Postprandial acylated ghrelin status following fat and protein manipulation of meals in healthy young women

Rima AL AWAR*, Omar OBEID*, Nahla HWALLA* and Sami AZAR†
*Department of Nutrition and Food Sciences, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon, and †Department of Endocrinology, American University of Beirut-Medical Center, Beirut, Lebanon

ABSTRACT

The aim of the present study was to investigate the postprandial effect of diet composition on circulating acylated ghrelin levels in healthy women. A randomized cross-over study of three experimental treatments was performed. A total of 11 healthy young women of normal body weight completed the study. All 11 subjects consumed three iso-energetic meals of different macronutrient composition, a balanced meal (50% carbohydrates, 30% fat and 20% protein), a high-fat meal (45% carbohydrates, 45% fat and 10% protein) and a high-protein meal (45% carbohydrates, 20% fat and 35% protein), for breakfast on separate days. The test meals were administered 1 month apart. Blood samples were withdrawn immediately before and at 15, 30, 60, 120 and 180 min after the test meal for measurement of plasma acylated ghrelin, as well as serum glucose, insulin and triacylglycerol (triglyceride) levels. Acylated ghrelin fell significantly after ingestion of both the balanced and high-protein meals. Ghrelin persisted at significantly lower levels than baseline for a longer duration following the high-protein meal (P < 0.05 at 15, 30, 60 and 120 min) compared with the balanced meal (P < 0.05 at 30 and 60 min). Moreover, acylated ghrelin levels correlated negatively with the postprandial insulin levels. In conclusion, postprandial changes in acylated plasma ghrelin depend on the macronutrient composition of the meal and are possibly influenced by insulin.

INTRODUCTION

Ghrelin was originally discovered in rat and human stomach extracts as the endogenous ligand for GHS-R (growth hormone secretagogue receptor). Rat and human ghrelin peptides consist of 28 amino acids, differing only by two amino acid substitutions [1]. Ghrelin is reported to be mainly present in two major molecular forms: acylated ghrelin, which contains an n-octanoyl modification at the third serine residue, and des-n-octanoyl ghrelin, which is the non-acylated form [2]. Human studies indicate that the contribution of octanoylated ghrelin to total ghrelin varies between 3 and 20% [3,4], suggesting that only a small amount circulates in the acylated form.

Ghrelin-producing endocrine cells are present in the gastrointestinal tract, being most abundant in the gastric oxyntic glands [5,6]. Circulating ghrelin is believed to have two major functions [7]: the potent stimulation of growth hormone release [1,8,9] and the involvement in the regulation of energy metabolism [10,11]. In rodents, ghrelin administration has been shown to stimulate food intake and weight gain, as well as adiposity, through increases in the respiratory quotient [11,12]. More recently, the addition of ghrelin has been shown to stimulate the differentiation of cultured rat preadipocytes, suggesting a direct adipogenic action on adipose tissue [13]. In humans, peripheral ghrelin administration at a concentration approx. 2–3-fold higher

Key words: acylated ghrelin, insulin, glucose, triacylglycerol (triglyceride), macronutrient, meal composition, protein content.
Abbreviations: BMI, body mass index; NPY, neuropeptide Y; REE, resting energy expenditure.
Correspondence: Dr Omar Obeid (email omar.obeid@aub.edu.lb).
than baseline also stimulates appetite and food intake [14]. The primary site of action of ghrelin was reported to be the arcuate nucleus [12]. It stimulates feeding through the orexin system and NPY (neuropeptide Y)/AGRP (agouti-related protein) systems, independently of each other [10,15–17]. It appears to antagonize leptin action in the regulation of the NPY system [10,15].

