Plasma Glycohydrolase Levels in Patients With Type 1 Diabetes at Onset and in Subjects Undergoing an Intravenous Glucose Tolerance Test

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The effect of hyperglycemia and insulin deficiency on the plasma level of lysosomal glycohydrolases, namely *N*-acetyl-β-D-glucosaminidase, β-D-glucuronidase, α-D-galactosidase, and α-D-glucosidase, was investigated. Two patient groups were assessed: (1) 28 children with type 1 diabetes at onset (fasting blood glucose, 444 ± 154 mg/100 mL; hemoglobin A_{1c}, 11.9% ± 2.4%; symptom duration, 15.9 ± 8 days; and absence of complications), (2) 14 adult subjects undergoing an intravenous glucose tolerance test (IVGTT), consisting of 8 non-obese subjects (body mass index, 26 ± 0.04 kg/m^2; fasting blood glucose, 82 ± 13 mg/100 mL; blood insulin, 6 ± 0.04 mU/L) and 6 obese subjects (fasting blood glucose, 97 ± 3.5 mg/100 mL; blood insulin, 27 ± 6 mU/L, with normal oral glucose tolerance test). Enzyme activity was determined with the fluorimetric method. The mean level of all evaluated enzymes was significantly increased in patients with type 1 diabetes at diagnosis compared with normal controls. Increased enzyme levels were also detected in the group of adults undergoing an IVGTT in whom hyperglycemia was accompanied by insulin resistance (ie, obese subjects). Glycohydrolase abnormalities appear to be related to insulin deficiency rather than hyperglycemia. Lysosomal apparatus abnormalities seem to be an inherent feature of diabetes that is present at disease onset. The possible role of insulin in the regulation of plasma glycohydrolase levels is discussed.

SEVERAL INDEPENDENT STUDIES have established a direct involvement of the lysosomal apparatus in diabetes.[1-9] Increased plasma levels of several lysosomal glycohydrolases have been observed in subjects with both insulin-dependent and adult-type diabetes mellitus. This increase parallels the degree of metabolic derangement, and enzyme values tend to normalize with disease control.[7,8] Erythrocyte plasma membrane glycohydrolases have also recently been shown to be potentially involved in the biology of the diabetic disease process.[9] The concordant behavior of these enzymes in different cellular compartments is an intriguing observation that has suggested several hypotheses.

Despite an extensive literature on the subject, the biochemical basis of the glycohydrolase abnormalities associated with diabetes remains poorly understood. Is it directly related to the hyperglycemia itself or is it rather a consequence of insulin deficiency? Is it detectable in the absence of complications? Could the different complications themselves, even if subclinical, be responsible? How can the simultaneous involvement of glycohydrolases in different cellular compartments be explained?

To try to address these issues, the plasma levels of some of the glycohydrolases were determined in recent-onset type 1 diabetes patients. The enzyme evaluations were performed at diagnosis, ie, prior to the institution of any form of therapy, and thus in the context of marked hyperglycemia, insulin deficiency, and substantial metabolic derangement. This situation has the additional advantage of avoiding the potentially confounding effects deriving from medium- and long-term complications, whether clinically evident or subclinical. Furthermore, the levels of some of the glycohydrolases have also been evaluated in nondiabetic subjects with normal or altered insulin secretion, in whom marked hyperglycemia was induced by an intravenous glucose tolerance test (IVGTT).

SUBJECTS AND METHODS

Chemicals and Other Products

The commercial chemicals were of the highest available purity. The water used was freshly redistilled in a glass apparatus. 4-Methylumbelliferone (4-MU), purchased from Fluka (Bucks, Switzerland), was recrystallized 3 times from ethanol. 4-MU-glycosides were purchased from Melford (Suffolk, UK).

