

Reduced Postprandial Concentrations of Intact Biologically Active Glucagon-Like Peptide 1 in Type 2 Diabetic Patients

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Incretin hormones importantly enhance postprandial insulin secretion but are rapidly degraded to inactive metabolites by ubiquitous dipeptidyl peptidase IV. The concentrations of the intact biologically active hormones remain largely unknown. Using newly developed assays for intact glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP), we measured plasma concentrations after a mixed breakfast meal (566 kcal) in 12 type 2 diabetic patients (age 57 years [range 49–67], BMI 31 kg/m² [27–38], and HbA_{1c} 9.2% [7.0–12.5]) and 12 matched healthy subjects. The patients had fasting hyperglycemia (10.7 mmol/l [8.0–14.8]) increasing to 14.6 mmol/l (11.5–21.5) 75 min after meal ingestion. Fasting levels of insulin and C-peptide were similar to those of the healthy subjects, but the postprandial responses were reduced and delayed. Fasting levels and meal responses were similar between patients and healthy subjects for total GIP (intact + metabolite) as well as intact GIP, except for a small decrease in the patients at 120 min; integrated areas for intact hormone (area under the curve [AUC]_{INT}) averaged 52 ± 4% (for patients) versus 56 ± 3% (for control subjects) of total hormone AUC (AUC_{TOT}). AUC_{INT} for GLP-1 averaged 48 ± 2% (for patients) versus 51 ± 5% (for control subjects) of AUC_{TOT}. AUC_{TOT} for GLP-1 as well as AUC_{INT} tended to be reduced in the patients ($P = 0.2$ and 0.07 , respectively); but the profile of the intact GLP-1 response was characterized by a small early rise (30–45 min) and a significantly reduced late phase (75–150 min) ($P < 0.02$). The measurement of intact incretin hormones revealed that total as well as intact GIP responses were minimally decreased in patients with type 2 diabetes, whereas the late intact GLP-1 response was strongly reduced, supporting the hypothesis that an impaired function of GLP-1 as a transmitter in the enteroinsular axis contributes to the inappropriate insulin secretion in type 2 diabetes. *Diabetes* 50:609–613, 2001

The intestinal hormones, glucose-dependent insulinotropic hormone (GIP) and glucagon-like peptide (GLP)-1, are both important messengers of the incretin effect, i.e., the enhanced insulin secretion after oral versus intravenous glucose administration (1). This has been clearly shown in animal experiments in which the genes encoding their receptors were deleted. In these experiments, the knockout animals had significantly reduced glucose tolerance compared with wild-type controls (2). Together, GIP and GLP-1 can completely account for the incretin effect (3). However, both hormones are quickly eliminated from the circulation, with their initial metabolism being mediated by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) (4–6). In addition to its localization in sites such as intestinal and renal brush border membranes, this enzyme is found also on the capillary surfaces and in a soluble form in plasma, giving it access to circulating hormones (7). It cleaves off the two NH₂-terminal amino acids, which, for both incretin hormones, abolishes biological activity (8,9). Whereas GLP-1 is rapidly degraded in the circulation, resulting in a clearance that exceeds cardiac output and an apparent half-life of 1–1.5 min (10), GIP is degraded more slowly, with a half-life for the intact hormone of 7 min (4). The truncated metabolites are eliminated more slowly, with half-lives of 4–5 and 17 min, respectively (4,10).

None of the existing assays for GIP in plasma distinguish between the intact hormone and its metabolites, and specific assays for intact GLP-1 have not been used for measurement of endogenous GLP-1. Although these non-discriminating assays are useful for the estimation of the rate of secretion of the hormones (which will be reflected in the sum of the concentrations of the intact hormones and their primary metabolites), they do not provide information about the impact of the hormones for the regulation of pancreatic islet secretion because this will be a function of the plasma concentrations of the intact biologically active hormones. Because the incretin function is known to be reduced or absent in type 2 diabetic patients (11), we in the present study measured the plasma concentrations of intact GLP-1 and GIP using newly developed assays to evaluate their potential roles for the incretin function in patients with type 2 diabetes and healthy subjects matched for age, sex, and BMI.

