Cephalic phase secretion of insulin and other enteropancreatic hormones in humans

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DURING THE PREABSORPTIVE MEAL phase, various exocrine and endocrine secretion products are released from the gastrointestinal tract (39). The preabsorptive phase may be subdivided into the cephalic and gastric phases (22). These are followed by the intestinal or absorptive phase, which is heralded by the appearance of nutrients in the portal blood. The cephalic phase is triggered by meal expectations, followed by visual, olfactory, and gustatory stimuli, and finally by oropharyngeal stimuli including masticatory and swallowing sensations. The gastric phase sets in when a food bolus enters the stomach from the esophagus. Stretching of the gastric walls activates mechanoreceptors on vagal afferents leading to the initiation of long vagovagal reflexes. The afferent signals of both the cephalic and the gastric phases are relayed to, and integrated in, the central nervous system prior to the activation of neurons in vagal motor nuclei projecting to the gastrointestinal target organs (7). Experimentally, the cephalic phase can be studied by sham feeding, classically involving an esophageal fistula preventing food from entering the stomach. In humans, this is mimicked by the “chew-and-spit” procedure (modified sham feeding, MSF), which is nearly as powerful as “adequate sham feeding” with respect to, e.g., gastric acid secretion (59). It is known that the pancreatic polypeptide (PP) cells of the pancreatic islets are innervated by cholinergic postganglionic vagal fibers and that PP levels increase markedly in response to vagal fibers and that PP levels increase markedly in response to sham feeding (56). Whether other enteropancreatic hormones are secreted in a preabsorptive, cephalic secretory phase has been debated for many years. Regarding insulin, which has been studied extensively, there appears to be a cephalic phase of insulin secretion in animals [rats (8–10, 57, 70), dogs (18), sheep (26)], but studies in humans have produced ambiguous results: some studies support the existence of a cephalic secretory phase (42, 51, 58, 66), whereas others do not (61, 63, 64).

In the present study, we investigated cephalic phase secretions elicited by MSF while, importantly, maintaining slightly elevated ambient glucose levels, which, apart from mimicking early prandial conditions, should facilitate any neurally mediated insulin response. The experiments were carried out in the presence and absence of atropine, on the assumption that vagally mediated responses might involve muscarinic transmission. This also allowed us to evaluate the importance of a muscarinic tone for fasting gastrointestinal hormone levels in healthy humans.

RESEARCH DESIGN AND METHODS

The study was conducted in accordance with the Helsinki II Declaration and was approved by the Ethical Committee of the Capital Region of Denmark (registration no. H-2-2011-062) and by the Danish Data Protection Agency (journal no. 2011-41-6381) and was registered at www.clinicaltrials.gov (ClinicalTrials.gov ID: NCT01534442). Oral and written informed consent was obtained from all participants.
**Participants**

Ten healthy male participants with no family history of enteral pathology or diabetes were screened by clinical examination and routine blood biochemistry (Table 1).

**Experimental Design**

Participants were studied in the morning on three separate occasions, all involving clamping of plasma glucose (PG) to 6 mmol/l. One day was a control day (n = 9) [saline + clamp (SAL + CLA)]; on another day (in random order) MSF was carried out during a saline infusion [saline + clamp + MSF (SAL + CLA + MSF)] or during an atropine infusion [atropine + clamp + MSF (ATR + CLA + MSF)]. Experiments were conducted at the Center for Diabetes Research, Gentofte Hospital, Hellerup, Denmark, in the morning after ~12 h of fasting and 24 h of abstinence from alcohol, exercise, and tobacco. Two intravenous catheters were placed in antecubital veins: one for infusions of isotonic saline/atropine and glucose, and one, in the contralateral arm, for blood sampling. Participants maintained a recumbent position for the duration of each experiment, and the arm catheterized for blood sampling was placed in a heating apparatus to arterialize blood samples.

