Glucose-Dependent Insulinotropic Polypeptide Augments Glucagon Responses to Hypoglycemia in Type 1 Diabetes

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Running title
GIP and GLP-1 during hypoglycemia in T1DM

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Abbreviations
AUC, area under curve; BMI, body mass index; DPP-4, dipeptidyl peptidase 4; FFA, free fatty acids; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; HbA1c, hemoglobin A1c; Type 1 diabetes mellitus, T1DM.
Abstract

Glucose-dependent insulinoïd polypeptide (GIP) is glucagonotrophic and glucagon-like peptide 1 (GLP-1) is glucagonostatic. We studied the effects of GIP and GLP-1 on glucagon responses to insulin-induced hypoglycemia in patients with type 1 diabetes mellitus (T1DM).

Ten male subjects with T1DM (C-peptide negative, age: 26±1 years (mean±SEM); BMI: 24±0.5 kg/m²; HbA1c 7.3±0.2%) were studied in a randomized, double-blinded, cross-over study, with 2-hour iv administration of saline, GIP or GLP-1. The first hour, plasma glucose was lowered by insulin infusion, and the second hour constituted a ‘recovery phase’.

During the recovery phase GIP infusions elicited larger glucagon responses (164±50 (GIP) vs. 23±25 (GLP-1) vs. 17±46 (saline) min·pmol/l, P<0.03) and endogenous glucose production was higher with GIP and lower with GLP-1 as compared to saline (P<0.02). On the GIP days significantly less exogenous glucose was needed to keep plasma glucose above 2 mmol/l (155±36 (GIP) vs. 232±40 (GLP-1) vs. 212±56 (saline) mg·kg⁻¹, P<0.05). Levels of insulin, cortisol, growth hormone, and noradrenaline, as well as hypoglycemic symptoms and cognitive function, were similar on all days.

Our results suggest that during hypoglycemia in patients with T1DM exogenous GIP increases glucagon responses during the ‘recovery phase’ after hypoglycemia and reduces the need for glucose administration.
Introduction

In recent years, it has become evident that glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) have glucose-dependent effects on glucagon secretion (1). While GLP-1 lowers glucagon levels during fasting and elevated blood glucose (1–3), GIP raises glucagon levels during fasting and hypoglycemic conditions (4–6). In most patients with type 1 diabetes (T1DM), the glucagon counter-regulatory response to insulin-induced hypoglycemia is deficient (7,8), and it is unknown to what extent the incretin hormones might affect this response. We therefore studied the individual effects of GIP and GLP-1 on the glucagon response to insulin-induced hypoglycemia in patients with T1DM without endogenous insulin secretion.

Research design and methods

Study design

This was a double-blinded, randomized, cross-over study comparing the effects of GIP, GLP-1 and placebo (saline) on the glucagon response to insulin-induced hypoglycemia in patients with T1DM. The study was conducted at Gentofte Hospital, University of Copenhagen. The study was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration number: H-D-2009-0078) and was conducted according to the principles of the Helsinki Declaration (Fifth revision, Edinburgh, 2000). The trial is registered with ClinicalTrials.gov (ID: NCT01739283).

Participants

We included 10 male patients (age: 26±1 years (mean±SEM); BMI: 24±0.5 kg/m²; glycated hemoglobin A1c (HbA1c): 7.3±0.2%) with T1DM (positive islet cell and/or glutamic acid decarboxylase-65 antibodies), treated with multiple doses of insulin (N=9) or insulin pump (N=1), without late diabetic complications, hypoglycemia unawareness and residual beta cell function (i.e. C-peptide negative following a 5 g-arginine stimulation test (9)).
Experimental procedures

