

# Glucose-Dependent Insulinotropic Polypeptide

## A Bifunctional Glucose-Dependent Regulator of Glucagon and Insulin Secretion in Humans

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**OBJECTIVE**—To evaluate the glucose dependency of glucose-dependent insulinotropic polypeptide (GIP) effects on insulin and glucagon release in 10 healthy male subjects (means  $\pm$  SEM) aged  $23 \pm 1$  years, BMI  $23 \pm 1$  kg/m<sup>2</sup>, and HbA<sub>1c</sub>  $5.5 \pm 0.1\%$ .

**RESEARCH DESIGN AND METHODS**—Saline or physiological doses of GIP were administered intravenously (randomized and double blinded) during 90 min of insulin-induced hypoglycemia, euglycemia, or hyperglycemia.

**RESULTS**—During hypoglycemia, GIP infusion caused greater glucagon responses during the first 30 min compared with saline ( $76 \pm 17$  vs.  $28 \pm 16$  pmol/L per 30 min,  $P < 0.008$ ), with similar peak levels of glucagon reached after 60 min. During euglycemia, GIP infusion elicited larger glucagon responses ( $62 \pm 18$  vs.  $-11 \pm 8$  pmol/L per 90 min,  $P < 0.005$ ). During hyperglycemia, comparable suppression of plasma glucagon ( $-461 \pm 81$  vs.  $-371 \pm 50$  pmol/L per 90 min,  $P = 0.26$ ) was observed with GIP and saline infusions. In addition, during hyperglycemia, GIP more than doubled the insulin secretion rate ( $P < 0.0001$ ).

**CONCLUSIONS**—In healthy subjects, GIP has no effect on glucagon responses during hyperglycemia while strongly potentiating insulin secretion. In contrast, GIP increases glucagon levels during fasting and hypoglycemic conditions, where it has little or no effect on insulin secretion. Thus, GIP seems to be a physiological bifunctional blood glucose stabilizer with diverging glucose-dependent effects on the two main pancreatic glucoregulatory hormones. *Diabetes* 60:3103–3109, 2011

The regulation of pancreatic islet function is crucial in glucose homeostasis (1). Despite intensive research, the regulation and function of the pancreatic  $\alpha$ - and  $\beta$ -cells in health and disease remain enigmatic (2). During the past 30 years, the involvement of several gut-derived peptides in the regulation of pancreatic islet secretion has been progressively uncovered. One such gut-derived factor is glucose-dependent insulinotropic polypeptide (GIP), a polypeptide hormone secreted from the small-intestinal K cells in response to nutrient intake (3). GIP is well founded as an incretin hormone potentiating insulin release from  $\beta$ -cells in healthy humans (4–6). In addition to insulinotropic effects, early

studies also delineated the glucagon-releasing properties of GIP by demonstrating that GIP stimulates glucagon secretion from  $\alpha$ -cells in the perfused rat pancreas at glucose concentrations  $<5.5$  mmol/L (4). However, a subsequent study was not able to reproduce the glucagon-releasing properties in humans during fasting and hyperglycemic conditions (5). Thus, additional investigations of the potential glucagonotropic effect of GIP was not pursued for nearly 25 years, until Meier et al. (7) used an improved immunoassay and demonstrated that GIP has dose-dependent glucagon-releasing properties when administered as bolus injections to healthy humans during euglycemia (plasma glucose [PG] 5.7 mmol/L). In contrast, using the same immunoassay, Vilsbøll et al. (8) reported GIP to be without glucagon-releasing properties when administered as a physiological infusion (1.5 pmol/kg/min for 30 min) in healthy humans clamped at euglycemia (5.1 mmol/L) as well as slightly elevated glucose levels (6 and 7 mmol/L, respectively). During overt hyperglycemic conditions, several studies have shown that GIP infusion does not have a glucagonotropic effect in healthy subjects (8–10). Consequently, although the relevance of GIP as an insulinotropic hormone in healthy individuals seems unquestionable, controversy exists regarding the glucagonotropic effects of GIP. We hypothesized that the glucagonotropic effects of GIP, such as its insulinotropic effects, are glucose dependent. Therefore, we aimed to evaluate, in the same individuals, the effects of GIP on plasma concentrations of glucagon and insulin at three distinct glycemic levels: hypoglycemia, euglycemia, and hyperglycemia.

### RESEARCH DESIGN AND METHODS

**Study protocol.** The study was approved by the scientific-ethical committee of the capital region of Denmark (reg. no. H-D-2009-0078), is registered with clinicaltrials.gov (clinical trial reg. no. NCT01048268), and was conducted according to the principles of the Helsinki Declaration II. Written informed consent was obtained from all participants before inclusion.

