

RECOMBINANT PORCINE LEPTIN REDUCES FEED INTAKE AND STIMULATES GROWTH HORMONE SECRETION IN SWINE

C.R. Barb,^{*1} X. Yan,^{**} M.J. Azain,^{***} R.R. Kraeling,^{*} G.B. Rampacek,^{***}
and T.G. Ramsay^{**}

^{*}USDA-ARS, Russell Agriculture Research Center, Athens, GA 30604,

^{**}Pennington Biomedical Research Center, Baton Rouge, LA 70808, and

^{***}University of Georgia, Athens GA 30602

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Two experiments (EXP) were conducted to test the hypothesis that porcine leptin affects GH, insulin-like growth factor-I (IGF-I), insulin, thyroxine (T_4) secretion, and feed intake. In EXP I, prepuberal gilts received intracerebroventricular (ICV) leptin injections. Blood was collected every 15 min for 4 hr before and 3 hr after ICV injections of 0.9% saline (S; $n = 3$), 10 μg ($n = 4$), 50 μg ($n = 4$), or 100 μg ($n = 4$) of leptin in S. Pigs were fed each day at 0800 and 1700 hr over a 2-wk period before the EXP. On the day of the EXP, pigs were fed at 0800 hr and blood sampling started at 0900 h. After the last sample was collected, feeders were placed in all pens. Feed intake was monitored at 4, 20, and 44 hr after feed presentation. In EXP II, pituitary cells from prepuberal gilts were studied in primary culture to determine if leptin affects GH secretion at the level of the pituitary. On Day 4 of culture, 10^5 cells/well were challenged with 10^{-12} , 10^{-10} , 10^{-8} , or 10^{-6} M [Ala¹⁵]-h growth hormone-releasing factor-(1-29)NH₂ (GRF), 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M leptin individually or in combinations with 10^{-8} and 10^{-6} M GRF. Secreted GH was measured at 4 hr after treatment. In EXP I, before injection, serum GH concentrations were similar. Serum GH concentrations increased ($P < 0.01$) after injection of 10 μg (21 ± 1 ng/ml), 50 μg (9 ± 1 ng/ml), and 100 μg (13 ± 1 ng/ml) of leptin compared with S (1 ± 2 ng/ml) treated pigs. The GH response to leptin was greater ($P < 0.001$) in 10 μg than 50 or 100 μg leptin-treated pigs. By 20 hr the 10, 50, and 100 μg doses of leptin reduced feed intake by 53% ($P < 0.08$), 76%, and 90% ($P < 0.05$), respectively, compared with S pigs. Serum IGF-I, insulin, T_4 , glucose, and free fatty acids were unaffected by leptin treatment. In EXP II, relative to control (31 ± 2 ng/well), 10^{-10} , 10^{-8} , and 10^{-6} M GRF increased ($P < 0.01$) GH secretion by 131%, 156%, and 170%, respectively. Only 10^{-6} M and 10^{-7} M leptin increased ($P < 0.01$) GH secretion. Addition of 10^{-11} and 10^{-9} M leptin in combination with 10^{-6} M GRF or 10^{-11} M leptin in combination with 10^{-8} M GRF-suppressed ($P < 0.05$) GH secretion. These results indicate that leptin modulates GH secretion and, as shown in other species, leptin suppressed feed intake in the pig. © Elsevier Science Inc. 1998

INTRODUCTION

The recently discovered protein, leptin, suppresses feed intake and stimulates metabolic rate and reproductive function in ob/ob genetically obese mice (1,2). Leptin receptors are expressed in the ventromedial and arcuate regions of the hypothalamus, pituitary, adipose tissue, ovary, and some additional organs (3,4). The two primary regulators of growth hormone (GH) secretion, GH-releasing factor (GRF), and somatostatin (SS), are produced in the arcuate and ventromedial hypothalamus in the pig brain (5). These same areas are also involved in food intake regulation. Thus, leptin may regulate feed intake and growth

in swine. Perhaps alteration in leptin secretion and/or receptors in growing swine could be used to enhance feed intake and growth. Porcine leptin was recently cloned and synthesized (Ramsay and coworkers, unpublished observations). Therefore, two experiments (EXP) were conducted to test the hypothesis that leptin modulates feed intake, GH, insulin-like growth factor-I (IGF-I), insulin, and thyroxine (T_4) secretion in the pig.

