

The effects of estradiol on β -endorphin, GnRH and galanin content in the oviduct and the uterus of ovariectomized gilts

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SUMMARY

Steroid hormones are known to affect synthesis and/or release of some peptides in the central nervous system and peripheral tissues. In the present study we determined changes in β -endorphin, GnRH and galanin contents in uterine and oviductal tissues of ovariectomized (OVX) gilts following treatment with estradiol benzoate (EB) at a dose inducing a preovulatory-like LH surge. Seven month old gilts (90-100 kg of body weight; BW) were used in the study. Four weeks after ovariectomy, experimental animals were injected intramuscularly with EB (15 μ g/kg BW) at 24 h (n = 5), 48 h (n = 6) or 72 h (n = 5) before slaughter. Three control gilts received corn oil vehicle. Tissues were sampled from the ampulla and isthmus of the oviduct and from the perioviductal, middle and paracervical regions of the uterine horn for determination of β -endorphin, GnRH and galanin content. Signifi-

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cant increases of β -endorphin content were found in all regions of the uterus either 24 h or 48 h after priming with EB. In oviductal tissue, β -endorphin concentration only tended to increase in response to EB. GnRH content in tissues originating from gilts receiving EB fluctuated from a stimulation in the ampulla of the oviduct and in the paracervical uterus to an inhibition in the middle part of the uterus. A significantly increased concentration of galanin in response to EB was observed exclusively in the paracervical part of the OVX pig uterus. The results suggest an involvement of β -endorphin, GnRH and galanin in the regulation of uterine function in pigs during the periovulatory period. *Reproductive Biology* 2003 3 (1): 63-80 .

Key words: pigs, estradiol, β -endorphin, GnRH, galanin, uterus, oviduct, ovariectomy

INTRODUCTION

In mammalian females, including sows, an increase in the plasma estrogen concentration during the late follicular phase leads to an initiation of the preovulatory LH surge [11] as well as structural and functional changes within the reproductive tract [13]. Estradiol administration to ovariectomized (OVX) gilts affects the GnRH/LH system, initially inducing a negative feedback, lasting approximately 50 hours, which is followed by a positive feedback and the preovulatory-like LH surge [2, 46]. In the central nervous system, estrogens may also influence activity of other peptidergic systems, e.g. β -endorphin, VIP, NPY and galanin [45]. Additionally, many neuropeptides appeared to be synthesized in peripheral tissues, including female reproductive tract, where they are suspected to participate in paracrine and/or autocrine regulations. The presence of β -endorphin [15, 16, 18], GnRH [16, 17], VIP and NPY [43] was described in tissues of the porcine uterus or in its secretions. Moreover, in female rats, galanin mRNA was established in the uterus, where its content fluctuated in response to exogenous estrogens [41]. In OVX gilts, a single injection of estradiol benzoate (EB), inducing a preovulatory-like LH surge, was able to affect both VIP and NPY concentrations in certain regions of the oviduct and/or the uterus [43]. However, prolonged priming of OVX gilts

with 17 β -estradiol neither influenced β -endorphin [15] nor GnRH [17] concentrations in uterine fluid.

The objective of the present study was to test whether priming of OVX gilts with EB, at a dose inducing a preovulatory-like LH surge, may change tissue content of β -endorphin, GnRH and galanin in the uterus and the oviduct.

MATERIALS AND METHODS

Chemicals

Estradiol benzoate was obtained from Polfa (Kutno, Poland). The antisera against β -endorphin (RAS 8616N), GnRH (RIN 7201), and galanin (RAS 7153) were purchased from Peninsula Laboratories Inc. (Belmont, CA, USA). The rabbit antisera against estradiol (BS/88/754) and porcine LH (Sz/Z/89/396) as well as the sheep antibody against rabbit γ -globulins (BS/86/o-L), used as the secondary antisera in radioimmunoassays of the peptides and LH, were described by Szafrńska et al. [37]. [2,4,6,7-³H] estradiol was from Amersham, UK. Radioactive iodine (¹²⁵I) for labeling the peptides and LH was purchased from Perkin-Elmer (Brussels, Belgium). Porcine β -endorphin and galanin were purchased from Peninsula Laboratories Inc. (Belmont, CA, USA). Other reagents were from Sigma (St. Louis, MO, USA) or ICN Biomedicals Inc. (Irvine, CA, USA).