**Subjects.** Ten healthy male subjects were included in the study, following a screening visit. Subject characteristics are presented in Table 1. At the screening visit, all potential subjects ( $n = 12$ ) underwent a physical examination and had standard hematological and clinical biochemistry parameters measured. Urine was sampled to determine the albumin-to-creatinine ratio. Exclusion criteria were having acute or chronic illnesses, taking ongoing medication, having first-degree relatives with diabetes, and showing repeated paraclinical abnormalities in hemoglobin, plasma liver enzymes (alanine or aspartate aminotransferases), creatinine concentration, or the urinary albumin-to-creatinine ratio.

**Design of the study.** Each subject underwent 6 experimental days (carried out in randomized order following a prespecified random-numbers table) within a 2-month period. Thus, for each subject, GIP or placebo (saline) was infused on 2 days during euglycemic clamps, on 2 days during 12 mmol/L hyperglycemic clamps, and on 2 days during hypoglycemic clamps, aiming at a PG level of 2.5 mmol/L. Subjects were instructed to maintain a carbohydrate-rich diet (at least 250 g of carbohydrate daily) and avoid alcohol and excessive eating for 3 days before each experimental day. In the morning of each experimental day, GIP or placebo (saline) was prepared (by M.C.) for infusion

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TABLE 1  
Subject characteristics

Characteristics (n = 10 male)	Median	Range
Age (years)	22	19–30
Height (m)	1.83	1.72–1.91
Weight (kg)	75	65–88
BMI (kg/m <sup>2</sup> )	22.3	19.8–25.4
Systolic blood pressure (mmHg)	130	113–142
Diastolic blood pressure (mmHg)	74	68–86
Pulse (beats per minute)	66	48–79
Fasting PG (mmol/L)	5.3	4.5–5.8
HbA <sub>1c</sub> (%)	5.4	5.1–5.8
Diabetes in family	None	—
Islet cell or GAD-65 antibodies	None	—

by mixing it with 1% human albumin in 0.9% saline. Subjects arrived at the laboratory after an overnight (10-h) fast having avoided strenuous physical activity the day before. Subjects were placed in a recumbent position and had a cannula inserted into a dorsal hand vein. The hand was placed in a heating box (55°C) throughout the experiment for the collection of arterialized blood samples. Another cannula was inserted into a contralateral cubital vein for glucose and hormone infusions through separate infusion lines. At time 0 min, a continuous intravenous infusion of either GIP (4 pmol/kg/min) or placebo (saline) was initiated. At time 15 min, the infusion rate was halved, and at time 60 min, the infusion was stopped. The variation in infusion rate was done with the intention to reproduce physiological concentrations of GIP normally observed after the ingestion of a mixed meal. The bolus in mL 50% glucose (wt/vol) administered at time 0 of the hyperglycemic clamp to elevate PG to 12 mmol/L was calculated as follows: (12 mmol/L – fasting plasma glucose) × 35 mg glucose × body weight (in kilograms). During the hypoglycemic clamp, insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) mixed with 1% human albumin was infused at a rate of 1.5 mU/kg/min for the entire study period (90 min). PG was measured bedside every 5 min, allowing the PG level to be clamped by an adjustable continuous infusion of 20% glucose (wt/vol).

**Peptides.** Synthetic GIP (PolyPeptide Laboratories, Wolfenbüttel, Germany) was prepared for infusion in humans by the independent capital region pharmacy in Denmark. The peptide was dissolved in sterilized water containing 2% human albumin (Statens Serum Institut, Copenhagen, Denmark), subjected to sterile filtration, and dispensed into vials similarly labeled and containing the

same volume as the placebo (saline) vials. All vials were subsequently blinded (by L.V.) by adding new labeling with numbers from the prespecified random-numbers table and stored frozen under sterile conditions until the day of the experiment.

**Blood specimens.** Arterialized blood was drawn at times –10, 0, 5, 10, 20, 30, 45, 60, and 90 min and distributed into chilled tubes containing EDTA plus aprotinin (500 KIU/mL blood; Trasylol, Bayer, Leverkusen, Germany) and a specific dipeptidyl peptidase 4 inhibitor (valine-pyrrolidide; final concentration of 0.01 mmol/L) for analyses of glucagon and intact GIP. For analyses of insulin and C-peptide, blood was distributed into chilled tubes containing heparin. All tubes were immediately cooled on ice and centrifuged for 20 min at 1,200g and 4°C. Plasma for GIP and glucagon analyses was stored at –20°C, and plasma samples for insulin and C-peptide analyses were stored at –80°C until analysis. For bedside measurement of PG, blood was distributed into fluoride tubes and centrifuged at room temperature immediately for 2 min at 7,400g.

**Analytical procedures.** PG concentrations were measured by the glucose oxidase method, using a glucose analyzer (model 2300 STAT Plus Analyzer; YSI, Yellow Springs, OH). Plasma concentrations of GIP and glucagon were measured by specific radioimmunoassay, as previously described (11). Plasma insulin and C-peptide concentrations were measured using a two-sided electrochemoluminescence immunoassay (Roche/Hitachi modular analytics; Roche Diagnostic, Mannheim, Germany).