**Atropine.** To block muscarinic signaling, atropine (Nycomed, Roskilde, Denmark) was given as an intravenous bolus of 1 mg at t = 0 min, followed by a maintenance infusion of 80 ng kg⁻¹ min⁻¹ (5) using isotonic saline as vehicle. To control for volume differences, isotonic saline (bolus and maintenance dose) was infused on the other days at the same rate as that of the atropine infusion. Participants were asked about antimuscarinic effects (e.g., dryness of mouth, palpitations, blurred vision). Oxygen saturation, electrocardiogram, heart rate, and blood pressure were monitored noninvasively. Participants were monitored for at least half an hour after discontinuation of the infusions.

**Hyperglycemic clamp.** For the 6 mmol/l hyperglycemic clamp (final PG concentration), a frequently adjusted infusion of a 20% dextrose solution was used (17). The clamp was established and maintained between t = 15 and t = 105 min. PG was measured bedside.

**Modified sham feeding.** The meal stimulus used for the MSF (chew-and-spit technique), performed between t = 45 and t = 60 min (56), consisted of an appetizing breakfast serving, including pancakes with jam, fried bacon, an egg omelet, yogurt with muesli and syrup, fruit salad, a bun with cheese, orange juice, and coffee. Participants had previously been carefully introduced to the procedure and were encouraged to sample and chew all elements but to spit all, including saliva. At the end of the MSF, what remained of the breakfast serving was removed from the room and participants flushed their mouths and spat out any saliva. PP secretion served as a positive control to evaluate the efficacy of the cephalic stimulus (55).

**Blood collection and storage of samples.** Blood for PG was collected into fluoride tubes and centrifuged immediately (7,400 g for 20 min at 4°C), plasma for PP, glucagon, GIP, and GLP-1 was stored at −20°C, while serum for insulin and C-peptide analyses was collected in dry tubes for coagulation (20 min at room temperature). After centrifugation (1,200 g for 20 min at 4°C), plasma for PP, glucagon, ghrilin, GIP, and GLP-1 were assayed as collections were clarified by centrifugation (1,200 g at 4°C). Total GIP was measured by using a COOH-terminally directed antibody code no. HYB 4305, which detects glucagon of pancreatic origin. Total GLP-1 levels were assayed by using antiserum code no. 89390, which requires the intact amidated COOH-terminus of the molecule (47). Glucagon was measured by using the COOH-terminally directed immunoassay code no. 4305, which detects glucagon of pancreatic origin (34). Total GIP was measured by using a COOH-terminally directed antiserum code no. 80 867 (41). Total ghrilin was measured with a RIA kit from Millipore (GHRT-99HK). Quality controls included in each assay fell within acceptable limits.

**Statistics and Calculations**

Results are presented as means ± SE. Data were tested by the D’Agostino and Pearson omnibus normality test. Two-way repeated-measures analysis of variance (ANOVA), followed by Tukey’s post-tests, was employed to test for differences in repeatedly measured values between days (n = 9) (e.g., PG, heart rate, hormone concentrations). Comparisons of more than two values were carried out by one-way ANOVA applying Tukey’s test for pairwise comparisons (n = 9). For nonnormally distributed data, a Friedman test was employed to compare multiple values, and a Wilcoxon test for paired difference was used for comparison between single values. A two-sided P value <0.05 was considered significant. Areas under the curve were calculated by the trapezoidal rule.

Statistical analyses were carried out with GraphPad Prism 6.0d for Mac OS X, GraphPad Software, La Jolla, CA.

**RESULTS**

**Heart Rate**

All participants experienced marked and immediate increases in heart rate in response to the muscarinic blockade induced by atropine (Fig. 1A). At t = 15 min, heart rate was significantly increased on the ATR + CLA + MSF day compared with the SAL + CLA + MSF and SAL + CLA day (88 ± 2 vs. 63 ± 2 and 59 ± 3 beats/min, P < 0.0001).

**Glucose**

PG levels were clamped on all experimental days at the desired 6 mmol/l level prior to the initiation of MSF. On the
MSF day, PG initially slightly exceeded the intended 6 mmol/l level (Fig. 1B). Thus, when comparing the SAL/CLA/MSF day with the SAL/CLA day, there was a small but significant PG difference (mean difference between 0.3–0.4 mmol/l) at t/25 and t/30 min (P < 0.01). Throughout the rest of the study period, PG levels were kept constant, with no significant differences between the days (Fig. 1B) [P = not significant (NS)].

