Effects of leptin on basal and GHRH-stimulated GH secretion from the bovine adenohypophysis are dependent upon nutritional status

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Abstract

We have shown recently that leptin modulates at least two aspects of anterior pituitary LH release in ruminants: basal and GnRH-mediated release. To test the hypothesis that leptin directly affects basal and GHRH-mediated GH secretion from the adenohypophysis, we examined the effects of various doses of recombinant ovine leptin (oleptin) on perifused adenohypophyseal (AP) explants and compared responses of tissues from control and fasted cows. Ten mature, ovariectomized and estradiol-implanted cows were assigned to one of two dietary groups: (1) normal-fed ($n=5$) and (2) fasted for 72 h ($n=5$). At the end of the fasting period, cows were euthanized and pituitaries were collected. Adenohypophyseal explants were perifused for a total of 6·5 h, including a 2-h treatment at 2·5 h with Krebs-Ringer bicarbonate buffer containing 0, 5, 10, 50, or 100 ng/ml oleptin, and a challenge with GHRH at 4·5 h. All doses of oleptin greater than 5 ng/ml decreased ($P<0·01$) basal GH secretion compared with controls in tissues collected from normal-fed cows. In contrast, GH release from AP explants from fasted cows treated with the lowest dose of oleptin was 28% ($P<0·002$) higher than control explants, but larger doses had no effect. Leptin caused an inversely related, dose-dependent increase in GHRH-mediated GH release in tissues from normal-fed cows. Marked increases ($P<0·01$–$P<0·001$) in GH release were observed for the 5 and 10 ng/ml oleptin, with lesser ($P<0·08$) and no effects observed at the 50 and 100 ng/ml doses respectively. In fasted cows, oleptin had no stimulatory effect on GHRH-induced GH release. Results show that leptin can act directly at the anterior pituitary level to modulate GH release, and this effect is dependent upon nutritional history.

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Introduction

Leptin, an adipocyte-produced hormone, regulates the secretion of several pituitary hormones in humans, rats, sheep, and cattle (Yu *et al.* 1997*a,b*, Roh *et al.* 1998, Amstalden *et al.* 2002*a*). Expression of leptin receptor mRNA has been demonstrated in the anterior pituitary gland and hypothalamus by RT-PCR (Zamorano *et al.* 1997), and leptin appears to have an important role in the control of growth hormone (GH) release (Roh *et al.* 1998, Iqbal *et al.* 2000).

Chronic undernutrition can markedly increase secretion of GH in both ruminant species and humans (Hull & Harvey 2002). However, in ruminants, the effects of short-term nutritional deprivation on GH secretion are less distinct than in monogastrics. Neither mean concentrations of serum GH nor GH secretory dynamics are affected by acute feed restriction in cattle; however, fasting reduces leptin gene expression and circulating leptin in association with reductions in secretion of insulin-like growth factor-I and insulin (Amstalden *et al.* 2000). Although Nagatani *et al.* (2000) demonstrated that 78 h of fasting in gonadectomized male sheep tended to increase mean concentrations of GH, its secretion was not stimulated by leptin administration. Moreover, continuous intracerebroventricular (i.c.v.) infusion of recombinant human leptin (hleptin) had no effect on circulating GH concentrations in ovariectomized, full-fed sheep (Henry *et al.* 1999) and normally-fed male rats (Vaugnat *et al.* 1998). On the contrary, a 7-day i.c.v. infusion of leptin increased mean concentrations and the amplitude of GH pulses in male rats (Tannenbaum *et al.* 1998), and enhanced circulating GH in chronically undernourished sheep (Henry *et al.* 2001). Furthermore, neuropeptide

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Y (NPY), a mediator of leptin action centrally, is elevated during undernutrition (McShane *et al.* 1992), suppresses luteinizing hormone (LH) secretion (Gazal *et al.* 1998), and stimulates GH release in ruminants (Thomas *et al.* 1999).