**Statistical analyses and calculations.** Results are reported as means ± SEM, unless otherwise stated. A two-sided P value <0.05 was used to indicate significant differences. When single P values were available, they were expressed instead of stating nonsignificance (NS). Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Repeated-measures ANOVA, followed by Bonferroni posttests, were applied to test for differences in repeatedly measured values between days (i.e., absolute PG and hormone concentrations). Area under the curve (AUC) values and incremental AUC (iAUC) values (i.e., baseline levels subtracted) were calculated using the trapezoidal rule. For paired comparisons between single values (e.g., between AUC values and baseline values, respectively), we used paired t tests or one-way ANOVA followed by Bonferroni posttests, if applicable. The insulin secretion rate (ISR) values were calculated by deconvolution of measured C-peptide concentrations and the application of population-based parameters for C-peptide kinetics, as previously described (12–14). ISR is expressed as picomoles of insulin secreted per minute per kilogram body weight.

RESULTS

**Glucose.** Mean PG concentrations during each of the 6 experimental days are displayed in Fig. 1, upper panel. Baseline PG concentrations did not differ between study days,

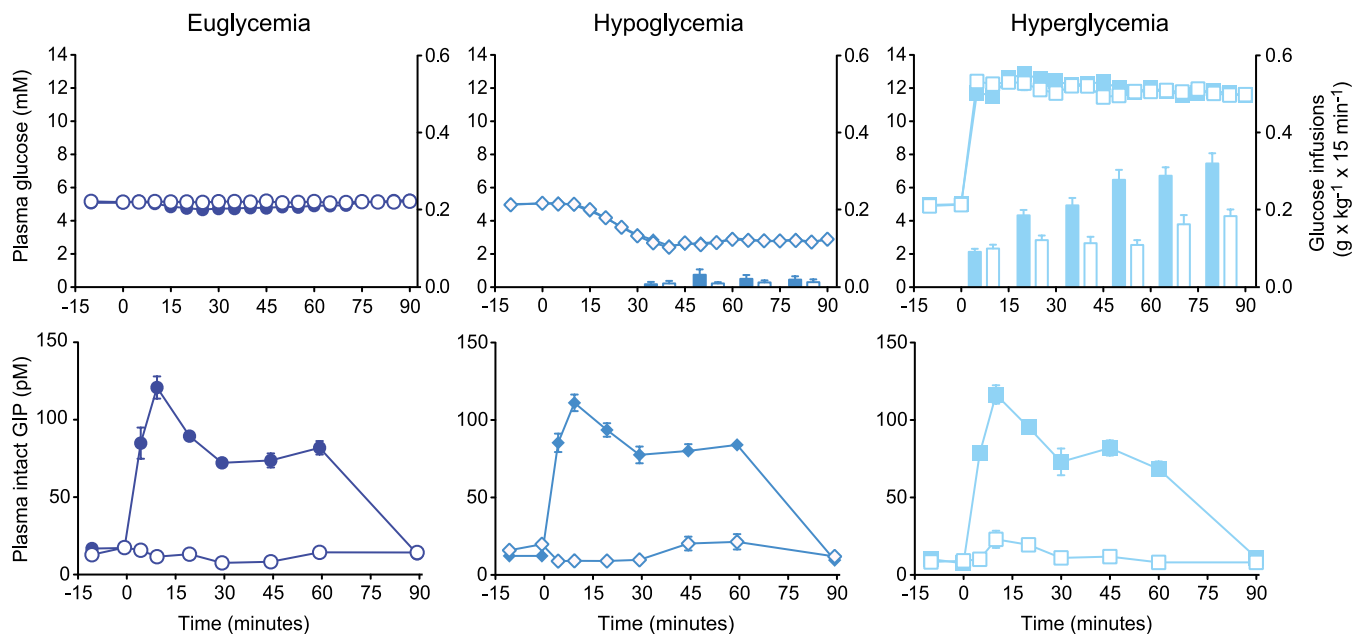


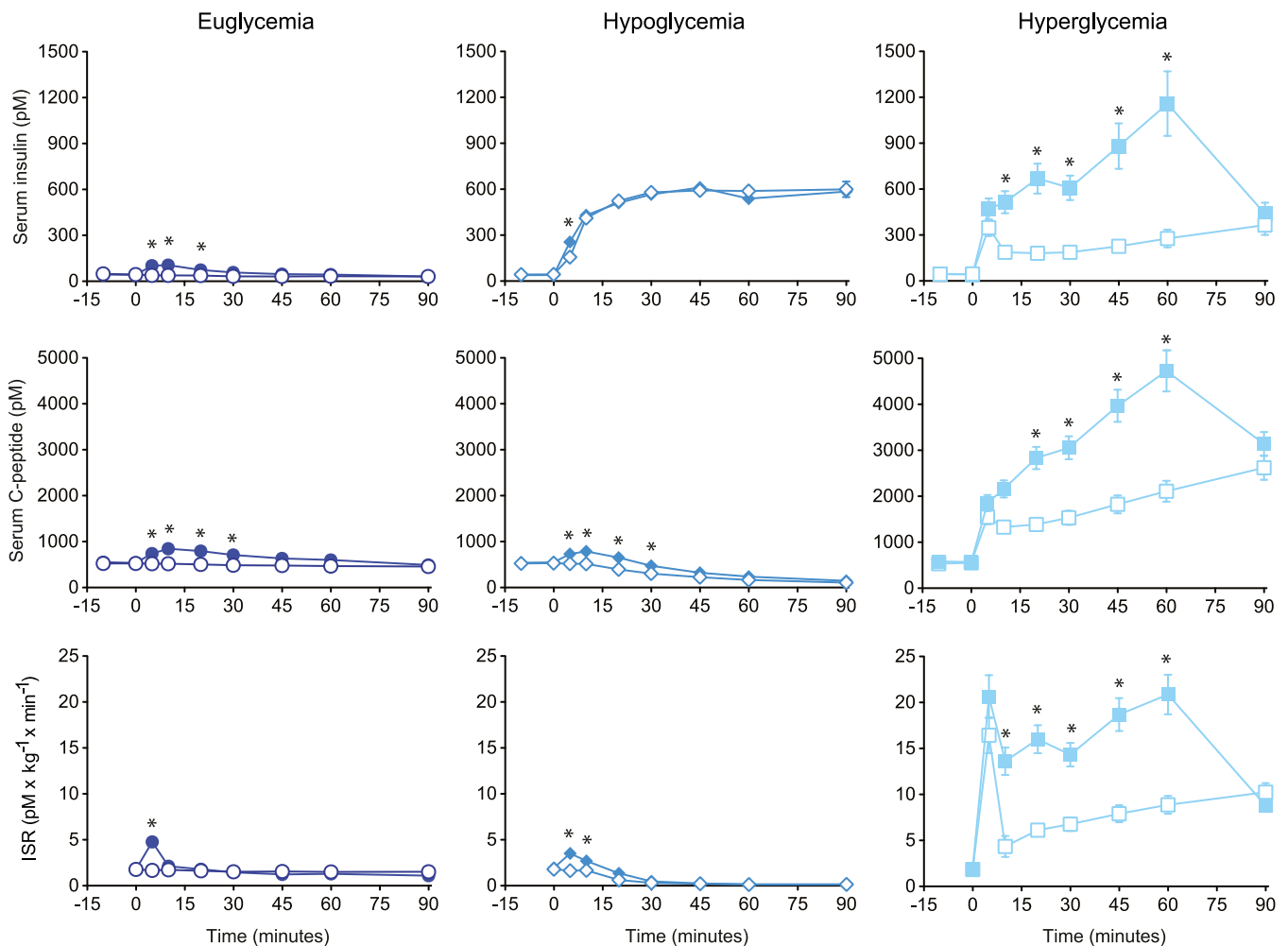
FIG. 1. Glucose and GIP plasma concentrations of glucose (upper panel) and GIP (lower panel) during euglycemia (dark blue curves, circles), hypoglycemia (blue curves, diamonds), and hyperglycemia (turquoise curves, squares) on days with GIP infusions (filled symbols) and days with saline infusion (open symbols). Concomitant glucose infusions (grams per body weight per 15-min time intervals) are depicted as bar graphs in the upper panel. Data are means ± SEM.