Each patient underwent three separate study days in randomized order with intervals of at least one week. Subjects were instructed to maintain a regular diet and avoid alcohol and physical activity for three days prior to each study day. Subjects were asked to wear a Guardian® REAL-time continuous glucose monitor (Medtronic A/S, Copenhagen, Denmark) for 2-4 days before each study day to monitor glycemic variability and facilitate therapeutic decisions in relation to food intake and insulin delivery. If patients had fasting plasma glucose levels between 5 and 9 mmol/l at the morning of the study day, two cannulas were inserted bilaterally into the cubital veins for infusions and blood sampling, respectively. Two hours before study start (i.e. time -120 min), an infusion of stable isotopes was initiated (D$_2$-glucose (priming dose 17.6 µmol×kg$^{-1}$ and continuous 0.5 µmol×kg$^{-1}$×min$^{-1}$) and D$_3$-glycerol (priming dose 3 µmol×kg$^{-1}$ and continuous 0.1 µmol×kg$^{-1}$×min$^{-1}$)) and at the same time basal insulin (Actrapid®, Novo Nordisk, Bagsværd, Denmark) iv infusion was initiated (dose 0.1 mU×kg$^{-1}$×min$^{-1}$). At time 0 min, a 60 min-iv infusion of human insulin (dose 1 mU×kg$^{-1}$×min$^{-1}$) was initiated to lower plasma glucose. Also at time 0 min, similar appearing iv infusions of either GIP (dose 4 pmol×kg$^{-1}$×min$^{-1}$), GLP-1 (dose 1 pmol×kg$^{-1}$×min$^{-1}$), or placebo (saline) were initiated and continued for the remainder of the study that day. Plasma glucose was measured bedside every 5 minute. An adjustable continuous infusion of 20% dextrose (w/v) was administered by a physician (SC) unaware of randomization allocation to keep plasma glucose above 2 mmol/l in the time interval 0-75 min and above 3.5 mmol/l in the interval 75-120 min. Hypoglycemic symptoms and cognitive function were assessed at baseline (time -60 min) and during hypoglycemia (time 65 min) using validated rating scales (10).

Analytical procedures

Steady-state glucose and glycerol concentrations and stable isotope enrichment were determined at time -45, -30 and -15 min. Arterialized blood was drawn at time -30, -15, 0, 5, 10, 20, 30, 45, 60, 75, 90, 105, and 120 min. Bedside glucose concentrations were measured by the glucose oxidase method, and concentrations of intact GIP, total GLP-1, and glucagon were measured by specific immunoassays.
as previously described (5,11). We used the assay for total GLP-1 to allow for comparison between GLP-1 concentrations in healthy volunteers (12) and during subcutaneous treatment with GLP-1 (13). Blood enrichment of D$_2$-glucose and D$_2$-glycerol was determined using gas chromatography mass spectrometry as previously described (14). Plasma insulin, C-peptide, cortisol, noradrenaline, and growth hormone were measured with commercial immunoassays.

**Calculations and statistical analyses**

To detect a real difference in glucagon responses between days of at least 2.5 pmol/l with a two-sided 5% significance level, a power of 80%, and a within-patient standard deviation of plasma glucagon measurements of 1.7 pmol/l (estimated from a previous study (5)), a sample size of 10 patients was found to be necessary. Area under the curve (AUC) and incremental AUC (iAUC) i.e. baseline level-subtracted AUC-values were calculated using the trapezoidal rule. Glucose rate of appearance (R$_a$) and glucose rate of disappearance (R$_d$) were calculated from the changes in glucose enrichment using a one-compartment, fixed-volume, non-steady-state model modified for use with stable isotopes and a pool fraction of 70 ml×kg$^{-1}$ (15). To test for differences in concentration time courses we used two-way repeated measures ANOVA followed by Holm-Šidák corrected post-tests. For comparisons of AUC values we used Friedman test followed by paired comparisons using Wilcoxon test. Statistics and graphical presentations were performed in Graphpad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). A two-sided P value <0.05 was used to indicate statistically significant differences.

Results are reported as mean±standard error of the mean (SEM) unless otherwise stated.

**Results**

**Plasma glucose**

Mean plasma glucose concentrations during each of the three experimental days are displayed in Figure 1a. Baseline plasma glucose concentrations (time -90 to 0 min) did not differ significantly
between study days ($P>0.79$) and the overall mean fasting value was $6.9\pm0.5$ mmol/l. Mean plasma glucose excursions from time 0 to 120 min also did not differ between days with a mean nadir of $2.7\pm0.1$ mmol/l reached at time 70 min. The total amount of glucose needed to prevent hypoglycemia differed significantly between days ($156\pm35$ (GIP) vs. $234\pm41$ (GLP-1) vs. $214\pm56$ (saline) mg $\times$ kg body weight$^{-1}$, $P<0.05$).

