LEPTIN AND LONG FORM OF LEPTIN RECEPTOR GENES
EXPRESSION IN THE HYPOTHALAMUS AND PITUITARY DURING
THE LUTEAL PHASE AND EARLY PREGNANCY IN PIGS

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Leptin is a polypeptide that plays a key role in the regulation of energy homeostasis and is also linked, among others, to mechanisms controlling reproductive processes. Data concerning the involvement of leptin in controlling reproductive functions at the level of hypothalamus and pituitary in the pig are limited. Therefore, in the present study, an expression of genes coding for leptin and long-form leptin receptor (Ob-Rb) was determined by a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) in the discrete areas of porcine hypothalamus (medial basal hypothalamus - MBH, preoptic area - POA, stalk median eminence - SME) and pituitary (anterior - AP and posterior/neural - NP parts) during the luteal phase of the cycle (days 10-12 and 14-16) and two early stages of pregnancy (days 14-16 and 30-32). Leptin gene expression in MBH was found to be higher in the mid- than in the late-luteal phase, whereas in other structures studied it remained unchanged during these periods. More pronounced differences were noted in expression of Ob-Rb gene, which was increased in MBH, AP and NP during the late-luteal phase in comparison to the mid-luteal one, whilst the relationship in the POA was reversed. In turn, during pregnancy, leptin gene expression in all tested areas of hypothalamus as well as Ob-Rb mRNA content in MBH were higher on days 30-32 than on days 14-16. In contrast, in the anterior pituitary, Ob-Rb gene expression was more pronounced on days 14-16 than during later stage of pregnancy. Comparison of leptin and Ob-Rb mRNA content in studied structures between the mid-luteal phase and days 14-16 of pregnancy revealed inhibition of leptin gene expression in almost all examined tissues (MBH, POA, SME, NP) during early pregnancy whereas Ob-Rb gene expression was inhibited in POA but stimulated in both parts of the pituitary during this stage.

In summary, obtained results suggest an involvement of leptin in the regulation of hypothalamic-pituitary axis activity during both the luteal phase of the cycle and early pregnancy in pigs.

Key words: leptin; leptin receptor; gene expression; hypothalamus; pituitary; pig
INTRODUCTION

Leptin is a peptide of 146 amino acid residues (16 kDa) and was initially found to be secreted by adipose tissue. The hormone is primarily known as a pivotal factor regulating food intake and energy homeostasis. However, numerous lines of evidence point to the participation of this peptide in the regulation of neuroendocrine axis and thereby, among others, reproductive processes in different species (1-4), including pigs (5, 6). This action takes place mainly at the level of the hypothalamus and pituitary. Gene expression for long-form leptin receptor (Ob-Rb) in these structures implies their sensitivity to leptin. Ob-Rb, thought to be the predominant signaling form, is one of alternatively spliced isoforms that differ mainly in the length of intracellular domains. These isoforms also include, apart from Ob-Rb, short forms (Ob-Ra, c, d, f), probably acting as transporters through physiological barriers, and soluble receptor (Ob-Re) consisting of only an extracellular loop (7).

Studies on leptin and Ob-Rb mRNA expression in porcine hypothalamus and pituitary were limited to prepubertal gilts and foetuses (8, 9). In our previous studies (10, 11), qualitative RT-PCR indicated a possibility of leptin and Ob-Rb gene expressions in the hypothalamus and pituitary of mature gilts. However, changes in transcript levels remain unknown in the pig during reproductive stages. Thus, the aim of this experiment was to extend our previous study and compare, using semi-quantitative RT-PCR, expression levels of leptin and Ob-Rb genes in pig hypothalamus and pituitary during mid- and late-luteal phase of the oestrous cycle as well as during two stages of early pregnancy (days 14-16 and 30-32).

MATERIAL AND METHODS

Experimental animals

The studies were carried out in accordance with the principles and procedures of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn. Sixteen gilts were assigned to one of four experimental groups (n=4 per group) as follows: days 10-12 and 14-16 of the cycle and days 14-16 and 30-32 of gestation. Within 10 min after slaughter, the hypothalamus had been divided into medial basal hypothalamus (MBH), preoptic area (POA) and stalk median eminence (SME) as described by Sesti and Britt (12), while the pituitary was separated into anterior (AP) and posterior (NP) parts. Additionally the adipose tissue samples were collected. All tissue samples were frozen in liquid nitrogen and maintained at -80°C until RNA isolation.

