

**SHORT-TERM MODIFICATION OF SEX HORMONES IS ASSOCIATED
WITH CHANGES IN GHRELIN CIRCULATING LEVELS IN HEALTHY
NORMALWEIGHT MEN**

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LIST OF THE ABBREVIATIONS

CPA: cyproterone acetate

DNG: dienogest

TE: testosterone enanthate

PLAC: placebo

PCOS: polycystic ovary syndrome

HOMA: Homeostasis Model Assessment

ANOVA: Analysis of Variance

ABSTRACT

The aim of this study was to evaluate the effect of selective and short term sex hormone modifications on ghrelin levels in normalweight eugonadal men undergoing hormonal contraceptive treatments. Seven men received an oral progestin (cyproterone acetate-CPA or dienogest-DNG) 10 mg/day for 3 weeks (CPA-DNG group), 7 CPA orally 5 mg/day in association with testosterone enanthate im 200 mg/week for 8 weeks (CPA-TE group) and 7 placebo for 8 weeks (PLAC group). Anthropometry and blood levels of LH, FSH, testosterone, estradiol, glucose, insulin and total ghrelin were evaluated. At baseline, no parameters differed among the three groups. After treatment, LH and FSH decreased in both CPA-DNG and CPA-TE groups, whereas they did not change in the PLAC group. Testosterone, and similarly estradiol, decreased in the CPA-DNG group to the hypogonadal range, increased in the CPA-TE group to supraphysiological concentrations and, as expected, remained unchanged in the PLAC group. Total ghrelin levels increased in the CPA-DNG, decreased in the CPA-TE and did not change in the PLAC group. No significant modifications in the other parameters were observed in any group, demonstrating that the short-term changes of circulating sex hormones are able to modify ghrelin levels.

These data therefore suggest that sex steroids are important regulators of ghrelin also in normalweight healthy men.

INTRODUCTION

Recent studies have suggested that androgens and ghrelin may reciprocally cross-talk. Gonadal tissues have in fact been proposed as a target for ghrelin action based on a high number of binding sites in both ovary and testis (1, 2). Studies in rats have shown that ghrelin reduces the hCG- and cAMP-induced testosterone secretion, in a dose-dependent manner, by down-regulating key enzymes of steroidogenic pathways (3). In addition, several data support the notion that gonadal tissues are also relevant sites of ghrelin production (1, 2, 4, 5) and that testosterone is a powerful regulator of ghrelin receptor expression (6). A series of recent studies performed by our group have strongly supported the concept that androgens may play an important role in the regulation of ghrelin blood concentrations also in humans. In particular, lower total ghrelin levels than those expected based on body weight were described in a group of obese women affected by the polycystic ovary syndrome (PCOS), a condition characterized by a hyperandrogenic state, and a highly significant negative correlation was found between total ghrelin and circulating androgens in both PCOS and obese controls (7). Subsequently, the interplay between androgens and ghrelin was confirmed by a further controlled follow-up study in a separate group of obese PCOS women demonstrating that long-term treatment with an anti-androgen agent, flutamide, significantly increased circulating total ghrelin levels, regardless of changes in body weight, fat topography and insulin sensitivity (8). The strong relationship between androgens and total ghrelin was also supported by the finding that overweight/moderately obese hypogonadal patients had significantly lower circulating total ghrelin levels than those observed in weight-matched and normalweight controls and that testosterone replacement therapy for six months restored ghrelin values to the normal range, regardless of changes in body mass, body fat and insulin sensitivity (9).

To further corroborate our hypothesis of the existence of a regulatory role of androgens on ghrelin, we examined ghrelin variations in a group of normalweight healthy eugonadal men undergoing two different hormonal contraceptive regimens in which a selective modification of sex hormones, in excess or in defect, occurred.

MATERIALS AND METHODS

Subjects and study design

This study was performed in the same subjects recruited in two previous pilot studies that we carried out to develop optimal hormonal male contraceptive regimens (10, 11). Briefly, two different treatments were administered, one consisting of a progestin such as cyproterone acetate (CPA) or dienogest (DNG) and the other of a progestin (CPA) plus testosterone enanthate (TE). In fact, when a progestin is administered at high dosages, a suppression of gonadotropins is produced with a consequent sperm suppression and a pathological decrease of testosterone levels to hypogonadal states (10). Moreover, a direct antiandrogenic activity has been hypothesized for DNG and CPA (10). Since previous studies have shown a similar ability of both CPA or DNG to suppress testosterone and gonadotropins (10), subjects treated with these two progestins were analysed together. In the second study (11), TE was combined with the progestin CPA, since it was previously found that the association of androgens and progestins seems to represent the most promising regimen to achieve optimal spermatogenic suppression and to avoid the hypogonadal status induced by the administration of the progestin alone. However, the high dose of TE administered in the study to further improve sperm suppression induced supraphysiological testosterone circulating levels (11).