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AUC, area under the curve; DPP-IV, dipeptidyl peptidase IV; GIP, glucose-dependent insulinotropic polypeptide; GLP, glucagon-like peptide; RIA, radioimmunoassay.

RESEARCH DESIGN AND METHODS

We studied 12 type 2 diabetic patients (10 men, 2 women; mean age 57 years [range 48–69], BMI 30.6 kg/m² [26.0–37.7], and HbA_{1c} 9.2% [7.0–12.5]) and 12 matched healthy subjects (mean age 56 years [50–70], BMI 31.0 kg/m² [26.3–37.9], and HbA_{1c} 5.6% [5.2–6]). Seven patients were treated with diet alone, whereas five were treated with diet and oral antidiabetic medications (sulfonylureas and/or biguanides). Six patients had a history of hypertension and were treated with thiazides, ACE inhibitors, and/or calcium antagonists. All type 2 diabetic patients were diagnosed according to the criteria of the World Health Organization (12,13). None of the patients had impaired renal function (normal serum creatinine levels [$<130 \mu\text{mol/l}$] and no microalbuminuria), proliferative retinopathy, or impaired liver function. None of the healthy subjects had a family history of diabetes, and all had a normal oral glucose tolerance test. All agreed to participate and gave oral and written consent. The study was approved by the Copenhagen County Ethical Committee 16 May 1997 (journal numbers in the committee: KA 97035 m), and the study was conducted according to the principles of the Helsinki Declaration.

All oral antidiabetic medications were discontinued 72 h before the study. After an overnight fast (from 10:00 P.M.), one cannula was inserted into the cubital vein for blood sampling. The test meal consisted of 50 g white bread, 50 g black bread, 10 g butter, 40 g cheese, 20 g jam, and 200 ml milk (34% fat, 47% carbohydrate, and 19% protein), comprising a total of 566 kcal (2,370 kJ), and the meal was consumed within 15 min. Venous blood was drawn 15, 10, and 0 min before and 15, 30, 45, 60, 75, 90, 120, 150, and 180 min after ingestion of the meal. Blood samples were placed in fluoride tubes for measurement of plasma glucose and in chilled EDTA tubes with aprotinin (500 KIU/ml blood; Trasylol; Bayer, Leverkusen, Germany) for peptide analyses. Tubes were immediately cooled on ice and centrifuged within 20 min at 4°C, and plasma was stored at –20°C until analysis.

Analysis. Plasma glucose concentrations were measured during the experiment using a glucose oxidase method in a glucose analyzer (Yellow Springs Instrument Model 23 A; Yellow Springs Instruments, Yellow Springs, OH). C-peptide concentrations were determined by radioimmunoassay (RIA) as described by Heding (14) using the polyclonal antibody M1230 (15). The detection limit is ~60 pmol/l, the intra-assay coefficient of variation is 5%, and the interassay coefficient of variation is 7.3%. Plasma insulin concentrations were measured using commercial enzyme-linked immunosorbent assay kits (Dako, Copenhagen, Denmark). The sensitivity of the assay was ~3 pmol/l, and the intra- and interassay coefficients of variation were 4–10% at 39–1.240 pmol/l.

