The Effect of Dipeptidyl Peptidase IV Inhibition on Growth Hormone-Mediated Vasodilation

Jessica K. Devin, MD MSCI

[redacted]

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1. Background and Previous Human Studies

Dipeptidyl peptidase IV (DPPIV) is a ubiquitously expressed cell surface serine protease which cleaves a dipeptide from the N-terminus of its polypeptide substrates containing a proline or alanine at the second residue. Interest in this peptidase and its pharmacological inhibition has arisen due to its proteolytic inactivation of the incretin hormones and the role this plays in glucose homeostasis. Selective DPPIV inhibitors improve glycemic control in patients with type 2 diabetes mellitus by decreasing the degradation of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).(1;2) The DPPIV inhibitor sitagliptin was approved by the FDA in 2006 as a once daily oral medication to lower blood sugar levels in patients with type 2 diabetes mellitus. Two additional DPPIV inhibitors have since received FDA approval: saxagliptin (2009) and linagliptin (2011). The direct and indirect effects of these inhibitors on cardiovascular function remain an active area of investigation.

GLP-1 also promotes endothelium-dependent vasodilation in the human vasculature in subjects with type 2 diabetes and stable coronary artery disease. In addition to preventing the degradation of the incretin hormones, DPPIV inhibitors prevent the degradation of other peptides with a penultimate proline or alanine including but not limited to the vasodilators substance P as well as BNP and the neurotransmitter neuropeptide Y.(3;4) Our group has previously demonstrated that five day treatment with sitagliptin significantly decreased mean arterial pressure in subjects with metabolic syndrome.(5) Others have reported that acute sitagliptin treatment reduces 24 hour ambulatory blood pressure in non-diabetic patients with mild to moderate hypertension.(6) It has recently been demonstrated in an animal model and *in vitro* that acute DPPIV inhibition leads to vascular relaxation via a GLP-1 independent mechanism involving nitric oxide release mediated by Akt dependent phosphorylation of endothelial nitric oxide synthase.(7)

DPPIV inhibitors could affect blood pressure and vascular function in part by their effect on the somatotropic axis. Pituitary growth hormone releasing hormone (GHRH), also known as growth hormone-releasing factor (GRF) is a substrate of DPPIV, which rapidly cleaves GRF(1-44)-NH_2 to the biologically inactive GRF (3-44)-NH_2 at Ala^2-Asp^3.(1;2) Conversion to GRF (3-44)-NH_2 is blocked *in vitro* by diprotin A, a DPPIV competitive inhibitor.(8) Human GRF is produced in the hypothalamus and stimulates the release of the growth hormone (GH) from somatotrope cells within the anterior pituitary gland; the active portion of the molecule resides in the first 29 amino acid residues. Opposing the actions of GRF, the hypothalamic hormone somatostatin inhibits the release of GRF.(9) Growth hormone circulates throughout the body exerting its

numerous effects both directly and indirectly via the hepatic production of insulin-like growth factor-1 (IGF-1). DPPIV proteolysis plays a major role in the degradation and inactivation of human GRF *in vivo*.(2) Thus, we hypothesize that inhibition of DPPIV will result in decreased GRF degradation and augmentation of the somatotropic axis *in vivo* in humans.

The direct vascular effects of GH and its downstream mediator IGF-1 are well-established. Systemic and arterial GH infusion increases forearm blood flow in healthy subjects independent of systemic and local IGF-1 production.(10;11) This vasodilation is reversed by infusion of the nitric oxide inhibitor, L-*N*-monomethylarginine (L-NMMA).(10) Furthermore, growth hormone receptors are present in abundance on human endothelial cells. Incubation of endothelial cells with GH *in vitro* increases the phosphorylation and activity of endothelial nitric oxide synthase (eNOS) resulting in increased formation of nitric oxide (NO). NO is formed by endothelial nitric oxide synthase (eNOS) within the endothelial cells of the vasculature and then diffuses to underlying smooth muscle cells where it increases vasodilation via cGMP production and decreases inflammation and platelet aggregation.(12-14) Growth hormone treatment also contributes to vascular integrity by enhancing NO-mediated mobilization of endothelial progenitor cells in healthy individuals.(15;16) The exact mechanism(s) through which GH regulates eNOS remain unclear. Evidence exists for both an IGF-1 mediated activation of eNOS involving the serine/threonine Akt phosphorylation-dependent kinase as well as a direct GH-mediated activation of both Akt and JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathways.(17)