**GIP and GLP-1**

Plasma concentrations over time of GIP and GLP-1 are shown in Figure 1. The respective plasma concentrations peaked at $134\pm4$ pmol/l during GIP infusion (Fig. 1b) and $219\pm19$ pmol/l during GLP-1 infusion (Fig. 1c).

**Insulin and C-peptide**

Serum insulin concentrations over time are presented in Figure 1d. Overall basal concentrations of serum insulin were similar on study days ($P=0.58$). After start of insulin infusion, levels of serum insulin increased significantly to overall mean peak levels of $810\pm129$ pmol/l, with no difference in mean values between study days ($P=0.52$). Mean C-peptide concentrations were below the detection limit at all time points (data not shown).

**Glucagon**

Plasma glucagon concentrations are presented in Figure 1e. Baseline levels of glucagon were similar on all study days ($P=0.29$). Glucagon responses expressed as AUC differed between study days ($P<0.001$). During the first hour the glucagon iAUCs amounted to $-88\pm38$ (GIP) vs. $-166\pm28$ (GLP-1) vs. $-49\pm20$ (saline) min$\times$pmol/l ($P<0.006$), and during the second hour, the glucagon iAUCs amounted to $164\pm50$ (GIP) vs. $23\pm25$ (GLP-1) vs. $17\pm46$ (saline) min$\times$pmol/l ($P<0.036$). Thus, during the insulin infusion (time 0-60 min) the glucagon iAUC was significantly lower on the GLP-1 days ($P<0.01$ vs. saline), whereas during the ‘recovery period’ (i.e. after termination of insulin infusion) there was a higher glucagon response on the GIP days ($P<0.02$ vs. saline).
**Glucose kinetics**

The time courses of glucose \( R_a \) and glucose \( R_d \) were similar on study days (Fig. 2a and 2b). GIP infusion increased endogenous \( R_a \) (significant at 75 min), whereas GLP-1 reduced endogenous \( R_a \) compared to saline (time 90-120 min) (Fig 2c). The glucose infusion rates also differed between days, with lower infusion rates on the days of GIP infusion at time 75 min (Fig. 2d).

**Free fatty acids, glycerol and glycerol kinetics**

The levels of Free fatty acids (FFA) were equally suppressed during the first hour of the study, but during the ‘recovery phase’ they increased more on the days of GIP infusion as compared to saline (Fig. 3a) and FFA iAUC was higher on the GIP days \((P<0.04)\). Plasma glycerol concentrations and glycerol \( R_a \) and \( R_d \) did not differ between study days (Fig. 3c and 3d).

**Hypoglycemia symptom score and cognitive function**

There were no differences in hypoglycemia symptom scores or cognitive function scores between the study days (Fig. 4a and 4b).

**Plasma cortisol, noradrenalin and growth hormone responses**

The plasma cortisol, noradrenalin and growth hormone concentrations did not differ significantly between study days (data not shown).

**Discussion**

We report that in patients with T1DM without residual beta cell function, GIP augments glucagon responses to experimental hypoglycemia and reduces the need for exogenous glucose administration (to prevent severe hypoglycemia) accompanied by a higher rate of endogenous glucose production (significantly higher at the time around maximal hypoglycemia, 75 minutes after study start) compared to placebo. We also report that GLP-1 infusions slightly suppress plasma glucagon during induction of hypoglycemia, with no additional need for exogenous glucose administration.
The mechanism underlying these findings could be the direct action of the incretin hormones on the pancreatic alpha cells as demonstrated in perfused rat pancreata (16). During perfusion with low levels of glucose (1.5 mmol/l), GIP had stimulatory and GLP-1 had inhibitory effects on glucagon secretion (16). The glucagonotropic effect of GIP is presumed to be mediated by activation of GIP receptors present on pancreatic alpha cells (17,18). The glucagon inhibitory effects of GLP-1 have been attributed to paracrine inhibitory signaling (somatostatin) from neighboring delta cells (16), or - more controversially - direct effects via GLP-1 receptors present on alpha cells (1,19).

