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The In Vitro Effect of Leptin on Growth Hormone Secretion from Primary Cultured Ovine Somatotrophs

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Although existing data suggest an influence of leptin on circulating levels of growth hormone (GH), the action site and properties of leptin are still controversial. Using primary cultured ovine pituitary cells, we studied the direct effect of leptin on the secretion of GH. Pituitary cells were dissociated by collagenase and subjected to Percoll gradient centrifugation to enrich the somatotroph population to 60–80% of cells. Treatment of primary cultured ovine somatotrophs with leptin (10^{-9}–10^{-7} *M*) for 30 min did not affect basal, GH-releasing hormone (GHRH) (10^{-7} *M*)- or GH-releasing peptide-2 (GHRP-2)(10^{-7} *M*)-stimulated GH secretion. Following treatment of cells for 1–3 d with leptin, GHRH-stimulated GH secretion was reduced and GHRP-2-stimulated GH secretion increased. The combined effect of GHRH and GHRP-2 on GH secretion was not altered by the treatment of cells with leptin for 3 d. GHRH receptor mRNA levels in cultured somatotrophs were decreased but GHRP receptor mRNA levels were increased by 3-d leptin treatment. These results suggest that leptin has a long-term effect on somatotrophs to reduce GHRH receptor synthesis leading to a decrease in GHRH-stimulated GH secretion. Leptin appears, however, to have an opposite effect on GHRP receptor synthesis leading to an increase in GHRP-stimulated GH secretion.

Key Words: Leptin; growth hormone; growth hormone–releasing hormone; growth hormone–releasing peptide; receptor; in vitro.

Introduction

Growth hormone (GH) is released from pituitary gland under dual control of hypothalamic somatostatin (SRIF) and GH-releasing hormone (GHRH). The cloning of the receptor from pituitary gland for the synthetic GH-releasing peptides (GHRPs) suggests the presence of an endogenous GHRP system that could be an important component in the regulation of GH secretion and synthesis *(1)*. Recent identification of ghrelin in rat stomach endocrine cells as an endogenous ligand specific for GHRP receptor further supports the view of an additional endogenous GH secretagogue other than GHRH in the control of GH release *(2)*. Factors such as age, gender, nutrition, sleep, and meals, influence GH release, which has been demonstrated in various species *(3,4)*. Compared to nonobese men, obese patients have defects in pulsatile GH secretion resulting in hyposomatotropism *(5,6)*. In addition, the GH response to GHRH is decreased in obese men and women, whereas fasting or weight loss tends to restore this response *(3,7,8)*.

Several lines of evidence have implicated the action of leptin on anterior pituitary function *(9–11)*. It was first reported in 1997 that leptin played a role in controlling hormone secretion in the anterior pituitary via a stimulation on nitric oxide (NO) release within the pituitary gland *(10)*. Leptin receptor–like immunoreactivity was also detected in 69% of ovine somatotrophs, but in only about 25% of gonadotrophs or corticotrophs *(12)*.

Leptin has been demonstrated in rat to restore pulsatile GH secretion after fasting *(13)*. Intracerebroventricular (icv) injection of leptin antibodies reduced GH secretion in rat *(14)* and icv injection of leptin protects against the inhibitory effects of restricted nutrition on GH secretion although this icv injection or infusion for 3 d did not change the GH levels in normal fed rats *(14,15)*. In sheep, as in humans, plasma GH levels are increased when body weight is reduced or under restricted nutrition *(16,17)*. Thus, one might expect that leptin would reduce rather than stimulate GH secretion. The present study was conducted to investigate the direct effects of leptin on pituitary GH secretion.

