EXPERIMENTAL STUDY

***In vitro* effect of leptin on LH release by anterior pituitary glands from female rats at the time of spontaneous and steroid-induced LH surge**

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Abstract

Objective: The purpose of this work was to study the direct effect of leptin on LH release by anterior pituitary glands from female rats at the time of spontaneous and steroid-induced LH surge.

Methods: LH responsiveness to leptin by pituitaries from rats killed in the afternoon (1500 h) at different stages of the 4-day estrous cycle (diestrus-1: D1; diestrus-2: D2; proestrus; estrus), ovariectomized (OVX; 15 days post-castration) and ovariectomized steroid-primed (OVX-E₂/Pg; pretreated with 5 µg estradiol and 1 mg progesterone), was evaluated *in vitro*. Hemi-adenohypophyses were incubated in the presence of synthetic murine leptin for 3 h.

Results: Addition of increasing concentrations of leptin (0.1–100 nmol/l) to the incubation medium of proestrus pituitaries produced a dose-related stimulation of LH release; the maximal increase to 315% of control was obtained with 10 nmol/l leptin. Leptin (10 nmol/l) enhanced LH release at all days of the estrous cycle, the greatest response occurring in proestrus (318%) and the lowest at D1 (123%). In order to evaluate the role of nitric oxide (NO) in the action of leptin on LH release, glands from proestrus rats were incubated in the presence of 10 nmol/l leptin with or without 0.3 mmol/l N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NO synthase (NOS). NMMA completely suppressed the stimulation of LH release induced by leptin. Leptin also stimulated LH release by pituitaries from OVX rats, and treatment with steroid hormones led to a marked increase in the response (OVX: 162% compared with OVX-E₂/Pg: 263%; $P < 0.05$). For comparative analysis, a similar experimental procedure was carried out using GnRH (10 nmol/l). Leptin acts at the pituitary level in a similar manner as GnRH, although with significantly lower potency.

Conclusions: These results confirm and extend previous reports regarding a direct action of leptin at the pituitary level, stimulating LH release by anterior pituitaries from female rats at the time of spontaneous and steroid-induced LH surge. In the female rat pituitary this leptin action is controlled by gonadal steroids and mediated by NO.

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Introduction

Leptin, a 16 kDa protein, is a hormone produced by adipocytes that, in addition to its involvement in the control of food intake, body weight and energy expenditure (1), has important effects on the reproductive system (2, 3).

Exogenous leptin administration advances the onset of puberty (4, 5), restores fertility in *ob/ob* mice (6, 7) and increases basal luteinizing hormone (LH) concentrations in fasted adult mice (8), rats (9, 10) and monkeys (11). LH secretion is increased by the central administration of leptin to ovariectomized estradiol-primed rats (12), whereas LH is suppressed and estrous cycles are disrupted by central infusion of leptin

antisera (13). Taken together, these observations suggest that leptin is a metabolic signal to the neuroendocrine reproductive system.

Although the mechanism(s) by which leptin regulates reproductive function remains to be fully characterized, evidence points to the hypothalamus as the main locus of its action (14–16). However, after demonstration of leptin receptors and the effects of leptin on *in vitro* systems, additional sites for leptin action, including the pituitary, testis and ovary (12, 17–20) have been suggested. The potential direct effects of leptin at the pituitary level, however, have been poorly characterized. In this regard, both stimulatory effects (12, 18) and the absence of a modulatory action (21) of leptin on LH, follicle-stimulating

hormone (FSH) and prolactin secretion by adult male rat pituitaries *in vitro* have been reported. Much less is known of the functional significance of leptin in the normal cycling female rat (13, 22).

The purpose of the present work was to study the direct effects of leptin on LH release by anterior pituitary glands from female rats at the time of spontaneous and steroid-induced LH surge. We also determined the influence of gonadal steroid hormones and the role of nitric oxide (NO) in the action of leptin on the anterior pituitary gland *in vitro*.

Materials and methods

Drugs

The source of the drugs used was as follows: gonadotropin-releasing hormone (GnRH): Bachem Inc. (Torrance, CA, USA); synthetic murine-leptin, 17 β -estradiol (E₂), progesterone (Pg) and N^G-monomethyl-L-arginine (NMMA): Sigma (St Louis, MO, USA).

