Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM

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Context: Complete loss of β-cell function in patients with type 1 diabetes mellitus (T1DM) may lead to an increased risk of severe hypoglycemia.

Objective: We aimed to determine the impact of C-peptide status on glucagon response and endogenous glucose production (EGP) during hypoglycemia in patients with T1DM.

Design and Setting: We conducted an open, comparative trial.

Patients: Ten C-peptide positive (C-pos) and 11 matched C-peptide negative (C-neg) patients with T1DM were enrolled.

Intervention: Plasma glucose was normalized over the night fast, and after a steady-state (baseline) plateau all patients underwent a hyperinsulinemic, stepwise hypoglycemic clamp with glucose plateaus of 5.5, 3.5, and 2.5 mmol/L and a recovery phase of 4.0 mmol/L. Blood glucagon was measured with a specific and highly sensitive glucagon assay. EGP was determined with a stable isotope tracer technique.

Main Outcome Measure: Impact of C-peptide status on glucagon response and EGP during hypoglycemia.

Results: Glucagon concentrations were significantly lower in C-pos and C-neg patients than previously reported. At baseline, C-pos patients had higher glucagon concentrations than C-neg patients (8.39 $\pm$ 4.6 vs 4.19 $\pm$ 2.4 pmol/L, $P = 0.016$, mean $\pm$ standard deviation) but comparable EGP rates (2.13 $\pm$ 0.2 vs 2.04 $\pm$ 0.3 mg/kg/min, $P < 0.391$). In both groups, insulin suppressed glucagon levels, but hypoglycemia revealed significantly higher glucagon concentrations in C-pos than in C-neg patients. EGP was significantly higher in C-pos patients at hypoglycemia (2.5 mmol/L) compared with C-neg patients.

Conclusions: Glucagon concentrations and EGP during hypoglycemia were more pronounced in C-pos than in C-neg patients, which indicates that preserved β-cell function may contribute to counterregulation during hypoglycemia in patients with T1DM. (***J Clin Endocrinol Metab*** **103: 1408–1417, 2018)**

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Abbreviations: AUC, area under the curve; AUC_{EGP}, area under the curve for endogenous glucose production; AUC_{GIR}, area under the curve for glucose infusion rate; $AUC_{GIRtotal}$, area under the curve for total glucose infusion rate; $AUC_{glucagon}$, area under the curve for glucagon; BMI, body mass index; C-neg, C-peptide negative; C-pos, C-peptide positive; CV, coefficient of variation; EGP, endogenous glucose production; GC, gas chromatography; GIR, glucose infusion rate; HbA1c, hemoglobin A1c; LOQ, lower limit of quantification; MS, mass spectrometry; PG, plasma glucose; Rd, rate of peripheral glucose disposal; SD, standard deviation; SW, Shapiro-Wilk test; T1DM, type 1 diabetes mellitus; TTR, tracer-to-tracee ratio.

Tight glycemic control in patients with type 1 diabetes mellitus (T1DM) decelerates the progression of microvascular complications such as retinopathy, neuropathy, and nephropathy (1) and can also reduce the development of macrovascular complications such as ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (2). However, achievement of more stringent treatment goals raises the risk of recurrent symptomatic and severe hypoglycemic events. Hypoglycemia is not only associated with an adverse clinical outcome but also has a negative impact on quality of life of affected patients (3).

In healthy people, glucagon and insulin produced from the α- and β-cells exert opposing effects on their target tissues, and their interaction plays a central role in glucose homeostasis (4). The defense mechanisms against hypoglycemia include reduction of endogenous insulin secretion and release of pancreatic glucagon, which in turn raises plasma glucose (PG) by increasing hepatic glucose output through stimulation of glycogenolysis and activating gluconeogenesis (5, 6).

In patients with T1DM, β-cells are destroyed by an autoimmune reaction leading to deficiency of endogenous insulin secretion. This might cause secondary abnormalities in the function of other pancreatic islet cells, like abnormal glucagon release by α-cells. Glucagon levels after oral food intake have been shown to be inappropriately high, which is associated with pronounced postprandial hyperglycemia in patients with T1DM (7, 8). In contrast, diminished glucagon response to hypoglycemia and insufficient stimulation of glycogenolysis and gluconeogenesis are major reasons for severe hypoglycemic events (9–11). Reasons for this disturbed glucagon response to hypoglycemia may be impaired PG sensing in the α-cells (11), autonomic dysfunction (12), or a loss of an insulin "switch off" signal from the β-cells (13), but the underlying mechanism is not yet fully understood.

Over time, different experimental setups, like various glucagon assays, have been used to gain a better understanding of glucagon levels in T1DM during hypoglycemia. Analytical methods for glucagon determination have been improving over the years, but commonly used assays are mostly unspecific and detect *N*-terminally extended or truncated forms of glucagon as well. The resulting and to some degree erroneous high glucagon concentrations may have compromised the interpretations of the role of glucagon in patients with T1DM (14, 15). Recently, a specific and highly sensitive glucagon assay that enables the specific detection of intact glucagon has become available (14, 16) and allows a reassessment of the role of glucagon during hypoglycemia in patients with T1DM.

