

Leptin inhibits testosterone secretion from adult rat testis *in vitro*

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Abstract

Leptin, the product of the *ob* gene, has emerged recently as a pivotal signal in the regulation of fertility. Although the actions of leptin in the control of reproductive function are thought to be exerted mainly at the hypothalamic level, the potential direct effects of leptin at the pituitary and gonadal level have been poorly characterised. In the present study, we first assessed the ability of leptin to regulate testicular testosterone secretion *in vitro*. Secondly, we aimed to evaluate whether leptin can modulate basal gonadotrophin and prolactin (PRL) release by incubated hemi-pituitaries from fasted male rats. To attain the first goal, testicular slices from prepubertal and adult rats were incubated with increasing concentrations (10^{-9} – 10^{-7} M) of recombinant leptin. Assuming that *in vitro* testicular responsiveness to leptin may be dependent on the background leptin levels, testicular tissue from both food-deprived and normally-fed animals was used. Furthermore, leptin modulation of stimulated testosterone secretion was evaluated by incubation of testicular samples with different doses of leptin in the presence of 10 IU human chorionic gonadotrophin (hCG). In addition, analysis of leptin actions on pituitary function was carried out using hemi-pituitaries from fasted adult male rats

incubated in the presence of increasing concentrations (10^{-9} – 10^{-7} M) of recombinant leptin. Serum testosterone levels, and basal and hCG-stimulated testosterone secretion by incubated testicular tissue were significantly decreased by fasting in prepubertal and adult male rats. However, a significant reduction in circulating LH levels was only evident in adult fasted rats. Doses of 10^{-9} – 10^{-7} M leptin had no effect on basal or hCG-stimulated testosterone secretion by testes from prepubertal rats, regardless of the nutritional state of the donor animal. In contrast, leptin significantly decreased basal and hCG-induced testosterone secretion by testes from fasted and fed adult rats. In addition, 10^{-9} M leptin inhibited LH and FSH secretion by incubated hemi-pituitaries from fasted adult males, whereas, at all doses tested, it was ineffective in modulating PRL release. Our results show that leptin, depending on the state of sexual maturation, is able to inhibit testosterone secretion acting at the testicular level. Furthermore, the present data suggest that the actions of leptin on the reproductive system are complex and are probably carried out at different levels of the hypothalamic–pituitary–gonadal axis.

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Introduction

The obese gene (*ob*) product, leptin, is a plasma protein hormone produced in the adipose tissue that plays a key role in the control of food intake and energy expenditure (Zhang *et al.* 1994, Campfield *et al.* 1995, Halaas *et al.* 1995, Pelleymounter *et al.* 1995, Weigle *et al.* 1995). Besides its well-known actions in the regulation of body weight, leptin has recently emerged as a metabolic link between nutrition and fertility. Several lines of evidence support this concept. First, it is well established that severe starvation is associated with impaired reproductive function (Warren 1983, Bergendahl *et al.* 1989, 1991, Rosenbaum & Leibel 1998) and reduced leptin levels (Nagatani *et al.* 1998, Vuagnat *et al.* 1998). Secondly, the absence of biological actions of leptin, due to mutations in

the *ob* gene (*ob/ob* mice) or the leptin receptor gene (*db/db* mice), leads to infertility (Jones & Ainsworth-Harrison 1957, Swerdloff *et al.* 1976, Batt *et al.* 1982). Thirdly, treatment of obese *ob/ob* mice with leptin, but not food restriction, stimulates the reproductive endocrine system and restores fertility in this animal model (Barash *et al.* 1996, Chehab *et al.* 1996, Mounzih *et al.* 1997). Furthermore, leptin administration advances the onset of puberty in normal female mice (Ahima *et al.* 1997, Chehab *et al.* 1997). Fourthly, immunoneutralization of endogenous leptin disrupts cyclicity in female rats (Carro *et al.* 1997b). In addition, leptin has also been implicated as an important factor for signalling the nutritional state to different neuroendocrine systems. In this regard, a role for leptin in the control of pulsatile growth hormone (GH) and luteinizing hormone (LH) secretion has been demonstrated

recently (Carro *et al.* 1997a,b, Nagatani *et al.* 1998, Vuagnat *et al.* 1998).

