RESEARCHREPORT

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ANIMAL SCIENCE

Title: Action of Leptin at the Hypothalamus for Reproductive Function
NPB#98-117

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I. Abstract

Twelve Meishan and twelve Yorkshire gilts were used to study the effects of diet and breed on leptin and LH concentration during estrous. Gilts were randomly assigned to a standard corn-soybean meal diet at approximately 2.5% of body weight or fasted. Estrus cycles for gilts were determined by observation of standing estrous in the presence of a boar. Two or more estrous cycles were observed to gain an estimate of the length, in days, of the estrus cycle for each. Twelve days before onset of expected estrus, jugular cannulas were inserted surgically and blood was collected at 08:00 and 20:00 hours. Three days prior to the onset of expected estrus, feed was removed and blood was collected every 4 hours. After standing estrus was established, blood was sampled every hour for the next 24-hour period and then collected every 4 hours for the next 36 hours until the pigs were killed humanely for collection of brain tissues. Fasting did not decrease basal LH (.47 ± .06 vs .57 ± .06 ng/ml) and but did decrease peak LH values (3.44 ± .51 vs. 5.18 ± .51 ng/ml; $P < .05$) in gilts during the estrous period. Fasted gilts also had decreased plasma leptin concentration during the protocol period when compared to fed gilts(1.88 ± .19 vs. 2.65 ± .19 ng/ml, respectively; $P < .01$) and lower peak leptin values (2.59 ± .51 vs. 5.22 ± .53 ng/ml, respectively; $P < .01$). Yorkshire and Meishan gilts had similar baseline values of LH prior to estrus, but Yorkshire gilts had lower LH peak values than did Meishan gilts (3.09 ± .51 vs. 5.53 ± .51 ng/ml, respectively; $P < .05$). There was no difference between plasma leptin values between breeds during the protocol period.

II. Introduction

Chinese Meishan pigs are noted for their early accretion of fatty tissue, precocious puberty, and large litter size compared with traditional breeds of pigs (i.e., Yorkshire). Meishan females usually attain puberty at 80 days of age and Yorkshire females at 160 days or when they attain about 22% of body weight as fat. Leptin, a newly discovered hormone from adipose tissue, plays a crucial role in controlling food intake, metabolism, and reproduction in animals (Houseneckt et al, 1998). Leptin serves as a positive metabolic signal to the reproductive system in the animal that has sufficient fat stores to meet metabolic demands. Nutrient deprivation arrests reproductive function, whereas extreme obesity also can lead to reproductive

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impairment. Critical events during the development of the fetus occur not only in the formation of the brain but prior to fertilization of the egg. These events begin with the chronological secretion of neuroactive peptides in the hypothalamus of the gilt. Previous work with the pig has shown that diet and energy during estrus acutely affect successful ovulation rates and implantation of eggs. Short-term feeding of high-energy diets increases ovulation rate, whereas 10 days of inanition (total fasting) blocks ovulation in the gilt. During feeding, plasma leptin concentrations vary in response to adequate energy intake; low energy abruptly decreases plasma leptin. We are interested in determining the effects of plasma leptin concentrations on the neuroactive peptide hormones and lipid-derived hormones necessary for high ovulation rates in pigs. Leptin is expressed at concentrations ten fold higher in breast milk than in the plasma of the lactating mother. Milk supplied during the suckling period is known to contain bioactive peptides that may influence growth. High concentrations of leptin in the plasma occur during the postnatal period of mice and decrease to adult levels after weaning. The feeding patterns of neonate mice suggest that they are resistant to leptin inhibition during the suckling period. Development of galanin (GAL), melanin-concentrating hormone (MCH), proopiomelanocortin (POMC), and neuropeptide Y producing neurons, which are influenced by leptin in the adult, change dramatically during development of the fetus and neonate. We wish to characterize the biochemical role of leptin via the leptin receptor during gestation and during postnatal development of the brain in obese (Meishan) and lean (Yorkshire) pigs. Animals must adjust their food intake and metabolism to maintain a reasonable level of fat stores to survive and reproduce. A regulatory system, or *adipostat*, has evolved to address this requirement by maintaining energy balance to within 0.2 of per cent calories consumed when food is abundant. Increased caloric intake increases basal metabolic rate and lipid synthesis. Leptin is a 167 amino acid peptide hormone secreted from adipose tissue and circulating in peripheral blood at concentrations that correlate positively to the amount of fat stores (Friedman, 1995). Leptin has a primary role in mediating changes in the hypothalamus to control food intake.

