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Original article

In vitro effect of leptin on anterior pituitary cells LH secretory activity during early pregnancy in pig

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Abstract

Leptin modulates reproductive activity but its potential influence on LH secretion from anterior pituitary (AP) cells during implantation period in pigs (days 14-16 of pregnancy) remained unexplored. This study focused on determination whether leptin affects basal and GnRH-induced LH secretion and intracellular accumulation and whether leptin receptor (OB-Rb) mRNA is expressed in the AP gland during implantation in pigs. Four individual AP glands were developed into separate primary cultures. $2x10^5$ cells/ml were preincubated (72 h) and next, for 3.5 h, experimentally treated with GnRH (100 ng/ml), leptin (10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M) alone, or given in respective combinations with GnRH. In the AP gland, OB-Rb mRNA expression was determined by real-time PCR method. Leptin activated LH secretion and its concentration-dependent effect was observed as stimulation shown in a full range tested (culture 1) and exhibited only at 10^{-6} M (culture 2). A pooled data analysis revealed that basal LH secretion increased at 10^{-9}, 10^{-7} and 10^{-6} M, but GnRH-induced LH release decreased at 10^{-6} M. Leptin down-regulated GnRH-induced LH secretion in all cultures, but only culture 3 exhibited sensitivity for all concentrations tested. Basal LH accumulation was activated in culture 1 (at 10^{-11} M) and inhibited in culture 4 (at 10^{-9} M). In the presence of GnRH leptin up-regulated LH accumulation with individual culture leptin-sensitivity (culture 1-3), while down-regulated LH accumulation in culture 4. Obtained data indicate that OB-Rb mRNA is expressed in the AP gland and leptin alone and in combination with GnRH specifically modulates LH activity during early pregnancy in pigs.

Key words: leptin, OB-Rb, GnRH, LH secretion/accumulation, pituitary, pig

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Introduction

Reproduction success depends on the coordinated action of the hypothalamus, pituitary and gonads with an array of central and peripheral signals interplay to modulate synthesis and release of several hormones of the reproductive axis. The secretory action of pituitary gonadotropes, which synthesize and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH), results from a complex neuroendocrine control network in which hypothalamic gonadoliberin (GnRH) plays a central role. This decapeptide conveys these regulatory signals down to the pituitary level to dictate the specific pattern of gonadotropin release. GnRH displays a bimodal pattern of release, episodic versus-surge-like where gonadal steroids play a crucial role. In both sexes the tonic mode of GnRH secretion is regulated by gonadal steroids negative feedback mechanism exerted both at the hypothalamic and pituitary level. In addition, adult females also exhibit a positive, estradiol-dependent feedback of GnRH release as a unique surge-like peak that evokes ovulation by means of the consequent surge of luteinizing hormone release (Counis et al. 2009, Naor and Huhtaniemi 2013). Besides GnRH and gonadal steroids, LH synthesis and release is also controlled by stimulatory and inhibitory influences of other hypothalamic-derived hormones such as: vasopressin, oxytocin (Bogacka et al. 2002), galanin, substance P, opioids (Estienne and Barb 2005), ghrelin, orexin (Kirsz et al. 2014) and recently discovered kisspeptin (Ezzat et al. 2010) also controls LH release. Extensive studies, primarily on ruminants and laboratory rodents, have established that leptin might be also directly or indirectly involved in the regulation of LH secretion acting at both the hypothalamic and anterior pituitary levels (Barb et al. 2004). Leptin, a 16-kDa protein named OB, encoded by the obese (*ob*) gene, is secreted mainly by adipocytes. Although initially leptin was recognized as a crucial regulator of food intake, body fat mass and energy expenditure (Pelleymounter et al. 1995) further studies revealed that this hormone is also implicated in the hypothalamic-pituitary regulation of the gonadotropin-gonadal axis, therefore it affects the reproductive processes (Chebab 2014, Perez-Perez et al. 2015). The effect of leptin is mainly mediated via its functional receptor – the long isoform (OB-Rb). It is capable of full signal transduction, acting mainly through JAK-STAT and MAPK (Fruhbeck 2006) pathways activation. Expression of OB-Rb has been detected in the hypothalamus and pituitary in the prepuberal gilts and mature pig (Lin et al. 2000, Siawrys et al. 2007, 2009). So far few studies focused on leptin effects on LH release from pig pituitary cells in pig (Barb et al. 2004, Nonaka et al. 2006, Kosior-Korzecka and Bobowiec 2007) but data concerning this aspect of leptin activity in pregnant pigs is still missing. Therefore, the current studies were designed to (1) examine the direct effect of leptin on basal and GnRH-induced LH secretion and accumulation in porcine AP cells on days 14-16 of pregnancy and (2) to determine whether AP cells express OB-Rb mRNA in this period of pregnancy.

