Correction by insulin added in vitro of abnormal membrane fluidity of the erythrocytes from Type 1 (insulin-dependent) diabetic patients

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Summary. Filtrability of erythrocytes obtained from uncontrolled Type 1 (insulin-dependent) diabetic patients is abnormal, but is corrected by insulin added in vivo or in vitro. As erythrocyte filtrability depends on several determinants, we chose to study a membrane property of erythrocytes from diabetic subjects. Membrane fluidity was studied by fluorescence polarization using a lipophilic probe, the diphenyl-hexatriene and the Coulter Epics V together with a laser Spectra-physics 2000. Fluorescence polarization values obtained for 31 normal subjects (0.253 ± 0.043 SD) and 31 uncontrolled Type 1 diabetic patients (0.231 ± 0.043 SD) were significantly different ($p < 0.01$). Insulin ($2.5 \cdot 10^{-9}$ mol/l) added in vitro increased the fluorescence polarization values of red cell membranes from diabetic patients (without insulin, fluorescence polariza-

tion values = 0.210 ± 0.032 SD; with insulin, fluorescence polarization values = 0.253 ± 0.024 SD, $p < 0.001$, $n = 15$), but had no effect on normal membranes (without insulin fluorescence polarization values = 0.255 ± 0.037 SD, with insulin, fluorescence polarization values = 0.251 ± 0.026 SD; $n = 12$).

Given a relationship between the lipid bilayer and membrane cytoskeleton proteins, this insulin-correctable abnormality of erythrocyte membrane fluidity may be an important determinant of the rheological behaviour of erythrocytes from diabetic patients.

Key words: Membrane, fluorescence polarization, erythrocytes, diabetes, insulin.

The erythrocytes of uncontrolled Type 1 (insulin-dependent) diabetic patients have abnormal rheological properties [1–4]. Modifications in the erythrocyte membrane could be the cause of these rheological abnormalities. Biochemical abnormalities have been described [5–7] in the membrane, and change in its viscosity have been demonstrated by the fluorescence polarization technique [7–9] or electron spin resonance [10]. However, these results have been challenged [11].

We have previously observed that the poor filtrability of erythrocytes from uncontrolled Type 1 diabetic patients was rapidly corrected by insulin added in vivo or in vitro [3, 4].

The aim of this study was therefore to verify whether insulin directly modified the membrane fluidity assessed by fluorescence polarization if such an abnormality was found in cells from diabetic patients. For this purpose, ghosts prepared from erythrocytes originating from uncontrolled Type 1 diabetic patients and matched controls were incubated with and without insulin. Fluorescence polarization was studied with a lipophilic probe, the 1,6 diphenyl-1,3,5 hexatriene (DPH).

Subjects and methods

The subjects were 31 Type 1 (insulin-dependent) diabetic patients, 20 males and 11 females, aged 26 to 64 years (mean $\pm$ SD = 44 ± 16), uncontrolled at the time of the study. None of the patients were smokers, and women did not use oral contraceptive agents. Diabetes duration

ranged from 1 to 26 years (mean $\pm$ SD = 9.6 ± 7 years). Two patients had macroproteinuria (i.e. > 500 mg/day) but serum creatinine did not exceed $100 \mu\text{mol/l}$ in any of the patients. Nine had background retinopathy at fundus examination after papillary dilatation. Patients with proliferative retinopathy or symptoms of cardiovascular disease were excluded from the study. Treatment consisted of two or three insulin injections per day except for two cases treated with one daily insulin injection. Glycosylated haemoglobin values, assessed by microchromatography at 23°C (Biorad Column) after an incubation at 37°C for 5 h in 10 vol of saline buffer 154 mmol/l with 2 mmol/l of glucose in order to eliminate the labile fraction, averaged $11.5 \pm 2.8\%$ (normal 4–8%). Capillary blood glucose value was checked in the fasting state before the insulin injection at 08.00 hours; the injection was postponed from its usual administration at 07.00–07.30 hours. If the capillary blood glucose value exceeded 11 mmol/l , a blood sample was obtained and collected on Na citrate, 0.11 mol/l . Mean plasma glucose value was $16.4 \pm 5.9 \text{ mmol/l}$ (range 11–21). Urine was checked for the presence of ketone bodies (Ketodistix R), which were detected in 8 cases. Each patient was matched for age and sex with a laboratory or hospital staff member, and blood samples from the diabetic patient and the control subject were obtained and processed the same day.