Pancreatic Polypeptide

On the SAL + CLA + MSF day, PP levels rose significantly in response to MSF compared with the SAL + CLA and ATR + CLA + MSF day and compared with baseline (t = 45 min) (Fig. 2B). Atropine suppressed PP levels throughout the experiments and completely abolished the response to MSF (Fig. 2, A and B).

GIP and GLP-1

GIP levels (Fig. 3A) were stable and unchanged in response to atropine, glucose, and MSF (two-way repeated-measures ANOVA, P = NS). GLP-1 levels (Fig. 3B) were stable and unchanged in response to atropine, glucose, and MSF on the sham feeding days (two-way repeated-measures ANOVA, P = NS). On the SAL + CLA day, GLP-1 levels decreased slightly late in the study period compared with baseline levels. In the same period GLP-1 levels were also slightly lower compared with the sham feeding days (Fig. 3B).

Insulin

Insulin levels increased in response to the glucose clamp (Fig. 4A). On the SAL + CLA + MSF day, the insulin concentration exhibited an early peak at t = 30 min, following which it declined but remained at a slightly but significantly higher level during the clamp period. During the period of MSF (t = 45 to t = 60 min), insulin levels showed slight random variations with no consistent pattern (Fig. 5A). On the individual days there was no significant difference in insulin levels over the course of the MSF period when tested by a repeated-measures one-way ANOVA.

C-Peptide

The initial insulin peak was accompanied by a significant increase in C-peptide levels (Fig. 4B), which lasted for the entire experimental period. During the period of MSF, the

Fig. 1. A: heart rate. B: plasma glucose. △, Saline (SAL) + plasma glucose clamp (CLA); ○, SAL + CLA + modified sham feeding (MSF) (SAL + CLA + MSF); □, atropine + CLA + MSF (ATR + CLA + MSF). Results are means ± SE. *SAL + CLA + MSF vs. SAL + CLA, P < 0.01.

Fig. 2. A: pancreatic polypeptide. B: pancreatic polypeptide (baseline subtracted). △, Saline (SAL) + plasma glucose clamp (CLA); ○, SAL + CLA + modified sham feeding (MSF) (SAL + CLA + MSF); □, atropine + CLA + MSF (ATR + CLA + MSF). Results are means ± SE. *SAL + CLA + MSF vs. SAL + CLA and ATR + CLA + MSF, P < 0.05; *SAL + CLA + MSF vs. t = 45 min, P < 0.05; ● ATR + CLA + vs. SAL + CLA and SAL + CLA + MSF, P < 0.01.
individual C-peptide excursions overlapped on all experimental days (Fig. 5B). On the individual days there was no significant difference in insulin levels when tested by a repeated-measures one-way ANOVA.

Glucagon

Baseline levels were similar on the SAL + CLA + MSF and ATR + CLA + MSF day but lower on the SAL + CLA day (Fig. 6A). On the ATR + CLA + MSF day, baseline glucagon levels were suppressed compared with SAL + CLA + MSF and SAL + CLA days (two-way repeated-measures ANOVA, \( P < 0.0001 \)), and the suppressive effect of atropine was maintained throughout the experiments (Figs. 5A and 6B). At \( t = 45, 47, \) and 51 min, glucagon levels were significantly higher on the SAL + CLA day compared with the SAL + CLA + MSF day (two-way repeated-measures ANOVA, \( P < 0.05 \)), ostensibly because of the slightly higher PG levels (\( t = 25 \) and \( t = 30 \) min) on the SAL + CLA + MSF day.

Ghrelin

From \( t = 15 \) min to the end of experiments, incremental ghrelin levels were significantly lower on the ATR + CLA + MSF day than on the other days (two-way repeated-measures ANOVA, \( P < 0.0001 \)) (Fig. 7B). There was no difference between ghrelin levels on the SAL + CLA + MSF and SAL + CLA days during the period of MSF. On the SAL + CLA and SAL + CLA + MSF days, ghrelin levels remained at baseline levels until cessation of the glucose infusion, after which levels increased compared with baseline levels (Fig. 7B).

