The role of efferent cholinergic transmission for the insulinoic and glucagonostatic effects of GLP-1

Astrid Plambock,1,2 Simon Veedfald,1,2,3 Carolyn F. Deacon,2 Bolette Hartmann,2 Tina Vilsbøll,1 Filip K. Knop,1,2 and Jens J. Holst2

1Center for Diabetes Research, Gentofte Hospital, University of Copenhagen, Hellerup, Denmark; 2The Novo Nordisk Foundation Center for Basic Metabolic Research and Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark; and 3Department of Surgical Gastroenterology and Liver Transplantation, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

Submitted 25 March 2015; accepted in final form 29 June 2015

Plambock A, Veedfald S, Deacon CF, Hartmann B, Vilsbøll T, Knop FK, Holst JJ. The role of efferent cholinergic transmission for the insulinoic and glucagonostatic effects of GLP-1. Am J Physiol Regul Integr Comp Physiol 309: R544–R551, 2015. First published July 1, 2015; doi:10.1152/ajpregu.00123.2015.—The importance of vagal efferent signaling for the insulinoic and glucagonostatic effects of glucagon-like peptide-1 (GLP-1) was investigated in a randomized single-blinded study. Healthy male participants (n = 10) received atropine to block vagal efferent transmission or saline infusions on separate occasions. At t = 15 min, plasma glucose was clamped at 6 mmol/l. GLP-1 was infused at a low dose (0.3 pmol·kg⁻¹·min⁻¹) from t = 45–95 min and at a higher dose (1 pmol·kg⁻¹·min⁻¹) from t = 95–145 min. Atropine blocked muscarinic, cholinergic transmission, as evidenced by an increase in heart rate [peak: 70 ± 2 (saline) vs. 90 ± 2 (atropine) beats/min, P < 0.002] and suppression of pancreatic polypeptide levels [area under the curve (AUC45–145: 598 ± 49 ± 8 (atropine) pmol/l · min, P = 0.0001). More glucose was needed to maintain the clamp during the high-dose GLP-1 infusion steady-state period on the atropine day [6.4 ± 0.9 (saline) vs. 8.7 ± 0.8 (atropine) mg·kg⁻¹·min⁻¹, P < 0.0023]. GLP-1 dose-dependently increased insulin secretion on both days. The insulinoic effect of GLP-1 was not impaired by atropine [C-peptide AUC45–145: 99 ± 8 (saline) vs. 113 ± 13 (atropine) nmol/l · min, P = 0.19]. Atropine suppressed glucagon levels additively with GLP-1 [AUC45–145: 469 ± 70 (saline) vs. 265 ± 50 (atropine) pmol/l · min, P = 0.018], resulting in hypoglycemia when infusions were suspended [3.6 ± 0.2 (saline) vs. 2.7 ± 0.2 (atropine) pmol/l, P < 0.0001]. To ascertain whether atropine could independently suppress glucagon levels, control experiments (n = 5) were carried out without GLP-1 infusions [AUC45–145: 558 ± 103 (saline) vs. 382 ± 76 (atropine) pmol/l · min, P = 0.06]. Our results suggest that efferent muscarinic activity is not required for the insulinoic effect of exogenous GLP-1 but that blocking efferent muscarinic activity independently suppresses glucagon secretion. In combination, GLP-1 and muscarinic blockade strongly affect glucose turnover.

GLP-1; vagus nerve; atropine; vagal efferent signaling; insulin; glucagon

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is secreted to the circulation from the intestinal L cells in response to meal ingestion (20). It has several effects, including augmentation of glucose-stimulated insulin secretion (insulinoic effect) (40), inhibition of glucagon secretion (glucagonostatic effect) (40), delay of gas-
elevated plasma creatinine, or urinary albumin-to-creatinine ratio. Participant characteristics are shown in Table 1.

**Experimental Protocol**

Participants were studied after a 12-h fast on two occasions separated by ≥48 h. They were instructed to maintain unchanged lifestyle and to abstain from alcohol and exercise in the 24 h leading up to each examination. Smoking was prohibited for the final 12 h. Participants were recumbent throughout the experiments. Intravenous catheters were inserted into antecubital veins in both arms: one for blood sample collection [catheterized forearm placed in a heating box (50°C)] and one for glucose, GLP-1, and saline or atropine infusions. Each participant was examined single-blinded on two separate occasions in randomized order with either atropine (ATR+CLA+GLP-1) or saline (SAL+CLA+GLP-1). On both days, GLP-1 was infused at two different rates, and plasma glucose (PG) levels were clamped at 6 mmol/l.

