Glucagon sensitivity and clearance in type 1 diabetes: insights from in vivo and in silico experiments

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Submitted 15 May 2015; accepted in final form 29 June 2015

Hinshaw L, Mallad A, Dalla Man C, Basu R, Cobelli C, Carter RE, Kudva YC, Basu A. Glucagon sensitivity and clearance in type 1 diabetes: insights from in vivo and in silico experiments. Am J Physiol Endocrinol Metab 309: E474–E486, 2015. First published July 7, 2015; doi:10.1152/ajpendo.00236.2015.—Glucagon use in artificial pancreas for type 1 diabetes (T1D) is being explored for prevention and rescue from hypoglycemia. However, the relationship between glucagon stimulation of endogenous glucose production (EGP) viz., hepatic glucagon sensitivity, and prevailing glucose concentrations has not been examined. To test the hypothesis that glucagon sensitivity is increased at hypoglycemia vs. euglycemia, we studied 29 subjects with T1D randomized to a hypoglycemia or euglycemia clamp. Each subject was studied at three glucagon doses at euglycemia or hypoglycemia, with EGP measured by isotope dilution technique. The peak EGP increments and the integrated EGP response increased with increasing glucagon dose during euglycemia and hypoglycemia. However, the difference in dose response based on glycemia was not significant despite higher catecholamine concentrations in the hypoglycemia group. Knowledge of glucagon’s effects on EGP was used to develop an in silico glucagon action model. The model-derived output fitted the obtained data at both euglycemia and hypoglycemia. Glucagon clearance did not differ between glucagon doses studied in both groups. Therefore, the glucagon controller of a dual hormone control system may not need to adjust glucagon sensitivity, and hence glucagon dosing, based on glucose concentrations during euglycemia and hypoglycemia.

A CUTTING-EDGE ARTIFICIAL PANCREAS (AP) system applied in type 1 diabetes (T1D) needs to prevent and treat hypoglycemia. This is a critical goal determined by the Food and Drug Administration in their recent guidance document for an AP system (50). To meet this goal, the main counterregulatory hormone glucagon may be utilized not only to “rescue” from but also to prevent hypoglycemia. Glucagon acutely stimulates hepatic glycogenolysis and thereby endogenous glucose production (EGP) (11–13, 32). However, although the dose response of glucagon on EGP (viz., hepatic glucagon sensitivity) in T1D is currently unknown during hypoglycemia, a recent study (20) did demonstrate a dose response at euglycemia that was modulated by prevailing insulin concentrations. Furthermore, whether this is contingent on prevailing glucose concentrations and whether glucagon clearance is influenced by changing glucagon concentrations are also undetermined. Filling such knowledge gaps in glucagon physiology are vital to inform and design a control algorithm where the intensity of the “controller” can be modulated based on the information obtained. For example, if hepatic glucagon sensitivity increases with hypoglycemia, a lesser dose of glucagon could evoke an equivalent glucose response than at euglycemia. On the other hand, if hepatic glucagon sensitivity does not alter with glucose levels, then the glucagon controller of the algorithm determining glucagon dose need not be influenced by glucose concentrations.

Hepatic sensitivity to increasing doses of glucagon has been shown to be similar between healthy controls and type 2 diabetics (30). To the best of our knowledge, in the only study to date in T1D, glucagon-induced changes to EGP were reduced in T1D (31). However, hepatic glucagon response was measured at only one dose of glucagon, and experimental conditions differed between groups. Additionally, both studies (30, 31) were conducted at euglycemia or mild hyperglycemia. This limits translational applicability of the information to an AP system that seeks to prevent and treat hypoglycemia in those with T1D, where glucose concentrations can frequently drop to hypoglycemic levels. In a recent study in nondiabetic dogs, hepatic glucose production in response to a single, high-dose glucagon infusion was shown to be higher during hypoglycemia than during euglycemia (33). However, human studies in T1D that would assess hepatic glucagon response at different glucose concentrations are lacking.

Furthermore, assessment of glucagon clearance is critical for the development of the glucagon controller of a dual hormone control system. Studies (1, 36) that have directly measured glucagon clearance differed in study designs, glucagon hormone preparations, and glucagon assay methodologies. Hence a reexamination of glucagon clearance in T1D using current glucagon formulation and assay methods is necessary.

The present studies were undertaken to determine whether EGP responses to three doses of glucagon differed at euglycemia vs. hypoglycemia in T1D. To minimize confounding effects of varying hepatic glycogen content between subjects, we preloaded hepatic glycogen with a weight-based glucose infusion prior to the experiments. We tested the hypotheses that 1) there is a glucagon dose response at both euglycemia and hypoglycemia, 2) hepatic glucagon sensitivity is greater at hypoglycemia than euglycemia, and 3) glucagon clearance increases with increasing glucagon concentration. Additionally, we also proposed a novel glucagon action model based on existing knowledge of glucagon physiology and tested the model with data obtained from the current study.
RESEARCH DESIGN AND METHODS

After approval by the Mayo Institutional Review Board, 34 TID subjects signed informed consent, while 29 subjects completed the study protocol. Inclusion criteria included non-pregnant adults between 18 and 65 yr, BMI < 40 kg/m², HbA1C ≤ 10% (85.8 mmol/mol), and creatinine ≤ 1.5 mg/dl, without micro- or macrovascular complications apart from stable background diabetic retinopathy. Twenty-five subjects were on insulin pump therapy, while the rest were on multiple daily injections with a basal bolus insulin regimen.

