

# Leptin modulates the corticotropin-releasing hormone (CRH) action on progesterone release from cultured rat granulosa cells

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## Abstract

**OBJECTIVES:** Corticotropin-releasing hormone (CRH) and leptin (LEP) are two neuropeptides involved in the regulation of food intake, energy homeostasis and stress response. These neuropeptides play an important role in the regulation of the hypothalamo-pituitary-gonadal (HPG) axis, too. It is known that leptin can affect the synthesis and release of CRH in the hypothalamus. There is no information about interactions between CRH and leptin directly in gonads. The aim of this study was to evaluate the effects of leptin and CRH on progesterone (P4) release from cultured rat granulosa cells obtained from mature rats in diestrus.

**METHODS:** Granulosa cell cultures were maintained with CRH, LEP, CRH + LEP and CRH-antagonist in varying concentrations. P4 supernatant concentrations were determined by RIA method.

**RESULTS:** CRH stimulated P4 release, CRH-antagonist had no effect on P4 release alone but inhibited the CRH-stimulated P4 release, and the effect of leptin depended on concentration and on time of incubation.

**CONCLUSION:** Our results show that leptin, beside its own influence, may effect on ovarian steroidogenesis through its interaction with effects of CRH activity. This is a new additional link between the stress response, body weight and reproductive functions.

## Abbreviations and units

CRH	corticotropin-releasing hormone
LEP	leptin
E2	estradiol
P4	progesterone
HPG	hypothalamus-pituitary-gonads
VMH	ventro-medial hypothalamus
PVN	paraventricular nucleus
WKY	Wistar-Kyoto
ir	immunoreactive
GC	granulosa cells
nM	nanomol
mins	minutes

## Introduction

Leptin is a hormonal product of the *ob* gene secreted by adipocytes, which in mammals plays a major role in controlling body fat mass. It has been reported that leptin reduces food intake [1, 2, 3] and increases energy expenditure [4]. Recent data indicate that leptin plays an important role in the regulation of the HPG axis. For example, leptin treatment rescued the sterility of genetically obese *ob/ob* mice [5, 6]. It accelerated the onset of puberty in female mice [7, 8] and in rats whose food intake was less than normal [9]. Furthermore, leptin administration to *ob/ob* mice stimulates all aspects of their reproductive endocrine system and restores their fertility [5, 10]. Other results show that in different animal models leptin inhibits the hormonally-stimulated ovarian steroidogenesis *in vitro* [11, 12, 13]. It seems to signal metabolic information to the reproductive system. Leptin may exert these effects at central and/or peripheral levels.

Corticotropin-releasing hormone is the other neuropeptide key involved in the regulation of food intake and energy homeostasis [14, 15]. A continuous administration of CRH into the cerebral ventricle reduces food intake and body weight in rats and monkeys [16, 17]. It is well-known that CRH has an inhibitory effect on the hypothalamus-pituitary-gonads (HPG) axis and reproductive functions. It has been proved that CRH administered centrally suppresses GnRH and gonadotropins secretion acutely in rodents and primates [18, 19, 20]. *In vitro*, CRH has been shown to suppress estrogen production from rat and human granulosa cells [21].

It is known that leptin can affect the synthesis and release of CRH. Heiman et al demonstrated that leptin can blunt the stress-induced activation of the HPA axis by inhibiting the CRH release from the hypothalamus [22]. Huang et al demonstrated that leptin prevented the induction of CRH synthesis in the PVN and activation of the PVN CRH neurons

observed in food-deprived *ob/ob* mice [23]. On the other hand, administration of leptin stimulated CRH secretion in the hypothalamus [24], activated hypothalamic CRH containing pathways [25], and increased CRH expression in paraventricular nucleus (PVN) and CRH-receptor type 2 mRNA expression in the ventro-medial hypothalamus (VMH) [26].

There is no information about interactions between CRH and leptin directly in gonads. The aim of this study was to evaluate the effects of leptin and CRH on progesterone (P4) release from cultured rat granulosa cells.