Materials and Methods

Experimental animals and tissue collection

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. The experimental animals were post-pubertal 5 crossbred pigs (Polish Landrace x Pietrain), aged 9-10 months, with body weight of 110-130 kg. Females were naturally bred on the second day of oestrus. Stage of pregnancy was detected by ultrasound scanning performed before slaughtering. Additionally, pregnancy status was confirmed by the presence of embryos obtained after flushing of uterine horns with 20 ml of sterile physiological saline.

The choice of 14-16 days of pig pregnancy resulted from crucial role of implantation process exerted in pregnancy maintenance. All tissues: pituitary and medial basal hypothalamus (MBH) were individually collected no later than 10 minutes after slaughter. The pituitary gland was dissected out from sella turcica located in the hollow of the sphenoid bone and AP lobe was separated from posterior neural lobe. MBH was defined as a block of tissue bordered rostrally by the optic chiasma, caudally by the mammillary body, laterally by the hypothalamic sulci and dorsally by 5 mm in-depth cut. Four isolated AP glands were individually placed in sterile Eagle's Medium (Biomed, Poland) containing antibiotics (penicillin and streptomycin; Polfa, Poland) and nystatin (Sigma-Aldrich, USA) and after enzymatic dispersion used for culture. For real-time PCR (qRT-PCR) analysis AP as well as MBH (as positive controls) excised from one pig were rapidly frozen in liquid nitrogen and stored at -80°C until extraction of total RNA (Sigma-Aldrich, USA). Blood samples collected from all pigs were centrifuged and stored at -20°C for the leptin plasma RIA assay.

Tissue isolation and culture of anterior pituitary cells

AP tissues from each animal were enzymatically dispersed and then cultured as described previously (Bogacka et al. 2002). The number and viabile cells was determined using a haemocytometer and trypan blue (MP Biomedicals, LLC, Santa Ana, CA) exclusion test. The viability of isolated cells exceeded 95%. The cells were diluted with McCoy's 5a medium (Sigma-Aldrich, USA) containing MEM-non-essential amino acids and vitamins (Sigma-Aldrich, USA), horse serum (10%; Biomed, Poland) and fetal calf serum (2.5%; Biomed, Poland) to $2x10^5$ cells/ml, finally placed into 24-well plates and incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. On the day of experiments cells were washed twice with fresh McCoy 5a medium and then incubated for 3.5 h in 1 ml of serum-free McCoy's 5a medium containing both 20 µM protease inhibitor – bacitracin (Sigma-Aldrich, USA) and respective tested substances: GnRH, leptin or both. As a positive control, cells were treated with 100 ng/ml of GnRH (Sigma-Aldrich, USA), whereas experimental incubations with 10^{-11}, 10^{-9}, 10^{-7}, 10^{-6} M leptin (Sigma-Aldrich, USA) were conducted alone or in co-treatment with 100 ng/ml of GnRH. For each experimental treatment three to six independent cells incubations were performed. After completing experimental protocol media were collected and stored at -20°C until assayed for LH. Cells were scraped, mixed with 1ml McCoy's 5a medium containing 0.2% Triton X-100 and bacitracin and obtained cellular suspension was homogenized using polytropic homogenizer (Janke & Kunkel, IKA Labortechnik, Germany) and frozen at -20°C until LH RIA.

RIA analysis

LH concentrations in the culture media and cellular homogenates were determined by RIA according to Ziecik et al. (1979) and Szafranska and Tilton (1993). The sensitivity of the assay was 0.08 ng/ml. The intra- and inter-assay coefficients of variation were 8.46% and 10.54%, respectively. Leptin concentrations in the plasma were determined using Multi-Species Leptin Radioimmunoassay Kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Assay sensitivity was 1 ng/ml; intra-assay coefficient of variation was 8.61 % (in one series).