The mechanism(s) whereby leptin regulates reproductive function remains to be fully characterised; however the sites for leptin action may include the hypothalamus, the pituitary, and/or the gonads. Compelling evidence points to the hypothalamus as the primary target of leptin (Schwartz *et al.* 1996, Elmquist *et al.* 1997). It is noteworthy that the hypothalamus is a key element in the control of food intake and neuroendocrine regulation of reproductive function. Leptin receptors are expressed in specific hypothalamic nuclei (Zamorano *et al.* 1997, Couce *et al.* 1997), and the ability of leptin to modulate the expression of several hypothalamic neuropeptides, including LH-releasing hormone (LHRH), somatostatin and neuropeptide Y (Schwartz *et al.* 1996, Quintela *et al.* 1997, Yu *et al.* 1997a,b, Sahu 1998) is well documented. Unlike its actions at the hypothalamic level, the potential direct effects of leptin on pituitary and gonadal function have been poorly characterised. Recent reports have shown that leptin increases basal and LHRH-stimulated LH secretion from hemi-pituitaries of normally-fed male rats (Yu *et al.* 1997a,b). Studies concerning the direct effects of leptin at the gonadal level have provided evidence for an inhibitory role of leptin on ovarian function (Spicer & Francisco 1997, 1998, Zachow & Magoffin 1997). However, to our knowledge, no study analysing the potential direct effects of leptin on testicular function has been reported.

Considering the relevant actions of leptin on the reproductive axis (see above), the present experiments were undertaken to test whether leptin can modulate basal and human chorionic gonadotrophin (hCG)-stimulated *in vitro* testosterone secretion from the testes of animals under two different nutritional states (normal-feeding and short-term starvation) and at two different ages (prepubertal and adult). In addition, we wanted to evaluate a potential direct regulatory role of leptin on basal gonadotrophin and prolactin (PRL) secretion by hemi-pituitaries from fasted adult male rats. Our data extend previous observations on the role of leptin in the control of pituitary function in the normally-fed rat (Yu *et al.* 1997a,b) as we monitored leptin actions on pituitary function of fasted animals. In this regard, evidence showing that the effects of leptin are dependent on the prevailing metabolic status has been presented previously (Carro *et al.* 1997a).

Materials and Methods

Animals and drugs

Prepubertal (30-day-old) and adult (75- to 90-day-old) Wistar male rats were purchased from Charles River (Criffa, Barcelona, Spain). On arrival, the animals were caged under constant conditions of light (14 h of light; lights on at 0700 h) and temperature (22 °C), and

acclimatised for 3 days with free access to standard laboratory animal food and tap water. Before initiation of experiments, prepubertal and adult animals were divided into groups of approximately similar weight (prepubertal males: 90.5 ± 3.6 g; adult males: 252.6 ± 7.2 g). In food-deprived groups, the animals had access only to water during the fasting periods: 60 h in prepubertal rats and 96 h in adult rats. Control, normally-fed animals received water and standard laboratory food which were available *ad libitum*. All experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union code for the care and use of experimental animals.

Human recombinant leptin was produced in *Saccharomyces cerevisiae* as described elsewhere (Considine *et al.* 1996), and kindly donated by Eli Lilly (Indianapolis, IN, USA). Highly purified hCG (Profasi HP500) was purchased from Serono (Madrid, Spain). LHRH was obtained from Sigma Chemical Co. (St Louis, MO, USA).

Tissue incubations

For the analysis of the direct effects of leptin on testosterone secretion, incubation of testicular tissue was carried out as described previously (Tena-Sempere *et al.* 1997), with minor modifications. Testicular samples were obtained from four experimental groups: (1) prepubertal fasted rats, (2) prepubertal fed rats, (3) adult fasted rats, and (4) adult fed rats. For static incubations, the animals were killed by decapitation and trunk blood was collected for serum hormone measurements. Upon death, the testes were removed immediately, decapsulated, and cut into pieces of approximately equal size (mean weight/piece: 373 ± 7 mg/piece from prepubertal testes, 2 slices/testis; 368 ± 5 mg/piece from adult testes, 4 slices/testis). Testicular slices (2 slices/well) were incubated in 2 ml Dulbecco's modified Eagle's medium (DMEM)/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 32 °C under an atmosphere of 5% CO₂-95% O₂. After preincubation for 1 h, the media were replaced with either fresh medium or medium containing increasing doses of leptin (10⁻⁹-10⁻⁷ M). In addition, to test the ability of leptin to modulate stimulated testosterone secretion, groups of testicular samples were challenged with different doses of leptin (10⁻⁹-10⁻⁷ M) plus hCG (10 IU) or with hCG alone. After 90 and 180 min, 100 µl aliquots from the incubation media were taken for testosterone measurement, as described below. The levels of testosterone in the media are expressed as normalised values per g incubated tissue.

