Glutamate Decarboxylase Autoimmunity and Growth Hormone Secretion in Type I Diabetes Mellitus

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Insulin-dependent (type I) diabetic patients are known to have an exaggerated growth hormone (GH) response to GH-releasing hormone (GHRH), which is hypothesized to be due to a decrease in somatostatin tone. The aim of the study was to ascertain the influence of the presence and activity of the autoimmune process involving a key enzyme (glutamic acid decarboxylase [GAD]) in the synthetic pathway of a neurotransmitter regulating somatostatin secretion, ie, gamma-aminobutyric acid (GABA), on the GH response to GHRH alone or combined with an acetylcholinesterase inhibitor, pyridostigmine (PD), in patients with type I diabetes mellitus. Twenty non-obese type I diabetic patients and 17 normal subjects underwent an intravenous (IV) injection of 100 μg GHRH(1-29)NH_2. Twelve of 20 diabetic subjects and all of the control subjects also underwent a second experimental procedure, administration of 120 mg oral PD 60 minutes before IV injection of 100 μg GHRH. Diabetic subjects with serum GAD antibody (GADA) levels more than 3 U (n = 10) showed significantly higher serum GH levels after GHRH injection as compared both with diabetic patients with GADA less than 3 U (n = 10) and with normal controls, whether expressed as absolute or peak values. GH peaks after GHRH were significantly (r_s = .46, $P < .05$) correlated with the level of GADA in the whole population of type I diabetic subjects studied. PD significantly enhanced the GH response to GHRH, in terms of both absolute and peak values, in patients without GADA (n = 6) and in normal subjects. On the contrary, PD failed to enhance the GH response to GHRH in diabetic patients with GADA (n = 6). Our findings suggest that autoimmunity may play a key role in determining the exaggerated GH response to GHRH in type I diabetes mellitus. The mechanism underlying this effect is hypothesized to be the production of antibodies to GAD, a key enzyme in the synthesis of GABA, and in turn a reduced GABAergic stimulatory tone on somatostatin production at the hypothalamic level.

SEVERAL EXPERIMENTS suggested the existence of a causal relationship between growth hormone (GH) and the development of diabetic microvascular disease.[1,2] Insulin-dependent (type I) diabetic patients are known to have abnormalities in GH secretion. Initial studies showed elevated 24-hour GH secretion in type I diabetic patients.[3,4] Recently, GH peak frequency and interpeak GH levels have both been found to be elevated in these patients.[5] Exaggerated GH responses to exogenous GH-releasing hormone (GHRH) have also been reported.[6-8] The possible hypothalamic origin of the defect leading to this GH hyperresponse to GHRH has been investigated using the acetylcholinesterase inhibitor, pyridostigmine (PD), which is hypothesized to decrease somatostatin tone.[9] These studies have demonstrated that diabetic patients with an exaggerated GH response to GHRH fail to respond to PD,[8,10] suggesting that the exaggerated response is due to a spontaneous decrease in somatostatin tone in type I diabetes.[11]

The mechanisms underlying the changes in hypothalamic somatostatin in type I diabetes remain to be explained. Type I diabetes is well recognized as an autoimmune disease.[12] Therefore, it could be hypothesized that in type I diabetic patients, decreased somatostatin tone may reflect damage induced by an autoimmune process either directly on somatostatin neurons or indirectly on neurotransmitters regulating somatostatin release. Interestingly, glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of gamma-aminobutyric acid (GABA),[13] has been identified as a major autoantigen of type I diabetes, being a target of both humoral and cell-mediated autoimmunity.[14-16] Experimental evidence suggests that GABAergic pathways may have an inhibitory role in the regulation of GH secretion.[17,18] Moreover, it has been hypothesized that these GABAergic influences may be mediated via either an enhancement of hypothalamic somatostatin[19] or a decrease in endogenous GHRH.[20]

