Brief Report

Direct Effects of Exendin-(9,39) and GLP-1-(9,36)amide on Insulin Action, β-Cell Function and Glucose Metabolism in Non-Diabetic Subjects

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Abstract: 191 Words

Paper: 2000 Words, 3 figures, 25 references

Clinical Trial: NCT01218633
Abstract

Exendin-(9,39) is a competitive antagonist of Glucagon-Like Peptide-1 (GLP-1) at its receptor. However, it is unclear if it has direct and unique effects of its own. We tested the hypothesis that Exendin-(9,39) and GLP-1-(9,36) amide have direct effects on hormone secretion and β-cell function as well as glucose metabolism in healthy subjects. Glucose containing [3-3H]-glucose was infused to mimic the systemic appearance of glucose after a meal. Saline (S), GLP-1-(9,36) amide (G), or Exendin-(9,39) at 30pmol/kg/min (Ex30) or 300pmol/kg/min (Ex300) were infused in random order on separate days. Integrated glucose concentrations were slightly but significantly increased by Exendin-(9,39) (365±43 vs. 383±35 vs. 492±49 vs. 337±50 mmol per 6hr, S, Ex30, Ex300 and G respectively, \( p = 0.05 \)). Insulin secretion did not differ amongst groups. However, insulin action was lowered by Exendin-(9,39) (25±4 vs. 20±4 vs. 18±3 vs. 21±4 \( 10^{-4} \text{ dl/kg(min per } \mu\text{U/ml)} \), \( p = 0.02 \)), resulting in a lower disposition index during Exendin-(9,39) infusion (1118±118 vs. 816±83 vs. 725±127 vs. 955±166 \( 10^{-14} \text{ dl/kg/min}^2 \text{ per pmol/l, } p = 0.003 \)). Endogenous glucose production and glucose disappearance did not differ significantly amongst groups. We conclude that Exendin-(9,39), but not GLP-1-(9,36) amide, decreases insulin action and disposition index in healthy humans.

Keywords: Insulin Secretion, Insulin Clearance, Glucagon Secretion, Incretin Hormones, GLP-1 Receptor
The incretin hormone Glucagon-Like Peptide-1 (GLP-1) arises by posttranslational processing of preproglucagon in the enteroendocrine L cells distributed throughout the intestine. GLP-1 secretion occurs within minutes of food ingestion, is a potent insulin secretagogue, and suppresses glucagon (1). However, the active form(s) of GLP-1 are rapidly deactivated by a serine protease dipeptidyl peptidase-4 (DPP-4) which cleaves the 2 N-terminal amino acids necessary for activation of the GLP-1 receptor (GLP-1R). This enzyme is widely distributed so that the half-life of active GLP-1 in the circulation is about 1 minute (2). The resulting metabolite GLP-1-(9,36) has been proposed as a potential antagonist of GLP-1R although at present there is no evidence of an effect of this peptide on insulin secretion (3).

Exendin-(7,39) is a naturally occurring analog of GLP-1-7,36 and is an agonist of the GLP-1R. This compound binds to GLP-1R with greater affinity than the natural ligand due to a nine amino-acid C-terminal sequence absent in native GLP-1 (4). On the other hand, Exendin-(9,39) which arises from removal of the 2 N-terminal amino acids, is a competitive antagonist of GLP-1 at the GLP-1R (5). It has been used to examine the effects of endogenous GLP-1 secretion on glucose homeostasis (6). While it is presumed that Exendin-(9,39) has no direct effects on glucose metabolism, it alters gastric emptying and capacitance through vagal mechanisms, thereby altering glucose tolerance independent of its ability to inhibit GLP-1-(7,36) effects on insulin and glucagon secretion (7; 8). A direct effect of GLP-1-(9,36) signaling on glucose metabolism has been reported (9).

The present studies were undertaken to determine whether Exendin-(9,39) and GLP-1-(9,36)-amide have direct effects on β-cell function, insulin action, glucagon secretion and glucose metabolism. We did so by infusing glucose in a manner that mimicked the systemic appearance of glucose after ingestion of carbohydrate. Since glucose was infused intravenously, this created a model that resulted in stimulation of insulin and suppression of glucagon in the
absence of a change in endogenous GLP-1 concentrations. Subjects were studied on four occasions receiving, in random order, saline, Exendin-(9,39) infused at 30pmol/kg/min and at 300pmol/kg/min and GLP-1-(9,36)-amide. Glucose turnover was measured on each occasion using [3-\(^3\)H]-glucose; insulin secretion and action were measured using the minimal model.