Total GIP was measured using the COOH-terminally directed antiserum R65 (16,17), which reacts fully with intact GIP and the NH₂-terminally truncated metabolite, GIP(3–42), but not with the so-called 8-kDa GIP, whose chemical nature and relation to GIP secretion is uncertain. The assay has a detection limit of <2 pmol/l and an intra-assay variation of ~6%. Intact biologically active GIP was measured using a newly developed assay. Antibodies were raised by immunizing rabbits with the synthetic sequence, GIP(1–10)-Cys (Genosys Biotechnologies [Europe], Cambridge, U.K.), coupled with keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, as described by Dyberg and Kofod (18). The assay is specific for the intact NH₂-terminus of GIP and cross-reacts <0.1% with GIP(3–42) or with the structurally related peptides GLP-1(7–36) amide, GLP-1(9–36) amide, GLP-2(1–33), GLP-2(3–33), or glucagon at concentrations of up to 100 nmol/l. Plasma samples were extracted with ethanol (70% vol/vol; final concentration), giving recoveries of synthetic GIP added to plasma of 84.8 ± 1.4%. Intra-assay variation was <6%, and interassay variations were ~8 and 12% for 20 and 80 pmol/l standards, respectively. Valine-pyrrolidide (0.01 mmol/l final concentration; a gift from Dr. L.B. Christensen, Novo Nordisk A/S, Bagsvaerd, Denmark) was added to the assay buffer to prevent NH₂-terminal degradation of GIP during the assay incubation. For both assays, human GIP (Peninsula Laboratories Europe, St. Helens, Meyerside, U.K.) was used as standard, and radiolabeled GIP was from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, U.K.). Separation of the bound from free peptides was achieved using plasma-coated charcoal (19). Plasma samples were assayed for GLP-1 immunoreactivity using RIAs that are specific for each terminus of the GLP-1 molecule: the NH₂-terminal assay measuring the concentration of intact surviving GLP-1 and the COOH-terminal assay measuring the sum of the intact peptide plus the primary metabolite. NH₂-terminal immunoreactivity was measured using antiserum 93242 (19), raised against proglucagon 78–87-Cys coupled with keyhole limpet hemocyanin as above, which cross-reacts ~10% with GLP-1(1–36) amide and <0.1% with GLP-1(8–36) amide and GLP-1(9–36) amide (19). The specificity of the assay was studied further by analysis of an acid-ethanol extract of porcine pancreas fractionated by gel filtration and assayed for the pancreatic proglucagon products as previously described (20). The assay identified three small peaks, one corresponding to glucagon and due to a 0.6% cross-reaction with this peptide, another corresponding to GLP-1(1–

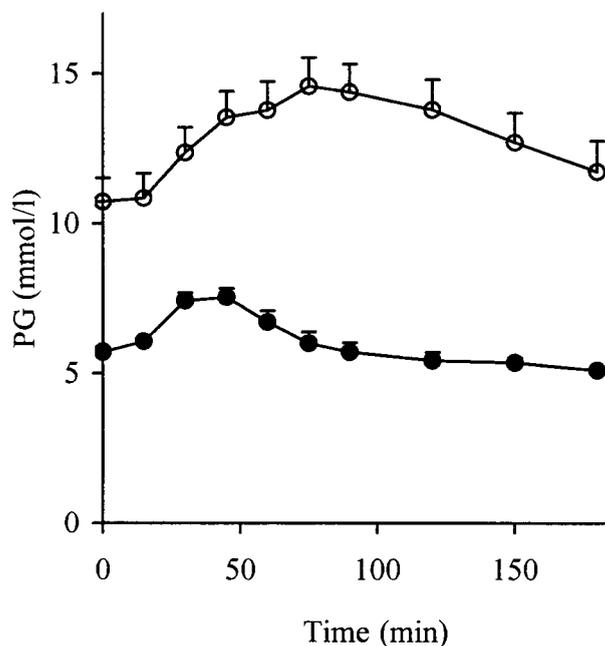


FIG. 1. Plasma glucose concentrations during the 180-min period after meal ingestion for type 2 diabetic patients (○) and healthy subjects (●). Data are means ± SE.

36) amide amounting to 10% of the concentration of this peptide when quantified by specific assays, and a final peak corresponding to 7.5% of the amount of proglucagon 72–158 (major proglucagon fragment) present at that position. In a similarly analyzed extract of porcine intestine, the assay exclusively identified fully processed GLP-1(7–36) amide and by 100% of the amount present (21). Standards and tracers were synthetic GLP-1(7–36) amide, and the specific activity of the tracer was 70 MBq/nmol. The assay has a detection limit of 2 pmol/l, an intra-assay variation of 6%, and an interassay variation of 16% at 40 pmol/l. Plasma was extracted with ethanol, and valine-pyrrolidide was added to the assay buffer as above. Recovery of added GLP-1 deviated <15% from the values expected when a 30% loss inherent in the extraction procedure was taken into account. For this recovery, addition of valine-pyrrolidide was essential. A 72-h preincubation of antiserum and sample/standard at 4°C was allowed before tracer addition (for 48 h) to enhance sensitivity. For separation, the charcoal method was used.