Incubation of anterior pituitaries was carried out as described above. Adult fasted males were killed by decapitation, and their anterior pituitaries were immediately

Table 1 Body weight and serum testosterone and LH levels in prepubertal (30-day-old) and adult (90-day-old) male rats after short-term starvation. The fasting period was 60 h for prepubertal and 96 h for adult animals. During these periods, paired control animals had access to standard laboratory food available *ad libitum*. Values are given as means \pm S.E.M. ($n=25-30$ animals/group)

	Prepubertal		Adult	
	Fed	Fasted	Fed	Fasted
Body weight (g)	101.5 \pm 2.5	78.9 \pm 1.4**	278.5 \pm 8.2†	215.6 \pm 6.2**†
Testosterone (ng/ml)	1.16 \pm 0.05	0.80 \pm 0.04**	2.67 \pm 0.12†	1.76 \pm 0.10**†
LH (ng/ml)	0.30 \pm 0.05	0.34 \pm 0.07	0.57 \pm 0.10†	0.34 \pm 0.07*

* $P \leq 0.05$, ** $P \leq 0.01$ vs corresponding control-fed group; † $P \leq 0.01$ vs corresponding prepubertal group (ANOVA followed by Tukey's test).

removed, dissected free of the posterior pituitary lobe, and halved. Hemi-pituitaries were preincubated for 1 h in 1 ml gentamicin-supplemented DMEM-F12 medium in a Dubnoff shaker (60 cycles/min) at 37 °C under an atmosphere of 5% CO₂-95% O₂. After preincubation, the media were replaced with either fresh medium or medium containing increasing doses of leptin (10^{-9} - 10^{-7} M) or LHRH (10^{-6} M). After 60 and 120 min, 50 μ l aliquots from the incubation media were taken for LH, follicle-stimulating hormone (FSH) and PRL measurements, as described below. Hormonal levels are expressed as normalised values per 10 mg incubated tissue.

Hormone measurements

Testosterone was measured from diethyl ether extracts of serum and tissue incubation media by RIA using ³H-labelled testosterone as tracer, as described elsewhere (Rodriguez-Padilla *et al.* 1987). LH, FSH and PRL levels were assayed in incubation media by specific RIAs, using kits supplied by NIDDK (Bethesda, MD, USA). Similarly, serum LH levels from fasted and fed, prepubertal and adult male rats were determined. The results are expressed in terms of LH-reference preparation (RP)-3, FSH-RP-2, and PRL-RP-3.

Statistics

Data are expressed as means \pm S.E.M. The values were homogeneous and no transformation was carried out. Results from testicular incubations were analysed for statistically significant differences using a two-way ANOVA, followed by Tukey's test. This procedure allows, for each age (prepubertal vs adult) and incubation time-point, the comparison of responses to different doses of leptin and nutritional states, and the interactions between these variables. Similarly, two-way ANOVA was used to assess significant differences in terms of body weight and serum testosterone and LH levels in the different experimental groups. Finally, data from pituitary

incubations were statistically evaluated using a one-way ANOVA, followed by Tukey's test. A value of $P < 0.05$ was considered significant.

Results

Assessment of leptin actions on testicular testosterone secretion *in vitro* was carried out in normally-fed and fasted animals. Food deprivation induced an average reduction in body weight of 21% and 24% from normally-fed prepubertal and adult male rats respectively (Table 1). Analysis of the endocrine background in the experimental groups was achieved by measuring serum LH and testosterone levels. As shown in Table 1, fasting induced a significant decrease in serum testosterone concentration in both prepubertal and adult male rats. However, a significant reduction in plasma LH levels was only observed in adult animals.