Acute and chronic states of energy balance appear to regulate ghrelin secretion. It is up-regulated under conditions of negative energy balance, such as fasting, weight loss and anorexia nervosa, and down-regulated under conditions of positive energy balance, such as obesity and food intake [18,19]. Moreover, plasma total ghrelin in humans increases before each meal and falls to nadir levels within 1 h after eating [20]. It is not clear whether nutrients inhibit ghrelin secretion by luminal routes [21], systemic routes [19] or via the release of enteric hormones (i.e., insulin and somatostatin) [22–29]. Studies in humans testing whether the macronutrient content of meals influences the postprandial ghrelin response are scarce. The responses of both acylated and total ghrelin to iso-energetic high-fat and high-carbohydrate meals have been investigated in women [30,31], and there is a general consensus that the substitution of dietary carbohydrate for fat causes lower postprandial ghrelin levels [30–32]. However, under extremely high-fat testing conditions (75–88 % of energy from fat), there is a discrepancy in the results as to whether a high-fat meal induces postprandial ghrelin suppression [30,31,33–36]. Therefore, the aim of the present study was to investigate the postprandial effect of three iso-energetic meals (high-protein, high-fat and balanced meals) on acylated ghrelin levels under normal physiological conditions. The meals were modestly modified in terms of macronutrient composition in order to be representative of a somewhat typical breakfast consumed by adult females. It is anticipated that the results will clarify which of the macronutrients are the metabolic candidates for the modulation of circulating ghrelin concentrations. The results may elucidate further whether particular changes in the macronutrient composition of the diet are necessary for the optimal management of ghrelin levels and thus appetite control and weight management. The impact of the different inhibitory effect of macronutrients on ghrelin levels on appetite has not been evaluated in humans.

**METHODS**

**Subjects**

Healthy young women (n = 11) of normal body weight were recruited for the study. Exclusion criteria were as follows: BMI (body mass index) > 25 kg/m² or < 18 kg/m², chronic medical or psychiatric illness, pregnancy, irregular menstrual cycle, use of oral contraceptives, use of tobacco products, alcohol consumption of more than one drink per day, regular intense exercise (> 1 h of aerobics three times/week), and a recent history of weight fluctuation (≥ 5 % of body weight within the last 3 months). None of the subjects had undergone gastrointestinal surgery.

**Experimental protocol**

The experimental protocol was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the Institutional Review Board at the American University of Beirut. All subjects gave their written consent after being fully informed about the nature and procedures of the study.

Prior to starting the experiment, the height and weight of subjects were measured as part of the inclusion criteria. REE (resting energy expenditure) of each subject was measured, after an overnight fast, using indirect calorimetry (Vmax Spectra 29 series; Sensor Medics Corporation), and their physical activity pattern was assessed by a questionnaire in order to estimate their total energy needs by multiplying REE by the appropriate physical activity level value. The lean body mass and percentage body fat for each subject were determined using dual energy X-ray absorptiometry (Delphi QDR series; Hologic).

The experiment used a within-subject repeated-measure design in which each subject served as her own control. All 11 subjects were tested three times, 1 month apart, in the follicular phase of three consecutive menstrual cycles during day 1–10 from menses. On the first visit, they arrived at the Nutrition Department of the American University of Beirut at 08.00 hours after a 10 h overnight fast, and an intravenous catheter was inserted into an antecubital vein by a registered nurse. At 08.30 hours, breakfast was served and consumed within 10 min. The experimental diets were as follows: balanced meal (50 % carbohydrate, 30 % fat and 20 % protein), high-protein meal (45 % carbohydrate, 20 % fat and 35 % protein) or high-fat meal (45 % carbohydrate, 45 % fat and 10 % protein). The basis of the 5 % difference in carbohydrate content was to enhance palatability. The energy content of the breakfast was 30 % REE of the subjects, ranging from 1465–2093 kJ (350–500 kcal) with a mean of 1716 kJ (410 kcal). Water intake was specified in order to achieve an energy density of 2.64–2.68 kJ/g (0.63–0.64 kcal/g) across all meals. The same food type was used in all meals; the amount of food consumed during the balanced, high-protein and high-fat meals at the 1674 kJ (400 kcal) level is shown in Table 1. The subjects had to consume their assigned breakfast within 10 min after meal onset and did not consume any food or beverage for 3 h post-meal ingestion. On the second and third visits, subjects underwent the same experimental procedures described above, but received an iso-energetic...
breakfast of different macronutrient composition. The order of the meals was randomized. For adaptation, the subjects were instructed to follow a 3-day pre-experimental weight-maintaining diet consisting of 50% carbohydrate, 30% fat and 20% protein, before each visit.