and the overall mean value amounted to  $5.0 \pm 0.1$  mmol/L. PG concentrations were similar during the paired days of similar glycemia ( $P = \text{NS}$ ). During the days with euglycemia, fasting PG levels were maintained throughout the study without glucose infusions. During the 2 days with hypoglycemia, mean PG declined similarly to a nadir of 2.4 mmol/L at 40 min, and similar amounts of glucose (51 and 53 mg glucose/kg for GIP and saline, respectively,  $P = 0.95$ ) were infused to clamp PG between 2 and 3 mmol/L for the remainder of the experiments. On the hyperglycemic days, fasting PG levels were raised to mean levels of  $12.1 \pm 0.3$  mmol/L (days combined), which were maintained throughout the study days using 786 and 1,372 mg glucose/kg ( $P = 0.01$ ) on saline and GIP infusion days, respectively.

**GIP.** Time courses for plasma concentrations of intact GIP are shown in Fig. 1, *lower panel*. Baseline values varied slightly between study days, with no significant difference between paired GIP and saline days with similar glycemic levels ( $P = \text{NS}$ ). During GIP infusions, plasma GIP concentrations reached high physiological (postprandial) levels with similar ( $P = 0.55$ ) peak values of  $121 \pm 8$  pmol/L (euglycemic clamp),  $111 \pm 6$  pmol/L (hypoglycemic clamp), and  $117 \pm 7$  pmol/L (hyperglycemic clamp). No significant

changes in GIP concentrations occurred during saline infusions ( $P = \text{NS}$ ).

