

Peripheral leptin administration alters hormone and metabolite levels in the young pig[☆]

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Abstract

The present study was conducted to determine if peripheral leptin administration can alter GH secretion or feed intake in young pigs. Six, 6 kg female pigs were fasted overnight and randomly chosen to receive porcine recombinant leptin or saline injections in a crossover design. Three leptin dosages were tested over a 10 day period, 100, 200 or 500 µg/kg body mass (L100, L200 or L500). Leptin was administered in 0.2% bovine serum albumin as a bolus injection into the carotid artery. Blood samples were obtained from the jugular vein over a 24 h period. Leptin delayed feeding in pigs treated with L200 and L500 ($P < 0.05$), while reducing overall intake in pigs treated with L100 ($P < 0.05$). L200 or L500 depressed blood glucose ($P < 0.05$). Plasma insulin levels were elevated by feeding in control animals, while insulin levels were depressed in pigs treated with L200 or L500 ($P < 0.05$). L200 elevated plasma growth hormone ($P < 0.05$) with three peaks apparent at 5, 8, and 13 h post injection. The ability for a single injection of leptin to produce significant changes in hormone and metabolite levels suggests that this peptide has a role in regulation of peripheral metabolism.

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1. Introduction

Central administration of leptin to the pig has been demonstrated to reduce feed intake and stimulate growth hormone (GH) secretion (Barb et al., 1998). Central administration of leptin to the sheep or rat has also produced similar responses, as well as changes in peripheral metabolism (Tannenbaum et al., 1998; Vuagnat et al., 1998; Henry et al., 2001; Morrison et al., 2001). Leptin has been proposed to bind to receptors in the hypothalamus to stimulate growth hormone releasing factor (GRF) release and subsequent stimulation of GH release (Barb et al., 2002). However, leptin has not yet been shown to be synthesized in brain tissue. Rather, stimulation of GRF release and somatostatin inhibition appear to be the result of transport of peripheral leptin across the blood brain

barrier with subsequent binding to hypothalamic leptin receptors (Banks et al., 1996; Golden et al., 1997; Thomas et al., 2001). Various transport mechanisms have been suggested, but at this time there has been no demonstration that leptin can cross the blood brain barrier in the pig (Barb et al., 2001). Ajuwon et al. (2003a,b) have recently reported that a recombinant human leptin analogue can elevate serum non-esterified fatty acids (NEFA), but no evidence for a response by central regulatory mechanisms was presented. Therefore, it is unknown whether peripheral leptin administration can alter central GH secretion or peripheral metabolism. The present study was conducted to determine if peripheral leptin administration can alter GH secretion or feed intake in the pig. The release of GH should have peripheral effects on metabolism, but this has yet to be documented for the pig.

2. Methodology

In vivo analysis of the mechanism of action of leptin is limited by availability of recombinant hormone. Recombi-

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nant leptin is not commercially available in quantity at a price appropriate to permit *in vivo* use. Recombinant porcine leptin was prepared and acquired from Dr. Arieh Gertler (Raver et al., 2000) for this study. The relatively small quantity of recombinant hormone available limits the size of pig that can be treated, as the hormone is administered peripherally on a per kilogram body mass basis. Due to the limitation of available porcine leptin, size of the pigs treated was limited to 6–8 kg to permit peripheral administration.

2.1. Animal procedures

Six, 6 kg female pigs (*Sus scrofa*, Landrace × Yorkshire × Hampshire × Duroc), purchased from the Agriculture Headquarters at the Texas Department of Criminal Justice, Huntsville, TX, were housed in individual steel cages at the USDA/ARS Children's Nutrition Research Center. Pigs were weighed daily and the total daily feed provided was calculated daily at 6% of body mass in kg. Pigs were fed twice daily at 08:00 (3% body mass) and 15:00 h (3% body mass) a diet containing 24% crude protein (Producers Cooperative Association, Bryan, TX, USA). Water was continuously provided. Catheterization of the carotid artery and jugular vein was performed using sterile techniques after an overnight fast as previously described (Wray-Cahen et al., 1997). Catheters were externalized and securely placed in jacket pockets. Pigs were given intramuscular injections of antibiotics (enrofloxacin, 2.5–5.0 mg/kg) for 2 days following surgery. Pigs were allowed to recover overnight before resuming their normal dietary regimen; although water was continually provided.