### Collection and preparation of blood samples

Blood samples were withdrawn immediately before (time 0) and at 15, 30, 60, 120 and 180 min after breakfast, and saline was infused following the withdrawal to keep the catheter patent. Blood was collected into tubes with EDTA as the anticoagulating substance for plasma preparation, as well as serum separator tubes with clot activator. After cloting, the samples were centrifuged at 2500 g for 15 min at 5°C to separate plasma and serum. The plasma was then acidified with 50 µl of 1 M HCl and 10 µl of PMSF (10 mg/ml of methanol) per 1 ml of plasma. Plasma samples were processed as quickly as possible and always kept on ice to prevent the breakdown of acylated ghrelin. All specimens were stored at −80°C until analysis.

### Biochemical analyses

Plasma ghrelin was measured in duplicate using a commercially available RIA (Linco Research) which determines the octanoylated (i.e. acylated) ghrelin levels. The limit of sensitivity was 10 pg/ml (100 µl sample size); intra- and inter-assay coefficients of variation were <6.0 and 9.0 respectively. Serum insulin was measured in duplicate using a commercially available RIA (MP Biomedicals). The lower detection limit was 4.2 µ-unit/ml; intra- and inter-assay coefficients of variation were <6.0 and 7.9 respectively.

For both ghrelin and insulin, all of a single subject’s samples were run in the same assay, and the order of the tubes in the assay was randomized. Both ghrelin and insulin measurements were performed in the Endocrinology Laboratory of the American University of Beirut-Medical Center. Serum glucose and triacylglycerol (triglyceride) levels were determined by commercial enzymatic colorimetric tests using glucose and triacylglycerol slide methods respectively, on a VITROS analyser (Ektachem DT60 II System; Johnson & Johnson Clinical Diagnostics).

### Statistical analyses

The data were analysed using SPSS for Windows, version 10.0.0. Data are means ± S.E.M, and a P value < 0.05 was considered statistically significant. Paired sample Student t tests were performed to determine changes in the variables over time after meal consumption. Differences in the responses of the variables to the three meals were analysed by two-way ANOVA with repeated measures, followed by post-hoc Tukey’s test. The Pearson’s product correlation test was used to analyse possible correlations among the variables.

### RESULTS

Table 2 shows the characteristics of the subjects at the time of enrolment. All subjects were healthy, non-obese and had similar body composition in terms of fat and lean body mass. The three meals were well-tolerated by all 11 subjects; there were no complaints regarding the palatability or size of the meals. None of the subjects experienced any particular discomfort during the withdrawal of blood. All 11 subjects were tested post-ingestion of the three meals.

#### Acylated plasma ghrelin

Fasting acylated ghrelin levels varied at the three times of testing within subjects (the mean coefficient of variation within subjects was 39.13%), although the subjects were only tested in the early and middle follicular phases of three consecutive menstrual cycles in order to control for possible hormonal interferences. Moreover, the subjects were advised to follow a 3-day pre-experimental diet in order to control their macronutrient and energy intake, as well as their eating pattern (three meals/day).

The mean fasting acylated ghrelin concentration across the three meals was not statistically different (Figure 1, upper panel). Mean plasma acylated ghrelin levels fell significantly at 30 and 60 min after the balanced meal, and
increased significantly above the fasting level at 180 min. The postprandial acylated ghrelin response following the high-fat meal was not statistically significant; this may have been the result of the low basal ghrelin levels observed with this intervention. However, a rapid and significant fall in the mean plasma ghrelin level was observed at 15 and 30 min after the high-protein meal, and the level remained significantly lower than baseline up until 120 min (Figure 1, upper panel).

Pooling the data from all three meals by two-way ANOVA with repeated measures revealed a significant main effect for time ($P = 0.000$), but not a main meal effect or interaction between meal and time.

**Serum insulin**

The mean fasting insulin concentration across the three meals was not statistically different (Figure 1, lower panel). In all three interventions, serum insulin concentration at 15, 30, 60 and 120 min was significantly elevated compared with baseline, and the magnitude of increase appeared to be lower in the high-protein group (Figure 1, lower panel). Two-way ANOVA with repeated measures revealed a significant main effect for time ($P = 0.000$), as well as interaction between meal and time ($P = 0.024$), but not a main meal effect.

