EXPERIMENTAL STUDY

***In vitro* pituitary and testicular effects of the leptin-related synthetic peptide leptin(116–130) amide involve actions both similar to and distinct from those of the native leptin molecule in the adult rat**

Manuel Tena-Sempere¹, Leonor Pinilla¹, Lucas C González¹, Juan Navarro¹, Carlos Diéguez², Felipe F Casanueva³ and Enrique Aguilar¹

¹Department of Physiology, Faculty of Medicine, University of Córdoba, Avda Menéndez Pidal s/n, 14004 Córdoba, Spain and Departments of ²Physiology and ³Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain

(Correspondence should be addressed to M Tena-Sempere, E-mail: fi1tesem@lucano.uco.es)

Abstract

The obese gene (*ob*) product, leptin, has recently emerged as a key element in body weight homeostasis, neuroendocrine function and fertility. Identification of biologically active, readily synthesized fragments of the leptin molecule has drawn considerable attention, as they may provide a powerful tool for detailed characterization of the biological actions of leptin in different experimental settings. Recently, a fragment of mouse leptin protein comprising amino acids 116–130, termed leptin(116–130) amide, was shown to mimic the effects of the native molecule in terms of body weight gain and food intake, and to elicit LH and prolactin (PRL) secretion *in vivo*. As a continuation of our previous experimental work, the present study reports on the effects of leptin(116–130) amide on basal and stimulated testosterone secretion by adult rat testis *in vitro*. In addition, a comparison of the effects of human recombinant leptin and leptin(116–130) amide at the pituitary level on the patterns of LH, FSH, PRL and GH secretion is presented. As reported previously by our group, human recombinant leptin (10^{-9} – 10^{-7} M) significantly inhibited both basal and human chorionic gonadotrophin (hCG)-stimulated testosterone secretion *in vitro*. Similarly, incubation of testicular tissue in the presence of increasing concentrations of leptin(116–130) amide (10^{-9} – 10^{-5} M) resulted in a dose-dependent inhibition of basal and hCG-stimulated testosterone secretion; a reduction that was significant from a dose of 10^{-7} M upwards. In addition, leptin(116–130) amide, at all doses tested (10^{-9} – 10^{-5} M), significantly decreased LH and FSH secretion by incubated hemi-pituitaries from adult male rats. In contrast, in the same experimental protocol, recombinant leptin (10^{-9} – 10^{-7} M) was ineffective in modulating LH and FSH release. Finally, neither recombinant leptin nor leptin(116–130) amide were able to change basal PRL and GH secretion *in vitro*. Our results confirm the ability of leptin, acting at the testicular level, to inhibit testosterone secretion, and map the effect to a domain of the leptin molecule that lies between amino acid residues 116 and 130. In addition, we provide evidence for a direct inhibitory action of leptin(116–130) amide on pituitary LH and FSH secretion, a phenomenon not observed for the native leptin molecule, in the adult male rat.

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Introduction

Leptin, the product of the *ob* gene, is an adipocyte-secreted plasma hormone involved in the control of food intake and energy expenditure that plays a key role in body weight homeostasis (1, 2). Moreover, leptin has recently emerged as a pivotal signal in the regulation of fertility and neuroendocrine function. The absence of biological actions of leptin, due to mutations in the *ob* gene (*ob/ob* mice), leads to infertility whereas leptin administration advances the onset of puberty,

maintains reproductive cyclicity despite acute fasting, and prevents sterility in *ob/ob* mice (2, 3). In addition, evidence for a stimulatory role of leptin in the control of growth hormone (GH) and luteinizing hormone (LH) secretion has been presented (4, 5). The mechanisms behind the wide range of biological actions of leptin are far from being completely elucidated, but compelling evidence points to the hypothalamus as the primary target for most of the metabolic and endocrine actions of leptin (6). However, based on the characterization of leptin receptor distribution and leptin effects on *in vitro*

systems, additional primary sites for leptin action have been suggested, including the pituitary, testis and ovary (7–11).

Different experimental approaches have been used to delineate the effects and mechanisms of the action of leptin. Among them, testing of the biological action of several synthetic peptides generated to replicate restricted areas of the leptin protein has helped to identify active domains of the molecule in terms of regulation of body weight and food intake (12, 13). Interestingly, it was shown recently that a fragment of the murine leptin protein comprising amino acids 116–130 (termed leptin(116–130) amide) displayed actions similar to those of the native molecule in terms of food intake and body weight in female *ob/ob* mice (13). In addition, we demonstrated recently that this synthetic peptide was able to elicit LH and prolactin (PRL) secretion in fasted adult male rats (14). As a continuation of our previous work (10, 14), the present experiments were undertaken to characterize the effects of leptin(116–130) amide on anterior pituitary secretion and testicular testosterone production *in vitro*. Comparative analyses revealed that the biological actions of leptin(116–130) amide on the pituitary–testicular axis involve effects both similar to and distinct from those of the native leptin molecule in the adult rat.

