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Leptin Acts at the Bovine Adenohypophysis to Enhance Basal and Gonadotropin-Releasing Hormone-Mediated Release of Luteinizing Hormone: Differential Effects Are Dependent upon Nutritional History[1]

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ABSTRACT

Recombinant ovine leptin (oleptin) stimulates an acute increase in the secretion of LH in fasted, but not in normal-fed, cows through an augmentation of the magnitude of individual pulses of LH. Herein, we tested the hypothesis that this effect could be accounted for by functional changes at the adenohypophyseal (AP) level. Eleven ovariectomized, estradiol-implanted cows were assigned to one of two dietary groups: normal-fed (n = 6) and fasted (fasted for 72 h; n = 5). After the animals were killed, the adenohypophyses were collected and AP explants were perifused with Krebs-Ringer bicarbonate buffer (KRB) for a total of 6.5 h, including a 2-h treatment at 2.5 h with KRB or increasing doses of oleptin and a challenge at 4.5 h with 50 ng of GnRH. To test for effects of leptin at the hypothalamic level, explants encompassing the medial basal hypothalamus-infundibular complex (HYP) were incubated in KRB alone (control) or in KRB containing 1000 ng of oleptin. Basal release of LH from AP explants treated with leptin was greater ($P < 0.02$) than that from control-treated explants in fasted, but not in normal-fed, cows. To the contrary, leptin-treated explants from normal-fed, but not from fasted, cows released more ($P < 0.001$) LH in response to GnRH than control-treated tissues. Neither fasting nor leptin affected ($P > 0.1$) the secretion of GnRH from HYP explants. These observations support the hypothesis that leptin modulates the secretion of LH in mature cows, to a large extent, by its direct actions at the AP. Differential manifestations of these effects are dependent upon nutritional history.

anterior pituitary, gonadotropin-releasing hormone, hypothalamus, leptin, luteinizing hormone

INTRODUCTION

Leptin, a 16-kDa product of the *ob* gene, plays a major role in communicating nutritional status to the central nervous system, including centers that control reproduction [1–4]. The leptin receptor (LR) is present at both hypothalamic and adenohypophyseal (AP) loci [5–8], and in vitro studies using explants collected from normal-fed rodents indicate that leptin can act directly at both sites [9, 10] to stimulate the release of GnRH and LH, respectively. However, in domestic ruminants, leptin has been shown repeatedly to stimulate LH secretion only during periods of negative energy balance. In addition, leptin-receptor mRNA increases in feed-restricted ewes [7]. In this context, restriction of nutrient intake for 2–3 d reduces concentrations of leptin concurrent with reductions in the frequency of LH pulses in castrated male sheep [11] and peripubertal heifers [12]. Leptin treatment prevents these reductions, suggesting an effect at hypothalamic centers that regulate pulsatile GnRH secretion [11, 13].

Importantly, leptin also stimulates increased LH secretion in animals in which short-term restrictions of nutrient intake do not measurably alter the pattern of LH release. For example, 2–3 days of fasting reduces expression of the leptin gene and markedly diminishes circulating concentrations of leptin, insulin, and insulin-like growth factor (IGF)-1 in mature ruminants without reducing mean concentrations of LH or the frequency of LH pulses [11, 14–16]. Nonetheless, 2–3 days of total feed restriction clearly hypersensitizes the mature cow to leptin, resulting in acute increases in the mean baseline and the magnitude of individual pulses of LH [16, 17]. This would suggest that the primary effects of leptin in this animal model are at the AP level. To test this hypothesis further, we examined in vitro the stimulatory effects of leptin on basal and GnRH-mediated secretion of LH from AP explants obtained from fasted and nonfasted cows. To assess coincident hypothalamic responses, the effects of leptin on GnRH secretion from hypothalamic-infundibular (HYP) explants were also examined.

MATERIALS AND METHODS

All animal-related procedures used in the present study were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System.

