Quantification of the Glycemic Response to Microdoses of Subcutaneous Glucagon at Varying Insulin Levels.

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OBJECTIVE

Glucagon delivery in closed-loop control of type 1 diabetes is effective in minimizing hypoglycemia. However, high insulin concentration lowers the hyperglycemic effect of glucagon, and small doses of glucagon in this setting are ineffective. There are no studies clearly defining the relationship between insulin levels, subcutaneous glucagon, and blood glucose.

RESEARCH DESIGN AND METHODS

Using a euglycemic clamp technique in 11 subjects with type 1 diabetes, we examined endogenous glucose production (EGP) of glucagon (25, 75, 125, and 175 μg) at three insulin infusion rates (0.016, 0.032, and 0.05 units/kg/h) in a randomized, crossover study. Infused 6,6-dideuterated glucose was measured every 10 min, and EGP was determined using a validated glucoregulatory model. Area under the curve (AUC) for glucose production was the primary outcome, estimated over 60 min.

RESULTS

At low insulin levels, EGP rose proportionately with glucagon dose, from 5 ± 68 to 112 ± 152 mg/kg (P = 0.038 linear trend), whereas at high levels, there was no increase in glucose output (19 ± 53 to 26 ± 38 mg/kg, P = NS). Peak glucagon serum levels and AUC correlated well with dose (r² = 0.63, P < 0.001), as did insulin levels with insulin infusion rates (r² = 0.59, P < 0.001).

CONCLUSIONS

EGP increases steeply with glucagon doses between 25 and 175 μg at lower insulin infusion rates. However, high insulin infusion rates prevent these doses of glucagon from significantly increasing glucose output and may reduce glucagon effectiveness in preventing hypoglycemia when used in the artificial pancreas.

DYSFUNCTIONAL COUNTER-REGULATION IN DIABETES

Beginning soon after the discovery of insulin, the role of the counter-regulatory hormone glucagon has been studied in light of its impact on glycogenolysis and gluconeogenesis (1). Although β-cell dysfunction is known to be the hallmark of type 1 and type 2 diabetes (2,3), α-cell dysfunction in both of these conditions may also contribute to the diabetic condition (4). The occurrence of α-cell dysfunction in diabetes is primarily due to loss of intrinsic β-cell control: insulin’s signaling is...
believed to be necessary for appropriate and timely release of glucagon during hypoglycemia, as well as for appropriate suppression of glucagon release during hyperglycemia (for example, after meals) (5). Conversely, isolated α-cell dysfunction has little effect on glucose regulation since normal β-cell function is sufficient to regulate glucose levels in the otherwise healthy individual (6). However, the administration of subcutaneous insulin, i.e., nonpulsatile, low portal-systemic ratio, does not sufficiently regulate glucagon release in people with diabetes (7). Additionally, subcutaneously delivered insulin has slow absorption and clearance profiles, even for fast-acting analogs (8). In patients who use insulin, hypoglycemia is accompanied by high insulin concentration at the time of low blood glucose, a nonphysiologic situation and a further cause of reduced glucagon effectiveness (7). For these reasons, endogenous glucagon release by the pancreas in people with diabetes cannot be relied upon in cases of imminent hypoglycemia after subcutaneous injections of insulin. Indeed, Lorenzi et al. (9) demonstrated in the 1980s that the glucagon response to hypoglycemia in people with type 1 diabetes was markedly reduced, especially in those with long-standing diabetes.

**GLUCAGON IN THE ARTIFICIAL ENDOCRINE PANCREAS**

With one early exception, most artificial pancreas systems over the last half century have been single hormone delivery with insulin for the control of type 1 diabetes (10–17). A number of control strategies have been used, along with the incorporation of glucoregulatory models (18–20), in the attempt to better match glycemic control and subcutaneous insulin delivery. However, despite the introduction of fast-acting insulin analogs, the delay in absorption and action of subcutaneous insulin remains one of the greatest hurdles to overcome in single-hormone closed-loop systems (21,22). The recently renewed inclusion of glucagon into artificial pancreas systems has led to a reduced risk of hypoglycemia during closed-loop control (23–26). Small (micromgram level) doses of glucagon have been shown by several research groups to minimize time spent in the hypoglycemic range (23,27). Yet a percentage of glucagon doses delivered are unsuccessful in preventing hypoglycemia, despite accounting for insulin on board (IOB) (27). Castle et al. (27) found that correcting glucagon dose based on IOB could account for ~46% of failures within the hour after a glucagon dose is delivered (37% of failures without accounting for IOB vs. 20% of failures with IOB-adjussting doses) (28). Factors such as sensor inaccuracy, glucagon degradation, glycogen depletion, and varying pharmacokinetic profiles have been offered as additional explanations (29), but the scarcity of data evaluating microgram doses of glucagon normally used in the setting of bihormonal closed-loop control leaves more questions than provides answers.