## Material and methods

Adult female Wistar-Kyoto (WKY) rats (220–250) were used throughout this study. They were maintained at 25°C under controlled lighting conditions (lights on at 07.00 h, lights off at 19.00 h), with food and water *ad libitum*. Vaginal smears were performed to assess the stage of the estrus cycle; only animals exhibiting two consecutive 4-day cycles were included in the study.

The ovaries from WKY rats in diestrus were collected under aseptic conditions. Isolated ovaries were washed with PBS, supplemented with a mixture of antibiotics and then they were rubbed through a sieve (mesh 50). Granulosa cells were treated with 0.15 collagenase and 0.1% hyaluronidase in Hank's buffer at 37°C for 30 mins and digested for the next 30 mins in a buffer containing: 0.02% EDTA, 0.1% glucose, 0.1% NaCl, 0.19% NaHCO<sub>3</sub> and 0.1% trypsin at 37°C. Dispersed cells were washed with culture medium (RPMI containing 0.5% BSA and 10% fetal calf serum) and seeded in culture medium in 24-well culture plates. The cells were then cultured for three days in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. After that period the medium was removed and the cells were cultured under serum-free conditions with CRH (Sigma), LEP (Pepro Tech EC LTD) and CRH-antagonist ( $\alpha$ -helical CRH [9–41]) (aCRF) (Sigma) in varying concentrations: 0.1, 1, 10 nM. The control culture group was cultured in physiological solution. Cell cultures were maintained for 60 and 120 mins. Culture supernatants were then decanted and stored until a hormone analysis. That method was based on the conditions described previously [27–31]. The experiments were repeated 3 times; 2 x 10<sup>5</sup>/ml cells were present in each culture.

P4 supernatant concentrations were determined by RIA method (Orion Diagnostica).

Data were expressed as means  $\pm$  SEM, and their statistical comparison was done by t-Student test.

## Results

CRH stimulates P4 release from rat ovarian granulosa cells (Fig. 1).

CRF antagonist has no effect on P4 release alone (Fig. 2) but inhibits the CRH-stimulated P4 release (Fig. 3).

Leptin in low concentrations (0.1 nM) stimulates P4 release and in higher concentrations (1, 10 nM) it inhibits P4 release (Fig. 4).

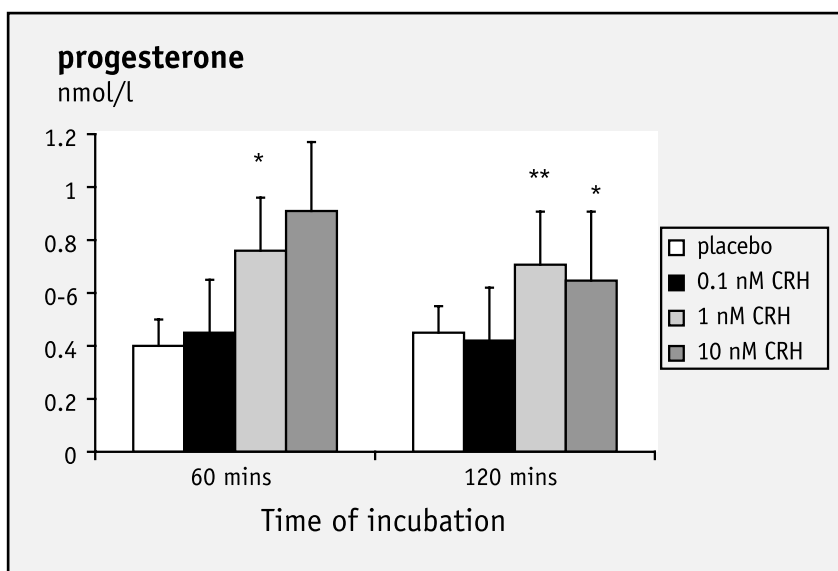
Low concentration of leptin (0.1 nM) inhibits CRH-stimulated P4 release (Fig. 5).

The effects of higher concentration of leptin (1 nM) depend on time of incubation: after 60 mins leptin inhibits and after 120 mins it increases the CRH-stimulated P4 release (Fig. 5).