Animal Model and Procedures

Eleven mature, ovariectomized cows, each bearing an estradiol implant to maintain circulating estradiol at 2–5 pg/ml, were used. This animal model has been used extensively by our laboratory and others [16–19] to study the effects of nutrition on neuroendocrine control of gonadotropin secretion in cattle. With this approach, implants provide a constant level

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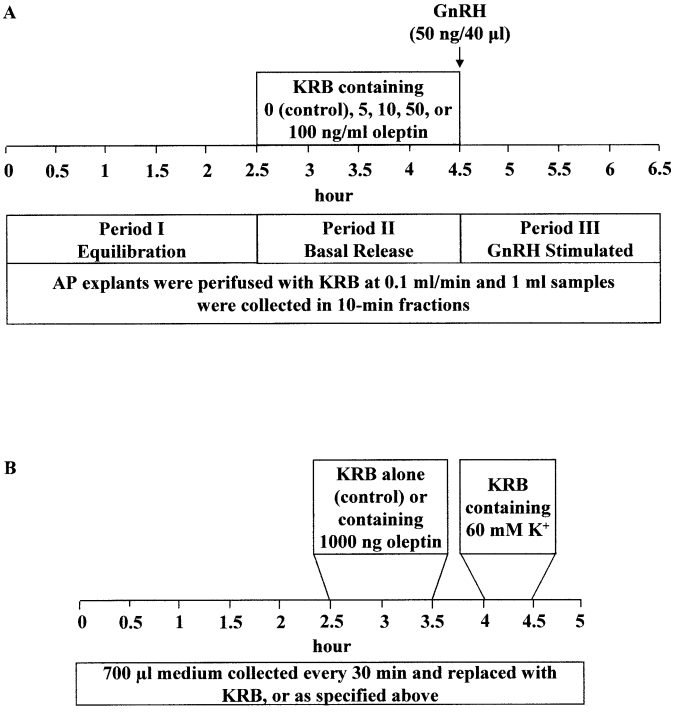

FIG. 1. Timeline for experimental procedures for AP (**A**) and HYP (**B**) explant incubations. Adenohypophyseal explants were perifused with KRB for 6.5 h. The first 2.5 h of perifusion served as an equilibration period (period I). Beginning at 2.5 h, explants were perifused with KRB containing 0, 5, 10, 50, or 100 ng/ml of oleptin for 2 h (period II: basal release). At 4.5 h, explants were challenged with 50 ng of GnRH and perifused for an additional 2 h (period III: GnRH-stimulated). Hypothalamic-infundibular explants were equilibrated in 0.7 ml of KRB for 2.5 h. Beginning at 2.5 h, explants were incubated in KRB alone or containing 1000 ng of oleptin for 1 h. Explants were treated at 4 h of culture with KRB containing 60 mM K^+ to induce release of GnRH as a test of viability.

of steroid (estradiol) negative feedback without the complications associated with ovarian cyclicity. Cows used in the current experiments had estradiol concentrations (mean ± SEM) of 4.35 ± 0.45 pg/ml.

Cows in moderately thin body condition (BC) (BC = 4; scale of 1–9) were fed once daily at 0700 h a diet formulated to provide 100% of the National Research Council (NRC) [20] requirements for maintenance for at least 2 wk before the beginning of the experiment. Each cow was assigned randomly to one of two dietary groups: normal-fed, in which cows were fed 100% of the NRC requirements to maintain body weight (n = 6); and fasted, in which cows were fasted for 72 h with free access to water (n = 5). On the day before the start of dietary treatments (Day −1), cows were fitted with jugular catheters (polyethylene tubing; inside diameter, 1.4 mm; outside diameter, 1.9 mm; Becton Dickinson, Parsippany, NJ) for intensive blood sampling. Cows were placed in stanchions, and blood was collected at 10-min intervals for 6 h on Days 0 and 3 of the experiment via an extension connected to the jugular catheter. Blood samples were dispensed into tubes containing 150 µl of a solution containing heparin (1000 IU/ml) and 5% EDTA and placed immediately on ice. Plasma was harvested by centrifugation and stored at −20°C until hormone analysis. At the end of the intensive blood sampling on Day 3, cows were killed by exsanguination following captive bolt stunning. Diencephalons were removed after disconnection of infundibuli from adenohypophyses. Adenohypophyses were removed from the sella turcica and kept on ice until tissue processing.