MATERIALS AND METHODS

Experiment (EXP) I. Eight crossbred prepuberal gilts, 80.6 ± 2.7 kg body weight (BW) and 150 d of age were implanted surgically with lateral intracerebroventricular (ICV) cannulas using the stereotaxic procedure of Estienne et al. (6) and Barb et al. (7). Animals were individually penned in an environmentally controlled building and exposed to a constant temperature of 22°C and artificial 12:12-hr light:dark photoperiod. Pigs were meal-fed daily at 0800 and 1700 hr a corn-soybean meal ration (14% crude protein) supplemented with vitamins and minerals, according to the National Research Council guidelines (8). One week after the last ICV surgery, all pigs were fitted with an indwelling jugular vein cannulae (9) 24 hr before treatment. On the day of the EXP, pigs were fed at 0800 hr and blood sampling started at 0900 hr. Blood samples were collected every 15 min for 4 hr before and 3 hr after ICV injections of 150 μl 0.9% saline (S), or 10, 50, or 100 μg of recombinant porcine leptin in 150 μl of S. One week later, the EXP was repeated with pigs reassigned to treatment such that no pig received the same dose as before, resulting in four pigs/dose of leptin or S. One S animal was eliminated from the study because of loss of patency of the jugular cannula. Serum was harvested and stored at -20°C until assayed for GH by radioimmunoassay (RIA). Hourly samples were assayed for IGF-I, T_4 , insulin, glucose, and free fatty acids (FFA).

Feed Intake. After the last sample was collected, feeders were placed in all pens and feed intake monitored at 4, 20, and 44 hr after feed presentation.

Expression and purification of the recombinant porcine leptin. Porcine leptin cDNA sequence representing the secreted porcine protein (amino acids 22 to 167) was amplified by PCR from a porcine leptin cDNA containing plasmid (PCR2.1) (Invitrogen, Carlsbad, CA) harboring the complete coding region (Ramsay et al., Genbank Accession No. U59894). The cDNA was subcloned in-frame into a procaryotic expression vector pGEX-2T (Pharmacia Biotech, Piscataway, NJ) and confirmed by DNA sequencing. The DNA construct was then transformed and overexpressed in *Escherichia coli* (JM109) after induction by IPTG. A crude protein extract containing the GST-leptin fusion protein was prepared by conducting sonification and precipitation. Sarkosyl and Triton X-100 were added to aid solubilization of the protein. Filtered protein extract was loaded onto a prepacked Glutathione Sepharose 4B column and washed with 30-bed volumes of PBS. The column was then incubated with thrombin solution (20 NIH units per liter culture in cleavage buffer, 20 mM Tris-HCl, pH 7.9, 140 mM NaCl, 10% Glycerol, 2.5 mM CaCl_2) according to the manufacturer's recommendations. Recombinant leptin protein was finally recovered in the flow through and the subsequent washes. Refolding of the protein was achieved by denaturing the protein in 4 M urea and dialyzing it successively against 3, 2, 1, and 0.5 M urea and three changes of PBS. Each dialysis step was performed for 12 hr in 10,000 MW cutoff tubing at 4°C against 50 volumes of solution. Purity of the recombinant protein was estimated by staining a SDS-PAGE gel with Coomassie Blue. Recombinant leptin was the only band detectable on the gel.

EXP II. Pituitary cells were studied in primary culture to determine if leptin affects GH secretion at the level of the pituitary. Two studies were conducted with six pituitary glands/study from crossbred prepuberal gilts 180 d of age and weighing approximately 105 kg. Pituitary glands were removed aseptically and ovaries were examined at slaughter.