**Methods**

**Subjects**

After approval by the Mayo Institutional Review Board, we recruited 11 healthy subjects (3 males, 8 females) with no history of prediabetes. Subjects were taking no medications other than oral contraceptives or stable doses of thyroid hormone. Fasting glucose was 4.62±0.13mmol/l and mean age was 31.0±2.1 years. All subjects were at a stable weight and did not engage in regular exercise. The mean weight and BMI was 82.1±7.1kg and 27.5±2.0kg/m\(^2\), respectively. Participants were instructed to follow a weight-maintenance diet containing 55% carbohydrate, 30% fat and 15% protein for at least three days prior to the initial study and then throughout the duration of the study. There was no prior abdominal surgery. Body composition was measured using dual-energy X-ray absorptiometry (DEXA scanner; Hologic, Waltham, MA) to determine lean body mass (48.3±3.1kg). No gastrointestinal symptoms were detected by the bowel disease questionnaire (10). The study was registered at www.clinicaltrials.gov. Identifier: NCT01218633. The use of Exendin-(9,39) and GLP-1-(9,36) was approved as Food and Drug Administration Investigational New Drugs (109555 and 109858 respectively).

**Experimental Design**

Participants were studied on four occasions in random order. On each occasion, subjects were admitted to the Mayo Clinic Clinical Research Unit at 1730 on the evening prior to study. Immediately following admission, subjects ate a standard mixed meal (10kcal/kg, 55% carbohydrate, 30% fat and 15% protein) and then fasted overnight. The following morning, an
18-gauge cannula was inserted in a retrograde fashion into a dorsal hand vein of the non-dominant arm. The hand was placed in a heated box (55°C) to enable sampling of arterialized venous blood. Another cannula was placed in the contralateral arm to enable infusion. At 0600 (-120), a primed continuous infusion of [3-3H]-glucose was initiated (10μCi bolus followed by 0.1μCi/min). At 0800 (0) a variable glucose infusion also labeled with [3-3H]-glucose was started so as to produce glucose concentrations similar to those observed after oral ingestion of 50g of glucose as previously described (11).

All infused glucose contained [3-3H]-glucose in amounts equal to the estimated baseline plasma glucose specific activity. In addition, the basal infusion of [3-3H]-glucose was altered so as to approximate the anticipated pattern of fall of glucose production in an effort to minimize changes in specific activity throughout the experiment.

On the saline control day, at 0800 (0 minutes), normal saline was infused at a rate of 0.1 ml/min (after a 0.4 ml bolus) for the 360 minute duration of the experiment (Saline). On the Exendin 30 day, at 0800, Exendin-(9,39) was administered as a bolus of 120pmol/kg followed by an infusion at 30pmol/kg/min (Exendin 30). On the Exendin 300 day, at 0800 Exendin-(9,39) was administered as a 1200pmol/kg bolus followed by infusion at 300pmol/kg/min. The GLP-1-(9,36) day differed from the other study days in that at 0800 GLP-1-(9,36) amide was infused at 1.2pmol/kg/min (after a 4.8pmol/kg bolus) (GLP). The order of the 4 study days was random.

**Analytical techniques**

Arterialized plasma samples were placed in ice, centrifuged at 4°C, separated, and stored at -20°C until assay. Plasma glucose concentrations were measured using a glucose oxidase method (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin was measured using a chemiluminescence assay (Access Assay; Beckman, Chaska, MN). Plasma glucagon and C-peptide concentrations were measured by radioimmunoassay (Linco Research, St. Louis, MO).
Calculations and Statistical Analysis

Specific activity was smoothed using the method of Bradley et al. (12). Glucose appearance and disappearance were calculated using non-steady-state Steele equations (13; 14) using the tracer infusion rate for each interval. The volume of distribution of glucose was assumed to equal 200 ml/kg and the pool correction factor to equal 0.65. Endogenous glucose production was determined by subtracting the glucose infusion rate from the tracer-determined rate of glucose appearance. All rates of infusion and turnover were expressed per kilogram of lean body mass.

Net insulin sensitivity ($S_i$), was estimated from insulin and glucose concentrations using the unlabeled minimal model. A global beta-cell responsivity index ($\phi$) was estimated from glucose and C-peptide concentrations by using the C-peptide minimal model, incorporating age-associated changes in C-peptide kinetics. Disposition indices (DI) were calculated as the product of $\phi$ and $S_i$. Hepatic Extraction (HE) was also calculated (15).

A repeated measures analysis of covariance was used to test whether fasting, peak, nadir, and integrated hormonal concentrations differed amongst the 4 study days incorporating BMI as a covariate. A compound symmetry correlation structure was assumed, and the Dunnett-Hsu multiple comparison method was used to compare each treatment with saline. A similar approach was used to assess effects on $S_i$ and DI. A $p$-value $\leq 0.05$ was considered significant.

The analyses used SAS® software version 9.3 (SAS Institute Inc., Cary, NC).

