CLINICAL STUDY

The negative association between total ghrelin levels, body mass and insulin secretion is lost in hypercortisolemic patients with Cushing's disease

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Abstract

Objective: Ghrelin exerts a wide spectrum of endocrine and non-endocrine actions. The stomach is the major source of circulating ghrelin levels that are negatively associated with body mass, insulin and glucose levels. The role of glucocorticoids in ghrelin secretion and action is still unclear.

Design: In 8 patients with Cushing's disease (CD, BMI 29.8 ± 1.6 kg/m²), 7 normal (NS) and 6 obese subjects (OB, BMI 32.9 ± 1.1 kg/m²) we studied: a) total ghrelin levels (every 15 min over 3 h) and their correlation with BMI, insulin, glucose, homeostatic model assessment (HOMA) index, ACTH and cortisol levels; b) GH, ACTH, cortisol, insulin and glucose responses to acylated ghrelin administration (1.0 µg/kg i.v. at 0 min).

Results: CD patients had BMI, insulin and glucose levels as well as HOMA index higher than those in NS ($P < 0.05$) but similar to those in OB. Despite this, total ghrelin levels in CD were similar to those in NS and both were higher ($P < 0.05$) than those in OB. No correlation was found among total ghrelin and BMI, insulin, glucose, ACTH and cortisol levels in CD patients. The GH responses to ghrelin in CD and OB were similar and both were lower ($P < 0.002$) than those in NS. In CD ghrelin induced exaggerated ACTH and cortisol responses clearly higher ($P < 0.005$) than in OB and NS. Ghrelin administration increased glucose in all groups; insulin levels showed slight decrease that was significant ($P < 0.05$) in OB only.

Conclusions: Hypercortisolism in humans is associated with impaired ghrelin secretion and action. In fact, total ghrelin secretion in CD is not reduced despite increased BMI, insulin and glucose levels, while the GH and ACTH responses to acylated ghrelin are clearly reduced and enhanced, respectively.

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Introduction

The stomach is the major source of ghrelin secretion, although it is also expressed in several other central and peripheral tissues including bowel and pancreas (1, 2). Ghrelin has been discovered as natural ligand of the orphan GH secretagogue receptor (GHS-R) type 1a, whose expression is concentrated in the hypothalamo–pituitary unit but also in other central and peripheral tissues, including the pancreas (3, 4). The widespread distribution of ghrelin and GHS-R expression agrees with the wide spectrum of endocrine and non-endocrine ghrelin actions (2, 5); indeed, ghrelin is much more than a natural GHS. At present, there is evidence suggesting that, among the most important ghrelin actions, there are central effects on appetite, energy balance, and peripheral metabolic

effects on insulin secretion, glucose and lipid metabolism (2, 6–8). Ghrelin secretion in humans undergoes important fluctuations during the day (9), it is stimulated by energy restriction and acetylcholine, while it is inhibited by somatostatin, insulin and glucose load (7, 10–13). Above all, it has been clearly demonstrated that ghrelin secretion is negatively correlated with body mass index (BMI) and insulin secretion; in fact, total ghrelin levels are increased in anorexia and cachexia while they are markedly reduced in obesity (14–20). In contrast to the body of information on the relationship between ghrelin and energy balance, the role of glucocorticoids on ghrelin secretion and action is still unclear. The marked impact of glucocorticoids on insulin and glucose metabolism as well as on GH secretion and body composition is well known. In fact, hyperinsulinism, insulin resistance and glucose

intolerance up to secondary diabetes mellitus, as well as marked GH insufficiency, are hallmarks of Cushing's syndrome, that is classically connoted by visceral adiposity and central obesity (21). Regarding ghrelin actions, it has already been reported that Cushing's disease (CD) is associated to unexpected ACTH and cortisol hyperresponsiveness coupled with reduced GH response to ghrelin as well as to synthetic GHS (22–24). Regarding ghrelin secretion and its metabolic actions as a function of glucocorticoid status, it has recently been reported in a group of patients with CD that ghrelin levels, evaluated as single morning measurements, were lower than in normal controls, confirming previous data (25), but increased after successful transphenoidal surgery, despite similar glucose and insulin levels (26).

In order to further clarify the relationship between ghrelin and glucocorticoid status, we studied ghrelin secretion as a function of several anthropometric and metabolic parameters, as well as the endocrine and metabolic response to acylated ghrelin, in patients with pituitary adrenocorticotrophic hormone (ACTH)-dependent Cushing's syndrome in comparison to a group of normal or obese age-matched controls.