Animals and experimental procedures

Crossbred gilts ovariectomized at approx. seven months of age (90-100 kg of body weight; BW) were used in the experiment. Four weeks following the ovariectomy, experimental animals were injected intramuscularly with EB (15 μ g/kg BW) at 24 h (n = 5), 48 h (n = 6) and 72 h (n = 5) before slaughter. Three OVX gilts received corn oil vehicle and served as control. Tissue samples were collected from the ampulla and isthmus of the oviduct and from perioviductal, middle and paracervical parts of the uterine horn. Tissues were homogenized in 0.5 M acetic acid at 4°C, incubated in boil-

ing water for 10 min, cooled and centrifuged for 20 min at $10\,000 \times g$. The supernatant was separated and the pellet was re-extracted two times. The supernatants were pooled and lyophilized. Peptides were isolated from the tissue extracts using octadecasilyl silica cartridges (Sep-Pak C 18, Waters Assoc., Deventer, Holland). The extracts resuspended in 2 ml distilled water were passed through the cartridges pre-washed with 10 mM HCl 10% acetonitrile. The retained peptides were subsequently eluted with 3×1 ml 10 mM HCl 50% acetonitrile containing 0.1% trifluoroacetic acid. The samples were then evaporated in a vacuum centrifuge, dissolved in an assay buffer and assayed for β -endorphin, GnRH and galanin contents using radioimmunoassay (RIA) procedures.

Additionally, blood samples were collected during the slaughter to confirm hormonal status of experimental gilts. The blood samples were allowed to clot overnight at 4°C . Samples were then centrifuged at $1\,800 \times g$ for 15 min, and serum was collected and stored at -20°C until assayed for estradiol and LH.

Radioimmunoassays

β -Endorphin. β -Endorphin-like immunoreactivity (β -END-LI) in tissue extracts was established by the double-antibody RIA procedure previously described by Przała et al. [30]. Rabbit antiserum against human β -endorphin exhibited equimolar cross-reactivity (100%) with β -endorphin and β -lipotropin, but did not show any cross-reactivity with α -endorphin, γ -endorphin, Met-enkephalin, ACTH and α -MSH. Porcine β -endorphin was used for iodination and standards. The sensitivity of the assay and the intra- and inter-assay coefficients of variation were 5 pg/tube (at 92% binding), 7.6% and 12.9%, respectively.

GnRH. The GnRH concentration in tissue extracts was determined by the RIA procedure previously described by Okrasa et al. [26]. The rabbit anti-GnRH serum did not cross-react with GnRH associated peptide (GAP) and adrenocorticotrophic hormone (ACTH). Radio-iodinated GnRH (acetate salt) was prepared by the iodogen method of Fraker and Speck [7]. The sensitivity of the assay was 1.6 pg/tube at 93% binding. The intra- and

inter-assay coefficients of variation for GnRH determinations were 8.4% and 12.3%, respectively.

Galanin. Tissue concentrations of galanin were determined by the double-antibody RIA method previously described by Ziecik et al. [45]. The antibody against galanin used in the assay did not cross-react with porcine secretin, neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP). The standard curve was linear for concentrations ranging from 3 to 100 pg/tube and the detection limit was 3 pg/tube. The intra- and inter-assay coefficients of variation were 8.1% and 13.7%, respectively.

Estradiol-17 β . Estradiol serum concentrations were determined using the steroid extraction and then RIA procedure [36]. The recovery of estradiol from the extracted samples was 88% and the sensitivity of the assays was 5 pg/tube. The intra-assay coefficient of variation was 9%.