The gene encoding the leptin defect in mice and the human analogue was first reported by Zhang et al, (1995). Subsequent experiments using recombinant leptin demonstrated the ability of leptin to decrease feed intake, decrease body weight, decrease adipose content of the body, influence circulating glucose, and influence basal metabolic rate in the *ob/ob* mouse (Campfield et al, 1995; Halaas et al, 1995; Pellymounter et al, 1995). Further experimentation demonstrated that leptin acts through the central nervous system (Malik and Young, 1996). *ob/ob* mice do not respond to abrupt changes in nutritional status with changes in NPY or CRH (Jang and Romsos, 1998). Leptin inhibits feeding behavior through modulation of neurons in the hypothalamus that secrete NPY, GAL, and MCH. Leptin administration into the third ventricle of rats will inhibit feed intake and even when feed intake has been stimulated by the ICV injection of NPY, GAL, and MCH (Sahu, 1998). Leptin acts by a receptor-mediated inhibition of NPY biosynthesis and release. The region of the hypothalamus involved in feeding has a unique "feeding" receptor, with NPY playing a crucial role in feeding behavior, food intake, carbohydrate preference, and metabolic and lipogenic rates. For example, centrally injected antibodies directed against NPY block normal onset of feeding, whereas long-term central administration of NPY induces obesity.

Neuropeptide Y is a 36 amino acid amidated peptide originally isolated from porcine brain. Neuropeptide Y is distributed widely in the mammalian brain and is involved in numerous functions including the control of feeding, growth, and reproduction. The highest concentrations of NPY are found in the hypothalamus and intracerebroventricular injection of NPY into this region increases food intake in pigs. Over-expression of NPY in the arcuate paraventricular has been linked to hyperphagia

and obesity as seen in Zucker and Wistar fatty rats, and obese mice. We have determined the immunohistochemical localization of NPY throughout prepubertal development in Meishan pigs, a Chinese breed known for superior reproductive characteristics (Pearson et al., 1996a). NPY-immunoreactivity (NPY-IR) in cell bodies and fibers is evident in many regions of the brain of 30-day fetus, including the basal telencephalon, hypothalamus, mesencephalon, pons, and medulla. Throughout prenatal development, cell bodies displaying NPY-IR increased in number and distribution in the brain. During postnatal development, the number of cell bodies displaying NPY-IR decreased. The arcuate nucleus of the hypothalamus showed a dramatic decrease in the number of immunoreactive cell bodies from day one to 20 days of age. Some additional increase in immunoreactivity occurs postnatally, especially in the periventricular hypothalamus and the hippocampus. The localization of NPY-IR in the hypothalamic structures that influence feeding, growth, and reproduction in several mammalian species supports the hypothesis that NPY is involved in controlling these production parameters in pigs. Galanin (GAL) is a 29 amino acid amidated peptide found in the mammalian brain that inhibits insulin secretion, stimulates food intake behavior, and stimulates secretion of growth hormone, LH, and LHRH. In these same brain coronal sections from Meishan pigs, we determined GAL immunoreactivity (GAL-IR) at these prenatal and postnatal stages of development (Pearson et al., 1996b). GAL-IR was present in the porcine brain in regions that participate in the control of food intake, growth, and reproduction in other mammalian species. The density and distribution of LHRH-IR cell bodies and fibers also was determined in brain coronal sections from these same Meishan pigs (Pearson et al., 1996c).

Leptin and Reproductive Function. Leptin has been shown to influence reproductive efficiency in mice, rats, and monkeys and has been correlated with reproductive parameters in humans. Male and female *ob/ob* mice are sterile and unable to reproduce through homozygous matings. Intraperitoneal injections of *ob/ob* mice with recombinant leptin has been shown to increase uterine and ovarian weight, LH plasma concentration, and improve follicular development in female *ob/ob* mice (Barash et al., 1996). Similar treatment of male *ob/ob* mice resulted in increased seminal vesicle and testes weight, FSH plasma concentrations, and fewer abnormalities of the seminiferous tubules. Furthermore, treatment of ob/ob mice with leptin is able to rescue the reproductive function of the sterile female (Chehab et al., 1996) and sterile male *ob/ob* mice (Mounzih et al.,1997). Leptin is also effective in accelerating the onset of puberty in normal female mice (Chehab et al., 1997; Ahima et al., 1997) and rats (Cheung et al., 1997).

Human plasma leptin concentrations vary during stages of menses (Hardie et al., 1997). Peak leptin concentrations have been correlated with peak progesterone levels in the luteal phase. In addition, plasma leptin has been correlated with post-ovulatory implantation potential in humans (Cioffi et al., 1997). These human data support our hypothesis that leptin regulates reproductive functions in the pig via previously established endocrine relationships.

Previous reports suggest that leptin is important for the control of NPY and CRH in the hypothalamus of *ob/ob* mice (Jang and Romsos, 1998). Leptin has been shown to directly inhibit NPY and CRH release in the hypothalamus when applied via ICV injection. As neonates mature there is a shift from non-responsive plasma NPY concentrations and high leptin production by adipose tissue at birth to a very responsive plasma NPY concentration and lower production of leptin by adipose tissue (Devaskar et al, 1997). Neuropeptide Y and CRH can both inhibit gonadotropin-releasing hormone secretion from the hypothalamus (Chrousos et al, 1998). The increase of leptin with a corresponding decrease of NPY, CRH, and, GAL

during the neonatal period suggest that leptin plays a role in the development of the neuroendocrine axis involved in reproduction.