**Serum glucose**

The mean fasting glucose concentration across the three meals was similar (Figure 2, upper panel). Serum glucose levels following the balanced meal were minimally affected throughout the intervention, and only the level at 60 min was significantly lower than that at baseline (time 0). In the high-fat meal, serum glucose levels increased at 15 and 30 min and then decreased below that at baseline. In the high-protein meal, serum glucose levels from 30 min onwards were lower than that at baseline. Two-way ANOVA with repeated measures across the three meals yielded a significant main effect for
time ($P = 0.000$), as well as interaction between meal and time ($P = 0.000$), but not a main meal effect. These data indicate that both circulating glucose and insulin change significantly across sampling times and that, in the three test conditions, both variables display significant differences over the samplings.

**Serum triacylglycerol**

The mean fasting triacylglycerol concentration across the three meals was not statistically different (Figure 2, lower panel). In all three interventions, serum triacylglycerol increased over time, but the magnitude of the increase appeared to differ between meals. In the balanced and high-protein meals, serum triacylglycerol levels at 60 min onwards were higher than that at baseline, whereas in the high-fat group the serum triacylglycerol level was significantly higher only at 120 min.

Two-way ANOVA with repeated measures across all three meals revealed a significant main effect for time ($P = 0.000$), but not a main meal effect or interaction between meal and time.

**Correlations among variables**

Fasting acylated ghrelin levels (mean of three interventions) did not correlate with any of the characteristics of the subjects, including BMI ($r = 0.111$, $P > 0.05$), lean body mass ($r = 0.221$, $P > 0.05$), percentage body fat ($r = 0.0.0.031$, $P > 0.05$) and REE ($r = 0.0.0.43$, $P > 0.05$). In addition, postprandial acylated ghrelin levels did not correlate with any of the characteristics of the subjects. Correlation analysis revealed a significant negative correlation between insulin and acylated ghrelin levels for the pooled data (all meals; Figure 3) and each of the three test meals, being most clear-cut in the following ascending order: high-fat ($r = -0.398$, $P < 0.05$), balanced ($r = -0.352$, $P < 0.01$) and high-protein ($r = -0.462$, $P < 0.001$) meals. Postprandial glucose and triacylglycerol concentrations did not show any significant correlations with ghrelin across all three meals.

**DISCUSSION**

The present study was designed to investigate whether meals of different macronutrient composition but with similar food types, energy content and weight have distinct effects on the postprandial secretion of acylated ghrelin in healthy young women of normal weight. The meals substantially differed in their amounts of protein and fat, and were not exaggerated in terms of energy, protein or fat content in order to be representative of a typical breakfast consumed by healthy young women. The major findings were: (i) a large variation in fasting acylated ghrelin levels existed both among subjects, as well as within subjects; and (ii) acylated ghrelin was significantly lower than the baseline level for a longer duration after a high-protein meal in comparison with a balanced meal.

In the present study, the subjects were healthy females matched for age, BMI and percentage body fat. The mean fasting acylated ghrelin level obtained was 138.83 pg/ml and the S.E.M. expressed as a percentage of the mean ghrelin concentration was 8.7 %, which is relatively low in comparison with other studies. Expressing the S.E.M. as a percentage of the mean total ghrelin concentration reveals a rather large percentage within studies that matched their subjects for age, BMI and gender. The range varies from 5.8–32.9 % for the studies that controlled for age and BMI [11,16,18,19,21,22,25], and 12.3–22.2 % for those that additionally recruited only females [30,37,38]. Thus the differences in subject characteristics, such as age, BMI, percentage body fat and gender, may not be the main factor responsible for the variation and it may be due to other characteristics that have not been accounted for. In addition, comparing mean total fasting plasma ghrelin concentrations across the aforementioned publications reveals a discrepancy that is approx. 3-fold; total fasting ghrelin levels range from 286.5–837.7 pg/ml. This is unlikely to be related to the use of different RIA kits to measure total ghrelin, because most of the above studies used the same commercially available RIA kit from Phoenix Pharmaceuticals, with the exception of a couple that were of the researchers’ own formulations [1,37]. Intra-individual differences in basal acylated ghrelin were found to be similar to those reported for total ghrelin and this remains unexplained as yet [31,35].

