EXPERIMENTAL STUDY

Effects of leptin on gonadotropin secretion in juvenile female rat pituitary cells

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Abstract

Objective: Leptin is an adipocyte-derived hormone, which is the product of the obese gene and it is thought to play important roles in pubertal development and maintenance of reproductive function in the female. In a study using adult male or female rats, it was found that leptin stimulated the secretion of gonadotropin directly from the pituitary in a dose-related manner. However, there is no study in juvenile female rats before puberty.

Methods: In this study, we cultured pituitary cells from 4-, 6- and 8-week-old female Wistar rats with leptin (0 – 10^{-7} mol/l) and gonadotropin-releasing hormone (GnRH) (0 or 10^{-8} mol/l). Basal or GnRH-stimulated secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and their synthesis within cells were determined by radioimmunoassay (RIA).

Results: Leptin induced bell-shaped dose–response curves of basal LH and FSH secretion from cultured cells of every age-group of rats studied. The most effective concentration of leptin on the basal secretion of LH and FSH from 6- and 8-week-old cultured pituitary cells was 10^{-10} mol/l. This leptin concentration was consistent with circulating physiological serum leptin levels at each age. As for juvenile 4-week-old pituitary cells, the most effective concentration was 10^{-11} mol/l which was lower than that of 6- and 8-week-old rats. It was consistent with the circulating serum leptin levels of 4-week-old rats. Also, the synthesis and the GnRH-stimulated secretion of LH and FSH were effectively controlled by leptin at concentrations similar to the serum leptin levels of given ages.

Conclusions: Leptin induced pituitary cells to synthesize and secrete both LH and FSH regardless of the presence or absence of GnRH. The concentration of leptin that induced the greatest synthesis and secretion of gonadotropins from pituitary cells changed around the pubertal period. The most effective leptin concentrations in each experiment were similar to the physiological serum leptin level at each animal age. These results indicate that leptin stimulates gonadotrophs not only in the pubertal and the mature period but also in the juvenile period before puberty. It is also conceivable that leptin may modulate the sensitivity of gonadotrophs until the appearance of GnRH stimulation, and may be the factor that brings about puberty onset.

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Introduction

It is well-known that gonadotropin-releasing hormone (GnRH) and gonadotropins are required for the onset of puberty (1). Once pulsatile GnRH secretion from the hypothalamus occurs, it raises serum gonadotropin levels gradually, thus inducing puberty. However, serum gonadotropin levels are already somewhat elevated during the juvenile period despite the absence of pulsatile GnRH secretion. The relatively high levels of gonadotropins in the juvenile period are probably due to stimulation by some factors that may render the pituitary gonadotroph cells ready for stimulation by GnRH. However, these mechanisms remain to be clarified.

It is clear that an adequate mass of adipose tissue is essential for the onset of puberty and maintenance of fertility. The classical studies by Kennedy (2) and Frisch and Revelle (3) established that the timing of sexual maturation is associated with body weight and composition. Zhang and collaborators (4) also showed that the increase of body weight is a fundamental factor for the onset of puberty. Leptin, a product of the obese gene, is secreted from adipocytes and plays an important role in the regulation of body weight and glucose metabolism. Serum leptin is related to the absolute amount of adipose tissue, body mass index or body weight and markedly increases in proportion to the degree of adiposity (5–8). Therefore, it is

strongly suggested that the increase in serum leptin level is associated with the onset of puberty.

There is some evidence that leptin plays an important role in reproduction through the hypothalamus–pituitary axis. Recently, Yu *et al.* demonstrated that leptin acts on the hypothalamus to stimulate the release of GnRH, thereby triggering gonadotropin release (9, 10). The subsequent release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) stimulates the secretion of gonadal steroid hormones, leading to the maturation of the reproductive system and the induction of puberty. Recently, we also demonstrated that leptin stimulates gonadotropin secretion from 6-week-old female rat pituitary cells (11). Our data indicated that leptin also acts on the pituitary during the period of puberty, but the mechanism of gonadotropin secretion during the juvenile period remains unknown.

In the present study, we investigated the effects of leptin on the secretion of gonadotropin from cultured pituitary cells of juvenile female rats in order to clarify the mechanism of puberty onset.

Materials and methods

Animals

All experiments were conducted in accordance with the ethical standards established by the Committee on Animal Care and Use of the University of Tokushima. Wistar female rats were obtained from Charles River Japan, Co. (Yokohama, Japan). The weights of 4-, 6- and 8-week-old female rats were 75–95 g, 130–145 g and 170–200 g respectively.