The severity of hypoglycemia in patients with T1DM correlates with diabetes duration (17–19). Patients with short diabetes duration [(C-peptide positive (C-pos)] had as many hypoglycemic events as patients with long diabetes duration [C-peptide neg (C-neg)], but these hypoglycemic events were less severe (17). Diabetes duration is associated with a loss of residual β-cell function, which indicates that the risk of occurrence of severe hypoglycemia increases with the loss of β-cell function and that C-peptide negativity is a major risk factor for developing severe hypoglycemia (20, 21). However, the mechanism of how residual β-cell function protects against severe hypoglycemia remains unclear, and the impact of C-peptide status on glucose release from the liver is controversial (22, 23).

We aimed to assess the impact of the C-peptide status in patients with T1DM on the glucagon response during a hyperinsulinemic hypoglycemic clamp by measuring glucagon levels with a specific and highly sensitive glucagon assay and by assessing the endogenous glucose production (EGP) with a stable isotope tracer technique.

Materials and Methods

Trial design

We conducted an open, comparative trial in matched C-pos and C-neg patients with T1DM, applying a hyperinsulinemic, stepwise hypoglycemic clamp. Written informed consent was obtained from all patients before any trial-related activities were started. The trial was approved by the local ethics committee of the Medical University of Graz, Austria (26-070 ex 13/14) and performed in accordance with Good Clinical Practice (24) and the Declaration of Helsinki (25).

Participants

All enrolled patients with T1DM had a history of T1DM with acute hyperglycemia and ketonuria and had a daily insulin requirement with either multiple daily insulin injections or continuous subcutaneous insulin infusion. Inclusion criteria were age 18 to 64 years, body mass index (BMI) 18.0 to 28.0 kg/m^2, and hemoglobin A1c (HbA1c) 6.0% to 9.5% (42 to 80 mmol/mol). Exclusion criteria were any late complications of diabetes, including hypoglycemic unawareness, any severe hypoglycemic event within 1 month before screening, and any relevant health risk during the hypoglycemic clamp.

At the screening visit, the cutoff for C-neg was determined as a fasting C-peptide below the lower limit of quantification (LOQ) of the assay (LOQ = 0.017 nmol/L) (26), and the cutoff for C-pos was determined as a fasting C-peptide of $\geq$0.05 nmol/L. We used the electronical diabetes database built by our department for screening. It provides information about all patients with diabetes (type 1 and type 2) who have given written informed consent for potential participation in clinical trials. We screened 1000 patients with T1DM and invited all 40 consecutive C-pos patients for a screening visit. Finally, 10 C-pos patients with T1DM were enrolled, and 11 C-neg patients with T1DM, also screened from the database, were

matched with regard to age, sex, weight, and BMI. The recruitment phase lasted from January 2014 until October 2015. No study patient withdrew his or her informed consent during the study.

Stepwise hypoglycemic glucose clamp with stable isotope tracer technique

On the evening before the study day, participants arrived at the Clinical Research Center at 20:00 hours for an in-house stay for ≥16 hours. All participants had been advised not to inject long-acting or intermediate-acting insulin after 08:00 hours (no ultra-long-acting insulin was used) and not to do any strenuous exercise after 10:00 hours. The last short-acting insulin injection was administered with the last meal at 17:00 hours. No hypoglycemic event (PG ≤3.9 mmol/L) was allowed to take place after 10:00 hours in the morning on the day before the study day; otherwise, the participant's visit was rescheduled.

A hand vein was cannulated for sampling of arterialized venous blood and remained wrapped in a heating blanket throughout the clamp. To achieve normoglycemia overnight, a hand vein in the contralateral arm was cannulated for a variable human soluble insulin infusion [40 U Actrapid (100 U/mL) (NovoNordisk, Copenhagen, Denmark) in 99.6 mL NaCl (154 mmol/L)], until 08:00 hours on the study day, or for safety reasons a glucose infusion (10%, Fresenius Kabi, Graz, Austria) until 05:00 hours on the study day. Overnight, PG was measured every 5 to 30 minutes depending on the glucose concentrations needed to keep the PG stable (PG target level of 5.5 mmol/L) and to avoid nocturnal hypoglycemia (defined as PG level ≤3.9 mmol/L). At 05:00 hours [6,6-$^2H_2$]-glucose solution (100 g/L; Euriso-Top, Saint-Aubin Cedex, France) was given intravenously with a bolus of 9.6 mg/kg/min for 1 minute and a constant rate of 0.08 mg/kg/min until the end of the clamp. From 05:00 until 07:30 hours, a PG level deviation of ±30% was allowed. Two and a half hours after infusion start, tracer glucose equilibration phase was reached and defined as baseline (low insulin, duration 30 minutes), while a variable low-insulin infusion was administered for stable PG levels (5.5 mmol/L, ±20% of PG deviation). At 08:00 hours, the hyperinsulinemic clamp was initiated by increasing the insulin infusion to a constant rate of 1.5 mU/kg/min (Fig. 1). PG was kept stable at normoglycemia with a variable glucose infusion rate (GIR) enriched with 4 mg [6,6-$^2H_2$]-glucose/mL for ~90 minutes. Then, the first PG plateau of 5.5 mmol/L was started and lasted 30 minutes. Afterward, the GIR was turned off and the PG was allowed to drop to a plateau of 3.5 mmol/L and afterward to nadir (target 2.5 mmol/L). Each PG plateau was kept stable with the GIR for 30 minutes. For safety reasons, PG was not allowed to drop below 2.2 mmol/L. Fifteen minutes after having reached nadir, the constant insulin infusion was stopped, and the GIR was tapered off to enable spontaneous recovery from hypoglycemia, if possible. If PG had not recovered 45 minutes after the insulin infusion was terminated, a constant intravenous glucose infusion (5.5 mg/kg/min) was initiated to reach the last PG plateau of 4.0 mmol/L (10 minutes) and finally normoglycemia. Throughout the clamp, participants continued fasting