Luteinizing hormone. Serum concentrations of LH were determined by the double-antibody RIA method described by Zięćik et al. [44]. The rabbit antibodies (Sz/Z/89/396) against porcine LH conjugated with ovalbumin were used at a final dilution 1: 1,800,000. The cross-reactions of the antiserum with other protein hormones were published by Szafrńska et al. [37]. The sensitivity of the assay and intra-assay coefficient of variation were 0.08 ng/ml and 6.7%, respectively.

Statistical analysis

Data analysis was performed using the Statistica program (StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance was used to determine within-time effects of EB treatment on estradiol-17 β and LH serum concentrations. Contents of studied peptides in examined tissues were subjected to two-way analysis of variance with region of the reproductive tract (oviduct-ampulla, oviduct-isthmus, uterus-perioviductal region, uterus-middle region, uterus-paracervical region) and time as the factors. Significant changes in peptide tissue contents between times within region and between regions within time were assessed by the LSD test.

RESULTS

The serum estradiol-17 β and LH concentrations are presented in table 1. Concentrations of estradiol-17 β in serum were significantly higher at 24, 48 and 72 h following EB injection in comparison to that found in vehicle-treated gilts (controls). Administration of EB suppressed serum LH concentrations at 24 and 48 h after the steroid injection ($p < 0.001$) to OVX gilts. In contrast, 72 h after the steroid treatment serum LH concentration was significantly ($p < 0.05$) increased as compared to that of control.

Mean basal concentrations of β -endorphin determined in tissues of different regions of porcine control oviducts and uterine horns insignificantly varied from 0.22 ± 0.01 to 0.76 ± 0.2 ng/g dry tissue (fig. 1). Concentrations of β -endorphin in the oviduct tended to increase after E_2 administration, but these elevations were not statistically significant. In the perioviductal and the middle part of the uterine horn, β -endorphin concentrations were increased significantly only at 24 h after treatment with EB in comparison to the respective control values (2.31 ± 0.78 vs. 0.22 ± 0.01 ng/g and 3.79 ± 1.36 vs. 0.54 ± 0.29 ng/g). In the paracervical part of the uterine horn, the mean β -endorphin content at 48 h after priming of gilts with EB was higher than that of vehicle-treated gilts (2.05 ± 0.25 vs. 0.29 ± 0.01 ng/g).

Table 1. Mean (\pm SEM) serum estradiol and LH concentrations in ovariectomized (OVX) gilts injected with corn oil (control) or estradiol benzoate (EB; 15 μ g/kg BW) at time 0. Hormone concentrations in OVX gilts treated with EB were compared to the respective control values.

Hormones	Control (n = 3)	Time after EB injection (h)		
		24 (n = 5)	48 (n = 6)	72 (n = 5)
Estradiol (pg/ml)	7.8 ± 0.5	64.2 ± 6.1 ***	40.1 ± 4.3 ***	32.4 ± 3.3 **
LH (ng/ml)	1.7 ± 0.5	0.2 ± 0.04 ***	0.4 ± 0.06 ***	3.59 ± 0.8 *