Effects of Leptin on Basal and GnRH-Mediated Release of LH from AP Explants

Adenohypophyses collected from each cow were dissected and sliced sagittally into approximately 0.5- × 2-mm strips. Four AP strips from each adenohypophysis were selected randomly, placed into each of five perifusion chambers, and perifused with Krebs-Ringer bicarbonate buffer (KRB) for 6.5 h using a multiple microchamber perifusion system (Endotronics, Inc., Coon Rapids, MN). Thus, AP explants from each cow were perifused with each dose of leptin tested or control media. In preliminary studies, it was determined that basal release of LH by AP explants was attained within the first 2.5 h of perifusion. At 2.5 h, AP explants were perifused for 2 h with KRB containing 0 (control), 5, 10, 50, or 100 ng/ml of recombinant ovine leptin (Dr. Arieh Gartler, The Hebrew University of Jerusalem, Jerusalem, Israel). After 4.5 h of perifusion, AP explants were challenged with 50 ng of GnRH (Sigma, St. Louis, MO) via an injection port in the chamber (Fig. 1A). In addition to investigating the effects of leptin on GnRH-mediated release of LH, the stimulation of AP explants by GnRH also made it possible to verify the viability of tissues based on specific stimulation of gonadotropin release.

Approximately 1-ml fractions of perifused media were collected at 10-min intervals and stored at −20°C until analysis for LH. In a separate study, we have reported simultaneous effects of leptin on basal and growth hormone (GH)-releasing hormone-mediated GH release from these same explants [21].

Effects of Leptin on GnRH Secretion from the Isolated Medial Basal Hypothalamus-Infundibular Complex

To isolate the medial basal hypothalamus (MBH), the entire HYP complex was transected sagittally into equal halves. A cut was made 2 mm lateral to the median sagittal cut for each half, followed by an anterior coronal cut extending from the optic chiasm to the anterior commissure. A posterior coronal cut was also made, resulting in a section containing approximately half the mammillary body. A final transverse cut containing two-thirds of the mammillary body was performed. Each half of the MBH connected to its infundibular half was incubated in 0.7 ml of KRB for 5 h. Medium was collected and replaced every 30 min. After a 2.5-h period of equilibration, HYP explants from each cow were incubated with KRB containing either 0 or 1000 ng of ovine leptin (oleptin) for 1 h, so that one half served as a control for the corresponding half (Fig. 1B). At 4 h of incubation, all explants were incubated with KRB containing 60 mM K^+ to test tissue viability, based on nonspecific stimulation of GnRH release [22].

Doses of recombinant oleptin used to treat HYP explants were chosen empirically based on quantities shown to have positive effects in comparable tissue collected from rodents [9] and on preliminary experiments using tissues collected from bulls and steers at slaughter. In these preliminary experiments, GnRH released from HYP explants collected from full-fed cattle was not affected by leptin (1–1000 ng of recombinant oleptin for 30–60 min). This observation was not totally surprising, because the stimulatory effects of leptin on LH release observed in vivo [11] were limited to fasted cows. Thus, we chose to use 1000 ng of oleptin in the current experiment.

Radioimmunoassays

Concentrations of GnRH in HYP culture media were determined by a validated assay as described previously [22]. Plasma concentrations of LH and concentrations of LH in AP perifused media were determined as reported previously [23]. Intra- and interassay coefficients of variation for these assays averaged 6.2% and 10.7%, respectively. Serum estradiol was assayed in extracted samples in a single assay as reported previously [24].