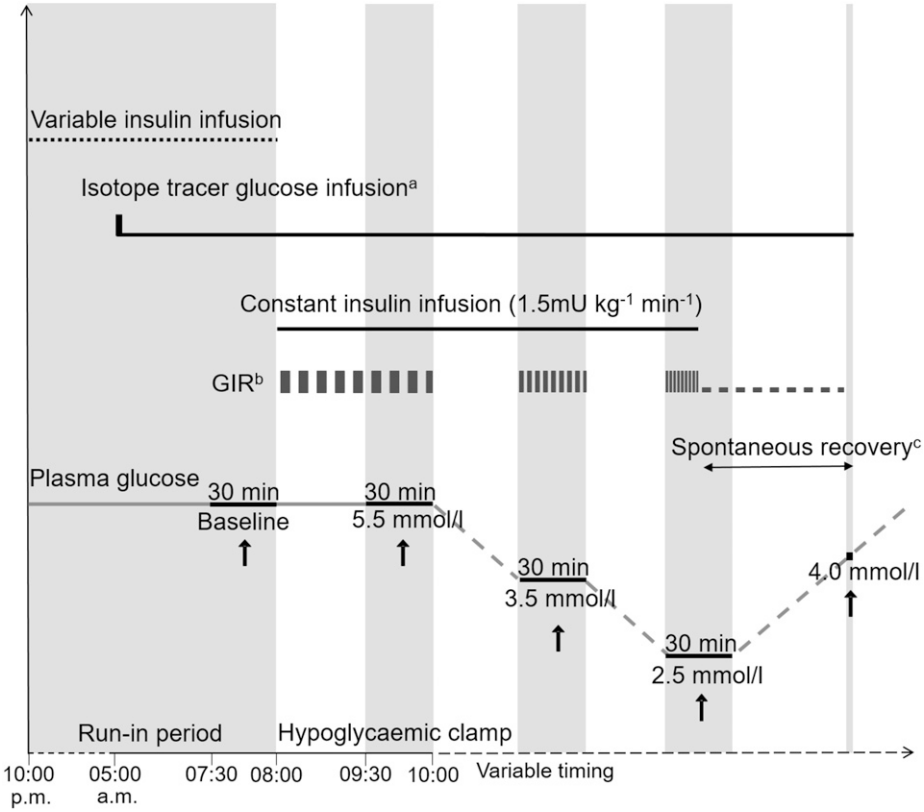

Figure 1. Hypoglycemic clamp with stable isotope tracer technique. [a]Isotope tracer glucose infusion: A bolus of 9.6 mg/kg/min of [6,6-$^2H_2$]-glucose was given for 1 minute and a constant rate of 0.08 mg/kg/min until the end of the clamp. [b]GIR was enriched with 4 mg [6,6-$^2H_2$]-glucose/mL. [c]Spontaneous recovery: After constant insulin infusion (1.5 mU/kg/min) was stopped, desired maximum time to reach plateau 4.0 mmol/L was 45 minutes. ↑, hypoglycemic response assessments include blood samples for glucagon and [6,6-$^2H_2$]-glucose, vital signs, hypoglycemic awareness, and hypoglycemic symptom tests.

(no food or beverages except water) and stayed in a supine or semisupine position. PG measurements were performed every 5 minutes from increasing insulin infusion (08:00 hours) until termination of the clamp. Blood sampling for measurement of [6,6-$^2H_2$]-glucose, glucagon, norepinephrine, and epinephrine concentrations was done at baseline and during the PG plateaus 5.5, 3.5, and 2.5 mmol/L at 0, 10, 20, and 30 minutes after the respective PG plateau had been reached. At the plateau 4.0 mmol/L, blood samples were taken at 0 and 10 minutes. Symptoms of hypoglycemia were evaluated with the Edinburgh Hypoglycemic Scale (27), and hypoglycemia awareness was assessed with the participants' responses (yes/no) to the question, "Do you feel any symptoms of hypoglycemia?"

