Anti-androgen treatment increases circulating ghrelin levels in obese women with polycystic ovary syndrome

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ABSTRACT. In a previous study we were the first to describe a negative correlation between circulating ghrelin concentrations and androgen levels in human plasma, suggesting an interaction between ghrelin and the endocrine regulation of reproductive physiology. We now investigated a potential direct regulatory influence of circulating androgens on plasma ghrelin levels. Fourteen obese women with polycystic ovary syndrome (PCOS) on a hypocaloric diet were randomly assigned to treatment groups (open-labeled design), receiving either placebo (no.= 7) or the antiandrogen flutamide (no.=7) for 6 months. Anthropometry, visceral (VAT) and subcutaneous (SAT) adipose tissue (quantified by computerized tomography), plasma hormone levels, insulin sensitivity indexes (Quantitative Insulin-Sensitivity Check Index-QUICKI) and Homeostatic Model Assessment applied to the oral glucose tolerance test ($HOMA_{OGTT}$) were evaluated at baseline and at the end of the study. Body weight decreased and insulin resistance indexes improved in both groups. A tendency toward a greater loss of VAT was observed in the flutamide group. Only in the flutamide group was a significant reduction of androgens levels observed. Plasma ghrelin levels significantly increased following treatment with flutamide, while ghrelin remained unchanged in the placebo group. We observed a negative correlation between changes of ghrelin levels and changes of androgen plasma concentration in the flutamide-treated group. In the same group a positive correlation was found between plasma ghrelin changes and insulin sensitivity as expressed by $HOMA_{OGTT}$. Analysis in a multiple regression model, however, showed that plasma ghrelin changes were mainly due to changes of androgen levels rather than improved insulin sensitivity. We, therefore, conclude that androgens are independent modulators of circulating ghrelin concentrations.

(J. Endocrinol. Invest. 26: 629-634, 2003)

INTRODUCTION

Ghrelin, an acylated peptide of 28 aminoresidues, is a novel hormone that has recently been identified in the stomach (1), following the previous cloning of the ghrelin (GHS-1a) receptor (2). Since ghrelin discovery (3), a rapidly growing body of scientific reports is describing multiple roles for this hormone. The distribution of the ghrelin receptor at hypothalamic and pituitary sites matches ghrelin's major biological roles in regulating energy balance (4-6) as well as activity of the hypothalamic-pituitary endocrine axes (7).

However, some earlier reports have documented that ghrelin binding sites are not restricted to central areas, but are also present in a large number of peripheral organs, pointing to a pleiotropic action pattern of ghrelin (8). Gonadal tissue has very recently been unmasked as a potentially important target of ghrelin action, since both ovary (8, 9) and testis (8, 10, 11) express ghrelin and the ghrelin receptor. Factors such as gonadotropins seem to influence both, steroid production and ghrelin secretion, therefore ghrelin may represent one more link functionally connecting the reproductive sys-

Key-words: Polycystic ovary syndrome, insulin resistance, androgens, flutamide, ghrelin.

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Accepted February 20, 2003.

tem with energy balance regulation in concert with leptin (9, 10). This hypothesis gains further support from our recent data in obese women with polycystic ovary syndrome (PCOS). In fact, we found that this cohort had lower ghrelin levels than expected based on a comparison with an equally sized group of body mass index (BMI)-matched obese women, who were in good health otherwise. Intriguingly, regardless of the presence of PCOS, an impressive negative correlation between ghrelin and androstenedione levels can be consistently observed, suggesting a strong link between synthesis or action of ghrelin and reproductive hormones (12). To further clarify these findings, we decided to investigate, in a separate group of women with PCOS, whether circulating ghrelin levels are influenced by flutamide, an anti-androgenic drug.

MATERIALS AND METHODS

Subjects and protocol

Fourteen obese women with PCOS (general characteristics are shown in Table 1) were enrolled in the study after giving informed consent. The study was approved by the Ethics Committee of the Local Medical Faculty. All subjects were outpatients attending the Endocrine Unit of the Department of Internal Medicine and Gastroenterology of the S.Orsola-Malpighi Hospital of Bologna. No thyroid, renal, liver, or cardiovascular dysfunctions, no previous gastrointestinal surgery and no diabetes [based on an oral glucose tolerance test (OGTT)] (13) were reported or found in the cohort examined.

