0013-7227/01/$03.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 86(8):3717–3723

Determinants of the Impaired Secretion of Glucagon-Like Peptide-1 in Type 2 Diabetic Patients

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To elucidate the causes of the diminished incretin effect in type 2 diabetes mellitus we investigated the secretion of the incretin hormones, glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide and measured nonesterified fatty acids, and plasma concentrations of insulin, C peptide, pancreatic polypeptide, and glucose during a 4-h mixed meal test in 54 heterogeneous type 2 diabetic patients, 33 matched control subjects with normal glucose tolerance, and 15 unmatched subjects with impaired glucose tolerance. The glucagon-like peptide-1 response in terms of area under the curve from 0–240 min after the start of the meal was significantly decreased in the patients (2482 ± 145 compared with 3101 ± 198 pmol/liter·240 min; $P = 0.024$). In addition, the area under the curve for glucose-dependent insulinotropic polypeptide was slightly decreased. In a multiple regression analysis, a model with diabetes, body mass index, male sex, insulin area under the curve (negative influence), glucose-dependent insulinotropic polypeptide area under the curve (negative influence), and glucagon area under the curve (positive influence) explained 42% of the variability of the glucagon-like peptide-1 response. The impaired glucose tolerance subjects were hyperinsulinemic and generally showed the same abnormalities as the diabetic patients, but to a lesser degree. We conclude that the meal-related glucagon-like peptide-1 response in type 2 diabetes is decreased, which may contribute to the decreased incretin effect in type 2 diabetes. (*J Clin Endocrinol Metab* 86: 3717–3723, 2001)

TYPE 2 DIABETES is characterized by hyperglycemia, insulin resistance, absolute or relative insulin deficiency, hyperglucagonemia, increased hepatic glucose production, and frequently accelerated gastric emptying and obesity (1). The positive influence of the incretin hormone glucagon-like peptide-1 (GLP-1) on the metabolic disturbances of type 2 diabetes, including stimulation of insulin secretion (2, 3) and inhibition of glucagon secretion (3, 4), hepatic glucose production (5, 6), gastric emptying (7, 8), and appetite (9, 10), has provided a rationale for its therapeutic use in type 2 diabetes. Furthermore, GLP-1 seems to exert trophic effects on the β-cell (11).

Several studies have documented the importance of GLP-1 for maintenance of normal glucose tolerance. Thus, GLP-1 receptor-deficient mice exhibit increased glucose levels and diminished insulin levels after an oral glucose challenge (12, 13). In healthy subjects, infusion of the GLP-1 receptor antagonist exendin-(9–39), during an oral glucose tolerance test, increased incremental glucose area under the curves (AUCs), and peak postprandial glucose levels (14). In type 2 diabetic patients the incretin effect is reduced or lost (15, 16). Glucose-dependent insulinotropic polypeptide (GIP) studies are inconclusive to date, with reports of both increased and decreased secretion in studies of diabetic patients compared with nondiabetic subjects (17), but the GIP effect on insulin secretion is decreased in type 2 diabetic patients (18, 19). In contrast, type 2 diabetic patients show a pronounced insulin response to parenterally administered GLP-1 (3, 19). The GLP-1 secretion patterns in type 2 diabetes and type 2 diabetes-related conditions are not clear. They has been reported to be increased (20) or reduced (21, 22) in obese subjects, to be higher in women than in men (23, 24), and to be increased (25, 26), decreased (24), or unaltered (19, 23, 27) in subjects with impaired or diabetic glucose tolerance. However, the early GLP-1 assays were unable to distinguish between GLP-1 from the gut and GLP-1-immunoreactive molecules from the pancreas [*i.e.* GLP-1-(1–36)amide or -(1–37) and major proglucagon fragment] as inactive by-products of glucagon secretion.

To determine the secretion and possible pathophysiological role of the incretins, GLP-1 and GIP, in type 2 diabetes, we subjected a heterogeneous group of type 2 diabetic (T2DM) patients and matched healthy subjects with normal glucose tolerance (NGT) as well as subjects with impaired glucose tolerance (IGT) to a meal test. We show that the GLP-1 response to a standard meal test in patients with T2DM is decreased, probably as a consequence of the diabetic state.

Abbreviations: AUC, Area under the curve; B, biguanide treatment; BMI, body mass index; D, treated with diet; GAD, glutamic acid decarboxylase; GADab, GAD antibodies; GIP, glucose-dependent insulinotropic polypeptide; IGT, impaired glucose tolerance; NEFA, nonesterified fatty acids; NGT, normal glucose tolerance; PG, plasma glucose; PP, pancreatic polypeptide; SU, sulfonylurea treatment; T2DM, type 2 diabetes mellitus.

Subjects and Methods

Subjects

The T2DM group was recruited from the diabetes out-clinic, whereas the groups with NGT or IGT, classified after an oral glucose tolerance test according to the WHO criteria of 1985, responded to an advertise-

ment in a local newspaper. None had a history of bowel disease, alcohol abuse, or, for the NGT/IGT subjects, diabetes among first degree relatives. According to the patients' medical records, they had normal serum creatinine, normal hepatic function, and no albuminuria.

The T2DM group consisted of 54 type 2 diabetic patients with a mean diabetes duration of 4.9 ± 5.6 yr (mean ± SD; see Table 1 for anthropometric data). Thirty-three control subjects with NGT were matched to the T2DM group (Table 1). A person to person match was not attempted, but when the subjects were divided according to body mass index (BMI; 20–25, 25–30, 30–35, and >35 kg/m^2) similar means, medians, and ranges for age, male/female ratios, and BMIs were obtained in each of the 4 groups for patients compared with volunteers. The IGT subjects had significantly higher BMI, but similar age and male/female ratio compared with the T2DM and NGT groups (Table 1).

All subjects agreed to participate after providing oral and written information. The study was approved by the ethical committee for Copenhagen and Frederiksberg Municipalities and was conducted according to the principles of the Helsinki Declaration.