Biochemical and hormonal blood analyses

P800 EDTA tubes (BD; Becton Dickinson, Franklin Lakes, NJ) were used to collect blood samples for the glucagon measurements. P800 EDTA tubes contain protease inhibitors and DPP-IV inhibitors and were selected based on the results of previously performed stability tests that showed a highly efficient inhibition of glucagon degradation (28). Samples were centrifuged for 15 minutes immediately after sample collection, stored at −80°C, and analyzed with a solid phase two-site enzyme immunoassay (Mercodia Glucagon ELISA; Mercodia, Uppsala, Sweden) (14, 16). The coefficient of variation (CV) for intra-assay variation was 3.3% to 5.1%, and the CV for interassay variation was 7.3% to 9.4%. The specificity for cross-reaction was with glicentin 0.8%, oxyntomodulin 4.4%, mini-glucagon <0.1%, glucagonlike peptide-1 <0.3%, glucagonlike peptide-2 <0.3%, and glicentin-related pancreatic peptide <0.0005%, and the detection limit was 1 pmol/L. All glucagon measurements were performed by blinded staff members in the laboratory.

EDTA plasma samples for detection of norepinephrine and epinephrine were stored at −80°C and were measured with a radioimmunoassay (DRG Instruments GmbH, Marburg, Germany). The plasma C-peptide concentrations were determined with a two-site sandwich immunoassay (ADVIA Centaur; Siemens Healthcare Diagnostics, Camberley, UK; LOQ 0.017 nmol/L). HbA1c was measured by high-performance liquid chromatography-ultraviolet (Menarini HA-8160; Menarini Diagnostics, Florence, Italy). No insulin measurements were done during the study.

Total PG concentrations for the clamp were measured at bedside with a glucose analyzer (Super GL; Dr. Müller Gerätebau GmbH, Freital, Germany; CV 2%). To determine natural glucose, [6,6-$^2H_2$]-glucose, and tracer-to-tracee ratio (TTR), blood samples were collected in sodium fluoride tubes, centrifuged immediately for 15 minutes at 4°C, and stored at −80°C, and plasma samples were prepared as described previously (29, 30). Standards were prepared by spiking the dialyzed plasma with well-known amounts of glucose (30 to 290 mg/dL) and [6,6-$^2H_2$]-glucose (0.5 to 5.8 mg/dL) as well as internal standard ([$^{13}C_6$]-glucose, [$^{13}C_6$, $^2H_7$]-glucose). The processed samples and standards (1 μL) were directly injected into the gas chromatography–mass spectrometry (GC-MS) (7890a GC, 7000b MS; Agilent Technologies, Santa Clara, CA) in splitless mode. Chromatography was performed with a flow rate of 2 mL/min and helium as carrier gas, with an HP-5MS 30 m × 250 μm × 0.25 μm GC column (Agilent Technologies) with the following temperature program: 110°C, hold 0.5 minutes, ramp 1: 25°C/min to 225°C. The analytes were detected with electron impact ionization in single ion monitoring mode at m/z 287 (natural glucose), m/z 288 ([$^2H_1$]-glucose), m/z 289 ([$^2H_2$]-glucose), m/z 293 ([$^{13}C_6$]-glucose), and m/z 300 ([$^{13}C_6$, $^2H_7$]-glucose). The natural glucose and [6,6-$^2H_2$]-glucose concentrations were quantified, and the TTR was calculated with the peak areas of the analytes. [6,6-$^2H_2$]-glucose measurement and calculation of TTR were also performed by blinded staff members.

Statistical analysis

Data are given as mean ± standard deviation (SD) unless indicated otherwise. The level of significance was set to $\alpha = 0.05$ for all tests. All parameters were tested for normality with a Shapiro-Wilk (SW) test. If data were distributed normally (SW test, $P \geq 0.05$), a t test was applied. Otherwise (SW test, $P < 0.05$), a Mann-Whitney U test was used. Average glucagon values were compared between C-pos and C-neg patients at baseline and at each PG plateau (5.5, 3.5, 2.5, and 4.0 mmol/L). Based on pilot data, the primary parameter was defined as average glucagon concentration measured at PG plateau 2.5 mmol/L. Within each group glucagon suppression was tested by calculating the individual difference between baseline and PG plateau 5.5 mmol/L, and the glucagon increase was assessed from PG plateau 5.5 to 2.5 mmol/L with a Wilcoxon test. Average PG, TTR, norepinephrine, and epinephrine were determined for each participant and each plateau, and resulting values were compared between the groups. Overnight average PG values were compared every 30 minutes between C-pos and C-neg patients with a Mann-Whitney U test.

Calculation for EGP and the rate of peripheral glucose disposal (Rd) were performed by blinded staff members to avoid a bias. EGP and Rd levels were calculated according to Powrie and the modified equation of Steele (29, 31, 32). Average EGP levels, Rd levels, and GIRs were calculated for each participant and each plateau in C-pos and C-neg patients, and the values from C-pos and C-neg patients were compared with each other. A paired t test was used to calculate EGP suppression within each group from baseline to plateau 5.5 mmol/L and to detect the EGP increase within each group between plateau 5.5 and 2.5 mmol/L. A Wilcoxon test was used within each group to assess the increase of the Rd from plateau 5.5 to 2.5 mmol/L. Norepinephrine and epinephrine blood samples were available only for 7 C-pos and 7 C-neg patients.