Statistical Analysis

Circulating concentrations of LH were analyzed by general linear mixed models for repeated measures using the mixed procedure (PROC MIXED) of the Statistical Analysis System (SAS 8.1; SAS Institute, Inc., Cary, NC). The frequency and amplitude of LH pulses were determined using both visual inspection and a pulse-detection algorithm, Pulsefit 1.2 [25]. Sources of variation were diet, day, and diet × day. Day was used as the repeated variable, and cow(diet) was used as the subject.

To test the effects of diet, leptin, and GnRH treatments on the release of LH from AP explants in vitro, the 6.5-h period of perifusion was subdivided in three periods (I–III) corresponding to equilibration (0–2.5 h), basal release (2.5–4.5 h), and GnRH stimulation (4.5–6.5 h), respectively. Hormone data obtained from perifused media were analyzed by the general linear mixed model (PROC MIXED) procedure of SAS. Sources of variation were diet, leptin treatment, period, and all interactions. Period was used as the repeated variable, and cow(diet) was used as the subject. The least squares means procedure was used to compare means when a significant F value was obtained. Because of differences in the release of LH by AP explants among perifusion chambers at the end of the equilibration period, covariate analyses were performed to test main effects during period II (basal release). Mean concentrations of LH in the last three

TABLE 1. Concentrations, frequency, and amplitude of LH pulses in normal-fed and fasted cows.[a]

	Normal Fed		Fasted	
Parameter	Day 0	Day 3	Day 0	Day 3
Concentration (ng/ml)	5.2 ± 0.1	5.9 ± 0.1	4.2 ± 0.1	5.3 ± 0.2
Frequency (pulses/6 h)	8.3 ± 1.2	10 ± 1.0	5.0 ± 1.4	7.0 ± 1.6
Amplitude (ng/ml)	1.6 ± 0.6	2.0 ± 0.5	1.7 ± 0.8	1.3 ± 0.2

[a] No dietary effect was observed. Values are presented as mean ± SEM.

samples collected during equilibration were used as the covariate (covariate 1). To test main effects during period III (GnRH stimulation), another covariate analysis was performed, using the mean of the last three samples of period II (basal) as covariate (covariate 2). To test cumulative (basal + GnRH) effects of leptin and GnRH stimulation on the amount of LH released by AP explants, an analysis was performed using covariate 1 to test main effects during period III.

To test the effects of leptin on GnRH release from HYP explants, data were analyzed by the general linear mixed model procedure of SAS (PROC MIXED). Sources of variation were diet, leptin treatment, period, and all possible interactions. Period was used as the repeated variable, and cow(diet) was used as the subject.

RESULTS

Does Leptin Modulate Basal or GnRH-Mediated LH Release from the Adenohypophysis?

As expected, mean plasma concentrations and mean frequency and amplitude of LH pulses did not differ ($P > 0.1$) between fasted and normal-fed groups before the cows were killed (Table 1). Using covariate analysis to adjust for differences in basal release of LH among perifusion chambers, it was observed that dietary treatment interacted with in vitro leptin treatments to influence the release of LH from AP explants. Leptin-treated AP explants from fasted cows had a higher ($P < 0.02$) basal release of LH than control-treated explants. Because effects of individual doses of leptin on the basal release of LH from AP explants of fasted cows did not differ, all doses of leptin were combined for presentation (Fig. 2). In explants of normal-fed cows, leptin did not affect ($P > 0.1$) basal release of LH (Fig. 2).