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

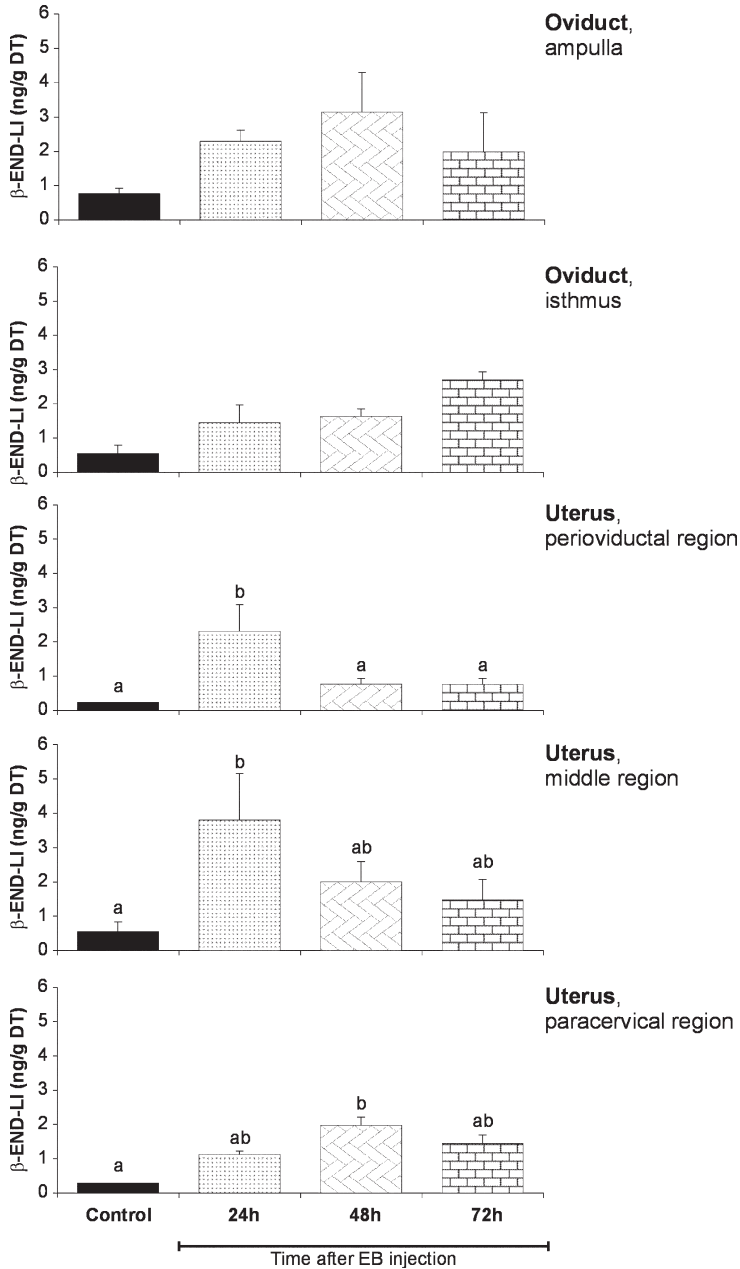


Fig. 1. Concentrations of β -endorphin-like immunoreactivity (β -END-LI; means \pm SEM) in different regions of the oviduct and the uterus (ng/g DT = dry tissue) of ovariectomized vehicle-treated (control) or estradiol benzoate (EB)-treated (15 μ g/kg BW) gilts. Bars with different superscripts are significantly different ($p < 0.05$).

Mean basal concentrations of GnRH varied from 0.57 ± 0.13 to 1.59 ± 0.44 ng/g (fig. 2) in analysed tissues of control gilts. In these animals, the middle part of the uterine horn contained higher GnRH concentration (1.59 ± 0.44 ; $p < 0.05$) than the perioviductal (0.61 ± 0.18 ng/g) and paracervical (0.57 ± 0.13 ng/g) uterus regions. There was no influence of EB administration on GnRH concentrations in the isthmus of the oviduct and the perioviductal part of the uterine horn. Increased tissue concentrations of the peptide were observed in the ampulla of the oviduct (3.31 ± 1.15 vs. 0.76 ± 0.17 ng/g) and the paracervical region of the uterine horn (1.32 ± 0.37 vs. 0.57 ± 0.13 ng/g) at 72 hours following priming of experimental gilts with EB comparing to the respective control values. In contrast, EB treatment significantly reduced GnRH content in the middle part of the uterine horn at all time-points. The lowest GnRH content was found at 24 h after EB injection (0.44 ± 0.11 ng/g).

