Leptin Stimulates Gonadotropin Releasing Hormone Release From Cultured Intact Hemihypothalami and Enzymatically Dispersed Neurons

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Leptin is a peptide released by adipocytes that has profound effects on central regulation of body metabolism. The present study represents an investigation into leptin effects on hypothalamic control of reproductive function, specifically on GnRH release. Adult male rats (gonadectomized or sham-operated) were used as donors of hypothalamic tissue that was used as intact hemihypothalami or as enzymatically dispersed hemihypothalami in a perifusion culture system. Continuous samples were collected at 10-min intervals for 8 to 10 hr and were assayed to measure temporal changes in GnRH release in response to various doses of leptin infused into the perifusion chambers. Leptin at the highest dose (10^{-8} M) resulted in consistent and significant stimulation of GnRH release. There were no effects of treatment for surgical preparation (gonadectomy versus sham) or tissue preparation (intact versus dispersed hemihypothalami). The results of this study support the hypothesis that leptin plays a direct stimulatory role in the regulation of GnRH release. This study describes an important step in our understanding of the mechanism that connects changes in basal metabolism with reproductive function. These results indicate an intact interneuronal network is unnecessary for these leptin effects, but does not exclude a role for interneuronal networks in this regulatory pathway.

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Leptin is a 167-amino acid protein with pronounced effects on body metabolism, via both peripheral and central targets (1). Originally identified as an adipocyte-derived signaling molecule with significant direct impacts such as limiting food intake and increasing energy expenditure (2), leptin also has neuroendocrine-based influence within the reproductive system, the thyroid, and the adrenal axis (3). The hypothalamus has been established as a site for leptin action by a number of different experimental approaches. Lesions to the hypothalamus induced obesity, while infusion of leptin to the hypothalamus induced anorexic changes (1, 4). Leptin specifically bound plasma membranes in the hypothalamus (4), and the leptin receptor (Ob-R) protein is synthesized in the hypothalamus (5, 6). Furthermore, Ob-R has been identified within the hypothalamus in brain regions rich in GnRH neurons such as the arcuate nucleus (in primates) (7, 8), the medial preoptic area (MPOA), and the median eminence (in primates and rodents) (7, 8), but rarely colocalized on GnRH neurons (7). While it is clear that leptin interacts with one or more targets within the hypothalamus, the mechanism of leptin action on the reproductive axis has not been fully resolved. In view of the common anatomical localization of GnRH neurons and some Ob-R, one likely candidate for coordination of leptin effects on the reproductive axis is action on cells producing the hormone GnRH.

The decapeptide GnRH directs release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in mammals, ultimately controlling reproductive function (9, 10). As such, it is a prime coordinator of subsequent events in response to a vast array of environmental inputs. Leptin, as a "signal of plenty" from fat tissues, would provide an assessment of energy stockpiles to aid in the accounting of reproductive readiness; the response to this accounting may be coordinated by release (or lack of release) of GnRH. Indirect evidence for this cascade of messages was seen *in*

vivo in mice and rats in which higher leptin levels advanced the onset of puberty (11, 12). Further, sterility in leptin-deficient rodents could be circumvented by leptin infusion (13, 14). The *ob/ob* mouse model displays profound weight gain that can be controlled by the addition of leptin (15, 16). Sterility in both male and female mice is another result of leptin deficiency in the *ob/ob* mouse (16). In some instances, fertility can be restored in male *ob/ob* mice through dietary restriction. While diet restriction fails to restore reproductive function to *ob/ob* female mice, the addition of leptin elevates gonadotropin levels and restores follicular development, resulting in fully fertile female mice (13, 16, 17). The connection between leptin and reproductive function, as well as the known interaction of leptin with the hypothalamus, directs attention to a possible central action of leptin in controlling GnRH levels. In this study we tested the hypothesis that one central target for leptin is GnRH-releasing neurons in the hypothalamus. The present study used both intact hemihypothalamic explants and enzymatically dispersed hypothalamic neurons in a perifusion system to demonstrate that leptin stimulates GnRH directly.