The aim of this study was to test the hypothesis of a possible influence of GAD autoimmunity on GH secretion in type I diabetes mellitus. Therefore, we investigated GH secretion in basal conditions and after GHRH and PD stimulation in type I diabetic patients characterized by the presence of circulating antibodies to GAD (GADA) and other disease-specific markers of autoimmunity, such as the classic islet cell antibodies (ICA)[21] and antibodies to the recently identified protein tyrosine phosphatase–like IA2 (previously denominated 37/40K) antigen.[22]

SUBJECTS AND METHODS

Patients and Controls

Informed consent was obtained before each test from all patients and controls. Twenty non-obese type I diabetic patients (plasma C-peptide level 6 minutes after 1 mg intravenous [IV] glucagon, <66.2 pmol/L) with normal liver and kidney function were studied. Type I diabetes was defined on the basis of clinical diagnosis. Patients had a mean age of 34 ± 2.9 years, with a mean duration of disease of 11.1 ± 2.2 years and a body mass index (BMI) of 22.6 ± 0.5 kg/m^2. None were taking any medications other than insulin (mixture of short- and intermediate-acting insulin by subcutaneous injection twice daily, before breakfast [6:45 AM] and dinner [6:45 PM]; mean daily insulin dosage, 0.62 ± 0.07 U/kg). Type I diabetic patients were on an appropriate diet (30 kcal/kg/d: 50% carbohydrate, 35% lipid, and 15% protein). Seventeen

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normal subjects matched with the diabetic patients for sex, age, and BMI served as controls (Table 1). All of the premenopausal women in the study were tested during the early follicular phase (two diabetic patients with GADA were menopausal). Background retinopathy, determined with retinal fluorescin angiography,[19] was present in five patients (three with GADA and two without GADA), and proliferative retinopathy was found in one patient with GADA. Microalbuminuria[20] was detected in six patients with GADA.

Methods

The study, which was approved by the local Ethics Committee, consisted of two experiments separated by intervals of at least 72 hours and performed according to a randomized single-blind protocol. On the day before each test, diabetic patients received their last insulin dose at 6:45 PM, and no further insulin was administered until the test ended the following day. After an overnight fast, each subject was admitted to the Clinical Research Unit at 8:00 AM. Patients rested in a recumbent position. A 30-minute period was allowed for stabilization after venipuncture. The catheter was kept patent by a slow saline infusion. Each subject underwent a bolus IV injection of GHRH(1-29)NH_2 (Geref, Serono, Italy) 100 μg in 2 mL saline. Twelve of 20 diabetic subjects (six with GADA: two men and four women; age, 36.5 ± 7.6 years; BMI, 22.7 ± 1.1 kg/m^2; hemoglobin A_{1c} [HbA_{1c}], 9.27% ± 0.6%; and six without GADA: five men and one woman; age 25.4 ± 4 years; BMI, 21.6 ± 1.2 kg/m^2; HbA_{1c}, 9.46% ± 1.2%) and all of the control subjects also underwent a second experimental session after at least 72 hours: oral PD (Mestinon; Roche, Basel, Switzerland) 120 mg was administered 60 minutes before IV injection of GHRH 100 μg.

Blood samples were taken for GH and glucose determinations at −75, −60 (time of oral PD administration), −45, −30, −15, 0 (time of IV GHRH), 15, 30, 45, 60, 90, and 120 minutes. GH secretory responses to the stimuli were expressed either as absolute values (micrograms per liter) or as peak levels (micrograms per liter).

Assays

Biochemistry and hormones. Plasma glucose level was measured by a glucose oxidase method (Beckman II analyzer; Beckman Instruments, Palo Alto, CA). HbA_{1c} level was measured by a chromatographic method (Bio-Rad, Milan, Italy; normal range, 3% to 6%). A commercial immunoradiometric kit (Allegro HGH; Nichols Institute, San Juan Capistrano, CA; intraassay coefficient of variation, ±4%; interassay coefficient of variation, ±7%; sensitivity, 0.06 μg/L) was used to measure circulating GH levels. Serum C-peptide levels (Byk-Gulden, Dietzenbach, Germany) and microalbuminuria (Sclavo, Siena, Italy) were measured with commercial radioimmunoassay kits. All samples from the same patient were assayed together in duplicate.

