Metabolism
Clinical and Experimental

VOL 48, NO 7 JULY 1999

Alterations in the Activity of Several Glycohydrolases in Red Blood Cell Membrane From Type 2 Diabetes Mellitus Patients

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The erythrocyte membrane in 71 patients with type 2 diabetes mellitus was assessed for glycohydrolase activity: *N*-acetyl-β-D-glucosaminidase, β-D-glucuronidase, α- and β-D-galactosidase, α- and β-D-glucosidase, α-D-mannosidase, and α-L-fucosidase. Only β-D-glucuronidase, α-D-glucosidase, and β-D-glucosidase showed markedly elevated levels with respect to the controls regardless of the presence of complications. Among the examined patients, those with good metabolic control (not yet submitted to any therapy) showed the same enzyme levels as the reference subjects, while the levels in patients with unsatisfactory metabolic control (treated with oral hypoglycemic and/or insulin) significantly differed from the control levels. For α-D-glucosidase and β-D-glucosidase, a correlation with glycemia and the parameters of metabolic control was also evidenced. Alterations of β-D-glucuronidase, α-D-glucosidase, and β-D-glucosidase were also ascertained in the plasma of the same diabetic patients according to the literature; each enzyme correlated with the other, either in plasma or in the erythrocyte membrane. This study shows a correlation between plasma and erythrocyte membrane levels for these three enzymes. The strict parallelism of the glycohydrolases in the two different compartments provides a profile of these enzymes in the pathology of diabetes.

INFORMATION on the various degradative enzymes in the erythrocyte plasma membrane, including the glycohydrolases[1-6] that were once thought to be exclusively located in lysosomes,[7] has become more readily available. It would seem that erythrocyte plasma membrane glycohydrolases are similar in several properties to glycohydrolases of lysosomal origin. It has been suggested that mature erythrocytes, despite lacking lysosomal particles, need these hydrolases to catabolize portions of their own membrane.[3]

Attention has been focused on erythrocyte plasma membrane glycohydrolases due to the possibility that these enzymes, given the appropriate conditions of pH and temperature, might become functional not only in degrading plasma glycoproteins but also in altering blood-group substances located at the membrane surface. As a consequence, the conformation of the membrane itself would change, probably with repercussions on the lifespan of the red blood cell.[3]

Based on the evidence that in diabetes mellitus, the erythrocyte plasma membrane undergoes alterations in its physicochemical, dynamic, conformational, and functional properties,[8-11] we performed a systematic study aimed at ascertaining the possible alterations of some of the glycohydrolases present in the membrane: *N*-acetyl-β-D-glucosaminidase ([β-GlcNAc-ase] EC 3.2.1.30), β-D-glucuronidase ([β-GlcA-ase] EC 3.2.1.31), β-D-galactosidase (EC 3.2.1.23), α-D-galactosidase (EC 3.2.1.22), α-D-glucosidase ([α-Glc-ase] EC 3.2.1.20), α-D-mannosidase (EC 3.2.1.24), α-L-fucosidase (EC 3.2.1.51), and β-D-glucosidase ([β-Glc-ase] EC 3.2.1.21).

Since the plasma levels of these enzymes are known to change in both type 1 and type 2 diabetes mellitus,[12-16] we decided to assay these enzymes in the erythrocyte membrane and the plasma of the same patients in an effort to reveal a correlation in the enzymatic activity of the two compartments.

SUBJECTS AND METHODS

Chemicals and Other Products

The commercial chemicals used in the study were the purest available. The water routinely used was freshly redistilled in a glass

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Submitted June 1, 1998; accepted March 14, 1999.

Supported by a grant (to A.L.) from the Italian Ministry of Education (40% contribution for 1995-1996).

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0026-0495/99/4808-0023$10.00/0

apparatus. 4-Methylumbelliferone (4-MU), purchased from Fluka (Bucks, Switzerland), was recrystallized from ethanol three times. 4-MU-glycosides were purchased from Melford (Suffolk, UK) and bovine serum albumin from Sigma (St Louis, MO).