RNA isolation and semiquantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from all collected tissues using the RNeasy Mini Kit (Qiagen, USA). The concentration of isolated RNA was determined on a spectrometer (Lambda Bio 10,
Perkin Elmer, USA) based on the optical density at 260 nm and its purity on the ratio 260/280 nm (1.6-2.0). RNA quality was checked by electrophoresis using a 1.5% (w/v) agarose gel and stained with ethidium bromide. Approximately 1 µg of RNA was reverse-transcribed into cDNA in a total volume of 20 µl with 0.5 µg oligo(dT)$_{15}$ primer (Roche, Germany) using the Omniscript RT Kit (Qiagen, USA) at 37°C for one hour and was terminated by incubation at 93°C for 5 min. Complementary DNA was amplified by polymerase chain reaction (GeneAmp PCR System 2400, Perkin Elmer, USA) in a total volume of 50 µl using the following primer pairs: 40 pmol of porcine leptin sense (5' ACA GAG GGT CAC CGG TTT GG 3') and antisense (5' TAG AGG GAG GCT TCC AGG AC 3') primers for the amplification of a 258-bp fragment; 40 pmol of porcine OB-Rb sense (5' TCG GAA GAT ATC AGT GTT GA 3') and antisense (5' TTG GGA TGC TCT GAT AA 3') primers for the amplification of a 382-bp fragment. To provide an appropriate internal control, coamplification of a 571-bp fragment of GAPDH mRNA was carried out in each sample using the primer pair: 10 pmol of GAPDH sense (5' CTG GCA AAG TGG ACA TTG TCG CC 3') and antisense (5' CTT GGC AGC GCC GGT AGA AGC 3'). Each reaction contained also 25 µl HotStartTaq Master Mix (2.5 U HotStartTaq DNA Polymerase, 1 x PCR buffer containing 1.5 mM MgCl$_2$ and 200 µM of each dNTP) and 5 µl of the first strand of cDNA. The optimal number of cycles, ensuring the termination of amplification for these genes in the log phase were established by primer dropping method (13) - 42 and 32 cycles were employed for leptin and GAPDH, and 38 and 28 cycles for OB-Rb and GAPDH, respectively. The PCR profiles consisted of an initial denaturing step at 95°C for 15 min, an appropriate number of cycles of denaturing at 94°C for 1 min, annealing at 63°C for leptin, and 58°C for OB-Rb for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Leptin primers (access No: AF026976) were complementary to positions 216-235 (F) and 454-473 (R) of the pig leptin gene sequence, leptin receptor primers (access No: AF0036908) were complementary to positions 7-26 (F) and 370-389 (R) of the porcine leptin receptor gene sequence, and GAPDH primers (access No: SSU48832) encompassed positions 28-50 (F) and 579-599 (R) of the pig GAPDH gene sequence. The adipose tissue was used as a positive control for leptin gene expression. Negative controls were reactions with total RNA without reverse transcriptase and with water instead of RNA. PCR products were electrophoresed on 1.5% agarose in Tris-borate buffer, visualized by ethidium bromide staining and pictures were saved as tif files by FOTO/Analyst Achiever software (Fotodyne, USA).

**Sequence Analysis**

RNA was reverse-transcribed and PCR-amplified as described above. PCR-amplified DNA was electrophoresed on 1.5% agarose in Tris-acetate buffer. After isolation from gel, DNA was sequenced (ABI Prism™ BigDye™ Terminator Cycle Sequencing kit, ABI Prism 3777 DNA sequencer, USA) in both directions to confirm the accuracy of amplification.