In contrast to observations made for basal release, all doses of leptin increased ($P < 0.001$) GnRH-mediated release of LH from AP explants of normal-fed, but not of fasted, cows (Fig. 3). In fact, in the fasted group, AP explants treated with 5 ng/ml of oleptin released less ($P < 0.001$) LH (1059 ± 55.4 ng/ml, ± SEM) in response to GnRH than control-treated explants (1133 ± 62.6 ng/ml, ± SEM), and all other doses had no effect on this variable. Because leptin increased basal release of LH from AP explants obtained from fasted cows before GnRH treatment, followed by nonsignificant numerical increases after GnRH, we examined the combined effects of leptin on basal and GnRH-stimulated release of LH. Leptin at doses of 50 (1416 ± 87.6 ng/ml, ± SEM) and 100 ng/ml (1455 ± 89.1 ng/ml, ± SEM) increased ($P < 0.001$) the overall (basal plus GnRH-stimulated) mean concentration of LH compared to control-treated explants.

Effects of Leptin on the Secretion of GnRH from the MBH-Infundibular Complex

Data from one normal-fed cow were not included in the analysis because of a lack of response to 60 mM K^+, suggesting a loss of tissue viability. Neither fasting nor leptin affected ($P > 0.1$) basal or potassium-stimulated release of GnRH from HYP explants (Fig. 4).

DISCUSSION

In the present study, we examined the hypothesis that the hypersecretion of LH induced by leptin in vivo in fasted, mature cows [16] could be explained by effects at the AP level. Significant effects of leptin on basal release of LH were observed in AP explants from fasted cows, on AP responsiveness to GnRH in normal-fed cows, and on overall release (basal plus GnRH-stimulated release of LH) in AP explants of fasted cows perifused with the highest doses of leptin (50 and 100 ng/ml). In contrast, we were unable to detect effects of leptin on the release of GnRH from isolated HYP explants obtained from either normal-fed or fasted cows.

As noted previously, 2–3 days of restricted feed intake does not affect mean concentrations of LH or its secretory patterns in mature ewes or cows [11, 14–16], although signs of metabolic stress, including decreased circulating insulin, IGF-I, and leptin, are clearly present. Similar metabolic constraints are observed in fasted heifers [12, 13] and estradiol-implanted wethers [11], but in these ruminant animal models, short-term fasting is able to reduce the frequency of LH pulses similar to that observed for monogastric species [26–28]. Nonetheless, feed-restricted, ma-

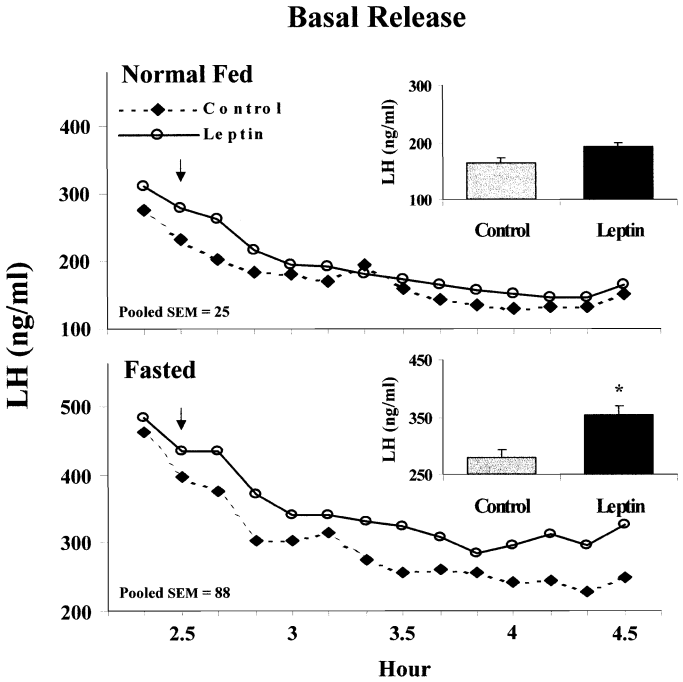

FIG. 2. Mean concentrations of LH in media collected from AP explants obtained from normal-fed (**Top**) and fasted (**Bottom**) cows and perifused (2.5–4.5 h) with KRB alone (control) or KRB containing 5, 10, 50, and 100 ng/ml of oleptin (combined). No effect ($P > 0.1$) of leptin was observed on the release of LH (basal release) from AP explants from normal-fed cows (**Top inset**). However, basal release of LH was elevated (*$P < 0.02$) in leptin-treated AP explants from fasted cows (**Bottom inset**). Arrows indicate the beginning of control (KRB alone) or leptin treatment.