Subjects

Seventy-one type 2 diabetic patients were selected from the diabetes outpatient Clinic of the Bassini Hospital of Cinisello Balsamo (Milan), their disease having been diagnosed according to World Health Organization criteria.[17] Some patients with good metabolic control (hemoglobin A_{1c} [HbA_{1c}] $\leq$7%) had no prior pharmacological treatment according to American Diabetes Association guidelines for diabetes treatment[18]; others were previously treated with oral hypoglycemic and/or insulin. All patients were on an appropriate diet. Thyroid, renal, and liver function tests were normal. Diabetic complications were evaluated through a medical history, clinical examination, fundoscopy, electrocardiogram, large-vessel Doppler ultrasonography, proteinuria and microalbuminuria, electromyography, and tests for the detection of autonomic neuropathy. Supine blood pressure was recorded, and hypertension was diagnosed for systolic blood pressure greater than 160 mm Hg and/or diastolic blood pressure greater than 90 mm Hg and/or in the presence of antihypertensive treatment. Height and weight were measured, and the body mass index (BMI) was calculated (kilograms per square meter). Seventy-one controls were selected from apparently healthy subjects who reported to the Laboratory of Clinical Chemistry and Microbiology of Bassini Hospital for scheduled hematological-chemical tests. None showed signs of metabolic, endocrinologic, or hemolytic diseases, hemoglobinopathies, or diseases of the major organs, and none had any family history of diabetes. Although they were appropriate for comparison to the diabetic subjects, the control group had a significant difference in mean age. It should be pointed out that no age- or body mass index–linked differences in enzyme activity have ever been verified neither in plasma[19] or in ghosts; nor has there ever been any correlation of ghost activities with the age and BMI in control or diabetic subjects. All the subjects provided informed consent for blood sampling. The main characteristics of the subjects are shown in Table 1.

Preparation and Storage of Plasma, Erythrocytes, and Erythrocyte Membranes (Ghosts)

Erythrocytes and plasma were prepared from heparinized venous blood (6 mL). After collection, the blood sample was immediately centrifuged for 15 minutes at 3,000 $\times$ *g*, the plasma was removed, ethylene glycol (30% vol/vol, final concentration) was added, and then it was stored at −20°C until assayed.[20] The buffy coat aspirated from the surface of the pellet was discarded, and the residual material was diluted (1:1 vol/vol) with phosphate buffer solution (PBS) at pH 7.4 and filtered with Leucostop 4LT-B filters (Baxter, Mizanolola, Moolena, Italy) to eliminate total platelet and leukocyte contaminants. The filtered solution, containing only erythrocytes, was centrifuged for 5 minutes at 1,200 $\times$ *g*, and the pellet was washed twice (1,200 $\times$ *g* for 5 minutes) with PBS at pH 7.4.

Erythrocyte membranes (ghosts) were prepared according to the method of Steck.[21] Briefly, erythrocyte lysis was performed by gently mixing (at 4°C for 20 minutes) cells diluted with 5 mmol/L PBS solution, pH 8.0 (1:40 vol/vol). The ghosts were centrifuged at 10,500 $\times$ *g* for 20 minutes, and the pellet was washed twice with a PBS solution, pH 8.0 (2.5 and 1.25 mmol/L, 1:40 vol/vol), and centrifuged under the same conditions.

Enzyme Assay

Glycohydrolase activity was determined fluorimetrically using the corresponding 4-methylumbelliferyl-glycosides as substrates. The determination on plasma was performed following the indications of Lombardo et al,[22] with a stable liquid material used for calibration purposes.[20] For ghost determination, the incubation mixture contained, in a final volume of 250 μL, 25 μL of the appropriate 50-mmol/L buffer system at the appropriate pH, 175 μL of the specific substrate aqueous solution, and 50 μL of the ghost preparation appropriately diluted with a saline solution. The mixtures were incubated in a shaker bath at 37°C for the established period. The reaction stoppage and fluorescence development were achieved by adding 750 μL of an alkaline solution (0.2 mol/L glycine-NaOH buffer containing 0.125 mol/L NaCl, pH 10.75). The control incubation mixtures (blanks) were set up using incubation mixtures without the ghost sample, which was incubated separately and added immediately before stopping the reaction. Enzyme activity is expressed as milliunits per liter of plasma or microunits per milligram of ghost proteins. Table 2 shows the conditions for optimized fluorimetric assay of all considered enzymes.

Other Methods

The protein content of ghost preparations was determined according to the method of Lowry et al,[23] using bovine serum albumin as the reference standard. Fasting venous plasma glucose and fructosamine (as 1-deoxy-1-morpholinofructose) were measured by colorimetric assay (Roche, Paris, France) and HbA_{1c} by immunoenzymatic assay (Roche).