GADA and IA2 antibodies. These antibody levels were measured by immunoprecipitation of in vitro–translated ^{35}S-methionine–labeled recombinant GAD_{65} and IA2 molecules, as previously described.[22,23] Results were converted to arbitrary units by extrapolation from a curve observed for each assay using a local standard designated as 100 U, tested undiluted and at 1:4, 1:16, 1:32, and 1:64 dilutions in a negative serum. The threshold for positivity, selected as the upper first centile of observations in 83 normal controls, was 3 U for both GADA and IA2 antibodies. Using this threshold level, only 1% of control subjects have been previously reported to show positivity for GADA.[23]

ICA. These antibodies were assayed in undiluted sera by indirect immunofluorescence on a 4-μm cryostat section of blood group O human pancreas as previously described.[21] Positive samples were subsequently titrated and quantified in Juvenile Diabetes Foundation (JDF) units.[24] The threshold for positivity was selected at 5 JDF U.

Statistical Analysis

Results are expressed as the mean ± SEM. Statistical comparison of absolute and peak GH values was performed with the nonparametric technique of Wilcoxon for paired (within-group) and unpaired (between-group) data. The Spearman rank correlation test was used to correlate GH peaks and GADA levels. A standard two-tailed Student's *t* test for paired or unpaired data was used for other calculations. *P* less than .05 was considered statistically significant.

RESULTS

GADA were detected in 10, ICA in nine, and IA2 antibodies in eight of 20 type I diabetic patients. The prevalence of GADA in our diabetic population was similar to that (~60%) previously reported in large groups of type I diabetic patients.[25] GADA were found in association with both ICA and IA2 antibodies in four patients, with ICA only in two, and with IA2 antibodies only in one. Seven patients had both ICA and IA2 antibodies.

No differences in age, sex, BMI, disease duration, insulin dose, and HbA_{1c}, ICA, and IA2 antibody levels were observed between patients with or without GADA. On the contrary, patients within the first year from diagnosis had a higher frequency of either ICA or IA2 antibodies than those with a longer duration of the disease.

In the whole population of type I diabetic patients, a slightly but nonsignificantly higher baseline GH level (4.25 ± 1.5 μg/L) and a significantly ($P < .05$) higher GH peak after GHRH (27.4 ± 2.8 μg/L) was observed as compared with the values in normal controls (baseline GH, 0.9 ± 0.2 μg/L; GH peak after GHRH, 16.4 ± 2.62 μg/L). Conversely, nonsignificant differences in GH responses to GHRH + PD were observed between

Table 1. Clinical Characteristics of the Subjects

Group	Sex	Age (yr)	BMI (kg/m^2)	Diabetes Duration (yr)	HbA_{1c} (%)	Insulin Dose (U/kg/d)	Basal GH (μg/L)	GADA (U)	ICA (JDFU)	IA2 (U)
Diabetic patients										
GAD⁺	5M/5F	39.4 ± 4.6 (19-64)	23.1 ± 0.8	11.3 ± 3.2 (1-34)	8.8 ± 0.4	0.68 ± 0.07	6.6 ± 2.5	39.4 ± 14.8* (3.3-130)	9.8 ± 2	13.3 ± 7
GAD⁻	9M/1F	30.2 ± 2.9 (16-46)	22 ± 0.8	11 ± 3.2 (1-28)	8.4 ± 0.7	0.56 ± 0.07	1.9 ± 0.6	1.5 ± 0.2 (0.4-2.5)	4.2 ± 1	2.8 ± 1
Normal subjects	13M/4F	26.4 ± 1.1 (23-36)	22.5 ± 1	—	—	—	0.9 ± 0.2	—	—	—

NOTE. Diabetic patients were subgrouped according to the level of circulating GADA: GAD⁺, GADA >3 U; GAD⁻, GADA <3 U.
Abbreviations: M, male; F, female.
*$P < .05$ *v* diabetic patients without GADA.

the whole population of type I diabetic patients and the normal subjects.