Gilts were considered prepuberal because their ovaries were devoid of corpora albicantia and corpora lutea. All subsequent procedures were performed under sterile conditions. The anterior lobe was dissected from each pituitary gland, cells were enzymatically dispersed and cultured as described previously (10). Briefly, after cells were dispersed and centrifuged, the cell pellet was resuspended in growth medium (Dulbecco's Modified Eagle's medium [DME] Ham's Nutrient mixture F-12 [F-12]) 90% [vol/vol; 4,150 mg/l glucose], and 10% [vol/vol] Fitton-Jackson medium [BGJb] (Gibco, Grand Island, NY) containing 2 mg/ml of BSA, 10 mM HEPES [pH 7.2], 100 U/ml of penicillin, 250 ng/ml of amphotericin B (Sigma Chemical Company, St. Louis, MO), and 100 μ g/ml of streptomycin to which was added 2% [vol/vol] fetal bovine serum (Pharmacia) supplemented with 100 ng/ml of cortisol, 1.0 ng/ml of human insulin, 10 μ g/ml of human transferrin (Sigma), 10 pg/ml of glucagon, 100 pg/ml of epidermal growth factor, 200 pg/ml of bovine parathyroid hormone (PTH), 400 pg/ml of triiodothyronine (Sigma), and 200 pg/ml of fibroblast growth factor (Collaborative Research, Bedford, MA). Cell viability and number were assessed by counting cells which excluded trypan blue on a hemocytometer. Culture medium was changed on Day 3 (day of seeding = Day 0 of culture) and replaced with serum-free growth medium as described before, except using DME containing 1,000 mg of glucose/l (Sigma). On Day 4 of culture, medium was discarded, plates were rinsed twice with serum-free medium, and 10^5 pituitary cells/well were cultured in 1 ml fresh medium without serum. Cells were challenged with 10^{-12} , 10^{-10} , 10^{-8} , or 10^{-6} M [Ala¹⁵]-h GRF-(1-29)NH₂, 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M leptin individually or in combinations with 10^{-8} and 10^{-6} M GRF. Cells were exposed to treatment for 4 hr, at which time the medium was harvested and quantified for GH. There were six wells per treatment per study.

Metabolite and Hormone Assays. Serum and media concentrations of GH were quantified by RIA (11). Assay sensitivity was 0.4 ng/ml. Intra- and inter-assay coefficients of variation were 3.2% and 13.6%, respectively. Serum IGF-I concentrations were quantified RIA (12). Assay sensitivity was 0.01 ng/ml. Intra- and interassay coefficients of variation were 3.2% and 3.5%, respectively. Total serum T₄ and insulin concentrations were quantified by RIA Kits (INCSTAR Corporation, Stillwater, MN). Samples were assayed for glucose using a glucose oxidase kit (Sigma) and FFA using a colorimetric assay kit (Wako Chemicals USA, Inc., Richmond, VA).

Statistical Analysis. To determine the effect of leptin on serum GH, IGF-I, T₄, glucose, FFA, and insulin concentrations across time and feed intake in EXP I, data were subjected to the general linear model split plot-in-time ANOVA procedure of the Statistical Analysis System (13). The statistical model included dose, pig, time, and replicate. Effects of dose and replicate were tested using replicate \times dose as the error term. Replicate \times dose was tested using pig within dose \times replicate as the error term. Time and time \times dose were tested using dose \times time \times replicate as the error term. Differences between treatment means within a time were determined by least-squares contrasts (13).

In EXP II, data were converted to percentage of basal secretion before averaging to minimize differences between replicates. To obtain an estimate of variation between control wells, medium GH concentrations for control wells were converted to a percentage of mean basal GH concentration. This was then used to calculate a SE for basal secretion. Basal secretion (control; C) was the amount of hormone secreted into the culture medium per 10^5 cells seeded/well in the absence of a secretagogue. Converted data were tested for homogeneity of variance using Hartley's F max test (14). Data were then subjected to a one way ANOVA and differences between means were determined by least-squares contrast (13).