Table 1 - *Anthropometric parameters and indexes of body fat distribution (measured by CT scan) (mean±SD) at baseline (B) and after 7 months of treatment (A) with hypocaloric diet plus placebo (PLAC) or flutamide (FLUT) in obese women with PCOS.*

Parameters	Time	PLAC	FLUT	*p* value
Age	B	27.0±3.9	26.9±6.3	NS
(yr)	A			
BMI	B	37.6±4.4	34.5±2.4	NS
(kg/m^2)	A	35.2±4.3[b]	31.8±3.1[b]	NS
Waist circumference	B	105±7	97±7	NS
(cm)	A	100±6[b]	92±7[a]	<0.05
TAT	B	581±74	589±63	NS
(cm^2)	A	522±46[b]	464±121[b]	NS
SAT	B	465±59	503±44	NS
(cm^2)	A	426±51[b]	404±105[a]	NS
VAT	B	117±35	86±28	NS
(cm^2)	A	95±14	60±31[b]	<0.05
VAT/SAT	B	0.21±0.06	0.17±0.05	NS
	A	0.18±0.04	0.15±0.08	NS

[a]$p<0.05$, [b]$p<0.01$ for comparison between values after treatment (A) vs baseline (B) within each group.
TAT: total adipose tissue; SAT: sc adipose tissue; VAT: visceral adipose tissue.

The presence of oligo/amenorrhea and hyperandrogenemia, defined by supranormal testosterone (T) levels (reference value in our laboratory 1.42±0.48 nmol/l), and confirmation of a polycystic ovary by ultrasound imaging (14) were the criteria to define the diagnosis. Diseases such as Cushing's syndrome and congenital adrenal hyperplasia were excluded by an overnight dexamethasone test (1 mg) and a quantification of 17-hydroxyprogesterone (17-OHP) levels after a 250 mg Synacthen-test. Body height and weight, waist and hip circumferences were measured as previously described (15). All 14 women presented with a body mass index (BMI) and a waist-to-hip ratio (WHR) greater than 30 kg/m^2 and 0.80, respectively. Body fat distribution was quantified by a standardized measurement at L4-L5 levels by computerized tomography (CT) (Siemens, Erlangen, Germany). Total adipose tissue (TAT), visceral (VAT) and sc adipose tissue (SAT) were measured as previously described (16). No medications were taken and no caloric restriction was made by the patients for at least 3 months before the study. Blood samples for hormones and glucose determination after an overnight fast between 8:00 h and 8:30 h followed by an OGTT [75 g of glucose (Curvosio, Sclavo, Cinisello Balsamo, Italy)] were drawn at the beginning and at the end of the study. At the start of the investigation, all patients were placed on a standardized hypocaloric diet (1200-1400 kcal daily) composed by 50% carbohydrates, 30% lipids and 20% proteins. After one month of dietary therapy, all subjects underwent a second anthropometric evaluation and CT scan. At that time-point of the study, no laboratory investigations were performed because loss of body water might have caused the initial reduction in body weight. In addition, the effects of acute negative energy balance rather than changes in body composition may be responsible for the changes in metabolic and hormonal parameters observed under these conditions (17). After the first month of hypocaloric diet, a treatment with flutamide (250 mg/os, twice daily) or placebo was added according to a randomization open-labeled design. Therefore, 7 patients were assigned to dietary plus flutamide treatment and 7 patients to dietary plus placebo treatment for 6 months. At the end of this period, anthropometrical, biochemical and hormonal (inclusive of an OGTT) parameters were repeated. Moreover, a new CT scan was performed.

Hormone assays and data analysis

Blood samples were immediately chilled on ice, centrifuged and serum or plasma aliquots were frozen at –80 C until assayed. All samples from individual subjects were analyzed in duplicate for each hormone. Plasma glucose levels were determined by the glucose-oxidase method, insulin, testosterone (T), androstenedione (A), sex hormone binding globulin (SHBG), and dehydroepiandrosterone sulphate (DHEA-S) were analyzed as previously described (18). Free T values were obtained by calculation from T and SHBG values following the method proposed by Vermeulen et al. (19). The intra-assay and inter-assay coefficients of variation were less than 3% and less than 10%, respectively. The quantitative Insulin-Sensitivity Check Index (QUICKI) was calculated according to the formula proposed by Mather et al. (20). The Homeostatic Model Assessment applied to the OGTT ($HOMA_{OGTT}$) was determined as suggested by Matsuda et al. (21). Human plasma ghrelin was measured with a commercially available radioimmunoassay (Phoenix

Pharmaceuticals, Mountain View, CA, USA) using ^{125}I-labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against the c-terminal end of human ghrelin. This assay recognizes both acylated and des-acylated ghrelin (22). The antiserum does not cross-react with any relevant peptide as previously shown (22, 23). Intra- and inter-assay CV's were below 5.3 and 13.6%, respectively.