Primary culture of rat anterior pituitary cells

We cultured rat anterior pituitary cells using a previously reported method (12). Rats were decapitated and the pituitary was removed. Pituitaries were cut into small pieces and washed in Dulbecco's modified Eagle's medium (DMEM; Nissui Co., Tokyo, Japan). The cells were then subjected to enzymatic dispersion for 45 min at 37°C using 0.25% trypsin and were then dissociated by pipetting with pancreatin at 37°C for 1 min, according to the previously reported method (9, 10). These cells were seeded in DMEM containing 10% fetal bovine serum, plated on 24-well culture dishes (Falcon Plastics, Los Angeles, CA, USA) at a density of 10^6 viable cells/well, and incubated for 48 h. Cell cultures were maintained at 37°C under a mixture of 95% air and 5% CO₂ at 100% humidity for 48 h.

After 48-h culture, the cells were washed 3 times with serum-free DMEM containing 0.1% bovine serum albumin and then incubated for 20 h in serum-free DMEM with leptin (0 – 10^{-7} mol/l) (Leptin Rat, Recombinant; R&D Systems, Minneapolis, MN, USA).

To estimate basal gonadotropin secretion, the supernatants were collected and assayed by radioimmunoassay (RIA). The cells were then washed 3 times with serum-free DMEM, and then continuously incubated in DMEM containing the same concentrations of leptin as stated above with or without 10^{-8} mol/l GnRH (Sigma, St Louis, MO, USA) for 4 h at 37°C. The supernatants were collected and assayed by RIA to estimate the GnRH-stimulated LH and FSH secretion.

The cells were then washed with serum-free DMEM. One milliliter Triton-X was added, the cells were removed from the dishes with a rubber remover, and the samples were sonicated (20 kHz, 160 W) at 30-s intervals for 5 min. They were collected and centrifuged at 10 000 *g* for 10 min at 4°C. The supernatants were collected and assayed by RIA for intra-cellular content of gonadotropins in order to estimate LH and FSH synthesis.

Measurement of hormones

Concentrations of LH and FSH were measured by double-antibody RIA using the National Hormone and Pituitary Program (NHPP, USA) kits. The intra- and interassay variations of LH were 3.8% and 5.2%, and those of FSH were 4.6% and 5.8% respectively.

Statistical analysis

The significance of differences between the various leptin experimental groups of animals was assessed using Student's unpaired *t*-test. LH and FSH concentrations were expressed as the percentage of the control value (mean $\pm$ s.e.m.). Moreover, the differences in the pattern of LH and FSH secretion curves among 4-, 6- and 8-week-old cells were compared using two-way ANOVA.

Results

Effects of leptin on basal LH and FSH secretion from cultured pituitary cells

Figure 1 shows the effects of leptin on basal LH levels secreted from cultured pituitary cells into the culture medium for 24 h. Leptin stimulated basal LH secretion at concentrations up to 10^{-11} mol/l (623%, $P < 0.01$ vs control) in 4-week-old pituitary cells. However, the effect of leptin diminished at higher concentrations and the results formed a bell-shaped dose–response curve (Fig. 1A).

Six- and eight-week-old cells also showed the bell-shaped dose–response curves (Fig. 1B and C). However, the concentration of leptin which induced the greatest basal LH secretion at these ages was 10^{-10} mol/l, and the increases in LH secretion were lower than that observed in 4-week-old cells (191% and 208% respectively; $P < 0.01$ vs control).

Figure 1 Effects of leptin (0 – 10^{-7} mol/l) on basal LH secretion from pituitary cells incubated *in vitro* for 24 h ($n = 8$). (A) Four-week-old pituitary cells. Basal LH levels exhibited a bell-shaped dose–response curve. The control level of LH (leptin free) was 35.3 ± 0.76 (mean $\pm$ S.E.M.) IU/l. The peak level markedly increased as the concentration of leptin increased to 10^{-11} mol/l (623%; $P < 0.01$ vs control). (B) Six-week-old cells. (C) Eight-week-old cells. LH levels in these two groups also exhibited bell-shaped dose–response curves. The control levels of LH (leptin free) in 6- and 8-week-old cells were 30.8 ± 1.81 and 18.0 ± 1.77 (means $\pm$ S.E.M.) IU/l respectively. They gradually increased as the concentration of leptin increased, reaching a peak value at a concentration of 10^{-10} mol/l (191 and 208% respectively; $P < 0.01$ vs control). The concentration of leptin which brought maximum secretion of LH changed from 10^{-11} mol/l to 10^{-10} mol/l as the ages of rats changed from the prepubertal period (A) to the pubertal (B) and adult (C) periods. There were significant differences in the shape of the curves between (A) and (B) and between (A) and (C). ($P < 0.05$, $P < 0.01$ respectively by two-way ANOVA). Results are expressed as percentage of the control value (mean $\pm$ S.E.M.); * $P < 0.01$, ** $P < 0.05$.