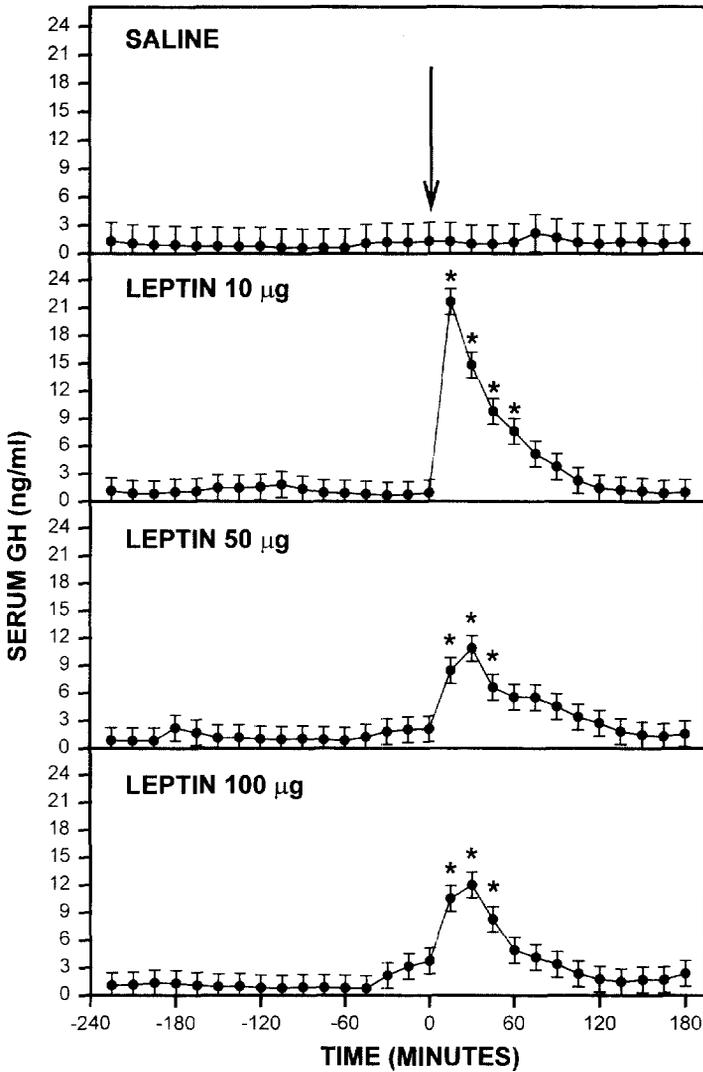


Figure 1. Serum GH concentrations (mean \pm SE) for pigs receiving ICV injection of saline ($n = 3$), 10 μ g ($n = 4$), 50 μ g ($n = 4$), or 100 μ g ($n = 4$) of leptin at time 0. Times at which effects of treatment were different from saline-treated animals are indicated * ($P < 0.01$).

RESULTS

EXP I. Before injection, serum GH concentrations were similar ($P > 0.1$) among groups and averaged 1.6 ± 1.5 ng/ml. Serum GH concentrations increased ($P < 0.01$) by 15 min after injection of 10 μ g (21 ± 1 ng/ml), 50 μ g (9 ± 1 ng/ml), and 100 μ g (13 ± 1 ng/ml) of leptin, compared with S (1 ± 2 ng/ml) treated pigs (Figure 1). The GH response to leptin was greater ($P < 0.001$) by 15 min after 10 μ g than after 50 or 100 μ g leptin. At 4 hr, feed intake was similar among groups. By 20 hr the 10, 50, and 100 μ g doses of leptin reduced feed intake by 53% ($P < 0.08$), 76%, and 90% ($P < 0.05$), respectively, compared with S pigs. By 44 hr, feed intake remained suppressed ($P < 0.05$) and averaged 62%, 66%, and 50% of control animals for the 10, 50, and 100 μ g doses of leptin, respectively (Figure 2). Serum IGF-I and T_4 were unaffected by leptin treatment