Erythrocyte ghosts were prepared at 4°C according to Hanahan and Eckholm [12]. Platelets and leucocytes were eliminated [13] by filtration on cellulose and Sigma cell 50. Erythrocytes were then haemolyzed by osmotic shock in Tris 11 mmol/l buffer. Haemoglobin free lysed cells were obtained by washing in Tris buffer and centrifuging at 20000 g for 40 min as many times as necessary. A microdosage of haemoglobin [14] was performed to verify that membranes were haemoglobin free. Membranes were resuspended at a concentration of $10^7/\text{ml}$ in Tris Hepes buffer with 0.25% bovine serum albumin as recommended by Williams et al. [15] and kept frozen at -20°C until use. The freezing and thawing procedure was inevitable due to geographical separation between hospital and fluorescence polarization laboratory. To verify if the freezing/thawing procedure could be responsible

for an artefact, a small series of experiments was performed on ghosts freshly prepared from 4 control subjects and 4 diabetic patients and repeated after freezing and thawing. After thawing the ghosts were incubated at 37 °C for 2 h in the same buffer with or without insulin (Actrapid, Novo, Paris, France) at $2.5 \cdot 10^{-9}$ molar concentration or with proinsulin ($2.5 \cdot 10^{-9}$ molar concentration) (a gift from Novo). The probe ($2 \cdot 10^{-3}$ molar solution of DPH in tetrahydrofuran) was first dispersed by 1000-fold agitative dilution in NaCl 154 mmol/l PBS buffer. Membrane suspension (0.4 ml) was mixed with 1 ml probe solution and incubated 30 min at 20 °C (16).

Fluorescence polarization was measured on individual cells with a Coulter Epics V cell sorter (Coulter Electronics, Hialeah Fla, USA) calibrated according to Capo et al. [17]. This instrument analyzed cells in aqueous suspension as they passed through the polarized beam of an argon-ion, with exciting light at 363 nm highly polarized in the vertical plane. The fluorescence emission was analysed simultaneously in two planes, parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the polarization plane of exciting light. The fluorescence polarization was then calculated by computer according to the following equation: $P = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}}$.

The fluorescence polarization value (P) for each subject was the mean obtained after the passage of 5000 or 10000 particles of 2 to 4 μ diameter. The Epics V was calibrated with the aid of half wave retardation plates. The incorporation of DPH into the membrane was indicated by an increase in fluorescence intensity as the probe moved from the aqueous phase to the hydrophobic domain of the membrane. DPH is localized almost exclusively within the hydrophobic domain of the lipid bilayer. The polarization of fluorescence is directly related to the rotational relaxation time of the fluorophore. A change in the local packing of the molecules surrounding the probe results, in an altered polarization of fluorescence value [18].

Statistical analysis

The Wilcoxon test for paired series was employed in the statistical analysis. Statistical significance was assumed at $p < 0.05$.

Results

The value of the fluorescence polarization (P) was significantly lower ($p < 0.01$) in the erythrocytes from the 31 diabetic patients ($P = 0.231 \pm 0.043$ mean $\pm$ SD) than in those from the matched controls ($P = 0.253 \pm 0.043$). An example of a histogram obtained with the 5000 determinations on ghosts from a diabetic patient and his control is shown in Figure 1.

Table 1 shows the effect of incubation in the presence of insulin. Fluorescence polarization values of normal erythrocyte ghosts were not significantly modified by incubation with $2.5 \cdot 10^{-9}$ mol/l insulin, but those of

ghosts from diabetic patients increased significantly ($p < 0.001$) and returned to normal (Fig. 2).