Procedure

After 3 d of discontinued antidiabetic medication and an overnight fast (10 h), the subjects consumed a mixed breakfast meal containing 2250 kJ (41.8% fat, 40.7% carbohydrate, and 17.5% protein; fiber content, 6.7 g). The meal was served with coffee or tea and ingested within 10–15 min. Blood was sampled from a needle in a forearm vein before the start and during the next 4 h as indicated in Figs. 1-3 and was distributed into fluoride tubes for analysis of plasma glucose (PG) and into EDTA/aprotinin tubes (6 mmol/liter EDTA and 500 kallikrein inhibitor units aprotinin/ml blood) for analysis of plasma concentrations of GLP-1, GIP, glucagon, insulin, C peptide, and pancreatic polypeptide (PP); fasting plasma concentration of nonesterified fatty acids (NEFA); glutamic acid decarboxylase (GAD) antibodies (GADab); and islet antigen antibodies (IA2ab). Tubes were immediately chilled in ice and centrifuged at 4 C within 10 min. Plasma was stored at −20 C until analysis.

The patients with T2DM were evaluated with respect to neuropathy status by 1) medical history; 2) physical examination; 3) biothesiometry; 4) cardiologic autonomic tests, *i.e.* orthostatic blood pressure measurement and deep breathing, standard 15-sec Valsalva maneuver, and 15-sec Valsalva maneuver with expiration against 40 cm water; the latter three tests evaluated by estimation of beat to beat variation (R-R interval) (29); and 5) electrophysiological evaluation of peripheral nerve function in 12 patients. The patients were divided into a group with no signs of diabetic neuropathy [class IIA, diabetic polyneuropathy consensus statement of 1993 (30)] and another group with manifest diabetic neuropathy (class IIB/IIC). Among patients with manifest neuropathy, 5 also had measurable autonomic dysfunction. Four patients could not be classified, 2 patients due to signs of incipient, but not manifest, neuropathy, and 2 due to lack of tests.

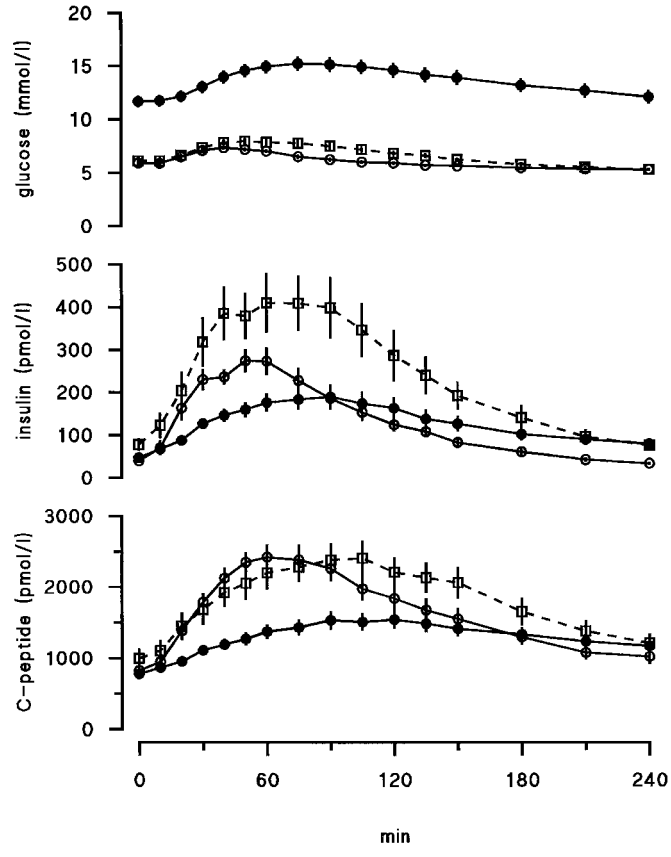

FIG. 1. Plasma glucose (*upper panel*), insulin (*middle panel*), and C peptide (*lower panel*) concentrations in T2DM patients (●), NGT subjects (○), and IGT subjects (□) during a 240-min meal test. The meal was started at time zero and finished in the 10- to 15-min period.

TABLE 1. Summary of the characteristics of the T2DM, NGT, and IGT groups reported as the mean ± SD (rows 1–6) and SEM (rows 7–13)

	T2DM	NGT	IGT	T2DM *vs.* NGT	ANOVA	ANOVA correcting for covariates
No. (M/F)	54 (44/10)	33 (27/6)	15 (12/3)			
Age (yr)	55.9 ± 8.0	56.2 ± 9.1	55.3 ± 6.8	NS	NS	
BMI (kg/m^2)	30.2 ± 5.3	29.6 ± 6.2	35.0 ± 5.3	NS	0.007[a]	
Treatment (D/SU/B/SU + B)	19/16/12/7					
HbA_{1c} (%)	8.4 ± 1.7	5.9 ± 0.4	6.1 ± 0.6	<0.001	<0.001[b]	<0.000[b]
Fasting PG (mmol/liter)	11.7 ± 4.0	5.9 ± 0.6	6.2 ± 0.6	<0.001	<0.001[b]	<0.000[sex,b]
Fasting plasma insulin (pmol/liter)	48 ± 5.0	40 ± 4.0	78 ± 15.0	NS	0.014[a]	NS[BMI]
Fasting plasma C peptide (pmol/liter)	778 ± 48	667 ± 42	999 ± 134	NS	0.025[c]	NS[BMI]
Fasting plasma glucagon (pmol/liter)	13.0 ± 0.8	8.4 ± 0.9	11.2 ± 1.6	<0.001	<0.001[d]	<0.001[b, BMI, sex]
Fasting plasma PP (pmol/liter)	33 ± 3.9	26 ± 2.9	24 ± 4.0	NS	NS	NS[sex, age]
Fasting NEFA (mmol/liter)	0.78 ± 0.04	0.78 ± 0.04	0.85 ± 0.07	NS	NS	NS
Fasting plasma GIP (pmol/liter)	12.7 ± 1.5	8.6 ± 0.7	9.8 ± 1.2	0.13	NS	NS
Fasting plasma GLP-1 (pmol/liter)	6.6 ± 0.5	4.9 ± 0.4	4.9 ± 0.4	0.037	NS	NS

The significances of differences between the age-, gender-, and BMI-matched T2DM and NGT groups were evaluated by means of Mann-Whitney tests, whereas comparisons involving also the unmatched IGT group were carried out by means of ANOVA (significant or near-significant covariates shown as superscripts).