Type I diabetic patients with GADA greater than 3 U showed slightly but nonsignificantly higher baseline GH levels compared with type I diabetic patients with low levels of circulating GADA (<3 U) and normal subjects (Table 1).

Moreover, diabetic subjects with GADA showed significantly higher absolute serum GH levels after GHRH at 30 and 45 minutes compared with diabetic patients without GADA (Fig 1A). This finding was confirmed by comparison of GH peaks after GHRH (33 ± 3.7 *v* 21.8 ± 1.8 μg/L; Fig 1B). GH responses to GHRH observed in diabetic patients with GADA were also significantly higher compared with those in normal controls when expressed as either absolute or peak values (Fig 1A and B). Conversely, no significant differences in GH responses to GHRH were observed between diabetic patients without GADA and normal subjects.

GH peaks after GHRH were positively correlated (r_s = .46, $P < .05$) with the level of GADA in the whole population of type I diabetic subjects studied. In detail, the overlap between the two subgroups of patients was represented by three of 10 patients without GADA having GH peaks after GHRH in the low range of patients with GADA (Fig 2). When patients were subgrouped according to GADA levels, no significant correlations were found between GH peaks after GHRH and GADA values in patients with or without GADA. Basal and GHRH-stimulated GH secretion did not correlate with any anthropometric, clinical, and glycometabolic parameters in our type I diabetic patients. No differences in GH secretion were observed in type I diabetic patients with regard to the presence of ICA and IA2 antibodies.

No significant differences in the GH response to GHRH + PD in terms of peak or absolute levels were observed between type I diabetic patients and controls or, within patients, between those with (n = 6) or without (n = 6) GADA. However, whereas in patients without GADA and control subjects, PD significantly enhanced the GH response to GHRH (GH peak from 19.7 ± 2.2 to 50.3 ± 8.1 μg/L, $P < .05$, and from 19.6 ± 2.3 to 38.2 ± 4.3 μg/L, $P < .05$, respectively), in patients with GADA, PD enhanced the GH response to a lesser and nonsignificant extent (from 33.0 ± 5.5 to 46.3 ± 9.8 μg/L, nonsignificant; Fig 3A and B). No significant correlations were found between GH peaks after GHRH + PD and GADA in the diabetic patients tested.

DISCUSSION

Diabetic patients have higher mean daily GH concentrations than normal subjects.[3] It has been suggested that this is due to the greater height and frequency of spontaneous GH peaks.[5] Moreover, in type I diabetes, GH responses to physical exercise,[26] L-dopa,[27] arginine, glucagon,[28] and GHRH[6,8,10] are exaggerated, as confirmed also by the present study. Indeed, it has been postulated that the disturbance in GH secretion observed in some type I diabetic patients might be due to decreased hypothalamic somatostatin tone.[6] This postulated reduction in somatostatin tone in type I diabetes may be due to a primary lesion of somatostatin neurons, as previously reported in experimental diabetes.[29] Alternatively, it can be suggested that alterations in other neuropeptides or neurotransmitters