**Insulin and ISR.** Time courses for serum insulin, C-peptide concentrations, and ISR values are presented in Fig. 2. Similar fasting values of insulin and C-peptide were observed on all experimental days. GIP infusion during euglycemic conditions resulted in a short-lasting (0–5 min) increment in ISR compared with saline ( $4.8 \pm 0.5$  vs.  $1.7 \pm 0.2$  pmol/L per kg/min,  $P < 0.0002$ ). Afterward, endogenous insulin secretion fell to, and remained at, basal levels. On the days of insulin-induced hypoglycemia, insulin levels rose similarly to plateau levels of  $\sim 600$  pmol/L (Fig. 2, *upper panel*). Initially (0–10 min), before PG started to decline, endogenous insulin secretion, assessed as ISR, was greater during GIP infusion compared with saline ( $3.5 \pm 0.7$  vs.  $1.6 \pm 0.2$  pmol/L per kg/min,  $P < 0.02$ ), but thereafter endogenous insulin secretion was suppressed (Fig. 2, *lower panel*). As illustrated in Fig. 2, establishment of the 12 mmol/L hyperglycemic clamp induced classical first- and second-phase insulin responses (as assessed by serum insulin concentrations and ISR values) during saline infusion. During concomitant GIP infusion, both first-phase ISR (time 5 min:  $20.6 \pm 2.3$  [GIP] vs.  $16.4 \pm 2.0$  [saline] pmol/L per kg/min,  $P < 0.052$ ), and especially second-phase



**FIG. 2.** Insulin, C-peptide, and ISR plasma concentrations of insulin (*upper panel*), C-peptide (*middle panel*), and ISR derived by deconvolution analysis (*lower panel*) over 90 min of GIP infusions (filled symbols) and saline infusions (open symbols) during euglycemia (dark blue curves, circles), insulin-induced hypoglycemia (blue curves, diamonds), and hyperglycemia (turquoise curves, squares). Data are means  $\pm$  SEM. Statistical analysis was done by repeated-measures ANOVA. \*Significant differences ( $P < 0.05$ ).

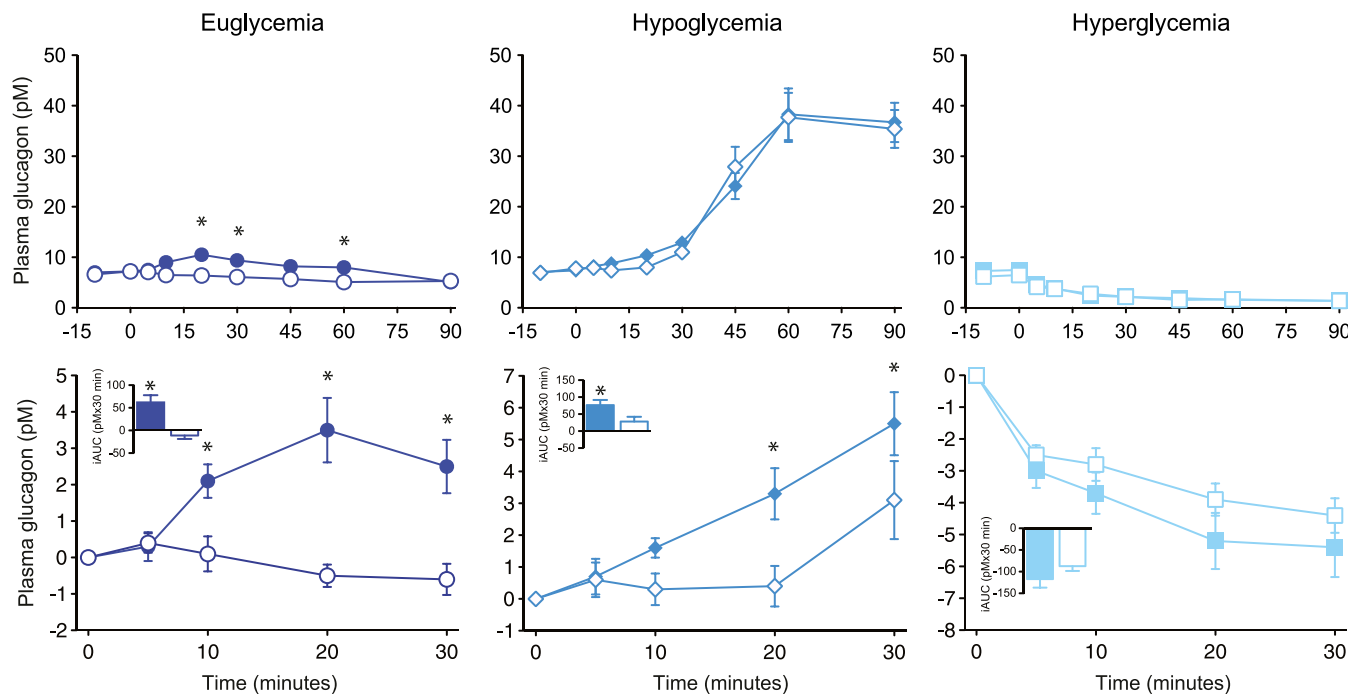
ISR (time 45 min:  $18.7 \pm 1.8$  [GIP] vs.  $7.9 \pm 0.9$  [saline] pmol/L per kg/min,  $P < 0.00001$ ), were potentiated (Fig. 2). **Glucagon.** Time courses for plasma glucagon concentrations during the 6 experimental days are presented in Fig. 3. Fasting levels of glucagon were similar on all study days. During the days of euglycemia, GIP infusion resulted in greater plasma glucagon concentrations at all time points from 10 to 60 min compared with saline infusions, and iAUC values amounted to  $86 \pm 44$  pmol/L per 90 min (GIP) and  $-100 \pm 21$  pmol/L per 90 min (saline), respectively ( $P = 0.003$ ). During the days of hypoglycemia, similar peak levels of glucagon were reached after 60 min (mean  $C_{max}$   $38 \pm 5$  [GIP] and  $37.7 \pm 5$  [saline] pmol/L, respectively,  $P = 0.81$ ). The iAUCs for the entire 90-min study period were similar ( $1,512 \pm 195$  vs.  $1,467 \pm 224$  pmol/L per 90 min,  $P = 0.72$ ). However, for the clinically relevant first half-hour of the hypoglycemic experiments (Fig. 3), the iAUC values during GIP and saline infusion, respectively, differed significantly ( $76 \pm 15$  and  $28 \pm 14$  pmol/L per 30 min,  $P = 0.02$ ). Upon establishment of the hyperglycemic clamps, plasma glucagon was suppressed (and remained so until the end of the experiments) with no effect of GIP compared with saline ( $-461 \pm 81$  vs.  $-371 \pm 50$  pmol/L per 90 min,  $P = 0.26$ ).

