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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Short title

Obestatin vs. ghrelin on basal and stimulated testosterone secretion

Summary

Background and Aim: The functional antagonism between obestatin and ghrelin in testis is under investigated. We investigated the ability of obestatin to counteract the inhibitory effect of ghrelin on basal and stimulated testosterone (T) secretion *in vitro*.

Materials and Methods: Testicular strips from adult rats were incubated with 10ng/ml and 100ng/ml of obestatin alone, ghrelin alone and obestatin + ghrelin. Obestatin modulation of stimulated T secretion was evaluated by incubation of testicular samples with 10ng/ml and 100ng/ml obestatin, ghrelin and obestatin+ ghrelin in the absence and presence of 10IU of human chorionic gonadotrophin (hCG).

Results: T concentrations in the hCG treated groups were significantly ($P < 0.0001$) high as compared to the control groups. Obestatin cause a significant increase in basal T secretion in a dose dependent manner, however obestatin at the both 10ng and 100ng/ml doses significantly ($P < 0.0001$) induced 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 opposes the inhibitory effect of ghrelin on T secretion under both basal and hCG stimulated conditions at all doses tested.

Conclusion: 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

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 still the exact mechanisms involved in the coupling of reproductive function and body energy store have been elucidated (Fernández-Fernández et al., 2004). Central and peripheral endocrine signals primarily involved in the control of energy balance, control reproductive functions by acting at different levels of hypothalamic pituitary–gonadal axis, thus providing basis for the link between energy homeostasis and fertility (Fernández-Fernández et al., 2006).

Ghrelin is a 28 amino-acid peptide that is characterized as the endogenous ligand of the growth hormone (GH)-secretagogue receptor (GHS-R) which 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 the 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 human (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 include 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 facilitate food intake while obestatin suppress food intake (Gualillo et al., 2003).

An *in vitro* experiment reported that obestatin antagonized ghrelin actions on GH secretion (Zizzari et al., 2007). It was evident that different factors with key roles in the growth axis and body weight homeostasis are potentially in part involved in the regulation of reproductive function through 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 the 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 blano-preputial separation, an external sign of pubertal development, and decreases circulating 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.

Material and Methods

Animals

Adult (125-135 days old) male Sprague Dawley rats (250-290g) were used in accordance with an experimental protocol approved by the ethics committee of College of Applied Medical Sciences, King Saud University. Animals were caged under standard conditions of light (12 hour light/12 hour 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 by (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 the 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). Animals were decapitated and testes were then immediately removed from scrotal sac and de-capsulated. Later on, testes were rapidly sliced into small pieces (approx. 100mg) 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 (Hiclone, Thermo Scientifics. Inc. USA) supplemented with 50 IU/ml penicillin and 50µg/ml streptomycin. After 30min 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 U.S.A) (supplemented with Aprotinin 500000KIU/L and disodium EDTA 1g/L) or combinations of both peptides at the dose 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% CO_2 and 95% air at 34 ^{0}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 vortex mixer and aliquots of 100 µl were collected for testosterone measurement. Aliquots were stored at -20 ^{0}C until 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±SEM. 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 10ng/ml and 100ng/ml was tested under both basal and hCG stimulated conditions. The hCG (10IU) hormone induces a significant increase in T concentration from testicular slices after 4 hours of incubation as compared to an

untreated control group (14.00±0.50 vs. 9.43±0.57 ng/ml. 100mg 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 secretions were measured after 10ng/ml and 100ng/ml of obestatin treatment of hCG-exposed testicular tissues ($p<0.05$ and $p<0.001$, respectively). On the other hand, Obestatin at 10ng/ml failed to modify basal T secretion, whereas, at the higher tested dose (100ng/ml), it significantly induces basal testosterone secretion $p<0.05$ (Table 1). These results showed that obestatin modify the basal T release *in vitro* in a dose dependent manner, however it stimulates the hCG-induced T secretion under both tested doses more or less with equal potency.

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 a concentration of 10ng/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 as compared to corresponding control groups (Table 1).

Obestatin counteract the suppressive effect of ghrelin on both basal and hCG induced testosterone secretion.

Treatment of the testicular tissue cultures with obestatin (in a dose 10ng/ml and 100 ng/ml) reverses the suppressive effect of ghrelin on testosterone secretion under both

basal and hCG stimulated conditions. The mean testosterone concentration measured in both 10ng/ml and 100ng/ml treated groups were more or less similar to the control group indicating that obestatin modulate the suppressive effect of ghrelin under basal conditions. Then we administered the combine doses of both peptides to the culture treated with 10IU hCG, obestatin at both tested doses significantly increased testosterone concentration as compared to ghrelin-alone treated groups and the mean testosterone concentration in combined-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 opposing effects of both peptides on reproduction. Therefore, we designed an in vitro study to demonstrate the effect of co-administration of both obestatin and ghrelin on both basal and stimulated testosterone production.