**Data Analysis**

Product yield was determined using GelScan for Windows ver. 1.45 (Kucharczyk, Poland). Data were expressed as a ratio of leptin or Ob-Rb mRNA relative to GAPDH in arbitrary units. Respective mean values were compared within luteal phase and pregnancy and additional comparison between mid-luteal phase (GnRH/LH secretory pattern characteristic for period of fully active corpus luteum) and early pregnancy (days 14-16) was performed. All data were analysed by one-way ANOVA and least significant difference (LSD) post hoc test and are reported as the mean ± SEM from four independent observations. Statistical analyses were performed using
RESULTS

Leptin gene expression in mid-luteal and late-luteal phase of the oestrous cycle

Semiquantitative RT-PCR analysis of leptin gene expression, performed in the discrete areas of the pig hypothalamus (MBH, POA, SME) and pituitary (AP, NP), revealed some differences in leptin mRNA level. In the MBH, leptin gene expression was decreased by 63% (p≤0.05) (Fig. 1A) on days 14-16 in comparison to days 10-12. Among examined areas of the hypothalamus, leptin mRNA expression in MBH and POA was more pronounced than in SME on days...
10-12 (by 440%; \( p \leq 0.01 \) and 454%; \( p \leq 0.01 \), respectively) and on days 14-16 of the cycle it was the highest in POA (by 205%; \( p \leq 0.05 \)) (Fig. 1A). Moreover, on days 14-16 of the cycle, leptin gene expression was higher by 419% (\( p \leq 0.05 \)) in the neural part of the pituitary than in the anterior one (Fig. 1B).

Leptin receptor gene expression in mid-luteal and late-luteal phase of the oestrous cycle

Ob-Rb gene expression was enhanced by 187% (\( p \leq 0.05 \)) in the MBH on days 14-16 in relation to the earlier stage of the luteal phase (Fig. 2A). Within the POA, Ob-Rb transcript expression was diminished by 81% (\( p \leq 0.05 \)) at the late-luteal phase compared to the level observed during days 10-12 of the cycle (Fig. 2A). During the mid-luteal phase, OB-Rb gene expression in POA increased by 1348% (\( p \leq 0.001 \)) and 2023% (\( p \leq 0.001 \)) compared to those in SME and MBH,
respectively (Fig. 2A). As shown in Fig. 2B, OB-Rb gene expression significantly increased during the late-luteal phase in the anterior (by 242%; \( p \leq 0.01 \)) and neural (by 133%; \( p \leq 0.001 \)) parts of the pituitary.

**Leptin gene expression on days 14-16 and 30-32 of pregnancy**

Significant differences in leptin gene expression during the two stages of pregnancy were observed in the discrete areas of the pig hypothalamus and pituitary. Expression of leptin gene in MBH, POA and SME was enhanced by 625% (\( p \leq 0.01 \)), 228% (\( p \leq 0.001 \)) and 362% (\( p \leq 0.05 \)), respectively, on days 30-32 compared to that observed during days 14-16 of pregnancy (Fig. 3A). Moreover, it was found that on days 14-16 of pregnancy, leptin gene expressions in POA vs. SME as well as AP vs. NP were higher by 300%

![Fig. 3. RT-PCR analysis of leptin mRNA in (A) hypothalamic (MBH - medial basal hypothalamus, POA - preoptic area, SME - stalk median eminence) and (B) pituitary (AP - anterior and NP - posterior parts) tissues of pregnant pigs. Upper panels: inverse image of agarose gel (MM - molecular marker, C - adipose tissue as a control); lower panels: densitometric analysis of leptin mRNA relative to GAPDH mRNA. Data are means (± SEM) for four animals. Significant differences: *(p<0.05), **(p<0.01), ***'(p<0.001) - between two stages of pregnancy within examined tissues; a, b (p<0.05) - between tissues on days 14-16 of pregnancy; 1, 2 (p<0.05) - between tissues on days 30-32 of pregnancy.](image-url)
On days 30-32 of pregnancy, MBH leptin transcript expression was larger by 65% (p≤0.01) and 370% (p≤0.001) compared to the expression detected in POA and SME, respectively; while leptin mRNA expression in POA was greater by 183% (p≤0.05) compared to SME (Fig. 3A).

