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The effect of orexin A on *CYP17A1* and *CYP19A3* expression and on oestradiol, oestrone and testosterone secretion in the porcine uterus during early pregnancy and the oestrous cycle

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ABSTRACT

Orexin A (OXA) is a hypothalamic neuropeptide known for its role in the regulation of food intake and arousal. It is also considered as a link between energy homeostasis and reproduction. Nevertheless, very little is known on the role of this peptide in the uterus. The objective of this study was to investigate OXA effect on oestradiol (E_2), oestrone (E_1), and testosterone (T) secretion by porcine endometrial and myometrial explants and gene expression of key steroidogenic enzymes involved in steroid production, namely cytochrome P450c17 (*CYP17A1*) and cytochrome P450 aromatase (*CYP19A3*), on days 10–11, 12 to 13, 15 to 16 and 27 to 28 of pregnancy and on days 10–11 of the cycle. In endometrium, OXA increased E_1 secretion on days 10–11 and 15 to 27 of gestation, and T release on days 12–13. A decrease in E_2, E_1 and T secretion was noted on days 27–28, 12 to 13 and 10 to 11 of gestation, respectively. OXA enhanced *CYP17A1* and *CYP19A3* expression on days 15–28 of pregnancy, whereas decreased their expression on days 10–13. In the myometrium, OXA increased E_1 secretion on days 10–16 of pregnancy, whereas inhibited the release of E_2 and T on days 10–11. *CYP17A1* and *CYP19A3* genes expression was enhanced on days 27–28 and 12 to 13 of pregnancy, respectively. The expression of both genes was suppressed on days 10–11 and 15 to 16 of pregnancy ($P < 0.05$). Our findings suggest that OXA, *via* its influence on steroidogenesis, may play a regulatory role in the uterus.

1. Introduction

Steroid hormones acts as main regulators of the cyclic changes within the female reproductive system, as well as in the processes involved in the establishment and maintenance of pregnancy. Recent studies using the pig model have indicated that the uterus is an important steroidogenic organ producing oestrogens and androgens *de novo*, in both, early pregnant and cyclic females [1–4]. Also the expression of key steroidogenic enzymes responsible for the production of oestrogens - oestradiol (E_2) and oestrone (E_1) as well as testosterone (T), namely *CYP17A1* and *CYP19A3* was noted in the porcine uterus during early pregnancy and the oestrous cycle [5–7]. One may conclude that the uterine production of steroids may supplement the amount of this hormones produced by the porcine embryos. It is also hypothesised that the uterine-derived steroids may be an alternative source of signals for pregnancy recognition and maintenance as well as for the initiation of implantation [1,8].

Although the reproductive success is largely dependent on the female's nutritional status, very little is known on the role of metabolic factors in the regulation of uterine secretory functions, for example steroidogenesis.

Orexins are postulated to be a part of a common endocrine system, which controls both, metabolism and reproduction. Orexin A (OXA) and orexin B (OXB), also known as hypocretins 1 and 2, are hypothalamic neuropeptides derived through proteolytic cleavage from a common 130-amino acid precursor molecule, prepro-orexin (PPO). The OXA and OXB consist of 33- and 28-amino acids, respectively. Their biological actions are mediated by two distinct,

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structurally similar, G-protein-coupled receptors: orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). OX1R is highly selective for OXA, whereas OX2R binds both orexins with a similar affinity [9,10]. Orexins were originally described as the regulators of food intake and energy homeostasis [10], and later identified as key modulators of the sleep-wake cycle and arousal [11]. There is a lot of data indicating that orexins may participate in the control of different endocrine axes, including the hypothalamic-pituitary-ovarian axis [12–19]. Smolinska et al. [20] observed the expression of *PPO*, *OX1R* and *OX2R* genes, as well as the presence of OXA, OXB, OX1R and OX2R proteins in the porcine endometrium, myometrium, conceptus and trophoblast during early stages of pregnancy. OXA was also found in the human placenta [21]. Furthermore, OX1R protein was identified in the syncytiotrophoblast and decidual cells of feline placenta, whereas OX2R was noted in cells constituting the endometrial glands [22].

Although the presence of the orexin system in the pregnant uterus was confirmed in the species mentioned above, there is general scarcity of data concerning the role of orexins in this organ.

We hypothesised that orexins may influence uterine steroidogenesis during the periods of pregnancy establishment and maintenance. To confirm this hypothesis, we explored the effect of OXA on *CYP17A1* and *CYP19A3* expression as well as on E_1, E_2 and T secretion by the endometrial and myometrial tissue explants harvested from pregnant pigs on days 10–11 of gestation (transuterine migration of embryos), days 12–13 (maternal recognition of pregnancy), days 15–16 (implantation) and days 27–28 (end of implantation), and from cyclic gilts on days 10–11 of the oestrous cycle (mid-luteal phase when corpus luteum activity levels are high and similar to those noted during pregnancy).