Statistical analysis

Results are reported as the mean values ± standard deviation (SD). Comparisons between the 2 study groups were analyzed at baseline and after 7 months. When significant between-group differences were observed post-treatment, we compared percent change from baseline between the group treated with placebo and the group treated with flutamide. All comparisons were performed by the Student's t-test. Simple and multiple regression analyses were used. Statistical evaluations were done with SPSS software (SPSS Inc. Chicago, IL)/PC + software package on a personal computer. *P* values of less than 0.05 were regarded as statistically significant.

RESULTS

At baseline, there were no significant differences between the randomized treatment groups for age, anthropometric and CT scan parameters (Table 1) or endocrine and metabolic variables (Table 2), confirming that the two cohorts were comparable. Basal ghrelin levels of patients were comparable to those described recently in our previous publication in a larger cohort of patients with PCOS (12). After 7 months of treatment, significant differences between the groups were detectable (Tables 1 and 2). Patients who received flutamide in addition to low calorie diet had smaller waist circumference, less VAT, lower levels of free T and A and higher plasma SHBG than the control group of women with placebo. Analysis of percent change from baseline showed that flutamide therapy resulted in improvements only in free T ($p<0.05$) and A ($p<0.05$). Both treatment groups showed similar changes in BMI, TAT, SAT, fasting insulin and insulin resistance indexes (QUICKI and $HOMA_{OGTT}$). Plasma ghrelin levels significantly increased in women treated with flutamide only, no changes in the placebo group were observed. Ghrelin concentrations in the two groups were significantly different at the end of the therapy (Table 2). Percent change from baseline confirmed that flutamide treatment increased ghrelin circulating levels more than dietary restriction alone ($p<0.01$). A significant negative correlation between percent change in ghrelin and changes of both A and free T was found in the entire population (r: –0.599, $p<0.05$ and r: –0.483, $p<0.05$, respectively). However, when the 2 treated groups were analysed separately, a significant negative correlation between changes of ghrelin and changes of both A and free T was observed only in the group treated with flutamide (Fig. 1A). Furthermore, the variation of plasma ghrelin positively correlated with changes in $HOMA_{OGTT}$ in the group treated with flutamide only, while the group treated with placebo did not show any significant correlation with changes in insulin sensitivity (Fig. 1B). As expected, the 2 groups having similarly modified BMI, no relationship between variation in ghrelin levels and BMI was found. The relationship between percent change in ghrelin and changes in androgens levels persisted even when $HOMA_{OGTT}$ value was taken into account in a multiple regression model (A, *t*: –2.303, $p<0.05$; free T, *t*: –0.206, $p<0.05$). On the other hand, after adjusting for both A and free T, no correlation could be found anymore between ghrelin and $HOMA_{OGTT}$ (*t*: 0.465, p=NS and *t*: 1.233, p=NS respectively), further underlining the close functional relationship between ghrelin and androgens.

Table 2 - *Sex hormones and SHBG blood concentrations, fasting glucose and insulin values, insulin resistance indexes and plasma ghrelin levels (mean±SD) at baseline (B) and after 7 months of treatment (A) with hypocaloric diet plus placebo (PLAC) or flutamide (FLUT) in obese women with polycystic ovary syndrome.*

Parameters	Time	PLAC	FLUT	*p* value
Total testosterone (ng/ml)	B	0.47±0.18	0.73±0.28	NS
	A	0.46±0.22	0.51±0.21[b]	NS
Free testosterone (pg/ml)	B	2.8±0.9	2.8±1.4	NS
	A	2.9±1.4	1.9±0.9[b]	<0.05
Androstenedione (ng/dl)	B	417±110	508±135	NS
	A	439±92	275±105[b]	<0.01
DHEAS (µg/ml)	B	1.5±0.7	2.1±0.8	NS
	A	1.4±0.8	1.3±0.5[b]	NS
SHBG (nmol/l)	B	17.4±5.2	28.7±9.6	NS
	A	18.0±7.2	29.0±10.8	<0.05
Glucose, fasting (mg/dl)	B	96.7±13.5	93.3±4.0	NS
	A	99.6±16.2	97.3±8.5	NS
Insulin, fasting (µU/ml)	B	15.7±11.7	15.6±14.8	NS
	A	7.8±10.6[c]	13.9±13.7[c]	NS
QUICKI	B	0.30±0.02	0.33±0.03	NS
	A	0.32±0.04[c]	0.34±0.04[c]	NS
$HOMA_{OGTT}$	B	2.3±1.4	2.6±1.0	NS
	A	3.5±2.7[c]	4.8±2.9[c]	NS
Ghrelin (fmol/ml)	B	103±22	108±59	NS
	A	103±26	165±71[a]	<0.05