Leptin receptor gene expression on days 14-16 and 30-32 of pregnancy

On days 30-32 of pregnancy, expression of OB-Rb gene in the MBH was increased by 330% (p≤0.05) in relation to days 14-16 and it was higher than that observed in POA during the same period (by 104%; p≤0.05) (Fig. 4A). However, in the anterior pituitary OB-Rb gene expression decreased on days 30-32 of pregnancy by 88% (p≤0.001), in comparison to the earlier stage. Furthermore, on
days 14-16 of pregnancy OB-Rb mRNA expression in the AP was larger by 105% (p≤0.05) in relation to NP (Fig. 4B).

Comparison of leptin gene and leptin receptor gene expression levels between mid-luteal phase (days 10-12) and early pregnancy (days 14-16) in the hypothalamus and pituitary in pigs

During early pregnancy leptin gene expression in the MBH (by 94%; p≤0.01), POA (by 92%; p≤0.05) and SME (by 89%; p≤0.001) as well as in the neural part of the pituitary (by 93%; p≤0.05) were significantly lower than on days 10-12 of the cycle (Fig. 5A, B).

OB-Rb gene expression detected on days 14-16 of pregnancy was lower by 88% (p≤0.05) only in POA, whereas in both parts of the pituitary, higher

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Fig. 5. Comparison of leptin mRNA expression in (A) hypothalamic areas (MBH - medial basal hypothalamus, POA - preoptic area, SME - stalk median eminence) and (B) pituitary (AP - anterior and NP - posterior parts) between mid-luteal phase of the cycle (days 10-12) and early pregnancy (days 14-16) in pigs. Upper panels: inverse image of agarose gel (MM - molecular marker, C - adipose tissue as a control); lower panels: densitometric analysis of leptin mRNA relative to GAPDH mRNA. Data are means (± SEM) for four animals. Significant differences: *p<0.05, **p<0.01, ***p<0.001.
expression was observed (by 457%; \( p \leq 0.001 \) and by 111%; \( p \leq 0.001 \) in AP and NP, respectively) compared to the mid-luteal phase of the cycle (Fig. 6 A, B).

**DISCUSSION**

The results of the present study demonstrate changes in leptin gene and leptin receptor gene expression in the hypothalamus and pituitary gland in the gilt during the oestrous cycle and early pregnancy as well as differentiated profiles of their expression between non-pregnant and pregnant animals. In cyclic gilts, differences between the two periods of the luteal phase particularly concerned OB-Rb gene expression in the hypothalamus and pituitary but leptin gene expression remained unchanged within most of studied structures (POA, SME, AP and NP), except MBH. In turn, during pregnancy (days 14-16 vs. 30-32) some differences were
noted for both leptin gene (POA, MBH, SME) and leptin receptor gene (MBH, AP) expression. Interestingly, comparison of these genes' expression between the mid-luteal phase of the cycle (days 10-12) and days 14-16 postcoitum indicates that the pregnancy induced inhibition of leptin gene expression in all tissues tested and stimulation of leptin receptor gene transcription in both lobes of the pituitary.

Leptin gene expression detected within the hypothalamus (MBH, POA, SME) in the luteal phase of the cycle and early pregnancy might suggest that also centrally synthesized leptin is implicated in the regulation of the hypothalamic-pituitary-gonadal axis (HPG). Caprio et al. (14) have implied that leptin is directly linked to reproductive processes through its actions at various levels of HPG via endocrine, paracrine and/or autocrine manner. Though the main source of leptin are differentiated adipocytes (15), leptin mRNA and leptin protein have been shown in rat hypothalamus and pituitary (16), human pituitary tumors (17) and mouse, sheep and human brains (18-20). In our study, leptin gene expression was detected in hypothalamic regions related to GnRH synthesis (POA, MBH) as well as in the area responsible for its release (SME). Recently, the neuronal localisation of leptin within the arcuate nucleus was established by a double-labeled immunofluorescent histochemistry (21). Sensitivity of the hypothalamus to leptin has been confirmed by determination of its receptor in previous studies. The leptin receptor was found in pigs (8, 10, 11), humans (20) sheep (22) and rats (23). Detailed immunohistochemical studies on pig hypothalamus revealed leptin receptor immunoreactivity in the supraoptic nucleus (SON), paraventricular nucleus (PVN), ventromedial nucleus (VMN), arcuate nucleus (ARC) as well as in POA (24). Our results demonstrate opposite changes in OB-Rb gene expression within MBH (increase) in comparison to POA (decrease) with progress of the luteal phase in pigs. The leptin receptor gene expression in the hypothalamus seems to be modulated by many factors, such as GnRH, orexin, CRF, somatostatin, POMC and leptin, since its correlation with gene expression of these neuropeptides was reported (9). According to studies on rats (25, 26) it is possible that also steroid hormones, e.g. oestrogens, are engaged in the regulation of hypothalamic Ob-Rb mRNA expressions. However, detailed discussion concerning hormonal regulation of both gene expression in porcine hypothalamus seems to be premature because of limited data concerning this topic.

