Effect of genetic variation in the GLP-1 Receptor on β-cell function during fasting and hyperglycemia in nondiabetic humans

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Abstract

Post-translational processing of proglucagon in enteroendocrine cells yields a highly conserved peptide hormone, Glucagon-Like Peptide-1 (GLP-1)[1,2]. Although GLP-1-based therapy is now used to treat type 2 diabetes (T2DM) and obesity[3], important questions about its physiological role remain[4]. It has a $t_{1/2}$ ~ 1 minute in the circulation due to rapid degradation by a ubiquitous peptidase – Di-Peptidyl Peptidase-4 (DPP-4)[5] and indeed, a significant proportion of endogenous GLP-1 is degraded close to the site of production[6]. Competitive antagonism of the GLP-1 receptor (GLP1R) with exendin-9,39 has little effect on postprandial insulin secretion – unless post-prandial GLP-1 concentrations are markedly elevated[7]. These conditions are typically only fulfilled after Roux-en-Y Gastric Bypass (RYGB)[8] and, to a lesser extent sleeve gastrectomy[9]. Nevertheless, data from our lab[10,11] and others[12] could suggest a physiologic role for GLP-1 secretion beyond the postprandial state.

We had previously shown that a non-synonymous Single Nucleotide Polymorphism (nsSNP) substituting an arginine for glutamine in *GLP1R* (rs3765467), increases insulin secretion in response to intravenous glucose alone as well as to exogenous GLP-1[13]. More recently, this variant has been shown to protect against T2DM and is associated with decreased fasting and, to a lesser extent, decreased postprandial glucose[14,15]. These and other findings support a role of endogenous GLP-1 (secreted during fasting and independently of meal ingestion) in islet function. We will use genetic variation at rs3765467 to probe the effect of endogenous GLP-1 secretion and of GLP-1 receptor agonism on islet function (independent of meal ingestion) *in vivo*.

I. Hypothesis and Specific Aims

Defects in α-cell[16] and β-cell function[17] are central to the pathogenesis of T2DM and to the progression from prediabetes through to T2DM[18]. There is accumulating evidence that α-cells can produce GLP-1 which in pharmacologic doses has potent anti-diabetic effects[4]. It has been hypothesized that this 'pancreatic' GLP-1 acts in a paracrine fashion to support islet function – at least early in the evolution of prediabetes to diabetes[19]. Indeed, the enzymatic machinery necessary for the production of GLP-1 from proglucagon is expressed in response to inflammation and caloric excess[20,21]. Islet GLP-1 content is increased by T2DM in humans. Moreover, our preliminary data, as well as recent genetic data[14,15] suggest that even in the absence of an oral challenge, GLP-1 receptor signaling can alter islet function. This may identify a therapeutic pathway to prevent or reverse islet dysfunction in T2DM. An additional important consideration is the role of glucagon signaling through GLP1R to stimulate insulin secretion. We will therefore use rs3765467 which alters affinity of GLP1R to its endogenous ligand[15] as a probe to compare the effect of high (AA) / low (GG) sensitivity to GLP-1 on glucose metabolism in the fasting state and during hyperglycemia (**Fig.1**). This will measure the contribution of GLP-1 (unstimulated by oral intake) to glucose homeostasis and decreased T2DM risk

We will therefore address the following specific aim: -

Specific Aim: Determine the effect of genetic variation in *GLP1R* (rs3765467) on β-cell function during fasting and hyperglycemia in nondiabetic humans.

1° Hypothesis: People with the diabetes-protective (AA) genotype at rs3765467 have enhanced insulin secretion in response to hyperglycemia than do people with the GG genotype

2° Hypotheses: (i) Between-genotype differences in response to hyperglycemia are abolished by GLP1R blockade with exendin-9,39 (ii) People with the AA genotype at rs3765467 have higher fasting insulin secretion than people with the GG genotype (iii) GLP-1 receptor agonism enhances fasting β-cell function to a greater extent in people with the AA genotype at rs3765467

II. Background and Significance

Diabetes mellitus causes morbidity, mortality and costs in excess of ~$170 billion/year[22]. The discovery of gut-derived proglucagon peptides, most specifically GLP-1[1], has fueled therapeutic innovations for T2DM[4]. Proglucagon is transcribed and translated from the glucagon gene (*GCG*) but its final fate depends on concomitant expression of prohormone-convertase (PC) enzymes[23]. 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[24]. IL-6[25], hyperglycemia[26] and FFA[20] 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**[27].