Additional parameters were the area under the curve (AUC) for glucose infusion rate (AUC_{GIR}), for glucagon ($AUC_{glucagon}$), and for endogenous glucose production (AUC_{EGP}). To calculate the AUC_{GIR}, $AUC_{glucagon}$, and AUC_{EGP} for each plateau, the trapezoidal method was applied. To calculate the area under the curve for total glucose infusion rate ($AUC_{GIRtotal}$), all AUC_{GIR} values at each PG plateau were added together. Linear regression was applied to $AUC_{glucagon}$ and AUC_{EGP}.

Results

Participant characteristics

Twenty-one men and women with T1DM were enrolled in the trial. Demographic and baseline characteristics are summarized in Table 1. Mean fasting C-peptide value was 0.16 ± 0.1 nmol/L for 10 C-pos patients and 0.0 ± 0.0 nmol/L for 11 C-neg patients.

Table 1. Demographic and Baseline Characteristics of C-Pos and C-Neg Patients With T1DM

Characteristic	C-Pos	C-Neg	*P*
Subjects, n	10	11	—
Sex, male/female	5/5	5/6	NS
Age, y	39.6 ± 13	37.4 ± 13	NS
Diabetes duration, y (range)	2.5 ± 2 (1–8)	23.9 ± 10 (11–37)	<0.001
BMI, kg/m^2	23.6 ± 1.8	25.0 ± 2.3	NS
C-peptide, nmol/L (range)	0.16 ± 0.1 (0.05–0.36)	0.00 ± 0.0[a] (0.00–0.01)	<0.001
HbA1c, %	7.3 ± 0.9	7.5 ± 0.8	NS
HbA1c, mmol/mol	56.3 ± 9.8	58.5 ± 8.7	NS
Daily basal insulin dose, U (range)	9.1 ± 4 (0–15)	24.5 ± 12 (11–50)	0.002
Daily bolus insulin dose, U (range)	17.7 ± 9 (5–36)	20.9 ± 4 (15–30)	NS
Total daily insulin dose, U (range)	26.9 ± 12 (10–51)	45.4 ± 14 (26–75)	0.017

Data are means ± SD (minimum–maximum). Kruskal-Wallis and Wilcoxon tests were used. Level of significance was set at $P < 0.05$.

Abbreviation: NS, not significant.

[a]LOQ of the assay = 0.017 nmol/L for C-peptide measurements.

PG levels, TTR, and GIR

No nocturnal hypoglycemic event occurred during the night before hypoglycemia was induced. C-pos patients needed less exogenous insulin during the night before hypoglycemia induction than C-neg patients. Mean PG levels did not differ at baseline (C-pos, 5.7 ± 0.4; C-neg, 5.7 ± 0.3 mmol/L), at glucose plateaus 5.5 and 4.0 mmol/L between the two groups (Fig. 2a). At glucose plateaus 3.5 and 2.5 mmol/L, mean PG levels were significantly higher in C-pos than in C-neg patients (difference in PG concentrations was 0.1, $P = 0.037$, and difference in PG concentrations was 0.2 mmol/L, $P = 0.011$, respectively). Mean TTRs were comparable in C-pos and C-neg patients at baseline and throughout the clamp at each glucose plateau (Fig. 2b). During the hypoglycemic clamp, AUC_{GIR} did not differ between C-pos and C-neg patients at all glucose plateaus (Fig. 2c). During recovery, which was defined as the time at glucose plateau 2.5 mmol/L (15 minutes after insulin infusion had been stopped) until the end of glucose plateau 4.0 mmol/L, AUC_{GIR} was significantly lower in C-pos than in C-neg patients (difference in AUC_{GIR} values was 102 mg/kg). $AUC_{GIRtotal}$ was comparable in both groups.

Glucagon concentrations, EGP, and Rd

Mean glucagon concentrations were significantly higher in C-pos compared with C-neg patients at baseline (difference in glucagon concentrations was 4.2 pmol/L) and during all glucose plateaus (plateau 5.5 mmol/L, difference in glucagon concentrations was 2.6; plateau 3.5 mmol/L, difference in glucagon concentrations was 3.3; plateau 2.5 mmol/L, difference in glucagon concentrations was 7.0; and plateau 4.0 mmol/L, difference in glucagon concentrations was 8.2 pmol/L). At plateau 5.5 mmol/L, suppression of glucagon concentrations was observed in both groups after insulin infusion was increased. From glucose plateau 5.5 mmol/L to nadir, glucagon levels significantly increased in both groups (Fig. 3a).

Mean EGP rates did not differ in C-pos and C-neg patients at baseline and at glucose plateaus 5.5 and 3.5 mmol/L. At glucose plateaus 2.5 and 4.0 mmol/L, EGP response to hypoglycemia was significantly higher in C-pos compared with C-neg patients (differences in EGP were 0.4 and 0.8 mg/kg/min, respectively). At glucose plateau 5.5 mmol/L suppression of EGP occurred in both groups, after insulin infusion had been increased (Fig. 3b).

