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COMMENT

The Relationship between Active Ghrelin Levels and Human Obesity Involves Alterations in Resting Energy Expenditure

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Ghrelin is a gastric hormone that exerts a stimulatory effect on appetite and fat accumulation. Ser(3) octanoylation is regarded as a prerequisite for ghrelin biological activity, although des-octanoylated forms may retain biological functions *in vitro*. Circulating ghrelin levels are usually low in obesity and in states of positive energy balance. Hence, the aim of our study was to analyze plasma active and serum total ghrelin levels in 20 obese (ages, 22–42 yr; body mass index, 41.3 ± 1.1 kg/m^2) and 20 lean subjects (ages, 22–43 yr; body mass index, 22.4 ± 0.6 kg/m^2) as well as their relationship to measures of glucose homeostasis, body fat, and resting energy expenditure (REE). The measured/predicted REE percentage ratio was calculated to subdivide groups into those with positive (≥100%) and negative (<100%) ratio values.

In obese patients, plasma active (180 ± 18 *vs.* 411 ± 57 pg/ml; $P < 0.001$) and serum total ghrelin levels (3650 ± 408 *vs.* 5263 ± 643 pg/ml; $P < 0.05$) were significantly lower when compared with lean subjects. Hence, ghrelin activity, defined as the proportion of active over total ghrelin levels, was similarly reduced in the obese state (6.1 ± 0.9% *vs.* 8.4 ± 1%; $P < 0.05$). There was a significant correlation between active and total ghrelin ($r = 0.62$; $P < 0.001$), and between total ghrelin and insulin ($r = -0.53$; $P < 0.001$) or insulin resistance using the homeostatis model of assessment-insulin resistance ($r = -0.49$; $P < 0.001$) approach. Significantly higher active ghrelin levels (214 ± 22 *vs.* 159 ± 30 pg/ml; $P < 0.05$) and ghrelin activity (8 ± 1.7% *vs.* 4.9 ± 0.9%; $P < 0.05$) were observed in patients with positive compared with negative measured/predicted REE ratio values.

Our study shows that obesity is associated with an impairment of the entire ghrelin system. The observation that ghrelin is further decreased in cases of abnormal energy profit adds new evidence to the relationship between ghrelin activity and energy balance in obesity. (*J Clin Endocrinol Metab* 89: 936–939, 2004)

GHRELIN IS A 28-amino acid gastric peptide stimulating pituitary GH secretion through the GH secretagogue receptor (GHS-R) and regulating feeding behavior and adiposity through neuronal mechanisms involving neuropeptide Y and Agouti-related protein (1–4). In humans, ghrelin administration increases hunger and food intake, reduces insulin secretion, and enhances energy intake by nearly 30% (5, 6). To be active, ghrelin requires n-octanoylation at serine 3 (1), although des-acylated ghrelin has been shown to inhibit apoptosis in cardiomyocytes independently of the GHS-R (7). Endogenous ghrelin secretion increases during acute fasting (8) and in food-restricted patients with anorexia (2). A decrease of ghrelin levels normally occurs in response to a standard meal (8), and in obesity, where ghrelin levels are on average 33% lower than normal (9), increase after weight loss induced by gastric bypass (10) and do not normally act in response to feeding (11). The recent generation of immunoradiometric assays recognizing circulating active and total ghrelin forms (12) may contribute to understanding the pattern and control of ghrelin secretion in human obesity. Hence, the aim of our study was to explore the relationship that exists between active and total ghrelin levels in obese and lean subjects, analyzing the relationship between the circulating ghrelin system and the degree of fat accumulation or energy balance.