[a]$p<0.05$, [b]$p<0.01$, [c]$p<0.1$ for comparison between values after treatment (A) vs baseline (B) within each group.
QUICKI: Quantitative insulin-sensitivity check Index; $HOMA_{OGTT}$: Homeostatic model assessment applied to the oral glucose tolerance test

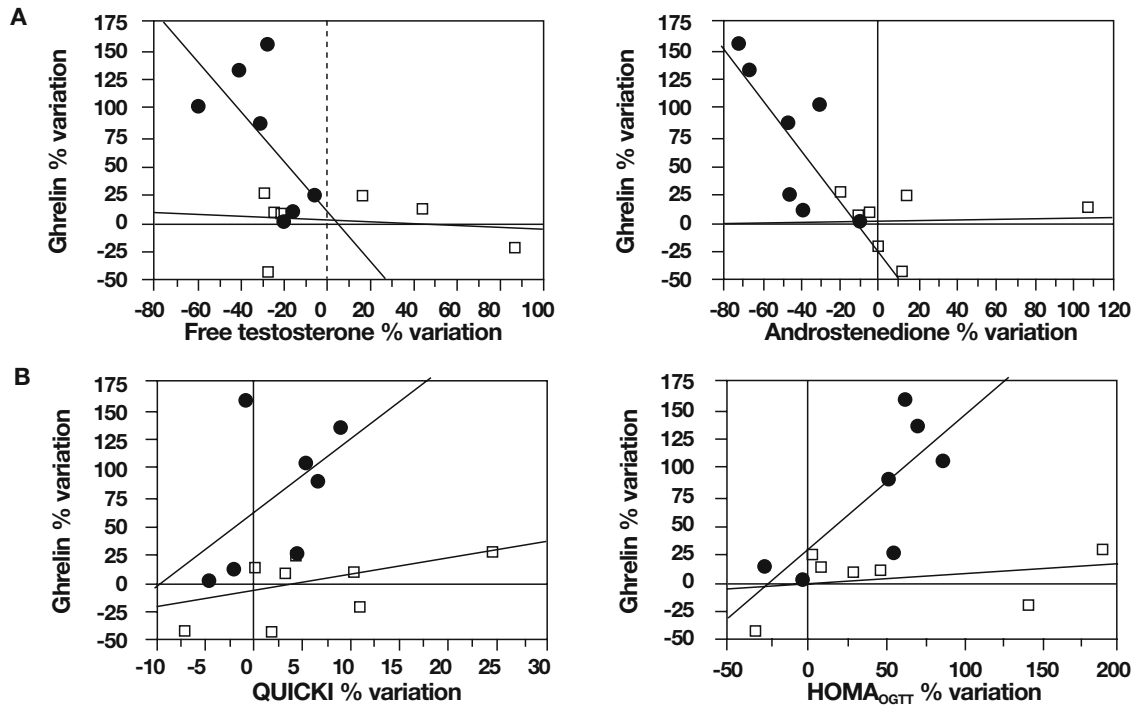

Fig. 1 - *A) Simple correlation coefficients between percent variation of plasma ghrelin and free testosterone and androstenedione during 7 months of therapy, inclusive of a month of hypocaloric diet followed by a 6-month period of hypocaloric diet combined with placebo (PLAC) or flutamide (FLUT) in obese women with polycistic ovary syndrome (PCOS). The relationships between percent variation of plasma ghrelin and free testosterone and androstenedione were statistically significant in obese PCOS women treated with FLUT (free testosterone r: -0.616, p<0.05; androstenedione r: -0.751, p<0.05). No significant relationships between percent variation of plasma ghrelin and free testosterone and androstenedione in obese PCOS women treated with PLAC were found (free testosterone r: -0.149, p=NS; androstenedione r: 0.039, p=NS).*
B) Simple correlation coefficients between percent variation of plasma ghrelin and insulin resistance indexes during 7 months of therapy, inclusive of a month of hypocaloric diet followed by a 6-month period of hypocaloric diet combined with PLAC or FLUT in obese women with PCOS. No significant relationships between percent variation of ghrelin and QUICKI were found in any group (PLAC r: 0.569, p=NS; FLUT r: 0.524, p=NS). The relationship between percent variation of plasma ghrelin and HOMA$_{OGTT}$ were statistically significant in obese PCOS women treated with FLUT (r: 0.767, p<0.05), whereas no significant relationship was found in those treated with PLAC (r: 0.289, p=NS).