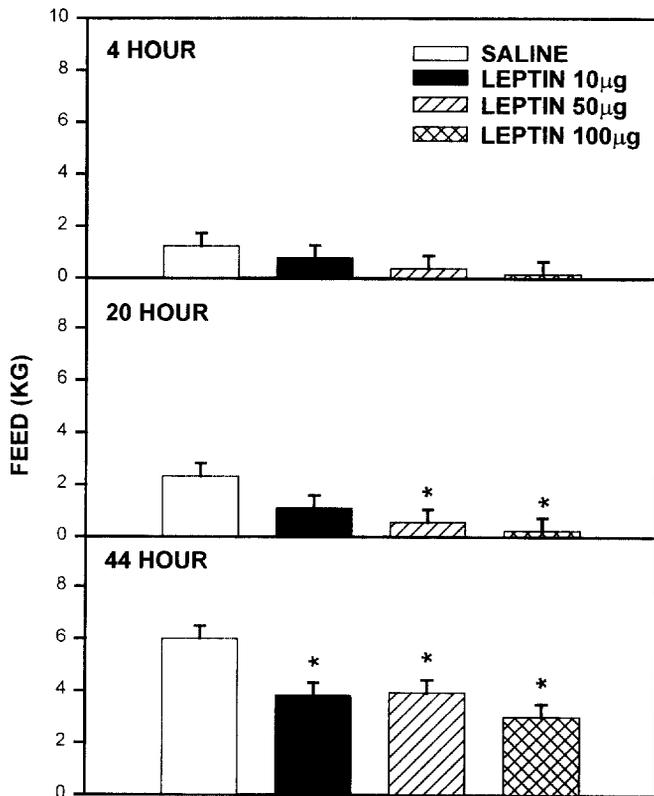


Figure 2. Cumulative feed intake (mean \pm SE) for pigs receiving ICV injection of saline ($n = 3$), 10 μ g ($n = 4$), 50 μ g ($n = 4$), or 100 μ g ($n = 4$) of leptin. Feed intake was monitored at 4, 20, and 44 hr after feed presentation. Times at which effects of treatment were different from saline-treated animals are indicated * ($P < 0.05$).

and averaged 63 ± 4 ng/ml and 43 ± 3 ng/ml, respectively, among the groups. Serum insulin concentration were similar among groups and averaged 26 ± 3 μ U/ml before treatment and 29 ± 4 μ U/ml by 1 hr after treatment. Serum glucose concentrations were similar among groups and averaged 89 ± 11 mg/dl. Serum FFA levels were unaffected by treatment and ranged from 141 ± 25 to 323 ± 29 μ Eq/l throughout the blood sampling period.

EXP II. Basal GH secretion (control; $n = 12$ wells) was 31 ± 2 ng/well. Relative to control at 4 hr, 10^{-10} , 10^{-8} , and 10^{-6} M GRF increased ($P < 0.01$) GH secretion by 131%, 156%, and 170%, respectively. Only 10^{-6} M (143%) and 10^{-7} M (147%) leptin increased ($P < 0.01$) GH secretion (Figure 3). Addition of 10^{-11} and 10^{-9} M leptin in combination with 10^{-6} M GRF or 10^{-11} M in combination with 10^{-8} M GRF suppressed ($P < 0.05$) GH secretion (Figure 4).

DISCUSSION

The discovery of the ob gene and anti-obesity effects of leptin seems to be a breakthrough in understanding the role adipose tissue plays in regulating food intake, body weight, and endocrine function. The intrinsic mechanisms through which leptin exerts its effects are poorly understood. It is currently hypothesized that leptin acts at the brain to reduce food intake, increase energy expenditure and alter endocrine activity (15,16). Leptin receptors are present in different hypothalamic nuclei that are involved in regu-

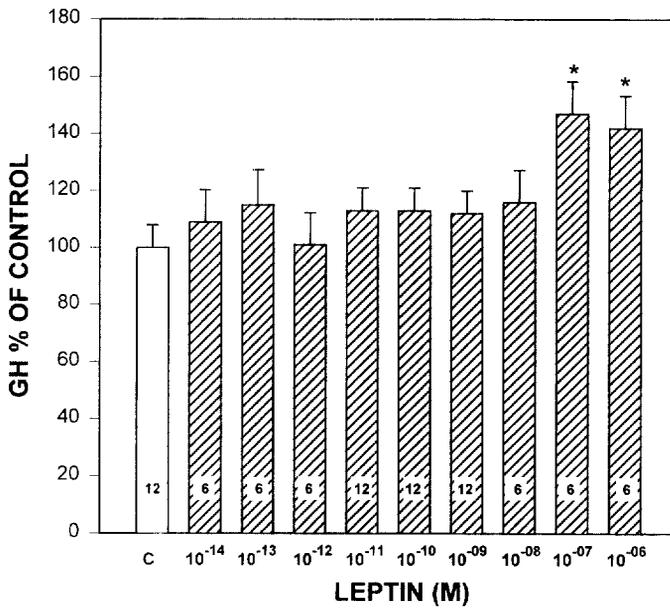


Figure 3. Effects of leptin on basal GH secretion. Values are the means ± SE for the two studies. Numbers in columns = number of wells per treatment. Control (C) = basal secretion in the absence of treatment. *Different from C (P < 0.01).

