Cephalic phase secretion of insulin and other entero-pancreatic hormones in humans

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Conflicts or Duality of Interest
The authors have no potential conflicts of interest relevant to this article.
ABSTRACT

Entero-pancreatic hormone secretion is thought to include a cephalic phase but the evidence in humans is ambiguous. We studied vagally-induced gut hormone responses with and without muscarinic blockade in ten glucose-clamped healthy males (age: 24.5±0.6 years (mean±standard error of the mean (SEM); body mass index: 24.0±0.5kg/m²; HbA1C: 5.1±0.1% /31.4±0.5mmol/mol). Cephalic activation was elicited by modified sham feeding (MSF, aka “chew and spit”) with or without atropine (1mg bolus 45min before MSF + 80ng/kg/min for 2h). To mimick incipient prandial glucose excursions, glucose levels were clamped at 6mmol/L on all days. The meal stimulus for the MSF consisted of an appetizing breakfast. Participants (9/10) also had a 6mmol/L-glucose clamp without MSF. PP levels rose from 6.3±1.1 to 19.9±6.8pmol/L (means±SEM) in response to MSF and atropine lowered basal PP levels and abolished the MSF response. Neither insulin, C-peptide, glucose-dependent insulintropic polypeptide (GIP) nor glucagon-like peptide-1 (GLP-1) levels changed in response to MSF or atropine. Glucagon and ghrelin levels were markedly attenuated by atropine prior to and during the clamp: at t=105min on the ATR+CLA+MSF compared to the SAL+CLA and SAL+CLA+MSF days; baseline-subtracted glucagon levels were -10.7±1.1 vs. -4.0±1.1 and -4.7±1.9pmol/L (mean±SEM), P<0.0001, respectively; corresponding baseline-subtracted ghrelin levels were 303±36 vs. 39±38 and 3.7±21pg/mL (mean±SEM), P<0.0001. Glucagon and ghrelin levels were unaffected by MSF. In spite of adequate PP responses, a cephalic phase response was absent for insulin, glucagon, GLP-1, GIP and ghrelin.

Trial Registration ClinicalTrials.gov ID: NCT01534442)
Keywords
Atropine  Cephalic phase  Hyperglycemic clamp  Insulin  Glucagon
Muscarinic blockade  Efferent vagal signaling  Vagus  Pancreatic polypeptide
Ghrelin

Abbreviations
ATR  Atropine
CLA  Clamp
DPP-4  Dipeptidyl peptidase 4
GLP-1  Glucagon-like peptide-1
GIP  Glucose-dependent insulinotropic peptide
MSF  Modified sham feeding
PG  Plasma glucose
PP  Pancreatic polypeptide
SAL  Saline
Introduction

During the preabsorptive meal phase, various exocrine and endocrine secretion products are released from the gastrointestinal tract (39). The pre-absorptive 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 vago-vagal 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 sham feeding (56). Whether other entero-pancreatic 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
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support the existence of a cephalic secretory phase (42, 51, 58, 66) while others do not (61, 63, 64). Studies of ghrelin (4, 24, 48, 58) and glucagon (1, 63) have generated equally ambiguous data.

The purpose of the present study was to investigate cephalic phase secretions, elicited by modified sham feeding (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, based 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 1A).
**Experimental Design**

Participants were studied in the morning on three separate occasions, all involving clamping of plasma glucose (PG) at 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, Denmark, in the morning after approximately 12 hours of fasting and 24 hours 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/min (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 anti-muscarinic effects (e.g. dryness of mouth, palpitations, blurred vision). Oxygen saturation, electrocardiogram, heart rate and blood pressure were monitored non-invasively. 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.
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(17). The clamp was established and maintained between $t = 15$ and $t = 105 \text{ min}$. PG was measured bedside.