Screening Visit

All subjects reported to the Clinical Research Unit (CRU) of the Mayo Clinic in the morning after an overnight fast. Written informed consent, medical history, and physical examination were undertaken to ensure that subjects were in good health and did not have any illnesses precluding enrollment. Blood testing and a normal ECG confirmed eligibility, and a pregnancy test was performed wherever appropriate to exclude pregnancy. Dieticians provided consultation to reinforce weight maintenance during the study and to ensure adherence to a 200-g carbohydrate daily diet. Body composition was measured using a DEXA scan (3, 4). Subjects meeting the enrollment criteria were randomized 1:1 into either euglycemia (5 mM) or hypoglycemia (<3.3 mM) study cohorts. Each subject was studied on three separate occasions with one of three glucagon infusion rates (0.65, 1.5, and 3.0 ng·kg⁻¹·min⁻¹) in random order. The studies were separated by at least a week.

Inpatient Study Visit

Day 1. Subjects reported to the CRU at 4:00 PM for the study visit within 4 wk of the screening visit. A standard mixed meal (10kcal/kg, 55% carbohydrates, 15% protein, and 30% fat) was provided at 5:00 PM. No additional food was ingested until the end of the study visit. Subjects administered their customary dose of bolus/premeal insulin according to their insulin-to-carbohydrate ratio, and the basal insulin patterns were continued. The basal insulin dose was not given in the evening for those on multiple daily injections. Two cannulae were inserted into forearm veins, one on each arm, for infusion and periodic blood draws during the study. At 9:00 PM, insulin pump therapy was discontinued and regular insulin infusion started intravenously with dose adjusted (49) to maintain euglycemia (~5 mM) or hypoglycemia (~3.3 mM) study cohorts. Each subject was studied on three separate occasions with one of three glucagon infusion rates (0.65, 1.5, and 3.0 ng·kg⁻¹·min⁻¹) in random order. The studies were separated by at least a week.

Day 2. At 5:00 AM, an 18-gauge cannula was inserted retrograde into a hand vein for periodic collection of arterialized venous blood for glucose, [3-3H]glucose, insulin, C-peptide, glucagon, and catecholamine measurements using the heated (55°C) hand vein technique. At 6:00 AM (~180 min), a constant infusion of somatostatin (60 ng·kg⁻¹·min⁻¹) and insulin (0.5 mU·kg⁻¹·min⁻¹) was started and continued for 6 h. Simultaneously, primed continuous (6 µCi bolus, 0.06 µCi/min for 180 min) infusion of [3-3H]glucose was started, and the rates were adjusted to mimic the anticipated changes in rates of EGP. A variable infusion of 50% dextrose containing [3-3H]glucose was also started simultaneously in amounts sufficient to maintain glucose at euglycemia (~5 mM) or hypoglycemia (~3.3 mM) until the end of the study at 12:00 PM. Plasma glucose was measured by YSI 2300 Stat Plus (YSI, Yellow Springs, OH) every 10 min throughout to maintain plasma glucose at target levels. At 9:00 AM (0 min), intravenous glucagon infusion was started at 0.65, 1.5 or 3.0 ng·kg⁻¹·min⁻¹ per randomization schedule and continued until end of study. Due to the known instability of glucagon in solution, the glucagon infusate was prepared shortly before the start of the glucagon infusion. To minimize adsorption to the syringe and tubings, all hormone solutions were prepared in albuminized saline. At end of the study, all cannulae were removed, and subjects were provided lunch and resumed their usual insulin programs prior to dismissal.

Analytic Methods

Plasma samples were placed on ice, centrifuged, separated, and stored at ~80°C until analyses. Plasma glucose concentration was measured using a glucose oxidase method (YSI). [3-3H]glucose specific activity was measured as described (3). Insulin was measured with a two-site immunoenzymatic assay performed on the DxI 800 automated immunoassay system (Beckman Instruments, Chaska, MN); glucagon was measured by a direct, double-antibody radioimmunoassay (Linco Research, St. Charles, MO); and catecholamines were measured by reverse-phase HPLC with electrochemical detection after extraction with activated alumina (ThermoFisher Scientific, Franklin, MA).

Calculations

All rates are expressed per kilogram of fat-free mass (FFM) per minute. Rates of glucose appearance and disappearance were calculated using the non-steady-state equation of Steele (40) during the medium-dose glucagon studies as the tracer-tracee ratios were not constant. Both steady-state and non-steady-state equations were used at low- and high-dose glucagon infusions. The calculated values were identical since tracer-tracee ratios did not change. EGP was calculated by subtracting the glucose infusion rate required to maintain glucose concentration from total glucose appearance. Hepatic glucagon sensitivity was determined by dividing the integrated iAUC (0–180 min) of EGP at each glucagon dose by the steady-state mean plasma glucagon concentrations. Glucagon clearance during the clamps was calculated by dividing the respective glucagon infusion rates by the increment in plasma glucagon concentrations (i.e., after subtracting baseline concentrations at t = 0).