The role of obestatin in male reproductive system is still not well studied despite the presence of obestatin expression on various testicular cells. Within the testis, obestatin immunoreactivity (irOBS) are detected in the Leydig and Sertoli cells, whereas, mild signals of obestatin were observed in rat testis; efferent ductules were the most immune reactive region for the peptide. Vas deferens and seminal vesicles showed intense obestatin labeling in addition to obestatin expression 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, 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 leads to significant increase in testosterone production and spermatogenesis. Furthermore, 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 as regarding the 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, reverses the inhibitory effect of ghrelin on basal and hCG induced T secretion in a dose dependent manner, as testosterone concentration was significantly higher in 100ng/ml obestatin plus ghrelin treatment group than testosterone concentration in ghrelin alone treated group and the mean concentration in the co administered group was more or less similar to the untreated control group. In order to evaluate the effect of obestatin on ghrelin induced suppression of hCG stimulates testosterone secretion, testicular tissues in the culture medium were exposed to 10ng/ml and 100ng/ml, obestatin and ghrelin along with 10IU 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 under stimulated conditions relating hypopeseal pituitary gonadal axis implication in controlling obestatin actions, but still the mechanism is not clear that whether the effect of obestatin is at 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 experiment internal reference in order to clarify the effect of combined peptide administration of a signal peptide treatment. This study extends the previous findings that beside opposite effect 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 were 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 mainly in regards to its role in controlling feeding behavior, the functions of the gastrointestinal tract and energy homeostasis at the hypothalamic level (Zhang et al., 2005), whilst its role in the regulation of reproduction remained less characterized and 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). Suggestion of a direct nature of the effect of obestatin in 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 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 study the effect at pituitary levels and combine administration of both peptides *in vivo.*

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

References

BARREIRO M, GAYTAN F, CAMINOS J, PINILLA L, CASANUEVA F, AGUILAR E, DIEGUEZ C & TENA-SEMPERE M. Cellular location and hormonal regulation of ghrelin expression in rat testis. *Biol reprod* **67:** 1768-1776, 2002.

CAMINOS J, TENA-SEMPERE M, GAYTAN F, SANCHEZ-CRIADO J, BARREIRO M, NOGUEIRAS R, CASANUEVA F, AGUILAR E & DIEGUEZ C. Expression of ghrelin in the cyclic and pregnant rat ovary. *Endocrinol* **144:** 1594-1602, 2003.

DUN SL, BRAILOIU GC, BRAILOIU E, YANG J, CHANG JK & DUN NJ. Distribution and biological activity of obestatin in the rat. *J Endocrinol* **191:** 481-9, 2006.

FERNÁNDEZ-FERNÁNDEZ R, TENA-SEMPERE M, AGUILAR E & PINILLA L. Ghrelin effects on gonadotropin secretion in male and female rats. *Neurosci Lett* **362:** 103-107, 2004.

FERNÁNDEZ-FERNÁNDEZ R, TENA-SEMPERE M, NAVARRO VM, BARREIRO ML, CASTELLANO JM, AGUILAR E & PINILLA L. Effects of ghrelin upon gonadotropin-releasing hormone and gonadotropin secretion in adult female rats: in vivo and in vitro studies. *Neuroendocrinol.* **82:** 245-255, 2006.

GAYTAN F, BARREIRO ML, CHOPIN LK, HERINGTON AC, MORALES C, PINILLA L, CASANUEVA FF, AGUILAR E, DIEGUEZ C & TENA-SEMPERE M. Immunolocalization of ghrelin and its functional receptor, the type 1a growth hormone secretagogue receptor, in the cyclic human ovary. *J. Clin. Endocrinol. Metab* **88:** 879-887, 2003.

GUALILLO O, LAGO F, GÓMEZ-REINO J, CASANUEVA FF & DIEGUEZ C. Ghrelin, a widespread hormone: insights into molecular and cellular regulation of its expression and mechanism of action. *FEBS Lett* **552:** 105-109, 2003.

HIZBULLAH SJ & AHMED S. Obestatin induces testosterone secretion from rat testis in vitro. *Afr J Biotechnol*. **10:** 3821-3928, 2013.

HOWARD AD, FEIGHNER SD, CULLY DF, ARENA JP, LIBERATOR PA, ROSENBLUM CI, HAMELIN M, HRENIUK DL, PALYHA OC & ANDERSON J. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273:** 974-977, 1996.