Animals were fasted overnight beginning at 18:00 h, prior to beginning the experiment and water was continually provided. Pigs were again weighed at 05:00 and randomly assigned to receive either leptin or saline injections. The experiment was repeated with three different doses of leptin and with saline serving as the control in a crossover design. The doses of leptin given per pig were 100 µg/kg body mass (L100), 200 µg/kg body mass (L200), 500 µg/kg body mass (L500). The initial dosage selection was based upon peripheral leptin injection studies in dogs (LeBel et al., 1999) and sheep (Nagatani et al., 2000). The basic experimental design was as follows. A baseline venous sample was obtained at 08:00 h before injecting leptin in physiological saline with 0.2% bovine serum albumin in a bolus dose into the carotid artery. Control pigs received an equal volume dose of sterile saline in the carotid artery. Pigs remained in cages with water provided while venous blood samples were obtained over a 24 h period. Feed was presented to each pig at 1 h following leptin injection with subsequent re-weighing of remaining food every 2 h. Feed intake between survey times was determined by subtraction from initial feed presented. Total feed intake for the total experimental

period (24 h) could then be calculated by subtraction of total feed consumed from the initial quantity presented. Venous blood samples were obtained every 15 min for the first 4 h, every 1 h from 4 to 12 h post-injection, and every subsequent 2 h from 12 to 24 h post-injection. Three milliliter of venous whole blood was obtained at each time-point, centrifuged, and plasma allocated for later analysis of concentrations of pGH (0.5 ml), free fatty acids (0.2 ml), and insulin (0.5 ml). Whole blood glucose was determined immediately. Following the 24 h study period ending at 08:00 h, catheters were secured within the jacket pocket and pigs resumed their regular dietary regimen. At approximately 68 h after the previous experiment, pigs were fasted again overnight in preparation for the next dosage trial. The identical experimental protocol was repeated as described above. However, the three pigs that received the control injection (saline) previously were administered porcine recombinant leptin, while the remaining pigs received saline, according to the crossover design. The experimental protocol was approved by the Animal Use and Care Committee of Baylor College of Medicine. The study was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Hormone analysis

Porcine GH was determined by a homologous radioimmunoassay (RIA) for porcine GH, as previously described (Campbell et al., 1988). Within assay variation was 9.9% while inter-assay variation for porcine GH was 12.7% with a sensitivity of 0.05 ng/tube. Porcine insulin was analyzed using a homologous RIA kit (Linco Research, Chesterfield, MO, USA). Intra-assay CV was 8.6% while the inter-assay CV was 6.7% for the insulin RIA, with a sensitivity of 2 µU/ml. Plasma leptin was assayed using a heterologous RIA kit (Linco Research) to confirm that leptin administration was successful. Intra-assay CV was 3.2% while the inter-assay CV was 12.3% for the leptin RIA, with a sensitivity of 1 ng human Eq/ml.

2.3. Metabolite analysis

Whole blood glucose was determined using a glucose oxidase procedure with a YSI 2300 Stat Plus glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Plasma non-esterified free fatty acids were analyzed using a kit (WakoChemical Co., Richmond, VA, USA).

2.4. Statistical analysis

Data were analyzed using repeated measure two-way analysis of variance with the main effects of time and treatment (SigmaStat Software, SPSS Science, Chicago,

IL, USA). Comparisons were made within each dose of leptin administered and not among the doses. Mean separation was detected by Student-Newman-Keuls test. Significant differences were defined at the 95% confidence level.

3. Results

Fasted animals were presented with feed one hour after leptin or saline injections. Animals treated with saline responded to presentation of feed by starting to eat immediately. Leptin treatment at L100 did not affect the pattern of feeding behavior (Fig. 1a, $P > 0.05$); however, it reduced total intake from 571 ± 25 to 432 ± 45 g ($P < 0.009$) during the 24 h period of measurement, while producing a small depression in blood glucose between 120 and 210 min post-injection (Fig. 2a, $P < 0.0001$). Three days later in a cross-over design, a dosage of L200 was tested according to the same experimental design. Feeding was delayed in leptin-treated animals by several hours (Fig. 1b, $P < 0.008$); however, accumulated feed intake over the entire 24 h of the experiment was similar between the two groups (Fig. 1b, $P > 0.05$). Blood glucose was depressed in these leptin-treated animals during the first 4 h after leptin administration (Fig. 2b, $P < 0.0001$), but recovered to levels observed

in control animals by 5 h post leptin administration. Pigs treated with L500 showed no interest in feeding during the first 2 h following feed presentation (Fig. 1c, $P < 0.0001$). The experiment was terminated after the first 3 h of leptin treatment as whole blood glucose levels had dropped to less than 40 mg/dl and animals were beginning to display symptoms of hypoglycemic shock (Fig. 2c, $P < 0.0001$). The leptin treated animals were immediately treated with an intravenous bolus of glucose (10%) to overcome the hypoglycemia.

