The effect of orexin B on steroidogenic acute regulatory protein, P450 side-chain cleavage enzyme, and 3 β -hydroxysteroid dehydrogenase gene expression, and progesterone and androstenedione secretion by the porcine uterus during early pregnancy and the estrous cycle¹

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ABSTRACT: The aim of this study was to investigate the effect of orexin B (OXB) on progesterone (P₄) and androstenedione (A₄) secretion by porcine endometrial and myometrial tissue explants and on the expression of key steroidogenic proteins and enzymes involved in steroid production. The hormones secretion and the expression of steroidogenic acute regulatory protein (*StAR*), P450 side-chain cleavage enzyme (*CYP11A1*), and 3 β -hydroxysteroid dehydrogenase (*HSD3B1*) were analyzed on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy and during the luteal phase of the estrous cycle (days 10 to 11). Endometrial and myometrial explants were cultured in vitro in the presence of OXB (1, 10, or 100 nM) and OXB (1, 10, or 100 nM) with 1 μ M of JNJ (OX2R antagonist).

Gene expression was examined by real-time PCR, and steroid secretion was determined by radioimmunoassay. Orexin B modulated *StAR*, *CYP11A1*, *HSD3B1* mRNA content depending on the type of uterine tissue, the applied OXB dose, and the stage of pregnancy or the estrous cycle ($P < 0.05$). Orexin B increased P₄ secretion in all stages of early gestation ($P < 0.05$). Orexin B enhanced the release of A₄ on days 12 to 13, 15 to 16, and 27 to 28 of gestation, whereas on days 10 to 11 of early pregnancy, A₄ secretion decreased in the endometrium and increased in the myometrium ($P < 0.05$). These results indicate that OXB affects the expression of key steroidogenic regulators and the secretion of steroid hormones in the porcine uterus during early pregnancy.

Key words: orexin B, pig, pregnancy, steroid hormones, steroidogenic enzymes, uterus

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INTRODUCTION

Orexin A (OXA) and orexin B (OXB), also known as hypocretin 1 and 2, are hypothalamic neuropeptides derived by proteolytic cleavage from prepro-orexin (PPO), a common 130-amino-acid

precursor molecule. Orexin A consists of 33 amino acids, OXB is composed of 28 amino acids, and 46% of the amino acids in OXB are identical to those in OXA (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A is fully conserved across mammalian species, whereas porcine and human OXB differs in only 1 amino acid (Sakurai et al., 1998; Dyer et al., 1999). The biological action of OXA and OXB is mediated by two G-protein-coupled receptors: orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). The binding properties of orexins receptors are varied. OX1R

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is highly selective for OXA, whereas OX2R has similar affinity for both OXA and OXB (Sakurai et al., 1998; Ammoun et al., 2003; McAtee et al., 2004). Kodadek and Cai (2010) reported that OX1R binds OXB with about 100-fold lower affinity than OXA.

Orexins were originally discovered in rat lateral and posterior hypothalamus which are involved in the control of food intake and energy homeostasis (Sakurai et al., 1998). Orexins were subsequently identified as modulators of arousal and sleep regulation (Chemelli et al., 1999). Orexins could be the link between energy balance and reproduction. Evidence for this hypothesis has been provided by many studies which demonstrated that orexins could participate in the control of the hypothalamic–pituitary–gonadal axis (Silveyra et al., 2007a,b; Kaminski et al., 2010a,b; Nitkiewicz et al., 2010, 2014; Maleszka et al., 2013; Smolinska et al., 2014). Orexins are involved in the regulation of testicular and ovarian steroidogenesis in rats and pigs (Barreiro et al., 2004; Silveyra et al., 2007a; Cataldi et al., 2012). However, the role of orexins in the uterus has not been fully elucidated to date. Smolinska et al. (2015) observed the expression of *PPO*, *OX1R*, *OX2R* genes and OXA, OXB, OX1R, OX2R proteins in the porcine endometrium, myometrium, conceptus, and trophoblast in the early stages of gestation. Orexin A was also found in human, canine, and feline placenta (Nakabayashi et al., 2003; Dall’Aglio et al., 2012, 2014), whereas OX1R was localized in the syncytiotrophoblast and decidual cells of feline placenta and OX2R was identified in human endometrial glands (Dall’Aglio et al., 2012). The presence of orexins and their receptors in the porcine uterus suggests that orexins play a physiological role in this organ.