Sample Size and Statistical Analysis

Preliminary data on mean (SD) of fasting rates of EGP was based on our previous study (30). The change from baseline to peak EGP was determined to be the primary outcome measure. It was assumed that the baseline and peak value would be highly correlated, but for sample size estimation we conservatively assumed that the correlation was 0.5 so that the standard deviation of the change from baseline would be 2.7. Based on an interpolation of this data, the changes from baseline in EGP in normal and type 2 diabetes were estimated to be 0, 14.7, and 17.6 µmol·kg⁻¹·min⁻¹ for glucagon infusions of 0.65, 1.5, and 3.0 ng·kg⁻¹·min⁻¹, respectively. The changes in the two
higher doses were greater than 100% and would be considered clinically relevant in this population. No preliminary data in humans exist on how the effect of hypoglycemia will impact changes in EGP, but it is hypothesized that these changes would be at least as large as those observed in the normoglycemia condition, based on a canine model (33). Accordingly, for the primary dose response test, we anticipated a maximum effect size of 6.5 [3.0 vs. 0.65 ng·kg⁻¹·min⁻¹ or (17.6 - 0)/2.7] and a minimum effect size of 1.07 [1.5 vs. 3.0 ng·kg⁻¹·min⁻¹, or (17.6 - 14.7)/2.7]. Sample size was based on this latter effect size. In particular, a sample size of 15 subjects would provide 80% power to detect an effect size of 1.07 with an \( \alpha \) of 0.05 (within glycemic group). For the between-group tests (tests of main effect of glycemic condition), we assumed that effective size of 1.07 would be the minimum clinical significant difference. Accordingly, 15 participants per glycemic condition would yield sufficient power. Thus, the total sample size for this study was determined to be 30 T1D.

The primary outcome measure for this study was the change in EGP as defined as the change from baseline to the maximum EGP observed during the first 60 min postinitiation of the glucagon infusion (“\( \Delta \)peak”). Data were modeled as a three-treatment (low-, medium-, or high-glucagon infusion), three-period cross-over design with a between-subject effect of randomized group (euglycemia or hypoglycemia), using a mixed model to account for correlation of observations within person (repeated measures) and participant withdrawal. There were some negative estimates of EGP, particularly around time 0 and 3 min, which were considered an artifact of the non-steady-state estimation equation. These negative values were treated as intermittent missing values, and, prior to analysis, these data points were imputed using the nonparametric random forest method (43). This approach to data imputation builds a random forest (an ensemble of regression trees) that can be used to predict the missing data. It is considered nonparametric since one does not need to specify the functional form of the EGP profiles. The approach is also iterative in that it repeatedly imputes the missing data and refits the data to the liver is governed by a first-order differential equation:

\[
\frac{\text{dG}}{\text{dt}} = a \cdot \text{G6P}
\]

with \( a \) representing dephosphorylation rate. G6P dynamics in the liver is governed by a first-order differential equation:

\[
\frac{\text{dG6P}}{\text{dt}} = \text{a} \cdot \text{G6P} - \text{b} \cdot \text{G6P}
\]

\( \text{a} \) and \( \text{b} \) represent dephosphorylation and phosphorylation rates, respectively.

In Silico Modeling

The hypothesized model describing glucagon action on EGP is shown in Fig. 8. Briefly, EGP is the result of dephosphorylation of glucose 6-phosphate (G6P):

\[
\text{EGP} = a \cdot \text{G6P}
\]

For variables that were essentially in steady state during the clamp (e.g., glucagon concentration, insulin concentrations, etc.), the mean of the measurements obtained during the clamp post-baseline was used. For the remaining measurements, the area under the curve was used (area under the curve is conceptually a weighted average). Similarly to EGP, missing data in these measurements also occurred, mostly due to availability of samples or laboratory/assay issues. A simplified imputation approach consisting of linear interpolation was used provided the proceeding and following values were observed. In the case of T3 missing values, the baseline (T0) value was carried forward. Likewise, if the T180 value was missing, the prior value (T120) was carried forward.

The main parameter of interest from the mixed model was the randomized group by glucagon interaction term. If this parameter was statistically significant (\( P < 0.05 \)), it would indicate that glycemia differentially affected the effect of the glucagon infusion rate. Post hoc comparisons based on model-based means (LS Means) were constructed to compare \( \Delta \)peak EGP (and other summary measures) between dose/glycemia combinations. Post hoc \( P \) values reported have not been adjusted for multiple comparisons, but a modified level of significance (0.05/3 = 0.0167) can be used to adjust for the three primary comparisons of interest (euglycemia vs. hypoglycemia for the three glucagon rates). Models were estimated using SAS v. 9.4 (Cary, NC). Random forest imputation for EGP was conducted using the missForest package using R v. 3.1.1(42).