Results

Plasma glucose, insulin, C-peptide and glucagon concentrations (Figure 1)

Fasting glucose concentrations did not differ amongst study days (5.2±0.1 vs. 5.1±0.1 vs. 5.1±0.1 vs. 5.1±0.1 mmol/l for the Saline, Exendin 30, Exendin 300 and GLP-9,36 study days respectively, $p=0.14$). Similarly, peak glucose concentrations did not differ (9.3±0.2 vs. 9.3±0.2
vs. 9.7±0.3 vs. 9.2±0.3 mmol/l, \( p=0.28 \). However, integrated area above basal glucose concentrations (AAB) differed slightly but significantly between study days (\( p=0.05 \), Upper Panel), so that glucose concentrations were higher in the presence of Exendin infused at 300pmol/kg/min compared to saline (365±43 vs. 492±49 mmol per 6hr, \( p=0.05 \)). Integrated glucose concentrations did not differ on the Exendin 30 and GLP-9,36 study days.

Fasting and integrated AAB insulin, C-peptide and glucagon concentrations did not differ amongst study days.

*Endogenous Glucose Production and Glucose Disappearance (Figure 2)*

Fasting rates of endogenous glucose production and disappearance did not differ amongst groups. In addition suppression of endogenous glucose production (Upper Panel) and stimulation of glucose disappearance (Lower Panel) did not differ amongst groups.

*Insulin Action, \( \beta \)-cell Responsivity, Disposition Index and Hepatic Extraction (Figure 3)*

Insulin action (\( S_i \)) differed amongst groups (\( p=0.023 \)) and was lower with Exe 300 than saline (18±3 vs. 25±4 \( 10^{-4} \) dl/kg(min per \( \mu \)U/ml), \( p=0.03 \)) and did not differ on the Exe 30 (20±4 \( 10^{-4} \) dl/kg(min per \( \mu \)U/ml)) or GLP-1-(9,36) (21±4 \( 10^{-4} \) dl/kg(min per \( \mu \)U/ml)) study days.

In contrast, no differences in beta-cell responsivity (\( \phi \)) were observed (31±3 vs. 29±3 vs. 27±4 vs. 30±2 \( 10^{-9} \) min\(^{-1} \), \( p=0.26 \) – Left, Lower Panel). This resulted in a Disposition Index which differed between study days (\( p=0.003 \) – Right, Upper Panel) and was lower on the Exe 30 day compared to saline (816±83 vs. 1118±118 \( 10^{-14} \) dl/kg/min\(^2\) per pmol/l, \( p=0.02 \)). This was also the case on the Exe 300 day (725±127 \( 10^{-14} \) dl/kg/min\(^2\) per pmol/l, \( p=0.002 \)) compared to saline.
Hepatic insulin extraction (Right Lower Panel) did not differ amongst groups (0.58±0.05 vs. 0.57±0.06 vs. 0.56±0.07 vs. 0.58±0.05, \( p=0.50 \)).

**Discussion**

In otherwise healthy subjects, under conditions where there is little endogenous incretin secretion, Exendin-(9,39) infusion leads to a decrease in insulin action with an accompanying decrease in disposition index. This ultimately results in a slight increase in glucose concentrations. Such alterations in insulin secretion and action were not observed with GLP-1-(9,36) and neither compound altered glucagon concentrations. These data suggest that some of the observed effects when Exendin-(9,39) is utilized as a competitive antagonist of GLP-1 at the GLP-1R are attributable to a direct effect of Exendin-(9,39), in addition to competitive antagonism of GLP-1 – with effects on incretin-mediated insulin secretion, gastric compliance and gastric emptying (7; 8) – effects that were not extant under the current experimental conditions.

Although no effect of Exendin-(9,39) on absolute \( \beta \)-cell responsivity (\( \phi \)) was observed, when \( \phi \) was expressed as a function of the prevailing level of insulin action, the resulting Disposition Index was impaired at both infusion rates, implying a failure of \( \beta \)-cell compensation to the decrease in insulin action. The mechanism by which this occurs is uncertain. One possibility is that inhibition of the actions of fasting concentrations of GLP-1 impedes compensatory insulin secretion. This would not explain the effect on insulin action given the absent effects of GLP-1-(7,36) on this parameter under similar experimental conditions (11).

Exendin has a unique interaction with the GLP-1R (4) but it is uncertain that insulin signaling can be modulated through ligand-GLP-1R interactions (16). Moreover, it seems that GLP-1-(9,36) has actions that are subject to interference by Exendin-(9,39) but are not mediated by the GLP-1R (17). Whether this novel, and alternate, signalling pathway can explain our
observations also remains unclear. No direct effect of GLP-1-(9,36), or indeed of Exendin-(9,39), on whole glucose metabolism was observed although this does not preclude small effects on specific tissue compartments such as the myocardium.