Results

Primary cultured ovine somatotroph-enriched cells were used. We first tested the acute effect of leptin on basal and

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GHRH-stimulated GH secretion. In incubation experiments, treatment of the cells with recombinant human leptin (*rh*-leptin, 10^{-9} –10^{-7} *M*) for 60 min had no effect on either 30-min basal GH release without any stimulation or 30-min maximal GHRH (10^{-7} *M*)-stimulated GH secretion (**Fig. 1**). Long-term treatment (24 h) of cells with *rh*-leptin was then tested at a dose of 10^{-7} *M*. After 24 h of treatment with *rh*-leptin, cell culture medium was replaced by incubation medium without leptin and 30-min basal or GHRH-stimulated GH secretion was assessed in the incubation medium. Such treatment with *rh*-leptin slightly increased basal GH secretion at a high dose of 10^{-7} *M rh*-leptin (**Fig. 2**). Treatment of the cells with 10^{-7} and 10^{-8}M *rh*-leptin for 24 h, however, significantly ($p < 0.05$) inhibited GHRH-stimulated GH secretion (**Fig. 2**).

GHRH-stimulated GH secretion was dose dependent, and the effect of 10^{-7} *M rh*-leptin on the dose response relationship of GHRH and GH secretion was investigated in ovine somatotrophs. GHRH stimulated GH secretion over the dose range of 10^{-9}–10^{-6} *M* ovine GHRH as shown in **Fig. 3,** and treatment with 10^{-7} *M rh*-leptin for 24 h reduced responsiveness of somatotropes to all doses of GHRH. Statistical analysis indicated a significant reduction in stimulation of 10^{-8}–10^{-6} *M* GHRH on GH secretion (**Fig. 3**).

We then tested the effect of leptin treatment on GH, GHRH receptor (GHRH-R), and GHRP receptor (GHRP-R) mRNA levels in cultured ovine somatotrophs. Twenty-four hours of treatment of somatotrophs with recombinant ovine leptin (*ro*-leptin, 10^{-7} *M*) significantly ($p < 0.01$) decreased the level of GH mRNA measured by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) (**Fig. 4**). For 3-d treatment, doses above 10^{-9} *M* of *ro*-leptin significantly ($p < 0.01$ or $p < 0.05$) reduced levels of GH mRNA (**Fig. 4**).

Similar to the alteration of GH mRNA, GHRH-R mRNA levels were reduced by treatment of cells with 10^{-7} *M ro*-leptin for 1 or 3 d ($p < 0.01$) (**Fig. 5**). Low doses of *ro*-leptin (10^{-8} and 10^{-9} *M*) treatment of cells for 3 d significantly ($p < 0.01$ or $p < 0.05$) reduced GHRH-R mRNA levels (**Fig. 5**).

GHRP-R mRNA levels were not significantly altered by 1-d treatment of cells with *ro*-leptin (**Fig. 6**). Three-day treatment of cells with *ro*-leptin (10^{-9} –10^{-7} *M*), however, significantly increased the GHRP-R mRNA levels ($p < 0.01$ or $p < 0.05$) (**Fig. 6**).

After 3-d treatment of cells with 10 n*M ro*-leptin, the GH response to GHRH (10^{-8} *M*) was significantly reduced ($p < 0.05$), whereas the GH response to GHRP-2 (10^{-7} *M*) was increased ($p < 0.05$) (**Fig. 7**). The combined effect of GHRH and GHRP-2 on GH release was not significantly changed by leptin treatment (**Fig. 7**).

Discussion

Leptin has been known to regulate neuroendocrine function by acting on the hypothalamus. There are, however,

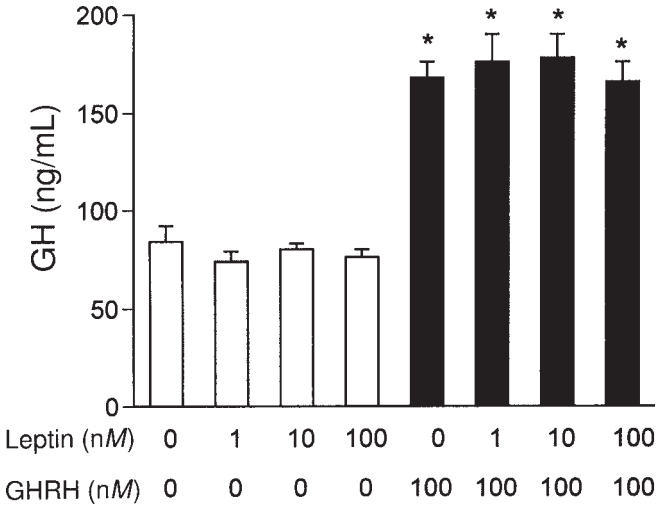

Fig. 1. Effect on the basal and 30-min GHRH-stimulated GH secretion by short-term treatment of leptin for 60 min (* $p < 0.001$ vs non-GHRH. (Adapted from ref. *11* with permission).

