Leptin is a multifunctional regulator in numerous tissues, including the pituitary. It is not known, whether the porcine pituitary is a source of leptin synthesis and possesses the leptin receptor protein. It is also unknown, if a relationship exists between expression levels of these proteins in the pituitary and physiological status of sows. Therefore, the aim of the study was 1] to examine, by Western-blotting analysis, the expression levels of leptin and the long form of leptin receptor (OB-Rb) in the porcine anterior (AP) and posterior (NP) pituitary gland during mid- and late-luteal phases of the oestrous cycle (days 10 - 12 and 14 - 16) as well as during two stages of early pregnancy (days 14 - 16 and 30 - 32); and 2] to localise, using in situ hybridisation method (ISH), the expression of leptin and OB-Rb genes in the pituitary gland in the above mentioned stages of the cycle and pregnancy. Western-blotting analysis showed that leptin protein expression in AP was higher in the late-luteal phase than in the mid-luteal phase, while OB-Rb protein expression in both lobes was higher in the mid-luteal phase. In turn, during pregnancy leptin protein content in AP and OB-Rb protein content in NP were more pronounced on days 14 - 16 than on days 30 - 32. Comparison of leptin and OB-Rb protein expression levels in AP between the mid-luteal phase and two periods of pregnancy showed, respectively, stimulation of leptin protein and inhibition of OB-Rb protein expressions during both examined stages of pregnancy. Taking AP from late-luteal phase as the point of reference, it was revealed stimulation of leptin expression during earlier period of pregnancy, whereas on days 30 - 32 of pregnancy both the hormone and its receptor expressions were diminished. In turn, comparison of leptin and OB-Rb protein expression levels in NP between the late-luteal phase and days 14 - 16 or 30 - 32 of pregnancy showed inhibition of leptin protein expression and stimulation of OB-Rb protein expression during pregnancy. Moreover, ISH studies localised leptin and OB-Rb mRNAs expression in the cells of AP as well as NP tissue during the two stages of the cycle and pregnancy. In conclusion, our findings suggest that leptin is produced within the pituitary in the pig and may participate in auto/paracrine manner in the regulation of this gland function during the luteal phase of the oestrous cycle and early pregnancy.

Key words: leptin, leptin receptor, pituitary, pig
INTRODUCTION

Leptin, discovered in 1994 as the ob gene product, is a protein with a molecular mass of 16 kDa secreted mainly by adipose cells (1). It plays a key role in the regulation of food intake and energy expenditure. Further studies revealed that leptin is also a multifunctional paracrine regulator in numerous tissues, including pituitary (2). Moreover, leptin is directly linked to reproductive processes through its actions at various levels of the hypothalamic-pituitary-gonadal axis (HPG) (3 - 5). In target tissues leptin binds to specific membrane receptors belonging to class I of the cytokine receptor family (6). One of six isoforms of leptin receptor, long form (OB-Rb) is thought to be capable of full signal transduction and acts via the JAK (Janus kinase)-STAT pathway (7). The expression of the OB-Rb gene has been detected within the anterior pituitary (AP) in a number of species, including rats (8), mice (9), sheep (10), pigs (11), men (12) which indicates the sensitivity of this gland to leptin. Siawrys et al. (11) localised OB-Rb mRNA also in the porcine posterior pituitary (NP). In turn, Morash et al. (13) detected leptin receptor immunoreactivity in the anterior and intermediate lobes, but not in the posterior lobe of the neonatal rat pituitary gland. Studies concerning leptin and OB-Rb gene expression in the porcine pituitary were performed in prepubertal, cyclic, pregnant animals and foetuses (11, 14, 15). Our previous experiments, using semiquantitative RT-PCR method (15), showed different patterns of leptin and OB-Rb mRNAs expression in both lobes of the porcine pituitary during the oestrous cycle and early pregnancy as well as the increase of the OB-Rb gene expression in the pituitary during pregnancy compared to the mid-luteal phase of the cycle. Moreover, in recent studies, using a quantitative real time-PCR method we also indicated the expression of the gene for the short form of leptin receptor (OB-Rs) in the pituitary (AP and NP) of cyclic and pregnant pigs (16). OB-Rs gene expression level in the posterior pituitary, like OB-Rb transcript content, was higher during pregnancy than the luteal phase of the cycle. However, leptin and leptin receptor protein expressions have not been examined in the pituitary of this species. Thus, it is still unclear, whether the porcine pituitary is sensitive to leptin (possesses leptin receptor protein) and able to produce this peptide. Therefore, the aims of the studies were: 1] to determine, by Western-blotting analysis, expression levels of leptin and long form leptin receptor proteins in the porcine pituitary gland (anterior - AP and posterior - NP lobes) during mid- and late-luteal phases of the oestrous cycle (days 10 - 12 and 14 - 16) as well as during two early stages of pregnancy (days 14 - 16 and 30 - 32) and 2] to confirm, using in situ hybridisation method, the expression of leptin and OB-Rb genes in the pituitary gland in the above mentioned stages of the cycle and pregnancy.

