INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino-acid tyrosine-rich peptide, which was first isolated from porcine brain extracts in 1982 (1). It belongs to “NPY family” of biologically active peptides, which also includes two gut hormones: peptide YY (PYY) and pancreatic polypeptide (PP) (1). In the pig, NPY is widespread in the structures of central nervous system (CNS; e.g. the hypothalamoneurohypophysial tract), as well as those innervating peripheral organs (e.g. ovary) (2, 3). The hypothalamus is considered to be the primary site for this neuropeptide production (2, 4-7). In cyclic pigs, much higher content of NPY was found in stalk-median eminence (SME) than in the preoptic area (POA) or medial basal hypothalamus (MBH) (4). From SME, NPY is transported via the hypophysial portal blood circulation to the anterior pituitary (AP). The immunoreactivity of NPY has been confirmed in the porcine pituitary gland (2). In addition, NPY mRNA or NPY-immunoreactivity was localized in different rat AP cells, i.e. thyrotropes (8) gonadotropes, somatotropes, corticotropes and lactotropes (9).

Neuropeptide Y affects target cells by activating various G-coupled receptors belonging to the rhodopsin-like superfamily (class 1) of receptors. In mammals, five subtypes of NPY receptors (Y1, Y2, Y4, Y5 and y6) were described (10, 11), but y6 receptor appeared to be functional only in mice and rabbits (11). NPY exhibits strong affinity to the Y1, Y2 and Y5 receptors (12, 13). NPY receptors are widely distributed in the porcine CNS, with predominant localization in the limbic system, olfactory system, hypothalamoneurohypophysial tract, corpus striatum and cerebral cortex (2), while data on NPY receptor subtypes in the pituitary gland are still lacking.

NPY system (the peptide and its receptors) is involved in the regulation of many physiological processes. One of the main roles of NPY is hypothalamic stimulation of food intake and control of energy balance, but recent studies have shown that it also takes part in the regulation of reproductive processes (14-18). Several in vivo and in vitro studies carried out mainly in rodents and ruminants reported that NPY may regulate LH secretion at the level of the hypothalamus by direct and/or indirect modulation of gonadotropin-releasing hormone (GnRH) system activity (15, 19-25). Numerous in vitro studies also indicated that the action of NPY on LH secretion may also take place at the anterior pituitary level in rats, rabbits, monkeys, but not necessarily in heifers and ewes (21, 26-32). In prepupal...
gilt, NPY did not change the basal and GnRH-induced LH secretion from AP cells (33). In mature, preovulatory gilt, NPY only decreased GnRH-induced LH secretion (34). However, studies pertaining to a role of NPY in the regulation of LH secretion at the pituitary level has not been carried out in pregnant pigs.

It has been suggested that NPY as an orexigenic peptide and simultaneously involved in the modulation of GnRH/LH system (as stated above) could be a link between nutrition and reproduction (15, 18). Numerous studies indicate that nutritional deficiency or excess can negatively affect functioning the reproductive system in cyclic as well as pregnant females (17, 35, 36). Pregnancy is associated with a positive energy balance, primarily due to an increase in food intake and it seems that the elevated activity of hypothalamic NPY system might be partly responsible for the physiological hyperphagia observed during pregnancy (37-39). Moreover, the plasma concentration of NPY appeared to be significantly higher in pregnant than in non-pregnant women (40). Collectively, these data suggest a potential participation of NPY in the physiological regulations associated with gestation period, including its influence on functioning the reproductive axis. During early gestation, progesterone (P₄) secretion by corpora lutea is necessary to maintain pregnancy and it has been demonstrated that in pigs on day 14 of pregnancy, LH begins to affect this steroid secretion (41). Moreover, one of the most critical period connected with the effectiveness of pregnancy in this species are days 14 – 16, when an implantation of embryos is undertaken (42). It has been reported that survival of porcine embryos is reduced by 17% within the first 18 days of pregnancy (43). For this reason, the regulation of LH secretion during this stage of pregnancy in the pig still requires better elucidation. We hypothesize that NPY is involved in the control of LH synthesis/secretion at the pituitary level during early gestation in pigs. Therefore, to verify this hypothesis, the present study was carried out with gilts on days 14 – 16 of pregnancy and aimed to: 1) examine the effects of NPY on basal and GnRH-induced LH secretion by isolated pituitary cells as well as LH contents in these cells and 2) detect the expression of gene coding for NPY and its receptors (Y1 and Y2) in the AP gland. Additionally, the presence of transcripts for genes encoding the β subunit of LH (β-LH) and GnRH receptor (GnRH-R) in the AP tissue was determined.

MATERIALS AND METHODS

Animals

The experimental animals were crossbred postpubertal pigs (n = 5), 9 – 10 months of age, with body weight of 110 – 130 kg. Females were naturally bred on the second day of estrus. 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 was connected with the beginning of implantation and its importance for the pregnancy maintenance.