**QUANTIFYING THE GLUCAGON RESPONSE**

The glycemic response to doses of intravenous glucagon has been studied extensively since its discovery, both as single dose injections or constant infusions (1,30,31). Like insulin, subcutaneously delivered glucagon has a delayed onset of action compared with intravenous delivery. Unlike insulin, however, the physiological effect of subcutaneously delivered glucagon has received little attention until more recently, evident by the scarcity of integrated glucagon absorption and action models (32,33). As a result, this research study was designed with the intent to quantitatively analyze the glucose response to small doses of glucagon delivered subcutaneously at varying steady-state insulin levels. The primary goal was to elucidate the interaction between insulin and glucagon in order to model the response of endogenous glucose production (EGP) at doses used in the artificial pancreas system. A secondary goal was to determine if high levels of insulin indeed can be overcome by increasing the dose of glucagon.

**RESEARCH DESIGN AND METHODS**

**Subject Recruitment**

Subjects were recruited from the Legacy Health Services outpatient clinics in Portland, OR, or from prior contact with our laboratory, between December 2011 and January 2013. Subjects were between 21 and 65 years of age with a diagnosis of type 1 diabetes for >12 months. Exclusionary conditions included pregnancy; ongoing cardiovascular, cerebrovascular, renal, or hepatic disease; any uncontrolled chronic medical condition; oral or parenteral corticosteroid use; immunosuppressant therapy; insulin or glucagon allergy; serum insulin antibody titer >100 μU/mL; or total insulin requirement >200 units/day. The research protocol was reviewed and approved by the Legacy Health Services institutional review board, and all subjects provided written informed consent. Sample size was chosen based on an estimated α error of 0.05, a power of 85%, to detect a 20% difference between groups, with an expected SD of 20% about the mean. Adverse events were monitored and reported by the principal investigator and coinvestigators.

**Study Materials**

Drugs included regular human insulin (Humulin R; Eli Lilly and Company) and octreotide (Sandostatin; Novartis) for intravenous infusion. Glucagon (Glucagen) was provided by courtesy of Novo Nordisk. Di-deuterated glucose (6,6-2H2-glucose, 98 atom % D isotopic purity) was purchased through Sigma-Aldrich (St. Louis, MO) for stable isotope infusion. Insulin was infused intravenously at a constant rate for the 10 h of each study day, although at a different rate on each day. Ooctreotide was prepared as 2 mg in 1,000 mL of 0.9% NaCl, supplemented with 1,330 mg of deuterated glucose and delivered at 0.45 mL/kg/h for the first nine studies. However, after the occurrence of gastrointestinal side effects (loose stools or nausea), the octreotide rate was subsequently lowered to 0.25 mL/kg/h with a resultant increase in the deuterated glucose supplementation to 2,337 mg per liter of fluid, in order to maintain the same infusion rate of deuterated glucose. All protocol changes were instituted after obtaining institutional review board approval. Additionally, each liter of 10% dextrose was supplemented with 800 mg of deuterated glucose (0.8% enrichment) in order to minimize dilution effects from infused glucose (34).

**Study Procedures**

Each subject underwent three studies on three separate days, each study lasting ~10 h. Subjects arrived between 7:00 and 8:00 A.M. on each day and were admitted to the Legacy Good Samaritan Hospital after having had breakfast at least 2 h before admission and...
having turned off their insulin pumps after their breakfast bolus. Subjects were fasted throughout the entire study to remove confounding from meals. On one arm, a double stop-cock system was attached. Arterialization of blood flow was accomplished by application of a heating pad to increase blood flow and allow for blood sampling up to every 5 min. On the other arm, insulin, octreotide, and 10% dextrose were infused. Insulin infusion was constant on each day, at one of three randomly assigned rates: 1) a minimum rate of either 0.01 units/kg/h or an average of the subject’s daytime basal rate, whichever was higher (designated “low”), 2) a rate of 0.05 units/kg/h (designated “high”), and 3) midway between the minimum and maximum rates (designated “medium”). These rates were determined at the time of screening. The lowest rate of insulin infusion was not set (for example at 0.01 units/kg/h) but rather was tailored to the subject’s usual basal infusion rate in order to achieve target glucose levels during the low insulin studies. An infusion rate less than their basal requirement would lead to prolonged hyperglycemia.