The porcine uterus is a steroidogenic organ. In a study by Franczak and Kotwica (2008), androstenedione (A_4), progesterone (P_4), testosterone (T), estrone (E_1), and estradiol (E_2) were released by the porcine uterus during early gestation and the estrous cycle. Other authors investigated the expression of key steroidogenic enzymes responsible for P_4 and A_4 production in the human endometrium: P450 side-chain cleavage enzyme (*CYP11A1*), steroidogenic acute regulatory protein (*StAR*), and 3 β -hydroxysteroid dehydrogenase (*HSD3B1*) (Rhee et al., 2003; Attar et al., 2009). Smolinska et al. (2016) reported on *CYP11A1* basal gene expression in porcine endometrium and myometrium during early pregnancy and the estrous cycle, whereas Kiewisz et al. (2014) observed the expression of *StAR* gene in the endometrium. Wojciechowicz et al. (2013) detected active 3 β -hydroxysteroid

dehydrogenase in the porcine uterus. There is evidence to suggest that uterine-derived steroids are important factors which participate in the recognition of gestation, beginning of implantation, and maintenance of pregnancy.

We hypothesized that OXB participates in the regulation of uterine steroidogenesis during early pregnancy and the estrous cycle. Therefore, the aim of this study was to investigate the effect of OXB on P_4 and A_4 secretion by porcine endometrial and myometrial slices and on the expression of key steroidogenic enzymes: *StAR*, *CYP11A1*, and *HSD3B1* on days 10 to 11 of pregnancy (transuterine migration of embryos), days 12 to 13 (maternal recognition of pregnancy), days 15 to 16 (implantation), days 27 to 28 (end of implantation), and during the luteal phase of the estrous cycle (days 10 to 11).

MATERIAL AND METHODS

Experimental Animals and Tissue Collection

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 (the number of ethical approval 91/2011DTN). Mature gilts (Large White x Polish Landrace) at the age of 7 to 8 months and the weight of 120 to 130 kg descended from private breeding farm were used in the study. Twenty-five animals were assigned to 1 of 5 experimental groups ($n = 5$ per group) as follows: 10 to 11, 12 to 13, 15 to 16, and 27 to 28 days of pregnancy and days 10 to 11 of the estrous cycle. Cyclic gilts were monitored daily for an estrus behavior in the presence of a boar. The day of onset of the second estrus was designated as day 0 of the estrous cycle. The phase of the estrous cycle was also confirmed on the basis of ovaries morphology (Akins and Morrisette, 1968). Insemination was performed on days 1 to 2 of the estrous cycle. Uteri from cyclic and early-pregnant animals were collected immediately after slaughter and placed in the ice-cold sterile phosphate-buffered saline (PBS) supplemented with 100 IU/mL penicillin and 100 μ g/mL streptomycin and transported to the laboratory on ice within 1 h for in vitro tissue culture. Pregnancy was confirmed by the presence and morphology of conceptuses. On days 10 to 11 and 12 to 13 of pregnancy, the uterine horns were flushed with 20 mL of sterile PBS to recover conceptuses. On days 15 to 16 and 27 to 28 of pregnancy, conceptuses/trophoblasts were dissected from the

endometrium. All slices of the uteri from days 15 to 16 and 27 to 28 of pregnancy used in this study were collected at the implantation sites.

Endometrial and Myometrial Tissue Explants Culture

Endometrial and myometrial tissue explants' cultures were performed based on technique described by [Franczak and Kotwica \(2008\)](#) with the modification of [Smolinska et al. \(2016\)](#). In brief, endometrial and myometrial tissues from the uterine horns were dissected, cut into small slices ($100 \text{ mg} \pm 10\%$), and washed 3 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., USA), 5% dextran/charcoal-stripped newborn calf serum (Sigma-Aldrich Co., USA), penicillin (100 IU/mL), and streptomycin (100 $\mu\text{g/mL}$). Cultures were preincubated for 2 h (37°C , 95% O_2 , 5% CO_2). To determine the effect of OXB on the expression of *StAR*, *CYP11A1*, *HSD3B1*, as well as on the secretion of P_4 and A_4 , endometrial and myometrial tissue slices were treated with OXB at the concentrations of 1, 10, and 100 nM alone or in the combination with 1 μM of JNJ10397049 (OX2R antagonist; Johnson and Johnson Pharmaceuticals, 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 [Barb and Matteri \(2005\)](#), [Borgland et al. \(2008\)](#), and [Perin et al. \(2014\)](#). All cultures were performed in duplicates in 5 independent experiments. At the end of experiment, culture media were collected and stored at -20°C until P_4 and A_4 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 preincubation 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 with its activity in the medium obtained after the destruction of endometrial and myometrial cells by homogenization (positive control for the cell death and maximal LDH release). Mean activity of LDH in the cultured slices after treatment period was $55.1 \pm 4.5 \text{ U/L}$ for the endometrium (1.8% of maximal release of LDH after total endometrial cells destruction) and $34.1 \pm 4.9 \text{ U/L}$ for the myometrium (1.7% of maximal release of LDH after myometrial cell destruction).