Subjects and methods

Subjects

Eight patients with CD (CD, 8 F, 37.5 ± 2.1 years, BMI 29.8 ± 1.6 kg/m²) were studied; these were all CD patients who have not been studied before. The diagnosis of CD was made by international criteria including: high levels of urinary free cortisol, normal or high plasma ACTH and serum cortisol levels, absent suppression of cortisol after low-dose dexamethasone test and adequate suppression of cortisol after a high-dose dexamethasone test. In 3 cases (cases 5, 6 and 8) inferior petrosal venous sinus sampling has also been performed, indicating the existence of a pituitary ACTH-dependent Cushing's

syndrome. Based on hormonal and MRI findings, CD patients were diagnosed as having: a) pituitary ACTH-secreting microadenoma (cases 1–3), b) pituitary ACTH-secreting macroadenoma (cases 4 and 7), c) pituitary ACTH-dependent Cushing's syndrome with normal MRI (cases 5, 6 and 8), all patients showed no evidence of hypopituitarism. Two CD patients had normal glucose tolerance (N), four patients showed impaired glucose tolerance (IGT) and two patients had Type 2 diabetes (DMII), respectively. None of the patients were taking medical treatment what would influence hormonal secretion during the test period. Clinical details of the patients are reported in Table 1. In five patients the diagnosis of ACTH-secreting pituitary adenoma was confirmed by immunostaining after transphenoidal surgery; three other patients refused surgery and were on medical treatment. Seven normal lean subjects (NS, 7 F, 30.6 ± 8.0 years, BMI 20.3 ± 1.7 kg/m²) and six subjects with simple obesity (OB, 6 F, 42.2 ± 5.1 years, BMI 32.9 ± 1.1 kg/m², waist circumference 102.2 ± 7.0 cm) were studied as control groups in their early follicular phase. The study had been approved by the Ethical Committee of the University of Turin and informed consent was obtained from all subjects. All subjects underwent the following two testing sessions, in randomized order with at least three days apart: 1) saline (250 ml infused for 180 min); 2) ghrelin (1.0 µg/kg acylated ghrelin i.v. bolus at 0 min). The testing sessions started between 0830 h and 0900 h after an overnight fast and 30 min after venous cannulation, kept patent by slow infusion of isotonic saline. All samples from individual subjects were analyzed together.

Assay

Blood samples were taken every 15 min from 0 up to 180 min (session 1) and from –15 up to 90 min (session 2). In session 1, total ghrelin, insulin and glucose levels were assayed at each time point, and HOMA index was calculated. In session 2, GH, ACTH, cortisol, insulin and glucose were assayed at each time point. Plasma

Table 1 Clinical and hormonal details of the patients with Cushing's disease.

Case	Sex	Age (years)	BMI (kg/m ²)	UFC (µg/24 h)	ACTH (pg/ml)	Cortisol (µg/l)	Glycemic State	MRI imaging
1	F	31	21	216	76.2	202.8	DM II	micro adenoma
2	F	29	35	183	88.6	258.8	DM II	micro adenoma
3	F	42	31	418	65.6	227.2	IGT	micro adenoma
4	F	43	35	109	54.0	133.3	IGT	macro adenoma
5	F	41	28	194	88.0	170.4	IGT	normal
6	F	42	31	112	55.3	290.1	N	normal
7	F	31	30	992	116.0	237.7	IGT	macro adenoma
8	F	41	27	497	34.2	191.7	N	normal
Mean ± S.E.M.		37.5 ± 2.1	29.8 ± 1.6	340.1 ± 105.4	72.2 ± 9.4	214.0 ± 17.7		

UFC, urinary free cortisol (mean of two measurements; 2.75 µg/24 h = 1 nmol/day).

ACTH plasma concentrations at 09.00 h (mean of two measurements; 0.22 pg/ml = 1 pmol).

Cortisol serum concentrations at 09.00 h (mean of two measurements; 2.7 µg/l = 1 nmol/l).

N, normal; IGT, impaired glucose tolerance; DMII, Type II diabetes.