Recently, we have shown that leptin modulates at least two aspects of anterior pituitary LH release in cattle: basal and gonadotropin releasing hormone (GnRH)-mediated release (Amstalden *et al.* 2002*b*). Although studies noted above confirm an influence of leptin on circulating concentrations of GH, the site of leptin action and the influence of nutritional status is not clearly understood. In the current study, we examined the direct effect of recombinant ovine leptin (oleptin) on basal and growth hormone releasing hormone (GHRH)-mediated GH secretion in perifused adenohypophyseal (AP) explants and compared responses of explants derived from normal-fed and fasted animals.

Materials and Methods

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

Animal model and procedures

Ten ovariectomized cows, each bearing a subcutaneous estradiol implant, were used. In the present study, mean (± S.E.M.) concentrations of estradiol in implanted cows were 4·35 ± 0·45 pg/ml. Hormonal implants provide a constant level of estradiol negative feedback without the complications associated with ovarian cyclicity. Ovariectomized, steroid-treated females and castrated, steroid-treated males have proven to be good models for studying the effects of nutrition on the neuroendocrine axis (Kile *et al.* 1991, Amstalden *et al.* 2002*a*). Experiments reported herein were conducted simultaneously with those that examined LH secretion and responses to GnRH as reported elsewhere (Amstalden *et al.* 2002*b*).

During a several-week pre-experimental period, cows were fed once daily at 0700 h and diets were adjusted so that all cows lost adequate body weight to achieve a moderate condition (body condition score (BCS) = 4; 1 = emaciated; 9 = obese). Body condition scores were adjusted down with the objective of increasing the sensitivity of the hypothalamic–pituitary axis to short-term fasting in this ruminant model (Amstalden *et al.* 2002*a*). After achieving a BCS of 4, cows were fed a diet formulated to provide 100% of the National Research Council (NRC 1996) requirements for maintenance for at least 2 weeks before the beginning of the experiment. Cows were each assigned randomly to one of two dietary groups: (1) normal-fed: cows were fed 100% of the NRC requirements for maintenance to maintain body weight (n = 5), and (2) fasted: cows were fasted for 72 h with free access to water (n = 5). One day before the start of dietary treatments (day 1), cows were fitted with jugular catheters (polyethylene tubing, 1·4 mm inside diameter, 1·9 mm outside diameter; Becton Dickinson, Parsippany, NJ, USA) 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. Blood samples were dispensed into tubes containing 150 µl of a solution containing heparin (1000 IU/ml) and 5% EDTA and placed on ice immediately. Plasma was separated by centrifugation and stored at − 20 °C until GH analysis. At the end of sampling on day 3, cows were euthanized humanely by exsanguination following captive bolt stunning. Brains were removed after disconnection of infundibuli and adenohypophyses were removed from the *sella turcica* and kept on ice until tissue processing.

Adenohypophyses were dissected and sliced sagittally into approximately 0·5 × 2 mm strips. Explants were perifused with Krebs-Ringer bicarbonate buffer (KRB) (pH = 7·4) for a total of 6·5 h using a multiple microchamber perifusion system (Endotronics Inc., Coon Rapids, MN, USA) (Fig. 1). At 2·5 h, AP explants were perifused for 2 h with KRB containing 0 (control), 5, 10, 50, or 100 ng/ml oleptin. After 4·5 h of perifusion, AP explants were challenged with 255 ng GHRH (Peninsula Laboratories, Inc., Belmont, CA, USA) injected via a tubing connection into the chamber and perifused for another 2 h. The dose of GHRH was based on preliminary experiments in this laboratory. Media were collected in 1 ml fractions and stored at − 20 °C until hormone analysis.

Radioimmunoassay (RIA) for GH

Plasma concentrations of GH and concentrations of GH in AP perifused media were determined as reported previously (Ryan *et al.* 1994). Intra- and interassay coefficients of variation for GH assays averaged 8·2 and 15·1% respectively.

