectrin and band 3. Patients in the IVb group demonstrated all clinic signs of HS. According to the values of T_A and T_G top temperatures the main membrane defect resided on spectrin, however this defect was apparently milder compared to that at IVa group.

An important hallmark of the erythrocytes of III and IV group of patients was the strong effect produced by DIDS on their T_G top temperature and resistance against thermal haemolysis. For example, binding of DIDS to the erythrocytes of IVa group of patients increased their T_G temperature from 60,6 ± 0,40 to 66,4 ± 0,23 °C (not shown) and their resistance against thermal haemolysis by 90% (Fig. 2). Similar increase in T_G and resistance against thermal haemolysis was obtained by binding of DIDS to the erythrocytes of the III group patients (not shown). Treating the erythrocytes of the IVb group of patients with DIDS also increased the T_G top temperature to about 66 °C and the resistance against thermal haemolysis by about 40% (not show). Latter results support the involvement of band 3 protein in the membrane event at T_G and in the resistance of anemic erythrocytes against thermal haemolysis.

4. Discussion

A recent study of ours has reported results on DIDS-treatment of erythrocytes from healthy donors (Ivanov et al., 2011). There was no effect on T_G and resistance against thermal haemolysis in case the DIDS-treatment was carried out suspending erythrocytes in isotonic medium of NaCl with slightly alkaline buffer (pH 7,8–8,2), generally used by investigators. This result is in full concord with the results of others (Chernitskii and Iamaikina, 1988; Lepock et al., 1989). Next, two other suspension media (isotonic solution of 100 mM NaCl and sucrose, and hypotonic solution of 100 mM NaCl) were used in order to transform the cells from discocytes into stomatocytes and to increase their volume. The DIDS-treatment of control erythrocytes suspended in latter media yielded an increase in T_G top temperature by 2.5 °C and enhancement of resistance against thermal haemolysis by 66%. These results were interpreted as a proof for the involvement of band 3 in the membrane event at T_G and in the heat target of thermal haemolysis. However, the need for shape transformation and volume increase as a

pre-requisite for the effect of DIDS-treatment remained not clear. It could be related to the model predicting that erythrocyte swelling generates elastic forces in the lipid bilayer that increases the free portion of band 3 molecules and their clustering (Pajic-Lijakovic et al., 2010).

In this study we used erythrocytes with hereditary destabilized band 3 as revealed by the strongly reduced T_G. DIDS-treatment of these erythrocytes was carried out in usual media (isotonic NaCl saline with slightly alkaline buffer) preserving the shape and volume of erythrocytes. Nevertheless, it yielded a strong increase in T_G top temperature by 5 °C and enhancement of resistance against thermal haemolysis up to 90%. These results strongly support previous claim for the involvement of band 3 in the membrane event at T_G and in the heat target of thermal haemolysis.

In addition, above results support the conception that the membrane event at T_G involved a pre-denaturational alteration in band 3. First, the inducing temperature of the T_G event was 60.7 °C, which is almost 6–7 °C in front of the denaturation temperature (67 °C) of band 3 transmembrane domain (Snow et al., 1978; Maneri and Low, 1988). Second, treatment of erythrocytes by DIDS causes a step-wise increase of the band 3 denaturation temperature by 13 °C (Snow et al., 1978), while the inducing temperature of the T_G event increased only by 2.5 °C in control erythrocytes (at specially designed conditions) and 5 °C in anemic erythrocytes. The nature of the pre-denaturational alteration in band 3, responsible for the hyperthermic activation of ion permeability and thermohaemolysis of erythrocytes remains to be elucidated.

5. Conclusion

Compared to control erythrocytes the membrane events at T_A and T_G top temperatures were found decreased to a variable extent in the anemic erythrocytes with membranopathy. DIDS-treatment of these erythrocytes restored the thermal stability of their membranes (T_G value) and strongly inhibited the thermally-induced haemolysis. Compared to the unfolding of band 3 transmembrane domain, the membrane event at T_G apparently involved a by far milder and preliminary alteration of this protein. The results proved the applicability of thermal impedance analysis for detecting anemia with hereditary membranopathy.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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Competing interests

The funding organization played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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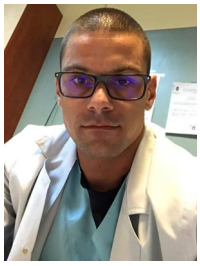

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