Proinsulin ($2.5 \cdot 10^{-9}$ mol/l) did not modify fluorescence polarization values of erythrocyte ghosts either from normal or diabetic subjects (Table 2).

The same results were observed in the small series using fresh membranes, where we found lower fluorescence polarization values in those from diabetic patients

Fig. 1. Histogram of fluorescence polarization value (P) of 5000 ghosts from one control subject and from one Type 1 diabetic patient

Fig. 2. Histogram of fluorescence polarization value (P) of erythrocyte ghosts from one Type 1 diabetic patient. 10000 ghosts were incubated for 2 h without insulin and 10000 ghosts with insulin ($2.5 \cdot 10^{-9}$ mol/l)

Table 1. Fluorescence polarization value obtained after 2 h of incubation at 37 °C without and with insulin ($2.5 \cdot 10^{-9}$ mol/l) of erythrocyte ghosts from control subjects and Type 1 diabetic patients (mean $\pm$ SD)

	Without insulin		With insulin
Control subjects (n = 12)	0.255 ± 0.037	NS	0.251 ± 0.026
	$p < 0.01$		NS
Type 1 diabetic patients (n = 15)	0.210 ± 0.032	$p < 0.001$	0.253 ± 0.024

Table 2. Fluorescence polarization value obtained after 2 h of incubation at 37 °C without insulin, with insulin ($2.5 \cdot 10^{-9}$ mol/l) and with proinsulin ($2.5 \cdot 10^{-9}$ mol/l) of erythrocyte ghosts from control subjects and Type 1 diabetic patients (mean $\pm$ SD)

Control subjects (n = 5)			Type 1 diabetic patients (n = 5)		
Without insulin	Insulin	Proinsulin	Without insulin	Insulin	Proinsulin
0.277 ± 0.023	0.277 ± 0.016	0.265 ± 0.018	0.195 ± 0.031	0.274 ± 0.009	0.192 ± 0.021

ients, correction by insulin *in vitro*, but no effect of the hormone on membrane from normal subjects (data not shown).

Discussion

The fluorescence polarization of DPH was measured in erythrocyte ghosts from Type 1 diabetic patients and normal subjects. The ghosts were frozen before use, and we observed virtually the same results with fresh and frozen thawed erythrocyte ghosts. Furthermore, the fluorescence polarization values from our control frozen ghosts ($P = 0.255 \pm 0.037$) were in a good agreement with those previously published using fresh erythrocytes by Beguinot et al. [19] and by Luly et al. [20].

We observed that erythrocyte membranes obtained from uncontrolled Type 1 diabetic patients had a decreased fluorescence polarization value compared to normal when using DPH as a probe. This finding corresponds to increased mobility of the probe in its hydrophobic environment in the deeper part of the lipid bilayer.

Several authors using a similar technique have shown abnormal fluidity in erythrocytes or other cells. Otsuji et al. [7] and Baba et al. [8] have demonstrated increased membrane microviscosity in the erythrocytes of Type 1 and Type 2 (non-insulin-dependent) diabetic patients with various degrees of control; fluorescence polarization values were correlated to cholesterol/phospholipid ratio in the plasma and the erythrocyte membrane, and were more disturbed in the group with high fasting plasma glucose. However, Hill and Court [11] observed no modification of the fluorescence polarization values of erythrocyte membrane in a group of diabetic children with varying degrees of control; neither did they find any modification in the cholesterol/phospholipid fraction of the erythrocyte membrane from their diabetic patients. Plasma membrane of hepatocytes from streptozocin-diabetic rats have been shown to have low fluorescence polarization values, suggesting an increase in membrane fluidity with the DPH probe [21]. Bryszewska and Leyko [9] demonstrated increased membrane viscosity by using a different technique, measuring the lateral mobility of a lipophilic probe (pyrene). Bryszewska et al. [22] found a higher membrane cholesterol/phospholipid ratio. These findings are in agreement with Otsuji et al. [7] and Baba et al. [8]. Kamada and Otsuji [10] used the electron spin resonance technique for studying erythrocyte membrane from Type 1 and Type 2 diabetic patients. They demonstrated decreased mobility of the stearic acid chains located in the deeper part of membrane. When the spin label was localized in the protein part of the membrane, a resistance to rapid binding was described in erythrocyte membranes from Type 1 diabetic patients [23]. Therefore, in spite of some contradictory results, it seems that the physical properties of the erythrocyte membrane of uncontrolled diabetic patients are abnormal.