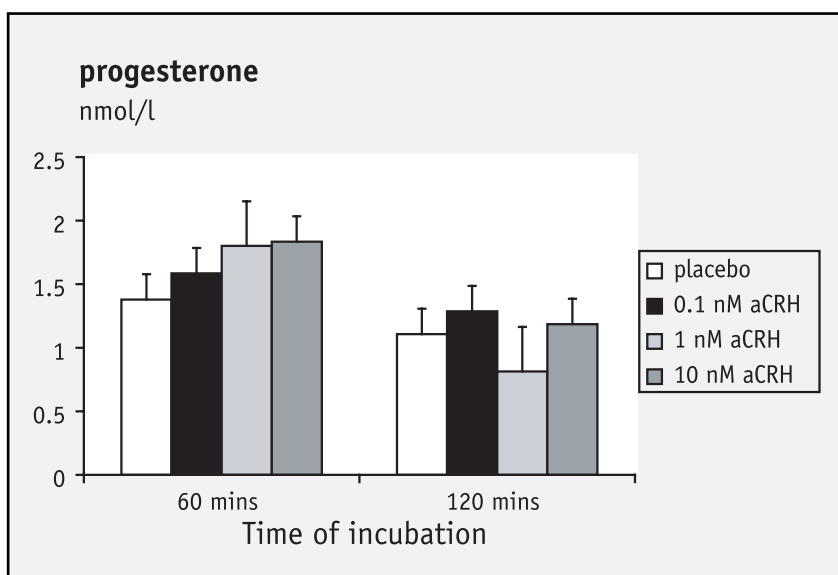
## Discussion

In the present study we have demonstrated the relations between CRH and leptin in their action on ovarian progesterone release. Many earlier experiments have shown that both these neuropeptides play an important role in direct regulation of gonadal steroidogenesis in humans and animals.

Previous studies have demonstrated the presence of immunoreactive (ir) CRH and CRH mRNA in the Leydig cells of the rat testis [32, 33]. Whereas CRH inhibits testosterone production by rat Leydig cells [33], it stimulates steroidogenesis in mouse Leydig cells in vitro [34]. The reason for this disparity is not known.