In a subset of participants (n = 5), who were available for this, two additional study days were carried out in which PG was clamped, and either atropine (ATR+CLA) or saline (SAL+CLA) was infused as before, but without the GLP-1 infusions.

**Infusions**

**Atropine.** Atropine (Nycomed, Roskilde, Denmark) was administered as a bolus of 1 mg (1 ml) at t = 0 min and, thereafter, as a constant infusion of 80 ng·kg⁻¹·min⁻¹ for 145 min, using a previously employed dosing regimen (5).

**Saline.** Sterile isotonic saline was given as a control, first as 1-ml "bolus" and, thereafter, at the same infusion rate as used for atropine for 145 min.

**Glucose.** Sterile 20% (wt/vol) glucose was infused from time 15 to 145 min at a variable rate, aiming to clamp PG at 6 mmol/l, as the effects of GLP-1 are more pronounced when glucose levels rise above fasting levels (35, 51).

**GLP-1.** Synthetic GLP-1 (7–36 amide) (PolyPeptide Laboratories, Wolfenbüttel, Germany) was dissolved in sterilized water containing 2% human albumin (Statens Serum Institut, Copenhagen, Denmark), subjected to sterile filtration, and dispensed into vials and stored frozen under sterile conditions until used in the experiment. The integrity and purity of the peptide were assessed by mass, sequence, and high-performance liquid chromatography analysis. On the days of the experiments, GLP-1 was diluted with 1% human albumin in saline (0.9%) and infused at 0.3 pmol·kg⁻¹·min⁻¹ (aiming at low physiological plasma concentrations) for 50 min, from 45 to 95 min. Thereafter, the dose was increased to 1 pmol·kg⁻¹·min⁻¹ (aiming at high physiological plasma concentrations) and continued for another 50 min, from 95 to 145 min. Participants were observed throughout the experiments and asked about any antimuscarinic side effects (e.g., dry mouth, palpitations, and blurred vision). Oxygen saturation, electrocardiogram, heart rate, and blood pressure were monitored continuously, and the participants were followed for at least half an hour after discontinuation of the infusions.

**Sampling**

Arterialized blood was drawn at 15, 10, and 0 min before and with 5-min intervals from 15 to 145 min and finally at 160 and 175 min after initiation of the atropine/saline infusion. The accumulated volume of blood drawn on each experimental day was ~220 ml. During the glucose clamp, PG was measured every 5 min, and hormones every 10 min. Blood was collected into chilled tubes containing EDTA and a DPP-4 inhibitor (valine-pyrrolidine, 0.01 mmol/l, final concentration, a gift from Novo Nordisk, Bagsværd, Denmark) for analyses of glucagon, total GLP-1, pancreatic polypeptide (PP), and somatostatin. Blood for C-peptide and insulin analysis was collected in dry tubes for coagulation (20 min at room temperature). After centrifugation (1,200 g; 20 min at 4°C) plasma for GLP-1, glucagon, PP, and somatostatin analyses was stored at −20°C, and serum for C-peptide and insulin analyses were stored at −80°C. For bedside PG measurement, blood was stored into fluoride tubes and centrifuged immediately (7,400 g; 1 min at room temperature).

**Laboratory Analyses**

PG was determined using a glucose analyzer [Yellow Springs Instrument (YSI) model 2300 STAT plus analyzer; YSI, Yellow Springs, OH] by the glucose oxidase method. Plasma samples for analysis of GLP-1, glucagon, PP, and somatostatin were extracted with ethanol (70% of final concentration) before analysis. Total GLP-1 levels were assayed using antisem 89390, which requires the intact amidated COOH terminus of the molecule (41). Glucagon was measured using the COOH terminally directed antisem 4305 (21). PP was determined using the midregion specific antibody code no HYB 347-07 (Statens Serum Institut, Copenhagen, Denmark), with human PP as a standard, and ¹²⁵I-labeled human PP as a tracer (Perkin Elmer, Boston, MA) (9). Somatostatin was measured using antisem 1758, which recognizes both somatostatin-14 and somatostatin-28 (18). Serum C-peptide and insulin concentrations were quantified by routine immunoassays (Siemens Healthcare Diagnostics, Ballerup, Denmark) using the ADVIA Centaur XP analyzer.