III. Stated Objective

Acute energy deprivation in the sow just preceding expected estrus abruptly decreases concentrations of circulating blood leptin, which increases the release of NPY that stimulates appetite and coincidentally blocks sexual receptivity (standing estrus). We hypothesize that neuronal circuitry of the NYP-, LHRH-, and GAL-secreting neurons necessarily communicate with leptin-binding neurons in discrete regions of the hypothalamus for expression of sexual behavior and pituitary gonadotropin release required for ovulation. To test this hypothesis, we will use our available lean (Yorkshire) and fat (Meishan) pigs to complete the following two specific aims:

Aim 1. Determine the relationship of acute changes in plasma leptin concentrations with those of estradiol, progesterone, LH, and follicle-stimulating hormone (FSH) secretion in gilts, and

Aim 2. Determine the localization and differences of receptors for NPY, LHRH, GAL, and leptin for (a) fasted and fed and (b) lean and obese gilts at the time of ovulation.

V. Procedures: Experimental design, methods and procedures.

Twelve Meishan and twelve Yorkshire gilts were used to study the effects of diet and breed on leptin and LH concentration during estrous. Gilts were randomly assigned to a standard corn-soybean meal diet at approximately 2.5% of body weight or fasted. Estrus cycles for gilts were determined by observation of standing estrous in the presence of a boar. Two or more estrous cycles were observed to gain an estimate of the length, in days, of the estrus cycle for each . Twelve days before onset of expected estrus, jugular cannulas were inserted surgically and blood was collected at 08:00 and 20:00 hours. Three days prior to the onset of expected estrus, feed was removed and blood was collected every 4 hours. After standing estrus was established, blood was sampled every hour for the next 24-hour period and then collected every 4 hours for the next 36 hours until the pigs were killed humanely for collection of brain tissues. All procedures used for the collection of blood and tissues were approved by the Iowa State University Animal Care Committee (Protocol 1-8-3764-3-S).

Plasma Assays for LH and Leptin

Blood was collected from the cannula, expressed into borosilicate tubes and treated with 10 U heparin (Sigma, St. Louis, MO) per ml blood collected and 25 µg aprotinin (Sigma, St. Louis, MO)/ml blood collected, and centrifuged on a bench-top clinical centrifuge to remove packed cells. Supernatant was removed, aliquoted into two separate containers, and stored at -80^{o}C until assayed. Plasma leptin concentrations was established using a multi-species leptin radioimmunoassay (RIA; Linco Research, Inc). Leptin hormone was measured in duplicate from 100 µl aliquots of plasma. Plasma or standard aliquots are pipeted into borosilicate tubes with 300 µl assay buffer and 100 µl multi-species leptin antibody (guinea pig derived polyclonal sera) and incubated at 4^{o}C for 24 hrs. ^{125}I-leptin is added to all tubes at a volume of 100 µl to all tubes incubated for 24 hrs at 4^{o}C; 1 ml of precipitating reagent (goat anti-guinea pig with glycerol) is added and tubes are centrifuged at 3,000 x g for 30 min. Supernatant was decanted and radioactivity of the precipitate determined in a γ-well spectrometer (Hewlett-Pakard).

Luteinizing hormone radioimmunoassay was carried out according to procedures currently being used by our laboratory (Anderson et al., 1981). Luteinizing hormone was measured from 300 µl aliquots of plasma, in duplicate, using highly purified porcine luteinizing hormone (NIH) for labeling with ^{125}I (Amersham) and for standards (36 ng - 2 pg). After dilution of ovine LH antiserum (1:40,000) with 1:400 normal rabbit serum, 200 µl was added to each assay tube containing the serum unknowns, standards, and controls and incubated at 4^{o}C for 24

hrs. ^{123}I-pLH was added and incubated at 4°C for 24 hrs. Next, 200 µl of a 1:45 dilution of goat derived anti-rabbit IgG (Cappel Labs., Inc) was added to each assay tube and incubated at 4 °C for 72 hrs. before centrifugation at 1000 x g for 30 minutes to precipitate the antibody/antigen complex. Supernatant was decanted and radioactivity of the precipitate was be determined in a γ-well spectrometer (Hewlett-Pakard).

Data were analyzed in a general linear model by PC-SAS (SAS Institute, Cary, NC).