Quantitative Real-Time PCR

Total RNA was isolated from all the tissue samples using peqGold TriFast isolation system (Peqlab, Germany). RNA concentration 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 ([Smolinska et al., 2016](#)). Specific primer pairs used to amplify parts of *CYP11A1*, *StAR*, *HSD3B1*, cyclophilin (peptidylprolyl isomerase A; *PPIA*), and β -actin (*ACTB*) genes are detailed in

Table 1. Characteristics of primers used in the study

Gene	Primer sequences	GenBank accession number	Complementary gene, nt	Primer, nM	Product size, bp	Reference
<i>StAR</i>	F: 5'-GGAGAGCCGGCAGGAGAATG-3' R: 5'-CTTCTGCAGGATCTTGATCTTCTTG-3'	U53020	416–435 575–599	300 300	184	Rusovici et al., 2005
<i>CYP11A1</i>	F: 5'-GTCCCATTTACAGGAGAAGCTCG-3' R: 5'-GGCTCCTGACTTCTTCAGCAGG-3'	X13768	291–314 451–472	300 300	182	Rusovici et al., 2005
<i>HSD3B1</i>	F: 5'-AGGTTCGCCCCGCTCATC-3' R: 5'-CTGGGCACCGAGAAATACTTG-3'	NM_001004049.1	95–111 144–164	300 300	70	Wojciechowicz et al., 2013
<i>PPIA</i>	F: 5'-GCACTGGTGGCAAGTCCAT-3' R: 5'-AGGACCCGTATGCTTCAGGA-3'	AY266299	219–237 269–299	300 300	71	Lord et al., 2005
<i>ACTB</i>	F: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' R: 5'-GAGGGGCGATGATCTTGATCTTCA-3'	U07786	266–289 608–631	500 500	366	Spagnuolo-Weaver et al., 1999

StAR = steroidogenic acute regulatory protein; *CYP11A1* = P450 side-chain cleavage enzyme; *HSD3B1* = 3β -hydroxysteroid dehydrogenase; *PPIA* = cyclophilin; *ACTB* = β -actin; F = forward; R = reverse.

Table 1. The constitutively expressed genes, *PPIA* and *ACTB*, were used as an internal control to verify the quantitative real-time PCR. Our preliminary study indicated that the expression of *PPIA* and *ACTB* was similar in the endometrium and myometrium and stable during the estrous cycle and pregnancy, as well as with or without treatments. The PCR reaction mixtures contained, 10 ng of cDNA, primers, 12.5 μ L Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and RNase-free water at the final volume of 25 μ L. Negative controls were performed using RNase-free water instead of cDNA. All samples were prepared in duplicates. Real-time PCR cycling conditions for *CYP11A1*, *StAR*, and *HSD3B1* were as follows: enzyme activation and initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 1 min, and elongation at 72 °C for 1 min. For *PPIA* reaction condition were as follows: 50 °C for 2 min, then enzyme activation and initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. The specificity of amplification was tested at the end of the reaction by melting-curve analysis. Products purity was confirmed by agarose gel electrophoresis. Calculation of the relative expression levels of *CYP11A1*, *StAR*, and *HSD3B1* was conducted based on the comparative cycle threshold method ($\Delta\Delta C_T$) described by Livak and Schmittgen (2001) and normalized using the geometrical means of the reference gene expression levels: *PPIA* and *ACTB*.

RIA of Steroid Hormones

Concentrations of A_4 were analyzed by RIA according to the method described by Dziadkowiec et al. (1982), whereas concentrations of P_4 were determined according to the method of Ciereszko et al. (1998). Cross-reactivities of the antiserum against A_4 and P_4 have been published previously (Szafranska et al., 2002). The no-extraction assay was used to monitor P_4 levels. Efficiency of extraction for the A_4 assay was $85.3 \pm 0.3\%$. The sensitivities of the assays for P_4 and A_4 were 1 and 2.2 pg/mL, respectively. The standard curve range was from 1 to 1500 pg/mL for P_4 , and from 1 to 500 pg/mL for A_4 . The intra-assay and inter-assay coefficients of variation were 1% and 7.36% for P_4 , and 2.1% and 5.1% for A_4 , respectively.