**DISCUSSION**

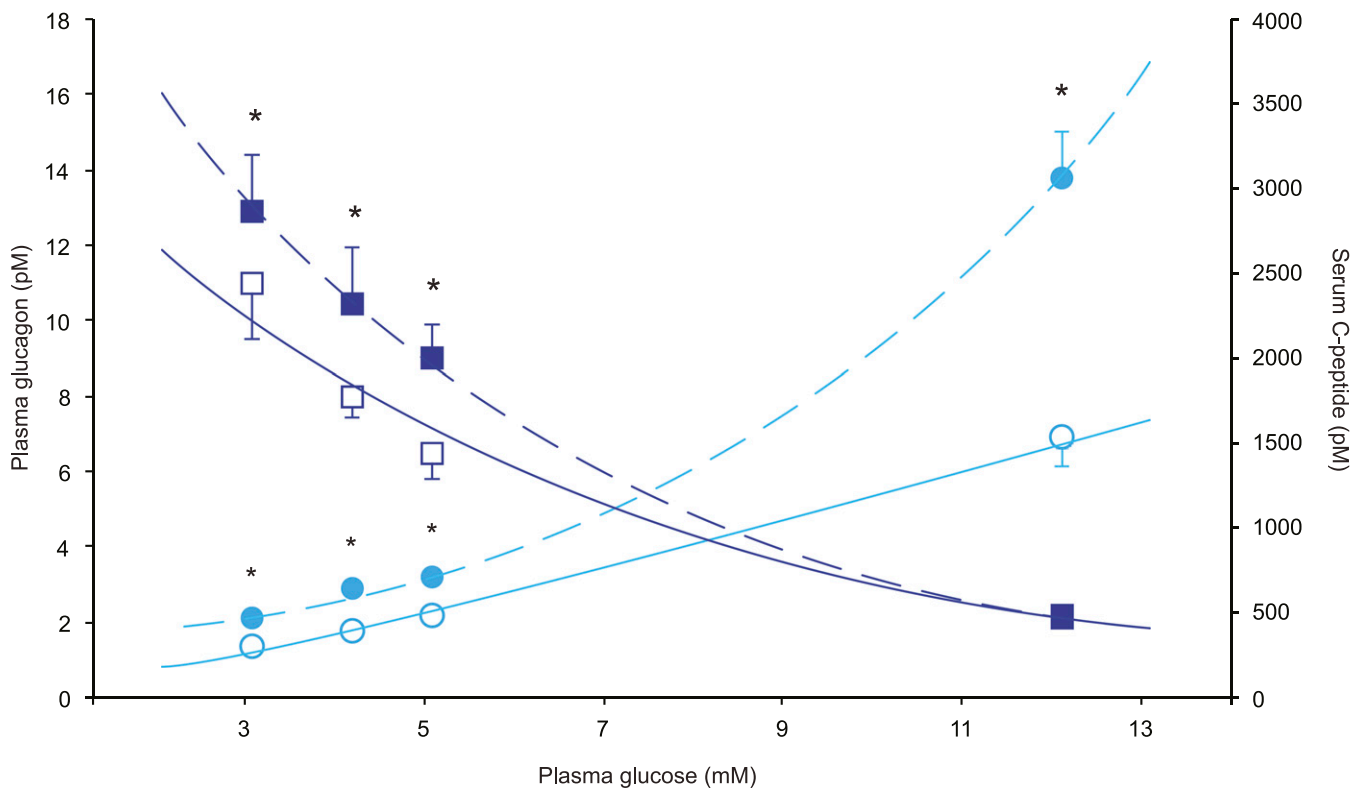
We examined the actions of GIP on the two main pancreatic hormones at three distinct glycemic levels and report the novel finding that GIP has inverted glucose-dependent effects on insulin and glucagon secretion. Thus, GIP exhibits glucagonotropic effects during fasting and hypoglycemic conditions when little or no effect on insulin secretion is exerted by the hormone. In contrast, GIP has no effect on glucagon secretion during hyperglycemia,