Patients and Methods

Twenty obese patients [10 males and 10 females; ages, 22–42 yr; body mass index (BMI), >30 kg/m^2] and 20 matched lean subjects (10 males and 10 females; ages, 22–43 yr; BMI <25 kg/m^2) were enrolled in this study after informed consent and approval by the local Ethics Committee. All subjects were nonsmokers and free from gastrointestinal, cardiovascular, or metabolic disorders. Testing was performed at 0800 h in fasting conditions and after voiding, 3 d after admission while patients were fed a balanced diet (30% lipids, 50% carbohydrates, and 20% proteins). Percentage of total body water (TBW), fat body mass (FBM),

Abbreviations: BIA, Bioelectrical impedance analysis; BMI, body mass index; CV, coefficients of variation; FBM, fat body mass; GHS-R, GH secretagogue receptor; HOMA-IR, homeostatis model of assessment-insulin resistance; LBM, lean body mass; pREE, predicted REE; REE, resting energy expenditure; RQ, respiratory quotient; TBW, total body water.

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and lean body mass (LBM) were determined by bioelectrical impedance analysis (BIA) (model BIA 101/S Akern, Florence, Italy). Patients with fluid overload according to vectorial analysis were excluded to minimize errors in estimating FBM and LBM in severe obesity (13). Respiratory quotient (RQ; VO_2/VCO_2), resting energy expenditure (REE; kcal/24 h), and predicted REE (pREE; kcal/24 h) were determined twice in a thermoregulated room (22–24 C) by computed open-circuit indirect calorimetry, measuring resting oxygen uptake and resting carbon dioxide production by a ventilated canopy (Sensormedics, Milan, Italy) at 1-min intervals for 30 min and expressed as a 24-h value. pREE was calculated by the Harris-Benedict formula (14). The calculated measured/pREE ratio was used to separate individuals into subgroups with negative and positive ratio values expressed as percentage (*i.e.* positive if ≥100%, negative if <100%). BMI was calculated as weight (kilograms)/height (meters^2).

Assays

Blood samples for active ghrelin assay were collected in plastic tubes containing EDTA and allowed to sit on ice for 10 min. Plasma was centrifuged, separated, and acidified with 1 N HCl according to the manufacturer's instructions, then stored at −80 C until assayed. Plasma active ghrelin levels and serum total ghrelin levels were measured by commercial RIAs (Linco Research, Inc., St. Louis, MO), using ^{125}I-labeled ghrelin as a tracer and a ghrelin antiserum raised either against Ser(3) octanoylated ghrelin for measurement of active ghrelin (100% specificity for ghrelin and ghrelin 1–10; <0.1% specificity for ghrelin 14–28 and des-octanoyl ghrelin) or against ghrelin independent of the octanoyl group for measurement of total ghrelin (100% specificity for ghrelin 14–28 and des-octanoyl ghrelin; <0.1% specificity for ghrelin 1–10). The detection limit for both assays was 10 pg/ml. Intra- and interassay coefficients of variation (CV) reported by the manufacturer were 4.4–10% and 14.7–16.7% for total ghrelin, respectively, and 6.5–9.5% and 9.6–16.2% for active ghrelin, respectively (for conversion to SI units: pg/ml × 3.371 = pmol/liter for active ghrelin; pg/ml × 3.189 for des-acylated ghrelin). Serum leptin levels were measured by commercial Linco RIA having a detection limit of 0.15 μg/liter and intra- and interassay CV as previously reported (15) (for conversion to SI units: μg/liter × 0.0625 = nmol/liter). Serum insulin levels were measured by chemiluminescence (Immulite 2000 Analyzer, Diagnostic Products Corp., Los Angeles, CA). Blood glucose, total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were measured by enzymatic methods (Roche Diagnostics, Mannheim, Germany). Insulin resistance was calculated by the homeostatis model of assessment-insulin resistance (HOMA-IR) approach, calculated as insulin (microunits per milliliter) × blood glucose (millimoles per liter)/22.5 (16). Ultrasensitive C-reactive protein was measured by the CRP (latex) HS Roche kit (Roche Diagnostics), having a sensitivity of 0.003 mg/dl, and intra- and interassay CV of 2.51–5.35% and 4.25–5.79%, respectively, as reported by the manufacturer.