Statistics

Circulating concentrations of GH 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, NY, USA). To test the effects of diet, leptin, and GHRH treatments on the release of GH from AP explants *in vitro*, following a 2-h equilibration period, the 4·5 h experiment was subdivided in three periods (I-III) corresponding to basal-pretreatment (2–2·5 h; period I), basal-leptin treatment (2·5–4·5 h; period II), and GHRH stimulation (4·5–6·5 h; period III) (Fig. 1). Hormone data obtained from perifusion media were analyzed using PROC

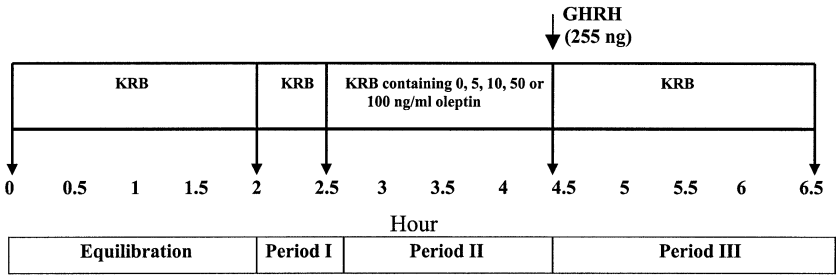

Figure 1 Time-line for experimental procedures. AP were perifused with KRB for 6·5 h. Following a 2-h equilibration period, the 4·5 h experiment was subdivided in three periods (I-III) corresponding to basal-pretreatment (2–2·5 h; period I), basal-leptin treatment (2·5–4·5 h; period II), and GHRH stimulation (4·5–6·5 h; period III). Beginning at 2·5 h, AP explants were perifused for 2 h with KRB containing 0 (control), 5, 10, 50, or 100 ng/ml oleptin. After 4·5 h perifusion, AP explants were challenged with 255 ng GHRH and perifused for another 2 h.

MIXED. 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 significant means procedure was used to compare means when a significant F-value was obtained. Because of differences in basal GH release by AP explants among perifusion chambers, covariate analyses were performed using mean GH release during period I (basal-pretreatment) as a covariate to examine the effects of leptin during period II.

Results

On day 3 of the experiment (66–72 h) and before euthanasia, mean serum concentrations of GH did not differ ($P>0·1$) between the fasted and normal-fed groups ($2·8 \pm 0·73$ and $2·2 \pm 0·34$ respectively).

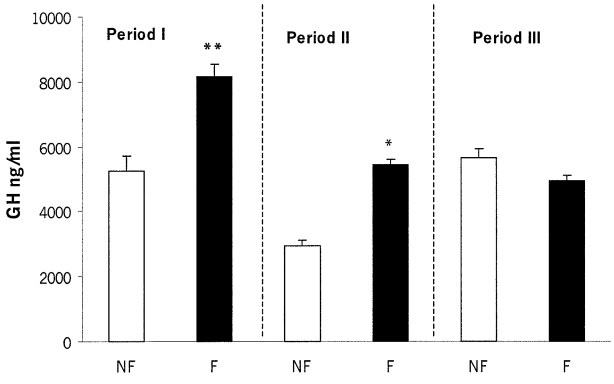

Figure 2 Mean (± S.E.M.) concentrations of GH in normal-fed (NF) and fasted (F) groups during periods I to III of perifusion. Mean concentrations of GH were higher in the perifusion media of the fasted group compared with the normal-fed group during periods I (basal-pretreatment) and II (basal-leptin treatment). $*P<0·01$, $**P<0·001$ denote differences between groups.

Adenohypophyseal explants collected from fasted cows released more GH during periods I ($P<0·001$) and II ($P<0·01$) compared with normal-fed animals (Fig. 2). All doses of oleptin greater than 5 ng/ml decreased ($P<0·01$) basal GH secretion (period II) compared with control-treated explants collected from normal-fed cows (Fig. 3). In contrast, basal GH release from AP explants of fasted cows treated with the lowest dose of oleptin (5 ng/ml) was 28% ($P<0·002$) higher than control-treated explants, but larger doses had no effect (Fig. 3).

