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Leptin concentration in blood and its hypothalamic binding are poorly related with amount of fat and growth rate in pigs*

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ABSTRACT

Three genetic groups of pigs - Polish Large White, Polish Landrace and line 990 - were investigated for leptin concentration in blood, the expression of the leptin receptor gene in the hypothalamus, and the binding of leptin to its hypothalamic plasma membrane receptors. These parameters were correlated with average daily weight gain and fatness traits. Mean leptin concentration differed slightly between investigated groups. Unexpectedly, the lowest concentration of the hormone was observed in line 990 with the highest subcutaneous fat deposition. Simultaneously, higher mean concentrations of leptin in blood and an elevated mean leptin binding in the hypothalamus were characteristic for breeds demonstrating higher body weight gains (Polish Landrace and Polish Large White). On the other hand, the analysis of correlation did not show any interdependence for the investigated parameters within the groups. These results suggest that the amount of the fatty tissue is not the only factor responsible for leptin secretion and that the intensity of anabolic processes (measured as daily weight gain) is poorly correlated with the expression of hypothalamic receptors for this hormone in the pig.

KEY WORDS: leptin, leptin receptor, pig, fat

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INTRODUCTION

Leptin, the product of the so-called obesity (*ob*) gene, is secreted by the adipose tissue and it can control body weight by the regulation of satiety centres in the hypothalamus (Zhang et al., 1994; Liu et al., 1997). Under experimental conditions it decreases feed intake (Barb et al., 1998; Velkoska et al., 2003). The amount of the produced and secreted leptin may depend on hormonal and nutritional factors (Zhang et al., 1994; Saladin et al., 1995; Cameron et al., 2000, 2003; McNeel et al., 2000). This substance informs the central nervous system about the metabolism of the fatty tissue and the amount of stored lipids (Campfield et al., 1995; Meister, 2000). Simultaneously, it may affect many other tissues because of widely distributed receptors (Hoggard et al., 1997). The distribution of leptin receptors in the central nervous system and different peripheral tissues suggests an important role played by leptin in organisms (Bray and York, 1998). Some known serious defects in leptin production (Zhang et al., 1994) or its receptor synthesis (Lee et al., 1996) result in the syndromes of obesity and diabetes, respectively.

Several literature data correlate the leptin level with the amount of the adipose tissue (Solin et al., 1997; Van Harmelen et al., 1998). Some authors investigated leptin concentrations or the expression of mRNA for leptin and its receptors in the pig and tried to describe their role during growth, fattening, and gestation (Guay et al., 2001; Berg et al., 2003; Cameron et al., 2003). Simultaneously, there are only scanty data presenting leptin binding to its receptors in animals (Margetic et al., 2002), and no data about leptin binding in pig tissues.

The aim of the present study was to analyse leptin concentration in blood and the expression of the hypothalamic leptin receptor in different breeds of the pig and their correlation with growth and fattening.

MATERIAL AND METHODS

Animals and traits

The analysed pigs of the Polish Large White - PLW (n=136), Polish Landrace - PL (n=123), and line 990 - L990 (n=186) were kept under standardized conditions at the Experimental Station in Pawlowice. Groups of gilts (usually four pairs of full-sibs), originating from four different litters but being progeny of a single boar, were fed *ad libitum* with a commercial mixed fodder up to 100 kg liveweight. During the fattening body mass gains and feed intake were controlled. Prior to slaughter animals were fasted for 24 h. Immediately after being slaughtered at about 100 kg of liveweight, according to the recommended station methodology, their blood and hypothalamuses were collected, and 24 h after slaughter

the amount of the fatty tissue was estimated. Abdominal fat was weighed and five backfat thickness measurements were taken - over the shoulder, at the last rib, and at *sacrum* (point I, II, and III). Moreover, the mean from the five above measurements was calculated.

Leptin assay

Leptin concentration in the blood serum was tested using the Multi-Species Leptin RIA kit Linco Research (USA).

Radioreceptor assays of leptin receptors in hypothalamus

Isolation of membranes. Right after collection tissues were stored at -80ºC. Because it was impossible to obtain sufficient amounts of membranes from a single hypothalamus for radioreceptor assays, pooled samples were used. Half parts of hypothalamuses (on average 7), originated from the offspring of the tested boar, were collected and used for isolation. The other part of each hypothalamus was necessary to isolate mRNA for the leptin receptor.

