Obestatin Modulates Ghrelin's Effects on the Basal and Stimulated Testosterone Secretion by the Testis of Rat: an In Vitro Study

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Received March 11, 2016

Accepted June 21, 2016

On-line October 26, 2016

Summary

The functional antagonism between obestatin and ghrelin in the testis is under investigation. We investigated the ability of obestatin to counteract the inhibitory effect of ghrelin on basal and stimulated testosterone (T) secretion *in vitro*. Testicular strips from adult rats were incubated with 10 ng/ml and 100 ng/ml of obestatin alone, ghrelin alone and obestatin + ghrelin. Obestatin modulation of stimulated T secretion was evaluated by incubation of testicular samples with 10 ng/ml and 100 ng/ml obestatin, ghrelin and obestatin + ghrelin in the absence and presence of 10 IU of human chorionic gonadotrophin (hCG). T concentrations in the hCG treated groups were significantly ($P < 0.0001$) higher than those in the control groups. Obestatin caused a significant increase in basal T secretion in a dose-dependent manner; however, obestatin at the both 10 ng/ml and 100 ng/ml significantly ($P < 0.0001$) increased hCG-stimulated T secretion. In contrast, ghrelin in a dose-dependent manner significantly ($P < 0.001$) decreased both basal and hCG-induced T secretion by testicular slices. Obestatin opposed the inhibitory effect of ghrelin on T secretion under both basal and hCG-stimulated conditions at all doses tested. In conclusions, administration of obestatin was able to antagonize the inhibitory effect of ghrelin on testosterone secretion *in vitro*.

Key words

Obestatin • Ghrelin • hCG • Testosterone • Testicular strips • Reproduction

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Introduction

In mammals, gonadal function critically relies on a complex regulatory network of autocrine, paracrine and endocrine signals. Although it has been known that conditions of negative energy balance are frequently linked to lack of puberty onset and reproductive failure, the exact mechanisms involved in the coupling of reproductive function and body energy store have not been elucidated (Fernández-Fernández *et al.* 2004). Central and peripheral endocrine signals that are primarily involved in the control of energy balance, also, control reproductive functions by acting at different levels of the hypothalamic-pituitary-gonadal axis, thus providing a basis for the link between energy homeostasis and fertility (Fernández-Fernández *et al.* 2006).

Ghrelin, a 28-amino-acid peptide that is characterized as the endogenous ligand of the growth hormone (GH)-secretagogue receptor (GHS-R), is an orexigenic peptide and a long-term regulator of energy homeostasis (Yang *et al.* 2008, Howard *et al.* 1996). Obestatin, the counterpart of ghrelin, is a 23-amino acid

anorexigenic peptide. It is produced by the enzymatic cleavage of pre-pro-ghrelin (Kojima *et al.* 1999, Caminos *et al.* 2003). Ghrelin and GHSR-1a has been localized in reproductive tissues, including the placenta, ovary and testis (Tena-Sempere *et al.* 2002). Within the testis, expression of ghrelin has been reported in Leydig cells (Barreiro *et al.* 2002). However, it is expressed in Sertoli cells in humans (Gaytan *et al.* 2003). Similarly, expression of obestatin has been reported in Leydig cells of the testis in rodents. Obestatin plays a functional role in the regulation of gastrointestinal and metabolic function through interaction with a member of the receptor family that includes receptors for ghrelin and motilin (McKee *et al.* 1997, Nogueiras *et al.* 2007, Kojima *et al.* 1999). Obestatin and ghrelin are functional antagonists of each other, as ghrelin facilitates food intake while obestatin suppresses food intake (Gualillo *et al.* 2003).

An *in vitro* experiment reported that obestatin antagonized the actions of ghrelin on GH secretion (Zizzari *et al.* 2007). It is evident that different factors with key roles in the growth axis and body weight homeostasis are potentially involved, in part, in the regulation of reproductive function in a paracrine or autocrine manner (Caminos *et al.* 2003). Concerning the involvement of obestatin in the reproductive functions is still scarce; however, it was found that obestatin significantly increased progesterone secretion in cultured porcine ovarian granulosa cells. Moreover, in adult male rats, it was reported that obestatin could induce testosterone secretion both *in vivo* and *in vitro* (Jahan *et al.* 2013, Jahan *et al.* 2011, Hizbullah and Ahmed 2013). On the contrary, ghrelin delays balano-preputial separation, an external sign of pubertal development, and decreases circulating luteinizing hormone (LH) and testosterone concentration (Martini *et al.* 2006). Therefore, this study was conducted to explore the probable effects of obestatin in modulating the inhibitory effects of ghrelin on basal and stimulated testosterone secretion in isolated strips of rat's testes.