Materials and Methods

Animals. The 18 adult male rats used in these experiments were obtained from Harlan Sprague-Dawley (Madison, WI). Weights ranged from 240 to 350 g. Rats were housed for 1 to 4 weeks in individual cages with controlled temperature (22°–24°C) and lighting conditions (lights on from 05:00–19:00 hr daily). Food and water were provided *ad libitum*. Rats were gonadectomized (GDX) or sham-operated (Sham) at least 10 days prior to experiments to allow clearance of circulating gonadal steroids. Surgery was performed under Nembutal anesthesia using aseptic techniques. On each day of an experiment, three rats were killed by decapitation between 08:00 and 09:30 hr and the hypothalami were removed within 2 min. These experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. This work was conducted under a protocol approved by the University of Wisconsin-Whitewater IACUC.

Cell and Tissue Preparation. The hypothalamus was surgically removed from the rat brain and was bisected down the midline as previously described (18). Briefly, the hypothalamus was delimited laterally by the hypothalamic fissures, anteriorly by a cut 2 mm anterior to the optic chiasm, posteriorly by the rostral portion of the mamillary bodies, and by a horizontal cut approximately 2 mm deep. Each hemihypothalamic section was immediately placed in a modified Krebs-Ringer bicarbonate buffer containing 2.2 mM $CaCl_2$, 154 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl$, 6.0 mM $NaHCO_3$, 10 mM glucose, 2 mM HEPES, 0.1% BSA, and 0.006% bacitracin, pH 7.40. From each animal, one hemihypothalamus was held intact in the medium and the corresponding hemihypothalamus was placed into medium and was enzymatically dispersed with 0.05% trypsin, as

described previously (18). Cell dispersion and viability were assessed by methylene blue dye exclusion. Immediately following dispersion, approximately 95% of cells were single or paired and alive; after 4 to 8 hr, over 95% of cells remained dispersed and alive.

Perifusion Procedures. An Endotronics Accusyst-S cell perifusion system (Cellex Biosciences, Minneapolis, MN) was used in these experiments. The medium was pumped at a flow rate of 100 μl/min through chambers containing rat hemihypothalami, either as intact tissue or as dispersed cells. Carbon dioxide flowed into the chamber area, stabilizing media pH at approximately 7.40 prior to media entry into incubation chambers. Experiments were conducted for 8 to 10 hr, with 1 ml of perifusate fractions being collected at 10-min intervals at 4°C. Samples were frozen and stored at −70°C until assayed by radioimmunoassay (RIA). At approximately 1.5-hr intervals, leptin at various concentrations (10^{-12} or 10^{-10} M or 10^{-8} M or vehicle) was infused into the chambers for 10 min. The infusion sequence was vehicle, 10^{-12}, 10^{-10}, and 10^{-8} M for 12 of the rats used, and was altered to 10^{-10} M, vehicle, 10^{-8}, and 10^{-12} M for the other six rats at 1.5-hr intervals. The second sequence was used to assess possible effects of potentiation of GnRH responsiveness by previous infusion of leptin. Since no effect of sequence of infusion was observed, both groups of animals were combined for the final analysis.

The highest dose of leptin used (10^{-8} M) represents approximately 10-fold higher concentration than normal rat serum levels of 12.5 ng/ml (19). Due to dilution and fluid dynamics within the perifusion chamber, the highest levels of leptin expected using our perifusion system with a 10-min infusion of 10^{-8} M leptin would result in tissue exposure to approximately twice the normal levels of leptin in rat serum.

GnRH RIA. GnRH concentration in perfusate was estimated by RIA, described elsewhere (18). Synthetic GnRH was used as trace and standard was purchased from Richelieu Laboratories (Montreal, PQ, Canada). Anti-GnRH antibody (R1245) was provided by Dr. T. Nett (Colorado State University, Fort Collins, CO). Sensitivity of the assay was 0.1 pg/tube at 95% binding. Intraassay and interassay coefficients of variation (CV) were 5.6% and 9.2%, respectively. Intraassay CV was determined in replicate samples, and interassay CV was determined by comparing four replicates from a GnRH pool included in each assay. Statistical analysis of summary data was performed using analysis of variance (ANOVA) with a Tukey-Kramer multiple comparisons *post hoc* analysis. Results were removed from the analysis only if tissue was determined unresponsive to KCl stimulation (total of three out of 36 chambers run).

Statistical Analysis. The GnRH response to leptin infusion was calculated as the peak GnRH response within 30 min of leptin or vehicle infusion minus the average of the three 10-min samples preceding infusion. The average

GnRH response at each dose was compared using ANOVA. By comparing the average change in GnRH release with both vehicle and leptin infusion, we were able to control for the underlying endogenous pulsatility in GnRH release characteristic of these preparations (18).