Plasma membranes were obtained according to the method of Havrankova et al. (1978), as described previously in details by Torlińska et al. (2002). The concentration of membrane proteins was measured using the method of Lowry et al. (1951). The final pellet of membranes was resuspended in a Tris - HCl buffer (0.05 mol/l; pH 7.4) containing 0.2 g/l bovine serum albumin (BSA). All chemical reagents originated from Sigma Chemicals Co. (USA).

Binding assay. Leptin binding activity was measured incubating 400 µl of membrane preparations (final concentration 0.5 mg protein/ml) with 50 µl of [3-^{125}I] - Tyr human leptin (final concentration 0.1 nmol/l) designed for radioreceptor assays (Biotrend, Germany) and 50 µl of unlabeled recombinant human leptin (final concentration 0-300 nmol/l; Peprotech, England). Incubation was carried out at 4ºC for 18 h.

Bound and free fractions of leptin were separated by centrifugation at 20000 × g for 6 min. Radioactivity of pellets was determined using a gamma counter. Data from competition binding studies were analysed by the Scatchard`s method (Scatchard et al., 1949) using the LIGAND Pc.v.3.1 computer program by Munson and Rodbard (1980).

Expression of mRNA for leptin receptors

Semiquantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from each porcine hypothalamus using the Trizol reagent (Invitrogen, USA). Extracted RNA was quantified at 260 nm. For each sample, RNA

TABLE 1
Primers and conditions used for PCR of OBR (total form of leptin receptor) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), following Guay et al. (2001)

Gene	Primer sequences	Length of PCR products	Annealing temperature
OBR	F 5'-GGCATATCCAATTACTCCTTGG-3' R 5'-AGTCCTCTTTCATCCAGCACTG-3'	486bp	58ºC
GAPDH	F 5'-CTGGCAAAGTGGACATTGTCGCC-3' R 5'-CTTGGCAGCGCCGGTAGAAGC-3'	571 bp	62ºC

integrity was verified by electrophoresis using 1% denatured agarose gel. The cDNA was generated by a Reverse Transcription System (Promega, USA). After a reverse transcription step (48°C for 45 min) and PCR activation (95°C for 5 min), the PCR conditions were as follows: 24 cycles at 95°C for 30 s, specific annealing temperature for 30 s and 72°C for 1 min, with the final extension step at 72°C for 7 min. The specific annealing temperatures and primer sequences for the total form of the leptin receptor (OBR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification are presented in Table 1. The yield of GAPDH and OBR PCR products from 20-32 cycles was measured to determine the linear amplification range. Control reactions were carried out without the addition of reverse transcriptase. The PCR products were electrophoresed on a 2% agarose gel followed by ethidium bromide staining and analysed with an image analysis system (Kodak, GelLogic 100) and related software (Kodak 1D 3.6).

Statistical analysis

Means and standard deviations for production traits and endocrine parameters were calculated. The Student's t-test was used to verify the hypothesis that there are no differences between genetic groups. Pearson's correlation coefficients between endocrine parameters and production traits were calculated. Data from the image analysis were expressed as the ratio of OBR relative to GAPDH.

RESULTS

The investigated pigs were slaughtered almost at the same mean body weight and the compared groups did not differ in terms of the feed conversion; however, the daily gains varied among the groups. The highest mean value of the daily gain was observed in the PL pigs; it was significant when compared to the PLW and L990 ones. The lowest mean value was noticed for the L990 pigs (Table 2).

TABLE 2

Mean values and standard deviations (in parentheses) for examined production traits and endocrine parameters according to genetic groups

Item	Polish Large White	Polish Landrace	Line 990
Number of animals	136	123	186
Weight at slaughter, kg	102.7 (1.7)	102.7 (1.9)	102.4 (1.6)
Feed conversion, kg/kg	3.0 (0.4)	2.9 (0.5)	3.1 (0.4)
Daily gain 25-100 kg, g	832^{Ab} (111)	882^{AC} (124)	807^{bC} (94)
Weight of abdominal fat, kg	0.55 (0.15)	0.58^{a} (0.19)	0.53^{a} (0.17)
Average backfat thickness/ 5 measurements/cm	1.48^{A} (0.31)	1.47^{B} (0.38)	1.75^{AB} (0.38)
Number of animals	128	110	152
Leptin concentration, ng/ml	2.40 (1.82)	2.51^{A} (1.23)	2.14^{A} (1.04)
Number of assays	6	6	9
Leptin binding, fmol/mg protein	3.34^{AB} (0.45)	1.87^{A} (0.84)	1.41^{B} (0.88)
Association constant K_A, 10^{10} mol/l	1.04 (0.48)	1.05 (0.39)	1.18 (0.69)
Number of animals	48	38	83
Relative content of the leptin rceptor mRNA, %	68.3 ± 4.8	69.2 ± 3.2	70.6 ± 2.6

means labeled with the same letter differ significantly between columns: capital letters $P \leq 0.01$ small letters$P \leq 0.05$