lating pituitary hormone secretion (3), and several reports have demonstrated the effects of leptin administration on the hypothalamic pituitary axis. Leptin treatment increased plasma concentrations of LH, FSH, and testosterone in fasted mice as well as ob/ob mice (1,16,17). Lastly, a direct effect of leptin on LH, FSH, and prolactin release from rat

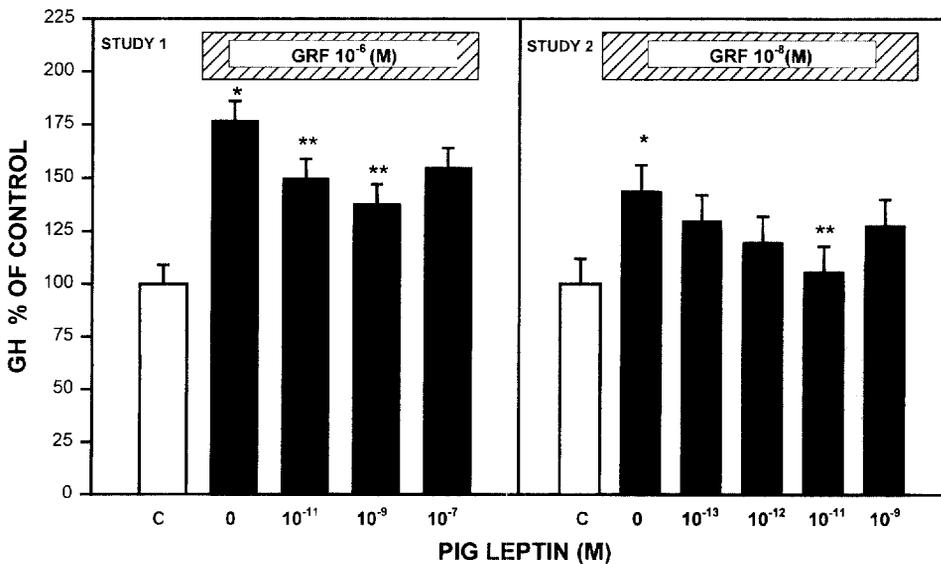


Figure 4. Interaction of leptin with GRF on GH secretion. Values are the means ± SE for each study (n = 6 wells/treatment). Control (C) = basal secretion in the absence of treatment. *Different from C (P < 0.01). **Different from GRF alone (P < 0.05).

pituitaries in culture was reported (17). Collectively, these data indicate that leptin plays a role in regulating the hypothalamic pituitary axis as well as food intake.

The thyroid axis is also influenced by leptin. Twice daily administration of leptin for 48 hr increased serum T_4 levels in fasted mice compared with fed controls (16). In addition, ICV leptin administration for 4 d in normal fed rats decreased serum T_4 levels and increased serum triiodothyronine concentrations (Azain et al., 1997 unpublished observations). The failure of leptin treatment to alter T_4 secretion in the current study may, in part, be related to species differences, nutritional status, and/or acute versus chronic treatment. Perhaps serum leptin levels must exceed or fall below a critical threshold before change in the neuroendocrine axis can be initiated.

In the present study ICV administration of leptin resulted in a marked increase in GH secretion in normal fed pigs with maximum concentrations occurring at 15–30 min post-ICV injection, similar to GH response to exogenously administered GRF (18). This is in contrast to a recent report by Carro et al. (19) in which central administration of leptin failed to increase GH secretion in normal fed adult male rats, but did reverse inhibition of GH secretion exerted by fasting. This paradox between the studies may in part be related to species, age, and/or sex differences. For example, in the rat fasting suppressed GH levels (20), whereas in the pig fasting elevated serum GH concentrations (21). Moreover, age and sex have a profound influence on the pattern of GH secretion in the rat and pig (22–24). We suggest that stimulation of GH secretion by leptin is attributable to a reduction in hypothalamic NPY release and a concomitant increase in GRF and/or decrease in SS release. In support of this idea, hypothalamic NPY release is stimulated by food deprivation in rats (25). Leptin receptors are present on NPY neurons in the hypothalamus (26) and immunocytochemical evidence demonstrated synaptic connection between NPY neuronal projections and SS neurons (25). Central administration of NPY inhibited GH secretion and stimulated SS release from hypothalamic tissue in vitro (27).