Materials and methods

Animals and drugs

Adult (75-day-old) Wistar male rats bred in the vivarium of our Institution were used. The animals were caged under constant conditions of light (14 h of light; lights on at 0700 h) and temperature (22 °C), with free access to pelleted food (Pacsa Sanders, Seville, Spain) and tap water. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals.

Human recombinant leptin was kindly donated by Eli Lilly (Indianapolis, IN, USA). Mouse leptin(116–130) amide was purchased from Bachem AG (Bubendorf, Switzerland). LH-releasing hormone (LHRH) was obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA).

Tissue incubations

For the analysis of the direct effects of recombinant leptin and leptin(116–130) amide on anterior pituitary secretion *in vitro*, incubation of pituitary tissue from adult males was carried out as described elsewhere (10). Briefly, upon decapitation of experimental animals, anterior pituitaries were immediately removed, dissected free of the posterior pituitary lobe, and halved. Hemi-pituitaries were preincubated for 1 h in

1 ml Dulbecco's modified Eagle's medium (DMEM)–Ham's F12 medium (1:1; Life Technologies, Grand Island, NY, USA) supplemented with 0.1 g/l gentamicin (Biological Industries, Bet-Haemek, Israel) in a Dubnoff shaker (60 cycles/min) at 37 °C under an atmosphere of 5% CO₂–95% O₂. After preincubation, the media were replaced either by fresh medium or medium containing increasing doses of leptin(10^{−9}–10^{−7} M) or leptin(116–130) amide (10^{−9}–10^{−5} M). As internal control for incubation conditions, a group of hemi-pituitaries was incubated in the presence of LHRH (10^{−6} M). After 60 and 120 min, 50 µl aliquots from the incubation media were taken for LH, follicle-stimulating hormone (FSH), PRL and GH measurements, as described below. Hormonal levels were expressed as normalized values per 10 mg incubated tissue.

Assessment of *in vitro* effects of leptin(116–130) amide on testosterone secretion was carried out using static incubation of testicular tissue from adult rats. For this purpose, testes were removed immediately upon decapitation of experimental animals, decapsulated, and cut into pieces of approximately equal size (mean weight/piece: 385 ± 7 mg/piece from adult testes, 4 slices/testis). Testicular slices (2 slices/well) were incubated in 2 ml gentamicin-supplemented DMEM–F12 medium in a Dubnoff shaker (60 cycles/min) at 32 °C under an atmosphere of 5% CO₂–95% O₂. After preincubation for 1 h, the media were replaced either by fresh medium or medium containing increasing doses of leptin(116–130) amide (10^{−9}–10^{−5} M). In addition, to test the ability of this leptin-related peptide to modulate stimulated testosterone secretion, groups of testicular samples were challenged with different doses of leptin(116–130) amide (10^{−9}–10^{−5} M) plus hCG (10 IU) or hCG alone. For comparative analysis, a similar experimental procedure was carried out using recombinant leptin(10^{−9}–10^{−7} M), in the presence or absence of hCG (10 IU). After 90 min, 100 µl aliquots from the incubation media were taken for testosterone measurement, as described below. The levels of testosterone in the media were expressed as normalized values per 1g incubated tissue.

Hormone measurements and statistics

Testosterone was measured from diethyl ether extracts of tissue incubation media by RIA using ³H-labelled testosterone as tracer. LH, FSH, PRL and GH levels were assayed in incubation media by specific RIAs, using kits supplied by NIDDK (Bethesda, MD, USA). The results are expressed using reference preparation (RP) LH-RP-3, FSH-RP-2, PRL-RP-3 and GH-RP-2 as standards.

Experiments were carried out in duplicate. Data are expressed as means ± S.E.M. (*n* = 8–12 samples/group). Results were analyzed for statistically significant differences using a one-way ANOVA, followed by Tukey's test. *P* < 0.05 was considered significant.

Results

Comparison of the effects of human recombinant leptin (10^{-9} – 10^{-7} M) and leptin(116–130) amide (10^{-9} – 10^{-5} M) on basal pituitary function of adult male rats was carried out. Assuming that the biopotency of the synthetic peptide is lower than that of the native molecule (13), a wider range of doses were tested for leptin(116–130) amide. Recombinant leptin, at all doses evaluated, failed to change basal release of LH, FSH, PRL and GH by incubated pituitary tissue. However, as a positive control, under similar incubation conditions 10^{-6} M LHRH elicited a significant 2.5- and 3-fold increase in LH and FSH secretion respectively (data not shown). In the same experimental set-up, leptin(116–130) amide was unable to alter basal PRL and GH release. However, at all doses tested, leptin(116–130) amide induced a significant inhibition of LH and FSH secretion by incubated hemi-pituitaries from adult male rats (Fig. 1).