Materials and Methods

Animals

Adult (125-135 days old) male Sprague Dawley rats (250-290 g) were used in accordance with an experimental protocol approved by the ethics committee of the College of Applied Medical Sciences, King Saud University. Animals were caged under

standard conditions of light (12 h light/12 h dark) and temperature (22-25 °C). These animals were acclimatized for three days with free access to food.

Tissue incubation

Assessment of the direct intra-testicular effect of obestatin and ghrelin upon basal and stimulated testosterone secretion *in vitro* was carried out by incubating adult rat testicular slices, as previously described (Tena-Sempere *et al.* 1999, Hizbullah and Ahmed 2013) with slight modifications. Based on our earlier findings that obestatin is a positive modulator of testosterone secretion and its effect depends on nutritional status, testicular tissues were obtained from normally-fed adult rats (n=9/treatment group) in the morning (8-9 AM) after overnight fasting (Jahan *et al.* 2011, Hizbullah and Ahmed 2013). The animals were decapitated, and the testes were then immediately removed from the scrotal sac and de-capsulated. Later, the testes were rapidly sliced into small pieces (approx. 100 mg) on an ice-cold glass plate. They were weighed and finally poured into 10 ml culture tubes containing DMEM/HEM F12 (1:1 ratio) medium (Hyclone, Thermo Scientific, Inc. USA) supplemented with 50 IU/ml penicillin and 50 µg/ml streptomycin. After 30 min of pre-incubation, the culture media in each tube were replaced with fresh media containing obestatin (mouse/rat, PGH-3891-PI, Peptides International, USA) or ghrelin (mouse/rat, Ana Spec USA) (supplemented with Aprotinin 500,000 KIU/l and disodium EDTA 1 g/l) or combinations of both peptides at the doses of 10 ng/ml and 100 ng/ml (Ob10, Ob100, Gh10, Gh100, Ob10+Gh10 and Ob100+Gh100 groups, respectively). The control group was replaced with only fresh media. Then, tissue cultures were preserved in 10 ml culture tubes under 5 % CO₂ and 95 % air at 34 °C. In order to evaluate the ability of obestatin and ghrelin to modulate stimulated testosterone secretion, testicular tissues were incubated with 10 IU Human chorionic gonadotropin (hCG) (Gonachore) alone in the medium (hCG control group). In addition to incubation with different doses of obestatin, ghrelin and obestatin plus ghrelin at the doses of 10 ng/ml and 100 ng/ml (Ob10+hCG, Ob100+hCG, Gh10+hCG, Gh100+hCG, Ob10+Gh10+hCG and Ob10+Gh100+hCG groups, respectively). At the end of the incubation period, culture tubes were placed in a vortex mixer and aliquots of 100 µl were collected for testosterone measurement. The aliquots were stored at -20 °C until the assay. The levels of testosterone in the

samples were expressed as normalized values per milligram of incubated tissue.

Hormone analysis

Testosterone concentrations were determined using specific EIA kits (Abcam plc, USA) according to manufacturer's instructions provided along with the kit.

Statistical analysis

Values are expressed as means $\pm$ SEM. The results from testicular incubations were analyzed for statistically significant differences among study groups by using one-way ANOVA with post-hoc Tukey's test on Graph Pad Prism 5 software.

Results

Stimulation of basal and hCG-induced T secretion by obestatin

In a previous laboratory work obestatin at 10^{-8} M showed significant increase in testosterone secretion *in vitro* (Jahan *et al.* 2013, Jahan *et al.* 2011, Hizbullah and Ahmed 2013). In the present investigation for

experimental internal reference, obestatin effect on testosterone secretion at 10 ng/ml and 100 ng/ml was tested under both basal and hCG-stimulated conditions. The hCG (10 IU) hormone induces a significant increase in T concentration from testicular slices after 4 h of incubation compared to an untreated control group (14.00 ± 0.50 vs. 9.43 ± 0.57 ng/ml, 100 mg of tissue, respectively, $P < 0.05$). This indicates that testicular tissues under *in vitro* culture conditions were responsive to hCG. Obestatin further induced hCG-stimulated T secretion in a dose-dependent manner, and significant increases in testosterone secretion were measured after 10 ng/ml and 100 ng/ml of obestatin treatment of hCG-exposed testicular tissues ($P < 0.05$ and $P < 0.001$, respectively). On the other hand, obestatin at 10 ng/ml failed to modify basal T secretion, whereas at the higher tested dose (100 ng/ml), it significantly induced basal testosterone secretion $P < 0.05$ (Table 1). These results showed that obestatin modifies the basal level of T release *in vitro* in a dose-dependent manner; however, it stimulates hCG-induced T secretion under both tested doses with approximately equal potency.

