A dose-response study examining the contribution of GLP-1 receptor signaling to Glucagon-stimulated insulin secretion

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Abstract

Glucagon is an important insulin secretagogue[1,2] in the concentrations found within the islet[3]. α-cell dysfunction as occurs in T2DM[4,5], in prediabetes[6,7] or in response to FFA elevation[23] results in increased glucagon concentrations. In these conditions glucagon signals through the GLP-1 receptor (GLP1R) present on β-cells[8] but its net contribution to insulin secretion is unknown. In this experiment we will determine the contribution of GLP-1R signaling to glucagon-induced insulin secretion *in vivo*. To do so, we will use a dose-response experiment for glucagon-stimulated insulin secretion. The experimental conditions will then examine how glucagon-stimulated insulin secretion is modified by blockade of GLP1R blockade using exendin-9,39. This approach will complement other experiments using a glucagon bolus known to stimulate β-cell function[21,22] in the presence and absence of GLP1R blockade.

I. Hypothesis and Specific Aims

Glucagon within the islet can signal the β-cell through GLP1R, and acts as an insulin secretagogue[3,8]. This signaling is blocked by exendin-9,39[9,10]. The relative importance of glucagon signaling through its cognate receptor or through GLP1R is unknown. Despite the lower affinity of GLP1R for glucagon[11], intra-islet concentrations of glucagon are sufficiently high to stimulate GLP1R[3]. The other situation where this may occur is in response to pharmacologic doses of glucagon as used for β-cell function testing[1,2] or raising peripheral glucagon concentrations above fasting values[5,12,13]. **The experiments proposed will characterize the role of GLP1R in glucagon's actions on the β-cell and the potential therapeutic role of dual (**GLP-1R and glucagon receptor[11]**) agonists for the treatment of T2DM and obesity**. The experiments will also provide an opportunity to test glucagon's actions on insulin secretion, independently of glucose concentrations.

We will therefore address the following specific aim: -

Specific Aim: Determine the contribution of *GLP1R* to glucagon-stimulated insulin secretion.

1° Hypothesis: Glucagon-stimulated insulin secretion is decreased by GLP1R blockade with exendin-9,39

2° Hypotheses: The contribution of GLP1R to glucagon-stimulated insulin secretion is more significant at higher glucagon concentrations

II. Background and Significance

Diabetes mellitus causes morbidity, mortality and costs in excess of ~$170 billion/year[14]. The discovery of gut-derived proglucagon peptides, most specifically GLP-1[15], has fueled therapeutic innovations for T2DM[16]. Proglucagon is transcribed and translated from the glucagon gene (*GCG*) but its final fate depends on concomitant expression of prohormone-convertase (PC) enzymes[17]. PC-1/3 is expressed in intestinal L-cells resulting in production of GLP-1. On the other hand, PC-2 is expressed in α-cells so that glucagon is the main derivative of proglucagon produced by these cells. However, PC-1/3 expression is inducible, suggesting that extra-intestinal synthesis and secretion of GLP-1 is possible[18]. IL-6[19], hyperglycemia[20] and FFA[21] seem to increase PC-1/3 expression in islets. **This raises the possibility that pancreatic production of GLP-1 modulates islet function in response to glucolipotoxicity and other metabolic stressors[9]**.

In pharmacologic concentrations, GLP-1 is a powerful insulin secretagogue. It also inhibits glucagon secretion[22], delays gastrointestinal transit and decreases appetite[23]. Endogenous concentrations of active GLP-1 are much lower and transient[24], partly due to the ubiquity of DPP-4. Considerable GLP-1 degradation occurs close to its site of production[25] resulting in a circulating $T_{1/2}$ ~1min[26]. This may explain why inhibition of GLP-1 actions at GLP1R using a competitive antagonist (exendin-9,39) has little effect on postprandial glucose metabolism[27,28]. Indeed, only one study to date has shown a post-prandial effect of exendin-9,39 in healthy humans[29] and this can be explained by increased gastric volume prior to meal ingestion[30,31]. On the other hand, after bariatric surgery when endogenous GLP-1 concentrations are raised several-fold, inhibition of GLP-1 action impairs insulin secretion and raises glucose concentrations slightly, but significantly[28].