COOH-terminal immunoreactivity of GLP-1 was measured as described previously (22) using standards of synthetic GLP-1(7–36) amide (i.e., proglucagon 78–107 amide) and antiserum 89390. The assay cross-reacts <0.01% with COOH-terminally truncated fragments and 83% with GLP-1(9–36) amide and has a detection limit <1 pmol/l. Intra- and interassay coefficients of variation were below 6 and 15%, respectively, at 40 pmol/l.

Statistical analysis and calculations. All results are presented as means ± SE. Statistical analysis was carried out as two-factor analysis of variance for repeated measurements with post hoc contrasting of patient results versus healthy subjects using the software Statistica. Areas under the curve (AUCs) were calculated using the trapezoidal rule and compared using the *t* test for paired data.

RESULTS

Fasting plasma glucose was 10.7 mmol/l (8.0–14.8) in the type 2 diabetic patients and 5.7 mmol/l (5.0–6.2) in the healthy subjects (Fig. 1). Plasma glucose increased during the first 75 min in the patient group, with a peak concentration at 14.6 mmol/l (11.5–21.5). In the healthy subjects, peak concentrations of plasma glucose occurred at 45 min and amounted to 7.5 mmol/l (6.0–9.2).

Time courses of insulin and C-peptide concentrations are presented in Fig. 2. After meal ingestion, peak insulin and C-peptide concentrations (in parentheses) occurred at 90 (75) minutes and amounted to 257 ± 37 pmol/l (1,935 ± 209) in the patient group. Corresponding results were 482 ± 76

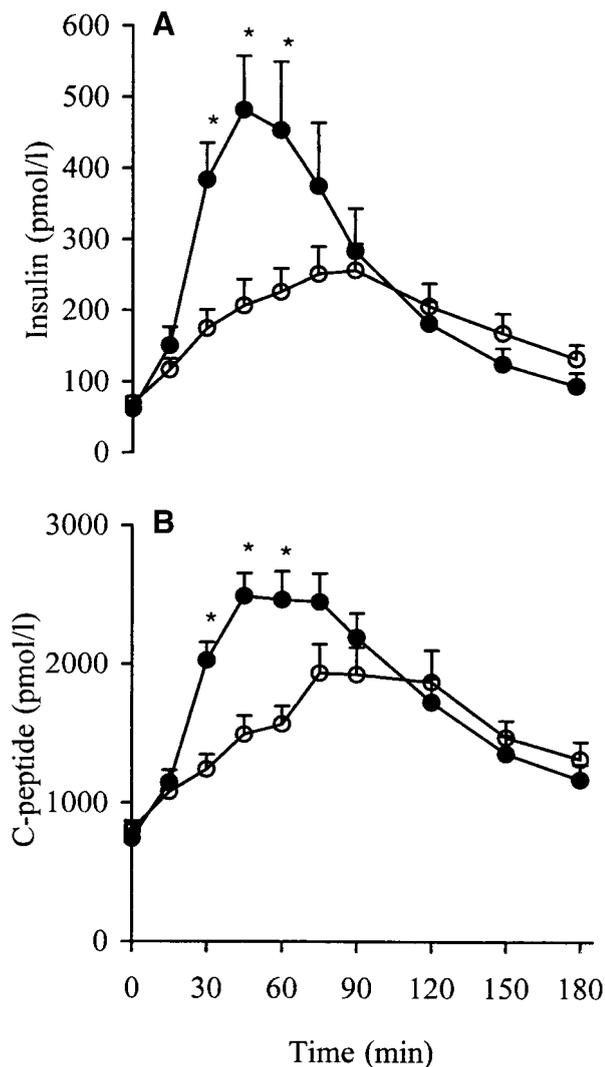


FIG. 2. Increase in plasma concentrations of insulin (A) and C-peptide (B) for type 2 diabetic patients (○) and healthy subjects (●) after ingestion of a mixed breakfast meal. Data are means \pm SE. * $P < 0.05$ for differences between type 2 diabetic patients and healthy subjects.

pmol/l ($2,491 \pm 165$), respectively, in the healthy subjects, with peak concentrations for both insulin and C-peptide occurring 45 min after meal ingestion. Both insulin and C-peptide concentrations were approaching basal levels at the end of the experiment. Analysis of variance for repeated measurements between the two groups followed by post hoc comparison showed significant differences between insulin and C-peptide responses in the two groups (Fig. 2).