Statistical Analysis

Tests of skewness and kurtosis showed significant differences from normal distribution, and nonparametric analysis techniques were used. The data were transformed into ranges, and the means were compared by Mann-Whitney and Wilcoxon tests. Multiple comparisons of the mean values were made by the Kruskal-Wallis test and, when necessary, adjusted with the Bonferroni correction; correlations were determined

Table 1. Main Characteristics of the Study Subjects

Group	No. of Subjects	Age (yr)	Duration of Disease (yr)	BMI (kg/m²)	Fasting Blood Glucose (mg/100 mL)	HbA_{1c} (%)	Fructosamine (μmol/100 mL)
Controls	71	46 ± 12		26.0 ± 0.05	89 ± 11	4.7 ± 0.7	228 ± 31
Total diabetic patients	71	60 ± 8	10.8 ± 7.9*†	30.2 ± 5.7	177 ± 58	7.4 ± 1.5*	336 ± 62*
Diabetics with complications	37	60 ± 7	12.8 ± 7.5*	30.5 ± 5.4	194 ± 68*	7.8 ± 1.7*	344 ± 67*
Diabetics without complications	34	60 ± 8	8.1 ± 7.1†	29.9 ± 6.1	154 ± 25	6.9 ± 1.0*	324 ± 54*
Diabetics without any therapy	8	60 ± 7	3.8 ± 3.9†	28.9 ± 4.1	140 ± 21	6.2 ± 0.4	278 ± 31
Diabetics treated with oral hypoglycemics	45	59 ± 8	9.6 ± 7.3*†	30.6 ± 6.4	177 ± 54	7.3 ± 1.4*	328 ± 42*
Diabetics treated with oral hypoglycemics and insulin	18	63 ± 6	16.9 ± 6.8*	29.9 ± 4.1	193 ± 73	8.2 ± 1.5*	381 ± 85*

NOTE. Values are the mean ± SD. HbA_{1c} reference interval, 4.0%-5.8%; fructosamine reference interval, 100-278 μmol/100 mL. For all considered parameters, all groups of diabetic subjects differed significantly from the controls ($P < .01$).

*$P < .05$ *v* diabetics without any therapy.

†$P < .05$ *v* diabetics treated with oral hypoglycemics and insulin.

Table 2. Conditions for Maximal Activity for the Fluorimetric Assay of Glycohydrolases in the Erythrocyte Plasma Membrane

Enzyme	pH for Maximal Activity*	Apparent Saturating Substrate Concentration (mmol/L)	Time Course at 37°C: Linear to (min)	Protein Range for Linear V/E Relationship (μg)
N-acetyl-β-D-glucosaminidase	4.3	4.0	150	6-65
β-D-glucuronidase	4.8	8.0	90	1-90
β-D-galactosidase	4.4	1.6	180	20-90
α-D-galactosidase	4.3	4.0	180	20-90
α-L-fucosidase	5.0	0.3	150	20-90
α-D-glucosidase	5.8	4.0	120	10-100
β-D-glucosidase	5.0	4.0	180	20-90
α-D-mannosidase	5.8	4.0	180	20-90

*Buffer providing maximal activity: citrate-phosphate, final concentration 50 mmol/L.

by the Spearman method. For all analyses, the SPSS/PC package was used.[24]

RESULTS

Table 3 shows the three glycohydrolases with relevant and statistically significantly increased ghost and plasma levels in type 2 diabetic patients with respect to controls. As stated in the Methods, the enzymatic activity is in no way influenced by the age of either group. There are significant differences in activity regardless of the presence or absence of complications. In the group of patients with good metabolic control and therefore not yet submitted to any pharmacological treatment, the enzymatic levels did not differ from those in the controls, whereas there was a significant difference for the patient groups that, having unsatisfactory metabolic control, were undergoing pharmacological treatment (hypoglycemics and/or insulin). The exception was β-GlcA-ase: the levels in patients under insulin therapy were indistinguishable from those in the controls and untreated patients (Table 3).

α-Glc-ase and β-Glc-ase, but not β-GlcA-ase, correlate with glycemia and the parameters of metabolic control (for α- and β-Glc-ase, respectively: with glycemia, $r = .41$, $P < .0001$ and $r = .45$, $P < .0001$; with HbA_{1c}, $r = .33$, $P < .002$ and $r = .52$, $P < .0001$; and with serum glycated proteins, $r = .25$, $P < .002$ and $r = .45$, $P < .0001$). Each of the erythrocyte membrane glycohydrolases mentioned correlates with its corresponding plasma form ($r = .25$, $P < .002$ for β-GlcA-ase; $r = .28$, $P < .0008$ for α-Glc-ase, and $r = .33$, $P < .0001$ for β-Glc-ase). None showed any correlation with the BMI, whereas each correlated with the other two both in the plasma and in the ghost.