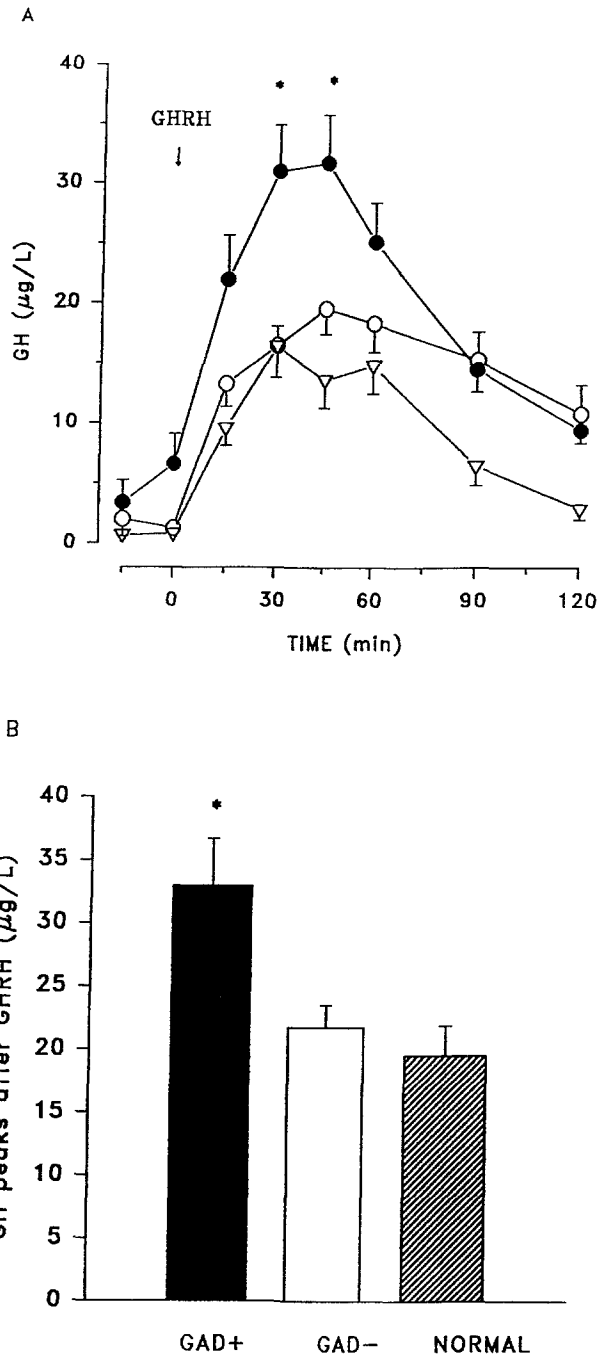

Fig 1. (A) Absolute serum GH levels (mean ± SEM) after IV GHRH in 10 type I diabetic patients with GADA (GAD+, ●), 10 diabetic patients without GADA (GAD−, ○), and 17 normal subjects (▽). (B) Peak serum GH levels (mean ± SEM) after IV GHRH in 10 GAD+ type I diabetic patients (■), 10 GAD− diabetic patients (□), and 17 normal subjects (▨). *$P < .05$ *v* GAD− diabetic patients and normal subjects.

physiologically[30] regulating hypothalamic somatostatin synthesis and secretion may occur in diabetes mellitus. GABA has been identified in the brain of several animal species, where it is now considered the major inhibitory neurotransmitter.[31] GABA has also been hypothesized to have a significant inhibitory role in the regulation of GH secretion in humans.[17,18] In fact, the GABAergic agent, sodium valproate, has been shown to inhibit

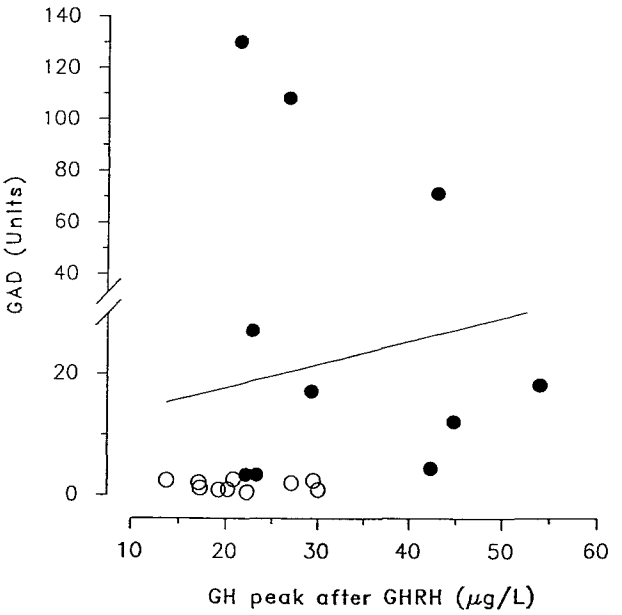

Fig 2. Correlation between GH peak values after GHRH and circulating GADA levels in a population of 20 type I diabetic patients with GADA (●) and without GADA (○). $r_s = 0.46$, $P = .04$.

the GH surge observed during physical exercise.[20] Moreover, administration of the GABA β-receptor agonist, baclofen, has been shown to inhibit the GH response to arginine,[19] an amino acid thought to decrease hypothalamic somatostatin.[32] Overall, GABA could be hypothesized to be implicated in the inhibition of stimulated GH secretion via an increase in hypothalamic somatostatin tone.

