Free fatty acids decrease circulating ghrelin concentrations in humans

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Abstract

Objective: Concentrations of the orexigenic peptide ghrelin is affected by a number of hormones, which also affect circulating levels of free fatty acids (FFAs). The present study was therefore designed to determine the direct effect of FFAs on circulating ghrelin.

Design: Eight lean, healthy men were examined for 8 h on four occasions using variable infusion rates (0, 3, 6 and 12 μl/kg per min) of intralipid to create different plasma FFA concentrations. Constant levels of insulin and GH were obtained by administration of acipimox (250 mg) and somatostatin (300 μg/h). At the end of each study day a hyperinsulinaemic-euglycaemic clamp was performed.

Results: Four distinct levels of FFAs were obtained at the end of the lipid infusion period (FFA LIPID: 0.03±0.00 vs: 0.49±0.04, 0.92±0.08 and 2.09±0.38 mmol/l; ANOVA, P<0.0001) and during hyperinsulinaemia (FFA LIPID+INSULIN: 0.02±0.00 vs: 0.34±0.03, 0.68±0.09 and 1.78±0.32 mmol/l; ANOVA, P<0.0001). Whereas, somatostatin infusion alone reduced ghrelin concentration by 67%, concomitant administration of increasing amounts of intralipid reduced circulating ghrelin by a further 14, 19 and 19% respectively (change in ghrelin: 0.52±0.05 vs: 0.62±0.06, 0.72±0.09 and 0.71±0.05 μg/l; ANOVA, P=0.04). No further reduction in ghrelin concentration was observed during hyperinsulinaemia.

Conclusion: FFA exposure between 0 and 1 mmol/l significantly suppresses ghrelin levels independent of ambient GH and insulin levels.

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Introduction

Ghrelin is a recently discovered gut peptide, which is the endogenous ligand for the GH secretagogue receptor (GHS-R) (13). When injected intravenously, ghrelin elicits a prompt rise in circulating GH (2, 25). Activation of hypothalamic GHS-Rs conveys the orexigenic effects of ghrelin, and in line with this, plasma ghrelin concentrations are high pre-prandially (3) and decline following ingestion of food (26).

Circulating ghrelin levels have been shown to be decreased by several hormones, e.g. somatostatin (18, 23), GH (4, 19) and insulin (7), but some controversy still exists as to whether they inhibit ghrelin release directly or indirectly. There is also evidence to support that oral as well as systemic exposure to some, but not all, macronutrients suppresses ghrelin secretion (5, 17). Since ghrelin stimulates food intake, it was originally hypothesized that ghrelin levels would be higher in conditions with a high food intake, such as obesity and hyperthyroidism, but several studies have described decreasing ghrelin concentrations with increasing body mass index (BMI; 10, 27) and ghrelin levels are reduced in hyperthyroid patients (20).

One common problem in all of these studies is that metabolism and circulating levels of many fuels, including free fatty acids (FFAs), are altered. It is therefore not known to which extent the changes observed are directly related to hormonal alterations or are secondary to metabolic changes. Interestingly, ghrelin levels and overeating is also dissociated in hyperthyroid patients as reported previously (20).

The purpose of the present study was therefore to observe the direct impact of a stepwise elevation of FFAs on ghrelin concentration in healthy humans. In order to isolate the effect of FFAs and avoid interference from other hormones, e.g. insulin and GH, a pancreatico-pituitary clamp was performed using somatostatin, subjects were then given constant amounts of insulin, glucagon

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and GH. We furthermore suppressed endogenous FFA release using acipimox and administered intralipid and heparin to obtain different FFA levels within the physiological range.

Material and methods

Subjects

Eight healthy men (Table 1) were studied on four occasions, each separated by at least 1 month. All participants were nonsmokers, not taking any medication, and had a stable weight for the previous 3 months. All were normotriglyceridemic and had normal blood pressure, normal haematological indices, liver and renal function. All participants gave their written informed consent after receiving verbal and written information concerning the study according to the Declaration of Helsinki II. The Aarhus County Ethical Scientific Committee approved the study.