JAHAN S, AHMED S & TAQVIM N. Possible modulation of testosterone secretion by obestatin in pubertal male rats. *Pak J Zool.* **43:** 799-803, 2011.

JAHAN S, SIDRAT T, AHMED S, WAZIR H & ULLAH K. Effect of obestatin on morphometry of testes and testosterone secretion in male rats. *Afr J Biotechnol.* **10:** 7717-7722, 2013.

KOJIMA M, HOSODA H, DATE Y, NAKAZATO M, MATSUO H & KANGAWA K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402:** 656-660, 1999.

MARTINI AC, FERNANDEZ-FERNANDEZ R, TOVAR S, NAVARRO VM, VIGO E, VAZQUEZ MJ, DAVIES JS, THOMPSON NM, AGUILAR E, PINILLA L,

WELLS T, DIEGUEZ C & TENA-SEMPERE M. Comparative analysis of the effects of ghrelin and unacylated ghrelin on luteinizing hormone secretion in male rats. *Endocrinol*. **147:** 2374-82, 2006.

MCKEE KK, TAN CP, PALYHA OC, LIU J, FEIGHNER SD, HRENIUK DL, SMITH RG, HOWARD AD & VAN DER PLOEG LH. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics* **46:** 426-434, 1997.

MORETTI E, VINDIGNI C, TRIPODI S, MAZZI L, NUTI R, FIGURA N & COLLODEL G. Immunolocalisation of ghrelin and obestatin in human testis, seminal vesicles, prostate and spermatozoa. *Andrologia*, 2013.

NOGUEIRAS R, PFLUGER P, TOVAR S, ARNOLD M, MITCHELL S, MORRIS A, PEREZ-TILVE D, VÁZQUEZ MJ, WIEDMER P & CASTANEDA TR. Effects of obestatin on energy balance and growth hormone secretion in rodents. *Endocrinol*. **148:** 21-26, 2007.

TENA-SEMPERE M, BARREIRO M, GONZALEZ L, GAYTAN F, ZHANG F-P, CAMINOS J, PINILLA L, CASANUEVA F, DIEGUEZ C & AGUILAR E. Novel expression and functional role of ghrelin in rat testis. *Endocrinol.* **143:** 717-725, 2002.

TENA-SEMPERE M, PINILLA L, GONZALEZ L, DIEGUEZ C, CASANUEVA F & AGUILAR E. Leptin inhibits testosterone secretion from adult rat testis in vitro. *J Endocrinol*. **161:** 211-218, 1999.

YANG J, BROWN MS, LIANG G, GRISHIN NV & GOLDSTEIN JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132:** 387-396, 2008.

ZHANG JV, REN P-G, AVSIAN-KRETCHMER O, LUO C-W, RAUCH R, KLEIN C & HSUEH AJ. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310:** 996-999, 2005.

ZIZZARI P, LONGCHAMPS R, EPELBAUM J & BLUET-PAJOT MT. Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents. *Endocrinol.* **148:** 1648-1653, 2007.

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

Treatments	*T concentration ng/ml. 100mg of tissue*	*% Reduction in T secretion*	*% Increase in T secretion*
Basal			
Control	9.43 ± 0.19	-	-
Obestatin 10ng/ml	10.46 ± 0.17	-	10.92
Obestatin 100ng/ml	11.70 ± 0.23	-	24.07 $^{*, c}$
Ghrelin 10ng/ml	7.69 ± 0.32	19.39$^{*, c}$	-
Ghrelin 100ng/ml	5.57 ± 0.20	40.93$^{***, c}$	-
Obs + Ghr 10ng/ml	9.88 ± 0.21	-	44.44 $^{**, a}$
Obs + Ghr 100ng/ml	8.83 ±0.13	-	58.52 $^{**, b}$
hCG stimulated			
hCG Control (10IU)	14.00±0.12	-	48.46$^{***, c}$
Obs 10ng/ml + hCG	16.38±0.19	-	17.0^{*,hc}
Obs 100ng/ml + hCG	19.64±0.18	-	40.27^{***,hc}
Ghr10ng/ml+ hCG	10.75±0.21	23.22^{*,hc}	-
Ghr 100ng/ml+ hCG	8.67 ± 0.19	38.1^{***,hc}	-
Obs + Ghr 10ng/ml+ hCG	13.97±0.13	-	29.9^{***,ha}
Obs + Ghr 100ng/ml+	13.24 ±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 10ng/ml, b=compared to ghrelin 100ng/ml, ha= compared to ghrelin 10ng/ml plus 10IU hCG, hb=compared to ghrelin 100ng/ml plus 10IU hCG, - indicate not applicable for particular analysis.