However, the implication that endogenous GLP-1 is of little relevance outside of bariatric surgery, is at odds with the conservation of the GLP-1 sequence across vertebrates[15]. GLP-1 may also contribute to the 'incretin effect' – the potentiation of post-prandial insulin secretion by gut (as opposed to I.V.) nutrient delivery. Salehi et al. reported that endogenous GLP-1 enhances postprandial insulin secretion[32]. However, the experiment

utilized a hyperglycemic clamp as opposed to the transient meal (or meal-like) challenges in our experiments (**Fig.1**)[28,33]. More germane to this proposal, they also observed an effect of GLP1R blockade on insulin secretion in response to intravenous glucose alone[32].

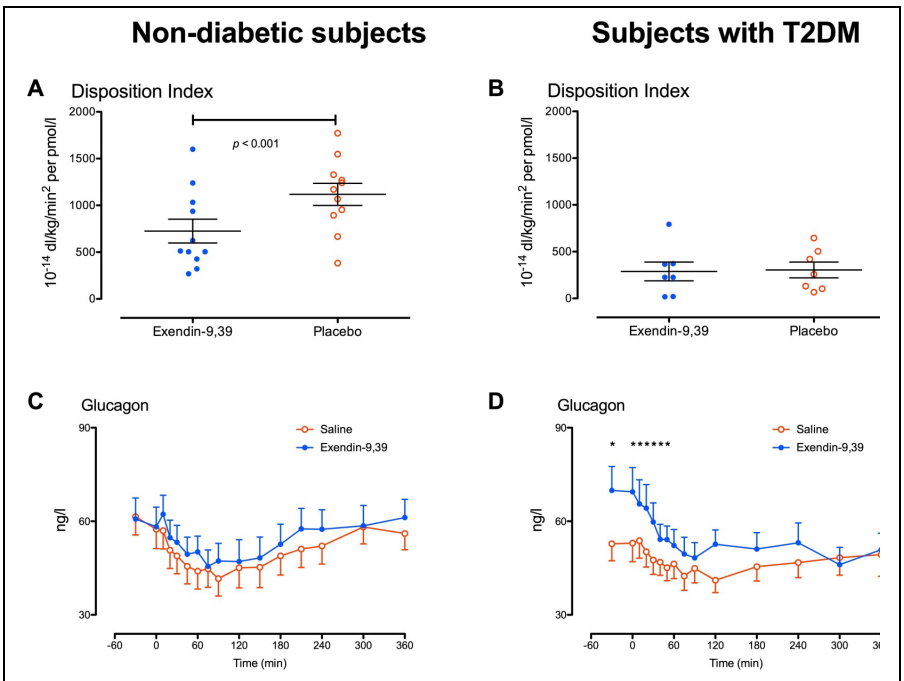

Figure 1: Effect of GLP-1 receptor blockade using exendin-9,39. At time 0, subjects received an intravenous glucose challenge. Exendin-9,39 raised post-challenge glucose concentrations (also fasting in T2DM). In people without diabetes, β-cell function (**A**) is impaired but this is not apparent (**B**) in people with T2DM. On the other hand, in non-diabetic subjects there is no effect on glucagon (**C**) whereas in T2DM, glucagon is increased during fasting and the first 50 minutes after the glucose challenge (**D**). * $p < 0.05$. Non diabetic data from Sathananthan, M., et al. (2013) *Diabetes* 62(8): 2752-2756

Infusion of exendin-9,39 to block GLP1R during an intravenous glucose challenge (that mimics the systemic appearance of a meal) impairs β-cell function in non-diabetic humans with little effect on glucagon secretion (**Fig.1a,c**)[33]. Similar effects are observed in cadaveric islets from people without T2DM (**Fig.2**). Conversely, in people with T2DM (**Fig.1b,d**) or in islets isolated from diabetic humans (**Fig.5**), there is no effect of exendin-9,39 on β-cell function, perhaps due to the degree of β-cell dysfunction. However, in T2DM, exendin-9,39 raises fasting glucagon and impairs its suppression by intravenous glucose *in vivo* (**Fig.4**). **Our preliminary data also suggests that GLP-1 contributes to islet function outside the postprandial period; the effects differ in T2DM compared to non-diabetic subjects (Fig.1)**.