In a small number of heterozygotes, we showed that a nsSNP (Arg131Glu – rs3765467) in *GLP1R* increases insulin secretion in response to glucose alone and to GLP-1 (**Fig.1**)[13]. This SNP, which substitutes arginine for glutamine increases affinity of the receptor for GLP-1[15], and has been associated with lower fasting glucose[15] and a decreased risk of T2DM[14,15]. Also of note, DPP-4 inhibition lowers fasting glucose (and glucagon) with minimal changes in fasting GLP-1 concentrations measured in the circulation[10]. These findings imply that manipulation of GLP-1 signaling in the fasting state can alter islet function and glucose homeostasis. **These observations raise the possibility that endogenous GLP-1 secretion (even in the fasting state when it is barely detectable in the circulation) alters islet function.**

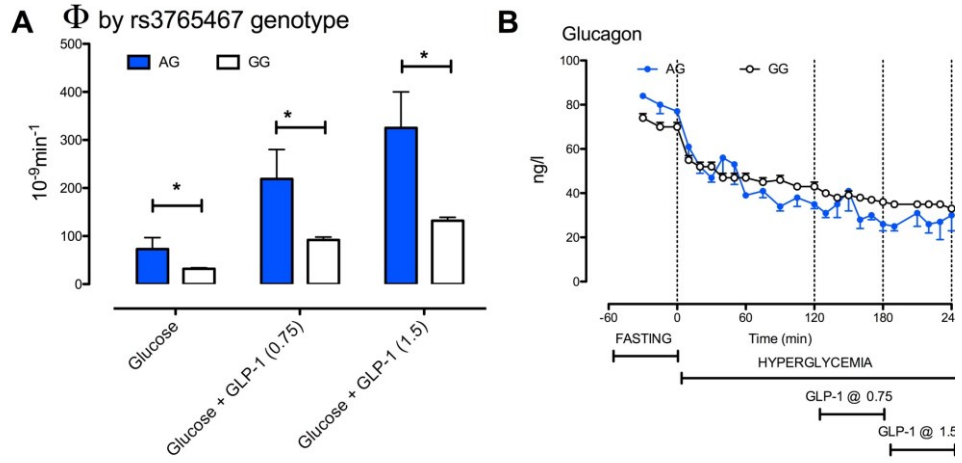


Figure 1: A prior experiment examined the effect of GLP-1 receptor polymorphisms on response to hyperglycemia alone and in combination with GLP-1 infusion (@ 0.75pmol/kg/min and @ 1.5pmol/kg/min). We demonstrated that rs3765467 increased responsivity to glucose alone and with GLP-1 (**A - *p < 0.05**). There is a trend to increased glucagon suppression but this will require further study (**B**). Data reanalyzed from Sathananthan, A., et al. (2010). *Diabetes Care* 33(9): 2074-2076.

The other consideration is that glucagon within the islet can signal the β-cell through GLP1R, and acts as an insulin secretagogue[28,29]. This signaling is blocked by exendin-9,39[27,30]. The relative importance of glucagon signaling through its cognate receptor or through GLP1R is unknown. Despite the lower affinity of GLP1R for glucagon[31], intra-islet concentrations of glucagon are sufficiently high to stimulate GLP1R[28]. The other situation where this may occur is in response to pharmacologic doses of glucagon as used for β-cell function testing[32,33] or raising peripheral glucagon concentrations above fasting values[34-36]. **The experiment will also 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[31]**) agonists for the treatment of T2DM and obesity**.

III. Preliminary Data

We have the necessary expertise and infrastructure to complete the proposed studies.

rs3765467	AA	AG	GG
N (%)	71 (11)	148 (23)	420 (66)

Table 1: Frequency of rs3765467 in the Biobank cohort who have expressed interest

1. Recruiting subjects with AA,AG and GG genotype at rs3765467: Utilizing the Mayo Biobank as before[37-39], we identified a cohort of subjects **(Table 1)** from which to recruit for the experiments in **Specific Aim 1**. Our sample from Olmsted County (location of Rochester, MN) is enriched in this SNP (similar to that of *TCF7L2* that we studied in this cohort) and motivated to participate in research e.g.: ~85% of subjects agreed to participate in a second experiment[39] after participating in a first[37]. **We have not observed between-sex differences in response to GLP-1, exendin-9,39 or to FFA but we will include sex as a covariate in our analyses and will balance recruitment for sex across groups**.

2. Use of exendin-9,39 and GLP-1: We have used these peptides extensively as attested by our preliminary data (**Fig.1-7**) and publication record[7,11,13,40-44]. We examined the effect of common genetic variation in GLP1R on response to GLP-1 and discovered that rs3765467 alters response to glucose and to GLP-1 (**Fig.1**)[13]. We have also developed models of GLP-1-induced potentiation of insulin secretion[45,46].