when it robustly potentiates glucose-induced insulin secretion. These findings help to explain most of the controversies, which exist in the literature, regarding the glucagonotropic effect of GIP. First, many of the negative results in healthy subjects could be related to the elevated glucose levels in studies using hyperglycemic clamping (9,10,15). Second, the low sensitivity of earlier glucagon assays could explain the negative glucagon responses seen in the earliest study (5,16). Third, in accordance with our results, a reexamination of the original data from the study by Vilsbøll et al. (8) revealed that short-duration GIP infusions (30 min) at a glycemic level of 5 mmol/L actually gave rise to significant glucagon responses compared with saline, but when the glucose levels were increased stepwise, first to 6 mmol/L and later to 7 mmol/L, the glucagon response was increasingly suppressed, resulting in the nonsignificant differences in total glucagon responses (GIP versus saline) reported in the original publication. Thus, on the basis of the collective human data, a glycemic threshold of  $\sim 5.5$ – $6$  mmol/L could exist, below which GIP mainly exerts glucagonotropic actions. Figure 4 illustrates that the bifunctional glucose-dependent role of GIP in vivo (in humans) at the physiological PG interval between 3 and 12 mmol/L bears resemblance to the results from perfused rat pancreas reported by Pederson and Brown (4) more than 30 years ago.

Although the combined glucagonotropic and insulinotropic effects of GIP seem to constitute a preserved physiological mechanism in rodents and humans alike, the physiological relevance of the dual hormonal regulating properties of GIP still remains to be elucidated. Several aspects of GIP physiology are interesting in this respect. Of note, GIP release is stimulated dose dependently not only by glucose but also by protein and fat ingestion and is, under physiological (postprandial) circumstances, always



**FIG. 3.** Glucagon plasma concentrations of glucagon during 90 min (upper panel) or the initial 30 min (lower panel) of GIP infusions (filled symbols) or saline infusions (open symbols) during euglycemia (dark blue curves, circles), insulin-induced hypoglycemia (blue curves, diamonds), and hyperglycemia (turquoise curves, squares). Insets in lower panel are the iAUCs of glucagon concentrations during the initial 30 min. Data are means  $\pm$  SEM. Statistical analysis was done by repeated-measures ANOVA followed by Bonferroni posttests or by paired *t* tests. \*Significant differences ( $P < 0.05$ ).



**FIG. 4. Glucagon and C-peptide.** The in vivo relation of plasma glucagon (dark blue curves, squares) and serum C-peptide (light blue curves, circles) to selected PG values between 3 and 12 mmol/L in the presence of stimulated GIP concentrations (broken lines, filled symbols) or basal levels (full lines, open symbols). Data are means  $\pm$  SEM. \*Significant differences ( $P < 0.05$ ) according to paired  $t$  tests.

present alongside the coincretin, glucagon-like peptide (GLP)-1 (17,18). Of interest, despite the glucose-dependent insulinotropic effects of both hormones, GLP-1, in contrast to GIP, has well-established suppressive effects on glucagon (19,20) and seemingly also the capability to induce hypoglycemia (21). Another discerning factor is that GIP signaling robustly has been demonstrated to be involved in fat metabolism in rodents and canine (22–26). Yet, although secretion of GIP is elevated in obese subjects (27) and enhanced by high-fat diets (28), the relevance of GIP in human fat metabolism remains more elusive (29). As central nervous tissue rely on stable blood glucose levels, we hypothesize that a role of GIP could be, through its glucagonotropic effect, to provide buffering against reactive hypoglycemia, especially after meals with high protein and fat content. A buffering effect on blood glucose levels is also highly compatible with a role for GIP as an anabolic hormone for adipose tissue and bone (29,30).