The experiments were performed in accordance with the principles and procedures of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn, Poland.

Anterior pituitary collection

The pituitary glands were individually collected no later than 10 minutes after the animal slaughter. Each of 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. AP glands were individually placed in sterile Eagle’s Medium (Biomed, Poland) containing antibiotics (penicillin and streptomycin; Polfa, Poland) and nystatin (Sigma-Aldrich, USA) for in vitro study or frozen in liquid nitrogen and transported to the laboratory for further analysis.

Experimental procedures

Tissue isolation and culture of anterior pituitary cells

AP tissues from each animal (n = 4) were enzymatically dispersed and then cultured as described previously (44, 45). The number and viable cells were determined using a haemocytometer and trypan blue (MP Biomedicals, LLC, Santa Ana, CA) exclusion test. The viability of isolated cells reached approximately 95%. The cells were dispersed in 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 2 × 10⁵ cells/ml, finally placed in 24-well plates and preincubated for 72 hours at 37°C in a humidified atmosphere with 5% CO₂. On the day of experiment, the 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 tested substances: GnRH (positive control), NPY or both. The cells were treated, or not (control), with NPY (Sigma-Aldrich, USA) at concentrations 10⁻⁸, 10⁻⁷, 10⁻⁶ M alone or in combination with 100 ng/ml of GnRH (Sigma-Aldrich, USA). After completion of the culture, media were collected and stored at –20°C until assayed for LH. Cells were scraped, mixed with 1 ml McCoy’s 5a medium containing 0.2% Triton X-100 and bacitracin, thereafter obtained cellular suspension was homogenized using polytropic homogenizer (Janke & Kunkel, IKA-Labortechnik, Germany) and frozen at –20°C until LH radioimmunoassay (RIA).

Radioimmunoassay analysis

LH concentrations in the culture media and cellular homogenates were determined by RIA according to Ziecik et al. (46) and Szafranska and Tilton (47). The sensitivity of the assay was 0.08 ng/ml. The intra- and inter-assay coefficients of variation were 7.21% and 10.54%, respectively.

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

Total RNA was extracted from the AP 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 (Quagen, USA) at 37°C for one hour. The real-time PCR analysis with the use of ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) was performed according to method described previously (48). cDNA samples were amplified in a final reaction volume of 25 µl containing 1 × SYBR Green PCR-Master Mix (Applied Biosystems, Foster City, CA, USA) and the respective primers using the following cycling program: 10 min at 95°C, followed by 15 s at 95°C, 1 min at 60°C or 55°C and 1 min at 72°C for a total of 40 cycles. The primer sequences and condition used in the study are showed in Tables 1 and 2.
**RESULTS**

In vitro effects of neuropeptide Y on basal and gonadotropin-releasing hormone-induced luteinizing hormone secretion or basal and gonadotropin releasing hormone-induced intracellular luteinizing hormone accumulation in porcine anterior pituitary cells

At all concentrations, exogenous NPY did not affect basal LH secretion (Fig. 1A). The lowest dose of NPY (10⁻⁸ M) significantly reduced (by 33%), while higher doses of NPY (10⁻⁷ and 10⁻⁶ M) only tended to inhibit GnRH-induced LH release when compared to the action of GnRH alone; by 26% and 20% (Fig. 1B). GnRH alone stimulated LH secretion by 119% in comparison to the control, but NPY (10⁻⁷ and 10⁻⁶ M) co-administered with GnRH (100 ng/ml) to the culture reduced this increase to 58% and 73%, respectively (Fig. 1B).

NPY altered neither basal nor GnRH-induced LH accumulation in the cells (Fig. 2). GnRH alone also did not affect LH accumulation comparing to the control (Fig. 2B).

The gene expression of neuropeptide Y and its receptors, β-luteinizing hormone and gonadotropin-releasing hormone receptor in porcine anterior pituitary gland

NPY and its receptor Y2 (the results for Y1 were not conclusive) as well as β-LH and GnRH-R transcripts were detected in the porcine AP gland on days 14 – 16 of pregnancy (Table 2). The products of real-time PCR were not revealed in the negative control samples (NYC).

**DISCUSSION**

The present study indicates that NPY may participate in a modulation of LH secretion at the level of anterior pituitary, but not affect the cellular accumulation of this hormone, in early pregnant gilts (on days 14 – 16). Moreover, expression of the genes coding for NPY and one of its receptors (NPY Y2) was established.