IV. Research Design and Methods

Subjects: We will recruit 40 nondiabetic subjects. We will use our prior methods[37] to identify and recruit subjects so that 20 will have the AA or AG genotype at rs3765467 while the remainder will have the GG genotype. Briefly, after approval from the Mayo Clinic Institutional Review Board, we will utilize the Mayo Clinic Biobank (a repository of 50,000 DNA samples collected to genotype individuals at rs3765467. The individuals genotyped will be selected from the Biobank cohort encompassing the age span of18-65 years. In addition, we will seek individuals with the appropriate genotype from the tapestry study and also by local advertising. Since this genotype is enriched in Asians and Southeast Asians, we will specifically request participation from these ethnic groups.

Pre-Screen
Subjects who express interest in participating will be contacted and invited to donate 5ml of blood for genotyping. Only subjects who have not been previously genotyped by biobank will need a pre-screen visit. If they have the requisite genotype, they will be then invited to participate in the full study. In this way we will recruit a total of 40 participants (**Table 1**). The subjects in each genotype group will be matched for age, gender and bodyweight as before[37].

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 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.

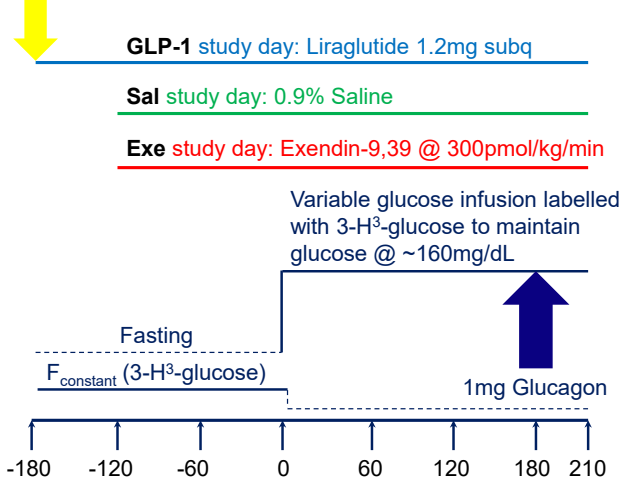


Figure 2: Experimental Design

Experimental Design (Fig.2): We will study 40 subjects a total of 3 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 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. At 1200 (180 min), glucagon will be administered as an I.V. bolus (1mg)[32,33]. Blood will be sampled till the end of the study (1230 – 210 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 -120 and 210 min.
On a separate study day (**GLP-1**) subjects will receive a subcutaneous injection of 1.2 mg of liraglutide (Victoza) at -180 minutes. A single use pen – as utilized in clinical practice will be used. On another study day, exendin-9,39 (**Exe**) will be infused at 300pmol/kg/min between -120 and 210 min. If nausea and / or vomiting occur in the first 3 subjects studied, the dose of liraglutide used will be decreased to 0.6mg.

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[13]. 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. The *GLP-1 model*[45,46], will be used to quantify the potentiation of insulin secretion by GLP-1 receptor agonism [40]. The model assumes that insulin secretion is linearly modulated by GLP-1 through an index (π), which quantifies the increase of insulin secretion due to (changes in) circulating GLP-1concentrations[13].

Statistical Analysis: An unpaired 2-tailed student *t*-test (parametric) or a Wilcoxon rank-sign test (non-parametric) will be used to examine differences between genotype groups for the primary endpoint (**Table 2**). Similar analyses for other response variables (e.g. hormone area under the curve) will be performed if indicated. Exploratory analyses of age, sex and weight effects on the study endpoints will also be undertaken.

Power Calculation: Assuming a similar effect size attributable to rs3765467 (>50% difference AG vs. GG - **Fig.1**)[13,45] and variation comparable to that previously observed[37,57], 20 subjects in each genotype group would provide 80% power (at a 2-sided 0.05 α level), to detect biologically significant, differences (**Table 2**).

Power Calculation	Mean ± SD	Detectable difference
(*n* = 20 / 20)		
Φ (10^{-9}min^{-1}) - hyperglycemia	149 ± 45	41 (28%)
Secondary Endpoints	**Mean ± SD**	**Detectable difference**
ϕ_b (10^{-9}) - fasting	8 ± 2	1.8 (22%)
Fasting glucagon (ng/l)	71 ± 17	15 (21%)
Fasting glucose (mmol/l)	5.0 ± 0.4	0.4 (8%)
Fasting EGP (μmol/kg/min)	14.4 ± 2.2	1.5 (10%)
Φ (10^{-9}min^{-1}) – GLP-1	316 ± 124	113 (36%)

Table 2: Means ± SD used to estimate power for Specific Aim 1

Interpretation: We will accept our 1° hypothesis if Φ during hyperglycemia alone (**Sal**) is increased in people with an A allele at rs3765467 compared to people with the GG genotype. This implies that genetic variation in *GLP1R* improves β-cell response to hyperglycemia. Our preliminary data (**Fig.1**) generated in a small no. of subjects suggests that we will accept our 1° hypothesis.