Although the source of fasting GLP-1 secretion is uncertain, complementary experiments in isolated human islets (and the literature[9,19,34,35]) strongly suggest an islet source for GLP-1 (**Fig.2**). O'Malley et al. have suggested that islet GLP-1 expression increases with the development of diabetes[36]. It is also notable that exposure of islets to FFA and hyperglycemia also increases GLP-1[18,37,38]. Our published[33] and unpublished (**Fig.1**) preliminary data, as well as genetic data[39] shows that GLP-1 signaling through its cognate receptor in the fasting state (or during an intravenous glucose challenge) plays a role in islet function. **Understanding the physiologic role of GLP-1 secretion independently of oral intake in humans will allow us to better modulate this pathway and prevent / treat islet dysfunction in prediabetes and T2DM.**

The other consideration is that glucagon within the islet can signal the β-cell through GLP1R, and acts as an insulin secretagogue[3,8]. This signaling is blocked by exendin-9,39[9,10]. The relative importance of glucagon signaling through its cognate receptor or through GLP1R is unknown. Despite the lower affinity of GLP1R for glucagon[11], intra-islet concentrations of glucagon are sufficiently high to stimulate GLP1R[3]. The other situation where this may occur is in response to pharmacologic doses of glucagon as used for β-cell function testing[1,2] or raising peripheral glucagon concentrations above fasting values[5,12,13]. **The experiments proposed will characterize the role of GLP1R in glucagon's actions on the β-cell and the potential therapeutic role of dual (**GLP-1R and glucagon receptor[11]**) agonists for the treatment of T2DM and obesity**.

Glucagon dysregulation is likely to be an earlier contributor to the pathogenesis of T2DM than has been appreciated[40]. More significantly, although I.V. administration of glucagon is used as a test of β-cell function[2], its role as an insulin secretagogue within the islet has been overlooked[3]. Glucagon can signal the β-cell through GLP1R in addition to the glucagon receptor[8] (and its action blocked by exendin-9,39)[10]. What role does this play in physiology and in T2DM? The experiments proposed will quantify the effects of exendin-9,39 on glucagon-stimulated insulin secretion. Complementary experiments will also explore whether glucagon-stimulated insulin secretion is altered by rs3765467, by T2DM and by acute FFA elevation to mimic metabolic stress.

III. Preliminary Data

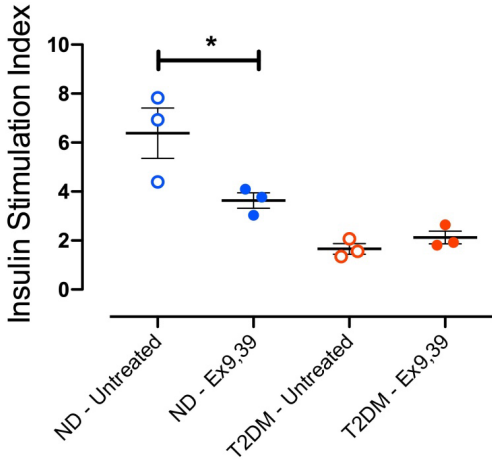

Figure 2: Acute GLP1R blockade with Ex-9,39 in isolated human cadaveric islets (obtained from Prodo labs) from non-diabetic subjects (ND) decreased insulin secretion in response to hyperglycemia. This was not apparent in T2DM. Glucose-stimulated insulin secretion was assessed by static incubation of islets at low (4mM) and high (16mM) glucose ± 100nM Ex-9,39. Insulin Stimulation Index represents fold change in insulin secretion at 16/4 mM glucose. *p<0.05 (n=3 technical repeats).

We[27,28] and others[41-43] have shown that GLP-1 in the postprandial period has little effect on post-prandial insulin secretion outside of RYGB. Intriguingly, a variant in GLP1R that increases response to GLP-1[44] has been shown to decrease risk of T2DM and lower fasting glucose[39,45]. Also, DPP-4i which raise postprandial concentrations of active GLP-1[46] also lower fasting glucose concentrations, suggesting that fasting GLP-1 secretion alters islet function. The literature[9,19,34,35] and our preliminary data (**Fig.1,2**), suggest a pancreatic source for GLP-1.

T2DM, inflammation and metabolic stress increase islet expression of GLP-1[19], perhaps as an adaptive function to preserve α- and β-cell function. Blockade of GLP1R with exendin-9,39 seems to have divergent effects in people with and without T2DM (**Fig.4,7**), perhaps because of the degree of β-cell dysfunction present (**Fig.4,5**). In **Specific Aim 2** (**Fig.9**) we will characterize differences (in people with and without T2DM) in islet function during fasting and hyperglycemic conditions attributable to endogenous GLP-1. We will also alter fasting FFA concentrations[40] to examine the role of GLP-1 during metabolic stress in people without T2DM.