Study design

The study protocol is depicted in Fig. 1. After an overnight fast of 10 h, subjects were studied for 8 h from 0700 h to 1500 h on four occasions with varying lipid (Intralipid 20%, Fresenius Kabi) infusions at 0 (saline), 3, 6 and 12 μl/kg per min. Intralipid is a fat emulsion consisting of triglycerides: 12% palmitic acid (C16:0), 4% stearic acid (C18:0), 21% oleic acid (C18:1n-9), 53% linoleic acid (C18:2n-6), 7% α-linoleic acid (C18:3n-3 and 3% others). (24). On each study day, infusion of heparin (Heparin ‘SAD’, 70 IE/kg per min) and somatostatin (300 μg/h) was initiated at t = 0 min as well as replacement with GH (2 ng/kg per min; Novo Nordisk) and glucagon (Glukagen, 1 mg/ml, 0.8 ng/kg per min; Novo Nordisk). Insulin (Novo Nordisk) was given at a rate of 0.08 mU/kg per min for the initial 6 h of the study and continued at a rate of 0.6 mU/kg per min during the 2 h clamp. Acipimox (Olbetam 250 mg, Pfizer) was administered at t = 0 min to suppress endogenous lipolysis. To avoid hypoglycaemia following the somatostatin infusion, glucose was infused from t = 60 to t = 140 min to maintain euglycaemia; the glucose infusions were identical on all four study days. Blood samples to assess the impact of FFAs on ghrelin were drawn from t = 330 to t = 360 min (referred to as ‘lipid’) after initiation of intralipid infusion, which was at least 3 h after discontinuation of glucose administration. After 6 h (t = 360 min), a 2-h hyperinsulinaemic-euglycaemic clamp (insulin infusion rate: 0.6 mU/kg per min) was performed to evaluate the impact of insulin on ghrelin concentration. Blood samples assessing the impact of FFAs on ghrelin with concomitant hyperinsulinaemia were drawn at t = 450 to t = 480 min (referred to as ‘lipid + insulin’).

Each subject initially underwent the study without intralipid infusion in order to estimate the amount of glucose required to avoid hypoglycaemia. The remaining three study days were performed in random order in a single-blinded manner. Unless specified otherwise, data referred to below were obtained with arterialized blood based on triplicate measurements during the last 30 min of the two study periods with 15 min intervals.

Measurements

Serum ghrelin was determined by an in-house RIA. In brief, breakable Maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 5°C with a polyclonal donkey antirabbit IgG (5 μg/ml, 250 μl per well; Sigma-Aldrich, Copenhagen, Denmark) dissolved in phosphate buffer (40 mM, pH 8.0). After coating, plates were washed once using 50 mM Tris–HCl buffer (pH 8.0) plus 0.9% (wt/vol) NaCl, 0.5% (vol/vol) Tween 20, and 0.05% (wt/vol) NaN3; the plates were then blocked for 2 h at room temperature with 300 μl of 40 mM phosphate buffer plus 1% BSA (Sigma-Aldrich), 0.05% (wt/vol) NaN3, and 0.6% (wt/vol) NaCl. We used 50 μl of standard

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**Table 1** General characteristics of the study participants. Values are mean±S.D.

<table>
<thead>
<tr>
<th>Participants at baseline</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.1±1.0</td>
</tr>
<tr>
<td>Weight (kilograms)</td>
<td>83.7±8.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.85±0.05</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>24.5±3.2</td>
</tr>
</tbody>
</table>

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(a serial dilution of recombinant human octanoylated ghrelin obtained from Novo Nordisk) or undiluted serum. In addition, 50 μl of 125I-labelled octanoylated ghrelin (10 000 c.p.m. per well (Peninsula Laboratories Inc., Division of Bachem, San Carlos, CA, USA) and 50 μl of a polyclonal ghrelin antibody (Novo Nordisk) were added. Standards were dissolved in assay buffer (40 mM phosphate buffer, 0.05% (wt/vol) NaN3, 0.9% (wt/vol) NaCl, and 2% (vol/vol) Tween 20) containing 5% BSA, whereas tracer and specific antibody were dissolved in assay buffer containing 0.2% BSA. All serum samples and standards were analyzed in duplicate except for nonspecific binding and standard zero, which were analyzed in quadruplicate. The plates were incubated for 2 days at 5 °C, washed three times, and counted for 5 min in a γ-counter. The lower detection limit was estimated to approximately 0.078 μg/l, the half-maximal displacement occurred at approximately 2 μg/l, and the upper standard was 10 μg/l.