Some comment needs to be made on β-GlcNAc-ase: we observed a significant statistical difference ($P < .05$) between patients with complications (32.8 ± 22.4) and without (24.2 ± 10.6). A more specific investigation revealed a greater increase in this enzyme in retinopathic patients, in ghosts and in plasma (39.7 ± 28.8, $P < .05$ and 35,850 ± 12,570, $P < .01$, respectively), compared with controls (27.5 ± 16.5 and 26,170 ± 6,430, respectively), with a positive and highly significant correlation coefficient ($r = .30$, $P < .01$). For the other enzymes, there was no difference among the specific complications.

DISCUSSION

Among the recognized enzymes present in the erythrocyte membrane are the glycohydrolases,[3,5,6] whose presence raises many questions about their origin and their hypothetical functions.

Glycohydrolases, once thought to be located exclusively in lysosomes,[7] have been extensively studied in the cells of different tissues and body fluids for their diagnostic significance, not only for some of the rare syndromes derived from inborn lysosomal pathology[25,26] but also for a number of acquired diseases, including diabetes mellitus.[12-16,27,28]

It has been pointed out that in the diabetic condition some of these enzymes show a correlation between the enzyme plasma level and the degree of metabolic compensation.[16,29] The glycohydrolases of the erythrocyte plasma membrane have been studied only in some hematological pathologies,[30,31] and no information is available in the literature about their involvement in diabetes mellitus type 1 or type 2.

Our investigation in type 2 diabetic patients clearly shows that some of the enzymes considered are involved in diabetic pathology. It is likely that the diabetic condition itself is responsible for the observed increase, given that the enzyme levels occur regardless of the presence of complications.

Table 3. Activity of Glycohydrolases in the Erythrocyte Plasma Membrane (ghost) and in Plasma of Type 2 Diabetic Patients With Reference to the Absence or Presence of Complications and to the Therapy

Group	No. of Subjects	β-D-Glucuronidase	α-D-Glucosidase	β-D-Glucosidase
Ghost				
Controls	71	504 ± 140	294 ± 76	15 ± 5.5
Diabetic patients				
All diabetics	71	626 ± 236†	364 ± 124†	27 ± 11.6†
Without complications	34	620 ± 225*	350 ± 130†	25 ± 9.8*
With complications	37	640 ± 250*	380 ± 120*	29 ± 13*
Without any therapy	8	580 ± 140	330 ± 80	21 ± 6.6
With oral hypoglycemic	45	670 ± 274*	370 ± 150*	27 ± 11*
With insulin and oral hypoglycemic	18	550 ± 125	350 ± 70†	30 ± 15*
Plasma				
Controls	71	2,092 ± 1,298	124 ± 23	4.0 ± 3.3
Diabetic patients	71	3,934 ± 2,767†	157 ± 47†	6.0 ± 5.4*

NOTE. Enzyme activities are expressed as μ/U/mg protein for ghosts and mU/L for plasma. Values are the mean ± SD.

*$P < .01$ *v* controls.

†$P < .001$ *v* controls.

It can be postulated that insulin itself acts specifically, perhaps in each case differently, on the activity, synthesis, and turnover of the different enzymes before the amelioration of metabolic control, as already demonstrated for other erythrocyte membrane enzymes.[32]

The data obtained show a parallelism between some glycohydrolases in plasma and the same enzymes in the erythrocyte membrane compartment and raise the question of a connection between them. Such parallelism could be explained by the alterations that occur in the erythrocyte membrane of diabetic subjects. In fact, in the diabetic condition, erythrocyte membrane proteins undergo conformational changes, probably due to nonenzymatic glycosylation, with repercussions on the physicochemical properties of the membrane itself.[9,10] It also has been highlighted that the integral membrane proteins tend to modify their interaction with the lipid environment.[10,11] Furthermore, membrane fluidity also undergoes change, which could be responsible not only for variations in the activity and properties of some enzymatic proteins but also for the process of vesicle formation.[33] Such a process provides an important means whereby protein sorting can occur, allowing the selective retention of certain proteins and the removal of others according to the way the proteins are anchored to the membrane itself.

Considering the different behavior of the glycohydrolases in the erythrocyte membrane in diabetes pathology, it can be hypothesized that such enzymes are associated in different ways to the same membrane. Some could be anchored through a glycan phosphoinositide bridge, as demonstrated for the sialidase,[6] and others could be weakly connected to the outer surface of the membrane and hence easily removed following a variation of the dynamic properties of the membrane. Still others could be wholly connected to the membrane itself. It cannot even be excluded that these enzymes may belong to different microdomains of the erythrocyte membrane.

In conclusion, the simultaneous alteration shown in the enzymatic levels of the two different compartments defines a profile of these enzymes in diabetes pathology, opening new perspectives into its study. Experiments are already in progress to ascertain the precise orientation and anchoring modality of glycohydrolases to the erythrocyte membrane.

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