MATERIALS 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, Poland. Mature gilts (Large
White x Polish Landrace), at 9 - 10 months age, weighing 90 - 110 kg, descended from private breeding were used. The animals were assigned to one of four experimental groups (n = 4 per group) as follows: 1 and 2 - gilts killed on days 10 - 12 and 14 - 16 of the oestrous cycle, 3 and 4 - gilts inseminated on the first or second day of oestrus and killed on days 14 - 16 and 30 - 32 of gestation. Pregnancy was confirmed by the presence of embryos/foetuses within uterine horns. Within 10 min after slaughter, the adipose tissue, mediobasal hypothalamus (MBH) and the pituitary gland were excised. The pituitary was separated into anterior and posterior parts. All tissue samples were frozen in liquid nitrogen and maintained at -80°C until Western-blotting and in situ hybridisation analysis.

**Western-blotting analysis**

The analysis was performed as described in a study by Smolinska et al. (17). The lobes of pituitary were homogenised in a buffer (pH 7.6) containing: 50 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovanadate, 0.02% sodium azide, 1% Triton X-100, 1 mM phenyl-methylsulphonyl fluoride and protease inhibitors: aprotinin (2 µg/ml), leupeptin (2 µg/ml) and pepstatin (1 µg/ml). Subsequently, homogenised tissues were incubated on ice for 30 min. After twice centrifugation at 10 000 g at 4 °C for 10 min, the supernatant containing the proteins was stored at -80 °C until required for use. Then 100 µg (for OB-Rb and actin) or 150 µg (for leptin) of supernatant proteins were resolved by SDS-PAGE (15% for leptin, 5% for OB-Rb and 12.5% for actin) and transferred to nitrocellulose membranes (0.45 µm). After blocking in Tris-buffered saline with Tween-20 (TBST buffer: 10 mM Tris, 150 mM NaCl, 0.05% Tween) containing 5% fat skimmed milk at 4 °C for 5 h, the membranes were incubated with either rabbit polyclonal anti-leptin antibody (1:500; PA1 – 052; Affinity BioReagents, USA) or goat polyclonal anti-leptin receptor antibody (1:100; C-20 sc – 1832; Santa Cruz Biotechnology, USA) or rabbit polyclonal anti-actin antibody (1:200; A 2066; Sigma, USA), diluted in TBST buffer, at 4 °C overnight. The membranes were then washed with TBST buffer and incubated with secondary alkaline phosphatase-conjugated antibodies (goat anti-rabbit for leptin and actin or rabbit anti-goat for OB-Rb; AP sc – 2057 and AP sc – 2949, respectively; Santa Cruz Biotechnology, USA) diluted to 1:5000 in TBST buffer at room temperature (RT) for 1.5 h. Nonspecific fetal calf serum (MP Biomedicals, USA) was used instead of primary antibodies to produce negative control blots. After incubation and washing with TBST buffer, immunoreactive bands were visualised using 4-nitroblue tetrazolium chloride (NBT, Promega, USA) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Promega, USA). Leptin/leptin receptor was quantified by scanning densitometry of immunoblots. As a positive control for leptin and OB-Rb protein analysis adipose tissue/mouse leptin and mediobasal hypothalamus (MBH) were used, respectively. The choice of MBH was based on studies indicating the presence of OB-Rb in pig MBH (11, 18).

**Statistics**

Product yield was determined using GelScan for Windows ver. 1.45 (Kucharczyk, Poland). Data were expressed as a ratio of leptin or OB-Rb protein content relative to actin protein in arbitrary units. Respective mean values were compared within studied periods as well as between the mid- or late-luteal phase and early pregnancy (days 14 - 16 – the beginning of implantation; or days 30 - 32 – the end of implantation). 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 Statistica program (Stat Soft Inc., USA). Values for P ≤ 0.05 were considered statistically significant.
In situ hybridisation analysis

