CLINICAL STUDY

Circulating obestatin levels and the ghrelin/obestatin ratio in obese women

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Abstract

Objective: We measured blood levels of obestatin, total ghrelin, and the ghrelin/obestatin ratio and their relationship with anthropometric and metabolic parameters, adiponectin and insulin resistance, in overweight/obese and normal-weight women.

Design: Outpatients Unit of Endocrinology of the S Orsola-Malpighi Hospital of Bologna, Italy.

Methods: Fasting obestatin, ghrelin, adiponectin and lipid levels, fasting and glucose-stimulated oral glucose tolerance test insulin, and glucose levels were measured in 20 overweight/obese and 12 controls. The fasting ghrelin/obestatin ratio was calculated; the homeostasis model assessment-IR (HOMA-IR) and insulin sensitivity index ($ISI_{\text{composite}}$) were calculated as indices of insulin resistance.

Results: Obese women had higher obestatin and lower ghrelin blood levels, and a lower ghrelin/obestatin ratio compared with controls. In all subjects, obestatin was significantly and positively correlated with total cholesterol and triglycerides, but not with ghrelin, anthropometric, and metabolic parameters. In the obese women, however, obestatin and ghrelin concentrations were positively correlated. By contrast, the ghrelin/obestatin ratio was significantly and negatively correlated with body mass index, waist, waist-to-hip ratio, fasting insulin, and HOMA-IR, and positively with $ISI_{\text{composite}}$ but not with adiponectin. None of these parameters were correlated with the ghrelin/obestatin ratio in the obese.

Conclusions: Increased obestatin, decreased ghrelin levels, and a decreased ghrelin/obestatin ratio characterize obesity in women. This supports the hypothesis that the imbalance of ghrelin and obestatin may have a role in the pathophysiology of obesity. On the other hand, some relevant differences between our data on circulating levels of obestatin and the ghrelin/obestatin ratio in obese subjects and those reported in the few studies published so far imply that further research is needed.

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Introduction

Obestatin is a recently discovered secreted peptide encoded by the preproghrelin gene (1). The biological activity of obestatin depends on the amidation at its carboxyl terminus. Originally, obestatin was proposed as a ligand of GPR39, an orphan receptor belonging to the ghrelin receptor family (1), but recent studies were not able to confirm this finding (2–5). Obestatin was also thought to display effects opposite to those of ghrelin (1, 6). However, recent studies did not support a role for obestatin either in the regulation of food intake or on body weight, energy expenditure control or growth hormone secretion (7–9).

The role of obestatin in the regulation of metabolism is still under debate. Recent data from Chanoine *et al.* (10) demonstrated the presence of obestatin in perinatal rat pancreas and its levels have been found to positively correlate with insulin concentrations in the postnatal pancreas. Even less clear is the physiological significance of

circulating obestatin levels as indicated in the few studies published so far. In children with Prader-Willi syndrome (PWS) (11), hematic obestatin levels were similar when obese PWS subjects were compared with normal obese subjects, and no correlation was found between circulating obestatin and insulin levels. Basal obestatin levels were positively correlated with body mass index (BMI) and glycemia in the PWS group. Obestatin levels have also been measured in a group of morbidly obese subjects when compared with a group of lean patients. Obestatin levels have been demonstrated to be lower in obese subjects when compared with lean subjects, showing a significant increase in the obese patients 6 months after gastric banding surgery (12). Accordingly, in a more recent study, Guo *et al.* (13) found that ghrelin and obestatin levels were lower but their ratio was higher in a mixed group of obese males and females, compared with their normal-weight counterparts, which raises some interesting points on the pathophysiological relevance of balance of these two peptides in obesity and related metabolic disorders.

Additional recent findings by Zizzani *et al.* (9) have demonstrated that, whereas fasting resulted in elevated ghrelin, obestatin levels were reduced, suggesting opposite effects on energy metabolism.

To shed light on the putative role of obestatin in obesity, we measured circulating blood levels of obestatin in a group of obese subjects when compared with lean ones. In order to study whether obesity may be characterized by an imbalance of the obestatin and ghrelin system, we also investigated the total ghrelin/obestatin ratio, and its relationship with anthropometry, the glucose–insulin system, lipid parameters, and adiponectin in the same groups of overweight/obese women and appropriate normal-weight controls.