Peripheral insulin concentrations represent the sum total of insulin secretion into the portal circulation and hepatic extraction as insulin appears in the systemic circulation. This is not a passive process and is affected by insulin secretion (18; 19). In rodents, GLP-1 appears to decrease insulin clearance (20; 21) but this is not the case in humans (22; 23). In the current experiment neither Exendin-(9,39) nor GLP-1-(9,36) altered insulin clearance (15).

Acute infusion of GLP-1-(7,36) in pharmacologic concentrations is associated with increased cortisol concentrations (11). To ensure that our observations were not explained by increased secretion of counterregulatory hormones, we measured Growth Hormone and Cortisol (as well triglycerides and free fatty acid) concentrations during the experiment (See Appendix). No significant differences in these concentrations were observed suggesting that effects on cortisol or Growth Hormone could not explain our observations. The time course of the effects of these hormones on glucose metabolism would also make this an unlikely explanation (24; 25). Of note, neither Exendin-(9,39), nor GLP-1-(9,36) lower glucagon, the latter observation implying that circulating GLP-1-(9,36) has little, if any, effect on the suppression of glucagon in the presence of hyperglycemia and hyperinsulinemia.

The current data indicate that Exendin-(9,39) causes a slight but significant decrease in both insulin action and insulin secretion that need to be taken into consideration when this agent is utilized as a GLP-1-(7,36) antagonist. It remains to be determined whether these effects are also present in individuals who already have impaired insulin action and insulin secretion (as in type 2 diabetes) and also whether they are more pronounced than those observed in the present experiment, with otherwise healthy non-diabetic subjects.
Acknowledgments

Author contributions: M.S. researched data and ran the studies; L.P.F. assisted with data collection and analysis; J.M.M measured Free Fatty Acid and Triglyceride concentrations, contributed to discussion and reviewed/edited manuscript; F.P. undertook mathematical modeling of insulin secretion and action; C.D.M. undertook mathematical modeling of insulin secretion and action; A.R.Z. undertook statistical analysis; C.C. reviewed/edited the manuscript; R.A.R. contributed to discussion and reviewed/edited manuscript; and A.V. researched data, wrote the manuscript. Dr. Adrian Vella 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.

Grant Support: We thank Merck for providing support for purchase of Exendin-(9,39) and GLP-1-(9,36) in this investigator-initiated study. The authors acknowledge the support of the Mayo Clinic General Clinical Research Center. Dr. Vella and Dr. Cobelli are supported by DK78646 and by DK82396.

Financial Disclosure: Dr. Vella has received research grants from Merck and Daiichi-Sankyo. He has consulted for Sanofi-Aventis, Novartis and Bristol-Myers Squibb. None of the other authors have disclosures.
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Figure 1: Glucose (Upper Panel), Insulin (Upper Middle Panel), C-Peptide (Lower Middle Panel) and Glucagon (Lower Panel) concentrations during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30) , Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days.

Figure 2: Rates of endogenous glucose production (Upper Panel) and glucose disappearance (Lower Panel) during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30) , Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days.

Figure 3: Insulin action ($S_i$ – Upper Left Panel), β-cell responsivity ($\phi$ – Lower Left Panel), Disposition Indices (DI – Upper Right Panel) and Fractional Extraction of Insulin (Lower Right Panel) during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30) , Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days.
Insulin Action - $S_i$

Disposition Index

Hepatic Extraction of Insulin

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Appendix

Direct Effects of Exendin-(9,39) and GLP-1-(9,36)amide on Insulin Action, β-Cell Function and Glucose Metabolism in Non-Diabetic Subjects

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\textbf{Keywords:} Insulin Secretion, Insulin Clearance, Glucagon Secretion, Incretin Hormones, GLP-1 Receptor
Appendix Figure 1: Growth Hormone and Cortisol Concentrations during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30), Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days.
Appendix Figure 2: Total GLP-1 concentrations during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30), Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days. As expected the total GLP-1 assay detected GLP-1-(9,36) during infusion. Intriguingly, total GLP-1 concentrations increased slightly but significantly from those observed during saline infusion in the presence of Exendin-(9,39) infusion at 300pmol/kg/min. The 10, 20, 30, 60 and 120 minute time points during GLP-1-(9,36) and Exe300 exhibited significant differences from the values observed during saline infusion.
Appendix Figure 3: Free fatty acid and Triglyceride Concentrations during the Saline, Exendin-(9,39) infused at 30pmol/kg/min (Ex 30), Exendin-(9,39) infused at 300pmol/kg/min (Ex 300) and GLP-1-(9,36)amide infused at a rate of 1.2pmol/kg/min (GLP-1-(9,36)) study days. No significant differences between study days were noted.