Total RNA isolation, cDNA synthesis and real-time PCR analysis

Total RNA was extracted from the tissues (AP and MBH) using fenozol (A&A Biotechnology, Poland) in accordance with the manufacturer's instructions. RNA purity and yield were determined spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies Inc., USA). 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 using the Omniscript RT Kit (Qiagen, USA) at 37°C for one hour. A qRT-PCR analysis was performed according to method described previously (Siawrys and Smolinska, 2012) with the use of ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNA samples were amplified in a final reaction volume of 25 µl containing 1x SYBR Green PCR-Master Mix (Applied Biosystems, Foster City, CA, USA) and the respective primers (400 nmol/l) using the following cycling program: 2 min at 50°C, 10 min at 95°C, followed by 15 sec at 95°C and 1 min at 59°C for a total of 46 cycles. The following primer sequences were used: long form of leptin receptor (GenBank acc no. NM001024587) forward 5' AAT CCT CCA GGA GAG CTG TTC ACA C 3' and reverse 5' CTA GGC TCT TGA AGG CTT TCT CAC AT 3; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank acc no. U48832;) forward 5' CCT TCA TTG ACC TCC ACT ACA TGG T3' and reverse 5' CCA CAA CAT ACG TAG CAC CAG CAT C 3'. MBH was used as positive control for OB-Rb mRNA expression.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using the Statistica 6.0 PL program (Stat Soft Inc., USA) and significant differences were determined by one-way ANOVA followed by the least significant difference (LSD) *post hoc* test. A value of $p<0.05$ were considered to be statistically significant.

Results

Leptin plasma concentration

Plasma leptin concentration was determined by specific RIA method. Obtained results revealed that during implantation period mean serum leptin level reached 3.92±0.40 ng/ml.