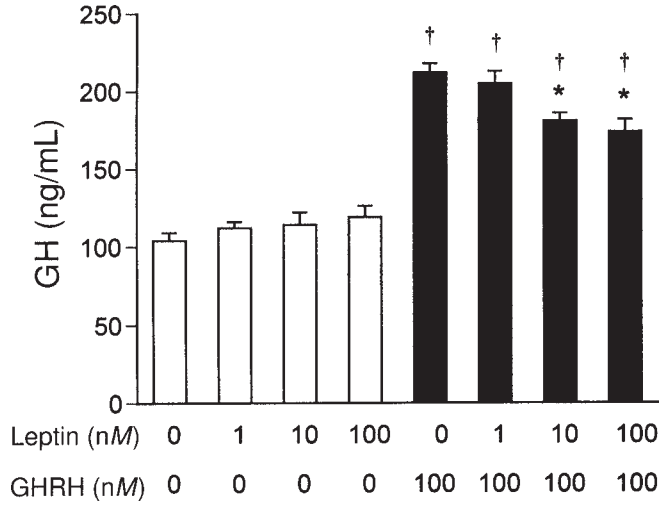

Fig. 2. Effect on the basal and 30-min GHRH-stimulated GH secretion by 24-h treatment of cultured cells with leptin ($^{\dagger}p < 0.001$ vs non-GHRH; $*p < 0.01$ vs GHRH and nonleptin treated). (Adapted from reference *11* with permission.)

contradictory reports about the action of leptin on GH secretion from the pituitary gland. Therefore, the present study was, designed to investigate the direct in vitro effect of leptin on the secretion of GH and the synthesis of GH, GHRH- and GHRP-R in ovine somatotrophs.

Leptin is secreted from adipose tissue and causes weight loss in rodents by reducing food intake and increasing energy expenditure *(18)*. Leptin has also been shown to regulate GH secretion in the rat *(13–15)* and pig *(19)* when administered intracerebroventricularly, suggesting a central mechanism of action. There are sparse data, however, on the direct action of leptin on the pituitary gland. The present study was conducted to investigate the short- and long-term effects of leptin on basal and GHRH-stimulated

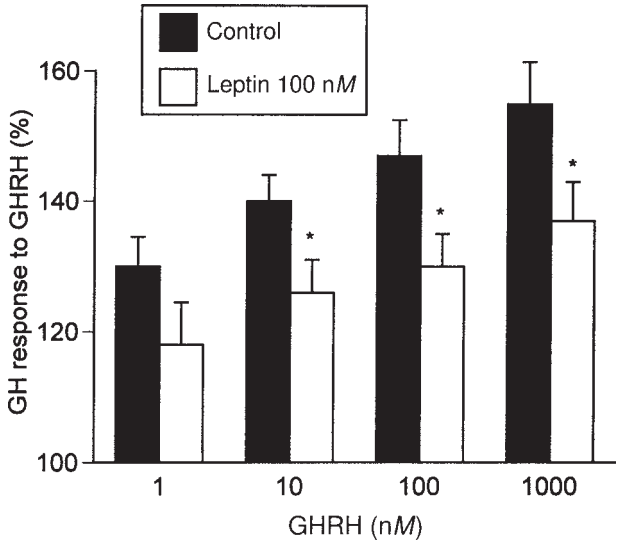

Fig. 3. Effect of 24-h treatment of cells with leptin on GH secretion caused by GHRH in a dose-dependent manner. The GHRH response is shown as percentage changes in relation to no treatment of GHRH *$p < 0.05$ vs control. (Adapted from ref. *11* with permission.)