DISCUSSION

We studied the effects of vagal activation and muscarinic blockade on enteropancreatic hormone release. We were unable to demonstrate a cephalic secretory phase for insulin, GIP, GLP-1, glucagon, or ghrelin. Interestingly, however, glucagon, ghrelin, and PP levels were suppressed by atropine, pointing to the existence of an independent tonic muscarinic regulation of these hormones.

Cephalic phase secretions of the gastrointestinal tract are initiated by activated vagal motor neurons.

The vagal nerve is capable of eliciting marked insulin secretion in the presence of permissive ambient PG levels, as demonstrated by ex vivo electrical stimulations of porcine pancreata via intact vagal trunks (33), vagal stimulations of in...
situ perfused canine pancreata (6), and in vivo stimulations of the vagal trunks of conscious calves (11) and anesthetized pigs (27–30, 32). Whether meal ingestion gives rise to cephalic phase insulin secretion via the vagus in humans remains uncertain despite a host of experimental data.

The experimental designs employed previously to demonstrate cephalic phase insulin secretion vary with regard to type, intensity, and duration of stimuli, not to mention choice of species. Moreover, the characteristics defining what investigators refer to as “cephalic phase insulin secretion,” such as timing, amplitude, and duration of the insulin excursions, are controversial. Importantly, since amplitudes of reported cephalic insulin responses are very modest compared with the peak postprandial levels, the biological significance of any such cephalic phase insulin release is questionable.

We carried out MSFs in healthy lean men in a calm and pleasant clinical setting. PG levels were clamped at 6 mmol/l to emulate early postprandial glucose excursions. In particular this would prime the pancreatic beta cells and, importantly, eliminate the risk of blurring a positive insulin secretion because of falling PG levels, resulting from the cephalic phase insulin response. Using this experimental approach, it was impossible to detect a cephalic phase of insulin secretion in our volunteers. Because of the slight initial PG overshoot on the SAL/CLA/MSF day, insulin and C-peptide levels on this day were higher at the onset of MSF. Nevertheless, during MSF, insulin levels were steady on all days, and without any marked, rapid rises, contrasting to the steep increase in PP levels. Rather, insulin levels oscillated slightly with a late upward drift, possibly representing a second phase insulin secretory response to the hyperglycemic clamp (17). We have included the individual insulin responses because they illustrate the absence of a common trend in response to the MSF.

The difficulties we and others have with respect to detecting cephalic phase insulin secretion may theoretically reflect the following: 1) cephalic phase release of insulin does not occur in humans, 2) blood sampling protocols are not appropriate to detect delicate events lasting only a few minutes with amplitudes just rising above baseline and spontaneous oscillations, 3) the experimental setting may for some be stressful (i.e., the expulsion of chewed foods may be disagreeable for some), 4) low ambient glucose levels may hinder insulin secretion, 5) high ambient glucose levels could potentially conceal a small cephalic phase response or possibly even abolish it owing to exhaustion of readily releasable, docked secretory vesicles.

Previous investigations have yielded ambiguous results. Teff et al. (64) studied healthy individuals on three occasions with 2 min of sham feeding using an aspartame-sweetened dessert and found a significant increase in insulin after 4 min. The taste of fat did not elicit cephalic phase insulin responses despite relevant increases in PP (15). Similarly, sweet taste did not elicit insulin responses (61). MSF of apple pie for 1 and 3 min did, however, elicit a significant rise in insulin (61).

In another study, Teff et al. (62) combined a gastric instillation of glucose with a 5-min MSF. This yielded no difference in insulin levels but incremental C-peptide levels differed significantly from the control experiment at $t = 4$ min. However, at that time glucose levels were also rising, making it difficult to ascertain what was due to MSF and what was caused by glucose. Similarly, Taylor and colleagues (60a) found no augmentation of insulin secretion despite a marked rise in pancreatic polypeptide when an intragastric glucose instillation was combined with a 30-min MSF. Lorentzen et al. (42) found that MSF increased glucose disposal but in an insulin/C-peptide-independent manner.