Interestingly, individuals with hypopituitarism experience increased cardiovascular and cerebrovascular morbidity and mortality as well as an increased prevalence of cardiovascular risk factors, despite adequate steroid and thyroid hormone replacement though lacking in growth hormone replacement.(18-20) The PI has demonstrated that adults with growth hormone deficiency also demonstrate alterations in plasma fibrinolytic balance, as compared to gender, age, and body mass index-matched controls.(21) Several prospective studies in adults with hypopituitarism have demonstrated that GH replacement improves cardiac function, intima-media thickness, endothelial function, and vascular reactivity as well as various biochemical markers of cardiovascular and metabolic disease including fat distribution and inflammatory cytokines.(22-27) Furthermore, GH deficient adults exhibit low levels of metabolites of systemic NO production; and GH replacement therapy normalizes biomarkers of NO.(16;28;29) Many of the adverse cardiovascular factors observed in GH deficient individuals are accordingly attributed to inadequate vascular NO bioavailability which can be reversed with GH treatment.

Patients with the cardiovascular risk factors of hypertension, hyperlipidemia, diabetes mellitus or tobacco use demonstrate endothelial dysfunction characterized by the inability of the endothelium to generate adequate amounts of NO.(14) Basal NO-mediated dilatation in the forearm arterial bed of patients with insulin-dependent diabetes is impaired as assessed by venous occlusion plethysmography.(30) The impairment in endothelial function in experimental animals and humans with diabetes mellitus is attributed to reduced NOS activity and/or NO availability as well as the accompanying increased production of reactive oxygen species. This characteristic endothelial dysfunction has been implicated in the pathogenesis of diabetic vascular disease.(31)

This aim promises to provide important new data regarding how DPPIV inhibition affects the somatotropic axis in healthy adults. Given the known effects that growth hormone and IGF-1 have on endothelial function as well as cardiovascular and metabolic markers, an augmentation of this axis may positively impact vascular function in the diabetic population. None of our currently available anti-glycemic agents offer this benefit. Data from the Framingham Heart Study demonstrates a negative correlation between circulating IGF-1 and insulin resistance in a community based sample.(32) GRF-induced GH secretion is blunted in premenopausal women with hyperlipidemia and insulin resistance.(33) Thus derangements in the somatotropic axis have previously been described in individuals at risk for the development of diabetes.

Adults with diabetes demonstrate heart disease death rates two to four times higher than adults without diabetes (“Diabetes Statistics” American Diabetes Association; www.diabetes.org); thus DPPIV inhibitors may potentially represent a novel therapy that is simultaneously able to confer both a glycemic and vascular benefit in this at-risk population. We therefore expect that findings from this study will lead to further investigations regarding currently unrecognized but significant vascular effects of this relatively new class of medications increasingly used in the diabetic population.

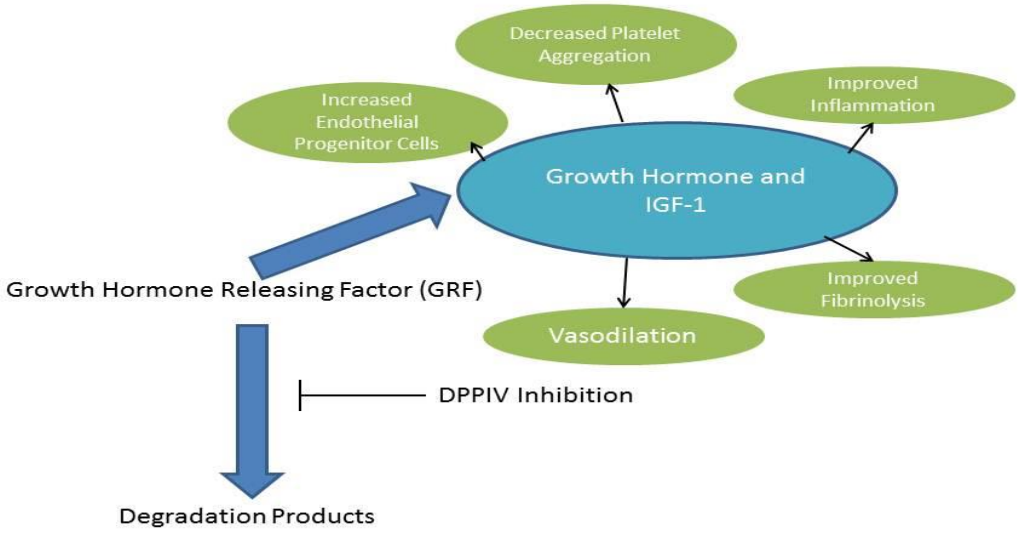
2. Rationale and Specific Aims

This study tests the hypothesis that DPPIV inhibition will favorably augment the somatotropic axis in healthy individuals by decreasing the degradation of GRF by DPPIV. This study specifically establishes the effect of acute DPP4 inhibition on arginine-stimulated GH secretion and GH-mediated blood flow in healthy humans. Arginine decreases inhibitory hypothalamic somatostatin secretion, allowing the primary stimulus of GH secretion to be GHRH. We will study healthy adults to obtain maximal GH signal in this proof-of-concept initial aim, given that obesity diminishes the GH response to pharmacologic stimuli.