Animals

Adult Wistar female rats bred in our laboratory were used. Animals were housed under controlled conditions of temperature (21 $\pm$ 1 °C) and lighting (14 h light–10 h darkness) and had free access to food and water. Estrous cycles were monitored by daily vaginal smears, taken between 0900 and 1000 h. Rats weighing 200–250 g and having exhibited at least three consecutive 4-day estrous cycles were used. Anterior pituitaries were collected from intact (diestrus-1 (D1), diestrus-2 (D2), proestrus or estrus) rats or from those that had been ovariectomized (OVX) 15 days earlier.

Incubation of hemipituitaries

Groups of six animals were killed by decapitation at 1500 h on different days of the estrous cycle or 15 days after ovariectomy. After removal of the posterior lobe, the anterior pituitary was divided into two pieces by a midline cut and each half was placed in a flask containing 1 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.25% BSA. Incubation was carried out in a Dubnoff metabolic shaker at 60 c.p.m. and 37 °C under an atmosphere of 95% O₂–5% CO₂. After a preincubation period of 30 min, hemipituitaries were incubated for 3 h in fresh medium alone or containing the test substances. At the end of the incubation period, media were separated, centrifuged and the supernatant kept frozen at –20 °C until assayed, and tissues were blotted and weighed. The culture media and chemicals were purchased from Sigma.

Experimental designs

Four experimental designs were used to analyze the *in vitro* effect of leptin on LH release. For comparative analysis, a similar experimental procedure was carried out using GnRH. In the first experiment, pituitaries from proestrus rats were incubated in the presence of increasing doses of GnRH (0.1–100 nmol/l) or leptin (0.1–100 nmol/l) in order to determine the dose–response relationship. In the second approach, the effect of GnRH or leptin on LH release throughout the estrous cycle was studied. Pituitary glands collected from animals on different days of the cycle were incubated for 3 h with or without 10 nmol/l GnRH or 10 nmol/l leptin. The third experiment focused on whether nitric oxide (NO) was involved in the action of GnRH and leptin on LH release. Pituitary glands from proestrus rats were incubated in the presence of 10 nmol/l GnRH or 10 nmol/l leptin, with or without (control groups) the addition of 0.3 mmol/l NMMA, a competitive inhibitor of nitric oxide synthase (NOS).

In the fourth set of experiments, the influence of gonadal steroids on GnRH- and leptin-induced LH release was examined. OVX rats were injected with 5 μ g E₂ in corn oil (0.1 ml s.c.) or vehicle on days 13 and 14 post-castration, at 1700 h, and with 1 mg progesterone in corn oil (0.2 ml s.c.) or vehicle on day 15 at 0900 h – a treatment that produces a well-defined preovulatory-type LH surge (23). The animals were killed on day 15 post-castration, at 1500 h. Pituitaries from steroid-primed (OVX-E₂/Pg) or oil-treated (control, OVX) rats were incubated in medium containing 10 nmol/l GnRH or 10 nmol/l leptin.

LH radioimmunoassay

LH was measured in media samples at two dose levels by double-antibody radioimmunoassay, with reagents supplied by the National Hormone and Pituitary Program, NIDDK, NIH. Hormonal values are expressed in terms of the reference standard rLH-RP-3. The intra-assay coefficient of variation (CV) for the LH assay was 4.8%, the corresponding inter-assay CV being 8.7%. The results were expressed as ng LH per mg pituitary gland.

Statistics

Data are expressed as means $\pm$ S.E.M. (n = 6 samples/group). Differences between experimental groups were analyzed by analysis of variance followed by Duncan's multiple range test. A level of P < 0.05 was considered statistically significant.

Figure 1 Dose–response relationship for GnRH and leptin. Release of LH from incubated anterior pituitaries of proestrus-afternoon rats in response to increasing doses (0.1–100 nmol/l) of GnRH or leptin. Hemiglands were incubated for 3 h in 1 ml of medium DMEM in the presence (filled symbols) or absence (C: control groups; open symbols) of GnRH (circles) or leptin (squares). Each point is the mean value of six hemiglands and the vertical lines indicate $\pm$ S.E.M. Differences between GnRH and leptin (0.1, 1.0, 10.0 and 100.0 nmol/l) were significant ($P < 0.05$). * $P < 0.05$: GnRH or leptin compared with the respective control (ANOVA followed by Duncan's test).