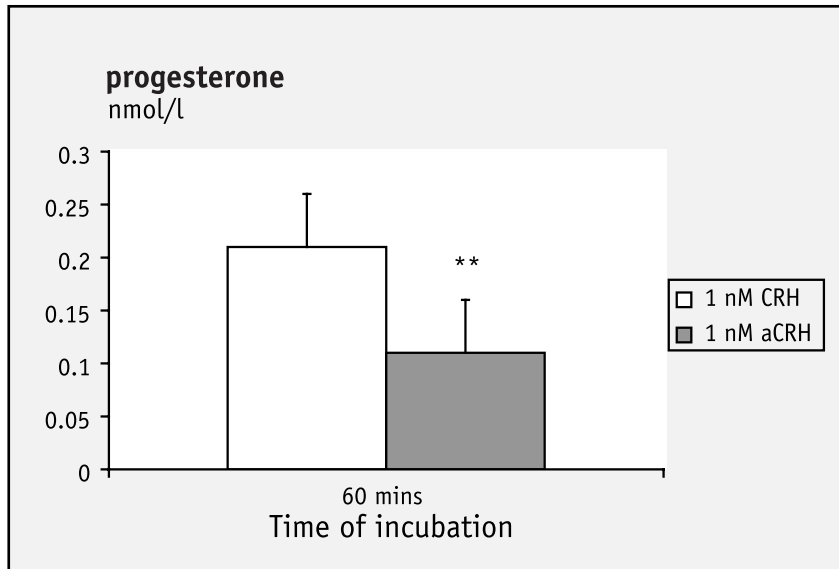


**Fig. 1.** The effect of CRH on progesterone release from granulosa cells  
\*  $p < 0.05$ , \*\*  $p < 0.01$

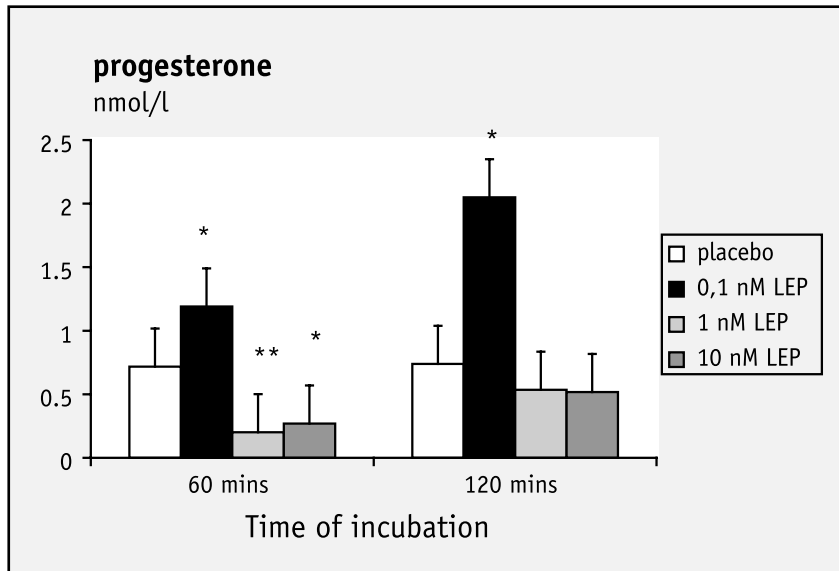


**Fig. 2.** The effect of CRH-antagonist on progesterone release from granulosa cells  
aCRH- CRH antagonist

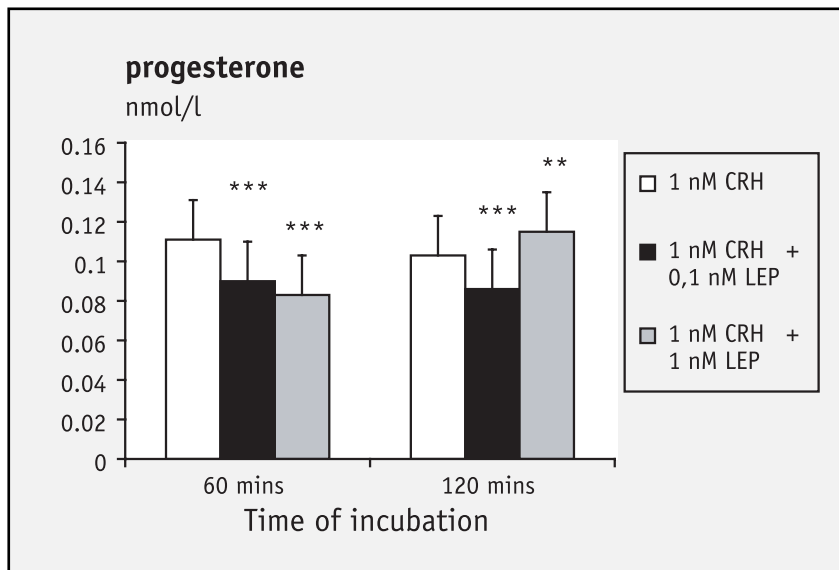
**Fig. 3.** The effect of CRH-antagonist on progesterone release from granulosa cells after CRH administration  
 \*\*  $p < 0.01$ , aCRH- CRH antagonist



**Fig. 4.** The effect of leptin on progesterone release from granulosa cells  
 \*  $p < 0.05$ , \*\*  $p < 0.01$ , LEP- leptin



**Fig. 5.** The effect of leptin on progesterone release from granulosa cells after CRH administration  
 \*\*  $p < 0.01$ ,  
 \*\*\*  $p < 0.001$



Rat and human ovaries contain immunoreactive irCRH in theca cells surrounding follicles, as well as in stroma cells, mature oocytes within antral follicles, and ovarian resident macrophages. In developing corpora lutea irCRH was detected in the cytoplasm of both small theca-derived and large granulosa-derived luteinized cells, which indicates that with luteinization CRH persists in theca-derived and appears de novo in granulosa-derived luteinized cells. Autoradiographically, CRH receptors were found in stroma and theca cells around follicles, as well as in cells of the cumulus oophorus. They were sparsely distributed within the corpora lutea but not in the granulosa layer of follicles [35, 36]. In situ hybridization characterized these ovarian CRH receptors as type I [37, 38].