Insulin and octreotide infusions were begun once the infusion catheter was available, in order to help achieve steady state quickly. Ten percent dextrose infusion was controlled by a proportional integral derivative algorithm (35), using a target glucose level of 85 ± 20 mg/dL. A 2-h run-in period was allowed for achievement of infusion steady states prior to the first dose of glucagon. Every 2 h after the run-in period, glucagon was delivered subcutaneously in a pseudo-random order based on blocks of four, in doses of 25, 75, 125, and 175 μg, varying the initial dose while keeping the same order. Each subject received the same glucagon dose order assigned during screening on each study day. Pseudo-randomization of glucagon doses resulted in the first dose as follows: 25 μg during eight studies, 75 μg during seven studies, 125 μg during nine studies, and 175 μg during five studies. Also, glucagon and insulin levels were drawn every 10 min during the 1st hour after each glucagon dose, and every 20 min during the 2nd hour. From a total of 116 batches of glucagon samples (4 per study from 29 studies), contamination was noted in 17 batches, in which the glucagon levels exceeded the upper limit of the assay. The remaining 99 batches were used for this analysis, 24 after the 25- and 75-μg doses, 26 after the 125-μg dose, and 25 after the 175-μg dose. Glucose levels were checked every 10 min (every 5 min if the previous check was <60 mg/dL) for the duration of each study utilizing the Hemocue 201 DM analyzer (Cypress, CA). Blood was drawn for measurement of di-deuterated glucose levels at the 0-min time point and every 10 min from the 60-min time point onwards.

**Laboratory Testing**

For analysis by gas chromatography–mass spectrometry, derivatization of glucose in blood samples was accomplished as follows (34):

1. 20 μL of plasma was placed into a 13 × 100 mm disposable glass test tube, to which 50 μL of water and 0.5 mL of ice-cold ethanol were added.
2. The mixture was then vortex mixed before centrifugation at 3,000 rpm for 10 min, with transfer of the supernatant, using gel-loading tips, to a 13 × 100 culture tube, and then allowing evaporation to dryness in a centrifugal evaporator (∼1 h).
3. To the dry vial, 50 μL of MOX reagent (2% methoxyamine-HCl in pyridine; Thermo Fisher Scientific, Ashville, NC) was added, and the vial was capped with a teflon-lined screw cap and then heated for 2 h at 80°C in a dry block heater.
4. After cooling, 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TCS) (Regis Technologies, Inc., Morton Grove, IL) was added to the vial, which was left to stand overnight at room temperature.
5. Using a fume hood, excess solvent was allowed to evaporate using dry nitrogen (∼30 min), and finally 100 μL of a 10% mixture of BHTFA + 1% TCS in dry n-decane was added to the vials.

**RESULTS**

**Baseline Characteristics**

Of 17 subjects initially screened, 11 met screening criteria and took part in the study. Table 1 shows the baseline characteristics of these 11 subjects. The mean age was 42 ± 11.5 years with a mean duration of diabetes of 23.9 ± 15.5 years, mean BMI of 27.7 ± 6.2 kg/m², mean basal infusion rate of 1 ± 0.3 units/h, and a mean HbA1C of 7.5 ± 0.9% (58 mmol/mol). Twenty-nine studies were completed in 11 subjects (2 subjects only completed two studies, and 1 subject only completed one study). All three drop-outs were voluntary and related to time constraints preventing the individual from returning for the repeat studies. A single pilot study was done to determine...
study parameters for the glucose infusion algorithm and is not included in the overall analysis.