These abnormalities could be related to a change in the biochemical composition of the lipid bilayer. An increase in cholesterol/phospholipid ratio and an increase in saturated fatty acids were shown in the same membranes by Otsuji et al. [7], Juhan-Vague et al. [6] and Bryszewska et al. [22]. The low value of the correlation coefficient between membrane cholesterol/phospholipid ratio and fluidity led Bryszewska et al. [22] to suggest that other factors like qualitative phospholipid changes may influence membrane fluidity in diabetes.

A close relationship between the lipid bilayer and the cytoskeleton [24–26] having been shown, a change in the lipid bilayer behaviour could have an effect on the cytoskeleton.

Incubation of the ghosts with insulin at nearly physiological concentration normalized the fluorescence polarization value of membrane from Type 1 diabetic patients but had no effect on the normal membrane. Proinsulin added at the same molar concentration as insulin did not show any effect on membranes from diabetic patients and controls.

These results have to be added to those of Bryszewska and Leyko [9], who observed with a different technique an increase in the mobility of the probe when erythrocyte membranes from diabetic patients were incubated with insulin. Luly et al. [20] were able to show an elevation of the fluorescence polarization value of normal erythrocyte membrane when incubated with physiological concentration of insulin. Dutta-Roy et al. [27] showed that the microviscosity of normal erythrocyte membrane and the filtration time of intact cells were reduced by insulin at physiological concentration in a dose-dependent manner; use of supraphysiological concentration of the hormone reversed the effect of the lower concentration of insulin. Proinsulin at different concentrations had no effect compared to insulin. It may be pointed out that, when plasma membranes from known insulin sensitive cells such as hepatocyte or adipocyte are used, incubation with insulin has been shown to decrease membrane fluidity [26, 28, 29]. These results, however, have not been found in all studies [30].

Therefore, the accumulation of results obtained with different techniques and different concentrations of insulin appears to confirm our previous proposition [3, 4] that insulin acts directly on the erythrocytes even while glucose transport across the membrane is not insulin-dependent, insulin receptors are scarcely distributed and protein synthesis does not occur.

It has been suggested that membrane fluidity is an important determinant in the behaviour of peptide hormone receptors [31]. Proinsulin was found not to produce the insulin effect at a similar concentration, arguing that insulin receptors mediate the change observed; however, the concentrations of insulin and proinsulin having not been manipulated, this conclusion must be retained with caution.

The role of qualitative change of phospholipid suggested by Bryszewska et al. [22] may be considered. In in

vitro insulin experiments no major change in lipid or protein composition may be expected. However, a change in phosphoinositide metabolism could take place, with the insulin acting on this metabolism [32]. It has been shown that these lipids are involved in membrane structure and maintenance of function [32–34], and a relationship between insulin-stimulated phosphoinositide turnover and Ca^{++} flux across the membrane known to be regulated by the membrane fluidity [35] was reported [33]. It is possible that insulin disturbs the phosphoinositide metabolism and then the membrane fluidity. The mechanism could be related to the protein kinase activities, key enzymes of the phosphoinositide metabolism [33] known to be regulated by insulin [36].

The link between insulin fixation to the erythrocyte receptors, modification of lipid bilayer and eventually of the protein cytoskeleton and modification of erythrocyte filtrability, remains to be elucidated.

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