The protocol used for ISH for leptin and leptin receptor mRNA was described in our previous study (17). Briefly, frozen sections (~ 6 µm) of porcine pituitary tissues (AP, NP) were attached to poly-L-lysine-coated slides (Menzel-Glaser, Germany) and kept at -80°C until required for use. Then the sections were fixed with 4% paraformaldehyde in PBS, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl, and dehydrated in graded ethanol (70%, 80%, 95%, and 100%). After air-drying at RT, a hybridisation solution mixture (50% formamide, 4xSSC, 10% dextran sulfate, 1xDenhardt’s solution, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml tRNA) which contains an antisense 35S-labeled probe (~ 1.0 x 10^6 cpm/slide; leptin starter sequence: 5' - TAG AGG GAG GCT TCC AGG AC -3'; OB-Rb starter sequence: 5' - TTG GGA TGC TGA TCT GA T AA - 3') was spotted on each slide. The slides were hybridised at 42°C for 22 h. The next day, the coverslips were removed, and the slides were washed in 1xSSC at RT, followed by four washes in 2xSSC/50% formamide at 40 - 45 °C and one washes in 1xSSC at RT. Next, the slides were dehydrated in graded ethanol and air-dried at RT. All slides were coated with emulsion (LM-1, Amersham Bioscience, UK), dried at RT and exposed at 4 °C for 21 days for leptin gene or 9 days for OB-Rb gene. Then samples were developed (Eastman Kodak, USA), and counterstained with hematoxylin/eosin. As negative controls sense probes were used. Localisation of mRNAs for leptin and its receptor was observed in the optic microscope (CH30/CH40, Olympus, Japan) and archived by digital camera (C-5060 WZ, Olympus, Japan) and program analySIS 5 Soft Imaging System (Olympus, Japan).

RESULTS

Western-blotting analysis of leptin protein expression

In the AP, the leptin protein expression increased by 284% (p≤0.001) on days 14 - 16 of the cycle in comparison to expression of that protein on days 10 - 12 (Fig. 1A). Moreover, on days 10 - 12 and 14 - 16 of the cycle the content of leptin protein in NP was higher than in AP by 984% (p≤0.001) and 255% (p≤0.001), respectively (data not presented).

On days 30 - 32 of pregnancy, leptin protein expression in AP was reduced by 67% (p≤0.001) compared to days 14-16 (Fig. 1A). Moreover, leptin expression in NP during the later period of pregnancy was higher by 279% (p≤0.001) than in AP (data not shown).

Both on days 14 - 16 and 30 - 32 of early pregnancy leptin protein expression in the AP was significantly higher than on days 10 - 12 of the cycle (by 664%; p≤0.001 and by 151%; p≤0.01, respectively). Moreover, on days 14-16 of pregnancy expression of leptin protein in AP was higher by 99% (p≤0.01) compared to days 14 - 16 of the cycle, whereas the expression on days 30-32 of pregnancy was lower by 35% (p≤0.01) than on days 14 - 16 of the cycle (Fig. 1A). Furthermore, leptin protein expression in the NP was reduced on days 14-16 and 30 - 32 of early pregnancy relative to days 14 - 16 of the cycle (by 32%; p≤0.05 and by 30%; p≤0.01, respectively) (Fig. 1B).

Western-blotting analysis of OB-Rb protein expression

During the late-luteal phase of the cycle (days 14 - 16), expression of the leptin receptor in AP (Fig. 2A) and NP (Fig. 2B) decreased by 40% (p≤0.001) and 88%
Fig. 1. Western-blotting analysis of leptin protein expression in the pituitary [A - anterior (AP) and B - posterior (NP) parts] of cyclic (days 10 - 12 and 14 - 16) and pregnant (days 14 - 16 and 30 - 32) pigs. Upper panels: transfer of leptin protein and actin protein on the nitrocellulose membrane (MM – molecular marker, adipose tissue and mouse leptin as a positive control); lower panels: densitometric analysis of leptin protein relative to actin protein. Data are means (± SEM) for four animals. Differences in protein levels were assumed as statistically significant for P<0.05 and marked with different letters.
(p ≤ 0.05), respectively, compared to the mid-luteal phase. Furthermore, on days 14 - 16 of the cycle the content of OB-Rb protein within AP was higher by 670% (p ≤ 0.001) than in NP (data not presented).

On days 30 - 32 of pregnancy, leptin receptor expression in NP was diminished by 57% (p ≤ 0.05) compared to the respective protein expression observed on days 14 - 16 (Fig. 2B). Furthermore, at the later stage of pregnancy OB-Rb protein content in AP was higher by 103% (p ≤ 0.001) than in NP (data not shown).