A later study by Teff (67) reported pancreatic polypeptide responses to modified sham feeding of liquid and solid foods. Albeit both glucose and insulin had been measured data were not included in the results section because “no significant increases in insulin or decreases in glucose were found in the studies presented”.

In yet another study by Teff et al. (63), individuals were tested on three occasions in a fixed order: a fasted experimental...
day, a sham feeding day, and a meal day. On the sham feeding day an early insulin peak was observed, but a similar increase was evident at $t/\sim 60 \text{ min}$, questioning the cephalic nature of the initial insulin peak.

In accordance with earlier findings in humans, atropine did not influence insulin secretion induced by intravenous glucose (25), and insulin levels were similar on the SAL + CLA and ATR + CLA + MSF days. In rhesus monkeys, atropine has been shown to blunt both first- and second-phase insulin secretion (16). A similar mechanism might account for the difference in insulin levels observed between the SAL + CLA + MSF and the ATR + CLA + MSF days, albeit that the small insulin peak apparent on the SAL + CLA + MSF day at $t = 30 \text{ min}$ could also be explained by the slightly higher PG level at the same time point.

We employed MSF, also known as the chew-and-spit technique (56). MSF does not include swallowing, esophageal, or gastric aspects of food ingestion, but only cephalic input as elicited by visual, olfactory, gustatory, and masticatory sensations. The MSF procedure achieves 48–68% of the maximum pentagastrin-induced acid response (40, 56), with no additional effect observed when extending MSF beyond 15 min (40). Gastric distension does not elicit insulin release, but it does provoke PP secretion (54). In the present study, MSF resulted in PP responses similar to those found by other investigators employing the same approach (56, 66). A rise in PP may be considered a surrogate marker of vagal activation and cholinergic signaling at the level of the pancreatic islets. However, being only a surrogate marker, an increase in PP does not necessarily have any bearing on a putative cephalic phase of insulin secretion (14).

Atropine lowered basal PP levels and abolished the PP response to MSF, indicating a decrease in basal cholinergic tone and loss of the MSF-elicited cholinergic activation of pancreatic PP cells. A contributing factor to the lower MSF-elicited PP responses on the ATR + CLA + MSF day could be the marked reduction in salivation caused by atropine, which would interfere with food sampling and appreciation. For some, the chew-and-spit procedure might be experienced as stressful. However, Teff et al. (63) found similar increases in catecholamine levels on fasting and MSF days, suggesting that an experimental setting, but not MSF in particular, may be perceived as stressful. Furthermore, in the present study heart

Fig. 6. A: glucagon. B: glucagon (baseline subtracted). △, Saline (SAL) + plasma glucose clamp (CLA) (SAL + CLA); ○, SAL + CLA + modified sham feeding (MSF) (SAL + CLA + MSF); ◊, atropine + CLA + MSF (ATR + CLA + MSF). Results are means ± SE. *ATR + CLA + MSF vs. SAL + CLA + MSF and SAL + CLA, $P \leq 0.01$; SAL + CLA + MSF vs. SAL + CLA, $P < 0.05$.

Fig. 7. A: ghrelin. B: ghrelin (baseline subtracted). △, Saline (SAL) + plasma glucose clamp (CLA) (SAL + CLA); ○, SAL + CLA + modified sham feeding (MSF) (SAL + CLA + MSF); ◊, atropine + CLA + MSF (ATR + CLA + MSF). Results are means ± SE. *SAL + CLA + MSF vs. ATR + CLA + MSF, $P < 0.0001$. 

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rates were not increased in connection with sham feeding, suggesting that this was not associated with an increased sympathetic drive. The inhibitory effect of hyperglycemia on PP secretion may also have attenuated MSF responses to some degree (45, 69).