total ghrelin levels (pg/ml) were assayed in duplicate for immunoreactive ghrelin concentration, by a commercially available RIA (Phoenix Pharmaceuticals, Mountain View, CA, USA) using ^{125}I -labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against the C-terminus of human ghrelin. This assay recognizes both acylated and deacylated ghrelin. The antiserum does not cross-react with any relevant peptide as previously shown. Intra- and inter-assay CV were below 5.3% and 13.6%, respectively. Serum insulin levels (mU/l; $1 \text{ mU/l} \times 7.175 = 1 \text{ pmol/l}$) were measured in duplicate by immunoradiometric assay (INSIK-5, Sorin Biomedica, Saluggia, Italy). The sensitivity of the assay was 2.5 mU/l. The inter- and intra-assay CV ranged from 6.2 to 10.8% and from 5.5 to 10.6%, respectively. Plasma glucose levels (mg/dl; $1 \text{ mg/dl} \times 0.05551 = 1 \text{ mmol/l}$) were measured by glucose-oxidase colorimetric method (Glucifix, Menarini Diagnostici, Florence, Italy). HOMA index was calculated with the formula: serum insulin (mU/l) $\times$ plasma glucose (mmol/l) /22.5. Plasma ACTH levels (pg/ml; $1 \text{ pg/ml} \times 0.2202 = 1 \text{ pmol/l}$) were measured in duplicate by immunoradiometric assay (Allegro HS-ACTH, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The sensitivity of the assay was 1 pg/ml. The inter- and intra-assay CV ranged from 6.9 to 8.9% and from 1.1 to 3.0%, respectively. Serum cortisol levels ($\mu\text{g/l}$; $1 \mu\text{g/l} \times 27.59 = 1 \text{ nmol/l}$) were measured in duplicate by RIA (CORT-CTK 125 RIA; Sorin Biomedica). The sensitivity of the assay was 0.4 $\mu\text{g/l}$. The inter- and intra-assay CV ranged from 6.6 to 7.5% and from 3.8 to 6.6% respectively. Serum GH levels ($\mu\text{g/l}$) were measured in duplicate by immunoradiometric assay (hGH-CTK IRMA, Sorin Biomedica). The sensitivity of the assay was 0.15 $\mu\text{g/l}$. The inter- and intra-assay CV ranged from 2.9–4.5% and from 2.4–4.0%, respectively. The hormonal responses are expressed as mean $\pm$ S.E.M. of absolute levels or areas under curves (AUC) calculated by trapezoidal integration.

Statistical analysis

The statistical analysis was carried out using non-parametric ANOVA (Friedman or Kruskal–Wallis test) and then Wilcoxon matched pairs test or Mann–Whitney U test as appropriate; correlations were carried out using the Spearman correlation coefficient. Results are expressed as mean $\pm$ S.E.M.

Results

BMI in CD (mean $\pm$ S.E.M.: $29.8 \pm 1.6 \text{ kg/m}^2$) was higher ($P < 0.05$) than in NS ($20.3 \pm 1.7 \text{ kg/m}^2$), but similar to that in OB ($32.9 \pm 1.1 \text{ kg/m}^2$). At baseline, HOMA index in CD (3.9 ± 1.4) was similar to that in OB (3.7 ± 0.9) and both were higher ($P < 0.05$) than in NS (2.5 ± 0.4). During saline infusion, insulin and glucose levels in CD

($2808.4 \pm 230.3 \text{ mU/l per h}$ and $14\,637.5 \pm 402.9 \text{ mg/dl per h}$, respectively) were higher ($P < 0.05$) than in NS ($2194.0 \pm 74.2 \text{ mU/l per h}$ and $13\,768.5 \pm 195.5 \text{ mg/dl per h}$, respectively), but similar to those in OB, ($3223.5 \pm 255.9 \text{ mU/l per h}$ and $14\,983.5 \pm 382.7 \text{ mg/dl per h}$, respectively) (Fig. 1). Total ghrelin levels in CD ($121650.0 \pm 13\,549.3 \text{ pg/ml per h}$) were more similar to those in NS ($125775.0 \pm 12\,194.3 \text{ pg/ml per h}$) and both were higher ($P < 0.05$) than in OB ($96\,840.0 \pm 6327.9 \text{ pg/ml per h}$) (Fig. 2). In contrast to OB, total ghrelin levels in CD did not correlate with BMI, insulin and glucose levels as well as HOMA index. Moreover, no correlation was found between total ghrelin, ACTH and cortisol levels in CD. The acute administration of acylated ghrelin induced a GH response in CD similar to that in OB (900.8 ± 285.2 vs $1317.9 \pm 294.0 \mu\text{g/l per h}$) and both were lower ($P < 0.002$) than that in NS ($4618.2 \pm 558.4 \mu\text{g/l per h}$). On the other hand, ACTH and cortisol responses to ghrelin in CD ($16\,439.6 \pm 2274.2 \text{ pg/ml per h}$ and $27\,677.4 \pm 1838.4 \mu\text{g/l per h}$, respectively) were clearly higher ($P < 0.005$) than in OB ($3910.6 \pm 971.2 \text{ pg/ml per h}$ and $17\,193.6 \pm 1508.0 \mu\text{g/l per h}$, respectively) and in NS ($2662.7 \pm 748.9 \text{ pg/ml}$ and $12\,299.4 \pm 19\,212.3 \mu\text{g/l per h}$, respectively). The ACTH and cortisol responses in OB and NS were in turn similar (Fig. 3). Ghrelin administration induced an increase of glucose levels in CD ($7851.0 \pm 437.6 \text{ mg/dl per h}$) similar to that in OB ($7775.6 \pm 238.3 \text{ mg/dl per h}$) and in NS ($6536.1 \pm 376.2 \text{ mg/dl per h}$). The ghrelin-induced glycaemic increase was coupled to a transient decrease of insulin levels in all groups although statistical significance ($P < 0.05$) was attained in OB only (Fig. 4).