-Aim 1A tests the hypothesis that acute DPP4 inhibition will increase stimulated GH secretion in healthy lean adults by decreasing the degradation of GHRH.

-Aim 1B tests the hypothesis that decreased degradation of GHRH during acute DPP4 inhibition will result in an increase in endothelium-dependent vasodilation mediated by GH, and independent from GLP-1.

This study promises to provide novel data regarding how this increasingly used class of anti-diabetic drugs could modulate vascular function. Given the known changes within the somatotropic axis in the setting of insulin resistance, further studies may wish to evaluate the effect of DPPIV inhibition in individuals with the metabolic syndrome.



3. Inclusion/Exclusion Criteria

Forty-two (fourteen per group) healthy, non-smoking subjects (31 females and 11 males), age 18 through 40 years of age will participate in this randomized, double-blind, placebo-controlled crossover study. Subjects taking medications other than multivitamins will be excluded. Pregnancy will be excluded in women of child-bearing age by serum β-HCG.

Inclusion Criteria

- Age 18 to 40 years inclusive
- BMI ≤ 25 kg/m^2
- For female subjects:
    - Status-post surgical sterilization, or
    - If of child-bearing potential, utilization of a barrier method of birth control following negative serum β-HCG at screening visit and on every study day

Exclusion Criteria

- Smoking
- Type 1 or Type 2 Diabetes Mellitus, as defined by a fasting glucose of 126 mg/dL or greater at the time of screening visit or the use of anti-diabetic medication
- Hypertension, as defined by an untreated seated SBP greater than 140 mmHg and/or an untreated DBP greater than 90 mmHg at the time of screening visit or the use of anti-hypertensive medication
- History of reported or recorded hypoglycemia (plasma glucose < 70 mg/dL)
- Pregnancy and/or Breast-Feeding
- Use of any medication other than multivitamin, including use of transdermal as well as oral hormone replacement therapy
- Anemia defined as hematocrit <35% at screening visit
- Cardiovascular or cerebrovascular disease, including history of myocardial infarction, history of congestive heart failure, history of stroke
- Pulmonary Hypertension
- Abnormal thyroid hormone levels (TSH) at the time of screening visit
- Abnormal serum IGF-1 at the time of screening visit
- Impaired renal function, defined as eGFR <60 mL/min/1.73M^2
- Impaired hepatic function (AST or ALT > 2 X upper limit of normal range)
- Treatment with an investigational drug in the 1 month preceding the study

4. Enrollment/Randomization

Subjects will be randomly assigned to treatment order **(*see below table*)** for each pair of visits using a block randomization algorithm with a block size of 2. [redacted] study biostatistician, will provide an allocation schedule. The Vanderbilt Investigational Drug Service will be responsible for the storage, preparation, and labeling of all

investigational agents and for maintaining accurate drug storage and dispending logs. A Clinical Research Pharmacist in the Vanderbilt Investigational Drug Service will devise standard operating procedures for the pharmacy to follow with regard to preparing, labeling, blinding, and dispensing study drug. The Investigational Drug Service will retain a secure set of sealed envelopes containing the treatment assignment.

Randomized subjects who do not complete the whole protocol for any reason will be replaced.