[a] $P < 0.05$, IGT *vs.* T2DM and NGT.
[b] $P < 0.05$, T2DM *vs.* NGT and IGT.
[c] $P < 0.05$, NGT *vs.* IGT.
[d] $P < 0.05$, T2DM *vs.* NGT.
[e] $P < 0.05$, NGT *vs.* T2DM and IGT.

Analytical methods

Plasma glucose concentrations were analyzed bedside using an analyzer (Beckman Coulter, Inc., Fullerton, CA).

Hormone analyses. The glucagon assay (RIA) is directed against the C-terminus of the glucagon molecule (antibody code no. 4305) and therefore measures glucagon of mainly pancreatic origin (25). Plasma concentrations of amidated GLP-1-(7–36) were measured by means of antibody code no. 89390 (RIA), which is highly specific for the C-terminus of GLP-1 and therefore measures the sum of GLP-1-(7–36)amide and its metabolite GLP-1-(9–36)amide (31). The detection limits and intraassay coefficients of the assays employed are 1 pmol/liter and less than 6% for both glucagon and amidated GLP-1 (antibody 89390), whereas the interassay coefficient of variation was less than 10%. GIP was measured using a C-terminally directed antibody (code no. R65; RIA), reacting 100% with human GIP, but not with so-called 8-kDa GIP (32, 33). The detection limit is 5 pmol/liter, the intraassay coefficient of variation is 9%, and the interassay coefficient of variation is 15–20%. For all three analyses plasma was extracted with ethanol (final concentration, 70%, vol/vol) before analysis. Insulin and C peptide concentrations were measured using commercial ELISA kits (code no. K6219 and K6218, respectively; DAKO Corp., Copenhagen, Denmark). PP concentrations were measured using a previously described specific RIA (34). Intra- and interassay coefficients of variation were less than 13% in the range of 16–50 and 30–75 pmol/liter, respectively.

Antibodies to GAD and IA-2 were measured by a radioligand binding assay, using full-length recombinant human GAD65 or IA-2 as previously described (35). The threshold for positivity was defined as 3 SD above the mean of 276 healthy Danish control individuals with normal glucose tolerance.

Lipids. NEFA were measured by an enzymatic spectrophotometric method as previously described (36).

Hemoglobin A_{1c} was measured at the laboratory of Steno Diabetes Hospital (Gentofte, Denmark), using an ion exchange HPLC method with an interassay coefficient of variation of 0.15 percentage points in the range of 4.7–11.3% (normal range, 4.1–6.4%).

Statistical analysis and calculations

Nonparametric statistical methods were generally used, *i.e.* Mann-Whitney's test for comparison of two groups (T2DM *vs.* NGT and neuropathy *vs.* nonneuropathy T2DM patients) and Kruskal-Wallace test for comparison of three groups (T2DM, NGT, and IGT). However, in the case of comparison of nonmatched groups in which correction for covariates would seem necessary (comparison between the different T2DM treatment groups; T2DM *vs.* NGT *vs.* IGT; neuropathy *vs.* nonneuropathy T2DM patients), we used multiple comparison ANOVA followed by a *post-hoc* test, least significant differences, and correction for significant covariates, such as BMI, gender, and age. Multiple regression analysis with the GLP-1 and the GIP response as the dependent variable was carried out as forward and backward regressions for T2DM and NGT separately, together, and with IGT included. No colinearity was apparent for the variables included in the regression analysis. In the case of non-Gaussian distribution in the ANOVA or of the dependent variable in the multiple regression analysis, data were logarithmically transformed.

AUC was calculated as incremental areas above zero, and incremental AUC was calculated as AUC above basal.

Results are presented as the mean ± 1 SD or as the mean ± SEM. The level of statistical significance was set at $P < 0.05$.

Results

GAD, IA2

Upon IA2ab analysis, 1 female patient of the 102 subjects (54 T2DM, 33 NGT, and 15 IGT) was marginally positive, but was most likely a true type 2 diabetic patient (fasting C peptide concentration of 534 pmol/liter). Upon GADab analysis, two subjects with NGT were marginally positive, and 1 male T2DM patient had a very high level of GADab. Although he had a fasting C peptide concentration of 1119 pmol/liter, he probably has late autoimmune diabetes of the adult. Neither of the subjects was excluded from the analysis.

NEFA

Fasting NEFA concentrations were the same for T2DM and NGT, whereas the level in IGT was a little higher (Table 1). There was no significance between groups.

Glucose

Plasma glucose concentrations (Fig. 1, *upper panel*) at all time points, including the fasting state, were significantly higher for T2DM compared with NGT and IGT and so were the AUCs. IGT values were higher than NGT values, however, significantly so only at 75 min (Tables 1 and 2).

Insulin and C peptide

T2DM and NGT had similar insulin and C peptide fasting levels (Table 1) and AUCs (Table 2), whereas IGT had higher levels. After correcting for BMI, only the difference between AUCs of IGT *vs.* T2DM remained significant. Peak insulin concentrations, insulin concentrations at 20–60 min, and C peptide concentrations at 20–105 min were significantly lower in T2DM compared with NGT and IGT (Fig. 1). The

TABLE 2. Hormone responses presented as the mean ± SEM of total areas under curves (AUC) during the 4-h meal test