Statistical Analysis

Statistical analysis was performed using Statistica program (StatSoft Inc., USA). All data

were analyzed by one-way ANOVA followed by the Fisher's least significant difference post hoc test and are reported as means $\pm$ standard error of the mean from 5 independent observations. Values for $P < 0.05$ were considered statistically significant.

RESULTS

The Effect of OXB on StAR Gene Expression in the Endometrial and Myometrial Tissue Explants

In the endometrial tissue explants, OXB stimulated *StAR* gene expression on days 12 to 13 (1, 10, and 100 nM) and 15 to 16 (1 and 100 nM) of pregnancy. An inhibitory effect of OXB on *StAR* gene expression was observed on days 10 to 11 (1, 10, and 100 nM), 27 to 28 (10 and 100 nM) of pregnancy and days 10 to 11 of the estrous cycle (100 nM). In samples incubated in the presence of JNJ, OXB increased *StAR* mRNA level on days 12 to 13 (10 nM) and 15 to 16 (10 nM) of pregnancy and decreased on days 10 to 11 (1, 10, and 100 nM) of gestation and days 10 to 11 of the cycle (10 and 100 nM). OX2R blocker stimulated *StAR* expression on days 12 to 13 and 15 to 16 of pregnancy, whereas inhibited on days 10 to 11 of gestation ($P < 0.05$; Figure 1A).

OXB increased *StAR* mRNA content in the myometrium on days 10 to 11 (1, 10, and 100 nM), 27 to 28 (1 nM) of pregnancy, and days 10 to 11 of the estrous cycle (10 nM). An inhibitory effect of OXB on *StAR* expression in this tissue was observed on days 12 to 13 (10 and 100 nM), 15 to 16 (1, 10, and 100 nM), and 27 to 28 (10 nM) of gestation. In samples incubated in the presence of JNJ, OXB stimulated *StAR* gene expression on days 10 to 11 (100 nM), 27 to 28 (10 nM) of pregnancy, and on days 10 to 11 of the cycle (1 nM), whereas inhibited on days 12 to 13 (1 and 10 nM) and 15 to 16 (1 and 100 nM) of gestation. The supplementation of media with JNJ caused an increase in the level of *StAR* mRNA on days 10 to 11 and 27 to 28 of pregnancy, and a decrease on days 15 to 16 of gestation ($P < 0.05$; Figure 1B).

The Effect of OXB on CYP11A1 Gene Expression in the Endometrial and Myometrial Tissue Explants

In the endometrial tissue explants, the stimulatory effect of OXB on *CYP11A1* expression was observed on days 27 to 28 (1, 10, and 100 nM) of pregnancy. In this tissue, OXB decreased concentration of *CYP11A1* mRNA on days 10 to 11 (1 nM), 12 to 13 (100 nM), and 15 to 16 (1, 10, and 100 nM) of pregnancy, and on days 10 to 11 of the estrous

Figure 1. The influence of OXB (1, 10, and 100 nM) in the presence or absence of the selective antagonist of orexin receptor type 2 (JNJ—JNJ10397049) on the relative expression of *Star* gene in endometrial (A) and myometrial (B) tissue explants harvested from pigs on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy, and on days 10 to 11 of the estrous cycle preincubated for 2 h and subsequently incubated for 24 h. The expression levels were normalized with the geometric mean expression of reference genes: cyclophilin and β -actin. Data are expressed as the mean $\pm$ standard error of the mean ($n = 5$). Bars with different letters are significantly different at $P < 0.05$.

cycle (10 and 100 nM). Supplementation of media with OX2R antagonist (JNJ) caused an increase in *CYP11A1* expression on days 27 to 28 (1 nM) of pregnancy and 10 to 11 of cycle (10 nM), and a decrease on days 10 to 11 (1, 10, and 100 nM), 12 to 13 (100 nM), and 15 to 16 (1, 10, 100 nM) of gestation, and on days 10 to 11 of cycle (1, 100 nM). JNJ

caused an increase of *CYP11A1* expression on days 27 to 28 of pregnancy and decrease on days 10 to 11, 12 to 13, and 15 to 16 of gestation, and on days 10 to 11 of estrous cycle ($P < 0.05$; Figure 2A).