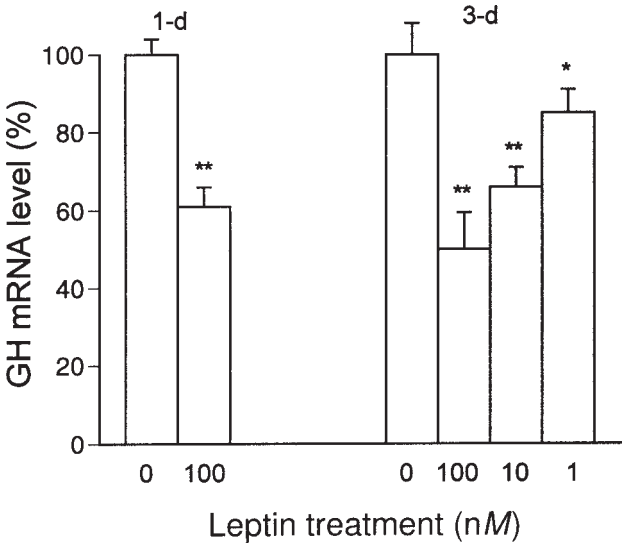

Fig. 4. The effect of 1- or 3-d treatment of cells with leptin on GH mRNA levels. GH mRNA levels given were corrected by glyceraldehyde-3-phosphate (GAPDH) mRNA levels in the same sample. *$p < 0.05$ vs control; **$p < 0.01$ vs control.

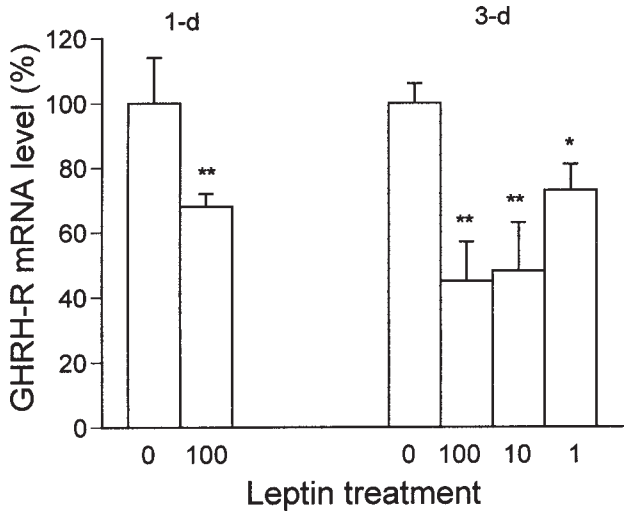

Fig. 5. Effect of 1- or 3-d treatment of cells with leptin on GHRH-R mRNA levels. GHRH-R mRNA levels given were corrected by GAPDH mRNA levels in the same sample. *$p < 0.05$ vs control; **$p < 0.01$ vs control.

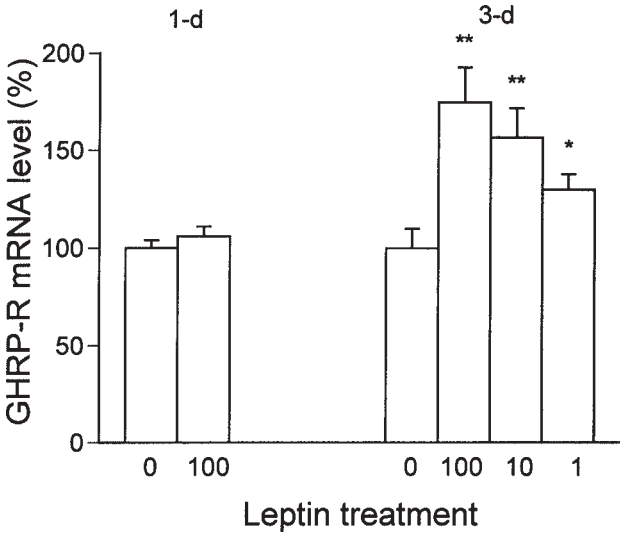

Fig. 6. Effect of 1- or 3-d treatment of cells with leptin on GHRP-R mRNA levels. GHRP-R mRNA levels given were corrected by GAPDH mRNA levels in the same sample. (*$p < 0.05$ vs control; **$p < 0.01$ vs control.