Calogero et al. [21] have shown an inhibitory effect of CRH on the basal estrogen production in human granulosa-luteal cells and FSH-stimulated estrogen production from rat granulosa cells (GC). In another study (GHIZ) CRH inhibited estradiol (E2) and P4 secretion from human granulosa-luteal cells [52]. In our experiment CRH stimulated P4 from rat granulosa cells. These findings are inconsistent with the absence of the CRH receptor on granulosa cells of maturing follicles in vivo [35, 36]. However, the presence of the CRH receptor on granulosa cells and the content of irCRH in the ovary may depend on age and on the phase of the estrus cycle. In immature rats total irCRH was undetectable both in control and acute stressed animals, while adult rats showed the highest values of irCRH at proestrus. An acute stress exposure induced a significant increase in irCRH ovarian contents only at proestrus, without affecting irCRH at the other phases of the estrus cycle [39]. In old rats (24 months) irCRH is 50% lower than in young rats (3–4 months) and stress was associated with a significantly lesser decrease in irCRH in the theca in old rats than in young rats (old 35% versus young 70%) [40]. Whereas Mastorakos [35] controlled cycling regularity only and Calogero's [21] rats were immature, we used normal cycling rats on diestrus day of the estrus cycle. It is possible that acquisition of CRH receptor and direct effects of CRH on ovarian steroidogenesis differ between phases of the menstrual cycle in women and estrus cycle in rats, and they may be different in animals at different ages. Thus, the ovarian CRH may be viewed as an autocrine and paracrine regulator of steroidogenesis.

Data about effects of leptin on hormone release from cultured granulosa cells differ between authors. Results of Kitawaki [41] indicate that leptin stimulates basal and FSH or IGF-1-stimulated E2 production; the production of progesterone does not change. However, in most studies leptin had an inhibitory

effect on the release of ovarian hormones. In GC obtained from immature rats, leptin antagonized the stimulatory effects of TGF-beta and IGF-1 on FSH-dependent estrogen production. Leptin did not alter basal or FSH-stimulated E2 secretion. The inhibitory effect of leptin was specific for E2 production since there were no effects on basal, FSH-, or FSH+IGF-1-dependent P4 levels [13, 42]. In the other study leptin suppressed the P4 synthesis costimulated by FSH and dexamethasone in the primary rat GC [43]. In human luteinized GC leptin inhibited human chorionic gonadotrophin-stimulated P4 production, but it did not alter basal steroidogenesis. Moreover, the inhibitory effect of LEP on HCG-stimulated P4 production was only manifested in the presence of insulin [44]. Karlsson et al. showed that leptin inhibited LH-stimulated but not basal production of estradiol in cultured human GC [45]. In bovine GC from small and large follicles, leptin attenuated insulin-induced but not basal E2 and P4 production. Furthermore, specific binding of <sup>125</sup>I-leptin was demonstrable in GC, and leptin did not compete for specific <sup>125</sup>I-insulin binding to GC [11].

In all these experiments leptin affected the stimulated P4 release. In our study leptin influenced the basal P4 secretion. This is possible because we used mature rats to culture the GC, in contrast to immature rats used by others authors. The differences between the species may also explain these disparities. In cited studies leptin inhibited stimulated P4 release only. We have also found this effect of leptin on basal release of P4, but only in higher concentrations (1 and 10 nM)—similar to those used by these authors [11, 13, 41–45]. However, leptin in concentration 0.1 nM stimulated P4 secretion in our study. Yu et al. observed this same effect [46] incubating with leptin explants from tissues of median eminence-arcuate nucleus taken from adult male rats. Leptin produced a significant increase in LH-RH release only at the lowest concentrations tested ( $10^{-12}$ – $10^{-10}$  M). As the leptin concentration increased, the LH-RH release decreased and it was significantly lower than the control release at the highest concentration tested ( $10^{-6}$  M).