In the myometrial tissue explants, an increase in *CYP11A1* mRNA content caused by OXB was observed on days 10 to 11 (10 nM) and 15 to 16 (1,

Figure 2. The influence of OXB (1, 10, and 100 nM) in the presence or absence of the selective antagonist of orexin receptor type 2 (JNJ—JNJ10397049) on the relative expression of *CYP11A1* gene in endometrial (A) and myometrial (B) tissue explants harvested from pigs on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy, and on days 10 to 11 of the estrous cycle preincubated for 2 h and subsequently incubated for 24 h. The expression levels were normalized with the geometric mean expression of reference genes: cyclophilin and β -actin. Data are expressed as the mean $\pm$ standard error of the mean ($n = 5$). Bars with different letters are significantly different at $P < 0.05$.

10 nM) of pregnancy and on days 10 to 11 days of the cycle (1 and 100 nM). A decrease in *CYP11A1* gene expression was noted on days 12 to 13 and 27 to 28 of gestation (10 and 100 nM). The addition of JNJ together with OXB to the media caused an increase in the content of *CYP11A1* mRNA on

days 10 to 11 (1, 10 and 100 nM), 15 to 16 (10 and 100 nM) of pregnancy, and on days 10 to 11 of the estrous cycle (10, 100 nM), whereas a decrease in *CYP11A1* gene expression was found on days 12 to 13 and 27 to 28 (1, 10, and 100 nM) of pregnancy. JNJ caused an increase in the expression of

CYP11A1 on days 15 to 16 of gestation and days 10 to 11 of cycle. JNJ also caused a decrease in the *CYP11A1* mRNA content on days 27 to 28 of pregnancy ($P < 0.05$; [Figure 2B](#)).

The Effect of OXB on HSD3B1 Gene Expression in the Endometrial and Myometrial Tissue Explants

In the endometrium, OXB caused an increase in the expression of *HSD3B1* gene on days 12 to 13 (100 nM) and 15 to 16 (1 nM) of pregnancy and on days 10 to 11 of the estrous cycle (10 nM). In this tissue, OXB decreased *HSD3B1* mRNA content on days 10 to 11 (10 nM) and 27 to 28 (10 and 100 nM) of gestation. In the samples cultured in the presence of JNJ, OXB stimulated *HSD3B1* expression on days 15 to 16 (10 nM) of pregnancy, whereas an inhibitory effect of OXB was observed on days 10 to 11 (10 nM) of pregnancy ($P < 0.05$; [Figure 3A](#)).

In the myometrial tissue explants, an increase in the *HSD3B1* mRNA content caused by OXB was observed on days 12 to 13 (10 and 100 nM) of pregnancy and days 10 to 11 of the estrous cycle (1 and 10 nM), whereas a decrease in the *HSD3B1* expression was noted on days 27 to 28 (10 nM) of gestation. The addition of JNJ to the media caused an increase in the content of *HSD3B1* mRNA under the influence of OXB on days 12 to 13 (1 nM) of pregnancy. JNJ caused an increase in the expression of *HSD3B1* on days 12 to 13 of gestation ($P < 0.05$; [Figure 3B](#)).

The Effect of OXB on P₄ Secretion by the Endometrial and Myometrial Tissue Explants

Secretion of P₄ by the endometrial tissue slices was enhanced under the influence of OXB on days 10 to 11 (100 nM), 12 to 13 (1 and 100 nM), 15 to 16 (10 nM), and 27 to 28 (1, 10, and 100 nM) of pregnancy, and on days 10 to 11 of the estrous cycle (1 and 100 nM). A decrease in P₄ secretion by the endometrium samples was caused by OXB (10 nM) on days 10 to 11 of the cycle. In samples incubated in the presence of JNJ, OXB (100 nM) stimulated P₄ release on days 10 to 11 of the cycle, whereas on the dose of 1, 100 nM inhibited on days 15 to 16 of gestation ($P < 0.05$; [Figure 4A](#)).

In the myometrial tissue slices, P₄ secretion was stimulated by OXB at the all doses used on days 10 to 11 of pregnancy. Progesterone release was also enhanced by OXB on days 12 to 13 (10 and 100 nM) and 27 to 28 (10 and 100 nM) of gestation, whereas suppressed at the dose of 1 nM on days 10 to 11 of the cycle. In the myometrial slices

incubated with OX2R blocker, OXB diminished P₄ secretion at the doses of 10 and 100 nM on days 10 to 11 of the estrous cycle. JNJ decreased progesterone release on days 15 to 16 of pregnancy ($P < 0.05$; [Figure 4B](#)).