GH secretion. Incubation experiments in this study demonstrate that long-term (1 or 3 d) leptin treatment of ovine somatotrophs suppresses GHRH-stimulated GH secretion. Such treatment, however, increases GHRP-stimulated GH secretion. GH secretion induced by combined GHRH and GHRP-2 was not changed by 3-d leptin treatment. By contrast, short-term (60 min) treatment of cells with leptin had no effect on the secretion of GH.

In obese individuals owing to a mutation associated with a truncated leptin receptor lacking both the transmembrane and intracellular domains combined with a high level of circulating leptin, pituitary dysfunction and GH deficiency have been observed concomitant with those changes *(20)*. This observation emphasizes the important role of leptin in pituitary function. The recent localization of leptin receptor isoforms in human pituitaries by RT-PCR and *in situ* hybridization provided direct evidence for a functional role of leptin in the human pituitary *(21,22)*. The long form of leptin receptor (OB-Rb) was detected in normal pituitary as well as in adenomas *(21)*, although another study found OB-Rb only in adenomas *(22)*.

In addition to humans, leptin receptors are known to exist in the pituitary gland of sheep *(12,23)* and rats *(24)*

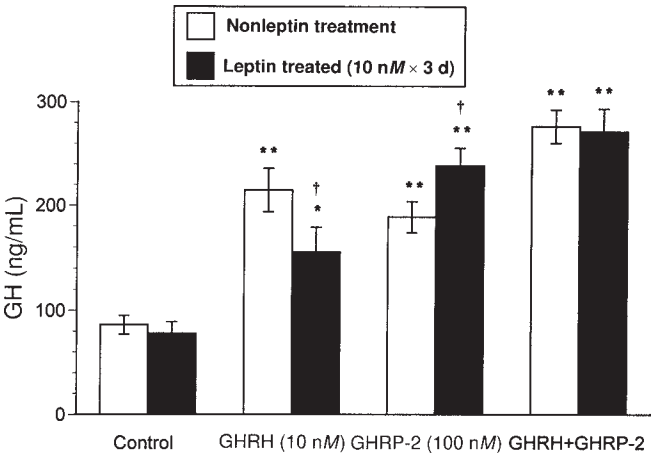

Fig. 7. Basal and 30-min GHRH-, GHRP-2-, or combined GHRH and GHRP-2-stimulated GH release. $*p < 0.05$ vs control; $**p < 0.01$ vs control; $^{\dagger}p < 0.05$ vs nonleptin treated with same stimulation of secretagogues.

and are upregulated in GHRH transgenic mice *(25)*. Because body mass index and several metabolic substrates have an effect on GH secretion *(26)*, a relationship between leptin and GH has been investigated in several species including human. The genetic *ob/ob* mouse has reduced plasma GH levels *(27)*. Intracerebroventricular administration of leptin antibodies reduces GH secretion in the rat *(28)*, and icv administration of leptin protects against the effects of restricted nutrition on GH secretion in the rat although this icv injection or infusion for 3 d did not change the GH levels in normally fed rats *(15,28)*. Most recently, however, it has been shown that a 7-d icv infusion (1.2 μg/d) increased both GH nadir levels and GH pulse amplitude in male rats *(13)*. These effects are most likely to be owing to action at the hypothalamic level. It has also been shown that 4-h treatment with leptin stimulated basal GH secretion and inhibited GHRH-stimulated GH secretion from pig pituitary cell cultures *(29)*. These in vitro data from the pig are similar to the observation reported here, but the leptin treatment in our experiments was much longer. The reported effects of an icv injection of leptin in pigs and infusion in rat are not in agreement with findings in sheep, in which no significant change in GH levels was found with icv leptin infusion *(30)*. In sheep, as in humans, plasma GH levels are increased when body weight is reduced and vice versa *(16)*. A similar relationship between body weight and plasma GH levels exists in humans *(17)*. Thus, one might expect that leptin would act on the pituitary somatotrophs to reduce GH secretion in sheep and humans. This appears to be true for GHRH-stimulated secretion from primary cultured ovine somatotrophs and may be synonymous with the reduced GH responses seen in obese individuals *(17)*.