Subjects and methods

Twenty overweight and obese women ($\text{BMI} > 28 \text{ kg/m}^2$, range 28.7–42.6) and 12 normal-weight controls ($\text{BMI} \leq 25 \text{ kg/m}^2$, range 19.03–24.9) were enrolled in the study. All women attended the Endocrinology Unit of S. Orsola-Malpighi Hospital for the treatment of obesity and related co-morbidities. None of them had diabetes, thyroid disease, endogenous hypercortisolism, hyperandrogenism, and/or other systemic disorders, including hypertension, renal, cardiac, or liver diseases, based on clinical examination and routine blood tests. Premenopausal women had regular menses. Three obese women were postmenopausal, according to the presence of amenorrhea for the previous 12 months, high gonadotropin levels, and an estradiol concentration $< 20 \text{ ng/ml}$. None of the subjects enrolled in the study had taken any drugs during the 3 months before the study, nor were dieting. Glucose metabolism was assessed to exclude type 2 diabetes by performing an oral glucose tolerance test (OGTT), as described below. The protocol was approved by the local ethics committee and all women gave their informed consent.

Anthropometry

Height, weight, and waist and hip circumference were measured according to previously standardized procedures (14), and the waist-to-hip ratio (WHR) was calculated.

Protocol study

Blood samples for insulin and metabolic parameters were drawn between 0800 and 0830 h after a 12-h overnight fast, after which an OGTT (75 g Curvosio, Sclavo, Cinisello Balsamo, Italy) was performed. Samples for glucose during the OGTT were obtained at baseline and 30, 60, 90, and 120 min thereafter, whereas those for insulin were obtained at baseline and 60 and 120 min after glucose load. Blood samples for ghrelin, obestatin, and adiponectin determination

were drawn into chilled tubes containing EDTA 2Na (1 mg/ml) and aprotinin (500 U/ml), immediately chilled on ice, and then centrifuged at 3000 *g* for 10 min at 4 °C. Plasma aliquots were subsequently frozen at -80°C until assayed.

Assays

Plasma glucose levels were determined by the glucose oxidase method after blood samples had been taken. Insulin, total cholesterol, HDL cholesterol, and triglyceride measurements were performed on blood samples as previously described (15). To investigate insulin sensitivity in basal conditions, the homeostasis model assessment (HOMA-IR) was calculated (16); in addition, from the results of the OGTT, the composite insulin sensitivity index ($\text{ISI}_{\text{composite}}$) was determined according to Matsuda *et al.* (17). Total ghrelin and adiponectin were measured in duplicate as previously described (14, 18). Obestatin levels were also measured in duplicate using a commercially available RIA kit (Phoenix Pharmaceuticals Inc., Mountain View, CA, USA) that uses ^{125}I -labeled bioactive obestatin as a tracer and a rabbit polyclonal antibody, after solid-phase extraction. Before extraction, 1 ml plasma was diluted with an equal volume of 1% TFA and centrifuged at 4 °C, 3000 *g* for 15 min. Acidified plasma was then loaded onto a C_{18} Sep-Pak-Vac, 200 mg/3 cc cartridge (Waters Corp., Milford, MA, USA) pre-equilibrated with 1 ml $\text{CH}_3\text{CN/water}$ (60:40) in 1% TFA. After three washes with 3 ml 1% TFA, absorbed peptide was eluted with 2.5 ml $\text{CH}_3\text{CN/water}$ (60:40) in 1% TFA. The eluted peptide was evaporated under nitrogen and dissolved in 250 μl RIA buffer. The intra-assay coefficient of variation (CV) was 2.3%. The intra-assay CV was 3% for insulin, 5.2% for adiponectin, 5.3% for ghrelin, and 2.3% for obestatin.

Statistical analysis

Glucose and insulin response to the OGTT was expressed as the incremental area under the curve (AUC incremental), which was calculated by the trapezoidal method after subtracting basal values. Statistical analyses were performed by running the SPSS/PC+ (SPSS Inc., Chicago, IL, USA) (19). ANOVA was applied to compare the two groups, as well as to evaluate the validity and strength of our sample size by computing the power of our main outcome measure (i.e. the ghrelin/obestatin ratio). Linear regression analysis was used to evaluate correlations between continuous variables. All data are expressed as mean $\pm$ s.d. Two-tailed $P < 0.05$ were considered as statistically significant.