The present study involved 21 men, aged 21-49 years, attending the Clinic of Obstetrics and Gynecology at the S. Orsola-Malpighi Hospital of Bologna in the frame of volunteers for hormonal contraception protocols in men. Enrolment criteria included an uneventful medical history, normal physical examination, normal routine blood chemistry, basal serum LH, FSH and testosterone levels within the normal range and normal seminal analysis according to the criteria established by the WHO (12). All volunteers signed a consent form to participate in the trial. The study was approved by the ethics committee of S. Orsola-Malpighi Hospital.

At baseline, all subjects underwent a physical examination and a blood analysis for hormone and biochemical measurements. Therefore, 7 subjects received an oral progestin, 5 of them CPA and 2

of them DNG, at a daily dose of 10 mg (CPA-DNG group) (10), 7 received CPA orally at a daily dose of 5 mg plus testosterone enanthate (TE) intramuscularly at the dose of 200 mg every week (CPA-TE group) (11) and 7 received placebo (PLAC group). Blood samples were taken and clinical data were recorded at weekly intervals throughout the study for checking the appearance of side effects. Samples and physical examinations at week 3 in the CPA-DNG group and at week 8 in both the CPA-TE and PLAC groups were selected for measurements in this study. The blood samples in the CPA-TE group were drawn the day after the administration of TE im.

All subjects were invited to maintain their regular diet during the study period.

Measurements

Physical examination included blood pressure, height, weight and waist circumference measurements. Body height was measured without shoes and body weight was assessed without clothes. Body fat distribution was evaluated by waist circumference that was obtained as the minimum value between the iliac crest and the lateral costal margin, using a 1-cm-wide metal measuring tape. Measurements were performed in each blood sample for reproductive hormones (LH, FSH, total testosterone, estradiol) and biochemical parameters (glucose and insulin), by assays according to previously described procedures (10, 11). Plasma glucose levels were determined by the glucose-oxidase method and insulin was analyzed as previously described (13). The Homeostasis Model Assessment (HOMA) insulin resistance index was calculated as proposed by Matthews et al (14). Human plasma ghrelin was measured with a commercially available radioimmunoassay (Phoenix Pharmaceuticals, Mountain View, CA, USA) using ¹²⁵I-labeled bioactive ghrelin as a tracer and a rabbit polyclonal antibody raised against C-terminal end of human ghrelin. This assay recognizes both acylated and des-acylated ghrelin (15). No cross-reactivity was found with most relevant peptides, as already previously described (15, 16). Intra- and inter-assay C.V.'s were below 5.3% and 13.6%, respectively.

Statistical analysis

Data are reported as mean values \pm SD, unless stated indicated. The variations of each parameter observed during the different therapies are expressed as Δ values, calculated as the difference (Δ) between the values obtained at the end of the treatments and those present in basal conditions.

Statistical analysis was performed by using the Analysis of Variance (ANOVA). All statistical analyses including correlation and multiple regression analysis were performed using StatView software (Abacus Concepts Inc., Berkeley, CA, U.S.). P values of less than 0.05 were regarded as statistically significant.

RESULTS

At baseline, there were no significant differences between the three groups in age, anthropometric, hormonal and metabolic parameters (Table 1). Body weight and waist circumference did not significantly change throughout the study period in any of the groups (Table 1). As expected, LH and FSH were significantly suppressed by CPA-DNG ($p < 0.01$ and $p < 0.001$, respectively) and CPA-TE ($p < 0.001$ for both) administration, whereas they remained unchanged after PLAC treatment (Table 1). However, changes in LH and FSH levels were significantly greater in the CPA-TE group than in the CPA-DNG group ($p < 0.01$). In the CPA-DNG group testosterone levels decreased to less than 2.5 ng/mL in all subjects, therefore within the hypogonadal range; whereas, in the CPA-TE group, testosterone significantly increased to supraphysiological concentrations, reaching circulating levels greater than 10 ng/mL ($p < 0.01$) (Figure 1). Estradiol changes during the CPA-TE and CPA-DNG treatment paralleled those of testosterone, being significantly increased in the former ($p < 0.01$) and significantly decreased in the latter ($p < 0.001$) (Figure 1). As expected, neither testosterone nor estradiol levels changed significantly during PLAC treatment (Figure 1).