In the type 2 diabetic patients, the mean basal plasma GLP-1 concentration was 7 ± 1 pmol/l measured using COOH-terminal GLP-1 assay and 7 ± 1 pmol/l with NH₂-terminal GLP-1 assay. Corresponding results in the healthy subjects were 11 ± 1 and 9 ± 1 pmol/l, respectively (Fig. 3). In the patient group, total GLP-1, representing the intact peptide plus the primary metabolite, increased to a peak value of 21 ± 1 pmol/l at 45 min and then slowly declined. In the control group, peak GLP-1 concentrations (26 ± 3 pmol/l) occurred at 120 min. The plasma concentrations of total GLP-1 differed significantly between patients and control subjects at 90 and 120 min ($P = 0.061$ and 0.012 , respectively).

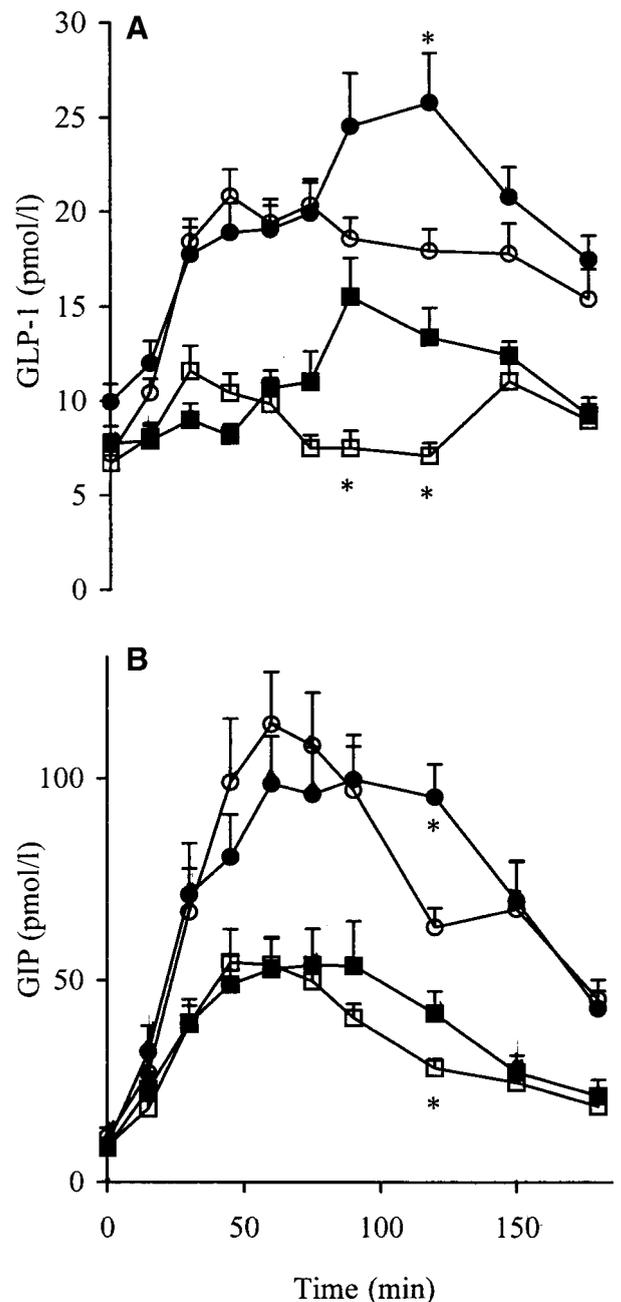


FIG. 3. GLP-1 concentrations (A) and GIP concentrations (B) using COOH-terminal assays; patients (○) and healthy subjects (●). NH₂-terminal assays; patients (□) and healthy subjects (■). Data are means \pm SE. * $P < 0.05$ for differences between type 2 diabetic patients and healthy subjects.