Results

The general characteristics of the overweight/obese women and those of the controls are reported in

Table 1 General characteristics, biochemistry, indices of insulin sensitivity, and adiponectin (mean $\pm$ s.d.) of obese women and normal-weight controls.

Parameter	Controls (n=12)	Obese (n=20)	P (ANOVA)
Age (years)	32.5 $\pm$ 5.71	37.3 $\pm$ 10.8	0.062
Body weight (kg)	60.4 $\pm$ 6.65	92.6 $\pm$ 12.8	0.001
Body mass index (kg/m ²)	23.03 $\pm$ 2.37	35.3 $\pm$ 4.19	0.001
Waist (cm)	75.4 $\pm$ 8.71	100 $\pm$ 7.77	0.001
Waist-to-hip ratio	0.75 $\pm$ 0.06	0.83 $\pm$ 0.05	0.004
Glucose (mg/dl)	86.7 $\pm$ 9.04	92.3 $\pm$ 9.58	0.004
Insulin (μ U/ml)	7.82 $\pm$ 7.90	11.2 $\pm$ 7.90	0.052
Glucose AUC (mg/ml per min)	2875 $\pm$ 2608	4572 $\pm$ 3164	0.158
Insulin AUC (μ U/ml per min)	4755 $\pm$ 5172	7491 $\pm$ 10 029	0.370
HOMA-IR	1.15 $\pm$ 0.39	2.81 $\pm$ 1.74	0.007
ISI _{composite}	10.10 $\pm$ 3.44	5.78 $\pm$ 3.23	0.002
Total cholesterol (mg/dl)	185 $\pm$ 15	202 $\pm$ 27	0.058
HDL Cholesterol (mg/dl)	72.0 $\pm$ 15.0	58.0 $\pm$ 13.4	0.012
Triglycerides (mg/dl)	71.4 $\pm$ 28.2	109.2 $\pm$ 55.5	0.047
Adiponectin (pg/ml)	25.3 $\pm$ 9.8	15.8 $\pm$ 13.8	0.046

Table 1. Age values were not significantly different between the groups, whereas BMI ($P < 0.001$), waist circumference ($P < 0.001$), and WHR ($P < 0.004$) values were, as expected, significantly higher in the overweight/obese than in the control group. The obese group had a significantly higher fasting glucose ($P = 0.004$), fasting insulin ($P = 0.052$) and HOMA-IR index ($P = 0.007$), lower ISI_{composite} ($P = 0.002$), HDL cholesterol ($P = 0.012$) and adiponectin ($P = 0.046$), and higher triglyceride ($P = 0.047$) concentrations (**Table 1**). The group of overweight/obese women was characterized by significantly higher circulating obestatin levels ($P = 0.045$) and a lower total ghrelin ($P = 0.033$); a significantly ($P = 0.002$) lower ghrelin/obestatin ratio was therefore found in overweight/obese women with respect to controls, with a power of 0.898 (**Fig. 1**).

Considering all subjects there was no significant correlation ($r = -0.022$, $P = 0.904$) between obestatin and ghrelin levels; a correlation between these two peptides was, however, detected in the obese women group ($r = 0.464$; $P = 0.039$). Moreover, obestatin levels were significantly and positively correlated with total cholesterol ($r = 0.472$; $P = 0.007$) and triglycerides ($r = 0.464$; $P = 0.009$), but not with anthropometric and other metabolic parameters. In obese and control subjects considered together, the ghrelin/obestatin ratio was significantly and negatively correlated with BMI ($r = -0.462$; $P = 0.008$), waist circumference ($r = -0.512$; $P = 0.003$), WHR ($r = -0.472$; $P = 0.006$), fasting insulin ($r = -0.355$; $P = 0.050$), and HOMA-IR ($r = -0.355$; $P = 0.050$), and positively with ISI_{composite} ($r = 0.460$; $P = 0.010$) but not with adiponectin levels ($r = 0.156$; $P = 0.395$) (**Fig. 2**). None of these parameters showed any significant correlation with the ghrelin/obestatin ratio in the obese group when considered alone.