**Insulin and Glucose Infusion Rates**

On average, subjects received 0.016 ± 0.006 units/kg/h (median of 0.014) during the low insulin studies (n = 10), 0.032 ± 0.003 units/kg/h (median of 0.03) during the medium insulin studies (n = 9), and 0.05 ± 0.00 units/kg/h (median of 0.05) during the high insulin studies (n = 10). Serum insulin levels were, on average, 17.6 ± 13.0 mU/L (median of 11.0 [IQR 9.7–24.6]) at the low infusion rate, 29.1 ± 8.9 mU/L (median of 28.1 [IQR 25.5–31.5]) at the medium infusion rate, and 46.0 ± 12.5 mU/L (median of 41.7 [IQR 37.5–46.8]) at the high infusion rate (Fig. 1A). Dextrose (D10%) infusion rate increased going from low (mean of 0.7 ± 0.5, median of 0.6 mg/kg/min [IQR 0.2–1]) to medium (mean of 2.9 ± 1.3, median of 3.2 mg/kg/min [IQR 1.9–4]) to high (mean of 4.5 ± 2, median of 5.1 μg/kg/min [IQR 2.9–6.2]) insulin infusion rates (Fig. 1B and D); P < 0.001 for between-group and linear trend analyses. Mean glucose levels during the low insulin studies were higher than those during the medium and high insulin studies (mean glucose after the initial 2-h run-in period: 150.8 ± 68.3, 92.9 ± 21.3, and 88.0 ± 16.0 mg/dL, respectively). The dextrose infusion algorithm used kept subjects within the target range 61% of the study time. Subjects spent, on average, 18 min in the hypoglycemic range (<70 mg/dL), or 3% of the total study time, and only one subject had a single venous glucose reading <50 mg/dL.

**Glucagon Levels**

The 60-min AUC and mean incremental change for serum glucagon matched well with glucagon doses used during each study; r² = 0.63 (Fig. 1C and E).

### Table 1—Baseline characteristics of study subjects

<table>
<thead>
<tr>
<th>Baseline characteristics (units)</th>
<th>Value* (n = 11)</th>
<th>IQR (25–75%)</th>
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<tbody>
<tr>
<td>Sex (% males)</td>
<td>54.5</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.0 ± 11.5</td>
<td>36.5–46.0</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>23.9 ± 15.5</td>
<td>11.0–32.5</td>
</tr>
<tr>
<td>HbA1c (% [mmol/mol])</td>
<td>7.5 ± 0.9 [58]</td>
<td>7.0–8.2 [53–66]</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>84.0 ± 19.0</td>
<td>69.1–93.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.7 ± 6.2</td>
<td>23.0–31.1</td>
</tr>
<tr>
<td>Basal insulin infusion rate (units/h)†</td>
<td>1.0 ± 0.3</td>
<td>0.8–1.3</td>
</tr>
</tbody>
</table>

*For all except sex; values expressed as mean ± SD. †Average of daily basal rates.
Linear regression identifies a 2,984.6 min · pg/mL increase in the glucagon AUC over 60 min for each 25-μg increase in the dose (P < 0.001), with a change in the peak serum level of 73.6 pg/mL for each 25-μg increase in the glucagon dose (P < 0.001). Average time to peak glucagon serum concentration was 23.2 ± 13.5 min for the 25-μg dose, 17.1 ± 8.1 min for the 75-μg dose, 19.6 ± 6.1 min for the 125-μg dose, and 20 ± 9.6 min for the 175-μg dose, with no significant difference across the doses.

**EGP**

EGP (measured by AUC) for each glucagon dose was calculated and stratified by insulin infusion rate. Two extreme outliers were excluded from the data analysis, as they were beyond the upper third SD level. Figure 2A shows the mean incremental change in EGP (time = 0 used as baseline) across each glucagon dose, and Fig. 2B shows 60-min AUC analysis with P values from linear regression analysis (without accounting for clustering) across glucagon doses. Table 2 and Fig. 2C separate mean EGP by glucagon dose and insulin infusion rate. Trends across the four dose groups were mirrored across the weight-adjusted doses and showed a significant rise in EGP for the low (P = 0.038; slope = 0.632 mg/kg per μg of glucagon) and medium (P = 0.04; slope = 0.59 mg/kg per μg of glucagon) insulin infusion rate experiments. However, as glucagon dose increased within the high insulin infusion rate group, there was no significant elevation in the EGP AUC. Results across all infusion rates show a mean increase in the AUC over 60 min of 20.7 mg/kg, for each 50-μg increase in the glucagon dose. The estimated dose-response curves across all doses, as well as for low and high insulin infusion rates, are plotted in Fig. 2D.