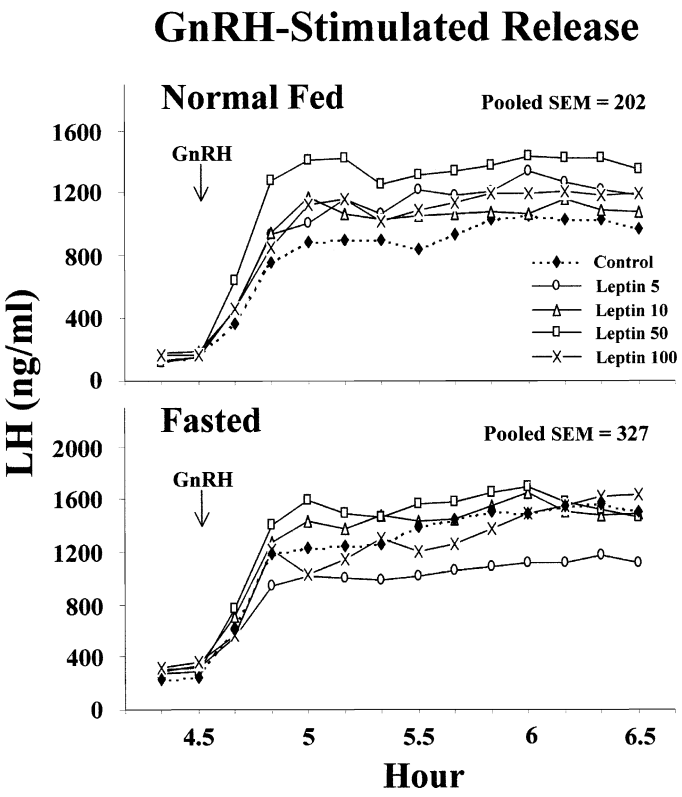

FIG. 3. Mean concentrations of LH in media after GnRH stimulation of AP explants of normal-fed and fasted cows perifused in the presence of either KRB alone (control) or KRB containing 5, 10, 50, and 100 ng/ml of oleptin. All doses of leptin increased ($P < 0.001$) GnRH-stimulated release of LH in AP explants collected from normal-fed (**Top**), but not from fasted (**Bottom**), cows. Adenohypophyseal explants from fasted cows perifused with 5 ng/ml of oleptin released less ($P < 0.001$) LH in response to GnRH than control-treated explants. Note the elevated baseline for leptin-treated compared to control-treated AP explants from fasted cows at the time of GnRH stimulation (4.5 h).