This is the first report demonstrating a direct effect of leptin on pituitary GH secretion. Leptin receptors were demonstrated in the pituitary of sheep (4), suggesting a physiological role for leptin in regulating GH secretion. However, only the 10^{-7} and 10^{-6} M leptin increased GH secretion in vitro in this study. These doses could be considered supra physiological and may not be physiologically relevant. More importantly leptin at doses considered to be physiological, suppressed GRF induced GH secretion. Yu et al. (17) reported that leptin increased FSH and LH secretion from the rat anterior pituitary in vitro. Taken together these data suggest the presence of leptin receptors on the secretory cells of the anterior pituitary. Further work is needed to elucidate the physiological role of leptin in modulating pituitary function.

The demonstration that recombinant porcine leptin reduced feed intake in a dose-dependent manner provides further evidence for the hypothesis that adipose tissue secretes a protein signal that acts on the central nervous system to regulate feed intake (2,28). The action of leptin on feed intake was still apparent at 44 hr after feed presentation. Similar studies in rodents showed that leptin injection into the lateral or third ventricle reduced food intake for up to 24 hr (29,30). These data strongly imply that leptin acts directly within the central nervous system.

The GH response to leptin, in the present study, was greater for the 10 μ g dose whereas, for feed intake the response was greater for the 100 μ g dose. This dichotomy, in part, may be attributable to separate neural pathway(s) involved in GH secretion and food intake regulation. Leptin acts to reduce the activity of arcuate NPY neurons and decrease release of NPY in the paraventricular nucleus and other brain locations involved in feed intake regulation (31). Growth hormone-releasing factor, SS (5), and NPY are produced primarily in the arcuate nucleus and ventromedial hypothalamus in the pig (32). More-

over, leptin and NPY may also affect other types of neurons (33), such as those modulating the sympathetic nervous system. Therefore, it is conceivable that leptin acts by different neural pathways to inhibit food intake and stimulate GH secretion.

It is well established that the anabolic activity of GH is mediated by serum IGF-I and IGF-I concentrations are GH dependent (34). It was hypothesized that serum IGF-I concentrations would increase after the leptin-induced increase in GH secretion. The lack of an increase in serum IGF-I concentrations after leptin treatment may be related to the length of the blood sampling period. Sillence and Etherton (35) reported a 4–6 hr lag before serum IGF-I concentrations began to rise after exogenous GH administration. Similar findings have been reported in humans (36). Thus, since blood samples were only collected for 3 hr after leptin treatment, the GH induced increase in serum IGF-I levels would have occurred after the blood sampling period.

Understanding the mechanisms that regulate food intake is of great relevance in the rapidly growing pig. Swine do not eat to gut fill, but stop eating based on other signals and behavioral mechanisms (37). Growth studies demonstrated that these animals have not reached their genetic potential for lean deposition. Therefore, understanding the leptin/GH axis is imperative to develop new methods to promote maximal growth and muscle accretion.

We have shown here that central administration of leptin inhibited feed intake and stimulated GH secretion and altered pituitary response to GRF. This is consistent with the idea that leptin is an important link between metabolic status, neuroendocrine system, and the growth process. How leptin achieves this link is poorly understood. A more detailed examination of the physiological role of leptin in regulating growth in the pig will be dependent on monitoring metabolic hormones that are influenced by leptin and by measuring circulating concentrations of leptin and associated changes in the GRF/GH axis.

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¹Corresponding author: Dr. C. Richard Barb, USDA, ARS, Animal Physiology, R.B. Russell Research Center, P.O. Box 5677, Athens, GA 30604-5677.

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