**Adverse Events**

There were a total of 42 reported adverse events throughout the study, none of which were severe. Nausea (38%), diarrhea (28%), and headache (21%) were the most frequent occurrences, with episodes of vomiting (10%) and weakness (10%) occurring less frequently. Hyper- and hypoglycemia occurred in 10% of cases as well.

**CONCLUSIONS**

In this study, at the low and medium infusion rates of 0.016 and 0.032 units/kg/h, the EGP response rose proportionately to the glucagon dose, with an average increase in AUC from 5 to 113 mg/kg (P = 0.038) and 14 to 75 mg/kg (P = 0.04). In contrast, during the high insulin infusion rate study, EGP values remained relatively flat (a nonsignificant rise of 18 to 26 mg/mg as glucagon dose increased). This finding suggests that at insulin serum concentrations of >40 mL/L, glucagon doses of 175 μg or lower are largely ineffective at increasing blood glucose levels.

Subcutaneous glucagon has become a useful tool in the bihormonal artificial pancreas, but in some cases, subjects in such studies develop hypoglycemia despite the administration of glucagon. Most cases of hypoglycemia experienced during the management of type 1 diabetes result from a high insulin effect. In the closed-loop setting, administration of glucagon when insulin effect is high would require scaling the dose upwards to compensate for prevailing insulin activity. Most glucoregulatory models currently used can predict insulin serum levels with relative certainty, and this information can then be used to appropriately dose glucagon during online running of an artificial pancreas system. Fear of instability in glucose control, with oscillations in glucose levels between hypoglycemia and hyperglycemia due to competition between insulin and glucagon remains a concern within the artificial pancreas community, but more importantly, the inability to prevent hypoglycemia due to prevailing insulin effect if glucagon is underdosed sets an important precedent for quantifying this relationship.

Glucagon’s primary action is upon increasing hepatic glucose output through glycogenolysis and, to a lesser extent, gluconeogenesis (30). Therefore, the quantification of glucagon action depends upon estimating this effect in vivo. The determination of EGP by tracer methods has evolved over the half century since first introduced (39). Although it remains difficult to accurately estimate EGP in non–steady-state conditions (such as after a dose of glucagon), the use of mathematical models for this estimation has proved useful in elucidating the quantitative relationship between insulin, glucagon, and glucose. Cherrington and colleagues (40) defined the action of intravenously delivered glucagon in the canine model, which is likely translatable to...
Table 2—Mean EGP AUC over the first 60 min after each dose

<table>
<thead>
<tr>
<th>Insulin infusion rate</th>
<th>Mean EGP AUC (±SD) over 60 min (mg/kg) by glucagon dose</th>
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<tbody>
<tr>
<td></td>
<td>25 µg</td>
</tr>
<tr>
<td>Low</td>
<td>5.05 (±67.9)</td>
</tr>
<tr>
<td>Medium</td>
<td>13.96 (±99.7)</td>
</tr>
<tr>
<td>High</td>
<td>18.82 (±53.6)</td>
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</tbody>
</table>

Acknowledgments. The authors thank Dr. Andrea Mari (University of Padova, Padova, Italy) for instruction and guidance into tracemaker analysis techniques and for the gracious use of his MATLAB software, GLUTRAN. The authors also thank Dr. Wayne Bequette (Rensselaer Polytechnic Institute, New York, NY) for fielding many questions about glucose infusion algorithms and Dr. Mike Lasarev (OHSU) for assistance with methods of statistical analysis. The authors especially thank the nursing and administrative staff of Legacy Good Samaritan Hospital (Portland, OR) for assistance during these studies.

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Duality of Interest. J.R.C. has a financial interest in Pacific Diabetes Technologies Inc., a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by OHSU. W.K.W. has a financial interest in Pacific Diabetes Technologies Inc., a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by OHSU. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. J.E.Y., J.R.C., P.A.B., and W.K.W. helped develop and implement the study protocol, performed data analysis, and assisted in the writing and editing of the manuscript. A.H. performed data analysis and provided substantial consulting for interpretation of data. D.L.B. and M.B. provided technical assistance during all studies and assisted in data analysis. J.E.Y. 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 analysis.

Prior Presentation. Data from this study were included in an abstract that was submitted to and presented as a poster at the 73rd Scientific Sessions of the American Diabetes Association, Chicago, IL, 21–25 June 2013.

References