Side effects

After ghrelin administration, four NS, three OB and one CD patient showed transient facial flushing, whereas five NS, four OB and six CD reported to be hungry at the end of the testing session.

Discussion

The results of the present study demonstrate that the negative relationship linking BMI, insulin secretion and resistance with ghrelin secretion is lost in hypercortisolemic patients with CD. In fact, in comparison with weight-matched obese subjects, patients with CD with the classical increase in body mass, glucose and insulin secretion, show circulating total ghrelin levels similar to those in normal lean subjects. The insulin and glucose responses to the acute administration of acylated ghrelin in patients with CD basically overlaps with the responses of either lean or obese control subjects. On the other hand, the findings of the present study confirm our previous data showing that, differently from obese patients, besides reduced GH response, hypercortisolemic patients

Figure 1 Insulin (IRI, mU/l; 1 mU/l $\times$ 7.175 = 1 pmol/l) and glucose (mg/dl; 1 mg/dl $\times$ 0.05551 = 1 mmol/l) levels and AUCs (mean $\pm$ s.e.m.) during saline infusion in patients with Cushing's disease (CD), obese patients (OB) and normal lean subjects (NS).

with CD also show an exaggerated ACTH and cortisol responses to ghrelin administration (24). It has been widely demonstrated that ghrelin secretion is negatively associated to BMI and insulin secretion. In fact, total

ghrelin levels are increased in anorexia and cachexia while it is markedly reduced in obesity (14–20). The reduction of total ghrelin levels in obesity is also confirmed by the present study. Obesity has been reported

Figure 2 Total ghrelin levels and AUCs (mean $\pm$ s.e.m.) during saline infusion in patients with Cushing's disease (CD), obese patients (OB) and normal lean subjects (NS).

Figure 3 GH, ACTH (pg/ml; 0.22 pg/ml = 1 pmol/l) and cortisol (µg/l; 2.7 µg/l = 1 nmol/l) levels and AUCs (mean ± s.e.m.) after acylated ghrelin (1.0 µg/kg i.v. at 0 min) in patients with Cushing's disease (CD), obese patients (OB) and normal lean subjects (NS).

Figure 4 Glucose (mg/dl; $1 \text{ mg/dl} \times 0.0551 = 1 \text{ mmol/l}$) and insulin (IRI, mU/l; $1 \text{ mU/l} \times 7.175 = 1 \text{ pmol/l}$) levels and AUCs (mean $\pm$ S.E.M.) after acylated ghrelin ($1.0 \mu\text{g/kg}$ i.v. at 0 min) in patients with Cushing's disease (CD), obese patients (OB) and normal lean subjects (NS).

to be connoted by some hypothalamic–pituitary–adrenal (HPA) axis hyperactivity (27) but evidence that total ghrelin levels are reduced points against any role of ghrelin in the subtle HPA derangement connoting this condition. The inhibitory action of insulin on ghrelin synthesis and secretion has been clearly demonstrated; in fact, total ghrelin levels are reduced either during euglycemic clamp studies or following insulin-induced hypoglycemia (12, 28–30). Nevertheless, ghrelin secretion has also been clearly shown to be inhibited after oral or intravenous glucose load (15, 31, 32). Despite the mechanisms underlying the inhibitory effect of insulin and glucose on ghrelin secretion are still a matter of debate, total ghrelin levels have been found to be negatively correlated with insulin secretion and resistance as well as with glucose levels (32, 33). In fact, total ghrelin levels have been shown to be reduced in diabetes mellitus type 2 (34) and surprisingly in diabetes mellitus type 1 (35). This strong negative relationship linking ghrelin secretion with body mass,