**Statistical Analyses and Calculation**

Results are shown as means ± SE, and a two-sided P value of <0.05 was taken to indicate significant difference. D’Agostino-Pearson omnibus K² normality test was used to determine whether data followed a normal distribution. Repeated-measures (RM) ANOVAs with treatment (SAL or ATR) and time as factors were used to assess differences in insulin levels. Post hoc comparisons adjusted for multiple comparisons by Bonferroni’s correction, were used to determine significant differences at individual time points. (e.g., PG and plasma hormone concentrations) and paired t-tests were used for comparisons between single values e.g., between baseline and total area under the curve (AUC) values. For comparison of single values not following a normal distribution, a Wilcoxon test for paired difference was used. For comparison of more than two values, a Friedman test was employed. Significant differences were then evaluated by Dunn’s post hoc test. AUCs were calculated using the trapezoidal rule. Total AUCs were calculated for the GLP-1 infusion periods on the GLP-1 infusion experimental days and the corresponding periods on the control days. Data from the GLP-1 infusion experiments and the atropine control experiments were analyzed separately. The statistical analyses were carried out using GraphPad Prism version 6.0 for Mac (GraphPad Software, La Jolla, CA).

---

**Table 1. Participant characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>10/0</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.8 ± 0.03</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.0 ± 0.5</td>
</tr>
<tr>
<td>Waist:hip-ratio</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>129 ± 2</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Family history of diabetes</td>
<td>None</td>
</tr>
<tr>
<td>Medication</td>
<td>None</td>
</tr>
</tbody>
</table>

Data are means ± SE. M, male; F, Female; BMI, body mass index; HbA1c, glycated hemoglobin A1c.
RESULTS

Heart rate. Atropine blocked cholinergic transmission, as evidenced by the increase in heart rate of ~20 beats/min (Fig. 1, A and B) occurring immediately after initiation of the atropine infusion. There was a treatment effect (P < 0.001) and a treatment × time effect (P < 0.0001). Post hoc analysis found significant differences from t = 15 min (*P < 0.0001). There was a treatment effect (P < 0.0001) and a treatment × time effect (P < 0.0001). Post hoc analysis found significant differences from t = 15 min (*P < 0.0001). Data are expressed as means ± SE.

Anti-muscarinic symptoms. On the ATR + CLA + GLP-1 day, all 10 participants reported dry mouth, five participants reported dysphagia, two participants reported blurred vision, and one reported a feeling of dry skin. On the ATR + CLA day, all five participants reported dry mouth.

PP. When comparing the GLP-1 infusion days (Fig. 2A) there was a treatment effect (P = 0.0043) and a treatment and time effect (P = 0.0028). Post hoc analysis yielded significant differences at t = 160 and t = 175 min (P < 0.002). The PP AUCs for the GLP-1 infusion period were significantly different (t-test, P < 0.0001) (Fig. 2B). On the control experimental days (Fig. 2C), there was a treatment effect (P = 0.020) and a treatment × time effect (P = 0.031). Post hoc analysis showed significant differences at t = 115 (P = 0.032) and 175 min (P < 0.0001). The AUC during the period corresponding to the GLP-1 period was not significantly lower than on the SAL + CLA day (Fig. 2D) (Wilcoxon, P = 0.063).

PG and glucose infusions. The 6 mmol/l clamp was maintained at the intended level on all experimental days (Fig. 3, A and C). However, there was a treatment × time effect (P = 0.0007), and post hoc analysis showed a significant difference when the clamp was suspended at t = 160 (P < 0.0001) and t = 175 min (P = 0.0082) (Fig. 3A). Notably, on the ATR + CLA + GLP-1 day (Fig. 3A), glucose levels dropped to levels in the hypoglycemic range (<3 mmol/l) in 7 out of 10 participants. At t = 160 min, glucose levels were 2.7 ± 0.2 (atropine) vs. 3.6 ± 0.2 (saline) mmol/l (P < 0.0001), and t = 175 min glucose levels were 2.9 ± 0.2 vs. 3.3 ± 0.2 mmol/l (P < 0.01) (Fig. 3A). The amount of glucose (mg/min/kg) needed to maintain the 6 mmol/l clamp was calculated for the steady state of the three infusion periods (glucose alone 35–45 min, low GLP-1 85–95 min, and high GLP-1 135–145 min). More glucose was needed to maintain clamp during the steady-state periods of both GLP-1 infusions (Fig. 3B), and more glucose was needed to maintain the clamp during the high-dose