In contrast to leptin protein, OB-Rb protein expression detected in AP on days 14 - 16 and 30 - 32 of pregnancy was lower by 30% (p ≤ 0.05) and 49% (p ≤ 0.001), respectively, than the expression in mid-luteal phase of the cycle. Moreover, the receptor expression in AP on days 30 - 32 of pregnancy was also reduced (by 16%, p ≤ 0.01) compared to days 14 - 16 of the cycle (Fig. 2A). In turn, OB-Rb protein expression detected in NP on days 14 - 16 and 30-32 of pregnancy was higher by 650% (p ≤ 0.01) and 220% (p ≤ 0.001), respectively, than the expression on days 14 - 16 of the cycle (Fig. 2B).

ISH localisation of leptin and leptin receptor mRNAs in porcine pituitary

ISH studies localised leptin and OB-Rb mRNAs in the cells of both pituitary lobes during the mid-luteal (days 10 - 12) and late-luteal (days 14 - 16) phases of the cycle as well as during the two stages of early pregnancy (days 14 - 16 and 30-32) (Fig. 3 and 4, respectively). Hybridisation was also found in adipose tissue used as a positive control for leptin and in MBH applied as a positive control for the leptin receptor. When the sense probe was used, no silver grains were detected in either the pituitary or control tissues.

DISCUSSION

The present study, using Western-blotting analysis, reveals for the first time that leptin and its receptor proteins are expressed in the pituitary gland (AP and NP) of the pig. Moreover, the results of this study demonstrate changes in leptin protein and leptin receptor protein expression in the porcine pituitary during the oestrous cycle and early pregnancy. The obtained results indicate that during early pregnancy, stimulation of leptin protein expression takes place in the AP, which confirm our earlier studies (15) suggesting leptin and OB-Rb gene expression in pituitaries of cyclic and pregnant pigs.

The detection of leptin protein expression within the pituitary during the cycle and pregnancy suggests that this gland, besides adipose tissue, is also one of leptin source. The localisation of leptin and its receptor mRNAs expression in the porcine pituitary, using hybridisation in situ method, confirms this hypothesis and supports earlier studies indicating both genes’ expression in porcine (15), rodent (9, 13, 19, 20), sheep (10) and human (12) pituitaries.
Fig. 2. Western-blotting analysis of leptin receptor protein expression in the pituitary [A - anterior (AP) and B - posterior (NP) parts] of cyclic (days 10 - 12 and 14 - 16) and pregnant (days 14 - 16 and 30 - 32) pigs. Upper panels: transfer of leptin receptor protein and actin protein on the nitrocellulose membrane (MM – molecular marker, MBH as a positive control); lower panels: densitometric analysis of leptin receptor protein relative to actin protein. Data are means (± SEM) for four animals. Differences in protein levels were assumed as statistically significant for P<0.05 and marked with different letters.
Interestingly, the expression pattern of leptin and OB-Rb proteins (present study) and expressions of corresponding genes (15) in the porcine pituitary during the same periods of the oestrous cycle and pregnancy are often quite different. For example, in contrast to leptin transcript level, the protein content in the AP increased during the later stage of pregnancy (days 30 - 32) compared to the mid-luteal phase of the cycle (days 10 - 12). In turn, unlike OB-Rb mRNA expression, the OB-Rb protein expression in AP was reduced on days 14 - 16 of pregnancy compared to days 10 - 12 of the cycle. This suggests the existence of the posttranscriptional processing of leptin and its receptor genes’ products and presence of the intracellular regulatory mechanism of the genes’ expression. An inverse relationship was also observed for the levels of leptin and its receptor in the pig pituitary, which implies homologous regulation of the leptin receptor by leptin itself. Similar homologous regulation of the leptin receptor, on the