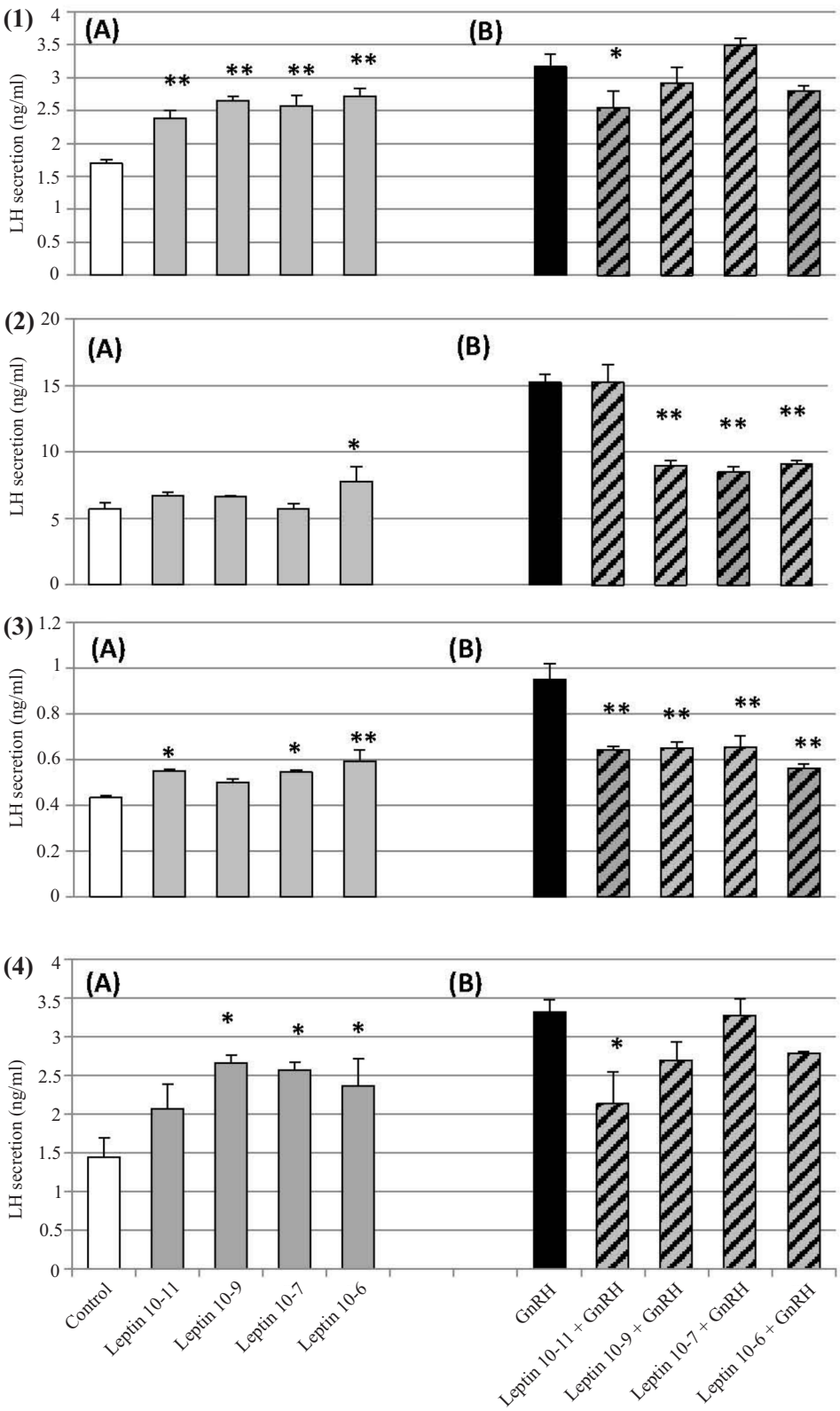

Fig. 1. The effect of leptin (10^{-11} – 10^{-6} M) on basal (A) and GnRH-induced (B) LH secretion by porcine AP cells on days 14-16 of pregnancy (1-4 = four independent experiments – separate analysis of individual pituitary primary cultures). Values are presented as the mean ± SEM. Control = basal release in the absence of treatment were 1.70 ± 0.06 ng/ml; 5.70 ± 0.49 ng/ml; 0.43 ± 0.01 ng/ml and 1.44 ± 0.26 ng/ml in four individual experiments, respectively. (A) *, ** different from Control ($p<0.05$ or 0.01); (B) *, ** different from GnRH alone ($p<0.05$ or 0.01).