glucose levels, insulin secretion and resistance would logically predict ghrelin hyposecretion in hypercortisolemic states, such as CD. However, a reduction of total ghrelin levels has also been reported in other conditions connoted by insulin resistance, such as PCO syndrome (36). However, our data show that CD patients, despite their classical metabolic feature, display mean total ghrelin levels (recorded over 3 morning hours) similar to those in age-matched lean controls, but higher than those in obese patients. It has to be emphasized that CD and obese patients were comparable in terms of BMI as well as insulin and glucose levels and even the HOMA index. Therefore, this evidence points towards the hypothesis that the hypercortisolemic state is able to counteract the negative impact of hyperinsulinism, insulin resistance, increased glucose levels as well as increased body fat mass on ghrelin secretion. These data agree with previous findings that showed a lack of correlation between ghrelin, BMI, glucose, insulin secretion and resistance in CD (25, 26). Although

other steroids such as androgens, are supposed to play a role in the modulation of ghrelin levels (37), the role (if any) of glucocorticoids in the regulation of ghrelin synthesis and secretion is still unclear. The stimulatory effect of corticosterone on ghrelin mRNA expression at the gastric and pituitary level in rats has been reported but these findings have not been confirmed by other studies (38, 39). As anticipated, it has recently been reported that in patients with CD, ghrelin levels evaluated as single morning measurements, were lower than normal controls (25, 26), but increased after successful trans-sphenoidal surgery, despite similar glucose and insulin levels (26). These data disagree with our present findings, but the difference in the study protocol (the evaluation of a single morning measurement instead of more prolonged observation during morning hours) could explain the discrepancy. In fact, it has been clearly demonstrated that ghrelin levels undergo marked circadian and interindividual variations (9), therefore it is a more appropriate method to observe ghrelin levels over a prolonged period of observation, as carried out in our present study. On the other hand, in agreement with our findings, Libe *et al.* 2005 failed to show any correlation between ghrelin, ACTH and cortisol levels, even though lower ghrelin levels have been found in their CD patients (26). Both the present study and Libe *et al.* 2005 show the lack of association between cortisol and ghrelin levels, this could be due to the small number of CD patients tested. Independent of the mechanisms underlying the relative ghrelin hypersecretion in hypercortisolemic states, such as CD, the main message of this study is that the exposure of chronically elevated glucocorticoid levels are able to counteract the major metabolic inputs that influence ghrelin secretion. Again, it has to be considered that important information about the relationship linking glucocorticoids and ghrelin secretion, should come from distinguishing total from acylated circulating ghrelin levels. Besides impaired ghrelin secretion, our results show that although the negative association between ghrelin, insulin and glucose levels is lost in CD, these patients present normal insulin and glucose responses to acute acylated ghrelin, suggesting that high glucocorticoid levels, which deeply impairs glycometabolic status, do not influence the metabolic response to ghrelin administration. It has been clearly shown that ghrelin is able to exert metabolic actions, influencing insulin secretion as well as glucose and lipid metabolism (2, 6, 7). In humans, acute ghrelin administration is followed by a transient decrease in insulin levels that is anticipated by a hyperglycemic effect (40, 41) and recent studies seem to indicate that these actions reflect the direct actions of ghrelin at the pancreatic, hepatic and adipose tissue level (2). In this context, our present findings suggest that the mechanisms underlying the ghrelin action on glycometabolic function are basically preserved during the chronic hypercortisolism state. On the other hand, we confirm that CD is associated with a decrease in somatotroph

and a clear cut increase in corticotroph responsiveness to acute ghrelin administration. In fact, these findings agree with our previous studies performed with ghrelin or synthetic GHS (22–24). The low GH response to ghrelin in hypercortisolemic states fits well with the generalized reduction of GH response to all known provocative stimuli of somatotroph secretion in CD. This is likely to reflect either pituitary or hypothalamic alterations (42, 43). Meantime, the low GH response to provocative stimuli including ghrelin in CD, a condition with surprisingly intact ghrelin secretion, is against the hypothesis that somatotroph deficiency in hypercortisolemic states reflects ghrelin insufficiency. The exaggerated ACTH and cortisol responsiveness to ghrelin as well as synthetic GHS, is peculiar in hypercortisolism due to pituitary ACTH-dependent Cushing's syndrome (22–24). In fact, the negative feedback effect of glucocorticoids on the ACTH response to GHS is still functional in ACTH-independent Cushing's syndrome as well as in normal subjects exposed to glucocorticoids (44). To explain the peculiar ACTH and cortisol hyper-responsiveness that connotes patients with CD, it has been hypothesized that it is likely to reflect the presence of GHS receptors on the ACTH-secreting pituitary tumor (45).

In conclusion, the results of the present study show that hypercortisolism in humans is associated with the peculiar impairment of ghrelin secretion and action. In fact, total ghrelin secretion in CD is not reduced despite increased BMI, insulin and glucose levels, while the GH and ACTH responses to acylated ghrelin are clearly reduced and enhanced, respectively.

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