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Euglycemia (n = 14)</th>
<th>Hypoglycemia (n = 15)</th>
<th>Total (n = 29)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>38.0 (13.3)</td>
<td>46.3 (14.8)</td>
<td>42.3 (14.5)</td>
<td>0.1108</td>
</tr>
<tr>
<td>Sex, %female</td>
<td>3 (21.4%)</td>
<td>7 (46.7%)</td>
<td>10 (34.5%)</td>
<td>0.1530</td>
</tr>
<tr>
<td>Height, cm</td>
<td>172.8 (5.8)</td>
<td>172.0 (6.8)</td>
<td>172.4 (6.3)</td>
<td>0.6783</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>83.9 (13.0)</td>
<td>77.9 (15.3)</td>
<td>80.8 (14.3)</td>
<td>0.1063</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.1 (4.1)</td>
<td>26.4 (5.3)</td>
<td>27.2 (4.8)</td>
<td>0.2056</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>57.9 (6.9)</td>
<td>54.3 (8.5)</td>
<td>56.0 (7.9)</td>
<td>0.2217</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>30.2 (8.3)</td>
<td>29.6 (9.3)</td>
<td>29.9 (8.7)</td>
<td>0.7934</td>
</tr>
<tr>
<td>FBG, mg/dl</td>
<td>146.2 (62.8)</td>
<td>169.1 (101.3)</td>
<td>158.1 (84.3)</td>
<td>0.8097</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>14.1 (1.4)</td>
<td>14.0 (1.3)</td>
<td>14.1 (1.3)</td>
<td>0.2054</td>
</tr>
<tr>
<td>HbA1C, %</td>
<td>7.6 (1.0)</td>
<td>7.5 (1.0)</td>
<td>7.5 (1.0)</td>
<td>0.8097</td>
</tr>
<tr>
<td>HbA1C, mM/mol</td>
<td>59.1 (11.0)</td>
<td>58.5 (11.1)</td>
<td>58.8 (10.8)</td>
<td>0.8097</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.9 (0.2)</td>
<td>0.9 (0.2)</td>
<td>0.9 (0.2)</td>
<td>0.4251</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>16.5 (5.1)</td>
<td>15.6 (3.9)</td>
<td>16.0 (4.5)</td>
<td>0.6932</td>
</tr>
<tr>
<td>TSH, mIU/l</td>
<td>1.9 (1.0)</td>
<td>2.1 (1.2)</td>
<td>2.0 (1.1)</td>
<td>0.6461</td>
</tr>
</tbody>
</table>
where \( \dot{G6P} \) is rate of glycogenolysis, \( \dot{Gng} \) is rate of gluconeogenesis, \( \dot{EGPb} \) is basal EGP, \( g6p0 \) is a parameter accounting for the rapid increase in EGP when glucagon rises above a given threshold. At baseline, it is assumed that \( Glys \) accounts for \( \approx 95\% \) and \( Gng \) for the remaining \( 5\% \) of the \( \dot{EGPb} \) (28).

Glycogenolysis is assumed to be linearly dependent on glucagon concentration (\( Gn \)) above a given glucagon threshold (\( Gth \)), and modulated by the so-called “evanescence” (E) effect, a well-described phenomenon for which, even at constant glucagon, insulin, and glucose levels, glucagon action tends to decrease with time (30, 32) (see also the experimental data plotted in Fig. 6, top):

\[
\dot{Glys} = \begin{cases} 
Glys_b + b \cdot (Gn - Gth) \cdot E & \text{if } Gn \geq Gth \\
Glys_b \cdot E & \text{if } Gn \leq Gth 
\end{cases}
\]

and

\[
E = \frac{1}{2} \left[ 1 - \tanh \left( \frac{t - t_0}{\tau} \right) \right]
\]

with \( b \) parameter governing the amplitude of glucagon action on glycogenolysis, \( t_0 \) determining the time of onset of the evanescence phenomenon, and \( \tau \) representing the time constant of the evanescent phenomenon. Glucagon effects on gluconeogenesis is delayed (\( XGn \)) and is prominent during hypoglycemia (14) and by other factor/s (OF) in euglycemic conditions. At variance with glycogenolysis, gluconeogenesis is assumed not to be affected by the evanescence phenomenon (14, 32, 33):

Fig. 1. plasma glucose, insulin, glucagon, and epinephrine concentrations obtained during euglycemia clamp period at low-dose (▲), medium-dose (□), and high-dose (●) glucagon infusions.

Fig. 2. plasma glucose, insulin, glucagon, and epinephrine concentrations obtained during hypoglycemia clamp period at low-dose (▲), medium-dose (□), and high-dose (●) glucagon infusions.
with \( c \) parameter governing the delay between plasma glucagon and glucagon action and \( d \) the amplitude of glucagon action on gluconeogenesis.

Model Identification The model was identified based on the average EGP measurements, using plasma glucagon as forcing function, in both euglycemia and hypoglycemia. Measurement error on EGP was assumed to be independent, Gaussian with zero mean and unknown constant standard deviation (a posteriori estimated). Plasma insulin was not included in the model, since it was maintained constant at the same level in all the studies. Parameter \( Gth \) was fixed to a population value of 80 pg/ml; the remaining parameters \( a, b, c, d, t0, \) and \( g6p0 \) were estimated from the data.

RESULTS

Subject Characteristics

Thirty-four subjects were screened for the study. Four subjects failed the screen visit (2 because of elevated C-peptide, 2 due to HbA1c). Thirty subjects were enrolled. One subject was excluded due to lack of venous access. Two subjects completed one study visit, while two others completed two study visits. All other subjects \((n = 25)\) completed three study visits. All data from subjects completing at least one study visit \((n = 29)\) were analyzed (Table 1).