**Modified sham feeding (MSF):** The meal stimulus used for the MSF (‘chew-and-spit’ technique), performed between $t = 45$ and $t = 60 \text{ min}$ (56), consisted of an appetizing breakfast serving, including pancakes with jam, fried bacon, an egg omelet, yoghurt 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 $(7,400 \text{ g for 1 min at room temperature})$. Blood samples for peptide analyses were collected at regular intervals between $t = -15$ and $t = 135 \text{ min}$. During the period of MSF ($t = 45$ and $t = 60 \text{ min}$), blood sampling was intensified ($t = 45, 47, 49, 51, 53, 55$ and $60 \text{ min}$). Blood for PP, glucagon, ghrelin, GIP, and GLP-1 analyses was collected into chilled tubes containing EDTA and a dipeptidyl peptidase 4 inhibitor (DPP-4i) (valine-pyrrolidide, 0.01 mmol/L final concentration, a gift from Novo Nordisk, Bagsværd, Denmark). Blood for insulin and C-peptide analyses was collected in dry tubes for coagulation (20 min at room temperature). After centrifugation $(1,200 \text{ g for 20 min at } 4^\circ C)$ plasma for PP, glucagon, ghrelin, GIP and GLP-1 was stored at -20°C, while serum for insulin and C-peptide analyses was stored at -80°C.
Laboratory analyses

PG was determined every 5 minutes during the clamp period and more frequently during MSF, by the glucose oxidase method using a glucose analyzer (Yellow Springs Instrument model 2300 STAT Plus Analyzer; YSI Inc., Yellow Springs, OH, USA). Serum insulin and C-peptide concentrations were quantified by routine immunoassays (Siemens Healthcare Diagnostics, Ballerup, Denmark) using the ADVIA Centaur XP analyzer at the Department of Clinical Biochemistry, Gentofte Hospital, Denmark. The intra-serial coefficients of variation were 3% for insulin and between 2.5-5% for C-peptide. Plasma for analysis of PP, glucagon, GIP and GLP-1 was extracted with ethanol (70% final concentration) before analysis. PP was assayed using a mid-region specific antibody code no HYB 347-07 (Statens Serum Institut, Copenhagen, Denmark), with human PP standards and \(^{125}\)I-labeled human PP (Perkin Elmer, Boston, MA, USA) (19). Total GLP-1 levels were assayed using antiserum code no 89390, which requires the intact amidated C-terminus of the molecule (47). Glucagon was measured using the C-terminally directed antiserum code no. 4305, which detects glucagon of pancreatic origin (34). Total GIP was measured using a C-terminally directed antiserum code no. 80 867 (41). Total ghrelin was measured using a RIA kit from Millipore (GHRT-89HK). Quality controls included in each assay fell within acceptable limits.

Statistics and calculations

Results are presented as means ± SEM. Data were tested by the D'Agostino & 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
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ANOVA applying Tukey’s test for pairwise comparisons (n=9). For non-normally 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 (AUC) were calculated using the trapezoidal rule.

Statistical analyses were carried out using Graphpad Prism 6.0d for Mac OS X, GraphPad Software, La Jolla, California, USA.

Results

Heart rate

All participants experienced marked and immediate increases in heart rate in response to the muscarinic blockade induced by atropine (Figure 1A). At \( t = 15 \) min, heart rate was significantly increased on the ATR+CLA+MSF day compared to 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 SAL+CLA+MSF day, PG initially slightly exceeded the intended 6mmol/L level (Figure 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 (Figure 1B) (\( P=NS\)).
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Pancreatic Polypeptide

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

GIP and GLP-1

GIP levels (Figure 3A) were stable and unchanged in response to atropine, glucose and MSF (two-way repeated-measures ANOVA, P=NS). GLP-1 levels (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 to baseline levels. In the same period GLP-1 levels were also slightly lower compared to the sham feeding days (Figure 3B).

Insulin

Insulin levels increased in response to the glucose clamp (Figure 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 (Figure 5A). On the individual days there was no significant difference in insulin levels over the course of the MSF period when tested by a RM one-way ANOVA.

C-peptide

The initial insulin peak was accompanied by a significant increase in C-peptide levels (Figure 4B), which lasted for the entire experimental period. During the period of
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MSF, the individual C-peptide excursions overlapped on all experimental days (Figure 5B). On the individual days there was no significant difference in insulin levels when tested by a RM 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 (Figure 6A). On the ATR+CLA+MSF day, baseline glucagon levels were suppressed when compared to 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 (Figure 5A and 6B). At \( t = 45, 47 \) and 51 min, glucagon levels were significantly higher on the SAL+CLA day compared to the SAL+CLA+MSF day (two-way repeated-measures ANOVA, \( P<0.05 \)); ostensibly due to 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, \( P<0.0001 \)) (Figure 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 to baseline levels (Figure 7B).
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Discussion

We studied the effects of vagal activation and muscarinic blockade on entero-
pancreatic 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, as amplitudes of reported cephalic
insulin responses are very modest when compared to the peak postprandial levels, the
biological significance of any such cephalic phase insulin release is questionable.