	T2DM	NGT	IGT	T2DM *vs.* NGT	ANOVA correcting for covariates
Glucose AUC (mmol/liter·240 min)	3289 ± 152	1452 ± 24	1591 ± 45	<0.001	$<0.001^{sex,a}$
Insulin AUC (10^3 × pmol/liter·240 min)	30.9 ± 3.9	31.1 ± 3.1	57.7 ± 9.8	0.13	$0.016^{BMI,b}$
C-peptide AUC (10^3 × pmol/liter·240 min)	315 ± 23	327 ± 20	442 ± 45	NS	$0.096^{BMI,b}$
Glucagon AUC (pmol/liter·240 min)	3585 ± 174	2386 ± 230	3108 ± 372	<0.001	$<0.001^{BMI,a}$
PP AUC (10^3 × pmol/liter·240 min)	29.6 ± 2.9	35.0 ± 3.9	29.1 ± 4.1	NS	NS^{age}
GIP AUC (10^3 × pmol/liter·240 min)	13.4 ± 0.7	16.0 ± 1.3	15.0 ± 1.4	0.047	$0.095^{BMI,sex,b}$
GLP-1 AUC (pmol/liter·240 min)	2482 ± 145	3101 ± 198	2765 ± 185	0.024	$0.011^{BMI,sex,a}$
GLP-1 AUC, incremental (pmol/liter·240 min)	907 ± 92	1927 ± 177	1587 ± 185	<0.001	$<0.001^{BMI,sex,a}$

The significances of differences between the age-, gender-, and BMI-matched T2DM and NGT groups were evaluated by means of Mann-Whitney tests, whereas comparisons involving also the unmatched IGT group were carried out by means of ANOVA with data logarithmically transformed in the case of non-Gaussian distribution (significant or near-significant covariates shown as superscripts). For insulin and C peptide, age is a significant covariate if BMI is also included as covariate.

[a] $P < 0.05$, T2DM *vs.* NGT and IGT.

[b] $P < 0.05$, T2DM *vs.* IGT.

time to reach the peak C peptide concentration was significantly delayed in T2DM compared with those in NGT and IGT. The delay in C peptide levels for IGT was not significant.

Glucagon

T2DM had significantly higher glucagon concentrations at all time points and significantly higher AUCs compared with NGT (Tables 1 and 2 and Fig. 2, *upper panel*). IGT tended to have higher values than NGT, significantly so at 20–75, 210, and 240 min.

PP

Fasting levels and AUCs of PP were similar for all groups, but in the curve for T2DM the early peak was reduced (Table 2 and Fig. 2, *middle panel*).

GIP

As shown in Table 1, the fasting GIP level in the T2DM was insignificantly higher than those in the NGT and IGT group. The GIP meal response (Table 2 and Fig. 2) was slightly, but significantly ($P = 0.047$), decreased in the T2DM compared with the NGT group, but this difference was absent in the BMI and gender-corrected ANOVA analysis for all three groups. Peak values were similar in all groups.

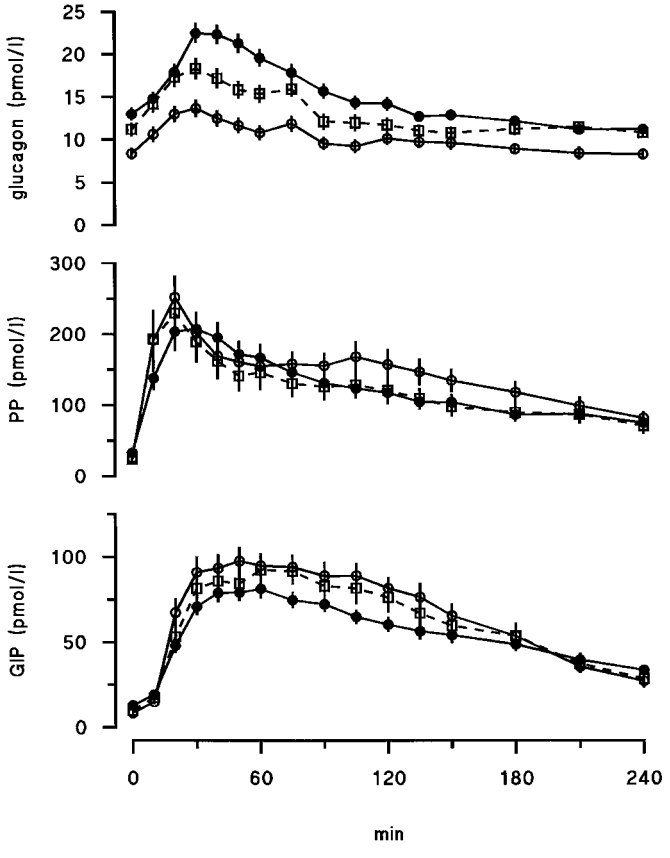

FIG. 2. Plasma glucagon (*upper panel*), PP (*middle panel*), and GIP (*lower panel*) concentrations in T2DM patients (●), NGT subjects (○), and IGT subjects (□) during a 240-min meal test. The meal was started at time zero and finished in the 10- to 15-min period.

GLP-1

Fasting GLP-1 concentrations were significantly higher in T2DM than in NGT (Table 1), but there was no difference between the three groups upon ANOVA, and no significant covariates were found. Postprandial GLP-1 levels (Fig. 3) and AUC were significantly decreased in T2DM compared with NGT (Table 2), and, upon ANOVA analysis correcting for BMI and gender, they were also decreased compared with IGT values. The GLP-1 AUC of the IGT group ranged between those of T2DM and NGT. The GLP-1 AUC was lower in males and decreased with increasing BMI. BMI- and gender-corrected GLP-1 AUC means were 2464 (T2DM), 2907 (IGT), and 3066 (NGT) pmol/liter·240 min (P = NS for NGT *vs.* IGT group). The incremental GLP-1 response in the T2DM group was even more impaired (Table 2), and here also IGT levels ranged between T2DM and NGT values.