The exact mechanism for the action of leptin on the pituitary gland is not clear, but NO and locally produced somatostatin may be involved. NO from folliculostellate cells has been shown to inhibit GHRH-induced GH secretion in rat pituitary cells *(31)*. It has also been reported that somatostatin is produced in the pituitary gland, and this may be related to basal and GHRH-induced GH secretion *(32)*. Because the present study was done in somatotroph-enriched (60–80%) cell cultures, the influence of these factors was most likely of minor importance. The present data are also consistent with the fact that GH levels are negatively correlated with leptin levels in human *(33)*.

The nature of the GH response to leptin (long-term rather than short-term) is consistent with the report that long-term but not short-term leptin treatment also inhibits somatostatin mRNA levels in fetal rat hypothalamic neurons and secretion of somatostatin from these neurons *(34)*. Such a reduction in somatostatin levels in rat by leptin infusion may partially mediate the GH-releasing activity of leptin *(13)*. In the present study, we examined the effects of *ro*-leptin on the levels of GHRH-R, GHRP-R, and GH mRNAs in ovine somatotroph-enriched cell cultures. GHRH-R and GH mRNAs were significantly reduced by the treatment of leptin, and such a reduction in both GHRH-R and GH mRNAs was time and dose dependent. One-day treatment of cells with only a high dose of leptin (10^{-7} *M*) significantly reduced mRNA levels encoding GHRH-R and GH, whereas 3-d treatment of cells with as low as 10^{-9} *M* ovine leptin significantly reduced the levels of GHRH-R and GH mRNAs. Theses results supply a logical explanation for the inhibitory effect of leptin on GHRH-stimulated GH secretion observed in ovine somatotrophs. It is therefore possible that reduced GH levels in obese patients may be owing at least partially to the reduction in the synthesis of GHRH-R and GH in somatotrophs. This also supports reports by others that the GH response to GHRH is decreased in obese men and women *(7,8)*. The degree of hyperleptinemia in obese individuals has been directly correlated with the extent of obesity and the reduction in GH levels *(35,36)*. GHRP-R mRNA levels in cultured ovine somatotrophs, however, were increased by the treatment of cells with *ro*-leptin. We also observed a slight increase in GHRP-2-stimulated GH secretion. The GH levels in incubation medium with a combined treatment of GHRH and GHRP-2 were not changed after 3-d leptin treatment. The physiologic or pathophysiologic roles of this increase in GHRP-R synthesis are not yet clear. This elevation in GHRP-R mRNA by leptin, however, suggests that GHRP may have a different function on the regulation of GH secretion to GHRH. Interestingly, to note a possible use of GHRP clinically in obese patients when a poor GH release to exogenous GHRH is obtained. We also noted in our experiments that the levels of GHRH-R and GHRP-R mRNA in cultured ovine somatotrophs were oppositely regulated by not only leptin but also the long-term treatment of GHRH or GHRP-2 (unpublished data).

The normal range of leptin in human plasma appears to be 5–20 ng/mL and is up to 600 ng/mL in obese individuals *(37,38)*. The doses of leptin used in the present study

(1–100 n*M*; 16 ng/mL to 1.6 μg/mL) were in the range of the plasma leptin concentration in obese patients. Leptin is secreted in pulses and in a circadian rhythm, with peak levels occurring shortly after midnight *(39)*, and leptin peak value may be higher than that observed so far. It is also possible that the local concentration of leptin may be higher in portal blood. Metabolic factors such as increased serum concentrations of insulin, free insulin-like growth factor-1, and free fatty acids may also influence GH secretion at both pituitary and hypothalamic levels *(40,41)*. Leptin may nevertheless be an important factor to blunt GH secretion in obesity.