Mean Rd was similar in both groups at baseline and during all glucose plateaus. After insulin was increased to the constant rate, Rd increased for both groups at glucose plateau 5.5 mmol/L. Rd significantly decreased from glucose plateau 5.5 mmol/L to nadir glucose in both groups (Fig. 3c). Linear regression revealed a significant correlation between EGP and glucagon (Fig. 4).

Norepinephrine and epinephrine

Mean norepinephrine concentrations (Fig. 3d) did not differ between both groups except for lower concentrations of norepinephrine at baseline in C-neg compared with C-pos patients. Mean epinephrine concentrations (Fig. 3e) were lower in C-neg patients at plateau 5.5 and 4.0 mmol/L than in C-pos patients.

Hypoglycemic awareness and hypoglycemic symptom scores

At glucose plateau 5.5 mmol/L, all participants answered "no" to the question "Do you feel hypoglycemic?" and at glucose plateau 2.5 mmol/L, 57.1% of the patients answered "yes," regardless of their C-peptide status. Hypoglycemic symptom scores increased in response to hypoglycemia, with no differences in score according to the C-peptide status, at any PG plateau

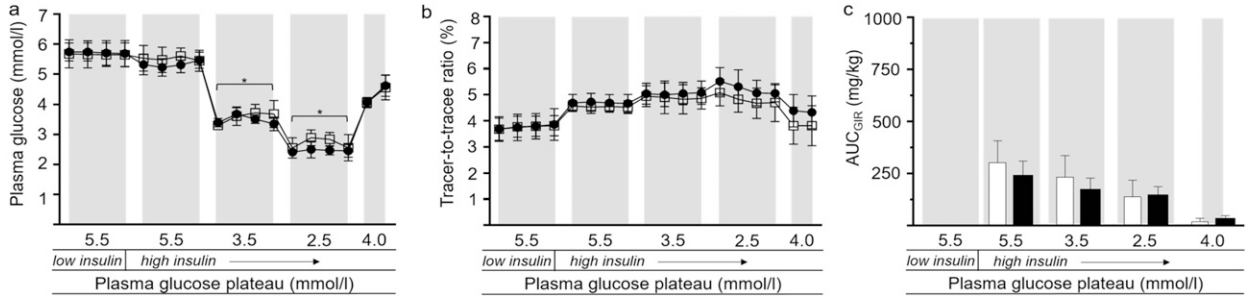

Figure 2. (a) PG concentrations, (b) TTR, and (c) AUC_{GIR} during hypoglycemic clamp at baseline (5.5 mmol/L, low insulin) and at each PG plateau (high insulin) in C-pos and C-neg patients. Four samples were taken at 10-minute intervals after patients reached baseline and the respective PG (5.5, 3.5, 2.5 mmol/L). Two samples were taken at 10-minute intervals at glucose plateau 4.0 mmol/L. Data are presented as means ± SD; C-pos patients, empty squares and white bars; C-neg patients, full circles and black bars; gray shaded areas, baseline and each PG plateau. AUC_{GIR} was calculated with the trapezoidal method. $*P < 0.05$.

or when PG decreased from plateau 5.5 mmol/L to 2.5 mmol/L.

Discussion

Our main finding was that induced hypoglycemia revealed significantly higher glucagon concentrations in C-pos than in C-neg patients and therefore might contribute to more pronounced EGP in these patients. Furthermore, significantly lower glucagon concentrations were detected in all patients at baseline and at each PG plateau than those found in previous studies (22, 23, 33, 34).

Glucagon levels in C-pos patients were significantly higher in euglycemia and throughout the hypoglycemic clamp than in C-neg patients. Interestingly, we found an insulin-dependent suppression of glucagon secretion from baseline to PG plateau 5.5 mmol/L in both groups. Furthermore, we observed in our C-pos patients a hypoglycemia-induced threefold to fourfold increase in glucagon secretion from PG plateau 5.5 mmol/L to nadir (3.3 to 9.9 pmol/L, difference of 6.6) and to recovery (3.3 to 13.9 pmol/L, difference of 10.6). In our C-neg patients, hypoglycemia-induced fourfold to eightfold increases in glucagon secretion from PG plateau 5.5 mmol/L to nadir (0.7 to 2.9 pmol/L, difference of 2.2) and to recovery (0.7 to 5.7, difference of 5.0) were seen (Fig. 3a). The findings of reduced but responsive glucagon levels at euglycemia and hypoglycemia in T1DM are in contrast to previous reports describing hyperglucagonemia independent of C-peptide status and glycemic levels (34, 35). Although in the current experiments we cannot establish the mechanism of the plasma glucagon levels, it is likely that α-cells in the islets of Langerhans remain responsive to insulin