With regard to the regulation of pancreatic islet hormone secretion, it is interesting that GIP during euglycemia causes significant glucagon responses with concurrent insulin responses. In addition, during hyperinsulinemic hypoglycemia, glucagon levels were higher during the first 30 min with elevated levels of GIP, despite concomitant higher peripheral and presumably pancreatic intraislet insulin levels. Insulin is a well-known inhibitor of  $\alpha$ -cell secretion, but the mechanism by which this inhibition occurs is not well described (2). A proposed mechanism is changes in paracrine  $\beta$ -cell secretion (i.e., insulin, amylin,  $\gamma$ -aminobutyric acid, zinc ions, etc.) as a director of glucagon secretion commonly referred to as the intraislet hypothesis (31). Thus, the current results demonstrate a dominant effect of GIP stimulation on glucagon release (presumably from

pancreatic  $\alpha$ -cells) over the proposed negative impact of insulin (and other products) secreted from neighboring  $\beta$ -cells at euglycemia and hypoglycemia. This is in line with previous results from our group demonstrating that the suppression of glucagon responses during GIP infusions at hyperglycemia does not depend on the concurrent rise in insulin responses, a conclusion drawn from studies in patients with type 1 diabetes who tested negative for C-peptide after an intravenous arginine test (i.e., without paracrine intraislet influence of insulin) (11). Therefore, the prevailing glycemia, seems to be of greater importance in the regulation of glucagon secretion. The consequences of these parallel insulin and glucagon responses at euglycemic levels were a slight lowering effect on PG. This is a probable consequence of the peripheral (i.e., nonhepatic) influence of insulin during these circumstances but certainly does not exclude some buffering effect of glucagon through hepatic glucose production. The influence of insulin on peripheral tissues was further demonstrated during the excursion toward hypoglycemia, where PG levels were almost completely superimposed on days with GIP and saline. Hence, in the presence of the rather non-physiological hyperinsulinemia inherent to the clamp in these healthy individuals, the stimulated glucagon levels for the first 30 min were not sufficient to modify the course of the downward-sloping PG curve. During the hyperinsulinemic clamp, the glucagon levels were increased by concomitant GIP infusion only in the physiological range of PG (i.e., in the range of 3.0–5.5 mmol/L), as shown in Fig. 4. The maximal glucagon levels reached (at the lowest PG levels) were similar on days with GIP and saline, probably as a result of maximal stimulation of glucagon secretion induced by hypoglycemia and activity in the autonomic

nervous system (observed clinically in most of the subjects by sweating).

The glucagonotropic effects of GIP could have pathophysiological consequences. It is well established that excess secretion of glucagon (weighed against a relative lack of insulin) in the postprandial and fasting state is a major determinant of diabetic hyperglycemia (1,32). Furthermore, it is evident that the hyperglucagonemia in type 2 diabetes cannot solely be explained by a lack of insulin (2). Intriguingly, in patients with type 2 diabetes the insulinotropic effect of GIP is severely deficient (9,15). Therefore, in these patients a glucagonotropic effect of GIP could be a factor adding to the mismatched insulin-to-glucagon ratio. The maximal difference in glucagon levels between GIP and saline on the days of euglycemia in the current study amounts to ~4 pmol/L, a difference that could be of clinical relevance in type 2 diabetic patients with insufficient opposing insulin secretion. This is supported by the observation that there is a similar difference (i.e., ~4 pmol/L) in fasting glucagon concentrations between individuals with type 2 diabetes and healthy control subjects (33). In further support of this notion, recent studies have shown that GIP infused in supraphysiological doses worsens postprandial hyperglycemia (34) and antagonizes the glucagon-suppressive effects of GLP-1 (35,36) in patients with type 2 diabetes. On the other hand, enhanced GIP action in type 2 diabetes could explain the improved glucagon response to hypoglycemia observed during treatment with the dipeptidyl peptidase-4 inhibitor, vildagliptin (37). The proposed role of GIP as a safeguard against hypoglycemia and its potential pathophysiological implications in type 2 diabetes could benefit from further exploration.

In conclusion, we have demonstrated glucose-dependent glucagonotropic effects of GIP in healthy humans. GIP has no effect on glucagon responses during hyperglycemia when it potentiates insulin secretion. In contrast, GIP increases glucagon levels during fasting and hypoglycemic conditions. Thus, GIP seems to be a physiological pancreatic islet regulator with diverging effects on the two main pancreatic glucoregulatory hormones insulin and glucagon.

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No potential conflicts of interest relevant to this article were reported.

M.C. contributed to the study design, researched data, and wrote and edited the manuscript. L.V. researched data and reviewed and edited the manuscript. J.J.H. and T.V. reviewed and edited the manuscript. F.K.K. designed the study and reviewed and edited the manuscript.

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