Fig. 1. A: time course of heart rate during infusion of saline (●) or atropine (○) combined with glucose clamp and glucagon-like peptide-1 (GLP-1) infusion. There was a treatment effect (P < 0.0001) and a treatment × time effect (P < 0.0001). Post hoc analysis found significant differences from t = 15 min (*P < 0.0001). B: time course of heart rate during infusion of saline (●) or atropine (○) combined with glucose clamp. There was a treatment effect (P < 0.0001) and a treatment × time effect (P < 0.0001). Post hoc analysis found significant differences from t = 15 min (*P < 0.0001). Data are expressed as means ± SE.

Fig. 2. A: time course of pancreatic polypeptide (PP) during infusion of saline (●) or atropine (○) combined with glucose clamp and glucagon-like peptide-1 (GLP-1) infusion. There was a treatment effect (P = 0.0043) and treatment and time effect (P = 0.0028). Post hoc analysis yielded significant differences (*P < 0.002). B: PP areas under the curve (AUCs) during the GLP-1 infusion periods (t-test, P = 0.0001). C: PP levels during infusion of saline (●) or atropine (○) and glucose clamp. There was a treatment effect (P = 0.020) and a treatment × time effect (P = 0.0306). Post hoc analysis showed significant differences at t = 115 (P = 0.032) and 175 min (P < 0.0001). D: PP AUCs for the time period corresponding to the GLP-1 infusion period (Wilcoxon, P = 0.063). Data are expressed as means ± SE.

Fig. 3. A: time course of plasma glucose (PG) during infusion of saline (●) or atropine (○) combined with glucose clamp and glucagon-like peptide-1 (GLP-1) infusion. There was a treatment × time effect (P = 0.0007). Post hoc analysis showed a significant difference at t = 160 (P < 0.0001) and t = 175 min (P = 0.0082). B: amount of glucose (mg/kg⁻¹·min⁻¹) needed to be infused to maintain the clamp at 6 mmol/l during steady state during saline/ atropine, glucose, and GLP-1 infusions (last 10 min of the infusion periods). There was a treatment × time effect (P = 0.019). Post hoc analysis yielded a significant difference between the glucose infusion rates during the high GLP-1 infusion (P = 0.0023). C: time course of plasma glucose (PG) during infusion of saline (●) or atropine (○) combined with the 6 mmol/l glucose clamp. There was no treatment × time effect. D: amount of glucose (mg/kg⁻¹·min⁻¹) needed to be infused to maintain the clamp at 6 mmol/l at steady state (last 10 min of the infusion periods) during the saline+clamp (SAL+CLA) and atropine+clamp (ATR+CLA) control experiments. There was no treatment × time effect. Data are means ± SE.
when GLP-1 infusions commenced, to plateau levels of 43 ± 2 pmol/L (low-dose GLP-1) and 122 ± 10 pmol/L (high-dose GLP-1) on the atropine day (Wilcoxon, P = 0.049). For the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days, there was a treatment × time effect (P < 0.0001), and post hoc analysis yielded significant differences at t = 115, 125, 135, and 145 min, (P < 0.02) (Fig. 6A). The insulin responses to the glucose clamp on the control days (Fig. 6C) were not affected by atropine when evaluated by ANOVA (treatment × time, P = 0.37) or expressed as AUCs (Fig. 6D).

**Glucagon.** The glucagonostatic effect of GLP-1 was apparent when inspecting the time course during both the low-dose and high-dose GLP-1 infusions (Fig. 7A). There was a treatment × time effect (P < 0.0001) and post hoc analysis yielded a significant difference at t = 175 (P < 0.001). In addition, the AUCs during the GLP-1 infusion period (t = 45–145 min) (Fig. 7B) were significantly lower on the atropine day (t-test, P = 0.018). For the ATR + CLA and SAL + CLA days (Fig. 7C), there was a treatment effect (P = 0.0005), and post hoc analysis showed there were significantly lower glucagon levels at t = 15 min on the ATR + CLA day. The AUCs for the 45- to 145-min period (Fig. 7D) were not significantly different (Wilcoxon test, P = 0.062).