Basal concentrations of galanin in vehicle-treated gilts varied across tissues from 2.23 ± 0.22 to 3.61 ± 1.12 ng/g (fig. 3) and did not significantly differ among studied regions of the oviduct and the uterus. Administration of EB had no influence on galanin concentrations in the oviduct as well as the middle and perioviductal regions of the uterine horn. However, in the paracervical part of the uterine horn, galanin content increased significantly at all examined time-points compared to that of control gilts (2.55 ± 0.32 ng/g).

DISCUSSION

The serum estradiol and LH concentrations found in OVX gilts and OVX gilts treated with estradiol have confirmed endocrine changes typical of an estradiol-induced LH surge model. In agreement with other studies [2, 46], the EB injection induced first negative feedback (serum LH 24 h and 48 h after EB treatment) followed by positive feedback (serum LH 72 h after EB treatment) on LH secretion.

In the present study, EB exerted a significant stimulatory effect on β -endorphin concentrations in all uterine regions tested. Significant responses of β -endorphin to EB in the uterus appeared either 24 h or 48 h after priming

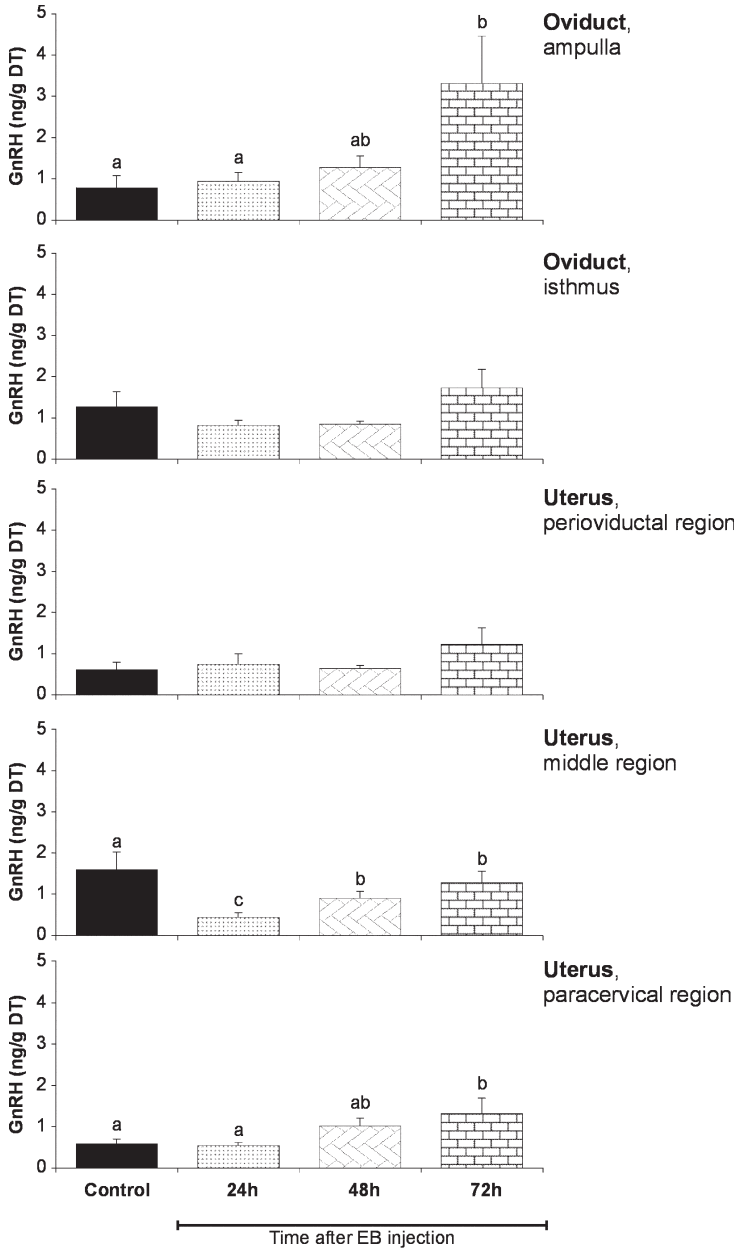


Fig. 2. Concentrations of immunoreactive GnRH (means \pm SEM) in different regions of the oviduct and the uterus (ng/g DT = dry tissue) of ovariectomized vehicle-treated (control) or estradiol benzoate (EB)-treated (15 μ g/kg BW) gilts. Bars with different superscripts are significantly different ($p < 0.05$).