Plasma Glucose, Insulin, Glucagon, and Catecholamine Concentrations

During the clamp, plasma glucose and insulin levels were not differentially affected by the combination of glycemia status and glucagon infusion levels \((P_{interaction} \leq 0.64)\) and \( P_{interaction} \leq 0.55 \), respectively). By design, glucose concentrations were statistically different between euglycemic and hypoglycemic conditions \((P_{interaction} = 0.001)\) but were not different across glucagon infusion levels within the randomized group \((P = 0.96)\) and \( P = 0.23 \) for

\[
Gng = \begin{cases} 
Gng_b + XGn \text{ in hypoglycemia} \\
Gng_b + OF \text{ in euglycemia}
\end{cases}
\]

(5)

with \( XGn \) obtained from glucagon concentration above a threshold with a chain of \( n \) compartments.

\[
\dot{X}_1 = \begin{cases}
-c \cdot X_1 + d \cdot (Gn - Gth) & \text{if } Gn \geq Gth \\
c \cdot X_1 & \text{if } Gn < Gth 
\end{cases}
\]

\[
\dot{X}_2 = -c \cdot X_2 + c \cdot X_1 \\
\ldots \\
\dot{X}_n = -c \cdot X_n + c \cdot X_{n-1}
\]

(6)

\[
XGn = -c \cdot XGn + c \cdot X_{n-1}
\]

Fig. 3. Glucose infusion rates (top) and specific activity (bottom) at euglycemia (left) and hypoglycemia (right) clamp periods at low-dose (●), medium-dose (□), and high-dose (●) glucagon infusions.
euglycemia and hypoglycemia, respectively). Likewise for the clamped mean plasma glucagon concentrations, there were significant differences between the protocol’s planned glucagon infusion rate ($P < 0.001$ for each pairwise comparison), but the differences in glucagon concentrations within each infusion rate were not different ($P = 0.78$, $P = 0.95$, and $P = 0.55$ for low, medium, and high infusion rates, respectively) between euglycemia and hypoglycemia (Figs. 1 and 2).

Epinephrine and Norepinephrine Concentrations

Epinephrine and norepinephrine concentrations followed a less succinct pattern. Specifically, although there was no overall interaction effect of randomized glycemia status and glucagon infusion rates, there were significant differences in epinephrine levels between euglycemia and hypoglycemia at each glucagon infusion rate ($P_{/H11005}/H11005 = 0.0018$, $P_{/H11005}/H11005 = 0.001$, and $P_{/H11005}/H11005 = 0.003$ for low, medium, and high infusion rates respectively). For each of these comparisons, the concentrations of epinephrine were approximately three times higher during hypoglycemia. Norepinephrine concentrations, in contrast, were not different among glycemia and glucagon infusion states (p-values ranged from 0.15 to 0.52 for all pairwise comparisons).

Glucose Infusion Rates

The AUC for glucose infusion rates over the 180-min study period was not differentially altered by glycemia across the glucagon infusion rates ($interaction = 0.77$). Within the euglycemia and hypoglycemia groups, glucose infusion amounts decreased as the glucagon infusion rate increased ($P_{/H11005}/H11005 = 0.0042$, and $P_{/H11021}/H11021 = 0.0039$), but within each glucagon infusion rate there were no differences between glycemia conditions.

Table 2. Outcome measures of 3 glucagon doses at euglycemia and hypoglycemia

<table>
<thead>
<tr>
<th>Variables</th>
<th>Glucagon Dose</th>
<th>Euglycemia ($n = 14$)</th>
<th>Hypoglycemia ($n = 15$)</th>
<th>$p**$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIR, AUC 0–180, $\mu$M/kg FFM</td>
<td>High</td>
<td>3235.1 (1926.9)</td>
<td>2,956.7 (1,732.9)</td>
<td>0.2783</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>4379.1 (1552.2)</td>
<td>3,397.8 (2,105.7)</td>
<td>0.7722</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>5073.2 (1358.5)</td>
<td>4,487.1 (1,383.6)</td>
<td>0.0042</td>
</tr>
<tr>
<td>iAUC EGP, 0–180, $\mu$M/kg FFM</td>
<td>High</td>
<td>5073.2 (1358.5)</td>
<td>4,487.1 (1,383.6)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>1306.3 (1055.4)</td>
<td>1,436.9 (862.3)</td>
<td>0.3465</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>506.8 (598.4)</td>
<td>843.7 (654.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>$\Delta$peak EGP, $\Delta$baseline to peak 0–60, $\mu$M/kg FFM</td>
<td>High</td>
<td>826.3 (1086.6)</td>
<td>1,366.2 (1,107.0)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>1169.1 (755.8)</td>
<td>782.1 (985.7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Glucagon sensitivity, iAUC EGP 0–180/mean glucagon concentration, ($\mu$M/kg FFM)/(pg/ml)</td>
<td>High</td>
<td>1.9 (3.3)</td>
<td>1.4 (2.4)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>4.1 (5.0)</td>
<td>7.8 (7.0)</td>
<td>0.3265</td>
</tr>
<tr>
<td>Glucagon clearance, glucagon infusion rate/mean glucagon concentrations, ml/kg/min</td>
<td>Low</td>
<td>18.3 (4.2)</td>
<td>18.8 (4.6)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

GIR, glucose infusion rate; EGP, endogenous glucose production; *Sample size of available data reflecting data analyzed from observed variables with missing values, due to missed blood draws and incomplete visits. **Group denotes the euglycemia or hypoglycemia randomized condition. Dose is a factor representing low, medium, or high levels of glucagon infusion. Period accounts for the ordering of presentation of glucagon doses within patient.
(\(P = 0.50, P = 0.19,\) and \(P = 0.48\) for low, medium, and high glucagon concentrations) (Fig. 3, top, and Table 2).