**Somatostatin.** Somatostatin levels were similar on the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days (data not shown), and on the SAL + CLA and ATR + CLA days (no treatment × time effect).

GLP-1 infusion on the ATR + CLA + GLP-1 day [treatment × time effect (P = 0.019), post hoc analysis (P = 0.0023)]. The amounts of glucose needed to maintain the clamp on the SAL + CLA and ATR + CLA days (Fig. 3D) were similar for all three periods.

**GLP-1.** GLP-1 levels (Fig. 4A) increased dose-dependently when GLP-1 infusions commenced, to plateau levels of 43 ± 2 pmol/L (low-dose GLP-1) and 122 ± 12 pmol/L (high-dose GLP-1) on the control (saline) day, (Friedman test followed by Dunn’s post hoc test, P < 0.0032); and to 41 ± 3 pmol/L (low-dose GLP-1) vs. 110 ± 5 pmol/L (high-dose GLP-1) on the atropine day (P < 0.032). The GLP-1 levels resulting from the infusions were not significantly different on the two GLP-1 days (no treatment or treatment × time effect). On the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days, the AUCs for the 45–145-min period on the saline + clamp (SAL + CLA) and atropine + clamp (ATR + CLA) days (Wilcoxon, P = 0.064) Data are expressed as means ± SE.

C-peptide. GLP-1 dose-dependently increased C-peptide levels (Fig. 5A). From the line curve, there appeared to be an augmented C-peptide response on the atropine day, with a treatment × time effect (P = 0.0001), and post hoc analysis yielding significant differences at time points 125, 135, and 145 min (Fig. 5A). However, the AUCs for the GLP-1 infusion periods on the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days were not significantly different (Wilcoxon, P = 0.19) (Fig. 5B). On the SAL + CLA and ATR + CLA days, there was a treatment effect (P = 0.041) and a treatment × time effect (P = 0.001), and post hoc analysis found significantly lower C-peptide levels on the atropine day from t = 60–160 min (Fig. 5C). However, the AUCs for the 45- to 145-min periods (the time period of GLP-1 infusions on the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days) were not significantly different (Wilcoxon, P = 0.13) (Fig. 5D).

**Insulin.** Insulin levels increased dose-dependently in response to GLP-1 on both days (Fig. 6A). The insulin AUCs (Fig. 6B) were significantly larger on the atropine day (Wilcoxon, P = 0.049). For the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days, there was a treatment × time effect (P < 0.0001), and post hoc analysis yielded significant differences (at t = 115, 125, 135, and 145 min, P < 0.02) (Fig. 6A). The insulin responses to the glucose clamp on the control days (Fig. 6C) were not affected by atropine when evaluated by ANOVA (treatment × time, P = 0.37) or expressed as AUCs (Fig. 6D).

**Somatostatin.** Somatostatin levels were similar on the SAL + CLA + GLP-1 and ATR + CLA + GLP-1 days (data not shown), and on the SAL + CLA and ATR + CLA days (no treatment × time effect).
In the present study, we aimed to pharmacologically block efferent vagal transmission to evaluate the importance of efferent signaling for the insulinotropic and glucagonostatic effects of GLP-1. We show that the dose used of atropine, a muscarinic blocker, efficiently blocks muscarinic transmission, and that the insulinotropic effect of GLP-1 is not attenuated by atropine. Rather, it seems that atropine may enhance the insulinotropic effect of GLP-1, seemingly enhancing the glucagonostatic effect of GLP-1.

Atropine has been used in earlier studies, investigating the influence of neural pathways on pancreatic function (2, 7). The effect of the vagus on the endocrine pancreas is thought to involve ACh acting on muscarinic receptors in the islets. In support of this notion, administration of ACh stimulates insulin and glucagon secretion and muscarinic and GLP-1-mediated regulation of alpha cell secretion (40). However, there was no attenuating effect of atropine, suggesting that efferent vagal cholinergic muscarinic activity is not a prerequisite for the insulinotropic effect of GLP-1 in the present experimental situation. Rather, insulin levels and C-peptide levels attained higher levels in the atropine group at 175 min, suggesting that glucagon secretion did respond to hypoglycemia despite muscarinic blockade. Taken together, the data point to an interaction between muscarinic and GLP-1-mediated regulation of alpha cell secretion (see below).