In prepubertal rats, testicular testosterone secretion *in vitro* was significantly lower ($P \leq 0.01$) than in the corresponding adult groups (normally-fed rats: 3.9 ± 0.32 vs 13.36 ± 1.34 ng/ml/g tissue after 180 min incubation). Sixty-hour starvation induced a significant decrease in basal and hCG-stimulated *in vitro* testosterone secretion by testes from prepubertal rats. However, leptin, at all doses tested, failed to modify basal testosterone release and it did not alter the testicular response to hCG in terms of testosterone secretion in fed or food-deprived groups at this age (Fig. 1). As in prepubertal animals, fasting (96 h) of adult males decreased basal and hCG-stimulated testosterone secretion *in vitro*. However, at this age, leptin was able to inhibit both basal and hCG-induced testosterone release by testes from both fed and starved rats (Fig. 2), although differences in the time-course and dose-dependency of this inhibitory effect were noted between groups. In the normally-fed group, at all doses (10^{-9} - 10^{-7} M) and both times (90 and 180 min) tested, leptin significantly decreased hCG-stimulated testosterone secretion. In addition, in this group basal testosterone release

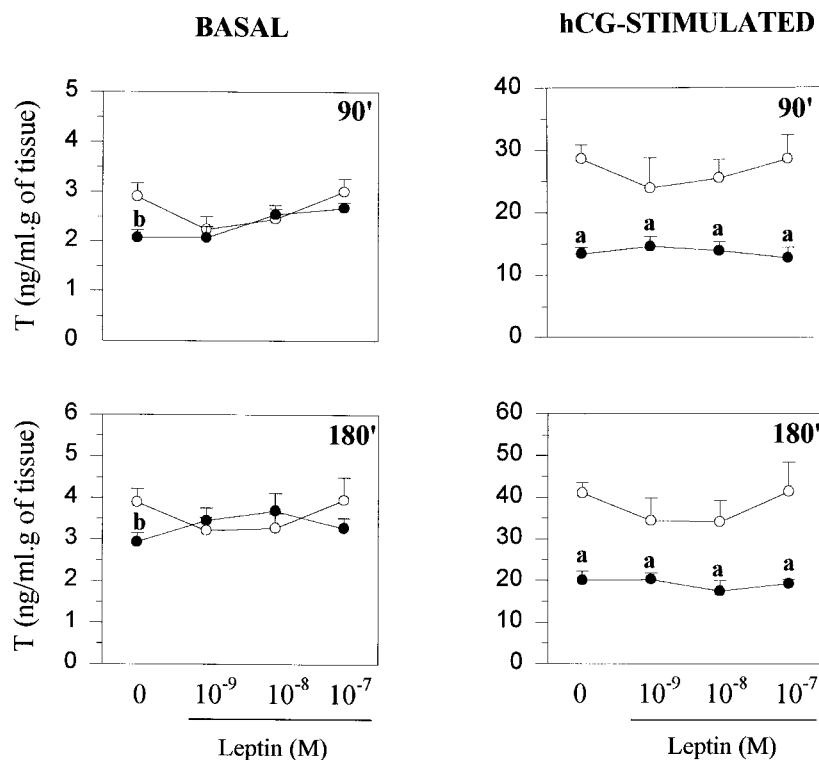


Figure 1 Effects of leptin on *in vitro* basal (left panels) and hCG-stimulated (right panels) testosterone (T) secretion in prepubertal male rats. Testes from normally-fed (○) and fasted (●) males were challenged with increasing concentrations of leptin alone (left panels) or leptin plus 10 IU hCG (right panels), and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (○) served as controls. Values are given as means \pm S.E.M. ($n=12$ samples/group). ^a $P<0.01$, ^b $P<0.05$ vs values from corresponding normally-fed groups (two-way ANOVA followed by Tukey's test).

was partially suppressed by leptin, an effect that was observed for all doses (10^{-9} – 10^{-7} M) after 90 min incubation, but only for the 10^{-9} M dose after incubation for 180 min. In the fasted group, 10^{-9} – 10^{-7} M leptin significantly inhibited hCG-stimulated testosterone secretion only after 180 min incubation. In addition, basal testosterone release by testes from fasted animals was reduced after 90 and 180 min incubation in the presence of 10^{-9} – 10^{-7} M leptin, although the decrease induced by 10^{-7} M leptin at 180 min was at the limit of statistical significance.

