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BENEFICIAL IMPACT OF DIPYRIDAMOLE AND DIDS ON ERYTHROCYTE MEMBRANE AT MEMBRANOPATHY

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ABSTACT

Spectrin and the band 3, major proteins of human erythrocyte membrane, are altered in patients with hereditary spherocytosis (HS). The band 3 is implicated in the permeability transition of erythrocyte membranes (EM) at 61°C (T_g), and its monomer is specifically stabilized by DIDS (4,4´-di-isothiocyanostilbene-2,2´-disulphonate) and Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-d] pyrimidine). PURPOSE. To study in vitro the impact of DIDS and dipyridamol on the band 3 in anemic patients as revealed by the changes in the T_g temperature. METHODS. We used thermal analysis of the impedance (70 kHz) of erythrocytes suspended in isotonic 30 mM NaCl/mannit media in order to detect permeability transition at T_g and the heat denaturation of spectrin at 49.5 °C. RESULTS: In patients (n = 8) with HS the T_g transition was detected at 56-57°C and accompanied by severe instability of EM after the spectrin denaturation temperature. DIDS (30 μM, irreversible) and dipyridamole (100 μM, reversible) increased the T_g to about 60°C and subdued the preceding membrane instability. CONCLUSION. Dipiridamole and DIDS both stabilized the structure of EM from patients with HS. Thermal dielectroscopy is useful test for population screening of asymptomatic forms of HS.

Key words: band 3, hemolytic anemia, membranopathy, thermal hemolysis.

INTRODUCTION

The integrity and mechanical properties of human erythrocyte membrane (EM) rely chiefly on the two major membrane proteins, spectrin and the band 3. They are frequently altered in some inherited anemic conditions such as hereditary spherocytosis (HS) (1-3).

Band 3 is an integral protein whose monomers are associated in dimmers and larger oligomers. According to microcalorimetric studies the monomer of band 3 has high structural stability as evident by its high temperature of denaturation, 68°C (4). In addition, the structural stability of band 3 monomer is strongly increased by DIDS and dipyridamole. DIDS is a membrane impermeable covalent amino reagent which at micromolar concentrations (<50 μM) specifically binds the dimer of band 3 and stepwisely increases the denaturation temperature of the monomer by 13°C (5). Similar, although reversible, effect on the stability of band 3 monomer is produced by dipyridamole (6). At anemic conditions caused by hereditary red cell membrane disorders the alteration in band 3 is not accompanied by a reduction in the denaturation temperature of band 3 monomer. The band 3 of EMs is implicated, however, in another milder, predenaturational thermally – induced membrane transition concerning the oligomeric structure of this protein (7). This permeability transition affects the ion permeability of EM and is responsible for thermal hemolysis. In healthy humans the permeability transition is centered at 61±0.2°C, the so called T_g temperature. Interestingly enough, for a limited group of patients with HS the T_g temperature had a values lowered by about 4-5°C (8). In the presented study we used larger group of patients with HS and studied the *in vitro* effect of DIDS and dipyridamol on the T_g

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temperature of the permeability transition in their EMs.

MATERIALS AND METHODS

Materials

Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-d] pyrimidine), DIDS (4,4´-di-isothiocyanostilbene-2,2´-disulphonate), sorbitol and NaCl were all purchased from Sigma, St. Louis, MO.

Impedance spectroscopy of the heated erythrocyte suspensions

The isolation and DIDS treatment of erythrocytes was described previously (7). The binding of DIDS to band 3 of EMs was carried out according to (9). To determine the permeability transition at T_g and the heat denaturation of spectrin at 49.5 °C we used thermal analysis of the impedance of erythrocyte suspensions as described previously (7).