DISCUSSION

In our present study, performed on a group of 14 PCOS patients, we found basal ghrelin levels of this new cohort to be very similar to those found in our previous study in 10 obese women with PCOS in which ghrelin was even lower when compared to a group of BMI and age-matched obese women (12). In agreement with recent data reported by another group (24), we therefore conclude that PCOS represents a pathological condition that is characterized by reduced circulating ghrelin levels. PCOS is a well-known epidemiologically relevant pathological condition showing hyperandrogenism, insulin resistance and hyperinsulinemia (25, 26). Frequently, PCOS is associated with obesity, particularly the abdominal phenotype (27). We started to study ghrelin levels in PCOS patients based on the hypothesis that circulating ghrelin levels might be associated with laboratory parameters reflecting insulin resistance. Therefore, based on what was reported previously in patients with severe insulin-resistance (22, 28), we were not surprised to detect a negative correlation, in our cohort of obese women with PCOS, between ghrelin and insulin resistance. However, surprisingly, as we have demonstrated in our previous report, long-term hypocaloric diet, even if associated with an insulin sensitizing agent, metformin, did not significantly affect ghrelin levels, while ameliorating insulin sensitivity (12). While this finding contradicts some earlier reports on an increase in plasma ghrelin levels following weight loss, we assume that the rather modest loss of body

fat in our study did not occur acutely enough to be reflected by significant stimulation of ghrelin secretion. Our data did not show any relationship between ghrelin and BMI. This finding is only apparently in contrast with the tight relationship between these two parameters found in other publications by us and by other Authors (22, 29, 30). In fact, at variance with these studies, in this work we investigated two groups made up of patients all affected by obesity and with similar BMI values. Our present data further confirm that, although both treatments (placebo and caloric restriction or flutamide and caloric restriction) improved insulin sensitivity, plasma ghrelin levels changed significantly in the flutamide group only. This drug was used based on its antagonistic effect at the level of the androgen receptor and its inhibitory influence on 17α-hydroxylase and 17,20-lyase, key enzymes involved in the androgen synthesis (31). Intriguingly, recent published findings already seem to indicate a new potential interactive role of ghrelin with the gonadal axis. In fact, ghrelin binding sites have been found in gonadal tissues (8) and ghrelin is solidly expressed in androgen-producing sites such as Leydig (11) and ovarian hilus interstitial cells (9). This indicates that gonadal tissues may represent a target of ghrelin action but also a site of ghrelin production suggesting possible interactions with steroidogenesis via paracrine or autocrine mechanisms. In support of this view, ghrelin has been shown to reduce the chorionic gonadotropin and the c-AMP induced testosterone secretion in rat testis by inhibiting enzymes involved in the steroidogenesis (10). It is still under debate whether ghrelin may directly interact with androgen synthesis or is rather indirectly modulating androgen secretion by influencing LH secretion and/or pulsatility (32). Anti-androgenic treatment in PCOS may serve as a model to further understand the molecular basis of the counterregulatory relationship between factors controlling energy homeostasis and the reproductive axis.

A solid increase in ghrelin secretion following long-term flutamide treatment represents the most intriguing finding of our study. We therefore propose that androgens are independent determinants of circulating ghrelin levels based on a strong negative influence on ghrelin secretion. However, it may also be possible, even though less likely, that anti-androgenic substances such as flutamide have a direct, androgen-independent, stimulatory influence on ghrelin secretion. Furthermore, it is possible that the modulatory influence of anti-androgens on plasma ghrelin concentrations involves modified ghrelin clearance rates rather than changed ghrelin secretory patterns.

The impact of androgens on ghrelin changes appears to be stronger than the influence of insulin resistance. In fact, our analysis only shows a significant negative correlation between insulin resistance and ghrelin in the group undergoing flutamide treatment. The lack of correlation, as based on the multivariate analysis between plasma ghrelin levels and insulin resistance indexes after adjusting for androgen levels, strongly suggests that sex steroids may be primarily involved in the regulation of circulating ghrelin levels.

While a causal role of ghrelin in the etiology of diseases presenting obesity and insulin resistance (such as PCOS) still remains largely speculative, data derived from the present clinical study suggest that sex hormones modulate circulating ghrelin levels (or even *viceversa*) in humans. In any case, earlier *in vitro* data mentioned above and the *in vivo* results presented in this study further support the existence of a physiologically relevant interplay between hormonal networks controlling the reproductive system and those regulating energy balance.

ACKNOWLEDGMENTS

We thank Dr. A. M. Morselli-Labate for statistical analysis and Ms. Susan West for reviewing the manuscript for English usage. This work was supported by the "Giovani Ricercatori" grant, sponsored by University of Bologna and by the grant offered by the Fondazione Cassa di Risparmio, Bologna, Italy.

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