Fasting glucose and insulin values and the HOMA index at the end of the treatments did not significantly change with respect to basal conditions in any of the groups (Table 1). However, the tendency toward an increment, although not significant, of fasting insulin and HOMA in the CPA-

TE group led to a significant differences in these two parameters among the three groups at the end of the treatment period ($p < 0.05$) (Table 1).

Basal total ghrelin levels were similar among the three groups, whereas significant differences were detectable after treatment ($p < 0.01$) (Figure 2). In fact, patients who received CPA-DNG significantly increased total ghrelin plasma levels ($p < 0.05$), which were conversely decreased in the group receiving CPA-TE ($p < 0.05$), without any significant changes in the PLAC group (Figure 2).

When all subjects were considered as a single group, no significant correlations were found between variations (expressed as Δ values) of total ghrelin and those of LH ($r: 0.213$, p NS), FSH ($r: 0.117$, p NS), fasting insulin ($r: -0.078$, p NS) and HOMA index ($r: -0.082$, p NS). However, importantly, total ghrelin Δ value variations were negatively correlated with those of both testosterone ($r: -0.637$, $p < 0.01$) and estradiol ($r: -0.479$, $p < 0.05$).

DISCUSSION

The rationale to examine ghrelin variations in a cohort of young normalweight eugonadal men undergoing short term hormonal contraceptive regimens was first based on the notion that significant changes in sex hormone levels can be achieved with these regimens, and secondly that in the short term these hormonal changes are probably insufficient to significantly modify body composition and body fat distribution (17), which are important factors regulating ghrelin circulating blood levels (15, 16). Therefore, the short term hormonal changes induced in this study were considered ideal to potentially separate ghrelin changes due to sex hormone variations from those that can be attributed to modifications in body mass and fat distribution, usually accompanying sex hormone variations in the long term, as can be observed in females with PCOS (7, 8) and in males with hypogonadism (9). Accordingly, we did not find any significant modification in anthropometric parameters in any of the groups investigated in this study. At variance, however, we did find a tendency toward a reduced insulin sensitivity in the group

receiving CPA-TE, which implies that supraphysiological testosterone levels may induce insulin resistance, even in the short-term period, as previously reported (18).

Basal ghrelin levels detected in all the groups of normalweight, eugonadal men investigated in this study were similar to those found in weight- and age-matched male subjects tested in a previous study (9), confirming that these subjects were representative of the general young male population. Moreover, as previously reported (7-9), we were able to demonstrate that changes in the androgen status were followed by significant modifications of ghrelin blood levels. In particular, the hypogonadal state induced by the administration of progestins (CPA or DNG) produced a significant increase in plasma ghrelin, whereas supraphysiological increases of testosterone levels, like those observed following the CPA-TE treatment, lead to a decrease of circulating ghrelin levels, which is consistent with the hypothesis that sex hormones may regulate ghrelin. However, the increase of ghrelin after short-term induction of a hypogonadal state in subjects treated with the CPA-DNG regimen represented an unexpected finding, since we previously showed that ghrelin concentrations in men with long-term hypogonadism were characterized by lower rather than increased circulating ghrelin (9). This discrepancy can probably be explained by the fact that chronic hypogonadism is characterized by several changes in body composition, including increased fat mass (particularly visceral fat), decreased lean body mass and several metabolic abnormalities, chiefly insulin resistance and associated hyperinsulinemia (17). On the contrary, we found that neither body weight, nor insulin levels and insulin sensitivity (measured by the HOMA index) significantly increased after the two months of CPA-DNG treatment, probably because of the short-term duration of the hypogonadal status. Conversely, the increase of ghrelin levels in the CPA-DNG group appeared to be related to changes in sex hormones.

The mechanisms by which sex hormones, particularly androgens, may regulate ghrelin expression and secretion are still poorly defined. Moreover, it is still undefined whether their regulatory capacity could be influenced by the interference of other hormones and factors. No transport proteins have yet been reported for ghrelin, so the possibility that androgens regulate ghrelin by