To analyse the role of leptin during growth and fattening, blood serum levels of leptin were measured, along with the expression of mRNA for the leptin receptor in the hypothalamus, and the binding ability of the hypothalamic membranes. All the results are presented in Table 2. Leptin levels did not differ much between the investigated groups. However, they were higher in the PL and PLW than in L990. The difference between the PL breed and L990 was significant. The weight of the abdominal fat was the lowest in L990, but generally it was very similar in all the investigated groups. Simultaneously, L990 showed a tendency for stronger peripheral fattening than the other breeds. Average backfat thickness was significantly higher in L990 than in the other groups.

The obtained results suggest that the mean leptin level in blood is higher in these groups which store more abdominal fat. On the other hand, the concentration of the hormone is not positively correlated with the amount of the subcutaneous fat. Pigs of L990 express the greatest thickness of backfat, but show the lowest concentration of the hormone.

Taking into consideration the daily weight gain, it was observed that higher values were present while higher concentrations of leptin were found. The high-

est daily gains and leptin levels were found in the PL and the lowest ones in the L990.

The investigated groups did not differ in the hypothalamic contents of the leptin receptor mRNA. Despite the fact that the expression of mRNA for hypothalamic leptin receptors was almost identical, the mean binding capacity of the hormone to its membrane receptors varied slightly and the breeds with faster growth rates (PL and PLW) expressed a higher binding ability.

The obtained data showed some differences in the mean leptin contents and leptin binding between the studied groups. On this basis some comparisons can be made and conclusions about mutual relations may be drawn. However, the analysis of correlation performed inside each group did not show significant relations between physiological parameters and the amount of stored fat and body gains in individual animals. The estimated coefficients of correlation were close to zero.

DISCUSSION

The role played by leptin in growth, fattening and obesity has been widely discussed (Solin et al., 1997; Qian et al., 1998; Van Harmelen et al., 1998; Kostalova et al., 2001). The cause seems to be clear. Seven years ago Schwartz et al. (1996) proved that the intracerebroventricular administration of leptin deeply and for many hours reduced feed intake by rats. Simultaneously, they showed that the leptin action on feeding behaviour was at least in part indirect because the injected hormone diminished in the brain level of mRNA for neuropeptide Y (NPY stimulates food intake) and increased the level of mRNA for the corticotrophin releasing hormone (CRH inhibits feeding). Taking into consideration the observations that the leptin level was stated in humans to be proportional to adiposity (Solin et al., 1997), these data showed a crucial role of leptin as a body weight-regulating factor. Because of this kind of action, in the late 1990ties leptin was even perceived as a cure for obesity. Despite the fact that many substances regulating food intake (e.g., orexins, ghrelin) have been described after the discovery of leptin, this hormone still focuses the interest of investigators. From the animal breeding point of view, leptin has arisen as an important hormone which can regulate the pig growth rate and time of fattening, beside its possible action on final meat quality (leptin diminishes fat storage). Cameron et al. (2003) even stated that in the pig breeding practice "...leptin concentration in blood could be used as a physiological predictor of genetic merit if there were significant responses to selection". The results obtained in this study indicate that not only there are differences in daily body gains and in the fat content between the investigated breeds, but also in these groups leptin concentration in blood and leptin binding in the hypothalamus is different. If we compare the mean thickness of the subcutaneous fat and the