The assay recognizes the COOH-terminal of ghrelin and as such determines acylated as well as des-acylated ghrelin. The intraassay coefficient of variation is below 3.9% and samples from each individual were analysed in one assay.

Levels of serum FFAs were determined using a commercial kit (Wako Chemicals, Neuss, Germany) and plasma glucose level was measured in duplicate immediately after sampling on a glucose analyzer (Beckman Instruments, Palo Alto, CA, USA).

A double monoclonal immunofluorometric assay (DELFIA, Perkin Elmer, Wallac, Turku, Finland) was used to measure serum GH. Insulin and C peptide were determined by commercial immunological kits (DAKO, Glostrup, Denmark; Immunoclear, Stillwater, MN, USA).

Statistics

Results are expressed as the mean±S.E.M. (parametric data) or median and range (non-parametric data). The Shapiro–Wilks test was used to test for normal distribution. Statistical comparisons between study days were assessed by repeated measures ANOVA. If the test was positive, a post hoc comparison was performed by means of a paired t-test or Wilcoxon sign rank test for non-parametric data. For the time series, the area under the curve was calculated by the trapezoidal method, and comparisons were made by ANOVA. P < 0.05 was considered significant.

Results

FFAs

Using varying infusions of heparin and intralipid, combined with inhibition of endogenous lipolysis by acipimox, we managed to obtain and maintain four distinct levels of FFAs throughout each of the four study days:

<table>
<thead>
<tr>
<th>Day</th>
<th>FFA (mmol/l)</th>
<th>Lipid</th>
<th>Lipid + insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Low FFA)</td>
<td>0.03±0.00</td>
<td>0.02±0.00</td>
<td></td>
</tr>
<tr>
<td>2 (Normal FFA)</td>
<td>0.49±0.04*</td>
<td>0.34±0.03†</td>
<td></td>
</tr>
<tr>
<td>3 (High FFA)</td>
<td>0.92±0.08**</td>
<td>0.68±0.09††</td>
<td></td>
</tr>
<tr>
<td>4 (Supra FFA)</td>
<td>2.09±0.38***</td>
<td>1.78±0.32†††</td>
<td></td>
</tr>
</tbody>
</table>

For basal conditions, overall ANOVA P < 0.0001. Post-hoc comparisons: *P < 0.0001 day 1 vs day 2; **P < 0.0001 day 2 vs day 3; ***P < 0.001 day 3 vs day 4; For clamp conditions, overall ANOVA P < 0.0001. Post-hoc comparisons: †P < 0.0001 day 1 vs day 2, ††P < 0.01 day 2 vs day 3, †††P < 0.05 day 3 vs day 4.

From baseline values of (FFA_BASELINE: 0.49±0.05 vs: 0.40±0.04, 0.55±0.06 and 0.48±0.03 mmol/l; ANOVA P = 0.11), plasma FFAs were clamped at four different levels referred to as low (∼50 micromol/l), normal (∼500 micromol/l), high (∼900 micromol/l) and supra (∼2000 micromol/l; Table 2). As expected, FFA levels were suppressed in all groups by hyperinsulinaemia.

Plasma glucose

Inhibition of GH and endogenous lipolysis by somatostatin and acipimox led to a fall in blood glucose reaching a nadir of ∼4 mmol/l after ∼30 minutes. Administration of exogenous glucose restored blood glucose levels to baseline values after which a gradual rise towards ∼6.5 mmol/l was observed.

Overall glucose levels did not differ significantly between the four study days: (AUCGlucose−480: 2646±107 vs: 2633±126, 2564±84, 2925±125 mmol/min per l; ANOVA P = 0.07).

Hormones (GH, insulin, C-peptide and cortisol)

Somatostatin suppression yielded comparable levels of insulin, GH, C-peptide and cortisol both at the end of the basal period and during the hyperinsulinaemic clamp (Table 3).

Ghrelin

Baseline (t = 0) ghrelin concentration did not differ significantly between the four study days (ghrelin: 0.76±0.04 vs: 0.76±0.07, 0.84±0.08, 0.82±0.05 μg/l; P = 0.56). As expected, after 6 h of somatostatin exposure, ghrelin concentration was markedly reduced on all study days at the end of the basal period. The extent to which ghrelin was suppressed, however, differed significantly showing an inverse stepwise relationship with the level of FFAs (Table 3).
The effect of hyperinsulinaemia on ghrelin levels was assessed by a hyperinsulinaemic-euglycaemic clamp and, quite surprisingly, ghrelin concentration was not suppressed but rather non-significantly elevated by hyperinsulinaemia on all study days. The suppressive effect of different levels of FFAs on ghrelin concentration, however, prevailed (Table 3).