The Effect of OXB on A₄ Secretion by the Endometrial and Myometrial Tissue Explants

In the endometrial slices, an increase in the secretion of A₄ in response to OXB was observed on days 15 to 16 (1 nM) and 27 to 28 (1 and 10 nM) of pregnancy, whereas a decrease on days 10 to 11 (1, 10, and 100 nM) of gestation. The addition of JNJ together with OXB to the culture stimulated A₄ release on days 10 to 11 of the cycle (10 nM), whereas inhibited A₄ secretion on days 10 to 11 (1, 10, and 100 nM) of pregnancy and days 10 to 11 of the cycle (1 nM). JNJ inhibited androstenedione secretion on days 10 to 11 of gestation and 10 to 11 of cycle ($P < 0.05$; [Figure 5A](#)).

In the myometrium, A₄ release was enhanced by OXB on days 10 to 11 (10 nM) and 27 to 28 (1 and 10 nM) of pregnancy. In the presence of JNJ, OXB stimulated A₄ production on days 10 to 11 (100 nM), 12 to 13 (1 nM), 15 to 16 (1 nM), and 27 to 28 (1, 10, and 100 nM) of gestation. A stimulatory effect of JNJ on androstenedione release was noted on days 10 to 11 and 12 to 13 of pregnancy ($P < 0.05$; [Figure 5B](#)).

DISCUSSION

The influence of orexins on the female reproductive system has been investigated for several years. Our previous study revealed the expression of all elements of the orexin system—*PPO*, *OX1R*, *OX2R* genes, and the presence of OXA, OXB, *OX1R*, and *OX2R* proteins in the porcine endometrium and myometrium during early pregnancy and on days 10 to 11 of the estrous cycle ([Smolinska et al., 2015](#)). We observed that gene expression was closely linked with the hormonal status of animals. The highest immunoreactivity of OXB was found in the myometrial longitudinal muscles on days 10 to 12 of the estrous cycle, in luminal endometrial epithelium on days 10 to 12 and 14 to 16, and in glandular epithelium and stroma on days 17 to 19 ([Nitkiewicz et al., 2012](#)). During gestation, the highest concentration of *PPO*, an OXB precursor, was found in the endometrium on days 12 to 13 and in the myometrium on days 30 to 32. The concentration of *OX1R* protein increased in the endometrium on days 10 to 11 of gestation and in the

HSD3B1 mRNA RELATIVE EXPRESSION

Figure 3. The influence of OXB (1, 10, and 100 nM) in the presence or absence of the selective antagonist of orexin receptor type 2 (JNJ—JNJ10397049) on the relative expression of *HSD3B1* gene in endometrial (A) and myometrial (B) tissue explants harvested from pigs on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy, and on days 10 to 11 of the estrous cycle preincubated for 2 h and subsequently incubated for 24 h. The expression levels were normalized with the geometric mean expression of reference genes: cyclophilin and β -actin. Data are expressed as the mean $\pm$ standard error of the mean ($n = 5$). Bars with different letters are significantly different at $P < 0.05$.

myometrium on days 27 to 28 of pregnancy. Orexin receptor type 2 protein concentration increased in the endometrium on days 15 to 16 and in the myometrium on days 27 to 28 of gestation (Smolinska et al., 2015). The above results suggest that the sensitivity of the endometrium and myometrium to orexins changes during gestation. The cited

findings could also explain the variable influence of OXA and OXB on uterine steroidogenesis in different periods of pregnancy and in different types of uterine tissue.

Steroid hormones are important for the establishment of proper endometrial sensitivity, recognition of pregnancy, and successful implantation.

PROGESTERONE CONCENTRATIONS

Figure 4. The influence of OXB (1, 10, and 100 nM) in the presence or absence of the selective antagonist of orexin receptor type 2 (JNJ—JNJ10397049) on P4 secretion by endometrial (A) and myometrial (B) tissue explants harvested from pigs on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy, and on days 10 to 11 of the estrous cycle preincubated for 2 h and subsequently incubated for 24 h. Data are expressed as the mean $\pm$ standard error of the mean ($n = 5$). Bars with different letters are significantly different at $P < 0.05$.