Statistical analysis

Data are expressed as mean ± SEM. Comparative analyses within and between groups were calculated by Mann-Whitney *U* test or two-tailed unpaired Student's *t* test after Welch's correction, when appropriate. In correlation and regression analyses, values of total and active ghrelin were log-transformed to compensate for the unskewed distribution. Relationships between variables were analyzed using Pearson's correlation coefficient or multiple linear regression analysis. Analyses were performed using SPSS 10.0 (SPSS, Inc., Chicago, IL) and Prism (Graphpad Sofware, Inc., San Diego, CA). Significance was set at $P < 0.05$.

Results

Measures of body adiposity and energy expenditure were significantly higher in obese subjects than in controls, whereas significantly lower active and total ghrelin levels were recorded in obese subjects compared with controls (Table 1). The resulting degree of ghrelin activity, defined as the proportion of active over total ghrelin levels, was similarly impaired in obese subjects (6.1 ± 0.9% *vs.* 8.4 ± 1%; $P < 0.05$), despite some overlap between the two study groups (range, 2.6–23% in lean and 2–17.3% in obese subjects). To evaluate the impact of energy expenditure on ghrelin levels, individuals were subgrouped by values of measured/pREE ratio. Obese patients with a positive ratio had higher active ghrelin levels (214 ± 22 *vs.* 159 ± 30 pg/ml; 63.3 ± 6.5 *vs.* 47.1 ± 8.9 pmol/liter; $P < 0.05$) and ghrelin activity (8 ± 1.7 *vs.* 4.9 ± 0.9%; $P < 0.05$) than patients with negative ratio values (measured/pREE ratio, 106 ± 2 *vs.* 97 ± 1; $P < 0.01$). No difference was noted among controls (data not shown).

A positive association existed between total and active levels (r = 0.62; $P < 0.001$; Fig. 1) that was completely explained by the control group (r = 0.70; $P < 0.001$). Total and active ghrelin levels were positively correlated with body water content and inversely correlated with leptin and measures of body fat (Table 2). Inversely, the negative correlation between total ghrelin levels and insulin or insulin resistance

TABLE 1. Anthropometric, adipose, metabolic, and biochemical parameters in lean subjects and obese patients

Parameters	Lean subjects	Obese patients
Age (yr)	31.7 ± 1.3	32.4 ± 1.6
BMI (kg/m^2)	22.4 ± 0.6	41.3 ± 1.1[b]
FBM (%)	22.2 ± 1.7	42.9 ± 1.5[b]
RQ (VO_2/VCO_2)	0.90 ± 0.01	0.86 ± 0.02[a]
REE (kcal/24 h)	1629 ± 53	2155 ± 85.6[b]
Total ghrelin (pg/ml)	5668 ± 644	3651 ± 408[a]
Active ghrelin (pg/ml)	411.8 ± 57.4	180.4 ± 18.5[b]
Ghrelin activity (%)	8.4 ± 1	6.1 ± 0.9[a]
Leptin (μg/liter)	7.9 ± 1.9	38.3 ± 5[b]
Insulin (μU/ml)	9.5 ± 1.3	13.7 ± 1.3[a]
Glucose (mg/dl)	85.2 ± 2.2	82.7 ± 1.7
Homa-IR	2 ± 0.3	2.8 ± 0.3[a]

Data are indicated as mean ± SEM. Conversion factors (metric units to SI units): active (acylated) ghrelin, pg/ml × 3.371 = pmol/liter, des-acylated ghrelin, pg/ml × 3.189 pmol/liter (total ghrelin measures both acylated and des-acylated ghrelin); leptin, μg/liter × 0.0625 = nmol/liter; insulin, μU/ml × 6.0 = pmol/liter; glucose, mg/dl × 0.056 = mmol/liter.

[a] $P < 0.05$.

[b] $P < 0.001$.