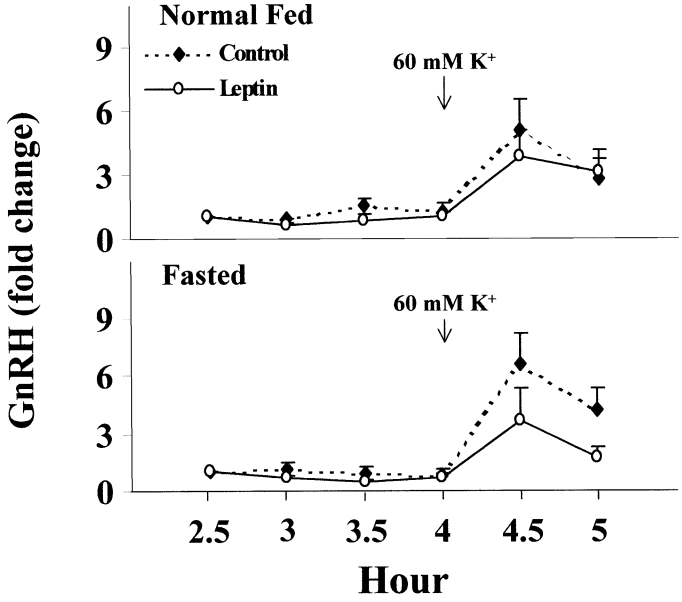

FIG. 4. Fold change in mean concentrations of GnRH in media collected from HYP explants of normal-fed and fasted cows. Hypothalamic-infundibular explants were incubated in 0.7 ml of KRB either alone or containing 1000 ng of oleptin at 2.5 and 3 h. Explants were incubated with KRB containing 60 mM K^+ at 4 h of incubation to test viability of tissue. No effects of diet or leptin were observed ($P > 0.1$).

ture cows are responsive to leptin treatment, and mean concentrations, mean baseline, and magnitude of LH pulses are increased [16].

Questions then arise as to whether stimulation of LH release by leptin is accounted for by effects at hypothalamic and/or AP levels. Most studies have proposed that leptin stimulates secretion of LH via interaction with neuronal systems and, ultimately, stimulation of GnRH secretory neurons. In models in which the frequency of LH pulses is diminished by feed restriction [11, 13], leptin likely stimulates GnRH release to prevent the effects of fasting. Alternatively, diminished release of LH during fasting could be explained by changes in the amplitude of GnRH pulses and GnRH's ability to trigger LH release, as observed previously in this laboratory [18] and in others [29] for other physiological contexts. However, under feed-restricted conditions, in which frequency of LH pulses is not affected, as observed in mature female ruminants [16], the stimulatory effects of leptin on LH secretion are more plausibly explained by a direct action at the adenohypophysis. In addition to the current results, evidence supporting a direct action of leptin at the level of the adenohypophysis includes the localization of leptin receptors on gonadotropes of ewes [8] and leptin stimulation of LH release from rat anterior pituitaries in vitro [9]. However, in contrast to the study performed using rat tissue [9], we observed no effect of leptin on basal release of LH from AP explants from normal-fed cows, suggesting important species differences in the acute interaction of nutritional status and leptin with gonadotropes in cattle. This view is supported further by our observation that LH release from primary cultures of bovine AP cells, maintained under optimal nutrient conditions, was not affected by leptin at doses of 1–1000 ng/ml (unpublished observations).

Increases in basal release of LH from AP explants from fasted cows treated with leptin appear to be different than what would be expected from a classical secretagogue. In fact, after the period of equilibration (initial 2.5 h of perifusion) and before GnRH stimulation (last 2 h of perifusion), basal release of LH tended to continue to decrease in all treatments, independent of dietary group. This steady decline in the release of LH during the basal-release period may reflect the absence of stimulation by endogenous GnRH. However, the decrease in LH release by AP explants from fasted cows treated with leptin was significantly smaller than control-treated explants. This effect was not observed in explants from normal-fed cows. It is possible that leptin may have stimulated an increase in basal metabolism of gonadotropes from fasted cows, resulting in an elevated release of LH compared to gonadotropes of control-treated explants. In support of this concept, increased glucose uptake has been observed in muscle and adipose tissue treated with leptin [30].

Mechanisms involved in the leptin-mediated increase in responsiveness to GnRH of AP explants from normal-fed cows are unknown. However, these effects could occur through several potential pathways, including effects on Ca^{2+} ion channels, an increase in the releasable pool of LH, and/or GnRH-receptor desensitization. All of these are important for cellular exocytosis of gonadotropins [31]. Leptin-receptor signaling involves activation of Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathways [32]. In addition, mitogen-activated protein kinase [32] and phosphatidylinositol-3 kinase [33] have also been involved in leptin-receptor signaling. In porcine

chromaffin cells, leptin caused a sustained increase of intracellular Ca^{2+} and activated inositol 1,4,5-triphosphate production [34], which are intracellular factors known to be associated with GnRH-receptor signaling and release of LH [35].