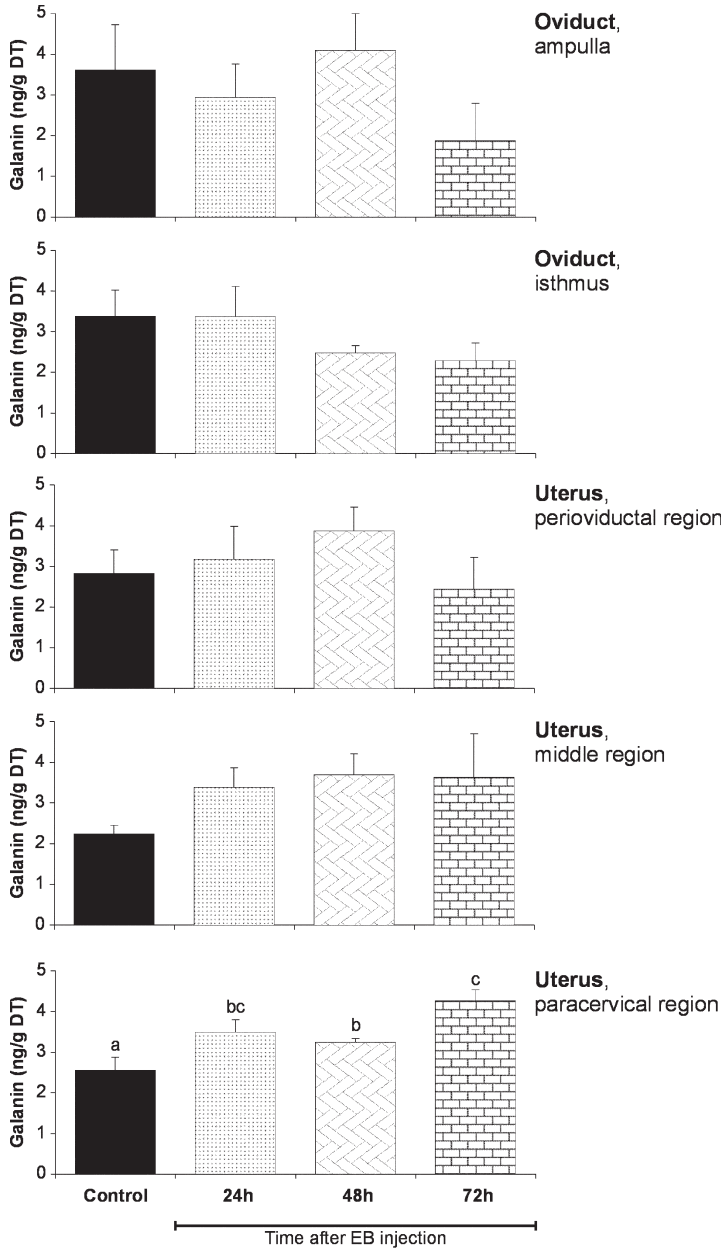


Fig. 3. Concentrations of immunoreactive galanin (means \pm SEM) in different regions of the oviduct and the uterus (ng/g DT = dry tissue) of ovariectomized vehicle-treated (control) or estradiol benzoate (EB)-treated (15 μ g/kg BW) gilts. Bars with different superscripts are significantly different ($p < 0.05$).

with the steroid. In oviductal tissue, β -endorphin content only tended to increase in response to EB. The results indicate that estradiol administered to OVX gilts as a bolus injection, inducing a preovulatory-like LH surge, is capable of increasing β -endorphin content in uterine tissues. Furthermore, this suggests that during the periovulatory period of the estrous cycle similar fluctuations of β -endorphin content in porcine uterine tissues may occur.