Despite random selection of animals for the two treatment groups (leptin or saline), plasma NEFA were always initially higher in those animals treated with leptin (Fig. 3a,b). There was no significant difference in plasma NEFA between control (saline) or leptin (L100) groups (data not presented, $P > 0.05$). Plasma NEFA declined with time following feeding from a peak of approximately 700 $\mu\text{Eq/l}$ to a nadir of 150 $\mu\text{Eq/l}$ approximately 300 min post-feed presentation. However, treatment with a moderate leptin dose (L200) resulted in an elevation in plasma NEFA following the presentation of feed, which did not decline until 5 h after leptin treatment (Fig. 3a, $P < 0.0001$). Following the presentation of food, plasma NEFA dropped in control animals while plasma NEFA remained elevated in animals treated with L500 (Fig. 3b, $P < 0.001$).

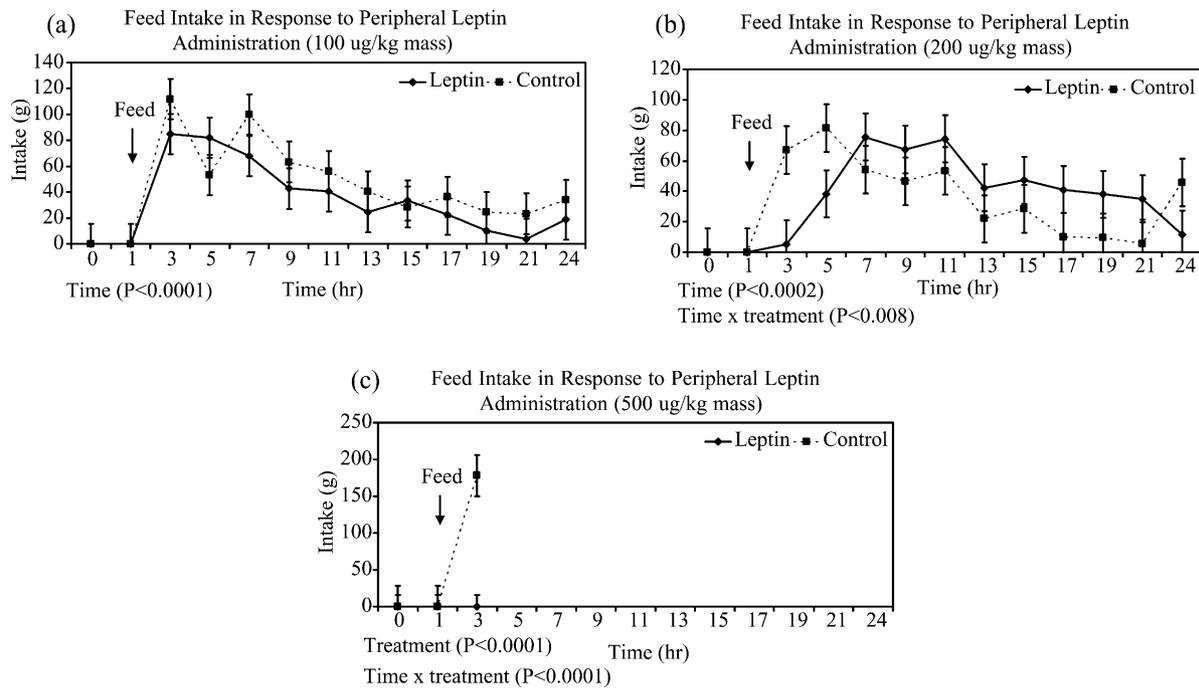


Fig. 1. Feed intake in female pigs in response to a single carotid injection of recombinant porcine leptin or saline. Feed was presented 1 h after leptin injection. Feed in the pen was weighed every 2 h and the difference calculated to determine the amount of feed consumed. Data are expressed as gram feed consumed between sampling time points ($n = 3$; mean \pm pooled S.E.M.). (a) 100 μg leptin/kg body mass dosage; significant effect of time ($P < 0.05$). (b) 200 μg leptin/kg body mass dosage; significant effect of time ($P < 0.05$), significant effect of treatment \times time ($P < 0.05$). (c) 500 μg leptin/kg body mass dosage; significant effect of treatment ($P < 0.05$). This experiment was terminated at 3 h post injection due to hypoglycemia of the pigs (see Fig. 2c). Thus, only one sampling point for feed intake was obtained.