Even though the stimulatory effects of leptin on the release of GnRH from rat hypothalamic explants (27) and POA tissue fragments of OVX prepubertal gilts (6) have been demonstrated, the question remains whether this action is direct, indirect or both. Our aforementioned data indicate the presence of the leptin receptor in those hypothalamic regions, which are directly involved in the regulation of gonadotrophin secretion. Localisation of leptin receptors on mouse immortalized GT-1 GnRH neurons (28) might suggest the possibility of direct action of leptin on GnRH-producing neurons. On the other hand, it was also suggested that leptin may exert its effects via NPY (29, 30), MCH (melanin-
concentrating hormone) (31), β-endorphin and α-MSH (32, 33). α-MSH is involved in the control of reproduction, among others preovulatory surges of LH and PRL in the rat, acting through MC4 receptors (34, 35).

Leptin involvement in the modulation of reproductive processes in gilts could occur not only at the hypothalamic, but at the pituitary level as well. The presence of leptin mRNA and leptin receptor mRNA in the pituitary gland was reported in rats (16, 23), mice (18), sheep (36) and humans (37), which further supports the premise that leptin may act as an autocrine/paracrine factor in this gland. Moreover, leptin is co-stored with pituitary hormones in the same secretory granules and is concomitantly released with them in a pulsatile manner (18, 38). In contrast, leptin is released from adipocytes immediately after synthesis (39). Interestingly, a study in women during the mid- to late-follicular phase of the menstrual cycle revealed that nocturnal increases in plasma leptin concentration were synchronous with those of LH and oestradiol (40). In our study a decreasing leptin gene expression within the pituitary (AP + NP) of the early pregnancy vs. the mid-luteal phase of the cycle was accompanied with a parallel increase of OB-Rb gene expression. It can not be ruled out, that inverse patterns of leptin and Ob-Rb gene expressions in the pituitary may result from homologues regulation of the receptor by leptin. This kind of hormonal regulation of Ob-Rb mRNA expression was described for rat testis (41), hypothalamus (42) and adrenal glands (43). It is possible that also other factors, produced locally or in the hypothalamus, take part in this regulation. Exemplarily, Ob-Rb mRNA expression in rat tissues can be affected by hCG, FSH and ACTH (41, 43) and this gene expression in porcine pituitary cells is dependent on GRF (44). Generally, leptin seems to modulate the functioning of HPG axis at the pituitary level but details concerning its regulatory effects have not been fully explained yet.

In conclusion, the presented data showed different patterns of hypothalamic leptin gene expression during the luteal phase of the cycle and early pregnancy in pigs with the characteristic its decrease on days 14-16 of pregnancy in comparison to the values observed in the luteal phase. Moreover, during this stage of pregnancy the increase of the OB-Rb gene expression in the pituitary was observed. The present results suggest an involvement of leptin in the regulation of reproductive axis at the level of hypothalamus and pituitary during the luteal phase of the cycle and early pregnancy in the pig. These data may also serve as basis for future profound studies concerning hormonal regulation of observed in these studies changes in both gene expressions in pig hypothalamus and pituitary. Moreover, further studies are required to determine the presence of leptin and its receptor in both the hypothalamus and pituitary.

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