**Specific Activity**

Glucose specific activity patterns were flat and unchanging during low- and high-dose glucagon infusions in both groups (Fig. 3, bottom). During medium-dose glucagon infusion, there were comparable rises in glucose specific activity in both groups during the first 30 min of the clamps before remaining reasonably flat thereafter.

**Endogenous Glucose Production**

The maximum change in EGP relative to baseline (\(t_0\)) that occurred during the first 60 min of the glucagon infusion was the primary outcome measure (Fig. 4, top, and Table 2). The peak increments in EGP increased (\(P < 0.0001\)) with increasing glucagon dose during euglycemia (1.9 ± 3.3 vs. 9.6 ± 5.6 vs. 15.1 ± 7.6 \(\mu\)M-kg FFM \(^{-1}\)·min \(^{-1}\), low vs. medium vs. high dose) and hypoglycemia (1.4 ± 2.4 vs. 10.4 ± 4.1 vs. 15.1 ± 7.6 \(\mu\)M-kg FFM \(^{-1}\)·min \(^{-1}\), low vs. medium vs. high dose). Likewise, the integrated EGP excursion (iAUC 0–180 min) increased (\(P < 0.0001\)) with increasing glucagon dose during euglycemia and hypoglycemia.

**Glucose Disappearance**

Integrated rates of whole body glucose disappearance did not differ within each group with increasing glucagon dosing. Likewise, there were no differences in integrated rates of glucose disappearance between euglycemia and hypoglycemia for any glucagon dose (Fig. 4, bottom, and Table 2).

**Hepatic Glucagon Sensitivity**

It is noteworthy that the interaction (or difference in the dose response profiles based on glycemia) was not significant (\(P = 0.99\)) despite higher catecholamine concentrations in the hypoglycemia cohort. There were no significant differences in peak increments in EGP by glycemia status within glucagon infusion rates (\(P = 0.83, P = 0.82,\) and \(P = 0.92\) for low, medium and high infusion amounts, respectively). The same conclusions were reached using the EGP incremental AUCs for the entire 180-min study period and the composite measure of glucagon sensitivity (Fig. 5).

**Glucagon Clearance**

Glucagon clearance, as defined by the mean clearance rate over the period of 30 to 180 min, were not differentially affected by glycemia status for the three glucagon dosages.
Glucagon clearance was not found to increase with increasing glucagon infusion rates in either glycemia state ($P = 0.88$ and $0.47$ for euglycemia and hypoglycemia, respectively; Fig. 6).

Model-based Estimation of Glucagon Action

Table 3 presents the estimated model parameters for each of the six study conditions. Figure 7 presents the fitted EGP time intervals.

(Interaction $P = 0.86$). Glucagon clearance was not found to increase with increasing glucagon infusion rates in either glycemia state ($P = 0.88$ and $0.47$ for euglycemia and hypoglycemia, respectively; Fig. 6).
Hypoglycemia glucagon level, also implying a level of saturation in glucagon on gluconeogenesis in hypoglycemia, was lower at the higher glucagon level in both euglycemia and hypoglycemia, implying a level of saturation in glucagon action. Similarly, parameter \(a\) governing the amplitude of glucagon action on glycogenolysis, was lower at the higher glucagon level in both euglycemia and hypoglycemia, implying a level of saturation in glucagon action. Similarly, parameter \(d\) governing the amplitude of glucagon action on gluconeogenesis in hypoglycemia, was lower at the higher glucagon level, also implying a level of saturation in glucagon action. Parameters \(t_0\) and \(\tau\) were higher at the low glucagon dose, implying a more rapid onset of evanescence at high glucagon concentrations. The lack of sensitivity parameters at low-dose glucagon reflects the relative inability of glucagon to stimulate EGP at low (baseline) concentrations.

**DISCUSSION**

We report that, in T1D, 1) there was a dose response of glucagon on EGP at both euglycemia and hypoglycemia; 2) hepatic glucagon sensitivity did not differ between euglycemia and hypoglycemia; and 3) glucagon clearance did not change with increasing glucagon concentrations. The tested glucagon doses, in the presence of a somatostatin pancreatic clamp, were selected on the basis of prior studies by us (30) and on the premise that the low, medium, and high doses of glucagon would represent rates that would reflect postabsorptive (12, 23), postprandial (23), and “stress” levels of glucagon concentrations (29), respectively. Furthermore, to minimize confounding due to unequal hepatic glycogen stores between subjects, we chose to shorten the duration of the overnight fast by “preloading” hepatic glycogen with a weight-based glucose infusion overnight in all subjects. While hepatic glycogen content following mixed meals has been shown to be reduced in poorly controlled T1D vs. healthy subjects (25, 27), we studied only well-controlled T1D in whom hepatic glycogen metabolism had been shown to be similar to that in controls (27). Furthermore, with frequent plasma glucose monitoring, we ensured that no subjects developed hypoglycemia overnight prior to the clamp studies, as such events would have also confounded data interpretation.

As previously observed in healthy and in type 2 diabetes subjects (30), the time course of EGP over 3 h of the clamp showed similar rapid onset but evanescent responses within a few minutes of commencement of medium- and high-dose glucagon infusions. This rise was followed by a plateau of ~1 h before EGP gradually fell to baseline within 90–120 min. The return to basal levels likely implies, in part, depletion of hepatic glycogen reserves such that continued glucagon stimulation failed to produce continued response. We did not measure hepatic glycogen reserves either indirectly (30) or directly (25), but the similarities in the temporal profiles of EGP with those in the prior report (30) supports the conclusion that the acute glucagon effect on EGP was due to its effects on glycogenolysis (25) rather than gluconeogenesis (32).