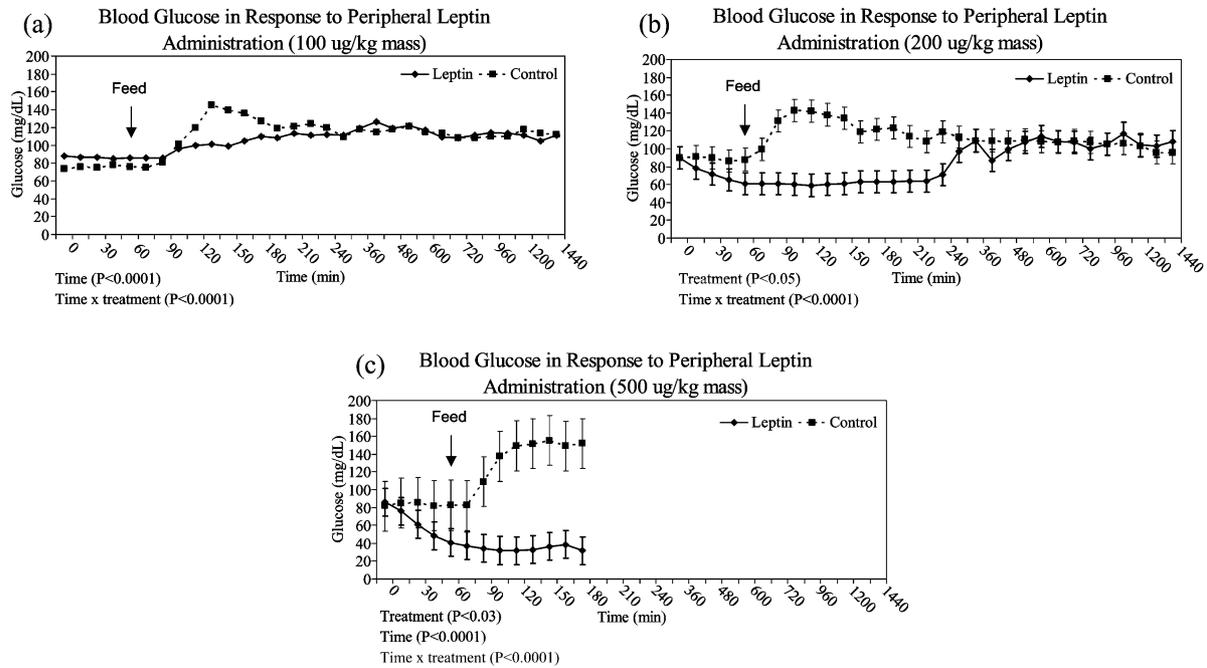


Fig. 2. Whole blood glucose concentration in female pigs in response to a single carotid injection of recombinant porcine leptin or saline. Data are expressed as milligram glucose per deciliter blood ($n = 3$; mean \pm pooled S.E.M.). (a) 100 μg leptin/kg body mass dosage; no significant effects ($P > 0.05$). (b) 200 μg leptin/kg body mass dosage; significant effect of treatment ($P < 0.05$), significant effect of treatment \times time ($P < 0.05$). (c) 500 μg leptin/kg body mass dosage; significant effect of treatment ($P < 0.05$), significant effect of time ($P < 0.05$), significant effect of treatment \times time ($P < 0.05$) (d).

Plasma insulin was unaffected by treatment of pigs with L100 (data not presented, $P > 0.05$). Treatment with L200 delayed the onset of an insulin response and that response was limited in comparison to animals treated with saline (Fig. 4a, $P < 0.003$). Plasma insulin levels were elevated by feeding in control animals, while insulin levels remained low during the first 3 h post injection in pigs treated with L500 (Fig. 4b, $P < 0.05$).

A heterologous RIA was used to confirm that the recombinant porcine leptin entered the blood stream in the injected pigs. Treatment with L100 produced a peak of 140 human Eq/ml by 15 min post-injection, declining rapidly by 45 min and clearance of the peptide by approximately 480 min post injection (Fig. 5). Treatment with L200 produced a similar curve to L100 with a peak at approximately 140 human Eq/ml (data not presented). Treatment with L500 resulted in plasma leptin levels peaking to 340 human Eq/ml and remained elevated throughout the first 3 h of the experiment (data not presented). The assay detected levels in the control animals, which ranged from 1.3 to 4.8 human Eq/ml across all three trials.

A leptin dose of L100 produced no significant difference in plasma growth hormone (data not presented, $P > 0.05$). Treatment with L200 produced a significant increase in overall GH level (Fig. 6a, $P < 0.05$) with three peaks apparent at 5, 8 and 13 h post injection. Plasma growth hormone was significantly higher in control animals ($P < 0.05$), increasing from approximately 2.5 to 10.5 ng/ml with feeding in control animals while animals

exposed to the high leptin dose showed no change in plasma GH (Fig. 6b).

4. Discussion

This is the first report of peripheral *in vivo* treatment of swine with leptin. The sampling population is small due to the limitations of recombinant protein synthesis. Young pigs were used also because of the limitation in availability of leptin.