The findings from the present study show that acylated ghrelin falls considerably after ingestion of a balanced meal, similar to previous studies which also confirmed postprandial total ghrelin suppression in response to a balanced meal [20,38–40]. A recent study has compared plasma concentrations of octanoylated and total ghrelin in the fasting and absorptive states and confirmed that a balanced meal significantly suppressed plasma concentrations of both forms of ghrelin to the same extent [41]. Thus both acylated and total ghrelin tend to respond in a similar manner to the consumption
of a balanced meal. The magnitude and duration of ghrelin suppression, however, differed across the studies and this may not be related to meal volume and energy value [39].

In the high-fat intervention, the low baseline levels of acylated ghrelin (in comparison with the balanced and high-protein interventions) may have masked the effect of the high-fat meal. The postprandial acylated ghrelin levels of the high-fat and high-protein meals were found to be similar and this may indicate that they have a similar effect on acylated ghrelin status. The relationship between fat ingestion and ghrelin was reported to vary between studies. Tentolouris et al. [30] reported that a breakfast with an exaggerated fat content [2227 kJ (532 kcal); 88% fat] failed to lower acylated ghrelin in lean female subjects, whereas others [31,33,34,36] observed a significant decrease in postprandial total ghrelin levels after a high-fat diet (75–100% fat). Postprandial total ghrelin levels were reported to be suppressed following high saturated [33,36] and unsaturated [34] fat meals, whereas that of acylated ghrelin was not affected by an unsaturated fat meal [30]. Therefore the relationship between ghrelin and fat intake remains unresolved, and it is not clear whether total and acylated ghrelin respond differently to high-fat meals.

To our knowledge, the present study is the first to investigate the acylated ghrelin response to a high-protein meal. Previous studies [33,35,36,42] have reported a significant rise or no change in total ghrelin following a high-protein meal (83–100% protein). The exaggerated high-protein content may be responsible for the stimulation of ghrelin observed by others or, as hypothesized for a high-fat meal, it may be that acylated ghrelin responds differently from total ghrelin after a high-protein meal. The decrease in acylated ghrelin after a high-protein meal had a different time pattern compared with a balanced meal, persisting at significantly lower levels than baseline for a longer duration following the high-protein meal (from 15–120 min compared with 30–60 min for the high-protein and balanced meals respectively). Moreover, acylated ghrelin increased significantly above the baseline level at 180 min after the balanced meal. This finding demonstrated that the substitution of dietary protein for fat results in a longer-lasting acylated ghrelin suppression. Owing to the orexigenic potency of ghrelin, our findings, combined with those of others [30,31,43], appear to be consistent with the principle of a higher satiating efficiency per kJ of energy ingested for dietary protein and carbohydrate compared with fat [44].

A significant negative correlation between the insulin and ghrelin levels was found across all three meals, suggesting a suppressant action of insulin on acylated ghrelin. The inverse regulatory relationship between insulin and ghrelin is consistent with previous findings, where an inhibitory effect of a physiological rise in plasma insulin on ghrelin was demonstrated [22,23,25], whereas two other studies failed to observe such a relationship [21,26].

The suppressant action of insulin on ghrelin secretion has been demonstrated to be mediated independently of its effect on glucose [24,25], as well as free fatty acid concentrations [23]. Among our findings, it must be noted, however, that the strength of the correlation between insulin and acylated ghrelin varied across the meals, being relatively weak following the ingestion of the high-fat meal (r = −0.298, P > 0.05), where high insulin levels were obtained but there was no significant decrease in acylated ghrelin. This may indicate that other mechanisms must be important for the modification of acylated ghrelin release. Interestingly, a recent study [45] in humans demonstrated that ghrelin levels can be suppressed by sham feeding as they are by real feeding, supporting a role for vagally mediated cephalic stimulation in the inhibition of ghrelin release.

In conclusion, postprandial changes in acylated ghrelin levels depend on the macronutrient composition of the diet. Under iso-energetic testing conditions, increasing the protein content appears to prolong the suppression of acylated ghrelin. However, the mechanisms responsible for acylated ghrelin regulation are still largely unknown.

ACKNOWLEDGMENTS

We thank Dr Riad Baalbaki for his assistance with the statistical analysis, Ms Carmen Hajj Shahin from the American University of Beirut-Medical Center Endocrinology Department for her laboratory assistance, and Miss Nancy El Helou, our phlebotomist. The study was supported by a grant from the University Research Board at the American University of Beirut.

REFERENCES


5 Date, Y., Kojima, M., Hosoda, H. et al. (2000) Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141, 4255–4261