<table>
<tr><th>Aim 1A
Randomization</th><th>Anticipated Result</th><th>Aim 1B Randomization</th><th>Anticipated Result</th></tr>
<tr><td rowspan="3">Sitagliptin vs. Placebo
(N=42)</td><td rowspan="3">Sitagliptin increases stimulated GH secretion and FBF</td><td>Group A (N=14)
Sitagliptin +
[L-NMMA vs. Placebo]</td><td>Endothelium-dependent vasodilation mediated by Arginine, GH, and GLP-1 blocked</td></tr>
<tr><td>Group B (N=14)
Sitagliptin +
[GH Receptor Antagonist vs. Placebo]</td><td>GH mediated vasodilation blocked</td></tr>
<tr><td>Group C (N=14)
Sitagliptin +
[GLP-1 Receptor Antagonist vs. Placebo]</td><td>Endothelium-dependent and independent vasodilation mediated by GLP-1 blocked</td></tr>
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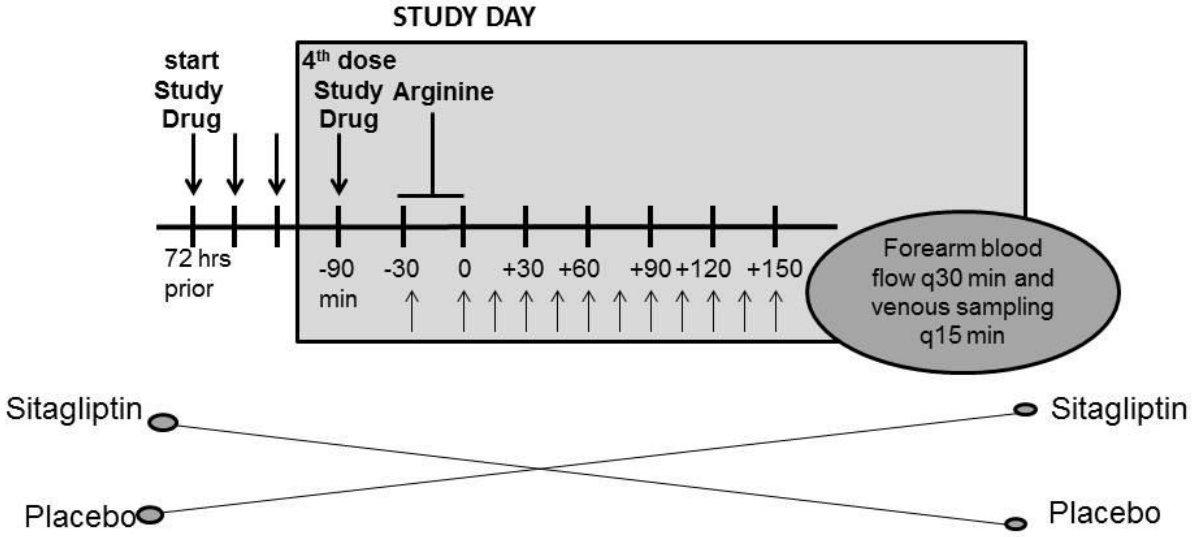
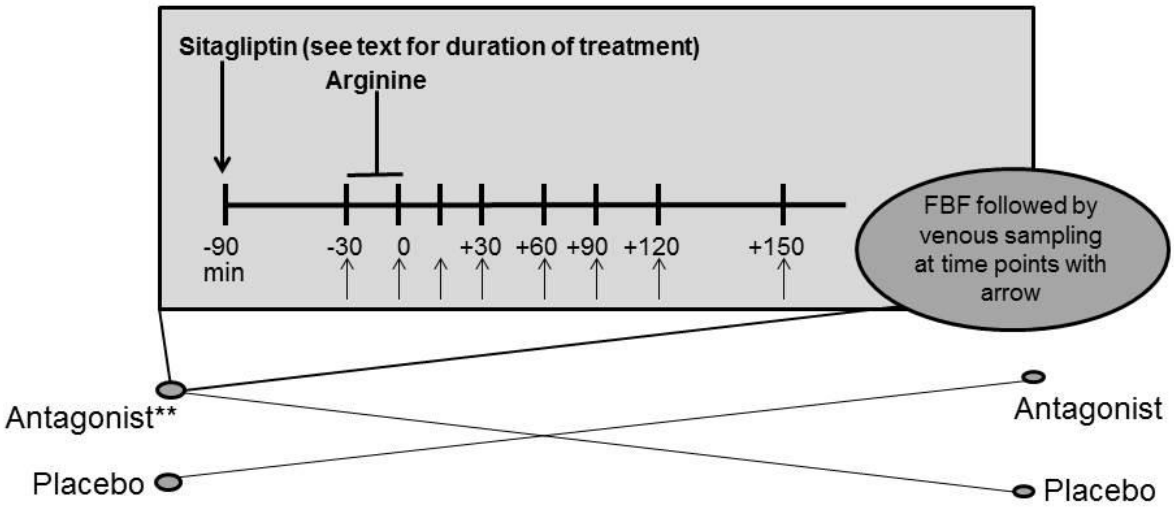
5. Study Procedures



Aim 1 A Study Protocol
(Two study days separated by a 2 week washout)