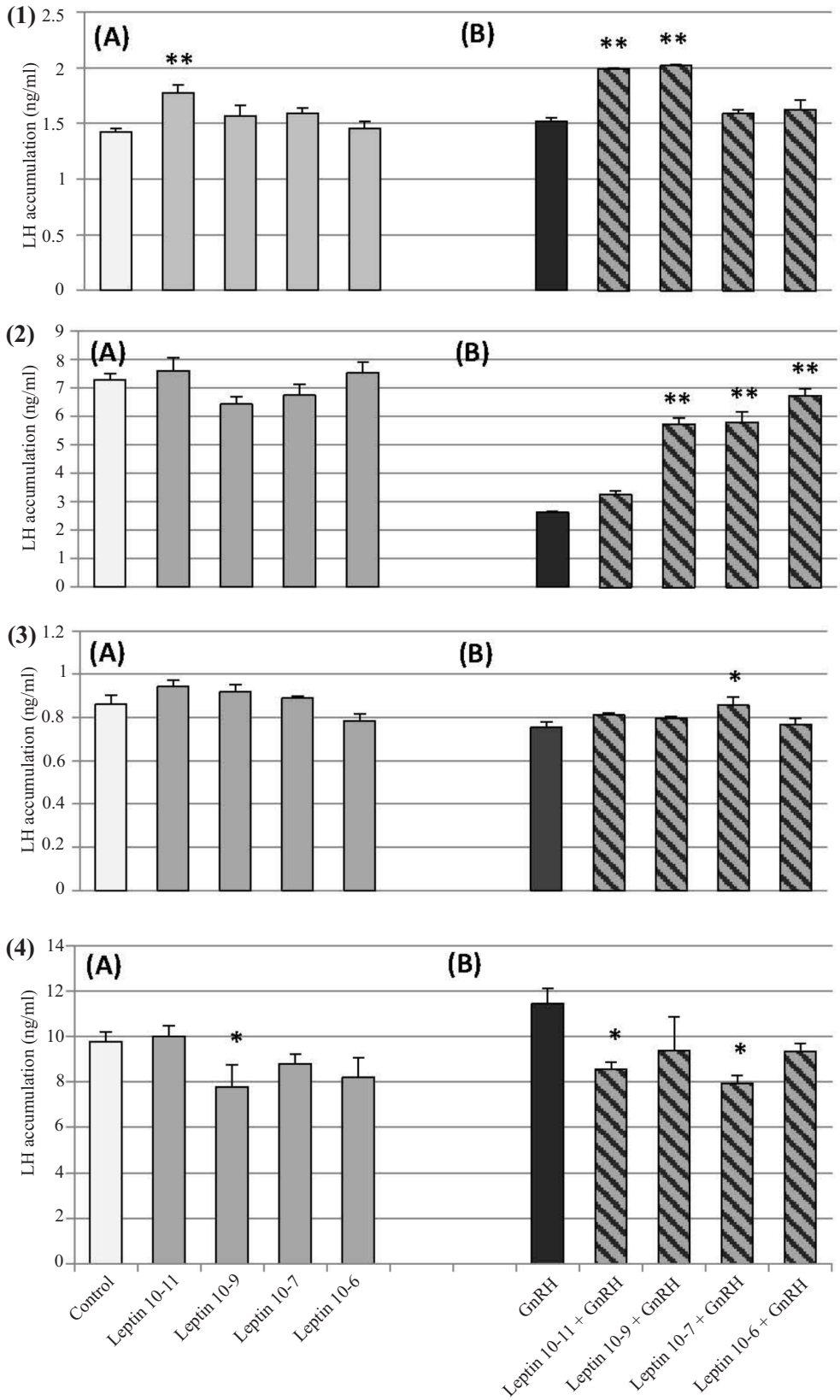

Fig. 2. The effect of leptin (10^{-11} – 10^{-6} M) on basal (A) and GnRH-induced (B) LH accumulation by porcine AP cells on days 14-16 of pregnancy (1-4 = four independent experiments – separate analysis of individual pituitary primary cultures). Values are presented as the mean ± SEM. Control = basal accumulations in the absence of treatment were: 1.43 ± 0.03 ng/ml; 7.28 ± 0.19 ng/ml; 0.86 ± 0.04 ng/ml; 9.76 ± 0.42 ng/ml in four individual experiments, respectively. (A) *, ** different from Control ($p<0.05$ or 0.01), (B) *, ** different from GnRH alone ($p<0.05$ or 0.01).

Effects of leptin on basal and GnRH-induced LH secretion from porcine AP cells *in vitro*

Obtained results indicate that, depending on individual pituitary primary culture, mean medium LH concentration ranged from 0.43 ± 0.01 ng/ml (culture 3) to 5.70 ± 0.49 ng/ml (culture 2) in control non-treated cells (Fig. 1). Exogenous leptin, at 10^{-6} M concentration, effectively activated LH secretion in all cultures tested, nevertheless a specific dose-response cells sensitivity was also observed. As shown in Fig. 1 only culture 1 responded positively for all tested leptin concentrations, whereas in culture 2 an enhanced LH secretion occurred only in the presence of 10^{-6} M leptin. Although in all cultures exogenous (100 ng/ml) GnRH significantly increased LH secretion in comparison to respective non-treated controls, a broad range of gonadoliberin-induced LH releasing activity was detected: with minimal 0.95 ± 0.07 ng/ml value found in culture 3 and maximal response, reaching 15.25 ± 0.58 ng/ml, found in culture 2. Presented data also revealed a down-regulatory effect of exogenous leptin on GnRH-induced LH secretion. This effect was observed in all cultures, however only culture 3 exhibited sensitivity to all leptin concentrations tested. In cultures 1 and 4 only 10^{-11} M leptin significantly reduced LH secretion as compared to respective GnRH-treated group.

Parallel to the analysis of LH secretion (ng/ml) in individual pituitary cell culture, a relative data representing a percent of leptin-induced changes vs control or GnRH-only treated groups (both taken as 100%, respectively) is also presented. As shown in Fig. 3, an up-regulatory effect of leptin was observed at all concentrations tested but statistical significance was achieved at three of them with basal LH secretion enhancement by 40%, 36% and by 48% found in the presence of 10^{-9}, 10^{-7} and 10^{-6} M of exogenous leptin, respectively. In contrast, pooled data concerning effects exerted on GnRH-stimulated LH release revealed that at all concentrations leptin reduced GnRH-induced LH secretion from these cells although only at 10^{-6} M that decrease (by 28%) reached statistical significance.