Leptin. The leptin used was obtained through a generous donation by Pfizer Inc. (Groton, CT) to Dr. Joe Kemnitz, Wisconsin Regional Primate Research Center and was used for a separate project (20). The remainder of the leptin was made available and used in the present study.

Results

Periodic leptin infusion into chambers containing hypothalamic tissue that was enzymatically dispersed or left as intact hemihypothalami resulted in very similar GnRH release profiles. Representative cases in which tissue or cells were initially infused with vehicle and the leptin was added in sequentially increasing concentrations are shown in Fig-

ure 1, A through D. These figures represent cases in which intact hemihypothalami and dispersed cells were isolated from the same rat (Fig. 1, A–D). The GnRH response profiles were comparable when the leptin infusion sequence was altered to nonsequential concentrations (data not shown). Further, there were no differences in the average response to any dose of leptin when the results of the two leptin infusion protocols were compared. The results from both infusion protocols were thus combined to present the summary data shown in Figure 2. It is important to note that GnRH release under these culture conditions typically results in pulsatile GnRH release without stimulation (18). Thus, all leptin versus control comparisons require the experimenter to account for endogenous GnRH pulsatility. This is done using a relatively large $n = 9$ per group and careful assessment of change in GnRH levels after leptin infusion.

The summary data in Figure 2 are representative of most individual cases, in that GnRH response to infusion of
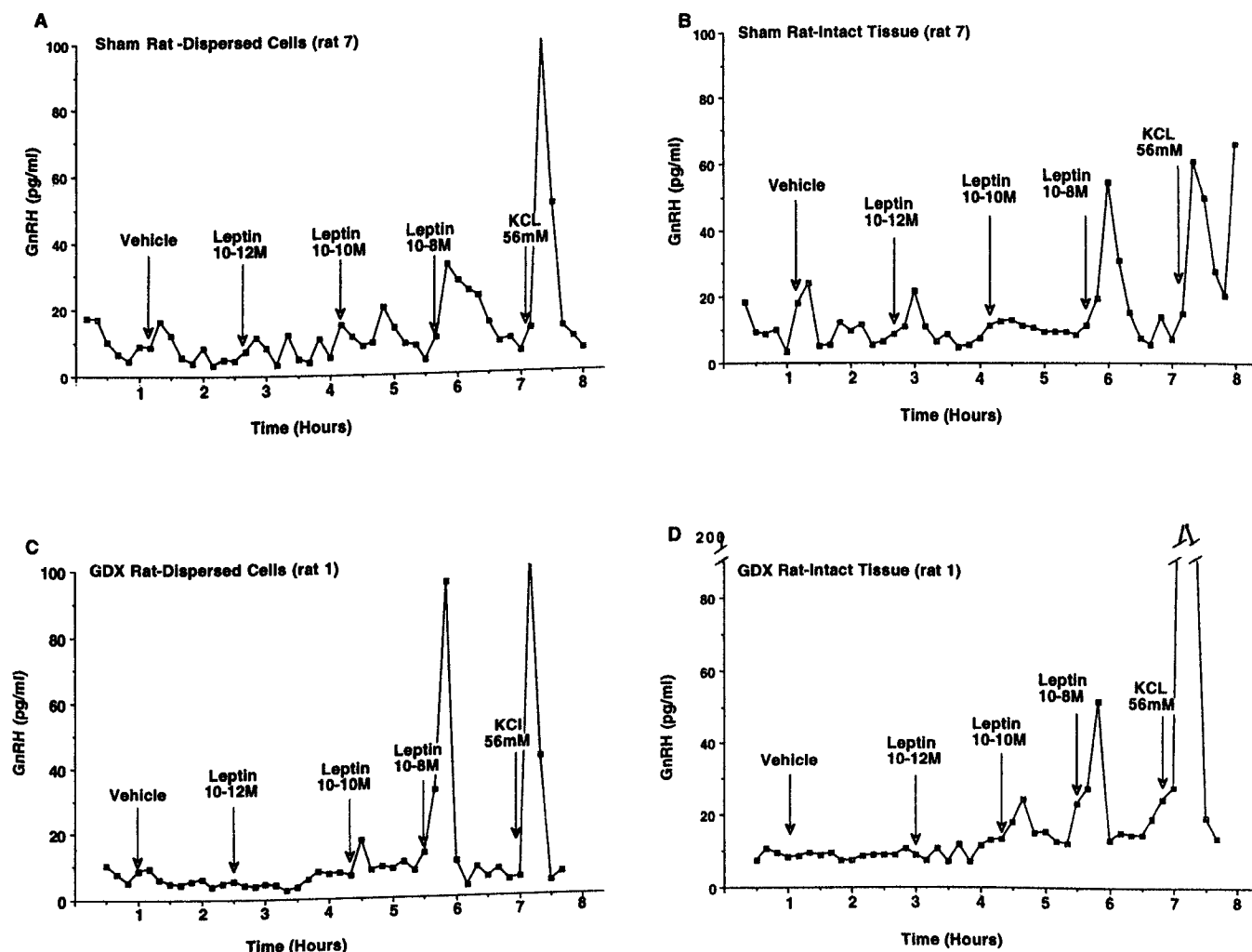