Insulin and hyperglycemia inhibit whereas glucagon stimulates EGP (32, 48). Hence, the portal venous insulin-to-glucagon ratio is a major determinant of EGP (32). To maximize the probability of matching portal insulin/glucagon ratio between subjects and groups, we carefully selected C-peptide-negative individuals, while the use of somatostatin ensured comparable inhibition of endogenous glucagon secretion. The concurrent weight-based glucagon infusions in both groups that were matched anthropometrically ensured that the measured periph-

![Graph](https://via.placeholder.com/150)

Fig. 6. rates of glucagon clearance during euglycemia (●) and hypoglycemia (■) clamp periods at low-, medium-, and high-dose glucagon infusions.

<table>
<thead>
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<th>Table 3. Glucagon model parameters</th>
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<tr>
<td>Parameter</td>
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<tr>
<td><strong>Euglycemia</strong></td>
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<tr>
<td>Rate of dephosphorylation</td>
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<td>Sensitivity of glycogenolysis to glucagon</td>
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<td>Rate constant of other factors</td>
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<td>Sensitivity of gluconeogenesis to glucagon</td>
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<tr>
<td>Time at which evanescence term = 1/2</td>
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<tr>
<td>Time constant of evanescence process</td>
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<tr>
<td>Prompt response of G6P following glucagon step</td>
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<tr>
<td><strong>Hypoglycemia</strong></td>
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<td>Rate of dephosphorylation</td>
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<td>Sensitivity of glycogenolysis to glucagon</td>
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eral (and thus unmeasured portal) glucagon concentrations were similar at each glucagon dose in both study cohorts. Additionally, the insulin dose was selected to achieve plasma insulin concentrations in the mid-physiological range (\(\approx 200 \text{ pM}\)) that not uncommonly occur in the postprandial state in T1D on the insulin pump after a premeal insulin bolus (23). Hence, the achieved portal insulin-glucagon milieu mimicked those that may be observed in real-life situations in T1D during both euglycemia and hypoglycemia. Therefore, one could reliably compare the EGP responses at each glucagon dose between the two study cohorts. Ideally, a perfect study design would have each subject perform all six study visits; that is impractical and burdensome on the subjects. Likewise, testing the effects of more than one dose of glucagon per visit would have confounded interpretation of the data, since hepatic glycogen content would have differed at each tested dose due to effects of the preceding glucagon dose on glycogen reserves. Although a higher insulin infusion rate could also have been tested, such a rate would have considerably dampened the glucagon effect on EGP, thereby obscuring the signal completely, as has recently been demonstrated during euglycemia (20), resulting in very high plasma insulin concentrations that may not be germane to the translational applicability of the study findings. However, the proposed glucagon model will need to be tested under varying insulin concentrations and suitably modified and validated.

It is noteworthy that the human kidney also contributes to EGP in dogs (10) and in humans (44), especially after a prolonged fast and exercise through stimulation of gluconeogenesis (46), because the kidneys do not have appreciable glycogen stores (5). Furthermore, it is also established that, unlike catecholamines that have a pronounced effect on renal glucose metabolism (44), glucagon does not influence renal glucose production either in vitro (7) or in vivo (45), even during hypoglycemia in T1D (9). Hence, our observed effects of glucagon on EGP can be attributed entirely to its effects on hepatic glucose production.

Glucagon degradation is mediated through its G protein-coupled receptors and also by the insulin-degrading enzyme located mainly in the liver and kidneys (15, 16). We report that the clearance rates of glucagon did not alter with increasing glucagon concentrations within the ranges studied and did not differ between the study cohorts. These results are similar to prior reports (1, 36). It is noteworthy that we measured glucagon clearance at steady state during pancreatic clamps using somatostatin to inhibit, at least in part, endogenous glucagon secretion (37, 39). We calculated glucagon clearance by dividing the glucagon infusion rates by the increment from baseline.
of steady-state plasma glucagon concentrations, as has been described before (2). This approach would minimize confounding that might be introduced by the glucagon assay cross-reacting with nonspecific substrates, especially at low glucagon concentrations. However, our results seemed to differ from a recent report (22), that measured glucagon clearance using stochastic modeling during closed-loop studies in T1D when glucagon was administered subcutaneously. While results obtained from this study are important to help create a model of glucagon effect on EGP that will ultimately lead to the development and validation of an informed glucagon controller in a dual-hormone AP system, additional studies are necessary to assess the pharmacokinetics and pharmacodynamics of subcutaneously administered glucagon in T1D.

In our study, the lack of an effect of hypoglycemia on EGP was intriguing. On the basis of a prior report in mongrel dogs (33), we had hypothesized that glucagon effects on EGP would be accentuated during hypoglycemia. Furthermore, elegant studies in dogs suggest that, unlike during euglycemia, when insulin effects on EGP dominate over those of glucagon (41), during hypoglycemia, glucagon effects on EGP are dominant over those of insulin (14). Additionally, the ratio of the hepatic glycogen metabolism enzyme activity favoring glycogenolysis was elevated during hypoglycemia compared with euglycemia in dogs (33). As anticipated, we observed elevated plasma epinephrine concentrations during hypoglycemia, which could, at least putatively, support our hypothesis of enhanced EGP response during hypoglycemia. However, this hypothesis was rejected by our findings. The disparate results could have been due to multiple reasons that include, but are not limited to, insulin and glucagon doses tested, analytic methods applied to estimate EGP (splanchnic balance vs. isotope dilution), species difference, etc. An additional important factor could also have been the degree and duration of hypoglycemia that was tested in our study. It is possible that maintaining clamp glucose at a lower level for a longer duration could have produced different results, but that would have likely been ethically inappropriate.