In addition, assessment of the effects of leptin(116–130) amide on basal and hCG-stimulated testosterone secretion *in vitro* was carried out after 90 min static incubation of testicular tissue, as this incubation time has previously been proven effective for demonstrating the direct inhibitory action of recombinant leptin on testosterone release (10). Moreover, in order to compare the potency of the leptin fragment and the native leptin molecule in suppressing testosterone secretion *in vitro*, evaluation of the effects of recombinant leptin under similar experimental conditions was included. In keeping with our previous reference (10), doses of 10^{-9} – 10^{-7} M leptin significantly decreased basal and hCG-induced testosterone secretion *in vitro*. As was the case for the native molecule, incubation of testicular samples from adult rats in the presence of increasing concentrations of leptin(116–130) amide (10^{-9} – 10^{-5} M) resulted in a significant inhibition of basal and hCG-stimulated testosterone secretion; an inhibition that appeared dose dependent: 10^{-9} M leptin(116–130) was ineffective whereas a significant reduction in basal and stimulated testosterone release was observed for 10^{-7} – 10^{-5} M doses (Table 1).

Discussion

In recent years, characterization of the biological effects and mechanisms of action of leptin has drawn considerable attention. Among the different experimental approaches, synthetic peptide technology has been used (12, 13). Testing of the biological action of several synthetic peptides generated to replicate restricted areas of the leptin protein has helped to identify those domains of the molecule that are responsible for its metabolic and endocrine effects (12–14). Moreover, the availability of biologically active, small, readily synthesized fragments of leptin could ease the characterization of the effects of the native molecule both in

Figure 1 Effects of recombinant leptin(left panels) and the leptin-related synthetic peptide, leptin(116–130) amide, (right panels) on *in vitro* LH, FSH, PRL and GH secretion in adult male rats. Hemi-pituitaries were incubated in the presence of increasing concentrations of human recombinant leptin(10^{-9} – 10^{-7} M) or leptin(116–130) amide (10^{-9} – 10^{-5} M). As the pattern of hormone release was similar after 60 and 120 min incubation, only data from the latter incubation are presented. Values are given as means $\pm$ S.E.M. ($n=8-10$ samples/group). ** $P<0.01$ vs control samples incubated in the presence of DMEM alone (ANOVA followed by Tukey's test).

physiological and pathological conditions and pave the way for development of therapies based on the use of potent leptin analogs. The ability of a fragment of the mouse leptin molecule, comprising amino acids 116–130, to mimic the actions of the native protein in terms of food intake and body weight gain, and to elicit LH and PRL secretion *in vivo* (13, 14), prompted us to evaluate its potential effects on anterior pituitary secretion and testicular testosterone production *in vitro*. In this sense, leptin receptors have been localized in these areas (7) and evidence has been presented for a direct regulatory role of leptin on testicular and pituitary function (8–10).

Incubation of testicular tissue in the presence of increasing concentrations of leptin(116–130) amide resulted in a dose-dependent inhibition of basal and hCG-stimulated testosterone secretion. These results confirm our previous data on the ability of leptin to inhibit testosterone secretion *in vitro* (10). Moreover,

Table 1 Effects of the leptin-related synthetic peptide, leptin(116–130) amide, on *in vitro* basal (–hCG) and hCG-stimulated (+hCG) testosterone (T) secretion in adult rats. To evaluate regulation of basal T secretion, slices of testicular tissue were incubated in the presence of increasing concentrations of leptin(116–130) amide (10^{-9} – 10^{-5} M). In addition, to test the ability of leptin(116–130) amide to modulate stimulated T secretion, testicular samples were challenged with different doses of the peptide (10^{-9} – 10^{-7} M) plus hCG (10 IU). For comparative analysis, results from incubations with recombinant leptin (10^{-9} – 10^{-7} M), in the presence or absence of hCG (10 IU), are presented. Testes incubated with medium alone served as respective controls. Values are given as means $\pm$ S.E.M. ($n = 10$ –12 samples/group).