Given the nature of the stimulus to insulin secretion provided by a hyperglycemic clamp, we expect that the main contributor to Φ will be ϕ_2 (2nd phase insulin secretion) and thought to represent *de novo* insulin synthesis and secretion in response to an ongoing insulinogenic stimulus[58]. Nevertheless, we will also compare differences in ϕ_1 (1st phase insulin secretion) which is thought to represent preformed insulin stored within insulin granules in the β-cell. To strengthen our conclusions we will also compare peak and mean (during the 150 – 180 min period) insulin secretion rates between genotype groups. We will measure total and intact GLP-1 as well as glucagon throughout the experiment to ensure that differences in endogenous GLP-1 and glucagon during the clamp[7,40], cannot contribute to differences in β-cell responsivity to hyperglycemia.

To confirm that between-group differences in β-cell function are due to endogenous GLP-1 acting through its cognate receptor, we will use exendin-9,39 (**Exe**), a competitive antagonist of GLP-1 signalling at GLP1R, as before[7,40]. We have already demonstrated that fasting GLP-1 concentrations do not change in response to an intravenous glucose challenge[13] and our preliminary data shows that in these experimental conditions, GLP-1

receptor blockade impairs β-cell function. If there is no difference in Φ between genotype groups on the **Exe** day we will accept our 2° hypothesis (i) which implies that between-group differences are GLP-1-mediated. A persistent difference in Φ between groups could suggest incomplete blockade of the GLP-1 receptor or intrinsic differences in islet function that develop because of lifelong differences in sensitivity to endogenous GLP-1. Based on prior experience with exendin-9,39 we believe the former scenario to be unlikely (see discussion in[7] and comparison to[59,60]). However, if this is the case, we will undertake additional studies using higher infusion rates of exendin-9,39. To examine the latter scenario, we would need to examine pancreatic specimen from individuals with different rs3765467 genotypes for differences in islet numbers and morphology using the Mayo Autopsy repository (See[61,62]).

People with the (A) allele at rs3765467 have lower fasting glucose than people with the diabetes-associated allele (G) in population studies[14,15], perhaps due to enhanced GLP-1 signaling. We will compare ϕ_b (a measure of the appropriateness of fasting insulin secretion for the prevailing fasting glucose)[58] between genotypes on the **Sal** day. We will accept our 2° hypothesis (ii) if indeed ϕ_b is higher in the group with a diabetes-protective allelle (A). In the fasting state glucose production should equal glucose disappearance. The liver is the main source of endogenous glucose production (EGP) and we would expect a lower EGP to accompany an increase in ϕ_b. On the other hand, increased fasting insulin secretion with no change in fasting glucose and EGP would imply hepatic insulin resistance[63]. This scenario is unlikely since to date, there is unconvincing evidence that GLP-1 alters insulin action[2,7,43]. It is also possible that under euglycemic conditions, GLP-1 may not stimulate insulin secretion. If this is the case, we will also compare glucagon concentrations to determine if decreased glucagon rather than increased insulin secretion is the mechanism underlying genotype-attributable differences in fasting glucose.

To ensure that the mechanism of lowered fasting glucose attributable to the (A) allele at rs3765467 is mediated by GLP-1 we will compare ϕ_b in the presence of exendin-9,39 (**Exe** study day) between genotype groups. Although we would expect between-group differences to be attributable to GLP-1-induced differences in ϕ_b (and therefore abolished by blockade of the GLP1R), we will also compare differences in fasting glucagon and EGP in the presence of exendin-9,39.

On the **GLP-1** study day, we will measure insulin secretion in the fasting state (ϕ_b) as well as in the hyperglycemic state (Φ). We expect to demonstrate that people with the A allele have a greater increase in Φ during the **GLP-1** day compared to the **Sal** day based on our preliminary data (**Fig.2**) in a small number of heterozygous subjects[13]. However, to test our 2° hypothesis (iii) we will compare ϕ_b on the **GLP-1** day. Although GLP-1-induced insulin secretion is glucose-dependent, we will determine if the increased affinity of GLP1R for GLP-1 conferred by the rs3765467 polymorphism can also alter responses to an agonist of the GLP-1 receptor, independently of glucose concentrations. We will accept our 2° hypothesis (iii) if this is indeed the case. Future experiments would be required to assess how fasting glucose concentrations interact with GLP-1 to alter insulin secretion in subjects with and without the A-allele at rs3765467. As an exploratory analysis, we will also compare the β-cell responsiveness to liraglutide (as quantified by π in our previously-described model[45,46]). If $\pi(A) > \pi(GG)$ this would provide further supporting evidence that rs3765467 increases GLP1R responsiveness to GLP-1 receptor agonism.

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