Diabetic neuropathy and autonomic dysfunction

The 15 (13 men and 2 women) patients with and the 35 (27 men and 8 women) patients without diabetic neuropathy had similar age, BMI, and hemoglobin A_{1c}. The patients with diabetic neuropathy had significantly higher fasting PG (13.5 ± 1.2 *vs.* 11.0 ± 0.6; $P = 0.09$, by Wilcoxon; $P = 0.015$, by ANOVA correcting for gender) and significantly lower insulin and C peptide responses (not shown). The GLP-1 meal response tended to be higher in the patients with neuropathy (2752 ± 285 *vs.* 2371 ± 176; $P = 0.26$ without and $P = 0.28$ with correction for BMI and gender). Incremental AUCs were similar. The GIP and PP responses did not differ significantly between groups. Of the 15 patients with neuropathy, 5 patients also had autonomic nerve dysfunction; of these, 1 had a GLP-1 AUC below, and 4 had a GLP-1 AUC above the group mean.

Treatment groups in T2DM

The 54 T2DM patients were treated with diet (D), sulfonylurea (SU), biguanide (B), or sulfonylurea and biguanide

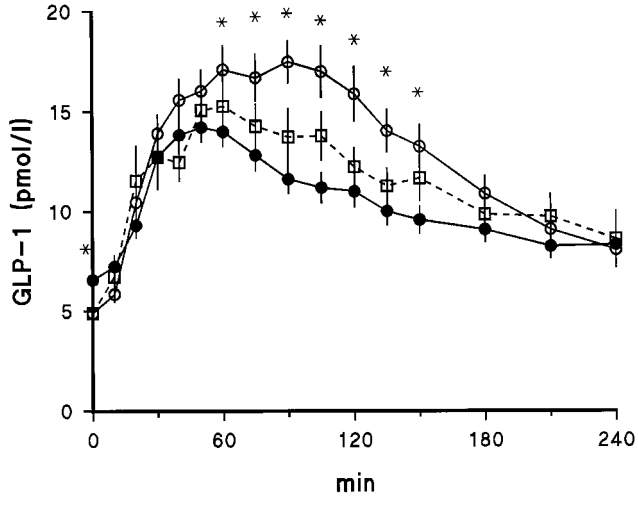

FIG. 3. Plasma GLP-1 concentrations in T2DM patients (●), NGT subjects (○), and IGT subjects (□) during a 240-min meal test. The meal was started at time zero and finished in the 10- to 15-min period. *, $P < 0.05$ between the T2DM and NGT group.

(SU+B; n = 19/16/12/7). In these groups SU patients were the oldest, had the lowest BMI, and, together with SU+B, had the highest fasting glucose. Group B patients were the youngest, and group D patients were the most well regulated. There was no difference with respect to diabetes duration. The GLP-1 responses in terms of AUC were significantly different between groups (D, 1964 ± 216; SU, 3151 ± 238; B, 2504 ± 337; SU+B, 2318 ± 283 pmol/liter·240 min), with the D group being significantly lower than the SU group and, after correcting for BMI, also significantly lower than the B group. Thus, the most well regulated T2DM patients had the lowest GLP-1 response. Multiple regression with GLP-1 AUC as the dependent factor showed that BMI (negative influence), fasting glucose (positive influence), and glucagon AUC (positive influence), but not any treatment modality, were determining factors of the GLP-1 response. The incremental GLP-1 response was not significantly different between any of the treatment groups after adjusting for BMI, age, and gender.

Covariates

As shown in Table 2, BMI was a significant covariate for fasting levels and AUCs of insulin, C peptide, glucagon (higher values with higher BMI), and for AUC for GIP and for AUC and incremental AUC for GLP-1 (lower values with increasing BMI). Gender was a significant covariate for fasting and AUC of glucose due to higher glucose values in T2DM females than in T2DM males and for AUC for GIP, AUC for GLP-1, and incremental AUC for GLP-1, for which females had higher values than males. Finally, PP increased with age.

Multiple regression analysis

Multiple regression analysis for the combined groups, T2DM/NGT, was conducted with GLP-1 AUC, incremental AUC for GLP-1, or GIP AUC as dependent variables. The independent variables were diabetic state, age, BMI, gender, NEFA, and AUCs of PG, insulin, glucagon, PP, and GLP-1/GIP, respectively. The AUC for C peptide could not be included due to colinearity. Fasting glucose was not included because the subjects were denoted T2DM or NGT. However, by Spearmann correlation analysis in the T2DM there was a significant positive correlation between GLP-1 AUC and fasting glucose ($r = 0.44$; $P = 0.0013$), *i.e.* the higher the PG, the higher the GLP-1 response.

With GLP-1 AUC as dependent parameter, forward and backward regression showed that a model with diabetes, male sex, BMI, insulin AUC, GIP AUC, and glucagon AUC explained 42% (adjusted r^2) of the variation of the GLP-1 response. Except for glucagon, which had a positive coefficient and thus was increasing the GLP-1 response, the rest of the parameters had negative coefficients, resulting in a negative influence on the GLP-1 response. The model of multiple regression analysis with incremental GLP-1 AUC as the dependent parameter differed only by leaving out insulin AUC and GIP AUC. With GIP AUC as the dependent parameter, a model with BMI, male sex, diabetes and GLP-1 AUC (all with negative coefficients) explained only 15% of the variation.

Discussion

In the present study the meal-stimulated GLP-1 response was measured in patients with type 2 diabetes and compared with that in carefully matched subjects with NGT. In addition, a group of subjects with IGT was investigated. We found that the meal-stimulated GLP-1 response, expressed as both the total AUC and the incremental AUC, was significantly decreased in patients with type 2 diabetes. Consistent with this, the subjects with IGT had GLP-1 responses in between those of the controls and the patients regardless of whether significant covariates (gender and BMI) were taken into account. A slight impairment of GIP secretion was also observed in the patients. As demonstrated in animal experiments, and for GLP-1 also in healthy volunteers, the incretin effect, for which GLP-1 and GIP are normally responsible, is important for the maintenance of NGT (12, 14, 37). Decreased incretin secretion, therefore, may worsen already existing diabetes mellitus or, theoretically, may contribute to the development of diabetes.