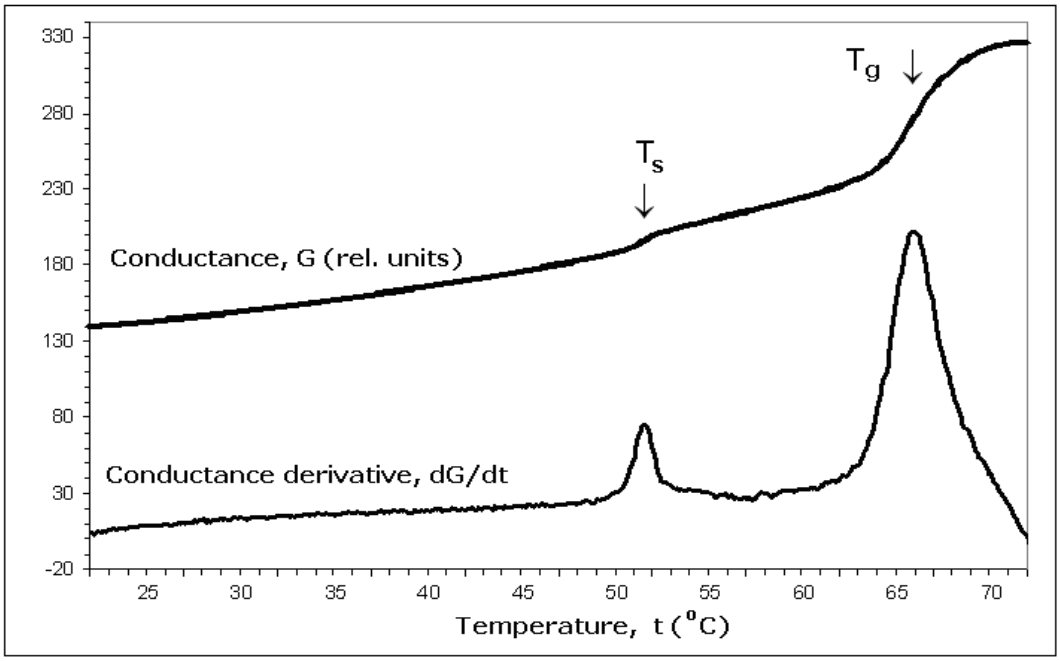

Figure 1. Temperature profile of the conductance, *G*, and temperature-derivative of conductance, *dG/dt* of erythrocytes suspended in isotonic 30 mM NaCl/mannit solution. The hematocrit, heating rate and frequency were 0.30, 2.5 °C/min and 70 kHz, respectively.

RESULTS

Temperature-induced variations in the conductance, *G*, of heated erythrocyte suspensions are shown in **Figure 1**. Superimposed over the continuous Boltzmann type temperature dependence of *G* two sharp, threshold changes were registered with mid-point temperatures T_s and T_g, respectively. These changes are better identifiable as sharp peaks on the derivative thermogram of suspension conductance. The two top temperatures, T_s and T_g, slightly depended on the heating rate applied and at heating rates extrapolated to zero their values were 49.5°C and 61.0°C, respectively. During repeated measurements of samples, taken from a same blood, the T_s and T_g temperatures displayed variations by ± 0.2 °C from respective mean values.

Previous studies evidenced that the peaks centered at the T_s and T_g top temperatures both correspond to thermally-induced structural transitions in the plasma membrane of erythrocytes (10). The change in the suspension conductance at T_s is explained by the effect of spectrin denaturation on the dielectric polarization of EM (11). According to microcalorimetric study (12) the heat denaturation of spectrin takes place at 49.5°C coinciding with T_s. The peak centered at T_g demonstrates the activated passive diffusion of cytosole ions due to membrane transition involving a mild, pre-denaturational alteration in the band 3 (7).

Compared to control ones, the T_g temperature was lowered by about 4 to 5°C for the membranes of erythrocytes with HS (**Figure 2**). Similar outcome was previously reported for a

smaller (n = 2) group of patients with HS (8). This finding is in line with the report (13) that ion permeability of erythrocytes with HS is increased. It possibly represents a distinctive feature of the erythrocytes from patients with HS and hereditary altered band 3.

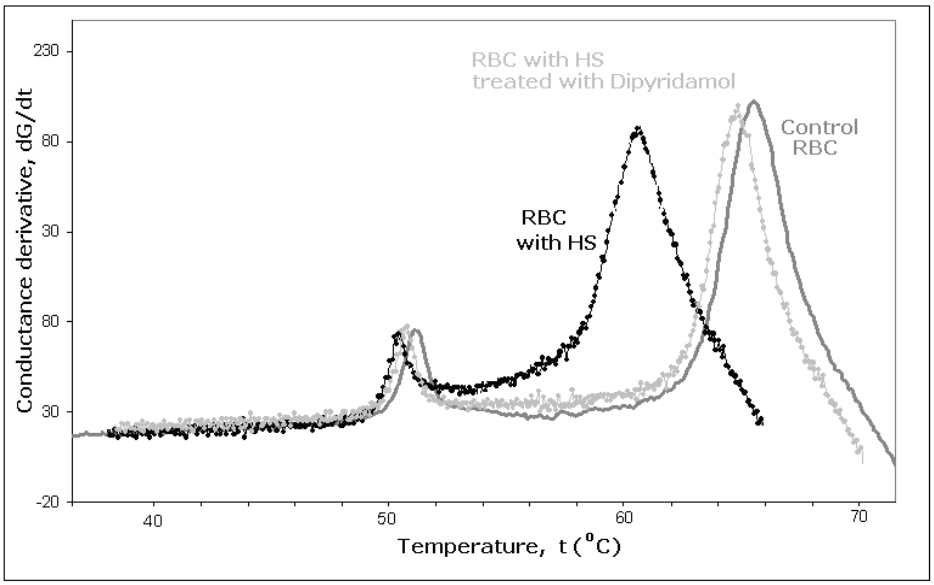

Figure 2. Temperature profile of *dG/dt* of erythrocyte suspensions. Dark gray curve - control erythrocytes, black curve - erythrocytes with moderate form of HS. Gray curve demonstrates the effect produced by Dipyridamol (100 μM) and DIDS (30 μM) on eryhrocytes with a moderate form of HS (n = 4). Other details as for **Figure 1.**

The *in vitro* treatment of such erythrocytes with DIDS (30 μM) significantly increased their T_g temperature close to that in control cells (**Figure 2**). This result is in concert with the report that the band 3 is important participant in the permeability transition in erythrocyte membrane at T_g (7). At micromolar concentrations (50–100 μM) the medecine dipyridamole (persantine, antistenocardine) also increased the T_g temperature. In contrast to DIDS, the effect of dipyridamole was reversible and concentration dependent (not shown).