Results

Dose–response relationship for GnRH and leptin

Responsiveness of pituitary glands from proestrus-afternoon rats, incubated in the presence of increasing doses (0.1–100 nmol/l) of GnRH or leptin, is shown in Fig. 1. As expected, basal release of LH was greatly stimulated by the addition of GnRH and the effect was proportional to the dose; the maximal increase to 1217% of control values was obtained with 100 nmol/l GnRH. Leptin also enhanced LH release in a dose-related manner, the maximal stimulation to 315% of control value being reached with 10 nmol/l leptin. A dose of 0.1 nmol/l GnRH was effective in inducing a significant increase in LH release (434% of control), whereas higher doses of leptin (1 nmol/l: 199% of control) were required to obtain a significant increase in LH release.

Effect of GnRH and leptin on LH release throughout the estrous cycle

On the basis of the above findings, a dose of 10 nmol/l GnRH or leptin was selected to test the responsiveness

Figure 2 Effect of GnRH and leptin on LH release throughout the estrous cycle. Release of LH from incubated hemipituitaries of 4-day cyclic rats in response to GnRH (10 nmol/l) or leptin (10 nmol/l) at different days of the estrous cycle. Animals were killed at 1500 h on D1, D2, proestrus (P) and estrus (E). The heights of the bars give the mean value of six hemiglands and the vertical lines indicate $\pm$ S.E.M. Differences between GnRH and leptin (D1, D2, P and E) were significant ($P < 0.05$). * $P < 0.05$: GnRH or leptin compared with the respective control (ANOVA followed by Duncan's test).

of pituitary glands collected from animals on different days of the estrous cycle (Fig. 2). As in our previous work (24), GnRH enhanced the release of LH at all stages of the cycle. The magnitude of increase varied according to the day of the cycle (D1: 253%; D2: 331%; proestrus: 813%; estrus: 326% of control), being significantly greater ($P < 0.05$) in the afternoon of proestrus than at the other times. A similar pattern of pituitary responsiveness was obtained with leptin, the greatest response occurring in the afternoon of proestrus ($P < 0.05$) and the lowest at D1 (D1: 123%; D2: 181%; proestrus: 318%; estrus: 176% of control).

Role of NO in the action of GnRH and leptin on LH release

The role of NO in the GnRH- and leptin-induced release of LH by anterior pituitaries from proestrus rats was studied. The competitive inhibitor of NOS, NMMA, in a single concentration of 0.3 mmol/l, was added to the media of non-treated hemiglands, and to hemiglands treated with either 10 nmol/l GnRH or 10 nmol/l leptin. The data in Fig. 3 show that NMMA had no detectable effect on basal LH, partially inhibited the GnRH-induced LH release, and completely suppressed the stimulatory effect of leptin on LH release.

Figure 3 Role of NO in the action of GnRH and leptin on LH release. Release of LH from incubated anterior pituitaries of proestrus-afternoon rats in response to NMMA. Hemipituitaries were incubated with either GnRH (10 nmol/l) or leptin (10 nmol/l), in the presence (+NMMA) or absence (–NMMA; control groups) of 0.3 mmol/l NMMA. The heights of the bars give the mean value of six hemiglands and the vertical lines indicate + S.E.M. Differences between with and without NMMA not significant in non-treated animals ($P > 0.05$), but significant ($P < 0.05$) in GnRH- and leptin-treated animals. * $P < 0.05$: with or without NMMA compared with the respective non-treated group (ANOVA followed by Duncan's test).

Influence of gonadal steroids on GnRH- and leptin-induced LH release

In order to evaluate the involvement of gonadal steroids, the effects of ovariectomy (OVX) and of ovariectomy followed by acute replacement of E_2 and progesterone (OVX- E_2 /Pg) on the GnRH or leptin responses of adenohypophyses *in vitro* were tested. Hemipituitaries from OVX or OVX- E_2 /Pg rats were incubated for 3 h in the presence of GnRH (10 nmol/l) or leptin (10 nmol/l). GnRH- and leptin-stimulated LH release was modified by the steroid treatment. It can be seen in Fig. 4 that pretreatment of the animals with steroid hormones led to a significant increase in the response to either GnRH (OVX: 323% compared with OVX- E_2 /Pg: 673%; $P < 0.05$) or leptin (OVX: 162% compared with OVX- E_2 /Pg: 263%; $P < 0.5$), although the magnitude of the increments did not reach those attained by proestrus pituitaries.