As anticipated, GLP-1 dose-dependently increased insulin secretion (40). However, there was no attenuating effect of atropine, suggesting that efferent vagal cholinergic muscarinic activity is not a prerequisite for the insulinotropic effect of GLP-1. This was necessary to unleash the full insulinotropic potential of GLP-1, because insulin secretion would otherwise be halted by the drop in PG caused by the insulin-mediated disposal of glucose (29, 35, 50, 51). Only small amounts of glucose were required to maintain the glucose clamp in the absence of exogenous GLP-1 infusion, while higher glucose infusion rates were needed during the low-dose and high-dose GLP-1 infusions. However, against expectations, more glucose was required during high-dose GLP-1 on the ATR+CLA+GLP-1 day than on the SAL+CLA+GLP-1 day. Further, in a subset of participants, PG dropped to hypoglycemic levels after termination of the clamp on the ATR+CLA+GLP-1 day. The explanation for the higher glucose infusion rates and post-clamp hypoglycemia is likely to involve the higher insulin levels and lower glucagon levels at clamp termination (t = 145 min). Identical glucagon levels were measured at 160 min and even higher levels in the atropine group at 175 min, suggesting that glucagon secretion did respond to hypoglycemia despite muscarinic blockade.

To evaluate the role of the efferent vagal pathway for the effects of GLP-1, we used cholinergic blockade and infused GLP-1 to attain both physiological and supraphysiological (42) concentrations during a permissive 6 mmol/l-PG clamp. Plateau levels of total GLP-1 were about 40 pmol/l (low-dose) and 120 pmol/l (high-dose), and similar on both experimental days. PG was clamped at 6 mmol/l to maintain the permissive glucose levels required for the glucose-dependent action of GLP-1 on insulin secretion (35, 51). This was necessary to unleash the full insulinotropic potential of GLP-1, because insulin secretion would otherwise be halted by the drop in PG caused by the insulin-mediated disposal of glucose (29, 35, 50, 51). Only small amounts of glucose were required to maintain the glucose clamp in the absence of exogenous GLP-1 infusion, while higher glucose infusion rates were needed during the low-dose and high-dose GLP-1 infusions. However, against expectations, more glucose was required during high-dose GLP-1 on the ATR+CLA+GLP-1 day than on the SAL+CLA+GLP-1 day. Further, in a subset of participants, PG dropped to hypoglycemic levels after termination of the clamp on the ATR+CLA+GLP-1 day. The explanation for the higher glucose infusion rates and post-clamp hypoglycemia is likely to involve the higher insulin levels and lower glucagon levels at clamp termination (t = 145 min). Identical glucagon levels were measured at 160 min and even higher levels in the atropine group at 175 min, suggesting that glucagon secretion did respond to hypoglycemia despite muscarinic blockade.

Taken together, the data point to an interaction between muscarinic and GLP-1-mediated regulation of alpha cell secretion (see below).

As anticipated, GLP-1 dose-dependently increased insulin secretion (40). However, there was no attenuating effect of atropine, suggesting that efferent vagal cholinergic muscarinic activity is not a prerequisite for the insulinotropic effect of GLP-1 in the present experimental situation. Rather, insulin levels and C-peptide levels attained higher levels in the atropine day. Most of our knowledge regarding pancreatic innervation comes from animal studies, showing the pancreas to be richly innervated by parasympathetic (vagal) and sympathetic nerves.
In general, vagal stimulation increases insulin, glucagon, and PP secretion (24, 27), whereas splanchnic nerve stimulation reduces insulin and increases glucagon secretion (23, 27). One recent study has indicated that human pancreatic islets may only be sparsely innervated (43) and that could perhaps explain why atropine did not inhibit the insulinotropic effect of GLP-1. The absent inhibitory effect of atropine in the present study would be consistent with the concept that intravenous infusions of glucose and GLP-1 act mainly directly on the β-cell independently of efferent vagal cholinergic muscarinic activity. However, infusion of glucose and GLP-1 into a peripheral vein, as used here, is not representative of the physiological situation, where high concentrations of endogenous GLP-1 (13, 19) may activate GLP-1Rs on afferent fibers (48). Moreover, parasympathetic efferent signaling is likely to include noncholinergic neurotransmitters remaining operative despite muscarinic blockade (22, 47, 33). Signaling by noncholinergic transmitters in the absence of cholinergic signaling might also explain the increase in C-peptide secretion.