The figure above illustrates the **Aim 1A** study protocol for **Groups A** and **C** (Group B has completed Aim 1A). Each subject will undergo two study days separated by at least a 2 week wash-out. Seventy-two hours prior to each study day, subjects will start once daily study drug (sitagliptin 100 mg by mouth or matching placebo), following a negative pregnancy test obtained in the Vanderbilt Clinical Research Center (CRC), as applicable. This duration and dose of sitagliptin was chosen to achieve steady-state with significant inhibition of DPP4 activity.(34) On each day, subjects will report to the Vanderbilt Clinical Research Center (CRC) in the morning after an overnight fast and having abstained from exercise that morning. Subjects will be studied in the supine position in a temperature-controlled room. All subjects will receive a peripheral venous

catheter in the non-dominant arm for infusion of arginine as well as a peripheral venous catheter in the contra-lateral arm for repeated blood sampling. Normal saline will be infused into the catheter in the non-dominant arm prior to arginine administration for purposes of keeping the vein open. Subjects will then be given their 4th dose of study drug. One hour after administration of study drug we will measure baseline forearm blood flow (FBF, see below) and obtain baseline venous blood samples (see below). We will infuse arginine (30 grams) intravenously over 30 minutes. We have chosen this dose of arginine, previously used in the clinical setting to diagnose GH deficiency, to yield a peak plasma GH level of 10 ng/mL 30minutes after completion of arginine infusion.(35;36) . Immediately following arginine administration we will repeat measurements of forearm blood flow every 30 minutes and venous blood sampling every 15 minutes up to 150 minutes after arginine infusion. The protocol will be repeated following a 2-week washout using the opposite study drug (sitagliptin or matching placebo). Both blood pressure and heart rate will be continuously monitored throughout each study day. At the completion of each study day subjects will be fed lunch prior to discharge from the CRC.



Aim 1B Study Protocol
**Antagonists include L-NMMA (Group A),
GH Receptor Blocker (Group B), GLP-1 Receptor Blocker (Group C)

The figure above illustrates ***Aim 1B.*** If an effect of sitagliptin on FBF is observed, subjects will be invited to return for 2 additional study visits (Study Visits 3 and 4) in order to elucidate the mechanism underlying the increase in FBF. Study Visit 3 will occur at least 8 weeks after Study Visit 2. Subjects will be given DPP4 inhibitor as well as double-blinded antagonist vs. placebo *(see previous table)* on each study days. In **Group B**, DPP4 inhibition will be given as a single oral dose (sitagliptin 200 mg) on each study day; a minimum of 4 weeks will separate study days. In **Groups A and C**, DPP4 inhibition will be given as 100 mg sitagliptin by mouth daily, starting 72 hours prior to each study day with the 4th dose given on the study day; a minimum two-week washout will separate study days. Venous sampling will occur following assessment of forearm blood flow. All other study procedures will take place as previously described. Details regarding the dose and administration of each antagonist are provided below.

Forearm Blood Flow Measurements

FBF will be measured using mercury-in-silastic strain-gauge plethysmography. The wrist is supported in a sling to raise the level of the forearm to above the level of the

atrium, and a strain gauge is placed in the widest part of the forearm. The strain gauge is connected to a plethysmograph (model EC-4, D.E. Hokanson, Issaquah, WA), calibrated to measure the percent change in volume and connected to a chart recorder to record flow measurements. For each measurement, a cuff placed around the upper arm is inflated to 40 mmHg with a rapid cuff inflator (model E-10, Hokanson) to occlude venous outflow from the extremity. The hand is excluded from the measurement of blood flow by inflation of a pediatric sphygmomanometer cuff to 200 mmHg around the wrist during measurement of FBF. Flow measurements are recorded for approximately 7 secs/15 secs, and the slope is derived from the first 3-4 pulses; 7 readings are obtained for each mean.

Venous Blood Sampling

Following measurement of FBF, venous samples will be obtained prior to arginine administration and immediately as the completion of arginine 30 minute infusion. Venous samples will then be obtained every 15 minutes up to 150 minutes following completion of arginine infusion in Aim 1A. In Aim 1B, venous samples will be obtained before and following arginine infusion at 8 designated timepoints. Samples may be analyzed for ADMA, arginine, GH, insulin, glucose, GLP-1, DPP4 activity and antigen, cGMP, PAI-1 antigen, tPA activity and IGF-1.

The dose of GH receptor antagonist (pegvisomant 80 mg given subcutaneously 72 hours prior to each study day in Aim 1B) was chosen as 80 mg of pegvisomant produces maximal drug levels 72 hours following administration and a reduction in free IGF-1 comparable to that observed in GH deficiency, thus indicative of efficient GH receptor blockade and a state of acute GH deficiency.(37;38) Pegvisomant is a GH analog that disables signal transduction through the GH receptor and thereby functions as a receptor antagonist. This selective blockade allows us to examine the effects of GH secretion in the absence of cofounders such as changes in IGF-1 secretion and body composition which may accompany chronic GH deficiency. This method of achieving acute GH blockade has been reported to safely and effectively study GH secretion in a variety of metabolic conditions and to evaluate the effect of GH secretion on cardiovascular risk markers.(37-39)

The dose of GLP-1 receptor antagonist (Exendin-(9-39) IV bolus of 7500 pmol/kg over 1 minute at time 0 (Aim 1B) followed by continuous infusion of 750 pmol/kg/min for 150 minutes) was chosen as this dose elicits blockade of GLP-1 action at its receptor in healthy volunteers during a fixed blood glucose, as measured by augmented post-prandial glucagon secretion, a blunted increase in post-prandial insulin secretion, and a significant increase in post-prandial GLP-1 secretion.(40-

42)The timing of Exendin-(9-39) infusion may be adjusted pending results of Aim1A to coincide with the timing of peak FBF response.

