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

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The role of efferent cholinergic transmission for the insulinotropic 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 insulinotropic 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 cholinergic 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 muscular, 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 during the GLP-1 infusions (AUC45–145): 492 ± 85 (saline) vs. 247 ± 59 (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 insulinotropic 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) mmol/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 insulinotropic 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 (insulinotropic 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 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, 1-glucagon, PP, and somatostatin were extracted with ethanol (70% of final concentration) before analysis. Total GLP-1 levels were assayed using antisemur 89390, which requires the intact amidated COOH terminus of the molecule (41). Glucagon was measured using the COOH terminally directed antisemur 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 antisemur 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).
RESULTS

Heart rate. Atropine blocked cholinergic transmission, as evidenced by the increase in heart rate of ~20 beats/min (Fig. 1, A and B) immediately after initiation of the atropine 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.

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 plasma 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 a 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.
GLP-1 infusions commenced, to plateau levels of 43 \( \pm \) 2 (low-dose GLP-1) and 122 \( \pm \) 11 \( \mu \)moll/l (high-dose GLP-1) on the control (saline) day, (Friedman test followed by Dunn’s test). GLP-1 and atropine combined with clamp experiments (\( n = 5 \)). When inspecting the time course during both the low-dose and high-dose GLP-1 infusions (Fig. 7A). There was a treatment \( \times \) 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 \( \times \) 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 \( \times \) time effect \( (P < 0.0001) \) and post hoc analysis yielded a significant difference at \( t = 175 \) (Fig. 7B). In addition, the AUCs during the GLP-1 infusion period \( (t = 45–145 \text{ min}) \) were significantly lower on the atropine day \( (t = 0.018) \). 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 \( \times \) time effect).

**C-peptide.** C-peptide levels increased dose-dependently on the control (saline) day, (Friedman test followed by Dunn’s post hoc test, \( P < 0.0032 \)) and to 110 \( \pm \) 5 \( \mu \)moll/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 \( \times \) time effect). On the SAL+CLA+GLP-1 and ATR+CLA+GLP-1 days, the AUCs for the GLP-1 infusion period (45–145 min) were not significantly different (Fig. 4B). GLP-1 levels were unchanged over the course of the control experiments (no treatment or treatment \( \times \) time effect) (Fig. 4C). GLP-1 AUCs were similar on the control days (Fig. 4D).

**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 \( \times \) 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 \( \times \) 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 \( \times \) time effect).

**C-peptide.** C-peptide levels increased dose-dependently (Fig. 5A). From the line curve, there appeared to be an augmented C-peptide response on the atropine day, with a treatment \( \times \) 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 \( \times \) 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).

**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 \( \times \) time effect).

**C-peptide.** C-peptide levels increased dose-dependently (Fig. 5A). From the line curve, there appeared to be an augmented C-peptide response on the atropine day, with a treatment \( \times \) 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 \( \times \) 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).
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 effenter 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 on 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

**DISCUSSION**

In the present study, we aimed to pharmacologically block effenter vagal transmission to evaluate the importance of vagal effenter 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 insulonotropic effect. Moreover, atropine suppressed glucagon, 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 [26 and reviewed in (1) and (27)], and this response can be abolished by atropine (26). However, parasympathetic actions on the pancreatic islets may also be exerted by noncholinergic neurotransmitters (22, 47, 33).

The atropine regimen used for the experiments had previously been used to block cholinergic transmission in humans (6), and was associated with typical side effects, including dry mouth and increased heart rate, and markedly suppressed plasma PP levels, indicating suppression of effenter vagal activity (10, 45).

To evaluate the role of the effenter 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

**Fig. 6.** A: time course of insulin during saline (●) and atropine (○) infusion combined with glucose clamp (6 mmol/l) and glucagon-like peptide-1 (GLP-1) infusion (circles; n = 10). 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. B: AUCs during the GLP-1 infusion periods. (Wilcoxon, P = 0.049). C: time course of insulin during saline (●) and atropine (○) infusion combined with glucose clamp (6 mmol/l) (n = 5). No treatment × time effect (P = 0.37). D: insulin AUCs for the 45- to 145-min period SAL+CLA and ATR+CLA days (Wilcoxon, P > 0.99). Data are expressed as means ± SE.
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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.

References

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