Because of the large number of very heterogeneous patients studied, it was possible to search for factors influencing GLP-1 secretion. Hyperglycemia *per se* is unlikely to be responsible for the decreased GLP-1 response, as indicated by an unexpected positive correlation between blood glucose and GLP-1 response in the T2DM group. Consistent with the significantly lower GLP-1 response in the T2DM compared with the NGT, diabetes was found to be a significant determinant for the GLP-1 response by multiple regression analysis. This is in agreement with results obtained by Vaag *et al.* (24), who studied a small group of monozygotic twins discordant for diabetes. Furthermore, gender and BMI turned out to both be determinants of the GLP-1 response in the multiple regression analysis and significant covariates in the ANOVA analysis. Males had a smaller response than females, in agreement with results presented by Nauck *et al.* (23) and Vaag *et al.* (24). The GLP-1 response decreased with the degree of obesity, consistent with the results reported by Ranganath *et al.* (21) and Näslund *et al.* (22). The negative relation between insulin AUC and the GLP-1 response was unexpected, but may illustrate a hitherto undescribed negative feedback effect of insulin on GLP-1 secretion. By multiple regression analysis, GIP had a barely significant, negative influence on the GLP-1 response. Thus, we found no support for a positive feedback mechanism of GIP on the L cell and, therefore, no support for the hypothesis that GIP promotes GLP-1 secretion as observed in rats (38). The strong positive influence of glucagon on the GLP-1 response found in the multiple regression analysis is also difficult to explain. It may be caused by a factor in the meal stimulating both the intestinal L cell and the pancreatic α cell. A glucose challenge does not promote glucagon secretion, but the protein content of meals does. Peptones (protein hydrolysates) have recently been shown to stimulate the L cell (39), and therefore, the relatively high protein content (17.5%) in the meal may be the link between the parallel glucagon and GLP-1 secretion.

In this study several factors were excluded as determinants of the GLP-1 response; hence, treatment with sulfonylureas or biguanides, neuropathy, and fasting NEFA concentrations did not seem to affect the GLP-1 response to a

detectable level. The diet-treated patients had the lowest GLP-1 response (total AUC), whereas the incremental GLP-1 response was not significantly different between any of the treatment groups, after adjusting for BMI, age, and gender differences. In the present study the 15 patients with diabetic neuropathy had an insignificantly higher GLP-1 response both with and without correction for covariates, and four of five patients with autonomic nerve dysfunction, who may have defective neural signaling, had GLP-1 responses above the mean. We conclude that the decreased GLP-1 responses are unlikely to be related to neural dysfunction in this patient group and, thus, do not support the results of Rocca *et al.* (40), who reported that vagal activity was important for GLP-1 secretion in rats. Recently, it was hypothesized that NEFA inhibit the L cell (41). However, in this study the fasting concentrations of NEFA were similar in the diabetic and NGT groups, and NEFA were not a significant determinant either in the multiple regression analysis including T2DM alone or in the total T2DM plus NGT group, apparently excluding NEFA as a major regulator of GLP-1 secretion.

A possible explanation for the decreased GLP-1 secretion may be a decreased gastric emptying rate, which hypothetically might increase the absorption in the proximal intestine resulting in less food reaching the distal intestine where the L cells are more numerous. Indeed, the opposite situation, increased exposure of carbohydrates to the distal intestinal mucosa by α-glucosidase inhibitors or accelerated gastric emptying, increases GLP-1 secretion (42, 43). However, the gastric emptying rate does not seem to exhibit consistent changes in T2DM and obesity, but is more often reported as delayed (44, 45). The gastric emptying rate in males is believed to be faster than that in premenopausal females, but this sex difference may disappear (46) (but probably not revert to the opposite) in the postmenopausal state. Almost all of the women participating in this study were postmenopausal, and therefore, gastric emptying rates would not be expected to explain the sex difference observed here. However, proximal absorption rates could hypothetically explain a decreased GLP-1 secretion in the patients. Obese subjects may have an increased proximal absorption rate (47), which could thus provide an explanation for the decreased GLP-1 secretion with increasing BMI. Proximal absorption rates in diabetic patients compared with nondiabetic subjects and in males compared with females have not been investigated to our knowledge.

Our finding of a decreased GLP-1 response in T2DM contrasts to earlier reports of unaltered (19, 23, 27) or even increased (24, 26) GLP-1 secretion in subjects with impaired or diabetic glucose tolerance. The discrepancy can at least partly be explained by use of different GLP-1 assays. The assays used in the studies by Fukase *et al.* (20, 26) and Ørskov *et al.* (25) were nonspecific and cross-reacted with several pancreatic GLP-1-containing peptide moieties such as the major proglucagon fragment and GLP-1-(1–37). Patients with type 2 diabetes and obesity have hyperglucagonemia, and as glucagon secretion is paralleled by a release of pancreatic GLP-1-containing proglucagon-processing products, the high levels of GLP-1 immunoreactivity in these two studies were probably due to hypersecretion of such products. The assay employed in the present study cross-reacts very little with other proglucagon products. It measures the COOH-terminus and, therefore, the sum of the biologically active intact molecule GLP-1-(7–36)amide and the primary inactive metabolite GLP-1-(9–36) amide. The use of this assay rather than an NH-terminal assay measuring only the intact, biologically active GLP-1 (48) is essential to estimate the rate of secretion of GLP-1, because the hormone is metabolized intravascularly and extremely rapidly (with an apparent half-life of 1–1.5 min and a clearance rate that greatly exceeds cardiac output). Thus, it is the sum of the concentrations of the primary metabolite and the intact hormone that reflects the secretory rate of the L cell. In fact, under certain circumstances peripheral concentrations of intact GLP-1 may remain constant despite increasing concentrations of metabolite (49). In agreement with this observation, recent research has demonstrated that the majority of GLP-1 secreted from the intestine in pigs is metabolized in the intestinal capillary bed before it enters the systemic circulation (50). Presumably, in this situation GLP-1 acts as a paracrine transmitter acting on mucosal nerve endings before being degraded (51), with the potential of activating pancreatic insulin secretion reflexly (52, 53). These recent findings underscore the importance of measuring the intact hormone as well as the primary metabolite for estimation of L cell activity.

We conclude that GLP-1 secretion is significantly impaired in type 2 diabetes, most likely as a consequence of the disease.