The production of GABA requires the presence of two key enzymes: GABA-T (4-aminobutyrate-2-oxaglutarate aminotransferase) and GAD. GAD converts glutamate to GABA by a decarboxylation reaction and is the rate-limiting step in the biosynthesis of GABA.[13] GAD has recently been demonstrated to be a major autoantigen in type I diabetes as a target of both humoral and cell-mediated autoimmunity associated with the disease.[14,15] Since GH hypersecretion is characteristic of type I diabetic patients[6-8] and, in contrast, is blunted in patients with type II diabetes,[33] it has been hypothesized that the disease-related autoimmune process could be involved in the derangement of GH neuroregulation observed in type I diabetes.[34]

This study was performed to evaluate whether a relationship between humoral autoimmunity against GAD and GH secretion in type I diabetes exists. Our findings showing that the presence of circulating GADA was linked to an increased GH response to GHRH in chronic type I diabetes patients support this hypothesis and further suggest a functional interference of GAD autoimmunity at the GABAergic system level. This functional abnormality of GH secretion was specifically associated with GAD autoimmunity, since no relationship was observed with the other two disease-specific autoantibody markers tested, ICA and IA2 antibodies.

This is the first evidence of a pathogenic effect of GAD autoimmunity in type I diabetes outside the endocrine pancreas. In fact, expression of GAD is not restricted to islet cells, since this enzyme is primarily expressed in the central nervous system with a distribution corresponding to that of the GABAergic system. However, no information was previously reported about a possible involvement of GAD-containing tissues other than pancreatic islets as targets of the autoimmune process associated with type I diabetes. The only example in which an extrapancreatic pathogenic effect of GAD autoimmunity has