The dose and timing of the nitric oxide synthase inhibitor, L-NMMA (3mg/kg priming IV infusion over 15 minutes given prior to Arginine infusion in Aim 1B and then continued at 6 mg/kg over an additional 120 minutes) has been selected to achieve peak hemodynamic response 15 minutes after priming dose and maintain hemodynamic effects for up to one hour after completion of infusion.(43;44) Doses up to 13.5 mg/kg IV have safely been given to healthy adults. (44-46) The elimination half-life of L-NMMA is approximately 60 minutes.(43) Co-infusion of a total dose of 6.5 mg/kg L-NMMA with L-arginine has not been shown to influence L-arginine-stimulated GH secretion.(47)

6. Risks

1. Repeated blood sampling from venous catheters may cause bleeding or infection.

2. Frequent blood draws can cause anemia.

3. Insertion of venous catheters may cause bleeding, bruising, or infection.

4. Arginine has previously been used to diagnose growth hormone deficiency (IV, 30 grams).(36) After completion of infusion, L-arginine plasma concentration rapidly decreases within 30 minutes to 47% of the maximum level. (43) Possible side effects include flushing, nausea and vomiting, headache, numbness and local venous irritation. Rarely, a burn-like reaction or injury to the skin requiring surgery has been reported. Rarely, allergic reaction has been reported.

5. Sitagliptin - Drugs like sitagliptin can cause lowered blood sugar (common) in individuals with high blood sugar (diabetes). This can result in dizziness, nausea, shaking, sweating, fast heartbeat, vision changes, headache, anxiety, tiredness, or confusion. It can rarely cause fainting, seizures, or coma. Other side effects are pancreatitis (damage to the pancreas), kidney damage (which can be life threatening), allergic reaction (which can be life threatening), rash, and liver damage (which can be life threatening). The FDA is warning that dipeptidyl peptidase-4 (DPP-4) inhibitors such as sitagliptin, saxagliptin, linagliptin, and alogliptin may cause joint pain that can be severe and

disabling. After the patients discontinued the DPP-4 inhibitor medicine, their symptoms were relieved, usually in less than a month. Some patients developed severe joint pain again when they restarted the same medicine or another DPP-4 inhibitor.

6. The dose of GH receptor antagonist (Pegvisomant 80 mg given subcutaneously 72 hours prior to each study day in Aim 1B, Group B only) was chosen as 80 mg of pegvisomant produces maximal drug levels 72 hours following administration and a reduction in free IGF-1 comparable to that observed in GH deficiency, thus indicative of efficient GH receptor blockade and a state of acute GH deficiency.(37;38) Pegvisomant is a GH analog that disables signal transduction through the GH receptor and thereby functions as a receptor antagonist. This selective blockade allows us to examine the effects of GH secretion in the absence of cofounders such as changes in IGF-1 secretion and body composition which may accompany chronic GH deficiency. This method of achieving acute GH blockade has been reported to safely and effectively study GH secretion in a variety of metabolic conditions and to evaluate the effect of GH secretion on cardiovascular risk markers.(37-39) Side effects in patients taking this medication on a daily basis may include pain and reaction at the injection site, flu-like symptoms, nausea, and diarrhea (common). Rarely, swelling, dizziness, sinusitis and elevations in liver tests have been reported with repeated use.

7. Exendin-(9-39) (IND [redacted])-is an investigational agent (Clinalfa®, Bachem Distribution Services; Weil am Rhein, Germany) that will be infused intravenously following dissolution in 0.25% human serum albumin. Salehi *et. al*. have not experienced any untoward effects with the use of this antagonist in healthy participants, those with Type 2 diabetes mellitus, or history of gastric bypass.(40;42;48) Deane *et. al*. reported that blockade of GLP-1 action was associated with accelerated gastric emptying and larger glucose excursions in the hour following meal intake(49). The dose of GLP-1 receptor antagonist (Exendin-(9-39) IV bolus of 7500 pmol/kg over 1 minute at Time 0 (Aim 1B) followed by continuous infusion of 750 pmol/kg/min for 150 minutes) was chosen as this dose elicits blockade of GLP-1 action at its receptor in healthy volunteers during a fixed blood glucose, as measured by augmented post-prandial glucagon secretion, a blunted increase in post-prandial insulin secretion, and a significant increase in post-prandial GLP-1 secretion.(40-42) The timing of Exendin-(9-39) infusion may be adjusted pending results of Aim1A to coincide with the timing of peak FBF response.