Table 1. Mean and SEM of testosterone concentration in *in vitro* testicular culture after 4 h of incubation (n=9) in different treatment groups.

Treatments	T concentration ng/ml 100 mg of tissue	% Reduction in T secretion	% Increase in T secretion
Control	9.43 $\pm$ 0.19	-	-
Obestatin 10 ng/ml	10.46 $\pm$ 0.17	-	10.92
Obestatin 100 ng/ml	11.70 $\pm$ 0.23	-	24.07*, c
Ghrelin 10 ng/ml	7.69 $\pm$ 0.32	19.39*, c	-
Ghrelin 100 ng/ml	5.57 $\pm$ 0.20	40.93***, c	-
Obs+Ghr 10 ng/ml	9.88 $\pm$ 0.21	-	44.44***, a
Obs+Ghr 100 ng/ml	8.83 $\pm$ 0.13	-	58.52***, b
hCG stimulated			
hCG Control (10 IU)	14.00 $\pm$ 0.12	-	48.46***, c
Obs 10 ng/ml+hCG	16.38 $\pm$ 0.19	-	17.0*, hc
Obs 100 ng/ml+hCG	19.64 $\pm$ 0.18	-	40.27***, hc
Ghr 10 ng/ml+hCG	10.75 $\pm$ 0.21	23.22*, hc	-
Ghr 100 ng/ml+hCG	8.67 $\pm$ 0.19	38.1***, hc	-
Obs+Ghr 10 ng/ml+hCG	13.97 $\pm$ 0.13	-	29.9***, ha
Obs+Ghr 100 ng/ml+hCG	13.24 $\pm$ 0.22	-	52.7***, hb

* $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, c – compared to untreated control, hc – compared to hCG control, a – compared to ghrelin 10 ng/ml, b – compared to ghrelin 100 ng/ml, ha – compared to ghrelin 10 ng/ml plus 10 IU hCG, hb – compared to ghrelin 100 ng/ml plus 10 IU hCG, - indicate not applicable for particular analysis.

Inhibition of basal and hCG-stimulated T secretion by ghrelin

In a dose-dependent manner, ghrelin significantly inhibited basal T secretion at the dose of 10 ng/ml and 100 ng/ml ($P < 0.05$ and $P < 0.0001$ respectively). The addition of ghrelin to the hCG-stimulated culture media at concentrations of 10 ng/ml and 100 ng/ml significantly inhibited hCG-stimulated T release by testicular slices (10.75 ± 0.192 ng/ml, and 8.67 ± 0.556 ng/ml, respectively, vs. 14.00 ± 0.50 ng/ml in control group). This shows that ghrelin significantly decrease both basal and hCG-induced T secretion compared to corresponding control groups (Table 1).

Obestatin counteracts the suppressive effect of ghrelin on both basal and hCG-induced testosterone secretion

Treatment of the testicular tissue cultures with obestatin (in the doses of 10 ng/ml and 100 ng/ml) reverses the suppressive effect of ghrelin on testosterone secretion under both basal and hCG-stimulated conditions. The mean testosterone concentrations measured in both the 10 ng/ml and 100 ng/ml treated groups were more or less similar to those of the control group, indicating that obestatin modulated the suppressive effect of ghrelin under basal conditions. Then, we administered the combined doses of both peptides to the culture treated with 10 IU hCG; obestatin at both tested doses significantly increased testosterone concentration compared to ghrelin-alone treated groups, and the mean testosterone concentration in combination-treated groups raised up to the level of hCG control group.

Discussion

The previous findings and receptor co-localization of obestatin in various testicular cells along with ghrelin prompted us to get insight into the opposing effects of the two peptides on reproduction. Therefore, we designed an *in vitro* study to demonstrate the effect of co-administration of obestatin and ghrelin on both basal and stimulated testosterone production.