Leptin caused an inversely related, dose-dependent increase in GHRH-mediated GH release (period III) in tissues from normal-fed cows (Fig. 4). A marked stimulation of GHRH-mediated GH release was observed in AP explants treated with 5 ($P<0·005$) and 10 ng/ml ($P<0·001$) oleptin, with a lesser trend ($P<0·08$) at 50 ng/ml, and no effect at the highest dose (Figs 4, 5). In fasted cows, oleptin had no stimulatory effect on GHRH-induced GH release (Figs 4, 5).

Discussion

This is the first report demonstrating a direct effect of leptin on pituitary GH secretion in cows, and showing clear differences in the ability of AP explants from normal-fed and fasted cows to respond to exogenous leptin.

Growth hormone secretion in ruminants is known to increase following chronic food restriction, whereas in rodents serum concentrations of GH decrease during undernourished states (Gluckman *et al.* 1987, Thissen *et al.* 1994). In the present study, differences in mean circulating concentrations of GH between the two dietary groups were neither observed nor expected after the relatively brief period of feed withdrawal (Amstalden *et al.* 2000, Nagatani *et al.* 2000). Increases in circulating GH concentrations have been observed in ruminants only after

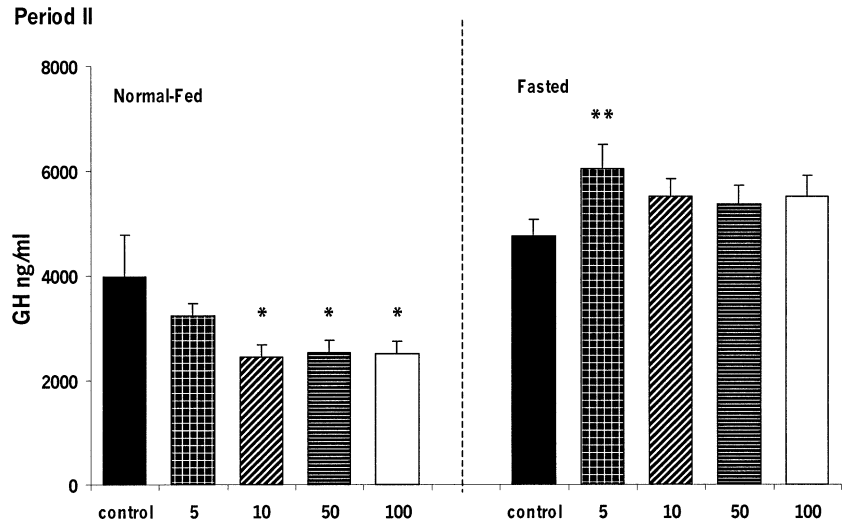

Figure 3 Effects of oleptin (0, 5, 10, 50 and 100 ng/ml) on mean (± S.E.M.) concentrations of GH in the perifusion media of AP explants from normal-fed and fasted groups during period II: $*P<0{\cdot}01$, $**P<0{\cdot}002$ compared with controls.

long-term feed restriction (Sejrsen *et al.* 1983, Ryan *et al.* 1994). However, under *in vitro* conditions, AP explants from fasted cows exhibited higher basal release of GH before and after leptin treatment compared with tissues from normal-fed animals, and this effect was exaggerated during periods I and II. Therefore, under *in vitro* conditions, pituitary somatotropes may be more sensitive to the interaction of leptin and previous nutrient restriction than *in vivo*.

Based upon increases in both receptor mRNA and protein levels (Baskin *et al.* 1998, 1999), it has been reported that fasting increases leptin receptor concentration in the arcuate nucleus (ARC) of the rat. Similarly, expression of the full-length leptin receptor (OBRb) in the ventromedial hypothalamus was found to be much greater in feed-restricted ewes than in ewes that were well-fed (Dyer *et al.* 1997). Explants from fasted animals in our experiment responded directly to leptin, with higher basal secretion of GH in response to the lowest dose (5 ng/ml) of leptin. This is consistent with a study by LaPaglia *et al.* (1998) in which leptin administration to fasted rats produced a threefold increase in GH mRNA,