In contrast, when GLP-1 was combined with atropine, glucagon levels were further suppressed to very low levels. The mechanism by which GLP-1 suppresses glucagon secretion is still incompletely understood. It could be direct, via activation of GLP-1Rs on the alpha cell, although reports of the existence of GLP-1Rs on the alpha cell are not consistent (17, 38). It is, however, well known that GLP-1 stimulates both insulin (12, 40) and somatostatin (30) secretion via GLP-1Rs on beta and delta cells, respectively. Thus, another possible mechanism of action for the glucagonostatic effect could be indirect, via effects on the release of insulin and/or somatostatin, which may then lead to an inhibition of glucagon secretion. In the present study, we can rule out insulin, because glucagon levels were already lower during the latter half of the low-dose GLP-1 infusion, at which time insulin and C-peptide levels were similar on both the SAL + CLA + GLP-1 and the ATR + CLA + GLP-1 days. Moreover, in a study of patients with Type 1 diabetes without endogenous insulin secretion, GLP-1 was still able to suppress glucagon secretion (32), suggesting that endogenous insulin is not a prerequisite for the glucagonostatic effect of GLP-1. On the other hand, somatostatin has been shown to potently suppress both insulin (3) and glucagon (34) release, suggesting a paracrine role for somatostatin in the regulation of islet hormone secretion (14). In support of this, both somatostatin immunoneutralization and somatostatin receptor subtype 2 antagonists reverse the inhibitory effect of GLP-1 on glucagon secretion from the isolated perfused rat pancreas (16). Thus, it seems likely that at least part of GLP-1’s glucagonostatic effect may be mediated indirectly via somatostatin. However, there is evidence that the pancreatic delta cells are also responsive to neural factors. Thus, vagal activation leads to an inhibition of somatostatin secretion (28), which can be abolished by atropine (25). The marked glucagonostatic effect of GLP-1 during blockade of cholinergic vagal transmission in the present study could, therefore, be caused by relieving a tonic vagal inhibition of pancreatic somatostatin secretion, thereby leading to higher intraislet somatostatin levels acting on the alpha cell. Unfortunately, this hypothesis cannot be tested by simply measuring plasma somatostatin levels, since somatostatin is secreted from endocrine delta cells and enteric neurons throughout the gastrointestinal tract (reviewed in Ref. 36). However, it remains plausible that the mechanism behind the marked glucagonostatic effect of atropine and GLP-1 combined may involve a local paracrine mechanism involving somatostatin.

**Conclusion**

In conclusion, efferent vagal cholinergic muscarinic activity does not seem to be a prerequisite for the insulinotropic effect of intravenously administered GLP-1. However, when GLP-1 was coinfused with atropine, glucagon levels were further suppressed, suggesting additive glucagonostatic effects. Further studies aiming to evaluate the importance of the vagal system in humans are clearly needed.

**Perspectives and Significance**

Our results suggest that exogenous GLP-1 interacts with muscarinic signaling mechanism to control insulin and glucagon secretion. Contrary to our hypothesis, muscarinic blockade did not block GLP-1-stimulated insulin secretion. Indeed, insulin secretion was augmented by atropine, whereas glucagon levels were further suppressed, supporting an important role for muscarinic regulation of islet function, whereas the actions of GLP-1 on islet function appear independent of cholinergic activity. A more detailed knowledge of the interplay between the cholinergic system and glucagon-like peptide is important because many drugs have anticholinergic side effects and because patients with diabetes of various etiologies may have autonomic dysregulation due to neuropathy. This study is one step toward a more complete understanding of diabetic pathophysiology and pharmacology.

**ACKNOWLEDGMENTS**

We thank Jytte Purtoft and Nina Kjeldsen from Center for Diabetes Research at Gentofte Hospital, University of Copenhagen, Hellerup, Denmark, together with Sofie Pilgaard and Lene Albæk from the Holst lab at The Novo Nordisk Foundation Center for Basic Metabolic Research, Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, for expert technical assistance and we thank all participants for spending time on this project.

**GRANTS**

The study was supported by a grant from The Danish Medical Research Council and the Novo Nordisk Foundation.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

EFFERENT CHOLINERGIC TRANSMISSION AND GLP-1