Discussion

Leptin, the hormonal product of the *ob* gene (1), has the potential to act as a metabolic signal to the reproductive system to reflect energy reserves (2, 3).

In the present work, we studied the direct effect of leptin on LH release by *in vitro* anterior pituitary glands

Figure 4 Influence of gonadal steroids on GnRH- and leptin-induced LH release. Release of LH from hemipituitaries of ovariectomized (OVX) and E_2 + progesterone-primed (OVX- E_2 /Pg) rats in response to GnRH (10 nmol/l) or leptin (10 nmol/l). The heights of the bars give the mean value of six hemiglands and the vertical lines indicate + S.E.M. Differences between OVX and OVX- E_2 /Pg not significant in non-treated animals ($P > 0.05$), but significant ($P < 0.05$) in GnRH- and leptin-treated animals. * $P < 0.05$: OVX or OVX- E_2 /Pg compared with the respective non-treated group (ANOVA followed by Duncan's test).

from female rats at the time of spontaneous and steroid-induced LH surge. In previous studies (12, 18), leptin was demonstrated to stimulate LH and FSH secretion by incubated hemipituitaries from adult, normally-fed male rats. We analyzed whether the effects of leptin in the control of LH secretion at the pituitary level can be modulated by the female estrous cycle.

Treatment of pituitaries from proestrus-afternoon rats with increasing doses of GnRH or synthetic murine-leptin caused dose-dependent increases in LH release. A dose of 0.1 nmol/l GnRH was effective in inducing a significant effect, whereas greater doses of leptin were required to obtain a significant stimulation of LH. Although the mechanisms by which leptin acts are far from being understood, leptin would be implicated in LH release through an autocrine or paracrine action, or both, as leptin receptor (OB-R) (25) and leptin (17, 26) are expressed by pituitary cells.

As reported earlier by our group (24), GnRH (10 nmol/l) stimulates the release of LH from incubated pituitary glands throughout the 4-day estrous cycle of the rat; the magnitude of increase varies according to the day of the cycle, being significantly greater in the afternoon of proestrus than at other times. Our current results show that a similar pattern of pituitary responsiveness was obtained with 10 nmol/l leptin, the greatest response occurring in the afternoon of

proestrus and the lowest at D1, although the magnitude of the effects was lower than with GnRH. Thus maximum responsiveness correlated with the proestrus discharge of the hormone, suggesting a modulatory effect of estrogen. It is generally held that there are two critically important estrogen-dependent processes that, together, mediate the LH surge-generating process: hypothalamic neurosecretion of a preovulatory GnRH surge, and a coordinate increase in pituitary responsiveness to this neurosecretory trigger. Indeed, permissive actions of estrogen on leptin production have also been shown. Estrogen was found to be involved in the regulation of leptin production in female OVX rats and humans (27) and regional variations in the regulation of *ob* gene expression by estrogen were demonstrated (27). Estrogens have been shown to stimulate leptin secretion by adipocytes *in vitro* (28, 29) – an effect that is observed only in adipocytes from women and not in samples from men (30). Leptin concentrations were decreased in OVX rats, and reverted to normal by estradiol supplementation (27, 29). Furthermore, leptin concentrations were found to be greater in women than in men (31) – a difference that has also been observed between female and male rats (22).