Fig. 3. In situ hybridisation analysis (dark-field images) of leptin mRNA in the pituitary (AP - anterior and NP - posterior parts) of cyclic (days 10 - 12 and 14 - 16) and pregnant (days 14 - 16 and 30 - 32) pigs. A bright-field photomicrographs depict histological preparation of tissues stained by hematoxylin/eosin. Insets – corresponding tissues hybridised with radiolabeled sense probe (sense) served as a negative control. A positive control of leptin gene expression was adipose tissue (AT). Magnification 250x.
transcript level, was described for the porcine pituitary (15) and previously for the rat hypothalamus (21) and testis (22). Furthermore, hypothalamic and peripheral factors may also participate in regulating both OB-Rb and leptin expression. It was shown that GHRH decreases long form leptin receptor expression in porcine anterior pituitary cells (23). On the other hand, GHRH added with oestradiol increased the percentage of pituitary cells with leptin protein or mRNA (24). Recently Akhter et al. (25) revealed that GnRH and estrogen added together increased the coexpression of leptin mRNA and gonadotrophins in rat anterior pituitary. They also detected changes in the percentage of AP cells expressing leptin protein relative to the phase of the cycle and period of pregnancy. It indicates that the ability of pituitary gland to produce leptin is dependent on hormonal status of animals. The same authors observed that AP leptin mRNA expression increases from metestrous to diestrous followed by decrease on the
morning of proestrous. They also noticed that cellular leptin protein level in AP was increased by GnRH treatment in proestrous rats and GnRH stimulated secretion of leptin by the cells from diestrous, proestrous and pregnant rats. The authors suggested that high level of AP leptin expression during proestrus and pregnancy support its participation during key events involved with reproduction. Results of our study also shown changes of leptin protein expression levels in the porcine pituitary between the oestrous cycle and pregnancy (increase of leptin protein expression level in AP during pregnancy) and confirm above hypothesis.

Leptin is produced by different pituitary cells. Colocalisation studies in humans with leptin and pituitary hormones revealed that 70% of ACTH cells, 21% of GH cells, 29% of LH cells, 33% of FSH cells, 32% of TSH cells, 64% of folliculostellate (FS) cells and only very few PRL cells (3%) were positive for leptin (12). In rats the major cell type colocalised with leptin was TSH cell (24%), whereas a smaller percentage of LH (4%) and FSH (3%) cells were positive for that protein. Moreover, less than 1% of ACTH, GH, PRL and FS cells expressed leptin by colocalisation studies (9). Leptin production in the pituitary gland was also noted in mice (9) and cattle (26). Cellular localisation of OB-R in the pituitary was indicated as well; however, like in the case of leptin, species differences in the localisation of this receptor were observed. Notably, in the rat anterior pituitary, GH-secreting cells almost exclusively possessed leptin receptors, while leptin receptors in TSH, LH, and FSH cells were difficult to localise (20). In the sheep, leptin receptors were found in GH (69%), ACTH (27%) and LH (29%) cells of pars distalis of the anterior pituitary as well as in LH cells (90%) of the pars tuberalis (27). Moreover, FS cells and various rat and mouse pituitary cell lines have also been reported to express leptin receptors (9, 28). It is still unclear, what type of cells in the porcine pituitary produces leptin and possesses OB-Rb. Results of the present study did not solve this problem.

The presence of the leptin receptor protein in the porcine pituitary might suggest that leptin is involved in the regulation of its function and, in connection with local leptin synthesis findings, further supports the hypothesis that leptin acts as an autocrine/paracrine factor within this gland. It is worth noting however, that also adipocyte-derived leptin may bind to the receptors and affect pituitary functions. In anterior pituitary leptin is involved in regulation of growth and differentiation of pituitary cells. High leptin concentration was reported to inhibit cell proliferation in human and rat pituitary cell lines (12). In vitro studies indicated that this hormone stimulated secretion of GH (29), LH, FSH, PRL (30-32) but inhibited TSH secretion (33). The leptin effect on the release of pituitary hormones may not only be direct, but also indirect. For example, it was shown that nitric oxide, whose production is affected by leptin, might be involved in the stimulation of LH, FSH and GH secretion (29, 32, 34). By hand of the pituitary hormones, leptin can effect on function of peripheral endocrine organs, including gonads, adrenals, thyroid and others. Moreover, it was found that leptin is co-stored with pituitary hormones in the same secretory granules and is
concomitantly released with them in a pulsatile manner (9, 35). Until now, it has not been known the possible role of leptin in posterior pituitary. Our studies showed that leptin is produced in this lobe of the pituitary and the level of leptin protein expression in the NP during the cycle and pregnancy is higher than in the AP. It is conceivable, that this hormone participates in the control of posterior pituitary function, among others in the regulation of oxytocin and/or vasopressin release. However, this hypothesis remains to be verified.

In summary, the presented studies indicated for the first time that leptin and its receptor proteins are expressed in the pituitary gland (AP and NP) in the pig and revealed different patterns of these proteins’ expression during the luteal phase of the cycle and early pregnancy. Moreover, the studies localised leptin and OB-Rb mRNAs expression in the cells of AP as well as NP tissue both during examined periods of the cycle and pregnancy. These findings suggest that leptin is produced in the porcine pituitary and may participate in auto/paracrine regulation of pituitary functions during the luteal phase of the oestrous cycle and early pregnancy.

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