Patient Population

Subjects with type 1 diabetes mellitus at onset. Since December 1997, we have analyzed 28 subjects. Their main characteristics (mean ± SD and range) were as follows: age, 7.9 ± 3.6 years (2 to 15); fasting blood glucose, 444 ± 154 mg/100 mL (180 to 700); hemoglobin A_{1c}, 11.9% ± 2.4% (6.8% to 17.7%); fructosamine as 1-deoxy-1-morpholino-fructose, 640 ± 142 mg/100 mL (309 to 899); and onset of initial symptoms, 15.9 ± 8 days (1.5 to 40) prior to evaluation. Fundus examination and microalbuminuria were negative in all patients. The diagnosis of diabetes was made at the Department of Pediatrics of the University of Padua (Italy) according to World Health Organization criteria.[10] The control population included 28 apparently healthy subjects with an age of 8.0 ± 5.9 years (range, 2 to 19), none of whom had a positive family history for diabetes. These subjects were seen at the Department of Pediatrics for scheduled laboratory evaluations.

Subjects undergoing an IVGTT. Fourteen adult subjects selected by the Department of Gerontological Research of the Center of Biochemistry of Ancona (Italy) underwent an IVGTT according to the method of Best et al[11] (0.33 g glucose/kg body weight for 3 minutes). Eight healthy control subjects (age, 38 ± 6 years; body mass index, 26 ± 0.04 kg/m^2; fasting blood glucose, 82 ± 13 mg/100 mL; blood insulin, 6 ± 2.4 mU/L) and 6 obese subjects with a normal oral glucose tolerance test (age, 40 ± 8 years; body mass index, 43 ± 12 kg/m^2; fasting blood

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glucose, 97 ± 3.5 mg/100 mL; blood insulin, 27 ± 6 mU/L) were evaluated. One obese subject with type 2 diabetes treated for 1 year with acarbose 120 mg/d (Glucoby; Bayer, Leverkusen, Germany) was also evaluated. All of these data are expressed as the mean ±1 SD.

Two days prior to the IVGTT, all subjects underwent an intravenous infusion of normal saline at the same volume and rate as the IVGTT. Blood samples were obtained immediately before glucose or saline infusion and at 5, 15, 30, and 60 minutes during the infusion.

The entire protocol was approved by the local bioethical committee. All specimens collected were negative for human immunodeficiency virus and hepatitis virus antibodies, and appropriate handling precautions were implemented. All examined subjects or their legal guardians provided informed consent for blood sampling.

Plasma Collection and Storage

Plasma was obtained immediately after blood sampling by centrifugation (15 minutes at 3,000× *g*) of blood (2 mL) anticoagulated with sodium citrate (0.2 mL 0.11-mol/L sodium citrate solution). Ethylene glycol (30% vol/vol, final concentration) was added to the plasma samples immediately after collection to preserve enzyme activity.[12] The samples were stored at −20°C until analysis.

Enzyme Assay

N-acetyl-β-D-glucosaminidase (EC 3.2.1.30), β-D-glucuronidase (EC 3.2.1.31), α-D-galactosidase (EC 3.2.1.22), and α-D-glucosidase (EC 3.2.1.20) were evaluated in patients with type 1 diabetes at onset, while only the enzymes known to be sensitive to short-term glycemic/insulinemic abnormalities,[8] ie, *N*-acetyl-β-D-glucosaminidase and β-D-glucuronidase, were evaluated in patients undergoing an IVGTT. Enzyme activity was measured fluorimetrically using 4-MU-glycosides as substrates. Plasma determinations were performed as previously reported[13] with a stable liquid material used for calibration purposes.[12] Glycohydrolase activities are expressed as microunits per milliliter of plasma.