**Statistical analysis**

Statistical analysis of data from in vitro studies was performed using the Statistica 13.1 PL program (Stat Soft Inc., USA) and significant differences were estimated by one-way analysis of variance (ANOVA), followed by Duncan test. All data were presented as the percentage (mean ± SEM) of basic secretion/accumulation (= 100%), established in control (or negative control samples (NTC)). The products of real-time PCR were not revealed in the negative control samples (NYC).
conducted with intact prepuberal gilts did not reveal any influence of NPY on both basal and GnRH-induced LH secretion. Studies of Elsaesser (34) and our results imply that an action of NPY (inhibitory) on LH release at the AP level in adult preovulatory and pregnant pigs requires concomitant activation of GnRH receptors. In the present study, we have confirmed transcriptional activity of genes encoding both GnRH-R and β-LH in pituitary gland of gilts on days 14 – 16 of pregnancy. Changes in pituitary expression levels of both genes or gene of GnRH-R have been shown in cyclic gilts or seasonal breeding swine (a wild boar and pig crossbred), respectively (49, 50). It seems that mechanism of inhibitory action of NPY on LH secretion found in our study may involve some changes (down-regulation) in concentration of GnRH receptors on gonadotropes. Generally, this assumption can be supported by studies of Parker et al. (51) performed with the rat, in which NPY potentiated GnRH-induced LH release by enhancing the number of GnRH binding sites on pituitary gonadotropes. Comparing results of studies conducted with mature gilts (preovulatory and pregnant) with those performed with prepubertal females, it seems that connection between NPY and LH secretion is dependent on the presence of steroid hormones. This suggestion is in line with the lack of NPY action on LH secretion by AP cells in prepubertal gilts. It is likely, that inhibitory effect of NPY on LH secretion - observed in our study on days 14 – 16 of gestation - may be connected with temporary slight reduction in P₄ plasma concentration. It was shown that in the pig during the first month of pregnancy, P₄ concentration in plasma enhances until day 12 of pregnancy, then slightly decreases until day 22 and stabilizes thereafter (52). In addition, it is worthy to emphasize that, in the present study, the expression of gene encoding NPY was confirmed in the porcine pituitary during early gestation, however open question remains how this expression is regulated under physiological or experimental conditions. Collectively, our and quoted above studies confirmed inhibitory potential of NPY in the modulation of LH secretion at the pituitary level in the pig and also revealed additional questions which require elucidation in further studies.

The action of NPY on LH secretion at the anterior pituitary level was also documented in other species, including rodents, monkeys and rabbits. In rats, different effects of NPY on LH secretion by isolated pituitary cells or explants were observed. A stimulatory influence of the peptide on basal secretion of LH was noted in OVX (53), proestrus (28) and immature with induced ovulation (54) rats, but in other studies this peptide was ineffective in diestrus (55), as well as in OVX (56) and proestrus (29) animals. As far as GnRH-induced LH secretion is considered, in most cases NPY had stimulatory (27,29, 56, 57) and sometimes inhibitory effects (58) or was without any influence (27). The stimulatory action of NPY on GnRH-induced LH release by AP cells was primarily found in proestrus rats, whereas suppressive in metestrus rats and it
seems that this neuropeptide exerts a positive influence to generate the preovulatory LH surge (27, 58). In turn, NPY augmented basal LH secretion by AP cells of intact macaque monkeys, but failed to alter it in the presence of GnRH as well as by the cells derived from OVX monkeys (26). It is therefore likely that, in this species, NPY may act at the AP level to enhance LH release in the presence of ovarian hormones without affecting the sensitivity of gonadotropes to GnRH stimulation. In rabbits, NPY caused a sustained or transient stimulation of LH release by pituitary fragments from intact and OVX rabbits, respectively (21). Interestingly, NPY failed to alter LH release at the AP level in ruminants under different conditions (30-32). The failure of the direct influence of NPY on: basal LH release in steers (30) and anestrous sheep (32) as well as GnRH-induced LH release in steers (30), anestrous ewes (32), OVX heifers and heifers in follicular and luteal phases of the estrous cycle (31) suggests that in the ruminants this neuropeptide does not significantly contribute to the physiological regulation of LH release at the level of the pituitary gland. Collectively, our present results and reported by others (21, 26-32, 56-58), indicate an existence of considerable differences between species in effects of NPY on LH secretion at the pituitary level.