We were not able to detect any differences between interventions in cognition, hypoglycemia symptoms, or other counter regulatory hormones. This is not surprising, as the glucose levels were similar on the three experimental days. Notably, the fact that glucose levels were kept above 2 mmol/l could explain the limited symptoms and hormonal responses to hypoglycemia. We demonstrated a minor GIP-dependent increase in free fatty acids and a non-significant increase in glycerol during the ‘recovery phase’ from hypoglycemia. This was unexpected, as a preponderance of evidence including human in vivo data support a role for GIP combined with insulin in stimulating postprandial lipogenesis (20,21). The observed paradoxical ‘lipolytic effect’ could therefore have depended on the low plasma glucose levels in combination with fairly low insulin levels and stimulated glucagon levels during the recovery phase after hypoglycemia.

A few considerations are worth mentioning. Firstly, we used doses of GIP and GLP-1 resulting in supraphysiological plasma levels. The plasma intact GIP levels of ~125 pmol/l were in the range that can be reached postprandially during DPP-4 inhibition (22). The total GLP-1 levels of ~200 pmol/l were similar or slightly lower than what can be used therapeutically in patients with type 2 diabetes (13). Secondly, even the maximal glucagon responses (i.e. on the GIP days) were deficient compared to the responses observed in healthy subjects under similar conditions (5), and in the range of what can be considered basal levels. Important for the interpretation of these modest changes, basal glucagon levels are located on the steep part of the dose-response-curve relating glucagon concentration to hepatic glucose output, as evidenced by studies where as little as 2-3 pmol/l changes in basal levels of
portal glucagon significantly increase hepatic glucose output (23). Lastly, the negative (albeit not statistically different from zero) endogenous glucose Ra values from time 75 to 90 minutes on the GLP-1 days and around zero on the GIP and saline days were likely caused by large abrupt glucose infusions leading to analytical and modeling limitations (24). Although these limitations may have been the same on all study days, the endogenous glucose production estimates should be interpreted with caution.

The present findings may have clinical relevance. Iatrogenic hypoglycemia is a major safety concern in patients with diabetes, and consequently minor improvements in hypoglycemia risk have attracted great public and pharmaceutical interest. The mean difference in glucose administration between the days of GIP versus saline in the present study amounted to 4 grams. For comparison, 15-20 g of glucose is commonly recommended as self-treatment of mild-to-moderate symptomatic hypoglycemia (25). Thus, both the hypoglycemia limiting effect of adding GIP to insulin treatment as well as glucagon reducing effect of GLP-1 (and GLP-1 receptor agonists) during low levels of glycemia merit further investigation.

Author contributions

MC designed the study, performed the data analysis, contributed to the clinical experiments and drafted the manuscript. SC contributed to the study design, data analysis and performed most of the clinical experiments. AHSU contributed to the clinical experiments and biochemical analyses. PLK contributed to the study design and data analysis. JF analyzed plasma for noradrenaline. GvH contributed to the study design and data analysis of stable isotope kinetics. JJH contributed to the study design and measurements of peptide hormones. FKK designed the study and contributed to the data analysis. All authors (MC, SC, AHSU, PLK, MMR, JF, GvH, FP, JJH, TV and FKK) revised and approved the final manuscript. MC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data.
Disclosures

SC became an employee in the Novo Nordisk Pharmaceutical Medicine Programme after the study was finished (September 2013). JJH has served as a consultant or advisor to Novartis Pharmaceuticals, Novo Nordisk, Merck, Sharp and Dome and Roche, and has received fees for lectures from Novo Nordisk, Merck, Sharp and Dome, and GSK. TV has received fees for being part of an advisory board from AstraZeneca, Boehringer Ingelheim Pharmaceuticals, Bristol-Myers Squibb, Eli Lilly and Company, GI Dynamics, Inc, Merck Sharp & Dohme, Novo Nordisk, Sanofi and Takeda, has received fees for lectures from: AstraZeneca, Boehringer Ingelheim Pharmaceuticals, Bristol-Myers Squibb, Eli Lilly and Company, Merck Sharp & Dohme, Novo Nordisk, Novartis, Sanofi, Takeda and Zealand Pharma, and has received research support from Novo Nordisk. FKK has received fees for consultancy or being part of an advisory board from AstraZeneca, Bristol-Myers Squibb/AstraZeneca, Eli Lilly, Gilead Sciences, Ono Pharmaceuticals, Sanofi-Aventis and Zealand Pharma, has received fees for lectures from: AstraZeneca, Boehringer Ingelheim Pharmaceuticals, Bristol-Myers Squibb, Eli Lilly and Company, Gilead Sciences, Merck Sharp & Dohme, Novo Nordisk, Ono Pharmaceuticals, Sanofi and Zealand Pharma, and has received research support from Sanofi-Aventis. The remaining authors (MC, AHSU, PLK, JF, MMR, GvH, FP) declare no conflicts of interest pertinent to this manuscript.