Discussion

Obestatin is a recently discovered peptide whose function was thought to be close to that of ghrelin due to the common origin of both peptides. However, after the first report describing a putative opposite effect of obestatin when compared to ghrelin (1), several recent studies seem not to confirm this proposal (5–9). The data reported in our study demonstrate that, in the

Figure 1 Obestatin, ghrelin, and the ghrelin/obestatin ratio in obese and control subjects.

Figure 2 Correlation coefficients between the ghrelin/obestatin ratio and several anthropometric and metabolic parameters in obese ($\square$) and normal-weight controls ($\bullet$). (a) $r = -0.468$, $P = 0.007$; (b) $r = -0.509$, $P = 0.003$; (c) $r = -0.470$, $P = 0.007$; (d) $r = -0.364$, $P = 0.044$; (e) $r = -0.362$, $P = 0.045$; (f) $r = -0.460$, $P = 0.011$.

presence of obesity, women had higher circulating levels of obestatin when compared with normal subjects. These findings are not, however, concordant with the data reported in the few studies performed so far. In fact, Hainer *et al.* (12) and, more recently, Guo *et al.* (13) found that obese subjects had lower rather than higher circulating levels when compared with normal-weight subjects. Based on available data, it is difficult to explain these disparate findings. All studies, including our own, did in fact use the same assay system, although in Hainer's study (12) the extraction procedure of obestatin in plasma was probably not performed. One interesting point is that the values of obestatin in the normal-weight control group we reported here are very similar to those reported by Guo *et al.* (13), which clearly indicates that the differences between the studies are related to the obese groups only. Obviously, differences in the obese status could partially explain such disparate obestatin values. In fact, Hainer *et al.* (12) investigated massive obese individuals, whereas Guo *et al.* (13) performed their study in Chinese subjects who are characterized by ethnic-specific values for BMI and waist circumference (20, 21). Interestingly, previous studies (13) did not find any sex differences in obestatin values, which makes it unlikely that our data depend on the fact that we included only obese and normal-weight control women. Much larger studies are therefore needed to resolve these debatable findings.

In our study, circulating obestatin levels correlated with total ghrelin only in the obese population and not in the normal one, whereas no correlation was found with any other hormonal and metabolic parameters, with the exclusion of total cholesterol and triglycerides. As reported before, previous studies performed in experimental animals have shown that obestatin may act as a satiety peptide, by decreasing food intake and reducing body weight in the short term (1, 6), although these findings have not been demonstrated in various other animal studies performed so far (7–9, 22). At present, the precise role of obestatin in the regulation of metabolic processes is still unknown; nonetheless, recent studies (9) performed in fasted and fed mice found that, whereas fasting resulted in elevated ghrelin levels, obestatin levels were significantly reduced, suggesting that the secretion of the two peptides is regulated in an opposing manner by the nutritional status. Indirectly, these data therefore seem to support our findings. Interestingly, in the same study, the authors found that exogenous obestatin *per se* did not modify food intake in fasted and fed mice; it did however inhibit ghrelin's orexigenic effects that were evident in fed mice only. These findings suggest that obestatin could modulate endogenous ghrelin actions. Additionally, previous studies (1) have shown that obestatin may inhibit jejunal activity and may suppress gastric emptying activity, which implies the possibility that

obestatin anorectic properties are related to peripheral mechanisms, possibly involving afferent vagal signals.

In this study, we confirmed that circulating ghrelin concentrations are lowered in the obese state, as previously reported (23), but an additional finding is that the ghrelin/obestatin ratio was lower in obese subjects than in normal-weight controls, which contrasts with the higher values reported by Guo *et al.* (13) in Chinese obese individuals. Despite the fact that this discrepancy cannot be resolved without additional more detailed studies, these findings seem to support the concept that disparate post-translational cleavage of preproghrelin into these two sibling peptides may be regulated differently in the presence of obesity or, alternatively, that the common regulatory factors are responsible for these still poorly defined coordinate changes of the ghrelin and obestatin system according to the nutritional status. This is further emphasized by the finding of Guo *et al.* (13) that the ghrelin/obestatin ratio was decreased after a morning mixed meal in both normal-weight and obese individuals, with a disappearance following the meal of the difference in the ghrelin/obestatin ratio between the two groups, further emphasizing the importance of the nutritional status on ghrelin and obestatin.