For intact GLP-1 (NH₂-terminal), a small but significant (analysis of variance) increase was seen in the patients during the first 30 min after meal ingestion, peaking at 12 ± 1 pmol/l and decreasing to fasting levels rapidly thereafter. In the control group, intact GLP-1 also increased significantly, but more slowly, peaking at 90 min (16 ± 2 pmol/l). The plasma concentrations of intact GLP-1 differed significantly between patients and healthy subjects at 75, 90, and 120 min ($P = 0.056$, 0.017 , and 0.017 , respectively). Total GLP-1 AUCs (mean \pm SE) were $3,113 \pm 167$ pmol \cdot min⁻¹ \cdot l⁻¹ in the type 2 diabetic patients and $3,599 \pm 277$ pmol \cdot min⁻¹ \cdot l⁻¹ in the healthy subjects

($P = 0.2$). For intact GLP-1 AUCs, the corresponding results were $1,606 \pm 133$ and $1,976 \pm 136$ pmol \cdot min $^{-1} \cdot$ l $^{-1}$ ($P = 0.07$), respectively.

The basal plasma GIP concentration was 11 ± 2.4 pmol/l in the patient group measured with COOH-terminal GIP and 9 ± 0.9 pmol/l with NH $_2$ -terminal GIP. Corresponding results for the healthy subjects were 9 ± 2 and 9 ± 1 pmol/l, respectively (Fig. 3). Peak concentrations of COOH- and NH $_2$ -terminal GIP occurred at 60 and 45 min in the patient group, respectively, after meal ingestion and amounted to 113 ± 13 and 55 ± 8 pmol/l. In the healthy subjects, the corresponding results were 100 ± 11 and 54 ± 9 pmol/l, respectively, with peak concentrations at 90 and 75 min. The plasma concentrations of total and intact GIP differed significantly between patients and control subjects at 120 min ($P = 0.025$ and 0.031 , respectively). Total GIP AUCs (mean \pm SE) were $13,092 \pm 1,268$ pmol \cdot min $^{-1} \cdot$ l $^{-1}$ in the type 2 diabetic patients and $13,597 \pm 1,342$ pmol \cdot min $^{-1} \cdot$ l $^{-1}$ in the healthy subjects (NS). For intact GIP AUCs, the corresponding results were $6,092 \pm 493$ and $6,938 \pm 942$ pmol \cdot min $^{-1} \cdot$ l $^{-1}$ (NS). In both groups, both GLP-1 and GIP concentrations returned toward basal levels at the end of the experiment.

DISCUSSION

This investigation revealed that the concentrations of intact GIP and GLP-1 were markedly lower than those measured with conventional assays, i.e., assays that cannot distinguish between the intact hormones and the metabolites resulting from degradation mediated by DPP-IV, illustrating the importance of using specific assays for measurement of the biologically active hormones. For GIP, approximately half of the total GIP concentration was constituted by intact GIP for both diabetic patients and healthy subjects, and this ratio did not change significantly throughout the 180 min of the investigation. The kinetics of GIP secretion revealed an insignificant tendency toward a more pronounced early rise and a decreased late phase in the patients just reaching significance at 120 min. The differences were small and probably of limited biologic significance. In a previous investigation of exogenous GIP, $\sim 45\%$ of the infused peptide was present in the intact form during 60 min of infusion (4). Thus, in the human studies of the physiological actions of GIP carried out so far, in which exogenous GIP was infused to concentrations mimicking meal responses of the endogenous hormone, these levels and hence the amount of peptide infused may have been at least twice as high as the true levels of intact hormone. However, the error generated by this approach is likely to be small because both exogenous and endogenous GIP are being metabolized at apparently similar rates. This relationship cannot, however, be expected to be constant under all circumstances, e.g., in patients with renal diseases, where differences in elimination of the two molecular forms might occur. Furthermore, in future studies of the function of GIP as an incretin hormone, e.g., in those using correlation analysis, it will be important to include the concentrations of the intact hormone rather than the intact plus inactive GIP. The degradation of GIP in plasma *in vitro* is very slow ($t_{1/2} = 75$ min) and is unlikely to explain why 50% of the hormone circulates in a truncated form. Rather, DPP-IV localized in the endothelial wall throughout the circulatory system may be responsi-

ble. However, further studies using the assay for the intact hormone are required to determine whether particular organs are predominantly involved in the degradation. Finally, truncated GIP has been extracted from the small intestinal mucosa (23), raising the possibility that part of the circulating metabolite is actually derived from the gut, as was recently reported for GLP-1 (21). The rate of secretion of GIP can be judged by both assays, but the secretory activity of the K-cell is probably best estimated by assay for total GIP because it reflects the total amount of GIP released, although the different $t_{1/2}$ for total and intact GIP will impede the interpretation of the results.