The combined effect of somatostatin and FFAs was assessed by the change in ghrelin concentration from baseline (t = 0 min) to the end of the designated periods of blood sampling ('lipid' and 'lipid + insulin', respectively). Since the amount of somatostatin infused was identical on all study days, we were able to factor out the independent effect of exogenous FFAs. Figure 2 shows the combined effect of somatostatin and increasing FFA concentrations on circulating ghrelin. Whereas somatostatin infusion alone reduced ghrelin concentration 67%, coadministration of increasing amounts of intralipid reduced circulating levels by a further 14, 19 and 19% respectively. (Change in ghrelin: 0.52 ± 0.05 vs: 0.62 ± 0.06, 0.72 ± 0.09 and 0.71 ± 0.05 mg/l; ANOVA P = 0.04). Post hoc comparisons of individual study days revealed that FFAs had to be increased from near zero to what is commonly regarded as the upper limit of the physiological range (~900 micromol/l) for the independent effect of FFAs to become evident.

### Leptin

Baseline (t = 0) leptin concentration did not differ significantly between the four study days (leptin: 3.46 ± 0.91 vs: 3.16 ± 0.77, 2.27 ± 0.51 and

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**Table 3** Circulating hormones under basal and clamp conditions. Values stated are mean ± S.E.M for parametric data, or median (25 and 75 percent quartiles) for non-parametric data. ANOVA (P-value) was used to test differences between study days. About there was a trend towards decreasing leptin concentration when FFAs were increased from 0 to 900 micromol/l. Serum ghrelin concentration was significantly suppressed from baseline levels on all study days, but more so with increasing FFA concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Day 1 (low)</th>
<th>Day 2 (normal)</th>
<th>Day 3 (high)</th>
<th>Day 4 (supra)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>26 ± 2</td>
<td>190 ± 11</td>
<td>37 ± 14</td>
<td>39 ± 14</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.43 ± 0.07</td>
<td>0.42 ± 0.08</td>
<td>0.42 ± 0.04</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td>169 ± 54</td>
<td>158 ± 61</td>
<td>165 ± 58</td>
<td>169 ± 58</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>2.24 ± 0.02</td>
<td>2.25 ± 0.03</td>
<td>2.27 ± 0.02</td>
<td>2.24 ± 0.02</td>
</tr>
<tr>
<td>Ghrelin (ng/ml)</td>
<td>0.24 ± 0.02*</td>
<td>0.25 ± 0.03†</td>
<td>0.27 ± 0.02†</td>
<td>0.24 ± 0.02†</td>
</tr>
</tbody>
</table>

*P < 0.05 vs day 1 (lipid), †P < 0.05 vs day 1 (lipid + insulin).
3.03±0.76 ng/ml; ANOVA P = 0.06). No significant change in leptin concentration was induced by either intralipid infusion alone (change in leptin concentration: 0.06±0.16 vs: 0.32±0.26, 0.09±0.19 and 0.1±0.13 ng/ml; ANOVA P = 0.34) or by intralipid and insulin combined (change in leptin concentration: 0.10±0.10 vs: 0.00±0.05, 0.00±0.06 and 0.01±0.13 ng/ml; ANOVA P = 0.71). There was no correlation between changes in ghrelin concentration and changes in leptin concentration. Likewise, no correlation was observed between ghrelin concentration and leptin concentration at the predetermined study periods (baseline, lipid infusion alone or lipid + insulin.)

**Discussion**

The purpose of the present study was to test the dose–response effects of FFA on serum ghrelin concentrations under conditions with stable insulin and GH levels. Our findings clearly show that FFAs decrease ghrelin dose-dependently, when FFA levels are below 1 mmol/l.