Acknowledgments

We thank Gertrud Petersson, Susanne Reimer, and Vladimira Tidsvilde (Hvidovre Hospital) and Hanne Mathiesen and Lene Albæk (Panum Institute) for technical assistance. Karina Lykke Rasmussen (Hvidovre Hospital) performed the biothesiometry, and Grete Hansen (Hvidovre Hospital) composed the meal. Thure Krarup, chief physician at Department of Internal Medicine F, Gentofte University Hospital, kindly allowed us to study six of his patients.

Received December 1, 2000. Accepted April 20, 2001.

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This work was supported by the Danish Diabetes Association, the Foundation of Poul and Erna Sehested Hansen, the Danish Medical Association Research Fund, the Danish Medical Research Council, and the NOVO Nordisk Foundation.

J.J.H. is a member of the Biotechnology Center for Signal Peptide Research.

References

1. **Alberti KGMM, Zimmet P, DeFronzo RA, Keen H** 1995 International textbook of diabetes mellitus, 2nd Ed. Chichester: Wiley & Sons
2. **Holst JJ, Ørskov AC, Schwartz TW, Nielsen OV** 1987 Truncated glucagon-like peptide-1, an insulin-releasing hormone from the distal gut. FEBS Lett 211: 169–174
3. **Nauck MA, Kleine N, Ørskov C, Holst JJ, Willms B, Creutzfeldt W** 1993 Normalization of fasting hyperglycemia by exogenous glucagon-like peptide-1(7–36 amide) in type 2 (non-insulin-dependent) diabetic patients. Diabetologia 36:741–744
4. **Ørskov C, Holst JJ, Nielsen OV** 1988 Effect of truncated glucagon-like peptide-1 (proglucagon 78–107 amide) on endocrine secretion from pig pancreas, antrum and stomach. Endocrinology 123:2009–2013
5. **Hvidberg A, Nielsen M-BT, Hilsted J, Ørskov C, Holst JJ** 1994 Effect of glucagon-like peptide-1 (proglucagon 78–107amide) on hepatic glucose production in healthy man. Metabolism 43:104–108
6. **Larsson H, Holst JJ, Ahrén B** 1997 Glucagon-like peptide-1 reduces hepatic glucose production indirectly through insulin and glucagon in humans. Acta Physiol Scand 160:413–422