In summary, the present data provide evidence for a direct action of leptin on pituitary somatotrophs to reduce GH and GHRH-R synthesis, leading to a reduction in GHRH-stimulated GH secretion. Leptin also increase GHRP-R synthesis in somatotrophs, suggesting a possible pharmaceutical use of GHRP in obese patients.

Materials and Methods

Ovine Pituitary Cell Preparation

Dispersed ovine pituitary cells were subjected to Percoll gradient centrifugation to enrich the somatotroph population to 60–80% of cells *(42)*. The cells were then cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% sheep serum and 2% fetal calf serum in 48-well culture dishes (1 to 2×10^5 cells/well), and the culture medium was changed every 2 to 3 d.

Leptin Treatment

Short-term treatment with leptin was performed on 4 to 5-d-old cultures. The culture medium was changed to incubation medium (DMEM with 0.5% bovine serum albumin) containing leptin (10^{-9}, 10^{-8}, 10^{-7}*M*) for 30 min prior to the addition of GHRH for a further 30 min. The incubation medium was then collected for GH radioimmunoassay (RIA).

In the long-term experiments, the cells were cultured for 3-4 days and then treated with leptin (10^{-9} to 10^{-7}M) for 24h or 72h. Culture medium was then changed to incubation medium for hormone assay under the challenge of GHRH for 30 min. The incubation medium was again collected for GH RIA. The total RNA from cultured cells after leptin treatment was extracted for GH, GHRH-R, and GHRP-R mRNA assays.

Semiquantitative RT-PCR

RT followed by PCR amplification was employed to detect ovine GH, GHRH-R, and GHRP-R mRNA. One microgram of total RNA sample extracted from each cell culture well was treated with deoxyribonuclease I to eliminate contamination of genomic DNA. RNA was reverse transcribed in a 20-μL RT reaction system containing random primers and AMV-RT. RT incubations were performed at 46ºC. Two microliters from the generated cDNA was used for subsequent PCR amplification. Ovine GH sequences are obtained from GeneBank (accession no. X15976). Ovine GHRH-R sequences were supplied by Dr. Gaylinn in Prof. Thorner's laboratory (University of Virginia Medical Center). Based on the conserved region from human, pig, and rat GHRP-R sequences, we have partially cloned the ovine GHRP-R (Genebank accession no. AF118636). The housekeeping gene GAPDH was used as internal control. For GH mRNA, we designed sense and antisense primers as follows: sense primer: 5'-CCTGCTCCTGGCTTTCACCCT-3'; antisense primer: 5'-ATCTTCCAGCTCCCGCATCAG-3'. For GHRH-R mRNA, we designed sense and antisense primers as follows: sense primer: 5'-GCCCGCCTTTCTTCTCTCAC-3'; and antisense primer: 5'-CTGGGCAATGTGGAGGCTAAG-3'. For GHRP-R mRNA, we designed sense and antisense primers as following: sense primer: 5'-ACCTCCTCTGCAAA CTCTTCC3'; and antisense primer: 5'-CACCCGGTACT TC TTGGACAT-3'. RT-PCR was performed as previously described *(43)*. Twenty-eight cycles of PCR amplification with 66ºC annealing temperature were used for GHRH-R, 32 cycles with 62ºC annealing temperature for GHRP-R, and 30 cycles with 60ºC annealing temperature for GH. GH, GHRH-R, and GHRP-R were coamplified with GAPDH. Twenty microliters of PCR products were run on a 2% agarose gel stained with ethidium bromide. The gel image was analyzed using National Institutes of Health image software, whose band intensity is expressed in pixels. The relative levels of ovine GH, GHRH-R, GHRP-R, and GAPDH mRNA were calculated for the intensity of ovine GH, GHRH-R, and GHRP-R corrected by GAPDH band, respectively, on each lane of the gel. The data shown in Figs. 4–6 represent the corrected density.

Hormone Assays

Ovine GH concentration in incubation medium was assayed by double-antibody RIA using materials supplied by the National Hormone and Pituitary Program of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Statistical Analyses

The data shown are means (±SEM) of three to four experiments. Comparisons were made between treatment groups using student's unpaired *t*-test. Data on percentage changes were analyzed using the Kruskal-Wallis H-test.

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