Effects of leptin on basal and GnRH-induced intracellular LH accumulation

At the level of basal LH accumulation exogenous leptin exerted a differentiated effect: with no influence observed in cultures 2 and 3, an up-regulatory action found in culture 1 (only at 10^{-11} M) and down-regulatory effect observed in culture 4 (only at 10^{-9} M). In the presence of GnRH, leptin was able to stimulate LH accumulation in pig anterior pituitary cells and individual cultures exhibited their specific leptin dose-sensitivity. As shown in Fig. 2 culture 2 responded positively to 10^{-9} to10^{-6} M leptin concentration, whereas in culture 3 only at 10^{-7} M leptin was effective. Interestingly, in culture 2 leptin effect on GnRH-induced LH secretion and LH intracellular accumulation was inversely-related. Indeed, its down-regulatory (at 10^{-9} 10^{-7} and 10^{-6} M) action exerted on GnRH-stimulated LH secretion (Fig. 1) was accompanied by simultaneous up-regulatory effect of leptin on LH accumulation in anterior pituitary cells (Fig. 2). In turn, in culture 4 leptin in the presence of GnRH down-regulated LH accumulation. In pooled data analysis no effect of leptin on basal and GnRH-stimulated LH intracellular accumulation was found (Fig. 3).

Localization of OB-Rb gene expressions in porcine AP gland

OB-Rb transcript was detected in porcine AP gland and in MBH, which served as a positive control, on days 14-16 of pregnancy. The negative control sample (NTC) did not reveal products of the qRT-PCR reaction. The obtained transcripts were sequenced (ABI Prism 3777 DNA sequencer) at the Institute of Biochemistry and Biophysics of the Polish Academy of Science and 95% homology to porcine OB-Rb sequence was confirmed.

Discussion

Presented results indicate a differentiated dose-sensitivity of individual anterior pituitary culture for exogenous leptin stimulation in respect to basal and GnRH-stimulated LH secretion and intracellular accumulation. Moreover, a dual and dose-dependent action of leptin on LH secretion was found with up-regulatory effect found when applied alone and down-regulatory when given in the presence of GnRH.

Presented data are in accordance with previous studies which indicated stimulatory action of leptin on basal LH release from pig AP gland (Barb et al. 2004, Nonaka et al. 2006). LH-activating leptin activity was also reported in anterior pituitary cells of female (Ogura et al. 2001, Tezuka et al. 2002) and male (Yu et al. 1997) rat hemi-anterior pituitaries. Pro-gonadotrophic stimulation was also shown in female rats at the time of spontaneous and steroid-induced LH surge (De Biasi et al. 2001) and leptin