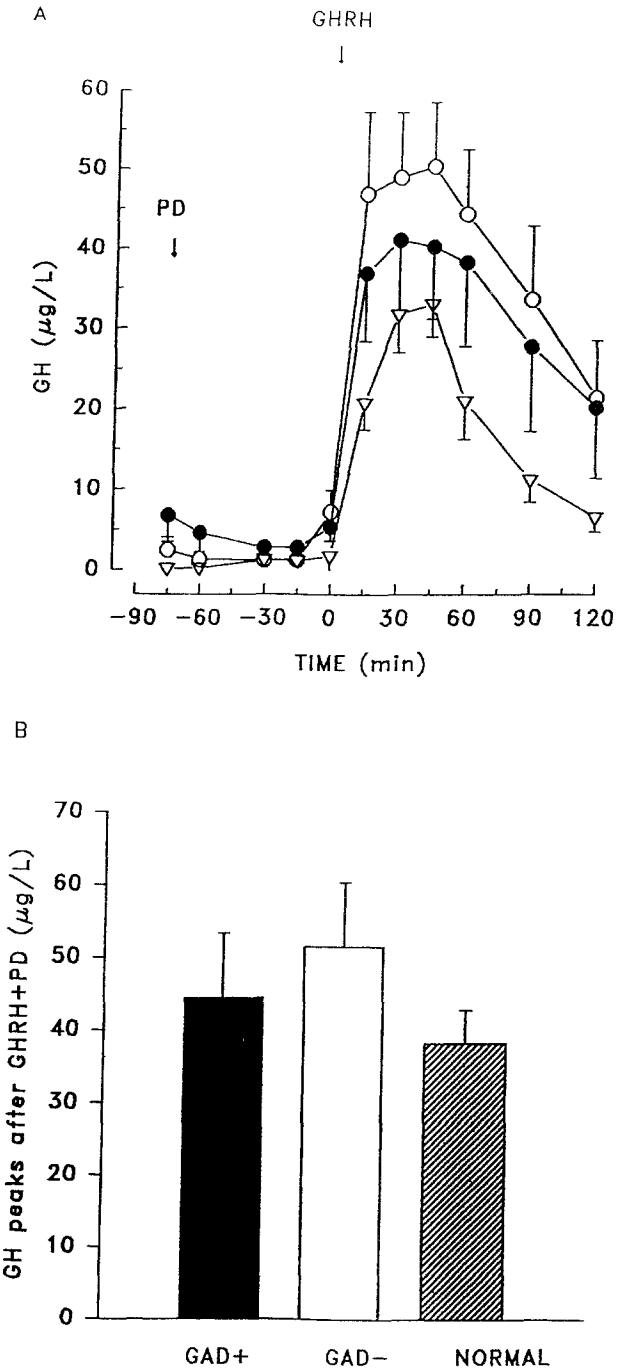

Fig 3. (A) Absolute serum GH levels (mean ± SEM) after IV GHRH + PD in 6 type I diabetic patients with GADA (GAD+, ●), 6 diabetic patients without GADA (GAD−, ○), and 17 normal subjects (▽). (B) Peak serum GH levels (mean ± SEM) after IV GHRH + PD in 6 GAD+ type I diabetic patients (■), 6 GAD− diabetic patients (□), and 17 normal subjects (▨).

been suggested is the stiff-man syndrome,[35] a disorder distinct from but frequently associated with type I diabetes. Nonetheless, although it is likely, functional evidence for the pathogenicity of GAD autoimmunity in stiff-man syndrome is still lacking. On the other hand, based on our results, a possible mechanism by which GAD autoimmunity may interfere with GH secretion in type I diabetic patients can be envisioned.

Evidence is increasing for an important role of cholinergic neurotransmission in GH secretion in normal man.[36] Experimental and human studies have suggested that the GH-stimulating action of cholinergic agents, such as PD, which also enhances the GH response to GHRH in normal subjects, is mediated by a decrease in the hypothalamic release of somatostatin.[9,37] The GH response to GHRH + PD in our type I diabetic patients with GADA was not higher than in normal subjects and patients without GADA and was not significantly enhanced as compared with the response seen with GHRH alone. Moreover, the GH response to GHRH + PD was not significantly correlated with GADA. These latter findings confirm the hypothesis that GAD autoimmunity may decrease GABA production at the central nervous system level and that, in turn, this may cause a reduction in hypothalamic somatostatin tone with increased GH secretion in type I diabetes. Alternatively, it has been recently reported that cholinergic and GABAergic neurons are strictly linked in the central nervous system, with evidence of GABAergic innervation on cholinergic neurons in the median septum.[38] Therefore, it can be hypothesized that altered GABAergic pathways, due to autoimmune aggression to GAD, may cause derangement in somatostatin secretion also indirectly via an increase of cholinergic inhibitory tone.

Several factors have been suggested as determinants of GH hypersecretion in type I diabetes, including microangiopathic complications,[7] duration of the disease,[8] and short-term[28] and long-term[8] glycometabolic control. However, none of these factors alone or even in combination have been able to completely account for the alterations in GH secretion observed in type I diabetes. In this context, no significant differences in glycometabolic control, disease duration, and insulin dose were observed in our study between patients with or without GADA. Finally, it cannot be excluded that the sex-based difference[39] between patients without GADA may play a role in the different GH responses to GHRH observed between groups.[5,39] Conversely, this sex-based difference can hardly explain the different GH responses to GHRH + PD between patients with and without GADA.[40]

In conclusion, our findings suggest that autoimmunity may play a role in determining the exaggerated GH response to GHRH observed in patients with established type I diabetes mellitus. The mechanism underlying this effect is hypothesized to be the autoimmune response against GAD, a key enzyme in the synthesis of GABA, and, in turn, a reduced GABAergic stimulatory tone on somatostatin release at the hypothalamic level.

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