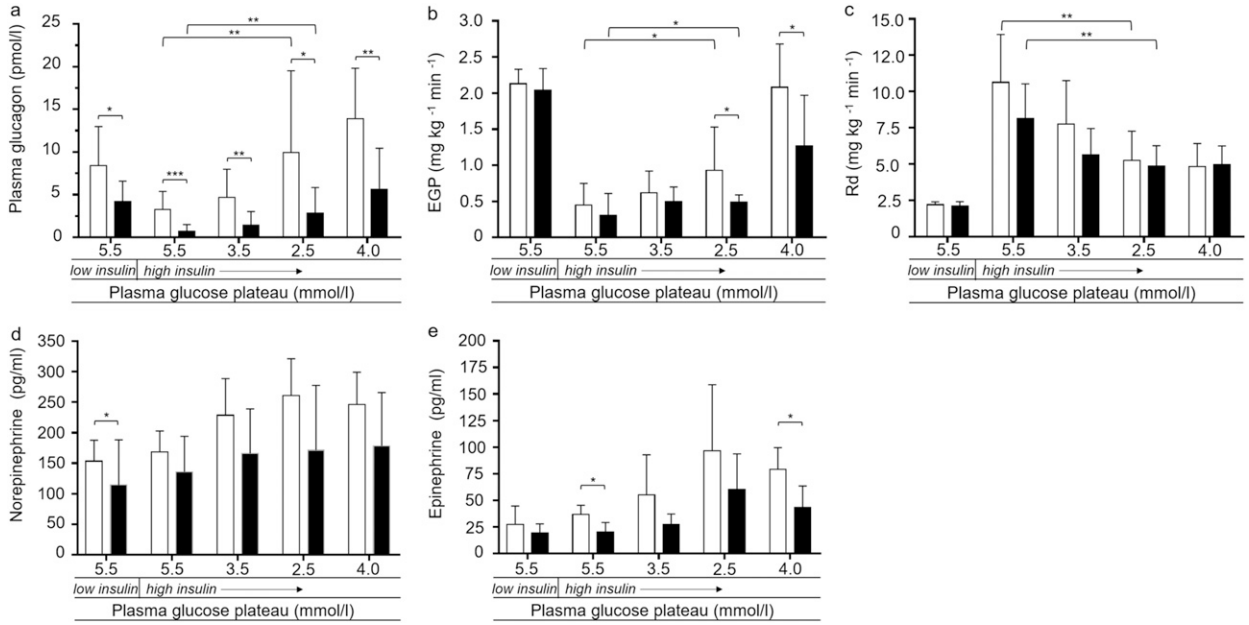

Figure 3. (a) Plasma glucagon concentrations, (b) EGP, (c) Rd, (d) norepinephrine, and (e) epinephrine at baseline (5.5 mmol/L, low insulin) and at each hypoglycemic clamp plateau (high insulin) in C-pos and C-neg patients. Data are means ± SD; C-pos patients, white bars; C-neg patients, black bars. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

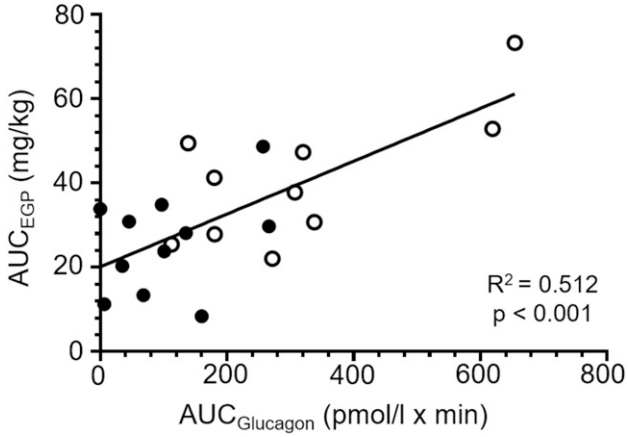

Figure 4. Linear regression of $AUC_{glucagon}$ and AUC_{EGP} in all patients with T1DM [n = 21; C-pos, empty circles (n = 10), C-neg, full circles (n = 11)]. $AUC_{glucagon}$, AUC of glucagon at plateaus 5.5, 3.5, 2.5 (standardized) and 4.0 mmol/L added together; AUC_{EGP}, AUC of EGP at plateaus 5.5, 3.5, 2.5 (standardized) and 4.0 mmol/L added together.

and to low glucose levels. This assumption is supported by our findings that C-pos patients exhibit higher glucagon levels than C-neg patients and by the positive glucagon levels staining in long-term patients with T1DM (36).

In line with our findings, Madsbad *et al.* (22) also observed higher glucagon levels with hypoglycemia in C-pos patients with T1DM compared with C-neg patients. However, they did not see any difference in glucose recovery between the two groups, and no EGPs were determined during their study. In contrast, we calculated the EGP and observed a greater EGP response at hypoglycemia (2.5 mmol/L) and recovery in C-pos than in C-neg patients. The reasons for these diverse results may lie in different hypoglycemic experimental setups. Madsbad *et al.* induced hypoglycemia with a constant insulin infusion, and they stopped the insulin infusion when patients had symptoms of hypoglycemia, regardless of their PG values. In contrast, we induced hypoglycemia stepwise depending on the patient's PG values (PG plateau 3.5, 2.5 mmol/L). Therefore, our clamp and the duration of hypoglycemia for each patient lasted longer, which could explain the different findings regarding glucose recovery.