IV. Results:

The plasma concentration of leptin in mice has been reported to decrease after 24 hours of fasting (Li et al, 1998). Plasma leptin in Yorkshire gilts responds similarly to fasting as did the plasma leptin of rats when fasted (Table 1). Yorkshire gilts had a plasma concentration of 1.84 ± 0.20 ng/ml leptin. The plasma concentration of leptin decreased to 1.35 ± 0.17 ng/ml leptin after three days of fasting. Decreases of plasma leptin concentrations in response to a fast are constant with data indicating that leptin mRNA production decreases in response to fasting in sheep and cows (Kumar et al., 1998; Tsuchiya et al., 1998).

Table 1. Plasma leptin concentrations in gilts during execution of feeding protocol[a].

Item	Fed	Fasted
Gilt	Leptin (ng/ml)[b]	
1553	1.57	1.43
1712	2.00	1.42
1714	1.93	1.30
1716	1.87	1.30
Mean	1.84[c]	1.35
Std Dev	0.20	0.17

[a]Gilts were fasted for 3 days.
[b]RIA measures plasma as human equivalents.
[c]Fed and Fasted values differ by P < .036.

Meishan and Yorkshire gilts had similar concentrations of LH at the initiation of the feeding protocol, 0.46 ± 0.06 and 0.58 ± 0.06, respectively (Table 2). Meishan gilts had a greater peak concentration of LH and a greater magnitude increase in plasma LH concentrations during estrus than that observed in Yorkshire gilts. The time that optimal LH concentration was achieved after initiation of the protocol was similar between the two breeds of gilts

Fed and fasted animals had similar plasma LH concentrations at the beginning of the feeding protocol (Table 2). Fasted gilts had, however, lower optimal plasma LH concentrations than did Yorkshire gilts. In addition, fasting did decrease the magnitude of the LH surge observed during. standing estrus. This data indicates that the protocol of three day fasting did have an effect on the onset and magnitude of the LH pulse during standing estrus.

Table 2. Luteinizing hormone concentrations of Yorkshire and Meishan pigs that were either fed or fasted for three days prior to expected estrus.

			LH (ng/ml plasma)		
Item[a]	**N**	**Baseline**	**peak**	**% change LH**[b]	**Time of LH peak**[c]
Breed					
Yorkshire	12	0.58 ± 0.06	3.09 ± 0.51*	502 ± 196**	126 ± 12
Meishan	12	0.46 ± 0.06	5.53 ± 0.51	1326 ± 196	125 ± 12
Diet					
Fed	12	0.57 ± 0.06	5.18 ± 0.51*	1145 ± 196	107 ± 12 *
Fasted	12	0.47 ± 0.06	3.44 ± 0.51	683 ± 196	144 ± 12

[a] Main effects of test are breed and diet.
[b] Increase in LH from baseline value to highest value measured during LH spike.
[c] Time was measured in hours after the initiation of feeding protocol.
* Values within treatment differ by P < .05.
** Values within treatment differ by P < .01.

Table 3. Leptin hormone concentrations of Yorkshire and Meishan gilts that were either fed or fasted for three days prior to expected estrus.

		Leptin (ng/ml plasma)[a]		
Item[b]	**N**	**Baseline**	**peak**	**% change leptin**[c]
Breed				
Yorkshire	12	2.32± .19	3.75 ± .53	67 ± 29**
Meishan	11	2.15 ± .19	4.05 ± .51	85 ± 30
Diet				
Fed	12	2.65 ± .19**	5.22± .53**	106 ± 30**
Fasted	11	1.88 ± .19	2.59 ± .51	47 ± 29

[a] RIA measures plasma as human equivalents.
[b] Main effects of test are breed and diet.
[c] Increase in leptin from baseline value to highest value measured during test spike.
* Values within treatment differ by P < .05.
** Values within treatment differ by P < .01.

Yorkshire and Meishan gilts had similar plasma leptin concentrations during the beginning of the protocol (Table 3). Yorkshire and Meishan gilts had similar peak values during the protocol period. The change in plasma leptin concentrations during the protocol period was greater for Meishan gilts than was the change observed during the same period for Yorkshire gilts.

Gilts that were fasted experienced a decrease in the plasma leptin concentration over the period that began with the protocol and ended with standing estrus as evidenced by their baseline values. Furthermore, gilts that were fasted had lower peak leptin values than did gilts that were fed over the protocol period. As a result the fasted gilts had a lower increase in leptin during the protocol period. These findings are consistent with published research demonstrating decreased leptin mRNA during periods of fasting for other domesticated animals (Kumar et al., 1998; Tsuchiya et al., 1998).

Leptin has been shown to affect the expression of NPY from NPY-containing neurons in the hypothalamus (Baskin et al., 1999). Furthermore, ICV injection of leptin is able to decrease fed intake inspite of galanin, POMC, and NPY. We are currently working to quantify the effect of fasting on the production of galanin, POMC, NPY, and leptin receptors in the brains of these two breeds of pigs.

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