The uterine β -endorphin production/secretion was studied in several previous experiments performed with pigs. Immunocytochemical studies of Li et al. [18] demonstrated the presence of β -endorphin in the surface and glandular epithelial cells of the endometrium with the highest accumulation in the apical portions of the cells. In addition, they found higher concentrations of β -endorphin in endometrial tissues of crossbred gilts on days 14-15 than on days 8-12 of both the estrous cycle and pregnancy. In another experiment, total content of immunoreactive (ir)- β -endorphin in uterine fluid, reflecting uterine secretion of the peptide, was studied between the 8th and the 14th day of the porcine estrous cycle and pregnancy [16]. The highest content of secreted β -endorphin was found in uterine fluids on days 10-11 and day 8 in Large White gilts and in Meishan gilts, respectively. Treatment of OVX gilts with progesterone stimulated uterine secretion of ir- β -endorphin. This effect was abolished when progesterone was co-administered with estradiol. No response to estradiol alone was observed [15]. In contrast, in our experiment the stimulatory effect of EB on β -endorphin content in the uterus was noted. This inconsistency may possibly result from differences in duration of estrogen treatment. We have applied a bolus EB injection (15 μ g/kg BW) while in the other study [15] OVX gilts were exposed to estradiol treatment for 30 days (100 μ g E₂/day). Such prolonged treatment might cause a down-regulation of estradiol receptors. Estradiol, unlike progesterone and dihydrotestosterone, was able to affect the release of ir- β -endorphin from the Ishikawa human endometrial cell line [20]. In this study an inhibitory effect of estradiol was dependent upon dose and time and was the greatest after a 4-day exposure to estradiol (10 nM). However, in the study of Wahlstrom et al. [42], detectable amounts of ir- β -endorphin were found in human endometrium at the secretory stage (the luteal phase of the menstrual cycle), but not at the proliferative one

(the follicular phase). Thus, the influence of steroid hormones on uterine β -endorphin secretion seems to be dependent on species and physiological status of the female organism.

In addition to the steroid milieu and locally produced CRH (corticotrophin releasing hormone) [5, 8, 21, 22], the mechanism controlling synthesis and secretion of β -endorphin in the uterus may involve some other factors, *e.g.* cytokines, prostanoids, oxytocin, prolactin and gonadotrophins. Cytokines (IL-1 and IL-6) and PGE₂ may act by controlling CRH gene expression in the uterus [47]. In turn, LH, FSH, hCG, oxytocin and prolactin were found to influence β -endorphin secretion *in vitro* at least by one type of porcine ovarian cells; luteal [30, 31], granulosa [9] or theca [10]. In rabbits, co-treatment with gonadotrophins, eCG and hCG, increased ir-Met-enkephalin content both in ovaries and in the uterus [19]. The interactions of endogenous opioid peptides, including β -endorphin, with other components of endocrine and paracrine regulatory loops affecting uterine function in the pig remain to be elucidated.

Knowledge pertaining to the role of endogenous opioids in the uterus is fragmentary so far. There are data suggesting the possibility of multidirectional action of opioids in this organ. Effects of opioid action may depend on the type of opioid receptor as well as species, age and physiological status. Opioids may exert many regulatory effects in the uterus affecting nitric oxide (NO) production by endometrial glandular epithelial cells [38]. The synthetic enkephalin analogue, [D-Met², Pro⁵]enkephalinamide, had an age-dependent inhibitory effect on cell proliferation in the rat uterus [39, 40]. Interestingly, this opioid peptide was capable of reducing the estradiol-stimulated human myometrial cell proliferation *in vitro* [12]. In addition, [D-Met², Pro⁵]enkephalinamide suppressed the estradiol-induced increases in the level of Fos protein and the binding of AP-1 proteins to DNA in rat uterine cells [27]. Moreover, κ -opioid receptor specific ligands may induce apoptotic processes in human endometrial tissue [4]. Studies performed *in vitro* revealed a potential involvement of opioids in placental endocrinology. For instance, opioid agonists, acting mainly through κ receptors, stimulated release of hCG [3] and human placental lactogen (hPL) [29] from trophoblastic cells. There are some implications of β -endorphin