Peripheral intravenous treatment of swine with 100 μg leptin/kg body mass produced a small change in overall intake but not the predicted inhibition or delay in feeding as observed by Barb et al. (1998) with intracerebroventricular administration. Although feed intake did not significantly differ during the first several hours after feed presentation, blood glucose levels were lower in leptin treated pigs. However, this might be ascribed to the lower level of feed intake (though not statistically different from control intake) between the second and third hour post injection, one hour after feeding.

The decision was then made to try a higher leptin dose to determine if an obvious hypophagia or delay in feeding could be induced. Feed intake was delayed and a relative hypoglycemia was apparent in pigs treated with 200 $\mu\text{g}/\text{kg}$ body mass, suggesting that the leptin may be crossing the blood brain barrier to affect the feeding centers in the hypothalamus, as reported for other species (Banks et al.,

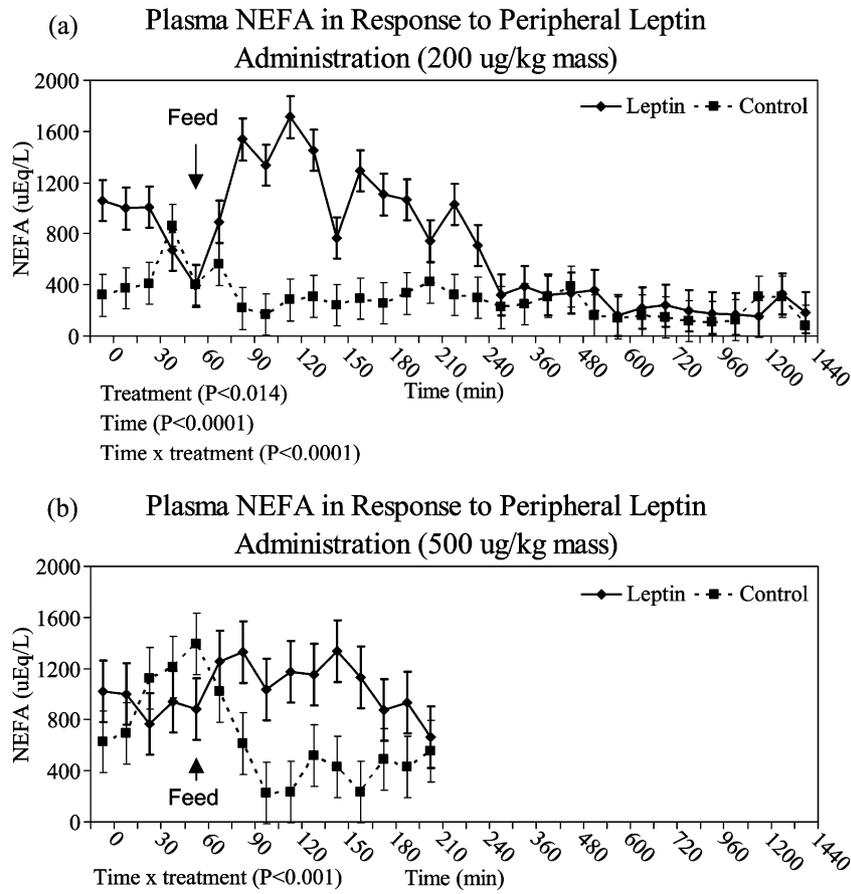


Fig. 3. Plasma non-esterified fatty acid concentration in female pigs in response to a single carotid injection of recombinant porcine leptin or saline. Data are expressed as microequivalents per liter ($n=3$; mean \pm pooled S.E.M.). (a) 200 μg leptin/kg body mass dosage; significant effect of time ($P<0.05$), significant effect of treatment \times time ($P<0.05$). (b) 500 μg leptin/kg body mass dosage; significant effect of treatment \times time ($P<0.05$).

1996; Golden et al., 1997; Thomas et al., 2001). The drop in blood glucose was apparent prior to feed presentation, therefore the response is likely a specific effect of the leptin treatment, as saline injected controls did not experience the drop in blood glucose despite a similar fast. While the leptin-treated animals continued to experience a decline in blood glucose after presentation of the feed, the control animals produced a rapid increase in blood glucose in accompaniment with feeding. Ajuwon et al., (2003b) did not observe a decline in blood glucose with pigs chronically treated with a human leptin analogue, although this might have been due to sampling at only one time point during the day. The present data demonstrate the blood glucose returns to control levels within 4 h of leptin injection.

Treatment with 500 $\mu\text{g}/\text{kg}$ body mass was predicted to be efficacious based upon studies in the sheep (Nagatani et al., 2000). However, the severe hypoglycemic response was unexpected as this was not reported with ICV (intracerebroventricular) administration to the pig (Barb et al., 1998). Again a decline in blood glucose was detected prior to feeding leptin treated swine. Concern for the welfare of the pigs terminated this experiment at approximately 3 h, with infusion of glucose.