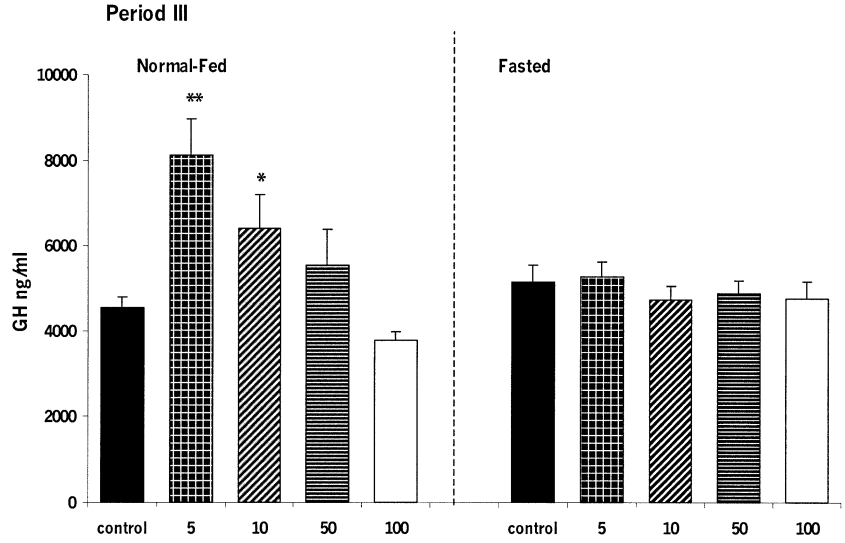

Figure 4 Effects of oleptin (0, 5, 10, 50 and 100 ng/ml) on GHRH-mediated GH release in the perifusion media of AP explants from normal-fed and fasted groups during period III. $*P<0{\cdot}005$, $**P<0{\cdot}001$ compared with controls.

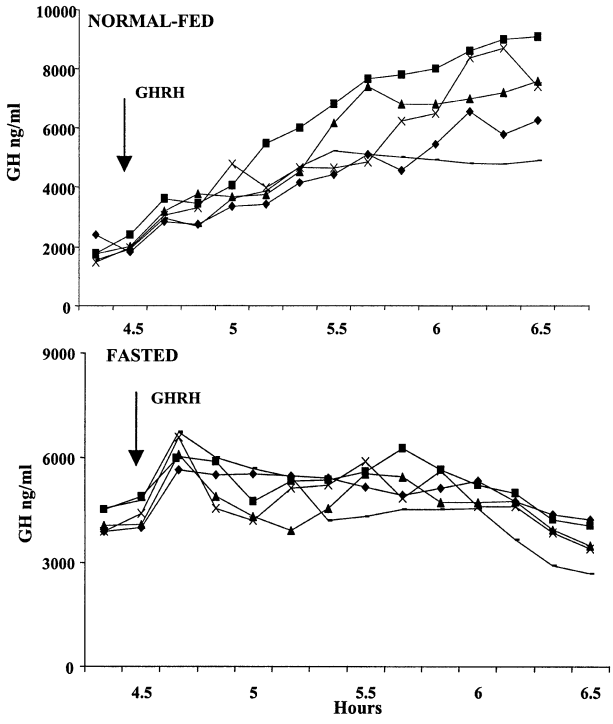

Figure 5 Temporal patterns of GH release after GHRH stimulation in the perifusion media of AP explants from normal-fed and fasted groups during period III. Control, ♦; 5 ng/ml oleptin, ■; 10 ng/ml oleptin, ▲; 50 ng/ml oleptin, X; 100 ng/ml oleptin, -.