mean leptin content between the groups, we can paradoxically draw a conclusion that the leptin level is inversely related to peripheral adiposity (however, positively related to the amount of abdominal fat). Following literature, the subcutaneous fatty tissue is a good source of leptin and it expresses higher levels of leptin mRNA and secretes more leptin than the visceral adipose tissue (van Harmelen et al., 1998). Additionally, in the PLW and PL pigs, which expressed higher levels of leptin in blood, the growth rate was not reduced, but seemed to be even higher. In the PLW group one can predict decreased daily gains due to the more efficient binding of leptin in the hypothalamus. These results indicate that there is a dissociation between the amount of the fatty tissue in adult pigs, the concentration of leptin and its action. A similar phenomenon was described in laboratory animals. Mooradian et al. (2000) stated that at the same adiposity of the rat leptin levels increase gradually during aging. On the other hand, while analysing the results obtained in this study from the physiological perspective, the lack of a positive correlation between the amount of fat and leptin concentration may be explained by the facts stated previously that this hormone acts not only centrally, but also exerts independent effects on the adipose tissue enhancing lipolysis (Rodriguez et al., 2003) and accelerating apoptosis (Gullicksen et al., 2003). So, it might be theoretically possible that genetically determined lower concentrations of leptin in the L990 pigs did not inhibit the storage of subcutaneous fat as much as it was observed in the other two investigated breeds of pigs.

Additionally, the interpretation of the leptin action in an organism is complicated by its direct effects not only on the hypothalamus and the adipose tissue, but also on endocrine glands, for example the pancreas (Nowak et al., 1998; Maćkowiak et al., 2001). The effect of leptin on beta cells of the pancreatic islets, where leptin receptors are found (Lin et al., 2000), and on the secretion of insulin *in vivo* and *in vitro* is another subject of investigations. Generally, the presence of the adipose tissue - pancreatic endocrine axis, which is an additional regulatory mechanism in animals, may make it difficult to explain the leptin action on growth and metabolism. Insulin, the main anabolic hormone in animals, may be in part involved in the indirect action of leptin. Thus, at the level of the whole organism we must be aware that the observed effects may be related with leptin action, but they may not be necessarily caused by leptin alone. Considering all the data obtained in this study and their possible explanations, it can be stated that in the investigated two breeds and one synthetic line the leptin level in blood and its action is dependent not only on the amount of stored fat. Thus, the observed differences may have genetic background. Quite a similar conclusion was drawn by Berg et al. (2003), but these authors showed also the possibility to apply the leptin concentration as a useful predictor of pig fattening. They observed a relationship between the blood concentration of leptin and the thickness of subcutaneous fat for six genetic breeds (Berkshire, Chester White, Duroc, Landrace, Poland China,

Yorkshire). It is difficult to compare directly those and our results, because the authors collected blood a day before slaughtering, whereas we did it just after slaughter. These factors may influence leptin concentration and our data may differ a little from those obtained by Berg and co-workers. However, in our opinion, the leptin level is rather a poor predictor of fattening and breeders should treat this endocrine parameter very carefully. Despite the observed differences between the PL, PLW and L990 pigs, we did not find any correlation between leptin, daily weight gains, and stored fat, when calculated for each group separately. On the other hand, one may claim that we measured the leptin level only at the "end point" of pigs' life, so the dynamics of leptin concentration changes during the whole life was not studied. Therefore, it may not be excluded that the leptin level might correlate with adiposity and body gains at earlier stages of development. To prove a possible correlation between the leptin concentration and fattening traits of the PLW, PL, and L990 pigs during growth, some additional investigations would have to be carried out.

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STRESZCZENIE

Stężenie leptyny we krwi i jej wiązanie w podwzgórzu wykazują u świn niską współzależność z ilością tłuszczu i przyrostami masy ciała

U świń należących do trzech grup genetycznych (wielka biała polska, polska biała zwisłoucha oraz linia 990) badano stężenie leptyny we krwi, ekspresję receptora leptyny i wiązanie leptyny z błonowymi receptorami podwzgórza. Parametry te korelowano z przyrostami masy ciała i ilością tłuszczu. Średnie stężenie leptyny różniło się nieznacznie pomiędzy badanymi grupami świń. Najniższe stężenie hormonu stwierdzono nieoczekiwanie u zwierząt linii 990, wykazujących największą tendencję do odkładania podskórnej tkanki tłuszczowej. Większe średnie stężenia leptyny we krwi i jej wiązanie w podwzgórzu były charakterystyczne dla ras o większych przyrostach masy ciała (wielka biała polska, polska biała zwisłoucha). Analiza korelacji nie wykazała jednakże wzajemnych zależności pomiędzy badanymi parametrami w obrębie badanych ras. Uzyskane wyniki sugerują, że ilość tkanki tłuszczowej nie jest jedynym czynnikiem odpowiedzialnym za sekrecję leptyny, a intensywność procesów anabolicznych (wyrażona przyrostami masy ciała) jest u świń słabo skorelowana z podwzgórzową ekspresją receptorów tego hormonu.