Except the reduction of T_g temperature, an additional membrane instability about the T_s temperature was apparent on the *dG/dt* curve of erythrocytes with HS (**Figure 3**). This membrane instability was demonstrated by a third peak with much broader half-height width than the spectrin denaturation peak at T_s. The amplitude of this peak was higher and its top temperature was lower in patients with severe form of HS while in patients with moderate condition this peak was poorly distinguishable (**Figure 3**). DIDS (30 μM) and dipyridamole (50-100 μM) both reduced significantly the amplitude of the third peak (not shown). Similar to the spectrin denaturation peak at T_s (11), the third peak had dispersive character as its amplitude was reduced with the frequency and it completely vanished above 200 kHz (not shown).

DISCUSSION

This study, combined with previous one (8), reports a 4-5°C reduction in the T_g temperature in erythrocytes of patients with HS. This could be explained taking into account that the two events, the permeability transition at T_g and thermal denaturation at 68°C, concern different types of band 3 structure. While the first event increase the olygomerization of band 3 (7) the latter event destroys the secondary and tertiary structure of this protein. Since the same erythroid isoform of band 3 is expressed in the acid secreting cells of kidney the genetically altered band 3 could also affect the regulation of urine acidity, according to (15).

The band 3 is phylogenetically preserved in most cells of vertebrate animals (14). Erythroid band 3 mutations cause 15–25% of cases of HS, and 100% of cases of southeast Asian ovalocytosis (1). HS affects 1 in 2000 people of Northern European ancestry and at least 1 in 5000 in general (1, 2, 16). Hence, the applied in this study method could be used for broad population screening.

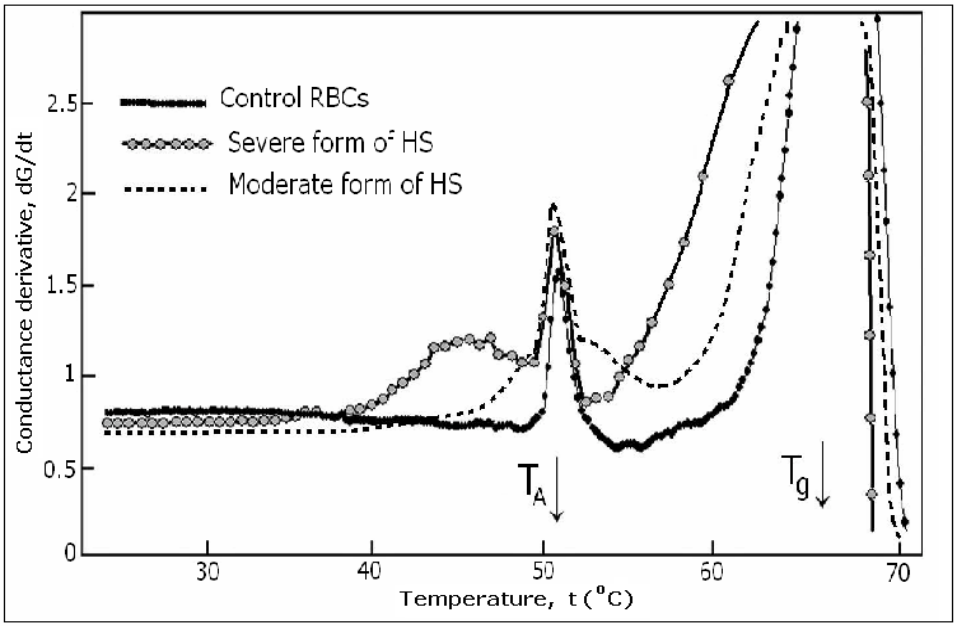

Figure 3. Temperature profile of *dG/dt* for erythrocytes with HS (n = 4) displaying varying severity, compared to control ones. Other details as for **Figure 1**.

CONCLUSSION

In contrast to microcalorimetry, the thermal analysis of suspension impedance detected the inherited red cell membrane disorder in band 3 protein of anemic erythrocytes. This was evident by the significant (4-5°C) decrease in the T_g temperature of the permeability transition implicating a mild change in band 3. This reduction in T_g temperature was largely compensated by dipyridamol and DIDS, two chemicals known to stabilize the structure of band 3.

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