8. L-N^G-monomethyl arginine citrate (L-NMMA) (IND #[redacted])- is an investigational agent (Clinalfa®, Bachem Distribution Services; Weil am Rhein, Germany) that will be infused intravenously. Side effects of systemic

administration include increase in blood pressure and decrease in heart rate. (3mg/kg priming IV infusion over 15 minutes given prior to Arginine infusion in Aim 1B and then continued at 6 mg/kg over an additional 120 minutes) has been selected to achieve peak hemodynamic response 15 minutes after priming dose and maintain hemodynamic effects for up to one hour after completion of infusion. (43;44)The elimination half-life of L-NMMA is approximately 60 minutes.(43) Co-infusion of a total dose of 6.5 mg/kg L-NMMA with L-arginine has not been shown to influence L-arginine stimulated GH secretion.(47) Doses up to 13.5 mg/kg IV have safely been given to healthy adults.(44-46)

7. Reporting of Adverse Events or Unanticipated Problems Involving Risk to Participants or Others

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All adverse events and instances of non-compliance with the protocol, subject withdrawals, and subject complaints will be formally summarized and reviewed by the Data and Safety Monitoring Board (DSMB) bi- annually. The DSMB will receive these in written format and review the study for appropriate and timely subject accrual, adherence to the protocol, and data accuracy and completeness. The DSMB will provide the PI (Dr. Devin) with written confirmation of receipt of this bi-annual summary as well as a recommendation with accompanying rationale for study termination or continuation. This written confirmation will be provided to the IRB and include results of the review and concerns, if any, regarding subject safety or study drug tolerability. The DSMB will ensure that all adverse event reporting to the IRB is in accordance with current policies. The members of the DSMB will be available for discussion of any questions regarding protocol adherence, consent issues, or adverse events. The DSMB will review final results of the study.

The DSMB is comprised of Drs. [redacted], [redacted], [redacted], and Dr. [redacted] (Group C only). Dr. [redacted] is a translational researcher and has experience with the use of sitagliptin to treat patients with diabetes. Dr. [redacted] is a translational researcher and has experience with the administration of vasoactive substances. All adverse events specifically related to the use of arginine and pegvisomant will be graciously reviewed as they occur with Dr [redacted], who as Director of Vanderbilt's Pituitary Center has extensive experience in the use of these agents to diagnose and treat patients with pituitary disease in the clinical setting. Dr. [redacted] is a clinical endocrinologist and translational researcher at the University of Cincinnati who has experience with the infusion of exendin (9-39) in clinical research protocols. Dr. [redacted] will serve as the DSMB chair.

All protocols will be reviewed and approved by the Vanderbilt IRB before any subject is enrolled. Dr. Devin, her mentor (Dr. [redacted]) and her co-investigators will

closely oversee the protocol. Any serious adverse events or toxicities will be reported to the Vanderbilt IRB as per policy and will immediately be reviewed with the DSMB. Any noncompliance with the IRB-approved protocol that increases risk or affects participants' rights, safety or welfare will be reported to the IRB within 10 working days of the investigators' knowledge and immediately reviewed by the DSMB.

Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. An adverse event will be classified as serious if it a) results in death, b) if life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect. All non-serious adverse events and instances of non-compliance with the protocol will be reported annually to the IRB at the time of continuing review.

Dr. Devin will review enrollment progress, all adverse events, protocol adherence data, and data quality entry at a minimum of every two weeks with her mentor (Dr. [redacted] Dr. Devin will review with Dr. [redacted] each enrolled subject's chart every two weeks for side effects and tolerability of the investigational drug. These findings will be included in the DSMB bi-annual report unless dictated otherwise above.

Summary Reports will be submitted to the IRB at least annually and will contain a) the number of adverse events and an explanation of how each event was handled, b) the number of complaints and how each complaint was handled, c) the number of subject withdrawals and an explanation of why the subject withdrew or was withdrawn, and d) the number of protocol violations (non-compliance with protocol) and how each was handled.

8. Study Withdrawal/Discontinuation

If at any time during the study, a subject develops any symptoms related to study participation that subject will be withdrawn from the study. If, in the opinion of the investigator, a subject is non-compliant, that subject will be withdrawn from the study. Subjects who are withdrawn will be followed until symptoms have resolved.