Besides its actions at the testicular level, the effects of recombinant leptin on basal pituitary function of adult fasted (96 h) male rats were evaluated. Leptin, in a dose-dependent manner, inhibited gonadotrophin secretion *in vitro*: 10^{-9} M leptin induced a significant decrease in LH and FSH release to the medium after 60 (data not shown) and 120 min incubation (Fig. 3), whereas 10^{-8} and 10^{-7} M leptin were ineffective. As a positive control, under similar conditions 10^{-6} M LHRH elicited a 3- and 2-fold increase in LH and FSH secretion respectively. In addition, the effect of leptin on PRL release *in vitro* was

also tested in the same experimental paradigm. Leptin, at all doses tested, failed to change basal PRL secretion by incubated hemi-pituitaries of adult fasted males (Fig. 3).

Discussion

Our data provide evidence for a direct inhibitory role of leptin in the control of basal and stimulated testicular testosterone secretion in adult rats. This inhibitory action appeared independent of the prevailing nutritional state, and was not observed in prepubertal rat testes. In addition, leptin, in a dose-dependent manner, selectively inhibited *in vitro* gonadotrophin secretion by hemi-pituitaries from adult fasted males. Several reasons prompted us to assess whether potential leptin effects depend on the age of the animal or on background leptin levels. First, testicular steroid secretion changes with age (Moger 1977, Corpéchet *et al.* 1981), and it is known that adult-type Leydig cells, responsible for testosterone production postnatally, show different developmental stages during

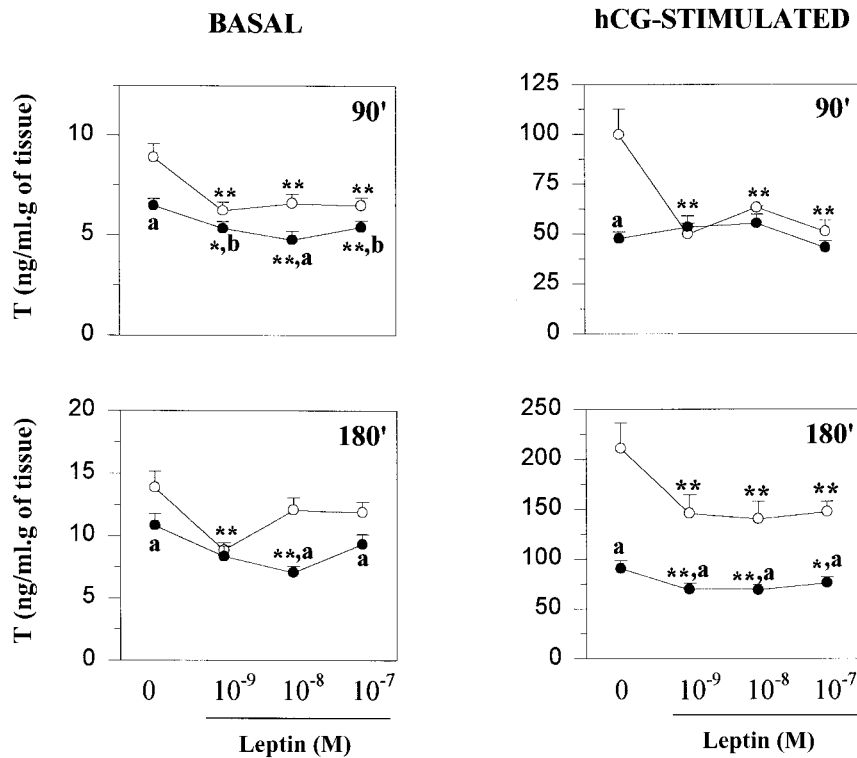


Figure 2 Effects of leptin on *in vitro* basal (left panels) and hCG-stimulated (right panels) testosterone (T) secretion in adult male rats. Testes from normally-fed (\circ) and fasted (\bullet) males were challenged with increasing concentrations of leptin alone (left panels) or leptin plus 10 IU hCG (right panels), and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (0) served as controls. Values are given as means \pm S.E.M. ($n=12$ samples/group). * $P<0.05$, ** $P<0.01$ vs values from corresponding controls (0); ^a $P<0.01$, ^b $P<0.05$ vs values from corresponding normally-fed groups (two-way ANOVA followed by Tukey's test).

pubertal and postpubertal maturation (Shan & Hardy 1992). Secondly, short-term starvation has been shown to induce a decrease in endogenous leptin levels in both male and female rats (Nagatani *et al.* 1998, Vaugnat *et al.* 1998) and evidence for the modulatory role of the metabolic state on the actions of leptin in the neuroendocrine control has been reported previously (Carro *et al.* 1997a).