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Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, and Lene Foged at the Clinical Metabolomics Core Facility, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark.
References


Figure legends

Figure 1. Plasma glucose and hormones
Plasma/serum concentrations of glucose (a), intact GIP (b), total GLP-1 (c), insulin (d), and glucagon (e) during insulin-induced hypoglycemia with iv infusion of GIP (red diamonds), GLP-1 (blue hexagons) or saline (white squares). Data are means±SEM. Cumulated glucose infusions are depicted as bar graphs (a). Statistical analyses were done with repeated-measures ANOVA and denote differences over time (A), differences between the groups (B), and differences owing to interaction between group and time (AB). Post-tests at individual time points were by Holm-Sidak's multiple comparisons tests. Significant differences are indicated by asterisks: *P<0.05, **P=0.001 to 0.01, ***P=0.0001 to 0.001, ****P<0.0001.

Figure 2. Glucose kinetics
The rate of glucose appearance (Ra) (a), rate of glucose disappearance (Rd) (b), rate of endogenous glucose production (c), and glucose infusion rate in 15 minute intervals (d) during insulin-induced hypoglycemia with iv infusion of GIP (red diamonds), GLP-1 (blue hexagons) or saline (white squares). Data are means±SEM. Statistical analyses were done with repeated-measures ANOVA and denote differences over time (A), differences between the groups (B), and differences owing to interaction between group and time (AB). Post-tests at individual time points were by Holm-Sidak's multiple comparisons tests. Significant differences are indicated by asterisks: *P<0.05, **P=0.001 to 0.01, ***P=0.0001 to 0.001, ****P<0.0001.

Figure 3. Serum free fatty acids and glycerol kinetics
Concentrations of plasma/serum free fatty acids (FFAs) (a), glycerol (b), glycerol rate of appearance (Ra) (c) and glycerol rate of disappearance (Rd) (d) during insulin-induced hypoglycemia with iv infusion of GIP (red diamonds), GLP-1 (blue hexagons) or saline (white squares). Data are means±SEM. Statistical analyses were done with repeated-measures ANOVA and denote differences over time (A), differences between the groups (B), and differences owing to interaction between group and time (AB). Post-tests at individual time points were by Holm-Sidak's multiple comparisons tests. Asterisk (*) indicates significant (P<0.05) difference.

Figure 4. Hypoglycemia symptoms and cognitive function score
Hypoglycemia symptom score (a) and cognitive function scores (b) depicted as the difference between baseline (time -60 min) and hypoglycemia (time 65 min) during infusion of GIP (red), GLP-1 (blue) or saline (white). Hypoglycemia symptoms were graded using the Edinburgh Hypoglycemia Scale, which is a Likert scale of symptoms scores from 1 (not present) to 7 (very intense). Cognitive functions scores are based on the Trail making test, which measures time to finish in seconds (panel b, left y-axis), and the Stroop test, which measures items named during a 45 second period (right panel, left y-axis). Results are depicted as box and whiskers (minimum to maximum). No significant differences between study days were observed.

Supplementary material Figure 1. Plasma cortisol, noradrenalin and growth hormone
Concentrations of plasma cortisol (a) and growth hormone (b) and noradrenalin (c) during insulin-induced hypoglycemia with iv infusion of GIP (red diamonds), GLP-1 (blue hexagons) or saline (white squares). Data are means±SEM. Statistical analyses were done with repeated measures ANOVA and denote differences over time (A), differences between the groups (B), and differences owing to interaction between group and time (AB).
Figure 1 - Plasma glucose and hormones

176x172mm (300 x 300 DPI)
Figure 2 - Glucose kinetics.
116x74mm (300 x 300 DPI)
Figure 3 - FFA and glycerol

121x81mm (300 x 300 DPI)
Figure 4 - symptoms and cognitive function
63x22mm (300 x 300 DPI)