interacting with its transport system is still speculative. On the contrary, as previously suggested (15), the putative role of insulin requires considerations. Insulin has in fact an inhibitory capacity on ghrelin expression and secretion, thereby reducing circulating ghrelin (19-21). In previous studies in both hyperandrogenic women with PCOS (7, 8) and hypogonadal men (9), we found a significant negative correlation between circulating insulin and ghrelin levels that, however, became insignificant after adjusting for androgen levels. In this study, a trend toward an increase of both insulin levels and insulin resistance was found in subjects receiving CPA-TE treatment, although no significant correlations were found between changes of ghrelin and insulin parameters. Moreover, as discussed above, insulin concentrations and the HOMA index did not significantly change after the achievement of a short-term hypogonadal state. How insulin affects plasma ghrelin concentrations remains to be established, although previous studies have suggested that ghrelin synthesis and secretion appears to be very sensitive to insulin action, particularly in the post-prandial state (21). Our data are not consistent with the relevant regulatory role of physiological basal insulin levels on ghrelin. We therefore speculate that the effects of androgens on ghrelin cannot be justified on the basis of insulin action and that other factors associated with chronic hyperandrogenism in women or chronic hypogonadism in men are probably involved to explain this phenomenon. They may involve both excess body fat or sustained insulin resistance state, just as occurs in women with PCOS (7, 8) and in men with long-term hypogonadism (9). The net increase of ghrelin concentrations in the CPA-DNG group remains unexplained based on the present knowledge, nevertheless it does not appear to be related to changes in insulin and body composition; it might instead reflect an acute adaptative phenomenon to compensate the reduced sex hormone availability.

Other factors potentially involved in the regulation of ghrelin were measured in this study. In fact, it has previously been suggested that gonadotropins may be involved in the regulation of ghrelin (1). Although a single measurement of LH and FSH may limit the reliability of the data, this possibility still appears unlikely. In fact, low levels of LH and FSH were observed both in the subjects with

low plasma ghrelin and in those with high circulating ghrelin levels and no significant correlations were found between changes of ghrelin and gonadotropins induced by the treatments. This is further supported by our previous findings demonstrating that the correction of hypogonadism by adequate testosterone replacement in a group of both hypergonadotropic and hypogonadotropic hypogonadal men restored ghrelin levels to normal values regardless of the gonadotropin status (9). Beside androgens, estrogens may represent potential additional factors involved in the regulation of ghrelin. Studies performed in rats (5, 22) and humans (23, 24) have in fact shown that estrogens may stimulate gonadal ghrelin expression and blood concentrations. In this study we showed that changes of estradiol paralleled those of testosterone and that both were inversely related to those occurring on ghrelin concentrations, raising the question as to whether the effect of testosterone on ghrelin is direct or mediated by estrogens themselves. Studies are in progress to clarify these possibilities. Finally, both the CPA-DNG and the CPA-TE treatments included progestin, so that a direct effect of progestins on ghrelin should be ruled out. This possibility has been suggested by one study performed in pregnant women (24), in whom plasma ghrelin concentrations were markedly decreased during the third trimester when a significant increment of progestin was present. This effect could be dose-dependent, therefore explaining the major impact observed in the CPA-DNG group, in which higher doses of progestins were administered, compared to the CPA-TE group. Alternatively, it cannot be excluded that the high dosage of testosterone administered in association with progestin only in the CPA-TE group could have counteracted the effect of progestin, since it has previously been found that TE given in combination with CPA led to an impairment of sperm suppression with respect to the group treated by CPA alone at the same dose, with no difference in serum gonadotropin levels between the two groups, indicating that testosterone may antagonize progestin actions (11).

In conclusion, the data reported here suggest that androgens play a regulatory role on circulating ghrelin levels even in the normalweight healthy men undergoing different hormonal contraception

regimens inducing a short-term defect or excess of androgens. Whether their mechanisms are direct or involve still undefined additional factors is a matter for further investigation.

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Legend to Figure 1.

Testosterone (left) and estradiol (right) levels at baseline and after therapy with placebo (PLAC) (ν), cyproterone acetate or dienogest (CPA-DNG) (μ) or cyproterone acetate plus testosterone enanthate (CPA-TE) (σ) in normal men. P values refers to changes observed within each group versus basal values. P values referring to the comparison among all 3 groups at baseline and at the end of the therapies were NS and < 0.0001 for all these parameters, respectively.

To convert testosterone to nanomoles/liter, multiply by 3.467; to convert estradiol to picomoles/liter, multiply by 3.671.

Legend to Figure 2.

Total plasma ghrelin concentrations at baseline and after therapy with placebo (PLAC) (ν), cyproterone acetate or dienogest (CPA-DNG) (μ) or cyproterone acetate plus testosterone enanthate (CPA-TE) (σ) in normal men. P values refer to changes observed within each group versus basal values. At baseline, ghrelin levels among the groups were similar, whereas values after treatment differed significantly ($p < 0.01$).

Table 1.