Interestingly, we also found that the ghrelin/obestatin ratio was negatively correlated with both BMI and indices of abdominal fat distribution. In the last few years, extensive research on ghrelin has produced evidence that this peptide may have important positive effects on feeding, since exogenous ghrelin administration stimulates appetite and food intake in both rodents and humans (24–26). In addition, there is evidence that ghrelin reduces energy expenditure (24, 27), fat catabolism, and lipolysis (24, 28), and may promote adipogenesis (24, 25, 28). These findings contrast with the negative correlation between ghrelin levels and BMI, and the fact that ghrelin and its agonists efficiently increase food intake in both obese and normal-weight individuals (29, 30). Since the mechanisms responsible for reduced ghrelin in obesity have not yet been elucidated (24), it could be conceptually hypothesized that disparate changes in circulating ghrelin and obestatin levels may represent adaptive modifications to obesity development, rather than primary defects, and that their alteration in circulating blood levels may reflect an imbalance of regulatory factors or mechanisms responsible, in turn, for their metabolic processes and action.

Intriguingly, the ghrelin/obestatin ratio was negatively correlated with several metabolic alterations. In fact, a negative correlation was found with fasting insulin and fasting HOMA-IR, whereas a positive one was found with ISI_{composite}. All the metabolic abnormalities reported in the women investigated in this study are commonly found in the presence of obesity, particularly the abdominal phenotype. These findings are therefore different with respect to the preliminary

data reported in children with PWS by Park *et al.* (11), who showed that the ghrelin/obestatin ratio during an oral glucose tolerance challenge was positively correlated with BMI and waist circumference, but no correlation was found either with plasma insulin or with ISI_{composite} (11). On the other hand, since the PWS is characterized, amongst others, by specific hormonal and behavioral abnormalities, such as increased circulating ghrelin, the presence of refractory hyperphagia, and a hypogonadic state, this model cannot apply to common obesity phenotypes.

The negative correlation of the ghrelin/obestatin ratio with insulin may be consistent with the putative negative regulatory effect of insulin on circulating ghrelin (31). Insulin excess, in fact, has been proven to decrease ghrelin concentrations in both rodents (32) and humans (33, 34). Although ghrelin itself has been found to decrease insulin secretion while inducing hyperglycemia in healthy normal-weight males (35), whether this may apply to a human pathophysiological condition rather than to a pharmacological effect remains to be clearly defined. Studies on the relationship between insulin resistance and ghrelin are conflicting (34), although there is evidence that ghrelin is significantly reduced in obesity only in the presence of insulin resistance and hyperinsulinemia, whereas this does not occur in insulin-sensitive obese individuals (34) and insulin is essential for meal-induced ghrelin suppression, even in type 1 diabetics (36). Whether an excess of insulin may conversely be responsible for increased obestatin levels is at present unknown. The significant correlation between the ghrelin/obestatin ratio and indices of insulin resistance and associated lipid abnormalities we found in the present study may be theoretically consistent with disparate effects of insulin on both ghrelin and obestatin secretion or metabolic processes. Studies in humans on these topics are not yet available. On the other hand, in the study performed in patients with the PWS, no relationship was found between obestatin and insulin blood levels or insulin resistance (11). The lack of correlation with adiponectin levels, however, makes the potential role of the balance between ghrelin and obestatin unlikely in determining insulin resistance, which characterizes obesity, particularly the abdominal phenotype.

In conclusion, this preliminary study shows that a decreased ghrelin/obestatin ratio characterizes obesity in women, supporting the hypothesis that the imbalance of ghrelin and obestatin may have a role in the pathophysiology of human obesity and may be secondary to the presence of insulin resistance and accompanying hyperinsulinemia. On the other hand, some relevant differences between our data on circulating blood levels of obestatin and the ghrelin/obestatin ratio in obese subjects and those reported in the few studies published so far imply that further extensive research in this new area is needed.

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