Insulin (7, 21) as well as GH (4, 19) have previously been shown to moderately suppress serum ghrelin levels even though the underlying mechanisms remain unclear. Since GH stimulates lipolysis (8), it seems plausible that elevated FFA levels could mediate the GH-induced inhibition of ghrelin. Contradicting this, however, hyperinsulinaemia lowers both the level of circulating FFAs and ghrelin, and the role of FFAs in the regulation of ghrelin secretion is thus not well established. To our knowledge, only one study has so far been designed to specifically investigate the independent effects of FFAs on circulating ghrelin (15) with the authors reporting no effect on circulating ghrelin after raising FFAs 2- to 3-fold to above 2 mmol/l. However, that particular study was characterised by a low number of subjects investigated (5). Moreover, the study was not designed in a manner allowing maintenance of stable and comparable levels of other hormones, notably GH and insulin. By contrast, the present study allowed an investigation of the effect of FFAs from very low levels and throughout the physiological range under appropriate control of both insulin and GH. Interestingly, the suppressive effect of FFAs on ghrelin observed in our study was only evident in the range from ~0 to 1.00 mmol/l and the results previously reported may therefore not conflict with ours: it is conceivable that elevation of FFA above 1 mmol/l do not further inhibit ghrelin. Still it should also be borne in mind that the well-known suppressive effect of somatostatin (18) on ghrelin concentration in our study resulted in ghrelin levels near the detection threshold, and we may therefore have been unable to detect any further reduction of ghrelin in the high physiological range of FFAs (>1.00 mmol/l). Furthermore it cannot be excluded that FFA under the present experimental conditions merely modifies the effect of somatostatin on ghrelin.

Experimental hyperinsulinaemia has been shown to decrease ghrelin concentration (1, 7, 21). The notion of insulin as a significant regulator of ghrelin secretion is furthermore supported by a number of cross-sectional studies describing an inverse relationship between obesity(9, 10, 22, 27) which is accompanied by high baseline levels of insulin, and ghrelin. However, to our knowledge, no insulin receptors have been identified on ghrelin secreting X/A-like cells in the gut. The present study differs from other studies in terms of the use of somatostatin and acipimox to control hormone levels and lipolysis and our inability to reproduce previous observations of insulin induced suppression of ghrelin levels during the clamp therefore in all likelihood relates to different experimental settings, in particular the precondition that ghrelin levels were very low prior to insulin exposure.

Interestingly, a number of conditions associated with high levels of FFAs are also characterized by reduced levels of ghrelin. Thus, lipodystrophy (14), hyperthyroidism (20) and obesity (27) are associated with markedly raised FFAs and low levels of circulating ghrelin. However, all three conditions are also associated with profound changes in a wide range of hormones, cytokines and metabolites and it is therefore impossible to ascribe the correlation solely to the action of FFAs.

We have recently reported that co-administration of GH and acipimox significantly reduces ghrelin levels in GH-deficient adults (29). In that study, we also observed an increase in leptin levels and it is possible that leptin counteracts the orexigenic effects of ghrelin (28). Lending further support to this notion, results from animal studies (rats) have indicated that as little as 3 h of intralipid infusion induces a significant increase in serum leptin levels as well as in leptin mRNA (6). However, FFAs induce insulin resistance and as expected, insulin levels were increased in rats infused with intralipid when compared with control animals. The observed effect of FFAs on leptin levels may therefore be indirect via insulin (which stimulates leptin production and secretion indirectly via its effects to increase glucose utilization and oxidative glucose metabolism in adipocytes; 11, 16). That we did not observe any effect of increasing FFA concentration on leptin concentration may therefore relate to our study design, where insulin levels were clamped at comparable concentrations on all study days by the somatostatin infusion.

It has also been reported that administration of a FFA synthase inhibitor suppresses ghrelin secretion from the stomach in mice (12). Inhibition of the hormone-sensitive lipase as well as FFA synthesis are usually encountered in the fed state, and high exogenous FFA levels will also feedback, inhibiting endogenous FFA synthesis and release. It could therefore be speculated that the secretion of ghrelin, which is an orexigenic peptide, during these conditions is
suppressed by a common signal indicative of energy surplus. Whether this signal is malonyl-CoA, the substrate of fatty acid synthase, as suggested by Hu et al. (12), remains to be investigated.

In conclusion, our data show that: 1) FFAs in the physiological range have an independent suppressive effect on ghrelin in humans, 2) this effect is evident with FFA level between 0 and 1 mmol/l and may wane at higher concentrations and 3) hyperinsulinemia does not suppress ghrelin under conditions of somatostatin and acipimox administration.

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