in the local, intrauterine modulation of immunological events during early pregnancy [47] as well as the control of myometrial contractility [5, 47]. This opioid peptide exerts its immunomodulatory effects in many ways [24] and may also manifest relaxant influence on uterine smooth muscles [6]. Li et al. [15, 16, 18] suggested that β -endorphin may play an important role in the regulation of early pregnancy in pigs. If β -endorphin production in the porcine uterus is elevated in response to pre-ovulatory increase of estrogens, like after the EB treatment in the current study, then we may speculate that this peptide – co-modulating uterine contractions – may facilitate efficient transport of gametes in the female reproductive tract [47]. Moreover, it is possible that during physiologically elevated estrogen secretion, β -endorphin – due to aforementioned antiestrogenic properties – may protect the uterine tissue from excessive action of these steroids.

The effect of EB on GnRH content in studied tissues ranged from a stimulation in the ampulla of the oviduct and in the paracervical uterus to an inhibition in the middle region of the uterus. In the study of Li et al. [17] injections of estradiol applied for 10 days did not affect GnRH secretion from the uterus of OVX pig. On the other hand, estradiol co-administered with progesterone augmented the stimulatory influence of the latter on uterine secretion of GnRH. Moreover, there was a markedly increased GnRH content in the pig uterine fluid during days 12 - 14 of pregnancy [16]. The authors suggested that this phenomenon could be associated with the modulation of local immune responses and enhancement of the implantation process. In the rat uterus, GnRH was able to inhibit estradiol-induced increases in activities of enzymes associated with cell proliferation, *i.e.* ornithine decarboxylase and glucosamine- 6-phosphate synthase [32, 33]. In prepubertal gilts, a single injection of a long-acting GnRH agonist, [D-Trp6, des-Gly10]-GnRH ethylamide], effectively reduced weights of both ovary and uterus at slaughter, four months after injection [34]. In rats, GnRH exerted an inhibitory influence on contraction of the non-pregnant uterus induced by acetylcholine and oxytocin as well as contraction of pregnant uterus evoked by oxytocin [23]. The responses of uterine GnRH to EB found in the present studies may imply a participation of GnRH in the modulation of uterine contractility during the periovulatory period in gilts.

In our study, galanin was found to be less susceptible to EB treatment than other tested peptides. Increased concentrations of galanin in response to EB occurred only in the paracervical region of the uterus. In female rats, treatments with a single dose of 17β -estradiol resulted in a rapid transient increase of galanin mRNA in the endometrium [41]. In the same study, a constant and prolonged exposure to estrogen, applied as diethylstilbestrol implants, also transiently elevated galanin mRNA in the uterus [41]. It has to be mentioned that galanin as well as galanin receptor mRNAs were found in the rat myometrium [25]. Moreover, the uterus appears to be innervated by galanin-immunoreactive nerve fibres, which are particularly abundant in the uterine cervix as it has been established in rats [28] and cows [14]. The results of previous studies performed on rats ascribe to galanin a stimulatory action on uterine contractility [1, 25, 35]. Other effects of galanin in the uterus remain to be determined.

In summary, EB treatment, at a dose capable of inducing a preovulatory-like LH surge, modulates β -endorphin, GnRH and galanin production by oviductal and/or uterine tissues of OVX gilts. The present results suggest an involvement of these peptides in the regulation of uterine function during the periovulatory period in gilts.

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