Glucagon effects on hepatic glucose metabolism are primarily mediated by their acute effects on enhancing glycogen phosphorylase enzyme activity and the gene expression and mRNA content of the catalytic component of the enzyme glycogen-6-phosphatase (6, 24). Furthermore, glucagon inhibits glycogen synthase activity and glycolysis while also stimulating the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, but in vivo such an effect is delayed (21). It is noteworthy that acute glucagon effects on EGP through stimulation of glycogenolysis is transient (30), possibly due to spike-decline effects of glucagon on cAMP (47). In contrast, glucagon effects on gluconeogenesis are delayed (28) and occur after 3 h. This is because there are no glucagon receptors on skeletal muscle or kidney and very few in adipose tissue; hence, glucagon cannot increase delivery of glucogenic amino acids, lactate, or glycerol to the liver to stimulate gluconeogenesis. Hence, although glucagon can enhance hepatic gluconeogenic machinery in the liver, it cannot increase availability of gluconeogenic precursors to the liver thus minimizing any acute effects on gluconeogenesis. Glucagon effects on EGP during hypoglycemia can be separated mechanistically into acute and prolonged phases. While the acute early rise in EGP is entirely attributable to glucagon effects on glycogenolysis (first 60–90 min), during prolonged hypoglycemia (>90 min), the rise in catecholamines increases EGP through gluconeogenesis secondarily to catecholamine-induced lipolysis and muscle lactate production by stimulation of glycolysis (6, 14, 24, 28, 32, 33).
Animal and human short-term clinical trials on the safety and efficacy of dual-hormone (insulin and glucagon) algorithms exist. The glucagon controllers have been based on glucagon pharmacokinetics obtained from the swine model (18) and from dual-hormone control systems (26). These reports have ranged from feasibility studies in a diabetic swine model (17) to recent studies by the same investigative group in T1D participants (19, 34, 35) with meal and exercise interventions during closed-loop control using algorithms based on the proportional-integral-derivative control system. A few other studies have also explored use of dual-hormone algorithms in T1D (8, 22). These studies have shown encouraging results as far as glucose control targets and hypoglycemia rates are concerned. However, longer-term outpatient studies that challenge refined and informed algorithms with real-life scenarios are necessary before widespread application in artificial pancreas systems.

The data reveal that, in individuals with T1D, although there is a dose response of glucagon on EGP, hepatic glucagon sensitivity is not increased despite the higher catecholamine concentrations during hypoglycemia. Furthermore, glucagon clearance does not alter with increasing glucagon dosing. Our observations have significant implications for the development of AP algorithms to manage patients with T1D. The results imply that the glucagon controller need not take into account the prevailing glucose concentrations in order to deliver glucagon to prevent and treat hypoglycemia. For example, higher hepatic glucagon sensitivity during hypoglycemia would have implied that a lower dose of glucagon would increase EGP and, hence, plasma glucose levels by a defined amount at hypoglycemia than at euglycemia. However, these data suggest that similar doses of glucagon would result in similar EGP increments and, hence, plasma glucose levels, at both hypoglycemia and euglycemia. This is an important concept, since one would not wish to compromise T1D patient safety by underdosing glucagon as prevailing glucose levels start to fall toward hypoglycemia.

Therefore, while more dynamic experiments on the effects of glucagon on EGP and its clearance, especially in the context of subcutaneous glucagon delivery, are necessary to refine the proposed novel glucagon action model, our finding that the model-derived output well predicted the experimental data is indeed promising. The suggestion from the model parameters that there is likely a saturation limit on acute effects of glucagon on glycogenolysis and, hence, EGP would limit overzealous dosing of glucagon to prevent or treat hypoglycemia in T1D in both open-loop and closed-loop therapies.

ACKNOWLEDGMENTS

We are deeply indebted to the research participants. Our sincere thanks to the staff of the Mayo Clinic Center for Translational Science Activities Clinical Research Unit (CRU), GI Motility Core, the CRU Mass Spectroscopy Laboratory, CRU Immunochromosomal Core Laboratory, and Pamela Reich, Chad Clark, Brent McConahay, Matthew Murphy, and Shelly McCrady-Spitzer. All persons mentioned above are at the Endocrine Research Unit, Mayo Clinic, Rochester, MN. Drs. Yogi Ch. Kudva and Ananda Basu are the guarantors of this work, had full access to all the data, and take full responsibility for the integrity of data and the accuracy of data analysis.

GRANTS

This work was supported by National Institutes of Health Grants R01 DK-085516 and DK-DP3 094331, and ULI TR-000135 from the National Center for Advancing Translational Science, a component of the National Institutes of Health (NIH). C. Dalla Man and C. Cobelli are partially funded by Italian Ministero dell’Istruzione, dell’Università e della Ricerca (Progetto FIRB 2009).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