The insulin response to feed intake and the changes in blood glucose were significantly altered by leptin administration. The low leptin dose (100 $\mu\text{g}/\text{kg}$ body mass) did not alter insulin secretion, as expected from the limited changes in blood glucose and feed responses in this treatment group. Paralleling the hypophagia and hypoglycemic response, insulin levels were unchanged in the pigs treated with the moderate or high leptin dose (200 or 500 $\mu\text{g}/\text{kg}$ body mass) and did not respond until the pigs began to eat. However, once the pigs began to eat, the subsequent insulin response was significantly less than was observed in control pigs when they initiated feeding. Leptin has been demonstrated to inhibit insulin secretion from pancreatic islets (Kieffer and Habener, 2000), which could account for this limited insulin response. Alternatively, the data may suggest that leptin enhances insulin sensitivity, as much less insulin was necessary to clear plasma glucose. Lin et al. (2002), have reported that ICV leptin injection improves peripheral insulin sensitivity in the diabetic rat. However, ICV administration to the pig did not produce any change in serum insulin or glucose levels (Barb et al., 1998).

The absence of available glucose would necessitate the use of NEFA as an essential fuel during this period of

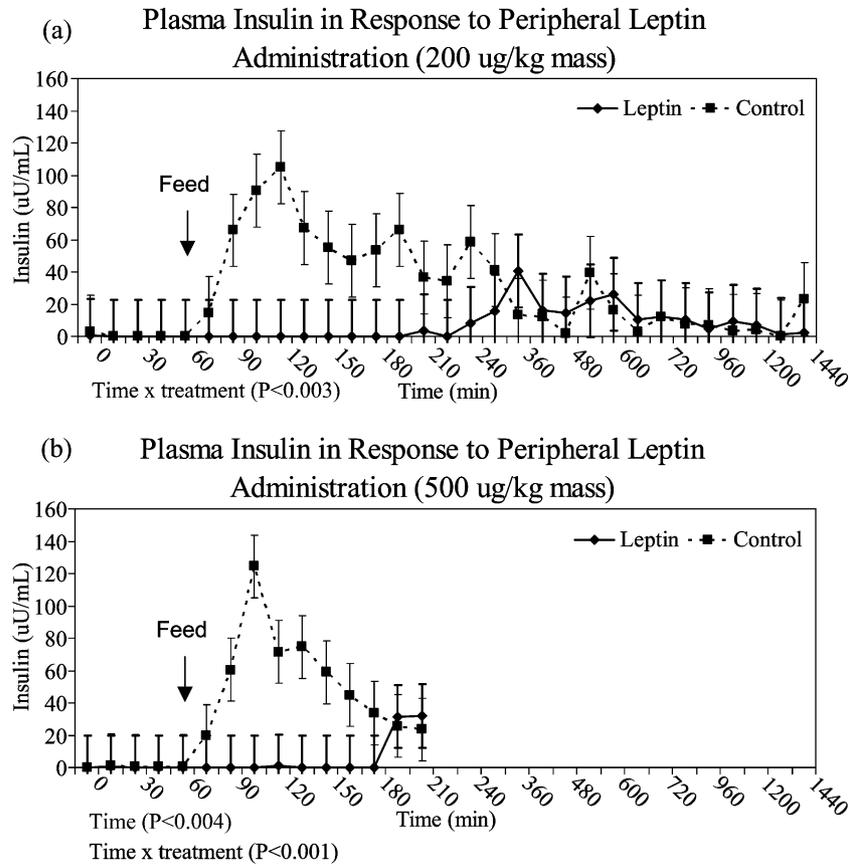


Fig. 4. Plasma insulin concentration in female pigs in response to a single carotid injection of recombinant porcine leptin or saline. Data are expressed as microunits per milliliter ($n=3$; mean \pm pooled S.E.M.). (a) 200 μg leptin/kg body mass dosage; significant effect of time ($P<0.05$), significant effect of treatment \times time ($P<0.05$). (b) 500 μg leptin/kg body mass dosage; significant effect of time ($P<0.05$), significant effect of treatment \times time ($P<0.05$).

relative hypoglycemia in the leptin-treated pigs as proposed by the glucose-free fatty acid cycle. This was confirmed with the moderate dose of leptin (200 $\mu\text{g}/\text{kg}$ body mass), which again maintained an elevated plasma

NEFA when feed intake and blood glucose were suppressed. In vitro studies have demonstrated that leptin can stimulate lipolysis in pig adipocytes (Ramsay, 2000; Ajuwon et al., 2003b). Reidy and Weber (2002) have