9. Statistical Considerations

Anticipated Results

DPPIV cleaves GRF at its penultimate alanine.(1;2) GRF stimulates endogenous GH secretion, potentially leading to an increase in the GH-mediated vasodilator response.(10;11) We expect that DPPIV inhibition (sitagliptin) will result in decreased degradation of GRF and an increase in stimulated GH secretion. ***(Aim 1A)*** We expect that this will increase forearm blood flow and that this effect will be prevented by pre-treatment with a GH receptor antagonist, but remain unaffected by administration of L-NMMA as well as GLP-1 receptor antagonist. ***(Aim 1B)***

Sample Size and Power Calculation

Aim 1A. Our preliminary data in females (N=4) demonstrate a difference in mean peak GH following arginine stimulation of 6.79 ng/mL (mean 14.07 ± SD 4.73 ng/mL Drug A vs. 20.86 ± 13.23 ng/mL Drug B). Sample size was calculated with PS Software using the design for a paired t-test with a 0.05 two-sided significance level.(50) A sample size of 28 females will have 80% power to *detect* a difference in means of 6.79 ng/mL assuming a 12.25 ng/mL SD of the differences. Accounting for a 10% drop-out between the 2 study days, we will enroll 31 females in this study. Our preliminary data in males (N=2) demonstrates a difference in mean peak GH in the opposite direction. Pre-menopausal women are more responsive to GHRH and secrete more GH per pulse than men, the latter is primarily a GHRH-mediated phenomenon.(51-53) Given the known sexual dimorphism in pulsatile GH secretion, we have chosen to study only enough men to allow us to estimate the difference in males. Studying 11 males will allow a half-width of the 95% confidence for the difference to be about 7.6 ng/mL. Enrolling forty-four subjects (32 females; 12 males) in Aim 1A divided amongst 3 groups in Aim 1B will allow each group to be adequately powered to detect a statistically significant difference in FBF (*see below*).

Aim 1B. Prior data indicates that a difference in forearm blood flow of the following is statistically significant $p<0.001$: baseline forearm blood flow mean ±SEM 4.4 ±0.2 vs 2.7 ±0.3 ml/min/100mLfollowing L-NMMA in n=59 subjects. (54) Conservatively assuming a correlation coefficient of 0.6 for the two measures on the same subject, the SD for the within subject difference would be 1.8. Assuming, for example, that sitagliptin+GH antagonist would achieve FBF of 2.7 ± 2.3 and sitagliptin+placebo would have FBF of 4.4±1.5 (mean±SD), a sample size of 11 completed subjects would have 80% power to detect this difference. Sample size was calculated with PS Software using the design for a paired t-test. Assuming a 10% drop-out in between the 2 study visits we will enroll 14 patients in each Group; gender distribution of the groups will be determined by the results from Aim 1A.

Data Analysis Plan

The primary endpoints are:

Aim 1A: Peak GH level following Arginine stimulation, assessed in a paired manner with and without sitagliptin.

Aim 1B: Repeated measurements of FBF following sitagliptin at the specified times will be assessed in a paired manner with and without antagonist.

Even though we do not expect any carry-over effect in this 2 by 2 crossover study with repeated measures based upon our prior experience with studies of sitagliptin, we will first test for a carry-over effect using the T-test approach proposed by Jones and Kenward.(55) Mixed effect models will be used to analyze the data with a random subject effect and with the treatment (sitagliptin vs placebo or sitagliptin+GH antagonist vs sitagliptin+placebo) and the time trend as measured at the above time points as fixed effects. The focus of this study will be the treatment effect and the time trend of the endpoints; however, mixed effect models also provide the flexibility of controlling for and evaluating covariates as needed by the study. The mixed effect models are robust in the sense that they can include subjects with missing data at some time points but not all time points to estimate the effects of interest. The above specifies the general model for most of the endpoints, however, there will be no time trend in the model for peak GH level. Besides the above evaluation of the treatment effect and the time trend through the regression models, specific inferences regarding effects of interest will be made by reporting a point estimate along with a 95% confidence interval. Hypotheses will be tested at the level of $\alpha=0.05$. This data analysis plan will be carried out using statistical software SPSS for Windows (Version 19.0, SPSS, Chicago) and the open source statistical package R (version 2.12, R Development Core Team, 2006).

10. Privacy/Confidentiality Issues

Clinical data, including clinical laboratory, will be entered by a member of the Key Study Personnel in a protected source database (RedCap). A unique identification case number will be used to protect the confidentiality of the study participants. The case numbers and participants' names will be included in the protected source database, accessible only by Key Study Personnel, but only case numbers will be included in any spreadsheet used for the statistical analysis.

11. Follow-up and Record Retention

The total duration of enrollment will be 2 years, and we anticipate completion of a manuscript within 3 years of initiation. All records will be retained for 7 years following publication of the data. After that time, records may be archived for an additional 5 years and then shredded.

Reference List

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