Overall, an inhibitory action of leptin on *in vitro* testosterone secretion was observed in both normally-fed and fasted adult animals. However, differences in the time-course and dose-dependency of the reported inhibitory actions of leptin were noted depending on the nutritional state. In the fed group, leptin, at all doses tested, decreased basal and hCG-stimulated testosterone secretion, although the effect on basal testosterone release was only evident after 90 min incubation. In food-deprived animals, hCG-stimulated testosterone secretion was inhibited by 10^{-9} – 10^{-7} M leptin only after 180 min incubation, whereas basal testosterone release decreased after 90 and 180 min incubation in the presence of 10^{-9} – 10^{-7} M leptin. Inter-

estingly, the effects of leptin at the testicular level appear to be opposite to those reported for the systemic administration of leptin in *ob/ob* male mice (Barash *et al.* 1996). A similar anomaly has been described for the female, where leptin administration increased serum LH and ovarian weight in female *ob/ob* mice, but it had a direct inhibitory action upon ovarian steroidogenesis (Zachow & Magoffin 1997, Spicer & Francisco 1998). Taken together, the current data point to a complex mode of action of leptin at multiple sites of the hypothalamic–pituitary–gonadal axis in its control of reproductive function.

It is noteworthy that the reported inhibitory effects of leptin appeared dependent on the state of sexual maturation as they were seen in adult but not in prepubertal animals. It is tempting to speculate that this phenomenon may represent a protective mechanism to ensure proper testosterone production around puberty, a period when a rise in circulating leptin levels occurs in humans and rodents (Chehab *et al.* 1997, García-Mayor *et al.* 1997). Conclusions regarding the mechanism(s) for the inhibitory