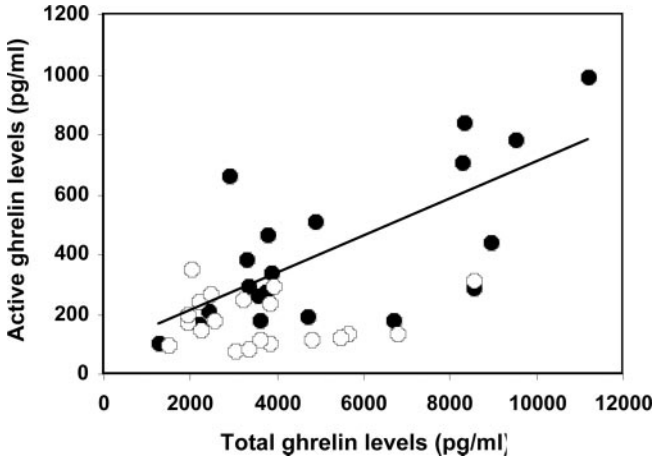

FIG. 1. Bivariate correlation analysis between total and active ghrelin levels in the group as a whole. Lean and obese subjects are indicated as *closed* and *open circles*, respectively. For conversion from metric units to SI units: pg/ml × 3.371 = pmol/liter for active (acylated) ghrelin; pg/ml × 3.189 for des-acylated ghrelin (total ghrelin measures both acylated and des-acylated ghrelin).

TABLE 2. Bivariate correlation analysis between total or active ghrelin levels and anthropometric, adipose, metabolic, and biochemical parameters in the study populations

Parameters	Total ghrelin	Active ghrelin
BMI (kg/m^2)	−0.40[b]	−0.53[c]
FBM (%)	−0.37[a]	−0.53[c]
TBW (%)	0.38[a]	0.55[c]
REE (kcal/24 h)	−0.33[a]	−0.34[a]
Leptin (mg/ml)	−0.53[b]	−0.43[a]
Insulin (mIU/ml)	−0.53[c]	−0.21
HOMA-IR	−0.49[c]	−0.14

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

was due to the obese group (insulin, r = −0.60, $P < 0.01$; insulin resistance as calculated by the HOMA-IR approach, r = −0.55, $P < 0.05$). In neither group was any correlation found between components of the ghrelin system and C-reactive protein or products of lipid homeostasis (data not shown). By multivariate analysis, TBW content appeared to better predict both active (T = 2.33; $P < 0.05$) and total ghrelin levels (T = 2.50; $P < 0.05$). Total ghrelin levels were also negatively associated with RQ values (T = −2.78; $P < 0.05$).

Discussion

The present study demonstrates that human obesity is associated with significantly lower levels of both acylated and des-acylated ghrelin and that a relationship exists between energy expenditure and components of the ghrelin system in obesity. The finding of a positive relationship between serum levels of total and plasma levels of active ghrelin is of interest and might be of future assistance in the study of the ghrelin system in lean subjects.

Ghrelin is a gastric hormone binding to the GHS-R, which partakes in the neuropeptide Y- and Agouti-related protein-mediated hypothalamic control of orexigenic signals (1–4). Although Ser(3) octanoylation is a prerequisite for ghrelin biological activity (3–6), des-octanoyl ghrelin variants have been additionally identified (12, 17) and found to exert novel antiapoptotic effects in primary adult and cultured rat cardiomyocytes (7). Hence, the aim of our study was to explore the role of acylated and desacylated ghrelin forms in human obesity, where circulating ghrelin levels are about 30% lower than normal; decline proportionately with increasing body fat, leptin, and insulin levels; and are far less responsive to postmeal inhibition than in controls (8–11). Using specific immunoassays (12) recognizing active (N-terminus) and total (N- + C-terminus) ghrelin levels, our analysis confirms the recognized association between obesity and low ghrelin levels (9) and reveals for the first time that both ghrelin forms are decreased in human obesity. Total and active ghrelin concentrations were 30 and 56% lower than normal, respectively, and these proportions also accounted for a significant decrease in ghrelin activity, which measured the fraction of active over total ghrelin levels. Findings similar to ours were obtained in genetically obese *ob/ob* and *db/db* mice, where total and active ghrelin levels were lower than in control littermates, whereas active ghrelin levels were comparatively more reduced at baseline and more responsive to feeding than total ghrelin levels (17). Taken together, these and our observations suggest that the regulation of the entire ghrelin system is altered in obesity and that acylated ghrelin could be more responsive to adipogenic signals than other molecular forms of ghrelin.