2. Material and methods

2.1. Experimental animals and tissue collection

Mature gilts (Large White x Polish Landrace) at the age of 7–8 months and weight of 120–130 kg descended from private breeding farm were used in the study. Twenty five animals were assigned to one of five experimental groups (n = 5 per group) as follows: 10 to 11, 12 to13, 15 to 16 and 27–28 days of pregnancy and days 10–11 of the oestrous cycle. Cyclic gilts were monitored daily for an oestrous behaviour in the presence of a boar. The day of onset of the second oestrous was designated as day 0 of the oestrous cycle. The phase of the oestrous cycle was also confirmed on the basis of ovaries morphology [23]. Insemination was performed with commercially available semen on days 1–2 of the oestrous cycle. Uteri from cyclic and early-pregnant animals were collected immediately after slaughter and placed in ice-cold sterile phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin and transported to the laboratory on ice within 1 h for *in vitro* explants tissue culture. Pregnancy was confirmed by the presence and morphology of conceptuses. On days 10–11 and 12 to 13 of pregnancy, the uterine horns were flushed with 20 ml of sterile PBS to recover conceptuses. On days 15–16 and 27 to 28 of pregnancy, conceptuses/trophoblasts were dissected from the endometrium. All slices of the uteri from days 15–16 and 27 to 28 of pregnancy used in this study were collected at the implantation sites. All studies were carried out in accordance with the ethical standards of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn, Poland.

2.2. Endometrial and myometrial tissue explants culture

Endometrial and myometrial tissue explants cultures were performed based on technique described by Franczak [2] with the modification of Smolinska et al. [8]. In brief, endometrial and myometrial tissues from the uterine horns were dissected, cut into small slices (100 mg) and washed three times in medium M199 (Sigma-Aldrich Co., USA). Obtained individual slices were placed into culture glass vials with 2 ml of medium M199 containing 0.1% BSA (MP Biomedicals Co.), 5% dextran/charcoal-stripped newborn calf serum (Sigma-Aldrich Co., USA), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cultures were preincubated for 2 h (37 °C, 95% O_2, 5% CO_2). In order to determine the effect of OXA on the expression of *CYP17A1* and *CYP19A3*, as well as on the secretion of E_2, E_1 and T, endometrial and myometrial tissue slices were treated with recombined human OXA (Sigma-Aldrich Co., USA) at the concentration of 1, 10 and 100 nM alone or in the combination with 1 μM of SB-3348667A (OX1R antagonist; Tocris, USA). Tissues incubated without any treatments were used as controls. The tissue explants were incubated with the treatments for another 24 h at the same conditions. The doses of orexins and selective antagonists of orexins receptors were chosen according to Barreiro et al. [24], Sasson et al. [25] and Small et al. [26]. All cultures were performed in duplicates in five independent experiments for each experimental group. At the end of experiment, culture media were collected and stored at −20 °C until E_1, E_2 and T concentrations were measured by radioimmunoassay (RIA). Endometrial and myometrial slices were snap-frozen in liquid nitrogen and stored at −80 °C for further RNA extraction and gene expression analysis. The viability of tissue explants was monitored by measuring lactate dehydrogenase (LDH) activity in medium at the end of 2 h-pre-incubation as well as at the end of the treatment period. The release of LDH was measured using Liquick Cor-LDH kit (Cormay, Poland), following the manufacturer's instructions. LDH activity during the tissue explants culture was compared to its activity in the medium obtained after the destruction of endometrial and myometrial cells by homogenization (positive control for the cell death and the maximal LDH release). Mean activity of LDH in the cultured slices after treatment period was 55.1 ± 4.5 U/L for endometrium (1.8% of maximal release of LDH after total endometrial cells destruction) and 34.1 ± 4.9 U/L for myometrium (1.7% of maximal release of LDH after myometrial cell destruction).

2.3. Quantitative real-time PCR

Total RNA was isolated from endometrial and myometrial tissue samples using peqGold TriFast isolation system (Peqlab, Germany). RNA quantity and quality were determined spectrophotometrically (Infinite M200 Pro, Tecan, Switzerland). One microgram of RNA was reverse transcribed into cDNA in a total volume of 20 μl with 0.5 μg oligo (dT)15 primer (Roche, Germany) using the Omniscript RT Kit (Qiagen, USA) at 37 °C for 1 h and was terminated by the incubation at 93 °C for 5 min. Quantitative real-time PCR analysis was performed using a PCR System 7300 (Applied Biosystems, USA) with SYBR Green as described previously [8]. Specific primer pairs used to amplify parts of *CYP17A1*, *CYP19A3*, cyclophilin (*PPIA*) and β-actin (*ACTB*) genes as well as temperature conditions of the real-time PCR reactions are detailed in Table 1. The constitutively expressed genes, *PPIA* and *ACTB*, were used as the internal control to verify the quantitative real-time PCR. Preliminary study indicated that the expression of *PPIA* and *ACTB* was similar in the endometrium and myometrium and stable during the oestrous cycle and pregnancy, as well as with or without treatments. The PCR reaction mixtures were containing 10 ng of cDNA, primers, 12.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and RNase free water at the final volume of 25 μl. Negative controls were performed using RNase free water instead of cDNA. All samples were prepared in duplicates. The specificity of amplification was tested at the end of