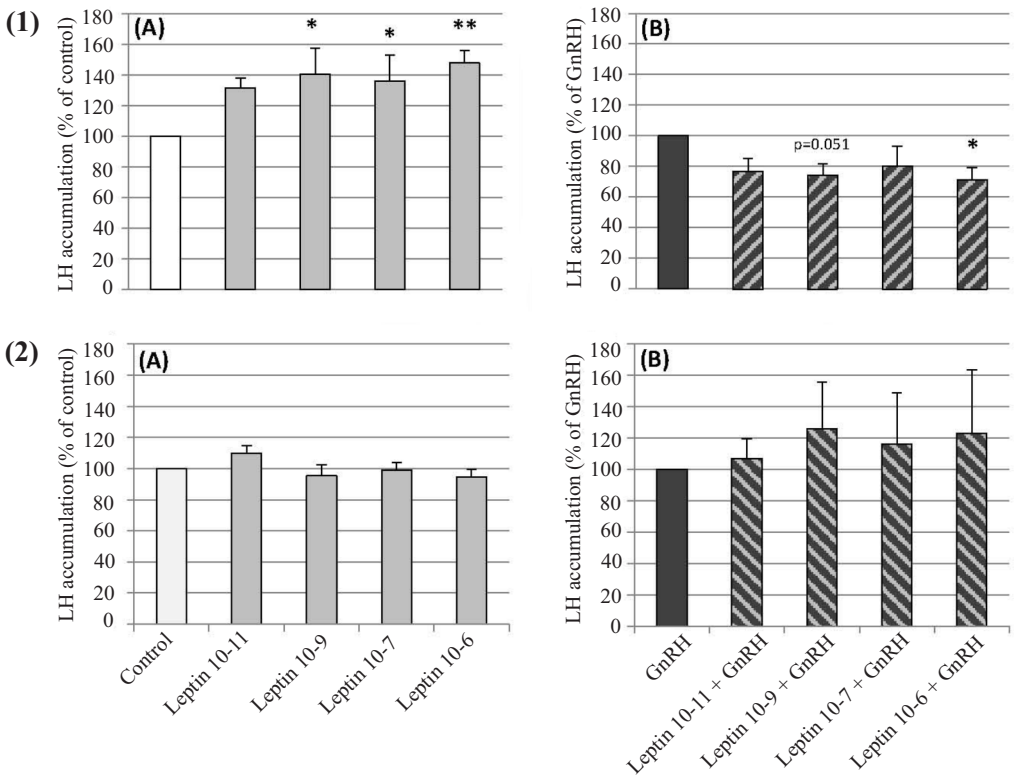

Fig. 3. The effect of leptin (10^{-11} – 10^{-6} M) on basal (A) and GnRH-induced (B) LH secretion (1) or accumulation (2) by porcine AP cells on days 14-16 of pregnancy (a pooled analysis of four independent experiments). Values are presented as a percentage (mean ± SEM) of basic LH release (1) or accumulation (2) established in (A) control group of cells (= 100%) or (B) GnRH (without leptin) group of cells (= 100%). (1) Control = basal release in the absence of treatment was 2.32 ± 1.16 ng/ml, GnRH-induced release (without leptin) was 5.67 ± 3.24 ng/ml. (A) *, ** different from Control ($p<0.05$ or 0.01); (B) * different from GnRH alone ($p<0.05$). (2) Control = basal accumulation in the absence of treatment was 4.83 ± 2.19 ng/ml, GnRH-induced accumulation (without leptin) was 4.08 ± 2.48 ng/ml. (A) Leptin-treated cultures were not different from Control; (B) Leptin-treated cultures were not different from GnRH alone.

concentrations maximally effective in basal LH release stimulation were similar to levels in juvenile, pubertal and mature female rats serum (Ogura et al. 2001, Tezuka et al. 2002).

Some data indicate a broad-range pituitary cells sensitivity to leptin stimulation. Indeed, leptin at concentration from 10^{-14} to 10^{-6} M, except for 10^{-12} and 10^{-7} M was shown to increase basal pituitary LH release (Barb et al. 2004) in prepubertal gilts. However, in another study on prepubertal barrows/gilts, leptin only at 10^{-8} and 10^{-7} M concentration evoked a stimulatory LH response (Nanoka et al. 2006). Also *in vitro* studies revealed that, at concentration from 10^{-11} M to 10^{-7} M of leptin increased basal LH release from juvenile female rat AP cells and observed effects showed a bell-shaped dose-response curve (Tezuka et al. 2002). In our present study, however, a culture-specific pattern of response for different leptin concentrations was observed with a 10^{-6} M being effective in LH basal release stimulation in four individual pituitaries-derived cultures.

Our individual and pooled data analysis revealed that in contrast to up-regulatory effects exerted by leptin given alone, its co-administration with gonadoliberin diminished GnRH-evoked LH releasing activity. Similarly, in study on prepubertal gilts Barb et al. (2004) reported a down regulatory activity of exogenous 10^{-7} M leptin on 10^{-8} M GnRH-stimulated LH release as well as 10^{-13} M leptin on 10^{-9} M GnRH-induced LH release whereas at other leptin concentrations tested no such effect was observed. Also in sows, leptin at concentration of 10^{-9} – 10^{-7} M increased, at 10^{-6} M decreased, whereas at 10^{-11} – 10^{-10} M did not affect GnRH-induced LH secretion from pituitary cell culture during follicular phase estrous cycle (Kosior-Korzecka and Bobrowiec 2007). Furthermore, stimulatory influence of leptin on GnRH-induced LH release from AP pituitary cells was reported in juvenile (Tezuka et al. 2002) and pubertal (Ogura et al. 2001) rats and observed effect expressed as a bell-shaped dose-response curve. No leptin activity was found in respect to the