Anthropometric, hormonal, and metabolic parameters at baseline and after therapy with placebo (PLAC group), cyproterone acetate or dienogest (CPA-DNG group) or cyproterone acetate plus testosterone enanthate (CPA-TE group) in normal men.

<i>Variable</i>	<i>Time</i>	<i>PLAC group</i>	<i>CPA-DNG group</i>	<i>CPA-TE group</i>	<i>P-value</i>
Age (years)	Baseline	27.6±1.6	28.2±1.9	27.1±1.4	NS
Weight (kg)	Baseline	72.0±10.0	69.8±4.7	70.4±8.7	NS
	After	73.5±10.1	68.2±3.6	70.4±8.2	NS
BMI (Kg/m ²)	Baseline	22.4±1.9	21.6±1.7	23.9±2.7	NS
	After	22.9±2.3	21.1±1.5	23.9±2.5	NS
Waist circumference (cm)	Baseline	81.8±7.6	80.6±7.7	83.8±7.7	NS
	After	83.4±7.1	81.1±7.5	83.9±7.5	NS
LH (IU/liter)	Baseline	4.25±0.88	3.76±0.77	3.59±1.44	NS
	After	4.23±0.96	2.09±0.89 ^a	0.03±0.02 ^b	< 0.0001
FSH (IU/liter)	Baseline	2.67±0.93	2.06±0.73	2.81±1.08	NS
	After	2.68±0.84	0.73±0.27 ^b	0.04±0.03 ^b	< 0.0001
Glucose, fasting (mg/dL)	Baseline	78.9±5.5	74.4±6.3	81.4±11.9	NS
	After	79.0±9.3	81.0±9.7	81.8±5.4	NS
Insulin, fasting (μU/mL)	Baseline	5.86±2.97	4.43±1.81	5.86±2.54	NS
	After	4.43±1.39	5.00±1.91	7.00±2.24	< 0.05
HOMA	Baseline	1.03±0.52	0.73±0.29	1.08±0.53	NS
	After	0.77±0.20	0.91±0.43	1.27±0.38	< 0.05

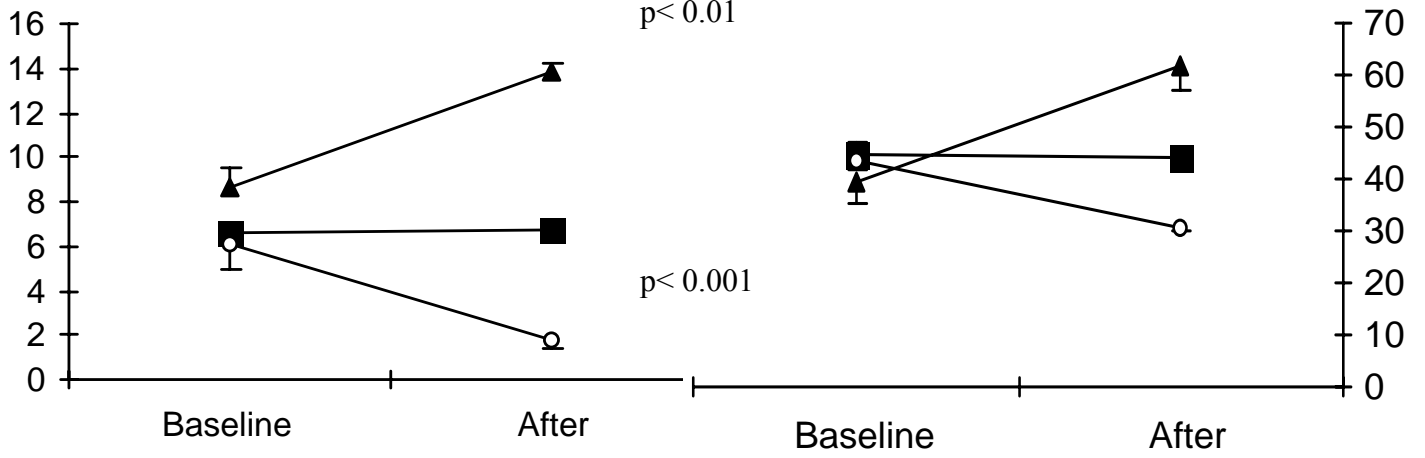
To convert glucose to millimoles/liter, multiply by 0.056; to convert insulin to picomoles/liter, multiply to 7.175.

^a p< 0.01, ^b p< 0.001 for comparison between values after treatment vs baseline within each group.

P values refer to the comparison among all 3 groups at baseline and at the end of the therapies.

Testosterone (ng/mL)

Estradiol (ng/mL)



Total ghrelin (fmol/mL)