In order to characterize further the involvement of gonadal steroids, the effect of ovariectomy and of ovariectomy and the acute replacement of E_2 plus progesterone on the *in vitro* adenohypophysis response to GnRH and leptin was tested. Compared with pituitaries from proestrus-afternoon rats, those from OVX rats reduced pituitary sensitivity. Thus, whereas in proestrus the increases in LH release induced by 10 nmol/l GnRH or leptin were 8.13- and 3.18-fold respectively, relative to the basal values, the increases in LH release in glands from OVX rats were 3.23- and 1.62-fold, in response to the same doses. Pretreatment of the animals with steroid hormones led to a significant increase in LH responsiveness to either GnRH or leptin. Thus the pituitary glands respond to GnRH and leptin more efficiently when E_2 and Pg are present, which can be correlated with the well-known regulatory effect of estrogen on LH release. Differences in leptin responsiveness might be related to changes in the pattern of leptin receptor isoform expression after estrogen treatment, as reported earlier in the brain (32). Multiple splice variants of leptin receptor have been found (33). In the central nervous system, the two major isoforms are OB- R_L , which has a long cytoplasmic domain and is believed to transduce the leptin signal (34), and OB- R_S , a short-form receptor which may act as transporter of leptin (35). It has been suggested that fasting and estrogen alter the balance between OB- R_L and OB- R_S , and that this could increase the sensitivity of discrete brain regions for circulating leptin (32). One cannot rule out that estrogen may exert a similar action to sensitize the pituitary to leptin. However, it is noteworthy that previous reports (17, 25) indicated that only the long isoform of leptin

receptor is present in normal mouse and rat pituitary, whereas the short isoform is not detected. Further work is clearly needed to resolve this issue.

NO is involved in the control of the hypothalamic–pituitary function, acting as an inter- and intracellular messenger (36, 37). Neuronal NOS (nNOS), the enzyme responsible for the production of NO from L-arginine (38) in the brain, is expressed in diverse hypothalamic areas (39) and in gonadotropes and follicle-stellate cells in the anterior pituitary gland (40). A physiological role of NO in the preovulatory LH surge was demonstrated by findings that inhibitors and antisense oligonucleotides to nNOS attenuate the steroid-induced (23, 41) and preovulatory LH surge (42). Evidence has also been provided that nNOS mRNA and protein concentrations in the preoptic area of the rat are increased on the afternoon of proestrus (43). In male rats, a direct stimulatory effect of NO on GnRH release *in vivo* and *in vitro* (44), and on the *in vitro* release of LH (45) has been reported. In addition, Yu *et al.* (18) found that the leptin-induced release of LH is mediated by NO.

The present experiments examined the role of NO in the action of leptin on LH release by incubating pituitaries from proestrus rats in the presence of NMMA, a competitive inhibitor of nNOS. The results, in agreement with those of Yu *et al.* (18), showed that NMMA completely suppressed the stimulation of LH release induced by leptin and partially inhibited that induced by GnRH. Therefore, it appears that GnRH and leptin stimulate LH release by activation of nNOS, leading to increased production of NO. Thus the production of NO at the hypothalamic and pituitary levels may be one of the key links in the complex control of the LH surge.

Increasing titers of plasma estrogen act as a primary stimulus for the preovulatory LH surge (46). At the pituitary level, the feedback actions of estrogen on gonadotropes involve both regulation of receptors and modulation of post-receptor events leading to LH secretion. On the basis of the results of this and other studies, we infer that, besides the positive feedback of estrogen secretion on the preovulatory GnRH surge, estrogen may also recruit leptin into the stimulatory cascade, by increasing pituitary leptin and OB-R expression, in order to potentiate the stimulatory signals to the gonadotrope. Furthermore, the actions of additional neurohormonal factors, such as neuropeptide Y, galanin and endothelin, enhance anterior pituitary sensitivity to GnRH. GnRH, which stimulates synthesis and release of LH and FSH, binds to receptors on gonadotropes, leading to increases in intracellular Ca^{2+} , by influx of extracellular Ca^{2+} and mobilization of intracellular pools, which serves to activate the calcium/calmodulin-dependent nNOS enzyme. Activation of nNOS in gonadotropes leads to increased production of NO, which, through as yet unidentified mechanisms, enhances exocytosis of LH. Leptin, like GnRH, may directly regulate LH via a functional OB- R_L

on gonadotropes, leading to increased production of NO by activation of the nNOS enzyme. In addition, progesterone leads to further amplification of LH surges. Thus hormonal signaling pathways controlling gonadotropin secretion are highly interactive networks that ensure the preovulatory LH surge necessary to trigger ovulation.

As a whole, this study provides further evidence that, undoubtedly, leptin and NO are members of the integrative mechanisms that mediate LH surge generation. The present results confirm and extend previous reports concerning the direct action of leptin at the pituitary level, stimulating LH release by anterior pituitaries from female rats at the time of spontaneous and steroid-induced LH surges. In the female rat pituitary, this action of leptin is controlled by gonadal steroids and mediated by NO.

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