The role of obestatin in the male reproductive system is still not well studied despite the presence of obestatin expression in various testicular cells. Within the testis, obestatin immunoreactivity (irOBS) is detected in the Leydig and Sertoli cells, whereas, mild signals of obestatin were observed in the rat testis; efferent ductules

were the most immune reactive region for the peptide. Vas deferens and seminal vesicles showed intense obestatin labeling; in addition, obestatin expression was observed in the prostate tissue. Ejaculated and selected spermatozoa were positive for obestatin in different head and tail regions (Dun *et al.* 2006, Moretti *et al.* 2013).

Previous laboratory investigations showed that, a single intravenous injection of obestatin increased testosterone secretion in adult male rats, whereas chronic infusion of obestatin to the rats at the onset of puberty led to a significant increase in testosterone production and spermatogenesis. Furthermore, a study of the direct effect of obestatin on testicular levels *in vitro* reveals that obestatin is a positive modulator of testosterone secretion and its effect is dependent on the nutritional status of the body (Jahan *et al.* 2013, Jahan *et al.* 2011, Hizbullah and Ahmed 2013).

Our hypothesis states that obestatin acts as a physiological antagonist for ghrelin regarding basal and stimulated T secretion. In order to evaluate whether obestatin can modulate ghrelin's suppression of basal and hCG-induced T secretion from adult male rats *in vitro*, we co-administered obestatin and ghrelin into the culture medium. Surprisingly, it was observed that addition of obestatin to culture medium reversed the inhibitory effect of ghrelin on basal and hCG-induced T secretion in a dose-dependent manner, as the testosterone concentration was significantly higher in the 100 ng/ml obestatin plus ghrelin treatment group than in the ghrelin-alone-treated group and the mean concentration in the co administered group was more or less similar to that of the untreated control group. In order to evaluate the effect of obestatin on ghrelin-induced suppression of hCG-stimulated testosterone secretion, testicular tissues in the culture medium were exposed to 10 ng/ml and 100 ng/ml, obestatin and ghrelin along with 10 IU hCG and hCG alone treatment group serve as control. Similar observations as basal effects were recorded under hCG-stimulated conditions herein effect of obestatin seems more pronounced in reversing the ghrelin's inhibitory effect on hCG-stimulated testosterone secretion. Results of this study, indicate that the effect of obestatin seems to be hCG-dependent as more pronounced effects seems to occur under stimulated conditions relating hypophyseal-pituitary-gonadal axis implication in controlling obestatin actions. However it is not clear whether the effect of obestatin is at a local gonadal level or is regulated by upstream targets. In the present experiments, we used both obestatin and ghrelin

alone treatment groups under both basal and hCG-stimulated conditions as an experimental internal reference in order to clarify the effect of combined signal peptide administration. This study extends the previous findings that in addition to the opposite effects of both obestatin and ghrelin on food intake, body weight, body composition and energy expenditure, obestatin also antagonizes the actions of ghrelin on testosterone secretion from adult rat testicular slices when both peptides are co-administered. Ghrelin negatively modulate testicular functions under low energy states while the opposite effect of obestatin on the gonads has been hypothesized (Dun *et al.* 2006, Moretti *et al.* 2013). Nevertheless, data concerning the physiological functions of obestatin are limited and are mainly in regard to its role in controlling feeding behavior, the functions of the gastrointestinal tract and energy homeostasis at the hypothalamic level (Zhang *et al.* 2005), while its role in the regulation of reproduction remains less characterized. We analyzed the involvement of this metabolic hormone in the direct control of testicular functions. Compelling evidence indicates that common regulatory signals are implicated in the integrated control of energy balance and reproduction (Tena-Sempere *et al.* 2002). The suggestion

of a direct effect of obestatin on testicular tissue was supported by the findings of Luque *et al.* (2014) which evidenced that obestatin had no effect on prolactin, LH, FSH, or TSH expression/release from pituitary cell cultures of rats and baboon.

In a conclusion, obestatin, as a peripheral signal for energy abundance, may play an important role in reproduction; conversely, ghrelin, as a peripheral signal for energy insufficiency, might play an opposite role. However, the analysis of the reproductive actions of ghrelin and obestatin remains largely incomplete, and further studies are required to clarify the effects at a pituitary levels and of the combined administration of both peptides *in vivo*.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saudi University, KSA for its funding this research group no (RGP- 193).

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