For GLP-1, the concentration of intact hormone was also $\sim 50\%$ of the total concentration, and the kinetics of intact and total GLP-1 showed similarities, at least for healthy subjects. However, compared with control subjects, a dramatic difference with respect to intact GLP-1 emerged for the type 2 diabetic patients in that healthy subjects showed a significant and prolonged response with a maximum around 90–120 min, whereas the intact GLP-1 concentrations between 75 to 120 min in the patients were in the preprandial level. It has previously been suspected that type 2 diabetic patients have an impaired secretion of GLP-1 particularly in the late phase of the meal response, as measured using a nondiscriminating assay (24). The impairment was similar to that observed here, where a significant difference between patients and healthy subjects occurred between 90 and 150 min. The markedly reduced response of intact GLP-1 in the patients observed here indicates that the impairment of GLP-1 secretion in type 2 diabetes is more severe than could be expected from the COOH-terminal response. Therefore, it must be suspected that impaired secretion of intact GLP-1 contributes to the impaired incretin effect and therefore probably also inappropriate secretion of insulin in type 2 diabetes. In our patients, insulin and C-peptide levels were similar in the late phase of the meal, when intact GLP-1 levels differed the most. However, because of their hyperglycemia, this insulin response must be considered inadequate and illustrates their β -cell secretory deficiency. Because it is well documented that GLP-1 significantly stimulates and may even restore to normal glucose-induced insulin secretion in patients with diabetes (25,26), it may be concluded that reductions in GLP-1 secretion, all others being equal, are likely to result in an impaired insulin secretion, or, in other words, had the GLP-1 response been normal, then the insulin response would have been greater and therefore more appropriate. Thus, the impaired GLP-1 response may explain part of the incretin deficiency in type 2 diabetes and thereby contribute to the inappropriate insulin secretion. On the other hand, the deficiency is probably a consequence of the diabetic condition rather than being a primary factor because it has been shown in identical twins discordant for diabetes that total GLP-1 secretion is lower in the diabetic twin (27) and that 24-h GLP-1 secretion is normal in first-degree relatives of diabetic patients (28).

As mentioned above, a significant proportion of newly secreted GLP-1 is degraded before it is released from the gut (21). Possibly, the diabetic state may both lead to an inhibition of L-cell secretion and an increase in the degradative activity of the gut. Thus, in a recent study (24) of a

large heterogeneous group of patients, postprandial total GLP-1 secretion was inversely related to the diabetic state and BMI (24), the latter of which could not apply here. Plasma DPP-IV activity was also measured and was not increased (24), but this may not necessarily reflect the tissue DPP-IV activity.

In various published reports over the last decade, GLP-1 concentrations have been reported to be both increased, unchanged, and decreased (29). The discrepancies probably mainly reflect the differences with respect to assay specificity, with the early assays reacting with all GLP-1-containing moieties including pancreatic forms (which will reflect the well-known hypersecretion of pancreatic proglucagon products in type 2 diabetes [30]) and subsequent assays reacting not only with intact GLP-1 but also its inactive metabolites. The marked difference between the concentrations of total and intact GLP-1 as well as the different response pattern uncovered here illustrate the importance of measuring the intact hormone when attempting to estimate the biologic impact of the endogenous hormone.

For both GIP and GLP-1, the differences regarding intact and total hormone in the fasting state were small and insignificant. However, the fasting values were low and close to the detection limits of the assays. Further studies using concentrated plasma samples and chromatographic analysis, as previously carried out for GLP-1 on single samples from healthy subjects (31), will probably be required to estimate the relationship between intact hormone and metabolites in the fasting state.

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