Glucagon is a known insulin secretagogue[1,2]. Within islets, glucagon can act on the β-cell through GLP1R in addition to its cognate receptor[3,10]. The experiments will provide an opportunity to characterize the GLP1R contribution to glucagon-stimulated insulin secretion using a range of glucagon infusion rates.

IV. Research Design and Methods

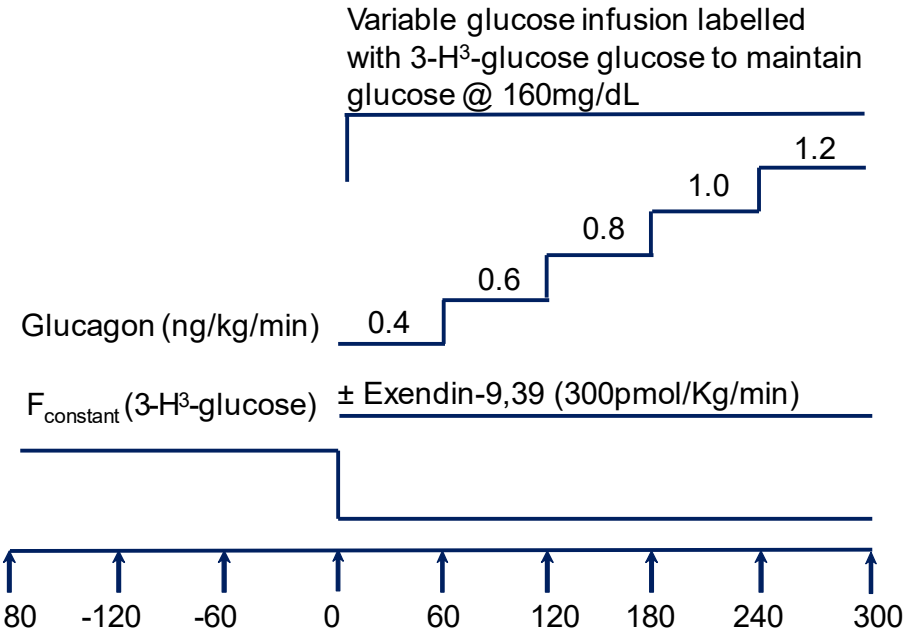

Figure 3: Experimental Design

Study Subjects: After approval from the Mayo Clinic Institutional Review Board, we will recruit 20 weight-stable, otherwise healthy subjects between the ages of 18-65, using local advertising and other methods employed previously[40]. Subjects will have no history of active disease or diabetes. Individuals who have expressed interest in participating in research will also be contacted and invited to participate in the study. **Screening Visit:** After an overnight fast of 8 hours, subjects will present to the Clinical Research Trials Unit (CRTU) at approximately 0700. Subjects will provide written, informed consent. To ensure they are otherwise healthy, subjects will undergo a history and limited physical examination; blood collection for complete blood count, fasting glucose, $HbA_{1c,}$ sodium, potassium, creatinine and urine collection to exclude pregnancy. An ECG will also be performed. The Minnesota Leisure-Time Physical Activity questionnaire will be used to assess habitual activity levels[47]. **Body Composition:** After informed written consent is obtained, body composition will be measured at the screening visit using dual-energy X-ray absorptiometry (iDXA scanner; GE, Wauwatosa, WI). All subjects will be instructed to follow a weight-maintenance diet (55% carbohydrate, 30% fat and 15%

protein) for at least 3 days prior to each study. **Screening OGTT:** At approximately 0900 subjects will ingest 75g glucose. Arterialized venous blood will be sampled over 2 hrs[48]. Glucose values at 0 and 120 minutes will be used to categorize glucose tolerance status. Subjects who have had a DEXA or OGTT in the last 3 months as part of the Vella Lab research studies or another Mayo research group, and weight is stable (+) or (-) 5 pounds, will not be retested and previously obtained values will be used.