Figure 1. Representative cases of GnRH release from perifusion of cultured dispersed cells (A and C) and intact hemihypothalami (B and D) in response to leptin infusion. Either vehicle or leptin (10^{-12}, 10^{-10}, or 10^{-8}M) dissolved in media were infused for 10 min at 90-min intervals. KCl at 56 mM was infused at the end of each experiment to test for viability of the LHRH neurons.
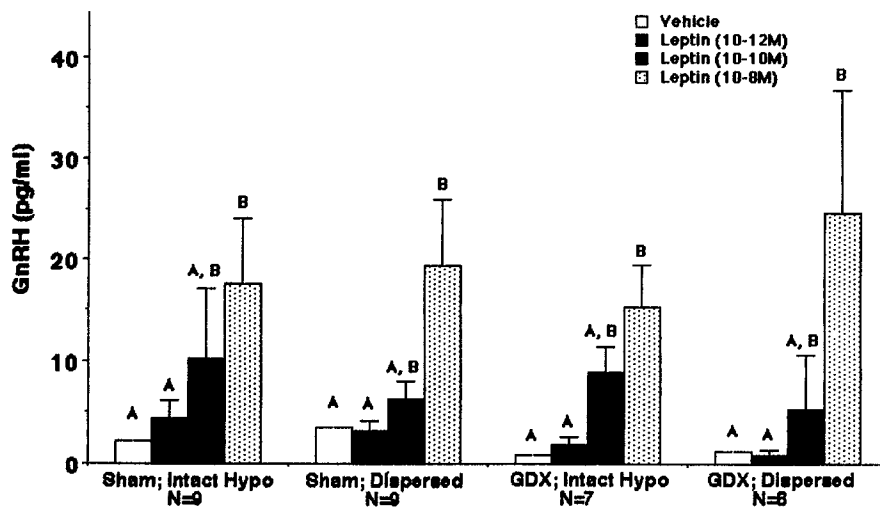
Figure 2. Summary data for leptin infusion. Columns with the same letters are not significantly different ($P > 0.05$). One-way ANOVA was used to compare leptin doses within a treatment group (i.e., GDX, dispersed), and two-way ANOVA was used to compare release profiles between the four treatment groups (sham, intact; sham, dispersed; GDX, intact; GDX, dispersed).

leptin at 10^{-12} M was not distinguishable from that of vehicle, in that at 10^{-10} M most cases showed a modest GnRH response (although not significant), and in that a dose of 10^{-8} M resulted in a clear stimulation of GnRH release. Comparisons between GDX and sham surgery animal sources, or between dispersed and intact hemihypothalami treatments, failed to show statistically significant differences at any leptin dose when compared via two-way ANOVA. The results are consistent with the idea that neither gonadal history of the excised tissue nor the integrity of the interneuronal network has an effect on responsiveness of the tissue to leptin infusion. To test viability of the GnRH neurons, each experiment concluded with a 56 mM KCl infusion to induce membrane depolarization and exocytosis. Three of 36 wells were unresponsive to this challenge and were eliminated from statistical analysis.

Discussion

The present study was designed to evaluate the effect of leptin on GnRH release from hypothalamic tissues. The perifusion system used here experimentally isolates the GnRH neurons from much of the vast array of feedback mechanisms and regulatory inputs that influence these neurons, but potentially confounds analysis of their responsiveness to external stimuli. This study is the first to clearly demonstrate that leptin stimulates GnRH release from hypothalamic tissue in a dose-related manner.