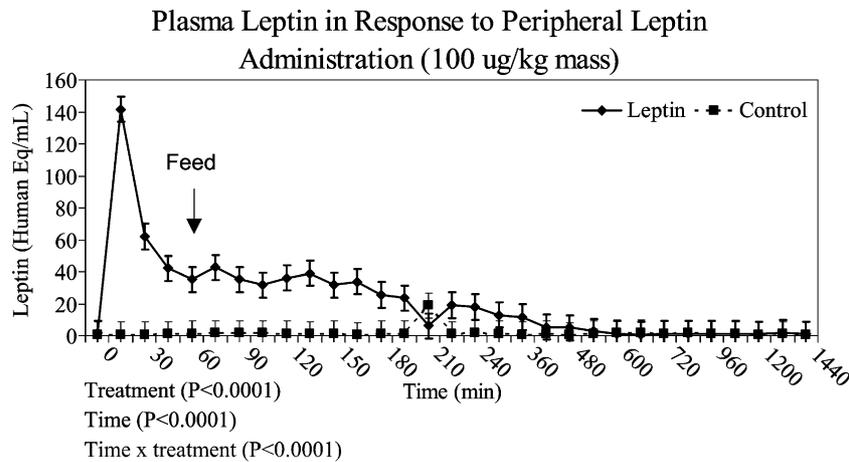


Fig. 5. Plasma leptin concentration in female pigs in response to a single carotid injection of recombinant porcine leptin (100 μg leptin/kg body mass) or saline. Data are expressed as human leptin equivalents per milliliter ($n=3$; mean \pm pooled S.E.M.). Significant effect of treatment ($P<0.05$), significant effect of time ($P<0.05$), significant effect of treatment \times time ($P<0.05$).

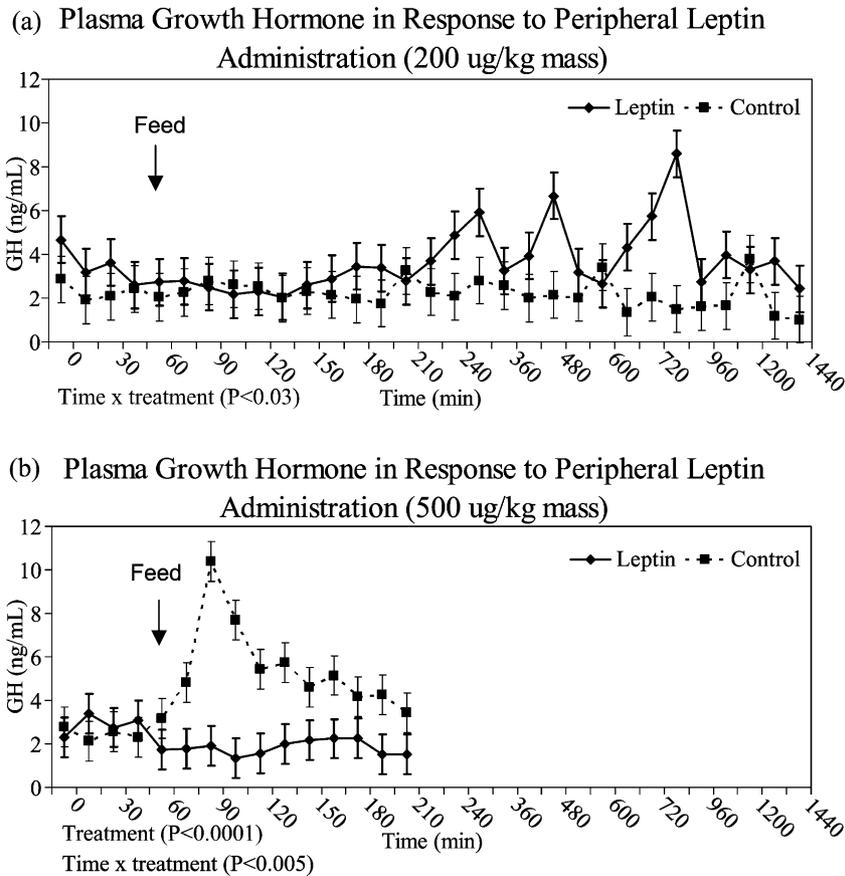


Fig. 6. Plasma growth hormone concentration in female pigs in response to a single carotid injection of recombinant porcine leptin or saline. Data are expressed as nanogram per milliliter ($n=3$; mean \pm pooled S.E.M.). (a) 200 μg leptin/kg body mass dosage; significant effect of treatment \times time ($P<0.05$). (b) 500 μg leptin/kg body mass dosage; significant effect of treatment ($P<0.05$), significant effect of treatment \times time ($P<0.05$).

reported that peripheral leptin administration increases *in vivo* lipolysis and fatty acid oxidation in rabbits, which suggests that leptin stimulation of lipolysis could contribute to changes in NEFA in the present study. Ajuwon et al. (2003b) have recently demonstrated that serum NEFA are elevated in swine following chronic peripheral treatment with a human leptin analogue to support the present data. The one confounding factor for interpretation of the NEFA data in the present study is the differences between the two groups of pigs prior to the actual injection of saline or leptin. Since the controls from the first experiment served as the leptin group in the second, with a switch again for the third dose, the consistent higher NEFA levels at time 0 for the leptin group cannot be explained.