Statistical Analysis

Parametric analysis was used since skewness and kurtosis testing was consistent with a normal distribution of the data. The comparison of the mean values and regression analysis were performed by ANOVA according to Snedecor and Cochran.[14] The SPSS/PC software package was used.[15]

RESULTS

Glycohydrolase Plasma Levels in Subjects at Type 1 Diabetes Onset

Table 1 summarizes the results of enzyme assays for the plasma of patients at type 1 diabetes onset. The mean levels of all glycohydrolases evaluated are significantly higher in diabetic subjects than in controls. Among the enzymes considered, the lowest increase was exhibited by *N*-acetyl-β-D-glucosaminidase (1.3-fold) and the highest by β-D-glucuronidase (4.8-fold); the latter was also the only enzyme to exhibit a positive correlation with age both in diabetic subjects ($P < .03$, $r = .41$) and in controls ($P < .05$, $r = .38$).

Table 1. Plasma Activity of Selected Glycohydrolases in Type 1 Diabetic Patients at Onset

	Control Subjects (n = 28)		Diabetic Patients (n = 28)	
Glycohydrolase	Mean ± SD	Range	Mean ± SD	Range
N-acetyl-β-D-glucosaminidase	22.7 ± 5.1	16-35	29.4 ± 9.7*	15-56
β-D-Glucuronidase	1.6 ± 0.4	0.9-2.4	7.8 ± 3.6†	1.8-15.5
α-D-Galactosidase	0.15 ± 0.03	0.09-0.19	0.31 ± 0.25†	0.03-1.2
α-D-Glucosidase	0.13 ± 0.02	0.10-0.17	0.21 ± 0.09†	0.07-0.48

NOTE. Enzymatic activity is expressed as mU/mL plasma.
*$P < .01$, †$P < .001$: diabetic patients *v* control subjects.

Glycohydrolase Plasma Levels During IVGTT

The effect of blood glucose and insulin on the plasma level of *N*-acetyl-β-D-glucosaminidase and β-D-glucuronidase was evaluated in parallel in subjects undergoing an IVGTT. Before the IVGTT, all evaluated subjects were normoglycemic. Eight of these individuals had normal insulin production (control subjects) and 6 were markedly obese and had high insulin values (obese nondiabetic subjects). Figure 1 depicts the behavior of the above-mentioned enzymes, blood insulin, and glucose values both before and after glucose infusion. The trend for the enzyme levels in the obese diabetic subject under acarbose treatment is also shown. Five minutes after IVGTT initiation, glycemia in all evaluated subjects increased to a maximum of 280 mg/dL, returning to near-baseline values within 60 minutes in the controls, while the obese subjects remained persistently hyperglycemic (about 200 mg/dL). The obese diabetic subject treated with acarbose had intermediate glycemia values.

Peak insulin values were reached 5 minutes after the IVGTT initiation in control subjects, while in obese subjects these values were reached after 15 minutes. In the latter, hormone levels remained persistently elevated throughout the observation period, as opposed to the controls, in whom the values returned toward baseline levels. In the patient treated with acarbose, the blood insulin trend closely approximates, with slightly higher values, that of the normal controls.

The activity of both glycohydrolases decreased by about 20% after 5 minutes in normal controls, remaining nearly unchanged throughout the observation period, while in obese subjects, the enzyme levels at the same time points increased by 30% and 10% for *N*-acetyl-β-D-glucosaminidase and β-D-glucuronidase, respectively. Moreover, the pattern of the enzyme levels differed significantly between the 2 groups (Fig 1). In the patient treated with acarbose, the time course of both enzymes is superimposable to that of the controls, with a decrease for *N*-acetyl-β-D-glucosaminidase after 5 minutes. No change in the plasma level of the above enzymes was observed during control experiments in which all subjects underwent intravenous saline infusion under the same conditions as the IVGTT.