7. **Wettergren A, Schjoldager B, Mortensen PE, Myhre J, Christiansen J, Holst JJ** 1993 Truncated GLP-1 (Proglucagon 78–107-amide) inhibits gastric and pancreatic functions in man. Dig Dis Sci 38:665–673
8. **Willms B, Werner J, Holst JJ, Ørskov C, Creutzfeldt W, Nauck MA** 1996 Gastric emptying, glucose responses, and insulin secretion after a liquid test meal: effects of exogenous glucagon-like peptide-1 (GLP-1)-(7–36) amide in type 2 (noninsulin-dependent) diabetic patients. J Clin Endocrinol Metab 81: 327–332
9. **Flint A, Raben A, Astrup A, Holst JJ** 1998 Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest 101:515–520
10. **Gutzwiller JP, Drewe J, Goke B, et al.** 1999 Glucagon-like peptide-1 promotes satiety and reduces food intake in patients with diabetes mellitus type 2. Am J Physiol 276:R1541–R1544
11. **Xu G, Stoffers DA, Habener JF, Bonner-Weir S** 1999 Exendin-4 stimulates β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276
12. **Scrocchi LA, Brown TJ, MacLusky N, et al.** 1996 Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide-1 receptor gene. Nat Med 11:1254–1258
13. **Pederson RA, Satkunarajah M, McIntosh CHS, et al.** 1998 Enhanced glucose-dependent insulinotropic polypeptide secretion and insulinitropic action in glucagon-like peptide 1 receptor −/− mice. Diabetes 47:1046–1052
14. **Edwards CMB, Todd J.F, Mahmoudi M, et al.** 1999 Glucagon-like peptide-1 has physiological role in the control of postprandial glucose in humans. Diabetes 48:86–93
15. **Tronier B, Dejgaard A, Andersen T, Madsbad S** 1985 Abscence of incretin effect in obese type II and diminished effect in lean type II and obese subjects [Abstract]. Diab Res Clin Prac 000(Suppl 1):s568
16. **Nauck M, Stöckmann F, Ebert R, Creutzfeldt W** 1986 Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. Diabetologia 29:46–52
17. **Krarup T** 1988 Immunoreactive gastric inhibitory polypeptide. Endocr Rev 9:122–134
18. **Elahi D, McAloon-Dyke M, Fukagawa NK, et al.** 1994 The insulinotropic actions of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (7–37) in normal and diabetic subjects. Regul Pept 51:63–74
19. **Nauck MA, Heimesaat MM, Ørskov C, Holst JJ, Ebert R, Creutzfeldt W** 1993 Preserved incretin effect of glucagon-like peptide 1 [7–36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabtes mellitus. J Clin Invest 91:301–307
20. **Fukase N, Igarashi M, Takahashi H, et al.** 1993 Hypersecretion of truncated glucagon-like peptide-1 and gastric inhibitory polypeptide in obese patients. Diab Med 10:44–49
21. **Ranganath LR, Beety JM, Morgan LM, Wright JW, Howland R, Marks V** 1996 Attenuated GLP-1 secretion in obesity: cause or consequence? Gut 38:916–919
22. **Fukase N, Manaka H, Sugiyama K, et al.** 1995 Response of truncated glucagon-like peptide-1 and gastric inhibitory polypeptide to glucose ingestion in non-insulin dependent diabetes mellitus. Effect of sulfonylurea therapy. Acta Diabetol 32:165–169
23. **Nauck M, Erpenstein A, Holst JJ, et al.** Release of GLP-1 [7–36 amide] after oral glucose in relation to glucose tolerance and sex. Proc of the 30th Annual Meet of the European Association for the Study of Diabetes. 1994; A118
24. **Vaag AA, Holst JJ, Vølund Aa, Beck-Nielsen H** 1996 Gut incretin hormones in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM): evidence for decreased glucagon-like peptide 1 secretion during oral glucose ingestion in NIDDM twins. Eur J Endocrinol 135:425–432
25. **Ørskov C, Jeppesen J, Madsbad S, Holst JJ** 1991 Proglucagon products in plasma from non-insulin dependent diabetics and non-diabetic controls in the fasting state and following oral glucose and intravenous arginine. J Clin Invest 87:415–423
26. **Naslund E, Gryback P, Backman L, et al.** 1998 Distal small bowel hormones: correlation with fasting antroduodenal motility and gastric emptying. Dig Dis Sci 43:945–952
27. **Ahrén B, Larsson H, Holst JJ** 1997 Reduced gastric inhibitory polypeptide but normal glucagon-like peptide 1 responses to oral glucose in postmenopausal women with impairs glucose tolerance. Eur J Endocrinol 137:127–131
28. **Bloom S, Till S, Sonksen P, Smith S** 1984 Use of a biothesiometer to measure individual vibration thresholds and their variation in 519 non-diabetic subjects. Br Med J Clin Res Ed 288:1793–1795
29. **Ewing DJ, Clarke BF** 1986 Autonomic neuropathy. Its diagnosis and prognosis. Clin Endocrinol Metab 15:855–889
30. **Anonymous** 1992 Proceedings of a consensus development conference on standardized measures in diabetic neuropathy. Summary and recommendations. Diabetes Care 15:1104–1107
31. **Ørskov C, Rabenhøj L, Wettergren A, Kofod H, Holst JJ** 1994 Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide 1 in humans. Diabetes 43:535–539
32. **Krarup T, Madsbad S, Moody AJ, et al.** 1983 Diminished immunoreactive gastric inhibitory polypeptide response to a meal in newly diagnosed type I (insulin-dependent) diabetics. J Clin Endocrinol Metab 56:1306–1312
33. **Krarup T, Holst JJ** 1984 The heterogeneity of gastric inhibitory polypeptide in porcine and human gastrointestinal mucosa evaluated with five different antisera. Regul Pept 9:335–346
34. **Schwartz TW, Holst JJ, Fahrenkrug J, et al.** 1978 Vagal, cholinergic regulation of pancreatic polypeptide secretion. J Clin Invest 61:781–789
35. **Petersen JS, Hejnæs KR, Moody A, et al.** 1994 Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. Diabetes 43:459–467
36. **Miles J, Glasscock R, Aikens J, Gerich J, Haymond M** 1983 A microflourometric method for the determination of free fatty acids in plasma. J Lipid Res 24:96–99
37. **Miyawaki K, Yamada Y, Ihara Y, Seino Y** 1999 Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. Proc Natl Acad Sci USA 96:14843–14847
38. **Roberge JN, Brubaker PL** 1993 Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop. Endocrinology 133:233–240
39. **Cordier-Bussat M, Bernard C, Levenez F, et al.** 1998 Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. Diabetes 47:1038–1045.l
40. **Rocca AS, Brubaker PL** 1999 Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. Endocrinology 140:1687–1694
41. **Ranganath L, Norris F, Morgan L, Wright J, Marks V** 1999 Inhibition of carbohydrate-mediated glucagon-like peptide-1 (7–36)amide secretion by circulating non-esterified fatty acids. Clin Sci 96:335–342
42. **Miholic J, Ørskov Ø, Holst JJ, Kotzerke J, Meyer HJ** 1991 Emptying of the gastric substitute, glucagon-like peptide-1 (GLP-1), and reactive hypoglycemia after total gastrectomy. Dig Dis Sci 36:1361–1370
43. **Qualmann C, Nauck MA, Holst JJ, Ørskov C, Creutzfeldt W** 1995 Glucagon-like peptide 1 (7–36 amide) secretion in response to luminal sucrose from the upper and lower gut. A study using α-glucosidase inhibition (acarbose). Scand J Gastroenterol 30:892–896
44. **Kong MF, Horowitz M** 1999 Gastric emptying in diabetes mellitus: relationship to blood-glucose control. Clin Geriatr Med 15:321–338
45. **Maddox A, Horowitz M, Wishart J, Collins P** 1989 Gastric and oesophageal emptying in obesity. Scand J Gastroenterol 24:593–598
46. **Hutson WR, Roehrkasse RL, Wald A** 1989 Influence of gender and menopause on gastric emptying and motility. Gastroenterology 96:11–17
47. **Wisén O, Johansson C** 1992 Gastrointestinal function in obesity: motility, secretion and absorption following a liquid test meal. Metabolism 41:390–395
48. **Deacon CF, Johnsen AH, Holst JJ** 1995 Degradation of glucagon-like peptide-1 by human plasma *in vitro* yields an N-terminally truncated peptide that is a major endogenous metabolite *in vivo*. J Clin Endocrinol Metab 80:952–957
49. **Ribel U, Larsen MO, Holst JJ, Deacon CF, Carr RD,** Dipeptidyl peptidase IV inhibitors: mechanism of action involves 'rescue' of the biologically intact version of both incretin hormones in the conscious pig. Proc of the 60th Scientific Sessions of Am Diabetes Assoc. 2000; A2
50. **Hansen L, Deacon CF, Ørskov C, Holst JJ** 1999 Glucagon-like peptide-1-(7–36)amide is transformed to glucagon-like peptide-1-(9–36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. Endocrinology 140:5356–5363
51. **Imeruz N, Yegen BC, Bozkurt A, Coskun T, Villanueva-Penacarrillo ML, Ulusoy NB** 1997 Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. Am J Physiol 273:G920–G927
52. **Nakabayashi H, Nishizawa M, Nakagawa A, Takeda R, Niijima A** 1996 Vagal hepatopancreatic reflex effect evoked by intraportal appearance of tGLP-1. Am J Physiol 271:E808–E813
53. **Balkan B, Li X** 2000 Portal GLP-1 administration in rats augments the insulin response to glucose via neuronal mechanisms. Am J Physiol 279:R1449–R1454