We will study 20 subjects a total of 2 times at least a week apart in random order. All subjects will be admitted to the Mayo Clinic CRTU at 1700 hours the evening before the study. Women who could become pregnant will have a urine collection for point of care pregnancy testing. Following ingestion of a standard 10 kcal/kg caffeine-free meal, subjects will fast overnight. The following morning at approximately 0530 (-210 min), a dorsal hand vein will be cannulated and placed in a heated Plexiglas box maintained at 55°C to allow sampling of arterialized venous blood. The contralateral forearm vein will be cannulated for glucose, and hormone infusions. At approximately 0600 (-180 min), a primed, (10μCi prime, 0.1μCi/min continuous) infusion containing trace amounts of glucose labeled with [3-^{3}H] glucose will be started and continued till 0900 (0 min). Subsequently, the infusion will be varied so as to mimic the anticipated pattern of fall of EGP to minimize changes in Specific Activity[49]. At that time another glucose infusion, also labeled with [3-^{3}H] glucose will commence and infusion rate varied so as to produce peripheral glucose concentrations of ~160mg/dL. A glucagon infusion at 0.4ng/Kg/min will start at 0900 (0 min), increasing by 0.2ng/kg/min every hour **(Fig.3**). Blood will be sampled till the end of the study (1400 – 300 min) when I.V. cannulas will be removed; participants will consume a late lunch and leave the CRTU. This will be referred to as the saline (**Sal**) day because 0.9% Saline will be infused between 0 and 300 min. On another study day, exendin-9,39 (**Exe**) will be infused at 300pmol/kg/min between 0 and 300 min.

Analytical Techniques: All blood will be immediately placed on ice, centrifuged at 4°C, separated and stored at -80°C until assay. *Glucose* will be measured using a Yellow Springs glucose analyzer. *Glucagon* will be collected in protease inhibitor-containing tubes (BD800, BD Franklin Lakes, NJ) and measured using an ELISA (Mercodia, Winston-Salem, NC). *C-peptide* will be measured using EMD Millipore (Billerica, MA) reagents. *Insulin* will be measured using a chemiluminescence assay with reagents obtained from Beckman (Access Assay, Beckman, Chaska, MN). *Total and Intact GLP-1* will be collected in protease inhibitor-containing tubes (BD800, BD Franklin Lakes, NJ) and measured using an ELISA (ALPCO Diagnostics, Salem, NH). *[3-^{3}H] glucose specific activity* will be measured by liquid scintillation counting after deproteinization and passage over anion- and cation-exchange columns[50].

Calculations: In addition to fasting, peak and nadir *hormone concentrations*, we will calculate area above basal (AAB) or area under the curve (AUC) during the experiment using the trapezoidal rule as previously described[50]. *S_i, Insulin Secretion Rate (ISR) and β-Cell responsivity (Φ)* indices will be calculated[51] from the glucose, insulin and C-peptide concentrations observed during the experiments using the minimal model[52], incorporating age-associated changes in C-peptide kinetics[53]. Φ is derived from the sum of ϕ_1 (1st phase insulin secretion) and ϕ_2 (2nd phase insulin secretion) as before[44]. Disposition index (DI) will express β-cell function as a function of the prevailing insulin action[51]. *Glucose disappearance (Rd)* will be calculated using steady state Steele equations[54,55] after *Specific activity* is smoothed as previously described[56]. *Endogenous glucose production (EGP)* will be calculated as the difference between the tracer determined rate of glucose appearance and the glucose infusion rate. All infusion rates will be expressed as Kg per lean body mass.

Power Calculation	Mean ± SD	Detectable difference
1° hypothesis (*n* = 20 - paired)		
Φ ($10^{-9}min^{-1}$) - hyperglycemia	149 ± 45	30 (20%)

Table 1: Means ± SD used to estimate power

Statistical Analysis: A paired 2-tailed student *t*-test (parametric) or a Wilcoxon rank-sign test (non-parametric) will be used to examine differences between **Sal** and **Exe** days for the 1° hypothesis (**Table 3**).

3) Power Calculation: Please refer to **Table 3**.

3) Interpretation: If glucagon stimulates insulin secretion by acting, at least partially, through GLP1R we would expect that exendin-9,39 will decrease the response to glucagon by blocking GLP1R. To test our 1° hypothesis, we will compare Φ during each glucagon infusion rate in the presence and absence of exendin-9,39. Assuming that glucagon concentrations as encountered in the islet are necessary to stimulate insulin secretion via GLP1R, we expect differences to be more marked at the higher glucagon infusion rates (2° hypothesis). The experiments will be conducted in relatively lean, healthy and insulin sensitive humans. Under these experimental conditions, we expect EGP to be suppressed and insulin secretion stimulated by hyperglycemia and glucagon infusion. The rising infusion rates of glucagon will enable us to examine the

effects of the latter on endogenous glucose production (EGP) and endogenous insulin secretion in the presence and absence of GLP1R blockade.

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