The results presented demonstrate the ability of leptin to directly stimulate GnRH release in enzymatically dispersed hemihypothalami in culture. Our cell dispersion method results in nearly complete dissociation of cell-cell connections (achieving ~95% single cells). These two observations provide some evidence for leptin exerting either a direct stimulatory effect on GnRH neurons, presumably through Ob-R independent action (7), or leptin inducing a paracrine or endocrine effect on surrounding hypothalamic cells that results in GnRH release. It is noteworthy that GnRH release profiles from dispersed cells and intact hemi-hypothalami are similar in shape and magnitude, suggesting

that dissociation of the cytoarchitecture of the hypothalamus has little effect on the responsiveness of cultured GnRH neurons to leptin infusion.

Our observation that leptin infusion stimulates GnRH release is consistent with other studies demonstrating effects of leptin on the reproductive axis. Leptin plays a role in LH secretion, either through direct pituitary action or indirectly through hypothalamic action on either GnRH neurons or interneurons that influence GnRH release. Infusion of leptin antisera into female rats decreases LH levels and interrupts reproductive cycles (21). In vivo perifusion of the stalk-median eminence with leptin at 10^{-4} and 10^{-6} M concentrations induced significant acute release of GnRH for 10 to 20 min in rhesus monkeys (22).

Modest food restriction can induce significant changes in release patterns of gonadotropins (23, 24). Suppressed pulsatile LH secretion observed in fasted animals can be restored by feeding (23) or by infusion of pulsatile GnRH in rats (25), sheep (26), and humans (27). These studies indicate a permissive role for leptin in the regulation of the reproductive axis, while our results suggest a stimulatory role.

One extensively discussed role for leptin action on the reproductive axis is that of a metabolic signal from fat stores to the brain. Such a signal would likely be involved in feedback of overall metabolic state, facilitating the return of reproductive fitness after such pathological/physiological conditions as anorexia nervosa, amenorrhea associated with highly trained athletes (gymnasts, skaters, and distance runners), or with puberty (24, 28, 29). Direct experimental evidence provides further details. Fasting for 2 days interrupted LH pulsatility in male monkeys. Treatment with leptin restored LH pulsatility in three of four monkeys, while vehicle restored LH pulsatility in zero of four control monkeys (7). Administration of leptin during undernutrition restores reproductive function in fasting female rats as well (30, 31).

Our results clearly indicate that under these culture conditions, leptin stimulates GnRH release. One contradictory

study using hypothalamic explants in static culture (32) failed to observe such consistent evidence of GnRH release stimulated by leptin. Mild, but significant increases in GnRH release with low doses of leptin (10^{-12} to 10^{-10} M) were followed by no effect (10^{-8} M) or a significant decrease in GnRH release (10^{-6} M) at higher doses. In each case, these effects were absent over the first 30-min of incubation and only observed in the second 30-min incubation period. This result differs in temporal response not only from that observed in the present study and in a recent *in vivo* study (31), but with the ordinary temporal response observed with other stimulatory molecules by the same group (32) using the same hypothalamic explant model (33). While these differences initially appear difficult to resolve, they may reflect methodological rather than physiological variation. Both *in vivo* push-pull perifusion (31) and *in vitro* cell perifusion (used in the present study) provide sequential samples from the tissue, whereas static culture methods (32) represent analysis of the average release over a large block of time, with significant disturbance of the culture at each media harvest.

Our results demonstrate that leptin infused into cultured hypothalamic explants or enzymatically dispersed hypothalami is capable of stimulating acute GnRH release. This observation does not preclude a permissive role for leptin in the reproductive axis via control of GnRH and/or LH release, as is suggested in a number of studies (reviewed in Ref. 34). We agree with these authors that a permissive role for leptin *in situ* remains the best explanation of the body of literature. Our study demonstrates the capacity for leptin to act as an acute stimulator of GnRH in a controlled experimental setting. Further, leptin having the same stimulatory effect on GnRH release in both dispersed GnRH neurons and in intact hemihypothalami suggests that we may need to reevaluate the role of the interneuronal network composed of neuropeptide Y (NPY), adrenergic, and opioid input that drives coordinated release of GnRH (4, 6, 7, 31, 32, 34).

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