The lack of increased responsiveness to GnRH in AP explants from fasted cows treated with leptin is unclear. However, it is well established that GnRH stimulation of gonadotropes is necessary for synthesis of gonadotropins [36]. Thus, it is possible that the increased basal release of LH observed in leptin-treated explants of fasted cows and lack of any GnRH stimulation during this period caused a diminution of readily releasable pools of LH that were not replenished.

Experimental models describing the temporal secretion pattern of GnRH by the hypothalamus have been developed in several large species. In sheep, this has been accomplished through collection of hypophyseal-portal blood [37]. In the mature cow, the anatomy of the cranium impairs practical application of this technique. However, collection of cerebrospinal fluid from the third ventricle has been shown by our laboratory and others to be a useful approach for determining patterns of GnRH secretion in cattle and sheep [18, 37], and studies are currently underway to investigate the effects of leptin using this approach. Incubation of HYP explants has also been used extensively to study the neuroendocrine regulation of GnRH release [38–40] in several species. In vitro incubations of isolated HYP tissue allows the investigation of factors acting directly at the hypothalamus. Hypothalamic explants used in the present experiment included the MBH connected to the infundibulum. The arcuate nucleus, which rests within the MBH, contains leptin receptors [7] as well as neuronal cell bodies that express mediators of the effects of leptin within the central nervous system, such as neuropeptide Y, agouti-related peptide, and POMC (precursor of α-melanocyte-stimulating hormone) [1]. Thus, explants used in the present experiment contain at least part of the neuronal systems that potentially mediate the effects of leptin on GnRH neurons. Using this approach, we were unable to detect effects of leptin on GnRH secretion from hypothalamus harvested from either normal-fed or fasted cows. In contrast, Yu et al. [9] have reported that leptin stimulates GnRH release from rat hypothalamic explants. Whether these discordant results are caused by species differences or the experimental methodology used to detect the effects of leptin is not clear.

Mechanisms that explain the inability of leptin to independently stimulate an increase in circulating concentrations of LH in cattle and sheep that are fed either maintenance [11, 41, 42] or growing diets [43] are not understood. However, the inability of leptin to stimulate directly basal secretion of LH in AP explants of normal-fed cows in the present study supports those findings. Syndromes involving the development of resistance to leptin have been observed in obese humans [44, 45] and rodents [46, 47], and evidence suggests that excessive activity of suppressor of cytokine signaling (SOCS)-3 activity is involved in leptin resistance [48, 49]. Inhibition of leptin signaling by SOCS-3 seems to involve suppression of JAK2 activation [48]. Whether a leptin-resistant condition occurs in ruminants that are under neutral or positive energy balance has not been determined; however, leptin is able to decrease feed intake in sheep [41, 42]. Moreover, the mass of ligand reaching target tissues must also be considered. We have reported recently that intravenously injected recombinant oleptin causes an inverse, dose-related increase in basal plasma concentrations of LH in ovariectomized, estradiol-implanted cows fasted for 60 h [17]. A dose of 0.2 μg/kg maximized the increase in LH, whereas doses of 2 and 20 μg/kg caused a lower response and no response, respectively. Reports in rodents [9] confirm that AP explants treated with high concentrations of leptin (10^{-5} M) lose their ability to respond with an increase in LH release. Therefore, the duration and/or amount of exposure to the hormone likely determines the development of resistance.

In conclusion, results from the current experiment support the hypothesis that leptin affects the secretion of LH in the mature cow, in part, by its direct action at the adenohypophysis. Leptin maintained higher basal AP release of LH in tissues obtained only from fasted cows, whereas increased responsiveness to GnRH was evident in AP explants obtained from normal-fed cows. This indicates that whereas leptin acts on gonadotroph function under both normal and fasting conditions, the manifestations of these effects are expressed differentially and will require further study at the cellular level to characterize in detail.

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