Franczak (2008) observed steroidogenic activity in the porcine endometrium and myometrium in the early stages of pregnancy and during the estrous cycle. Other authors determined the expression of key steroidogenic enzymes in the uterus. The presence of *CYP11A1*, *StAR*, and *HSD3B1* transcripts was noted in the human endometrium (Rhee

et al., 2003; Attar et al., 2009). The expression of *CYP11A1* and *HSD3B1* was also observed in the murine endometrium during gestation (Ben-Zimra et al., 2002). In a pig model, the *StAR* gene was expressed in the endometrium on days 12 and 16 of the estrous cycle and during gestation (Kiewisz et al., 2014). Smolinska et al. (2016) reported the

ANDROSTENEDIONE CONCENTRATIONS

Figure 5. The influence of OXB (1, 10, and 100 nM) in the presence or absence of the selective antagonist of orexin receptor type 2 (JNJ—JNJ10397049) on A_4 secretion by endometrial (A) and myometrial (B) tissue explants harvested from pigs on days 10 to 11, 12 to 13, 15 to 16, and 27 to 28 of pregnancy, and on days 10 to 11 of the estrous cycle preincubated for 2 h and subsequently incubated for 24 h. Data are expressed as the mean $\pm$ standard error of the mean ($n = 5$). Bars with different letters are significantly different at $P < 0.05$.

expression of the *CYP11A1* gene in the porcine uterus on days 10 to 11 of the estrous cycle and during early gestation. Wojciechowicz et al. (2013) described differences in the expression of the *HSD3B1* gene and the activity of 3β -hydroxysteroid dehydrogenase in the porcine endometrium and myometrium during early gestation and the estrous cycle. Other authors linked OXA and OXB with

steroidogenesis in different tissues. Ramanjaneya et al. (2009) and Wenzel et al. (2009) analyzed the influence of orexins on steroidogenesis in the human adrenocortical cell line H295. Cataldi et al. (2012) observed that OXA and OXB reduced P_4 secretion in rat luteal cells. Barreiro et al. (2004) demonstrated that OXA enhanced testosterone production in rat testes in vivo and in vitro. The effect of

orexins on adrenal steroidogenesis was also investigated in rats (Malendowicz et al., 2001) and pigs (Nanmoku et al., 2002). To the best of our knowledge, we demonstrated for the first time the influence of OXA and OXB on the expression of key steroidogenic enzymes, *CYP71A1* and *CYP19A3*, and on the secretion of estradiol, estrone, and testosterone in the porcine uterus during early pregnancy and the estrous cycle (Kiezun et al., 2017; Kaminski et al., 2018). The cited studies suggest that the uterus could be the target organ for orexins, and that uterine steroidogenesis, which is linked with early recognition of pregnancy and implantation, could also be influenced by orexins. This study revealed that OXB modulates the expression of key steroidogenic enzymes, *StAR*, *CYP11A1*, and *HSD3B1*, and the secretion of P_4 and A_4 by endometrial and myometrial tissue explants harvested from pregnant pigs during maternal recognition of pregnancy and embryo implantation, and from cyclic pigs during the luteal phase of the cycle.

This study was undertaken to investigate the effect of OXB on the gene expression of protein and key enzymes which are rate-limiting in steroidogenesis. The following protein and enzymes were analyzed: *StAR*, a protein transporter controlling cholesterol transport inside mitochondria, *CYP11A1*, a mitochondrial enzyme responsible for the first step in steroid biosynthesis, namely, the conversion of cholesterol to pregnenolone, and *HSD3B1* which catalyzes the biosynthesis of P_4 from pregnenolone and A_4 from dehydroepiandrosterone. This study was also performed to determine the influence of OXB on P_4 and A_4 secretion. The results did not provide direct evidence that OXB influences the expression of key steroidogenic enzymes and hormone secretion, but certain trends supporting the above hypothesis were observed in different stages of pregnancy and in different types of uterine tissue. Orexin B could participate in the establishment of pregnancy and implantation by stimulating the release of P_4 from endometrial slices in the early stages of pregnancy and from myometrial slices on days 10 to 11, 12 to 13, and 27 to 28 of gestation. Orexin B also stimulated the secretion of A_4 on days 15 to 16 (beginning of implantation) and 27 to 28 (end of implantation). In this study, OXB enhanced *StAR* gene expression in the endometrium on days 12 to 13 (maternal recognition of pregnancy) and 15 to 16 (beginning of implantation). These stages of gestation are critical for embryo survival; therefore, by stimulating an increase in *StAR* mRNA content in these periods, OXB creates favorable conditions for initiating the