For *N*-acetyl-β-D-glucosaminidase, the specific activity values were similar between control and obese subjects, but for β-D-glucuronidase, the obese group had significantly higher values compared with the controls (3.1 ± 0.5 and 1.4 ± 0.2 mU/mL, respectively, $P < .001$), confirming the previous observation that plasma levels of this enzyme are influenced by the body mass index.[16]

DISCUSSION

The involvement of the lysosomal apparatus in diabetes was reported for the first time in 1965 by Woollen and Turner,[17] and many studies since then have further characterized this phenomenon.[1-8] The increase in glycohydrolases is a well-recognized occurrence in diabetic patients and is found even in the absence

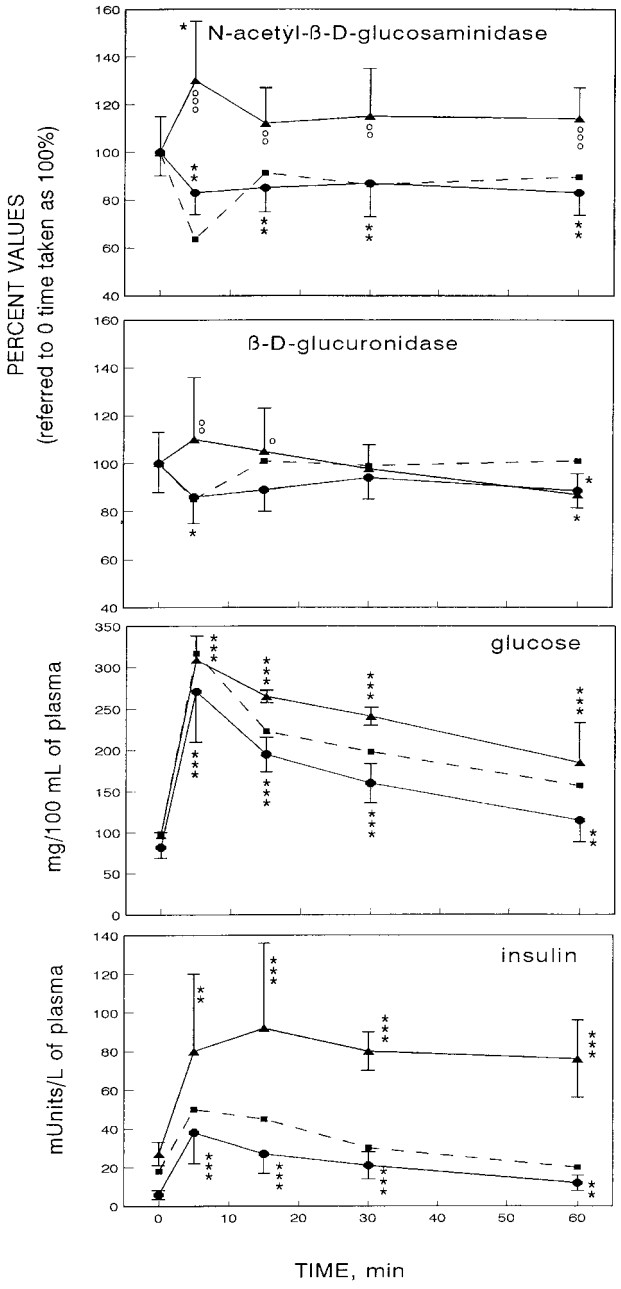

Fig 1. Plasma level of *N*-acetyl-β-D-glucosaminidase and β-D-glucuronidase after an IVGTT, expressed as percent values (mean ± SD) relative to the activity obtained before starting the IVGTT (time 0) defined as 100%. Glucose and insulin blood levels before and after the IVGTT are also reported. ●, Control subjects; ▲, obese subjects; ■, obese diabetic subject under acarbose therapy. *$P < .05$, **$P < .01$, ***$P < .001$ *v* time 0; °$P < .05$, °°$P < .01$, °°°$P < .001$ obese *v* control subjects.

of clinically evident complications.[7,8] More recently, erythrocyte membrane glycohydrolase involvement has also been reported in this context.[9]

Several hypotheses have been formulated to explain the nature of the increase in the plasma level of these enzymes. According to some investigators, the alteration of the glycidic component of glycoprotein that occurs during the disease could modify their distribution between the intracellular and extracellular environment.[18,19] It has also been proposed that the diabetic condition itself and the associated long-lasting metabolic derangement, in addition to facilitating the release of lysosomal enzymes in the extracellular fluid, could interfere with the mechanisms that control their half-life.[20,21]

To our knowledge, no study to date has attempted to clarify whether these alterations are the consequence of the hyperglycemic condition or the lack of insulin or are related to the presence of complications, even if subclinical.