The stimulation of growth hormone secretion by peripheral leptin treatment (200 $\mu\text{g}/\text{kg}$ body mass) confirms previous data from Barb et al. (1998), using ICV administration to swine. Baratta et al. (2002), demonstrated that leptin stimulates GH expression and secretion from porcine anterior pituitary cell *in vitro*, with a maximal response to 10 nM leptin. The present study used concentrations of recombinant pig leptin that produced a serum concentration at its peak of at least this amount in the peripheral blood. While

the leptin assay used in the present study is heterologous and thus the actual values may not be accurate, it does demonstrate that antibody reactive peptide reached the vasculature.

The protocol for these experiments used a carotid injection to maximize leptin presentation to the blood brain barrier, which produced a rapid rise in serum leptin. The present study could not demonstrate an immediate GH secretory response to leptin administration. An increase in serum GH required approximately 6 h prior to detection, paralleling the initiation of feeding and normalization of blood glucose. The metabolic and hormonal changes associated with feeding in these fasted animals may contribute to the observed increase in GH secretion (Barb et al., 2002). The second peak of GH secretion at 8 h parallels a spike in insulin secretion in leptin treated swine and confounds evaluation of leptin's role in GH secretion. However, the third peak and largest peak of GH secretion occurs following normalization of all blood parameters and is more likely the result of the actions of leptin.

This would imply that transport across the blood brain barrier in the pig is quite slow. Unfortunately no analysis of transport systems at the pig blood brain barrier has

been performed. Studies using the sheep choroid plexus indicate that a saturable transport system exists, along with a non-saturable transport system (Thomas et al., 2001). Alternatively, it may suggest that growth hormone secretion may be the consequence of feedback from signals produced in response to leptin binding to peripheral receptors. Leptin receptor mRNA have been detected in numerous peripheral tissues in the pig, which implies the presence of receptors in the peripheral tissues (Lin et al., 2000). Growth hormone secretion in response to leptin treatment may feedback on the adipocyte to affect leptin expression. Growth hormone inhibits the expression of leptin in porcine adipose tissue (Spurlock et al., 1998). Thus a feedback loop between leptin and growth hormone has been proposed for the pig (Ramsay, 1999; Barb et al., 2001). Ajuwon et al. (2003a) did not demonstrate a GH response to chronic peripheral treatment in swine with a human leptin analogue; however, samples were only obtained at a single time point on the day of sampling. This may have precluded detection of a GH response, as the present data demonstrates the pulsatility of this GH response to leptin.

The ability for a single injection of leptin to produce significant changes in hormone and metabolite levels suggests that this peptide has a role in regulation of peripheral metabolism. However, the dosages used in the present study produced supra-physiological serum leptin levels (approx. 40–140 ng leptin (human Eq)/ml). Serum leptin levels in the pig have been reported to be <10 ng/ml (Qian et al., 1999). As with many recombinant proteins, we are uncertain whether this recombinant protein is optimally folded, which might severely limit its efficacy, thus requiring such a high dosage to produce endocrine and metabolic responses. Interpretation, therefore, of a physiological role for leptin must be made with caution. The data indicate that this preparation of recombinant leptin requires a dosage of >100 µg/kg body mass to affect the monitored parameters, while a dosage of <500 µg/kg body mass is necessary to avoid the negative effects of hypoglycemia. Whether this dosage range produces similar results with chronic leptin treatment in the pig is unknown. However, the data from studies in humans and rodents would suggest that chronic leptin treatment in swine may have significant effects on peripheral metabolism, either by affecting peripheral organs directly or indirectly through leptin uptake and binding in the central nervous system. The data from Ajuwon et al. (2003b) support a chronic metabolic response to leptin treatment, as evident in elevation in adipose tissue lipolysis, increase in serum NEFA and changes in gene expression within the adipose tissue following treatment with a leptin analogue. The requirement for an extended time period following peripheral leptin administration to affect growth hormone secretion suggests that leptin may affect the regulatory pathways for growth hormone secretion primarily through peripheral feedback.

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