The close relationship seen between total and active ghrelin in lean subjects, but not in obese patients, is potentially relevant, because this finding may indirectly reflect the different regulation of acylated ghrelin synthesis in the obese state. Because active ghrelin levels constitute a functional parameter of ghrelin activity, the correlation described herein could be of future help in blood sampling procedures and usage of frozen serum samples in similar study groups, although larger characterization studies are necessary to substantiate our current findings. The use of immunoassays discriminating different molecular ghrelin forms is also of interest with regard to the circulating form(s) of ghrelin. Recent evidence that matrix-bound ghrelin is able to interact with high-density lipoprotein subtypes in human plasma (18) indirectly suggested the existence of carrier-bound ghrelin forms in the circulation. Our study also showed an inverse relationship between ghrelin and leptin levels that likely reflected the inverse correlation seen between ghrelin levels and body fat also shown by others (9). Despite the lower accuracy of BIA with respect to dual-energy x-ray absorptiometry in measuring body fat mass (9), in our study patients with fluid overload according to the vectorial analysis were excluded to minimize the impact of severe obesity on the accuracy of BIA measurements. The finding of a negative correlation between total ghrelin levels and insulin or insulin resistance in obesity is unprecedented and requires further analyses.

One peculiar aspect of our results is the atypical relationship noted between components of the circulating ghrelin system and energy expenditure in obesity. In rodents, ghrelin infusion promotes weight accrual by increasing food intake and by decreasing energy expenditure and fat catabolism (3, 4, 19). This effect is primarily due to an increase of caloric intake and RQ, suggestive of a switch from fatty acid oxidation to glycolysis leading ultimately to fat deposition (3, 19). Although it would be expected that ghrelin might intervene endogenously to correct states of impaired energy balance, the relationship between ghrelin and energy expenditure in obesity constitutes a matter of debate. In rats carrying leptin transgene expression at multiple hypothalamic sites, the observed decrease in adiposity coexists with an increase in both ghrelin secretion and thermogenic energy expenditure (20). Human studies on lean monozygotic twins failed to observe significant correlations between plasma ghrelin levels and states of either positive or negative energy balance (21). The relationship between ghrelin and energy balance becomes even less significant in obesity, where ghrelin levels happen to be unresponsive to feeding (11). Our analysis stratified by values of measured/pREE ratio showed that active ghrelin levels and ghrelin activity were higher among obese patients with a positive index than those with a negative index. This unexpected reciprocity suggests that ghrelin secretion is decreased in obesity in cases of impaired energy expenditure. Speculatively, it could be interpreted as an obesity-related compensatory mechanism

acting to contain the orexigenic signals afferent to the brain. Determining the relationship between ghrelin secretion and energy balance after weight loss may significantly contribute to clarifying the role of basal metabolic rate on the regulation of adipogenic signals.

In conclusion, the present study shows that obesity is associated with a reduction of the entire ghrelin system encompassing acylated and des-acylated molecular forms, and that states of negative energy balance may further contribute to reducing ghrelin activity in obesity. The underlying mechanism(s) remain to be elucidated. Our observations, however, add new evidence to the hypothesis that ghrelin activity is closely related to energy balance and plays a relevant role in regulating hunger and satiety stimuli afferent to the brain.

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