To verify some of these hypotheses, we tested a well-defined and ''clean'' diabetic population consisting of insulin-dependent subjects at diagnosis prior to any treatment and thus characterized by marked hyperglycemia, insulin deficiency, and an absence of late complications. Although the metabolic deterioration was present for only a very short period, a significant increase in plasma enzyme levels was observed. The increase was particularly elevated for β-D-glucuronidase, confirming a previous report[8] indicating that this enzyme is more sensitive for monitoring the metabolic compensation status. Since we studied a group of patients at diagnosis, we can exclude the presence of complications as having any significant role in these enzyme elevations.

To clarify whether the observed increases could be ascribed to acute hyperglycemia per se or to prolonged insulin action deficiency, glycohydrolases were also studied in nondiabetic subjects with normal and abnormal insulin secretion undergoing an IVGTT. While in control subjects the plasma levels of both enzymes were slightly decreased, patients with insulin resistance had increased levels, suggesting normal insulin action as the most important factor responsible for the maintenance of normal glycohydrolase plasma levels.

Interestingly, an obese diabetic subject treated with acarbose and maintaining normal blood glucose who underwent an IVGTT with a normal insulin response had enzyme levels superimposable to the controls. The role of insulin in maintaining normal plasma glycohydrolase levels is further supported by recent findings on the mechanism of action of acarbose. In fact, it has been shown that this pseudotetrasaccharide, in addition to its well-known inhibitory effect on intestinal α-glucosidase, localizes at the level of the pancreatic islet lysosomes regulating glucose-induced insulin secretion.[22]

The effect of insulin in maintaining low glycohydrolase plasma levels might be explained on the basis of the potential of this hormone to increase, in some cell types, the translation to the plasma membrane of cytoplasmatic vesicles containing both the glucose transporter and the receptor for mannose-6-phosphate.[23] This latter receptor is well known to be involved in the specific release and reuptake of glycohydrolases by the cell.[24,25]

The increase of lysosomal enzymes in the plasma of diabetic subjects could also be explained on the basis of the results of cytogenetic studies performed on lymphocytes in culture.[26,27] It has been shown that in diabetes, these cells undergo alterations of the lysosomal apparatus resulting in extralysosomal localization of some enzymes. These enzymes could be more easily secreted from cells, and the insulin deficit, with the resulting decrease in mannose-6-phosphate receptor, would interfere with their turnover between extracellular and intracellular compartments. The impairment of lysosomal function observed

in diabetes may concur with hyperglycemia to predispose these patients to infection.[28]

The recently observed parallel increase of the same glycohydrolases at the erythrocyte plasma membrane level in diabetic subjects[9] suggests that the involvement of the enzymes may be part of the disease process. However, since these enzymes are weakly anchored to the erythrocyte membrane (Goi and Massaccesi, personal unpublished observation, April 1998), it is possible that their increased plasma concentration may facilitate their adhesion to the red blood cell membrane. In addition, the conformational and fluidity alterations of the erythrocyte membrane associated with diabetes[29,30] may concur with the insulin deficit to alter vesicle formation.[31,32] As a consequence, the different glycohydrolases present in the plasma might have difficulty reaching the intracellular milieu.

In conclusion, our study strongly suggests that lysosomal abnormalities resulting in increased plasma levels of the enzymes they contain are a feature of the diabetic disease process arising at disease onset and probably related to insulin deficiency.

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