By using the chew-and-spit approach, activation of vagal efferents is accomplished without concurrent nutrient absorption and intestinal peptide secretion. Although no swallowing was reported or observed during MSF, our application of the hyperglycemic clamp might have masked accidental swallowing, which would otherwise have been detectable from rising PG levels. Indeed, if a cephalic phase of insulin secretion had been demonstrated, the possibility of swallowing as the underlying factor would have to be entertained. However, we did not observe any acute insulin or C-peptide increments beyond those elicited by the hyperglycemic clamp with superimposed spontaneous oscillations of insulin (23, 50). Moreover, GIP and GLP-1 levels did not increase during or after MSF. A rise in GIP and GLP-1, peptides secreted by enteroendocrine cells of the small intestine in response to luminal nutrients (36), might have questioned the cephalic nature of stimulations or alternatively suggested the existence of a cephalic phase of GIP and GLP-1 secretion. The absence of a cephalic phase of GLP-1 secretion is in accord with other human data (2, 21, 46).

Only a single animal study, conducted in rats by use of an unusual approach, has suggested the existence of an anticipatory GLP-1 response (68). Furthermore, atropine did not affect the GLP-1 baseline concentrations.

Although MSF did not affect glucagon levels, baseline concentrations were significantly suppressed by atropine, suggesting the presence of cholinergic tone. Glucagon levels decreased following the initiation of the glucose infusion on all days but remained lower during the ATR + CLA + MSF experiments, indicating independent modes of glucagon suppression. The influence of the vagal nerve on glucagon secretion has previously been investigated. Stimulation of the dorsal vagal trunk in dogs increased portal glucagon levels, a response that was blocked by atropine (38). In pigs (27) and dogs (3), electrical stimulations of the combined vagal trunks increased glucagon levels, but in both these experiments atropine had no effect on the glucagon responses to vagal stimulation (3, 28), suggesting peptidergic signal mediation (3). In dogs, intrapancreatic injections of acetylcholine caused glucagon levels to increase, and this response was blockable by atropine (37), whereas glucagon secretion from isolated porcine pancreata was decreased by acetylcholine (33). In humans, muscarinic blockade has been found by some investigators to suppress glucagon in both the basal (12) and the postprandial state (65), while others have found no suppressive effect of atropine on glucagon (13, 20, 53). In these studies, PG levels were not clamped. In one of the studies, atropine was administered before baseline sampling (20). In the second study, a fixed, continuous dextrose infusion was employed with or without confuision of atropine to markedly elevate (but not clamp) PG (53). In the third study no difference in glucagon levels was identified; however, when inspecting the line curve a decline in glucagon levels is suggested (13).

Ghrelin levels were markedly suppressed by atropine but were unaffected by MSF. In earlier studies, a similar effect of atropine was reported in fasting healthy individuals (43, 44). In another study, ghrelin was suppressed during somatostatin infusion (52). Plasma levels of somatostatin are normally quite low and steady, suggesting that only high local concentrations play a role for ghrelin secretion. Interestingly, vagal stimulation has been shown to suppress somatostatin release from the gastric fundus of pigs (35), and atropine is reported to abolish this effect of vagal stimulation in the gastric antrum and the pancreas (31, 35). The attenuated ghrelin levels during atropine infusion in the present study might, therefore, be due to the loss of tonic vagal suppression of fundic somatostatin secretion. A similar mechanism in the pancreas could explain the suppression of glucagon during the atropine infusion. We found no independent effect of hyperglycemia and resulting insulinemia on ghrelin levels, confirming previous findings (52).

In conclusion, despite performing MSF at fixed glucose levels to emulate postprandial glycemia we were unable to convincingly demonstrate a cephalic secretory phase for insulin, GIP, GLP-1, glucagon, or ghrelin in healthy humans. Muscarinic blockade markedly suppressed PP, glucagon, and ghrelin levels, suggesting a role of vagal tone in the regulation of these hormones, possibly by a common mechanism involving paracrine regulation via somatostatin.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.V., A.P., and J.J.H. conception and design of research; S.V. and A.P. performed experiments; S.V. analyzed data; S.V. and J.J.H. interpreted results of experiments; S.V. prepared figures; S.V., A.P., C.F.D., B.H., F.K.K., T.V., and J.J.H. edited and revised manuscript; S.V. drafted manuscript; S.V., A.P., C.F.D., B.H., F.K.K., T.V., and J.J.H. approved final version of manuscript.

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