These results indicate that in very low concentrations leptin may stimulate and in higher concentrations may inhibit the P4 release from rat GC. Because we are the only authors who obtained GC from mature rats, it is possible that mature GC may be more sensitive to leptin's action. This effect may also depend on the phase of the estrus cycle.

For today, the relationship between leptin and CRH was studied only in the context of appetite. Leptin seems to reduce food intake and body weight at least partially due to enhancement of the anorectic

effect of CRH. It increased CRH mRNA expression in PVN [26, 47] and CRHR-2 mRNA expression in VMH, which is a satiety center—a possible target of anorectic CRH effects [26]. Injection of leptin into the third ventricle prevented the fasting-induced reduction of CRH mRNA in PVN [48] and increased the hypothalamic CRH content, whereas CRH antagonist attenuated the anorectic effect of leptin [49]. In concentrations 0.1–10 nM leptin stimulated the CRH secretion in primary cultures of neonatal (5–6 day old) rats' hypothalamus [24, 50]. These findings are consistent with the reported presence of leptin receptors in the rat hypothalamus and other brain areas involved in energy balance [47], and they strongly suggest that leptin may regulate the appetite, at least in part, by directly modulating the secretion and action of CRH in the hypothalamus. We have encountered only two papers, which showed an adverse effect of leptin on CRH synthesis and on release from the hypothalamus [22, 23]. In Huang's study [23] leptin prevented the induction of CRH synthesis in PVN and the activation of the PVN CRH neurons in food-deprived ob/ob male mice. In the other study, in isolated rat hypothalami, leptin blocked the CRH release stimulated by decreasing the glucose of the buffer. In this same experiment leptin did not alter the secretion of ACTH from rat primary cultured pituitary cells, but in vivo it blunted the plasma ACTH and corticosterone responses to restraint stress [22].

To reconcile these divergent findings, it can be assumed that the rapid action of leptin to decrease the readily releasable storage pool of hypophysiotropic CRH is independent of actions of leptin to influence hypothalamic CRH mRNA in the PVN. The second reason may be different actions of leptin on different hypothalami cultures: cell culture or only isolated hypothalami, rat or mice tissue, mature or immature, male or female animal, action on stimulated or basal release of CRH.

In spite of much information about the separate influence of leptin and CRH on HPG axis [5–13, 18–21], there is no information about the relationship between leptin and CRH acting on this axis. The purpose of the present study was to understand this relationship in P4 release from GC obtained at diestrus day from adult rats. In our study low concentrations of leptin inhibited CRH-stimulated P4 release. The effects of higher concentrations of leptin depended on time of incubation: after 60 mins leptin inhibited and after 120 mins it increased the CRH-stimulated P4 release.

Heiman et al. [22] demonstrated that leptin can blunt the stress-induced activation of the HPA axis, and is capable of exerting this effect through inhibition of stimulated CRH release in the hypothalamus.

Thus, if stress can change CRH content in the ovary [39, 40] and leptin can blunt the stress-induced activation of the HPA axis by inhibiting the CRH release from the hypothalamus [22], our results show that leptin may blunt the stress-induced changes in gonadal steroidogenesis through its interaction with effects of CRH activity directly in the ovaries. These findings, coupled with observations that leptin and cortisol show an inverse circadian rhythm [51], strongly support the existence of a closed, bi-directional circuit between the HPA axis function and adipose tissue metabolism and provide an additional important link between the human stress response, body weight and reproductive function regulations.

The effect of these interactions on ovarian steroidogenesis and hormone release, similar to effects of each hormone alone, may depend on species, age, gender, phase of estrus cycle, metabolic and hormonal status of organism (doses of substances, art of stressors, time of action in experiments), and many other factors.

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