indicating the potentially important role of leptin in regulating GH gene transcription and/or stability, as well as GH secretion. However, AP explants from normal-fed cows exhibited reduced GH secretion with most of the leptin doses when compared with control tissues. *In vivo* experiments using well-fed sheep (Henry *et al.* 1999, Morrison *et al.* 2002) and rats (Carro *et al.* 1997) found no effect of leptin on circulating GH concentrations. The observed increase in GH secretion from AP explants of fasted cows, and the decreased secretion of GH from tissues of normal-fed cows observed in our study, could have resulted from differences in leptin receptor expression and a down-regulation of OBRb expression in explants from well-fed cows. Moreover, the effect of leptin on somatotropes may also be dose-related, a phenomenon observed in perfused rodent pancreas. A low dose of leptin has been shown to have either no effect or to increase basal release of insulin in isolated rodent islets (Tanizawa *et al.* 1997), whereas higher doses of leptin suppressed basal (Emilsson *et al.* 1997, Kieffer *et al.* 1997) and glucose-stimulated (Pallett *et al.* 1997) insulin secretion from islets isolated from both normal or *ob/ob* mice and rats. These observations may help to explain the increase in secretion of GH observed after treatment of explants from fasted cows with the lowest dose of oleptin, and the lack of effect when higher doses of oleptin were employed. Data are also consistent with a previous study with fasted cows (Zieba *et al.* 2003) in which leptin had a dose-dependent, bimodal influence on the normal-sensing mechanism of the pancreas, and on basal secretion of LH from the anterior pituitary. In both cases low doses of leptin (0·2 and 2·0 μg/kg) optimized endocrine responses, while the highest doses (20 μg/kg) attenuated them. High leptin concentrations can have detrimental effects on leptin receptor number or on signaling molecules. However, whether high doses of leptin can prevent oligomerization of receptors, thus inducing desensitization or leading to a loss of nuclear STAT3 activation, still needs to be clarified.

Growth hormone is produced in the anterior pituitary gland as a 22-kDa polypeptide and is secreted into the circulation under the regulation of GHRH, somatostatin, NPY and Ghrelin (Frohamn *et al.* 2000, Kojima *et al.* 2001). Several studies *in vivo* and *in vitro* have implicated both GHRH and somatostatin in leptin-mediated GH secretion in rats (Quintela *et al.* 1997, Carro *et al.* 1999). Studies with normal-fed rats provide a more direct demonstration of mechanisms underlying the GH-stimulating activity of leptin. Short-term i.c.v. administration of leptin increased the expression of GHRH mRNA by 62%, decreased somatostatin mRNA by 41% in the hypothalamus, and increased GH synthesis in the adenohypophysis (Cocchi *et al.* 1999). In our experiment, leptin-treated explants from normal-fed cows perifused with low doses of leptin released larger amounts of GH in response to GHRH compared with control-treated explants. Similar positive effects of exogenous leptin on GHRH-induced GH release were observed in primary monolayer cultures of rat pituitary cells (Mizuno *et al.* 1999). However, opposite results have been reported by Roh *et al.* (2001) in which a negative effect of leptin on GHRH-stimulated GH secretion was observed with a reduction in GHRH receptor synthesis in somatotropes from ovine primary cell cultures. This paradox may be due, in part, to fundamental differences in physiology of somatotropes maintained in short-term (explants) compared with long-term (primary) culture.

In explants harvested from fasted cows, leptin did not affect GHRH-mediated GH release. Fasting has been shown to reduce hypothalamic GHRH mRNA expression in rats (Bruno *et al.* 1992, LaPaglia *et al.* 1998). However, neither fasting nor fasting and leptin altered the hypothalamic content of the GHRH peptide (LaPaglia *et al.* 1998). This supports the concept that the effects of leptin on GH secretion are mediated, at least in part, at the pituitary level. Furthermore, Baratta *et al.* (2002) have demonstrated that leptin exerts direct effects on GH gene expression and secretion, and this activity is modulated by nitric oxide.

In summary, results of our experiments show that leptin can act directly at the anterior pituitary level to modulate GH release, and this effect is dependent upon nutritional history. Specifically, the fasted state accelerates basal

secretion of GH and this effect is potentiated by leptin. Conversely, the effects of leptin in the normal-fed state are seen only during GHRH stimulation. Together, these observations are remarkably similar to those that we observed for the effects of leptin on basal and GnRH-mediated LH release in these same explants (Amstalden *et al.* 2002*b*). In those experiments, leptin maintained higher basal release of LH in tissues obtained from fasted cows, whereas increased responsiveness to GnRH was evident only in AP explants collected from normal-fed cows.

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