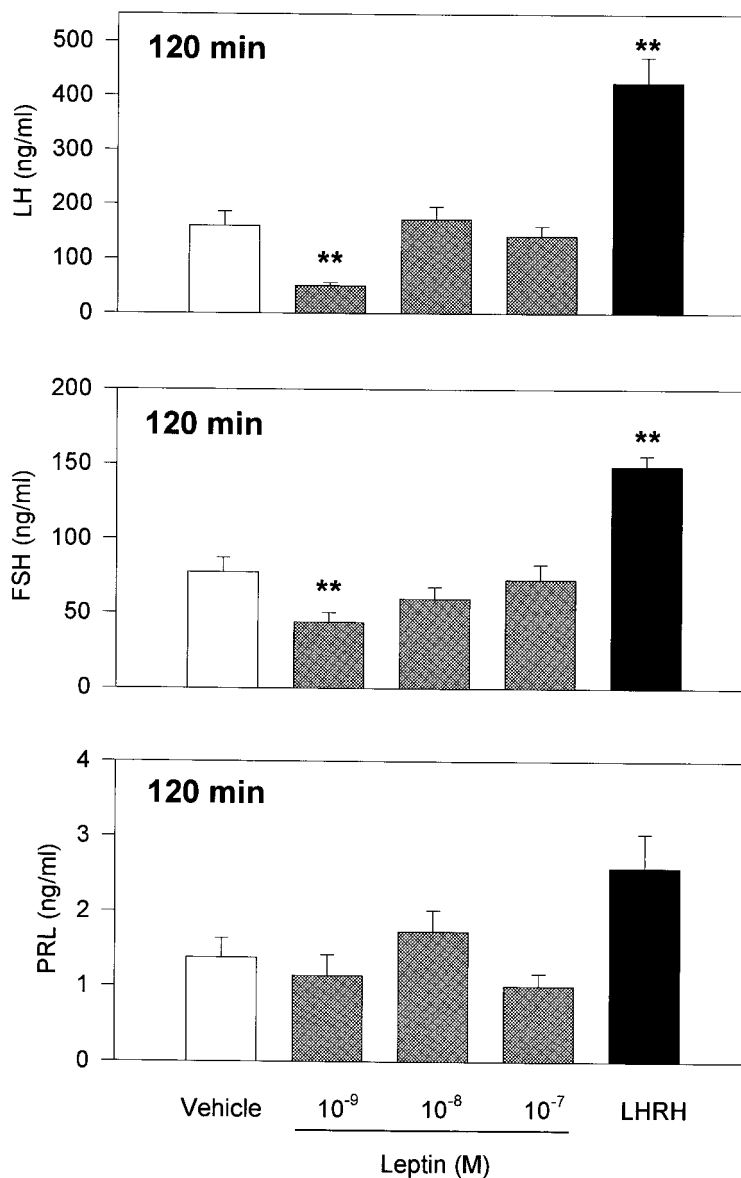


Figure 3 Effects of leptin on *in vitro* basal LH, FSH, and PRL secretion in adult fasted male rats. Hemi-pituitaries from adult fasted animals were incubated in the presence of increasing concentrations of leptin (10^{-9} – 10^{-7} M) or LHRH (10^{-6} M). As the pattern of hormone release was similar after 60 and 120 min incubation, only data from the latter time-point are presented. Values are given as means \pm S.E.M. (10 samples per group). ** $P < 0.01$ vs control samples incubated in the presence of DMEM alone (one-way ANOVA followed by Tukey's test).

actions of leptin cannot be drawn on the basis of the present data. Interestingly, a recent report showed that murine recombinant leptin attenuated the ability of insulin-like growth factor (IGF)-I to elicit testosterone release from isolated rat Leydig cells but failed to modify basal and hCG-stimulated testosterone production (Valenti *et al.* 1998). Although clear-cut differences in the

experimental approaches exist, our present data using whole testicular tissue open up the possibility that, at least partially, the actions of leptin are due to its ability to modulate the paracrine network involved in the control of basal and stimulated testicular steroidogenesis.

In our animal model, fasting of prepubertal and adult rats induced a significant decrease in serum testosterone

levels as well as in the capacity of testes to secrete testosterone *in vitro*, under both basal and hCG-stimulated conditions. However, a concomitant reduction in circulating LH levels was only evident in adult fasted rats. The possibility of age-related changes in the pattern of gonadotrophin response to fasting cannot be ruled out. However, speculation on this topic is hampered by the limited sensitivity of conventional RIAs for LH measurement. Indeed, variations in the magnitude of the decrease in LH after fasting in adult rats have been reported depending on the assay method used (Perheentupa *et al.* 1995).

In a previous report, leptin was demonstrated to stimulate LH and FSH secretion by incubated hemi-pituitaries from adult, normally-fed male rats (Yu *et al.* 1997a). We aimed to extend this observation, and determine whether the effects of leptin at the pituitary level in the control of gonadotrophin secretion can be modulated by the prevailing nutritional state. Basal LH and FSH secretion by hemi-pituitaries from adult fasted males was significantly inhibited by 10^{-9} M leptin, whereas basal PRL secretion was not modified. Surprisingly, in our incubation system, 10^{-8} and 10^{-7} M leptin did not change basal gonadotrophin release, suggesting that the inhibitory actions of leptin on LH and FSH secretion are carried out in a narrow range of doses. In addition, when compared with data from Yu and co-workers (1997a,b), our results suggest that the effects of leptin at the pituitary level on the control of gonadotrophin secretion depend on the nutritional background. The mechanism(s) underlying such a phenomenon is unclear but may involve the presence of different leptin receptor isoforms. Two major putative isoforms of leptin receptor (termed leptin- R_L and leptin- R_S), with different biological roles, have been identified (Tartaglia 1997), and the balance of their expression in rat brain is altered by fasting (Bennett *et al.* 1998). Thus, it is tempting to speculate that the reported switch in the effect of leptin *in vitro* on LH and FSH release may depend on the differential expression of leptin receptor isoforms under different nutritional states.

In conclusion, our results show that leptin is able to inhibit *in vitro* basal and hCG-stimulated testosterone secretion by adult rat testes. This effect is not dependent on the prevailing nutritional state, and is not observed in prepubertal animals. In addition, leptin, in a dose-dependent manner, selectively inhibits *in vitro* gonadotrophin secretion by hemi-pituitaries from adult fasted males. Overall, our present data suggest that the actions of leptin on the reproductive system are probably carried out at different levels of the hypothalamic–pituitary–testicular axis.

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