In view of the anatomy of the pancreatic islets, one would expect in the basal euglycemic state that in C-pos patients with T1DM, the higher insulin concentrations at the level of the α-cell would suppress glucagon secretion, which would result in lower blood glucagon levels in C-pos T1DM compared with C-neg patients with T1DM. However, available published research comparing hormone levels between healthy subjects and patients with T1DM suggests that the reverse is the case (37–39). This research supports our findings of higher glucagon concentrations at baseline in C-pos than in C-neg patients with T1DM and suggests that in C-pos patients, the possibly higher insulin concentrations at the level of the α-cells may not suppress glucagon secretion.

Another impaired counterregulatory mechanism to decreasing PG in patients with T1DM is attenuated secretion of epinephrine and norepinephrine concentrations. In healthy subjects, the epinephrine response to hypoglycemia has also a stimulating effect on the EGP and limits glucose utilization by insulin-sensitive tissues (40, 41). It has been reported that the epinephrine response to hypoglycemia in healthy subjects and patients with new-onset T1DM remained intact, whereas patients with long-standing T1DM showed diminished epinephrine response (9, 42). However, we did not find a statistically significant difference between C-pos and C-neg patients regarding the epinephrine and norepinephrine response to hypoglycemia, but we found a tendency toward higher epinephrine and norepinephrine levels in C-pos compared with C-neg patients. Based on these results we assume that the epinephrine response might also contribute to the differences in the EGP response.

Several well-controlled clinical intervention studies have been performed aiming to prevent or postpone T1DM in people at risk and preserve residual β-cell function from autoimmune destruction (43–45). So far, these interventions have not been able to stop the autoimmune destruction of β-cells, but residual β-cell function has been preserved for a certain time (44–46). C-peptide status has been associated with severity of hypoglycemia in patients with T1DM, but the mechanism by which residual β-cell function and its impact on α-cells protect from severe hypoglycemia is still controversial (35, 37). It has been suggested that the inability of α-cells to produce adequate amounts of glucagon during hypoglycemia and the risk of severe hypoglycemic events is increasing with the loss of β-cell function (17, 18). In a previous study (23), the diabetes duration of patients with T1DM (7.8 $\pm$ 3.6 years) was comparable to that of our C-pos patients (range 1 to 8 years). However, our C-pos patients had higher glucagon levels and higher EGP values. We assume that the observed glucagon concentrations and the EGP might have been even more pronounced during hypoglycemia in newly diagnosed patients or patients with shorter diabetes duration.

The inclusion criteria for our C-pos patients was a fasting C-peptide level of $\geq$0.05 nmol/L, and it was challenging to find such patients for the study. Nevertheless, after recruitment the mean C-peptide level of our C-pos patients was 0.16 nmol/L. This level indicates a residual amount of endogenous insulin secretion that will remain for a certain period of time and that definitely is observed in all patients with T1DM. To ensure enrollment of patients with T1DM, the enrolled patients had a history

of T1DM with acute hyperglycemia and ketonuria, a daily insulin requirement, and a normal BMI.

We induced hypoglycemia by applying a hyperinsulinemic, stepwise hypoglycemic clamp and determined EGP with a stable isotope tracer technique. Although the tracer enrichment differed between plateaus, it remained stable within each plateau, which allowed us to apply the modified Steele equation to calculate EGP and Rd (31). Unfortunately, we cannot present data for insulin concentrations during the clamp, but based on results from Hother-Nielsen *et al.* (47) and Bell *et al.* (48), we assume that because of the high insulin infusion during the clamp and the likely suppression of endogenous insulin secretion, the insulin levels were comparable in both groups. During the hypoglycemic clamp, C-pos patients had higher mean PG levels at plateaus 3.5 and 2.5 mmol/L than C-neg patients. The fact that it was more difficult to reach glucose nadir in C-pos patients than in C-neg patients might be attributed to the greater counterregulation of higher glucagon levels and therefore higher EGP during hypoglycemia in C-pos than in C-neg patients. Furthermore, patients with T1DM with an increased glucagon release during hypoglycemia also had an increase of EGP, which strongly suggests a relationship between glucagon and EGP (Fig. 4). Two patients showed substantially high glucagon response in comparison with the other patients, but the glucagon response in healthy subjects is even higher than the two highest found in our study. C-pos patients spanning a wider range of glucagon response to hypoglycemia would have been desirable for the study. Of note, C-pos and C-neg patients showed similar glucose requirements, the same Rd, and the same hypoglycemic awareness and symptoms during hypoglycemia.

A limitation of the study is that it lacks a glucagon stimulation test to stimulate endogenous insulin secretion for C-peptide quantification. However, detecting fasting C-peptide concentrations is a validated standard. Another limitation of this study is the lack of a nondiabetic control group.

In conclusion, induced hypoglycemia revealed significantly higher glucagon concentrations and might contribute to more pronounced EGP in C-pos than in C-neg patients, which indicates that preserved β-cell function may contribute to counterregulation during hypoglycemia in patients with T1DM. Additional studies are needed to elucidate the role of the C-peptide status in the pathophysiology of glucagon secretion and its impact on the EGP to counteract hypoglycemia in patients with T1DM.

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