We carried out MSFs in healthy lean males 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. Due to 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 upwards 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 due to exhaustion of readily-releasable, docked secretory vesicles.

Previous investigations have yielded ambiguous results. Teff et al 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(64). The taste of fat did not elicit cephalic phase insulin responses despite relevant increases in PP
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(15). Similarly, sweet taste did not elicit insulin responses (61). MSF of apple pie for one and three min did however elicit a significant rise in insulin (61).

In another study, Teff et al 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 (62). Similarly, Taylor et al. 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 (49). Lorentzen et al found that MSF increased glucose disposal but in an insulin/c-peptide independent manner (42).

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

In yet another study by Teff et al, 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=60 min questioning the cephalic nature of the initial insulin peak (63).

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
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apparent on the SAL+CLA+MSF day at t = 30 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 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 (63).
Furthermore, in the present study heart 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. Though 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 using an unusual
approach, has suggested the existence of an anticipatory GLP-1 response (68).
Further, atropine did not affect the GLP-1 baseline concentrations.

While 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
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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 (20, 53) (13). 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 co-infusion 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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Author contributions

SV designed and authored the protocol, carried out the experiments, analyzed the data, prepared figures and wrote the manuscript. AP designed and authored the protocol, carried out the experiments, revised the manuscript. CFD and BH analyzed the data and revised the manuscript. FKK and TV contributed to the design of the protocol and revised the manuscript. JJH designed the protocol, analyzed the data and revised the manuscript.

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Legends:

Figure 1: (A) Heart rate (B) Plasma glucose. Triangles = saline (SAL) + plasma glucose clamp (CLA), Circles = SAL+CLA+ modified sham feeding (MSF) (SAL+CLA+MSF), Squares = atropine+CLA+MSF (ATR+CLA+MSF). Results are means±SEM.

Figure 2: (A) Pancreatic polypeptide (B) Pancreatic Polypeptide (baseline subtracted) Triangles = saline (SAL) + plasma glucose clamp (CLA), Circles = SAL+CLA+ modified sham feeding (MSF) (SAL+CLA+MSF), Squares = atropine+CLA+MSF (ATR+CLA+MSF). Results are means±SEM.

Figure 3: (A) Glucose-insulinotropic polypeptide (GIP) and (B) Glucagon-like peptide 1 (GLP-1) and Triangle = saline (SAL) + plasma glucose clamp (CLA), Circle = SAL + CLA + modified sham feeding (MSF), Square = atropine + CLA + MSF. Results are means±SEM.

Figure 4: Insulin (A) and C-peptide (B), time courses. Triangle = saline (SAL) + plasma glucose clamp (CLA), Circle = SAL + CLA + modified sham feeding (MSF), Square = atropine + CLA + MSF. Results are means±SEM.

Figure 5: Insulin (A) and c-peptide (B), individual excursions during modified sham feeding. Triangle = saline (SAL) + plasma glucose clamp (CLA), Circle = SAL + CLA + modified sham feeding (MSF), Square = atropine + CLA + MSF. Results are means±SEM.

Figure 6: (A) Glucagon, (B) Glucagon (baseline subtracted). Triangles = saline (SAL) + plasma glucose clamp (CLA) (SAL+CLA), Circles = SAL+ CLA + modified
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662  sham feeding (MSF) (SAL+CLA+MSF), Squares = atropine + CLA + MSF
663  (ATR+CLA+MSF). Results are means±SEM.
664  Figure 7: (A) Ghrelin (B) Ghrelin (baseline subtracted). Triangles = saline (SAL) +
665  plasma glucose clamp (CLA) (SAL+CLA), Circles = SAL+ CLA + modified sham
666  feeding (MSF) (SAL+CLA+MSF), Squares = atropine + CLA + MSF
667  (ATR+CLA+MSF). Results are means±SEM.
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Table 1

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<td>24.0±0.5</td>
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<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>
</tbody>
</table>
A

Pancreatic polypeptide (pmol/L)

Time (min)

-15 0 15 30 45 60 75 90 105 120 135

SAL/ATR  CLA

Infusions end

B

Δ Pancreatic polypeptide (pmol/L)

Time (min)

-15 0 15 30 45 60 75 90 105 120 135

SAL/ATR  CLA

Infusions end

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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