Basal FSH secretion from 4-week-old pituitary cells was stimulated at leptin concentrations up to 10^{-11} mol/l (224%, $P < 0.01$ vs control). The effects of leptin on FSH secretion from 6- and 8-week-old cells were increased by leptin at concentrations up to 10^{-10} mol/l (139% and 147% respectively; $P < 0.05$ and $P < 0.01$ vs control). However, at higher concentrations, these effects diminished in all age groups (Fig. 2A, B, C).

Figure 2 Effects of leptin (0 – 10^{-7} mol/l) on basal FSH secretion from pituitary cells incubated *in vitro* for 24 h ($n = 8$). (A) Four-week-old cells. Basal FSH levels exhibited a bell-shaped dose–response curve. The control level of FSH (leptin free) was 69.0 ± 2.18 (mean $\pm$ S.E.M.) IU/l. The peak level increased as the concentration of leptin increased to 10^{-11} mol/l (224%; $P < 0.01$ vs control). (B) Six-week-old cells. (C) Eight-week-old cells. FSH levels in these two groups also exhibited bell-shaped dose–response curves. The control levels of FSH (leptin free) in 6-week-old and 8-week-old cells were 36.7 ± 4.14 and 60.6 ± 2.18 (means $\pm$ S.E.M.) IU/l respectively. They increased as the concentration of leptin increased reaching a peak value at a concentration of 10^{-10} mol/l (139 and 147% respectively; $P = 0.02$, $P < 0.01$ vs control). It was observed that the peak changed from 10^{-11} mol/l to 10^{-10} mol/l at the time of puberty onset. There were significant differences in the shape of the curves between (A) and (B) and between (A) and (C) (both $P < 0.01$ by two-way ANOVA). Results are expressed as percentage of the control value (mean $\pm$ S.E.M.); * $P < 0.01$, ** $P < 0.05$.

Effects of leptin on intracellular LH and FSH content in 4-week-old pituitary cells

Intracellular LH content in 4-week-old pituitary cells after 24-h treatment with leptin increased in accordance with increased leptin concentrations up to 10^{-11} mol/l (194%, $P < 0.05$ vs control), but these effects diminished at higher concentrations of leptin (Fig. 3A) producing a bell-shaped dose–response curve.

Similar results were observed for intracellular FSH content (Fig. 3B).

Figure 3 Effects of leptin ($0-10^{-7}$ mol/l) on intracellular LH and FSH content in 4-week-old pituitary cells incubated *in vitro* for 24 h ($n = 8$). (A) LH level exhibited a bell-shaped dose-response curve. The control level of LH (leptin free) was 66.8 ± 17.8 IU/l. The LH level increased as the concentration of leptin increased to 10^{-11} mol/l (194%; $P < 0.05$ vs control). (B) FSH level also exhibited a bell-shaped dose-response curve. The control level of FSH (leptin free) was 22.8 ± 4.36 IU/l. The FSH level increased as the concentration of leptin increased to 10^{-11} mol/l (176%; $P < 0.05$ vs control). Results are expressed as percentage of the control value (mean $\pm$ S.E.M.); * $P < 0.05$.

Effects of leptin on the GnRH-stimulated LH and FSH secretion from 4-week-old pituitary cells

We examined the secretion of LH and FSH stimulated by GnRH (10^{-8} mol/l) in 4-week-old pituitary cells (Table 1). These cells showed a good response to GnRH. LH and FSH secretion increased under GnRH stimulation by twenty and fifteen times respectively.

Table 1 LH and FSH secretion from cultured 4-week-old pituitary cells incubated *in vitro* for 4 h without (–) or with (+) GnRH (10^{-8} mol/l). Results are means $\pm$ S.E.M. ($n = 8$ in each group).

	GnRH (–)	GnRH (+)
LH (IU/l)	4.97 ± 0.64	$101.73 \pm 13.31^*$
FSH (IU/l)	14.28 ± 0.70	$214.15 \pm 13.90^*$

* $P < 0.01$.

Moreover, GnRH-stimulated LH and FSH secretion increased as leptin concentrations increased up to 10^{-11} mol/l (170% and 135% respectively; $P < 0.01$ vs control). However, these effects decreased at higher concentrations of leptin. GnRH-stimulated LH and FSH secretion in 4-week-old pituitary cells also exhibited bell-shaped dose-response curves (Fig. 4A, B).

Discussion

It is commonly thought that the onset of puberty requires good nutritional condition. Puberty occurs when body weight reaches a so-called critical weight (2, 3). It is likely that leptin is one of the critical factors that triggers the onset of puberty because leptin from adipose tissue may inform the hypothalamus and the pituitary that the energy store has reached an appropriate level for reproduction.