	T secretion (ng/ml · g tissue)			
	Control	10^{-9} M leptin(116–130)	10^{-7} M leptin(116–130)	10^{-5} M leptin(116–130)
–hCG	8.36 $\pm$ 1.02	7.72 $\pm$ 1.16	4.51 $\pm$ 0.69**	5.03 $\pm$ 0.58**
+hCG	94.15 $\pm$ 18.5	64.5 $\pm$ 10.14	54.3 $\pm$ 7.71**	52.5 $\pm$ 3.75**

	T secretion (ng/ml · g tissue)			
	Control	10^{-9} M leptin	10^{-8} M leptin	10^{-7} M leptin
–hCG	8.91 $\pm$ 0.60	6.35 $\pm$ 0.72**	6.61 $\pm$ 0.46**	6.42 $\pm$ 0.36**
+hCG	101.2 $\pm$ 13.7	59.92 $\pm$ 10.52*	55.25 $\pm$ 6.36**	51.45 $\pm$ 5.31**

* $P < 0.05$; ** $P < 0.01$ vs values from corresponding control groups (ANOVA followed by Tukey's test).

the data presented herein allow the localization of this biological action to a domain of the leptin protein between amino acid residues 116–130. This domain appears as an important functional area of the molecule as it is involved in relevant metabolic and endocrine actions of leptin (13, 14 and present results). Interestingly, the effectiveness of leptin(116–130) in suppressing testosterone secretion *in vitro* was lower than that of the native molecule, as 10^{-9} M doses were inhibitory only for the latter. The decreased biopotency of leptin(116–130) amide has also been reported for its effects on food intake (13), and likely depends on a lower affinity for the cognate receptor when compared with the intact protein.

Comparison of the effects of recombinant leptin and its active fragment on basal pituitary function *in vitro* revealed differences in the action of the two molecules. Leptin(116–130) amide induced a significant inhibition in LH and FSH secretion by incubated hemi-pituitaries whereas it was ineffective in terms of PRL and GH release (Fig. 1). The latter finding is in good agreement with previous reports on the lack of action of leptin on pituitary GH secretion *in vitro* (4, 15). In addition, these results are in line with the ability of leptin(116–130) amide to partially suppress basal LH secretion by pituitaries from ovariectomized, steroid-primed female rats *in vitro* (16), and are in keeping with our previous findings on the selective inhibition by recombinant leptin of basal LH and FSH release by pituitaries from adult fasted male rats (10). Present results, however, are in contrast to data obtained in normally fed animals using the complete leptin protein. On this point, both stimulatory effects (8, 9) and the absence of modulatory action (present results) of leptin on LH, FSH and PRL secretion by adult male rat pituitaries *in vitro* have been

reported. The reasons behind such a discrepancy are unclear as similar experimental settings were used in these studies; yet differences in terms of rat strain and type and doses of leptin used were noted. Overall, however, a striking difference between the effects of recombinant leptin and leptin(116–130) amide on basal gonadotropin secretion can be outlined. The mechanism(s) underlying such a phenomenon may involve the presence of different leptin receptor isoforms. In this sense, several leptin receptor subtypes, with different biological roles, have been identified and the balance of their expression in rat brain appears to be regulated by hormonal and metabolic signals (17). In such a scenario, it is tempting to speculate that the net action of leptin on basal pituitary function derives from the activation of different subsets of receptors, and that the conflicting observations reported above may be due to differences in the binding affinities of the leptin molecule and its active fragment for different receptor isoforms. Interestingly, the identification of distinct patterns of response to recombinant leptin and leptin(116–130) amide in terms of basal LH and FSH secretion, reported herein, may help to understand the mechanism behind the controversial data gathered on the direct actions of leptin on anterior pituitary secretion under different experimental conditions (see above). In this sense, evaluation of the effects of leptin(116–130) amide on LH and FSH secretion by pituitaries from adult, fasted males may prove helpful to further extend our present data on the structure–function relationship of the leptin molecule in terms of regulation of basal gonadotropin secretion.

It is worthy of note that the effects of leptin(116–130) amide *in vitro* were different from those observed

in vivo, as the leptin fragment inhibited LH secretion and failed to alter PRL secretion by incubated hemipituitaries whereas it stimulated LH and PRL release when systemically administered to adult male rats (14). This observation, together with the lack of effect of recombinant leptin on pituitary secretion *in vitro*, reinforces the contention that the predominant actions of leptin in the control of anterior pituitary secretion are carried out mainly at the hypothalamic level (4, 7, 8). In addition, our data point to a complex mode of action of leptin at multiple sites of the gonadotropic axis that likely involves both inhibitory and stimulatory effects.

In conclusion, our results further demonstrate the ability of leptin to inhibit testosterone secretion *in vitro*, and implicate a domain of the molecule between amino acid residues 116–130 in this action. In addition, based on comparative analyses, we propose that the biological action of leptin(116–130) amide on the pituitary–testicular axis involves effects both similar to and distinct from those of the native leptin molecule in the adult rat.

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