modulation of LH release from GnRH-activated hemi-anterior pituitaries of adult male (Yu et al. 1997) as well as proestrus rats (Peters et al. 2009). Leptin was also shown to enhance GnRH-mediated secretion of LH from AP explants of normal-fed, but not of fasted, cows (Amstalden et al. 2003). Taken together, available data clearly indicate the existence of the precise mechanism responsible for physiological status-dependent gonadotrophic cells sensitivity for leptin stimulation.

In pooled data analysis no effects of leptin on basal and GnRH-induced LH accumulation in AP cells were shown. Opposite results were reported in rats in which increase of basal and GnRH-induced cellular LH level was dependent on applied leptin concentration (Ogura et al. 2001, Tezuka et al. 2002) with 10^{-10} M being effective in basal whereas 10^{-10}, 10^{-9} and 10^{-8} M in GnRH-induced stimulation of intracellular LH accumulation (Ogura et al. 2001). An existing differences between studies concerning gonadotrope cells response for exogenous leptin stimulation may result both from age and reproductive/endocrine status of tested animals, their metabolic state as well as from specific applied experimental protocol (e.g. type and duration of cell culture, doses of treatments). Moreover, also specific cellular composition of each culture might contribute for their experimentally observed differentiated leptin dose-sensitivity. Nevertheless, taking collectively, available data clearly indicate that leptin participates in the regulation of LH release from gonadotrope cells and its effect is dual: stimulatory, when related to basal secretion, but can be inhibitory in respect to GnRH-mediated release.

Leptin acts on LH secretory activity via its specific receptor and is able to autoregulate its own receptors expression (Di Yorio et al. 2008) and pituitary-derived leptin may act as an autocrine/paracrine factor within AP gland. Studies, primarily on ruminants and laboratory rodents, have established that leptin receptor is expressed in all types of AP cells, including gonadotropes (Iqbal et al. 2000, Sone et al. 2001). However, to date no data on leptin receptor localization in porcine AP specific cell subtypes are available. Present research confirmed the presence of OB-Rb in the AP gland in the pregnant gilts. In previous studies both long and short isoforms of leptin receptor in AP gland in the pregnant as well as cyclic pigs were detected (Bogacka et al. 2006, Siawrys et al. 2007) and changes in leptin receptor expression observed during different stages of gestation and estrous cycle were suggested to be related to the physiological fluctuation of gonadal steroid status in these animals (Kaminski et al. 2006, Siawrys et al. 2007).

In our study also leptin plasma level in pregnant gilts was determined. Obtained result revealed that in contrast to well recognized pattern found in humans and rodents, porcine leptin plasma concentration has not changed during the estrous cycle as well as between gestation and the cycle (unpublished own data). Serum leptin concentration varied significantly during the menstrual cycle in women with higher leptin levels observed in the luteal than in the follicular phase (Ahrens et al. 2014). Cycle-dependent changes in leptin concentration have been also reported in female rats with a peak value appearing at proestrous (Fungfuang et al. 2013) what indicates a cycle-specific interplay among leptin and reproductive hormones (E_2, P_4, LH, FSH) concentrations (Ahrens et al. 2014). In humans and rodents serum leptin levels were shown to increase during pregnancy due to enhanced leptin synthesis by both maternal and fetal adipose tissues as well as by trophoblast (Henson and Castracane 2000). In pregnant sows, however, serum leptin concentration was not sensitive to body weight enhancement (Estienne et al. 2003, Metges et al. 2012) what indicates a differences in leptin physiology between species.

In conclusion, in this study we found that anterior pituitary primary cultures derived from individual pituitaries obtained from pigs at early pregnancy maintain a differentiated dose-sensitivity to exogenous leptin stimulation in respect to LH secretion. Collectively, a dual and dose-dependent action of leptin on LH secretion was found with up-regulatory effect observed when applied alone and down-regulatory when given in the presence of GnRH. These data indicate that leptin alone, as well as in combination with gonadoliberin, can specifically, at the pituitary level, modulate LH secretory activity during early pregnancy in pigs.

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