In ob/ob mice which lack leptin secretion and show obesity and anovulation, chronic leptin treatment not only reduces food intake and body weight but also restores puberty and fertility (13, 14). It is also well known that extreme weight loss or gain due to intense exercise, malnutrition or overfeeding disturbs reproductive function and causes infertility in both rodents and humans. In normal adult female rats, LH pulsatility was suppressed by anti-leptin serum (15), suggesting that leptin stimulates hypothalamic GnRH pulsatility. In the adult rat pituitary, leptin produces a dose-related increase in LH and FSH at low/normal concentrations (9), but not at high concentrations (9, 16). Therefore, it is assumed that leptin also stimulates the pituitary, and that excessive leptin concentration also interferes with reproductive function. For example, hyperleptinemia caused by extreme obesity may disrupt pituitary function and impair gonadotropin secretion during the peripubertal and postpubertal period in humans (17). Our data showing bell-shaped dose-response curves in gonadotropin secretion stimulated by leptin support these findings.

Body weight of rats increases with age. Serum leptin levels also increase in parallel with age and body weight, and serum concentrations of estradiol begin to rise at 6 weeks of age in rats (11). Vaginal opening and follicular growth of female rats also appear from 5 to 6 weeks of age (18). Therefore, it is considered that 6 weeks of age corresponds to the pubertal period. In addition, 4 and 8 weeks of age correspond to the juvenile and the mature period respectively. According to our previous report, physiological serum leptin levels of female rats was 10^{-10} mol/l in pubertal 6-week-old rats, and 10^{-11} mol/l in juvenile 4-week-old rats.

Leptin stimulates LH and FSH secretion from cultured female rat pituitary cells, and these effects show a bell-shaped dose-response curve. In this study, we demonstrate that leptin concentrations able

Figure 4 Effects of leptin (0 – 10^{-7} mol/l) on GnRH-stimulated LH and FSH secretion in 4-week-old pituitary cells incubated *in vitro* for 4 h (GnRH: 10^{-8} mol/l) ($n = 8$). (A) LH level exhibited a bell-shaped dose–response curve. The control level of LH (leptin free) was 101.7 ± 13.3 IU/l. The LH level increased as the concentration of leptin increased to 10^{-11} mol/l (170%; $P < 0.01$ vs control). (B) FSH level also exhibited a bell-shaped dose–response curve. The control level of FSH (leptin free) was 214.2 ± 13.9 IU/l. The FSH level increased as the concentration of leptin increased to 10^{-11} mol/l (135%; $P < 0.01$ vs control). Results are expressed as percentage of the control value (mean $\pm$ S.E.M.); * $P < 0.01$, ** $P < 0.05$.

to stimulate gonadotropin secretion were similar to the circulating leptin levels at 4 weeks old (10^{-11} mol/l), and at 6 and 8 weeks old (10^{-10} mol/l). The changes in circulating serum leptin concentrations around puberty suggest a physiological role of leptin in modulating pituitary gonadotroph cell sensitivity from the juvenile to the mature period of animal life.

In normal girls, serum leptin levels increase before the rise of reproductive hormones which are related to puberty (5, 6, 19). In juvenile female mice, injection of leptin induced earlier maturation of the reproductive system (20–23). In our study, leptin stimulated the secretion of gonadotropin in juvenile 4-week-old rats. These findings suggest that leptin plays a physiological role in the juvenile period.

In the pituitary, the production of gonadotropin might be induced both directly and indirectly through modification of the sensitivity to GnRH. In the present study, juvenile pituitary cells of 4-week-old rats showed a good response to leptin at a concentration equivalent to the circulating juvenile serum level,

with or without GnRH. In fact, GnRH secretion is rarely seen in 4-week-old rats. In the juvenile period, leptin may render gonadotrophs ready to be stimulated by even small amounts of GnRH.

Leptin receptors are expressed all over the body (24), including the pituitary (25). However, taking into account possible differences in density of leptin receptors, leptin may mainly affect the hypothalamus rather than the pituitary. Apparently, initiation of GnRH secretion is an important factor for puberty onset. Leptin may exert actions on the GnRH neuron modifying the frequency and magnitude of GnRH pulses directly or indirectly through induction of nitric oxide or suppression of neuropeptide Y (26–30). The role of leptin may be more important in the hypothalamus than in the pituitary during the pubertal period.

In conclusion, we demonstrate that leptin acts directly on pituitary cells even in the juvenile period before the occurrence of GnRH secretion. The maximum effects of leptin on gonadotrophs is manifest at physiological serum concentrations from the juvenile to the mature period. The present data suggest that leptin stimulates the secretion of gonadotropins and modulates the response of the pituitary in the juvenile period before puberty until the appearance of GnRH secretion which strongly drives the hypothalamus–pituitary–gonadal axis in the pubertal period.

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