The differences observed in studies, concerning the NPY influence on LH secretion in vitro may result from species, age and endocrine status (e.g. steroid milieu) of tested animals as well as from specific methodological procedures applied (e.g. cultures of isolated cells or tissue, static or perfusion culture systems, duration of culture and various doses of treatments). Moreover, final effect of NPY on LH secretion by pituitary cells may depend on the NPY receptor subtypes involved in its action. Although at least four functional Y receptor subtypes (Y1, Y2, Y4 and Y5) have been identified in mammals, their participation in the regulation of reproductive axis function remains poorly defined. In female rats, the presence of Y1, Y2 and Y5 mRNAs in the AP gland was demonstrated (59, 60) and it was suggested that the up-regulation of Y1 receptor by estrogens may enable an augmentation of GnRH-induced LH secretion in response to NPY (59). It was also considered that, in this species, Y2 receptors may be involved in stimulatory actions of NPY on LH secretion from the pituitary in the presence of GnRH (22). Previous studies generally revealed the presence of NPY receptors in the porcine hypothalamus, infundibulum and neurohypophysis, but not adenohypophysis (2). In the current study, the expression of NPY Y2 receptor was found in the porcine AP gland. This suggests a participation of the receptor in the modulation of LH secretion from anterior pituitary cells by NPY. Nevertheless, further experiments concerning an involvement of particular subtypes of NPY receptors in the regulation of LH secretion at the pituitary level are needed.

It has to be mentioned that NPY is also involved at the AP level in the modulation of other pituitary hormones secretion.
This neuropeptide stimulates basal GH secretion in vitro from AP cells of prepubertal gilts, whereas increases or inhibits this hormone secretion by the cells in the presence of higher and lower doses of GHRH, respectively (33). Neuropeptide Y has also affected the pituitary in vitro secretion of FSH in rats (27, 58, 61) and rabbits (21), PRL in rats (55, 59), but did not exert any effect on the secretion of ACTH in sheep (62) and rats (63) as well as TSH in rats (64). Collectively, the results of our study and quoted above papers evidently confirm participation of NPY in para- and/or autocrine regulation of secretory activity of the anterior pituitary gland.

The hypothalamus is considered to be a primary site of NPY action on the activity of GnRH/LH system. Barb et al. (18) revealed that central administration of NPY decreases serum LH concentrations and LH pulse frequency in OVX prepubertal gilts. Generally, based on studies performed with different species, there are many reports indicating that this neuropeptide is involved in the regulation of LH secretion at the hypothalamic level by modulating the activity of GnRH neuronal system (15, 18-23, 54). Morphological studies have demonstrated that NPY neurons come in close contact with GnRH neurons in the medial preoptic area - POA (the principal site of GnRH synthesis), the arcuate nucleus of the hypothalamus (a major hypothalamic site of NPY expression and the site of the GnRH pulse generator) and in the median eminence - ME (the site of GnRH release into the portal vessels delivering hypothalamic regulatory substances to the anterior pituitary) (65-69). NPY is able to exert both stimulatory and inhibitory actions on GnRH neurons, which appeared in many studies to be steroid-dependent (20, 21, 26, 54, 70). It was suggested that mainly NPY Y1 and Y2 receptors are involved in the control of GnRH secretion (22, 25, 70, 71), although the contribution of Y4 and Y5 is not excluded (72-74). Moreover, NPY also like another appetite hormone e.g. orexin-A, may take part in the metabolic control of reproduction (23, 75-77). This neuropeptide is considered to be an important regulator of the nutrition-induced suppression of GnRH/LH system and its inhibitory action during undernutrition may be associated with elevated hypothalamic expression of NPY mRNA (75). The increased level of mRNAs for NPY or NPY receptors in the hypothalamus was also reported pregnant females (37-39, 75) and it could be partly responsible for the physiological hyperphagia (39) as well as may participate in the reduction of GnRH/LH secretion during pregnancy (e.g. observed in the present study). Therefore, our and other studies collectively confirm that the NPY action at the anterior pituitary level connected with the modulation of LH secretion plays a complementary role in relation to its participation in the hypothalamic control of the GnRH/LH system.

In conclusion, the present study indicates inhibitory effect of NPY on LH secretion by porcine pituitary cells in response to GnRH, but does not confirm any effect on cellular accumulation of this gonadotropin on days 14 – 16 of pregnancy. The obtained results suggest the participation of NPY in the modulation of LH secretion at the pituitary level in pigs during early pregnancy. The action of NPY seems to be exerted, at least partially, via NPY Y2 receptors, but this problem needs further more detailed studies including the use of specific agonists and/or antagonists of all NPY receptor subtypes.

Acknowledgements: This research was financed by the Ministry of Science and Higher Education (project: N N311 098634), grant from University of Warmia and Mazury in Olsztyn (020600.0205) and statutory funds (project: No. 12.610.005-300).

Conflict of interests: None declared.

REFERENCES


52. Hoving LL, Soede NM, Feitsma H, Kemp B. Embryo survival, progesterone profiles and metabolic responses to an increased feeding level during second gestation in sows. *Theriogenology* 2012; 77: 1557-1569.


Received: April 24, 2018
Accepted: October 30, 2018

Author’s address: Prof. Gabriela Siawrys, Department of Animal Anatomy and Physiology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, 1A Oczapowskiego Street, 10-719 Olsztyn-Kortowo, Poland. E-mail: gabri@uwm.edu.pl