production of steroid hormones. Smolinska et al. (2016) reported that the basal mRNA expression of *CYP11A1* did not differ in the porcine endometrium harvested on days 12 to 13, 15 to 16, and 27 to 28 of pregnancy. Our research indicates that OXB has an inhibitory effect on *CYP11A1* mRNA content in the endometrium in the early stages of pregnancy, excluding days 27 to 28 when *CYP11A1* expression was stimulated by OXB. At the end of implantation on days 27 to 28, steroid hormones are required to complete the implantation process and promote embryonic development (Kodaman et al., 2004). For this reason, by enhancing the expression of *CYP11A*, OXB increases the production of pregnenolone, a precursor for steroid hormones. Our unpublished data demonstrated that the basal concentrations of *HSD3B1* mRNA in the endometrium remained stable on days 12 to 13, 15 to 16, and 27 to 28 of pregnancy and was significantly higher on days 10 to 11 of gestation. The current study revealed that on days 15 to 16 of pregnancy, OXB stimulated *HSD3B* expression in the endometrium, which is necessary for increased secretion of P_4 and A_4 . It is worth noting that the highest content of OX2R protein and, consequently, the highest endometrial sensitivity to OXB were observed on days 15 to 16 of gestation (Smolinska et al., 2015).

McAtee et al. (2004) and Lebold et al. (2013) reported that JNJ-10397049 is a high-affinity selective OX2R receptor antagonist and can be successfully used in the studies concerning this particular receptor and its agonists. In previous studies, the OX2R antagonist, JNJ-10397049 was most often used at the dose of 1 μ M and significantly inhibited orexins effect (Borgland et al., 2008; Perin et al., 2014). Cataldi et al. (2012) used extremely high-dose 10 μ M of JNJ-10397049 to achieve a full blockage of OX2R. The OX2R antagonist used in the present experiment did not completely block the influence of OXB on gene expression and steroid secretion. It can be assumed that in this case the concentration of the blocker (1 μ M) added with OXB was too low. We also cannot exclude eventuality of auto- and paracrine influence of orexins produced by the cultured tissue explants, which could contribute to insufficient blockage of OX2R.

In the present study, we obtained some unexpected results, for example, the increased *StAR* expression in OXB 10nM + JNJ-10397049 group, and decreased when the tissues were treated with OXB 1nM + JNJ-10397049 or with OXB 100nM + JNJ-10397049. This case could be explained by a phenomenon called hormesis. In the work of Calabrese and Baldwin (2001, 2003), the authors

focused on dose-dependent response to treatment. According to these authors, the fundamental nature of the dose response is neither linear nor threshold and could be rather described as U-shaped or inverse U-shaped. There are examples indicating that the same treatment can have both stimulatory and inhibitory effects, which depends on its concentration. In most cases, hormesis is associated with toxicological and pharmacological studies. However, there are some data showing U-shaped response to various factors in steroidogenesis studies (Gregoraszczyk et al., 2008; 2011). Another example of unobvious results was observed in case of A⁴ secretion, where OXB (10 nM) combined with JNJ increased the hormone release, whereas OXB at the same concentration added alone did not change A⁴ level and JNJ alone decreased the hormone release. It can be suggested that OXB despite of low affinity to OXR1 could act through these receptors when OXR2 is blocked. JNJ-10397049 is in vitro 600-fold more selective for OXR2 than OXR1 (McAtee et al., 2004). JNJ-10397049 added alone to the cultures was able to change gene expression and steroid concentrations via abolition of stimulatory or inhibitory effect of OXR2 signaling. Similar effect was observed by other authors in their in vivo studies on rats (Silveyra et al., 2007b) and in our previous studies concerning effect of OXA and OXB on uterine steroidogenesis (Kiezun et al., 2017; Kaminski et al., 2018).

In conclusion, we have shown that OXB could affect steroidogenesis in the porcine uterus during early pregnancy by modulating the gene expression of *StAR*, *CYP11A1* and *HSD3B*, and the secretion of P₄ and A₄. The obtained results demonstrated that the influence of OXB on gene expression and hormone secretion is determined by the type of cultured uterine tissue, the applied dose of OXB, and day of pregnancy and the estrous cycle. The role of OXB in the porcine uterus remains insufficiently investigated, and our results could fill the existing knowledge gap. In our earlier studies, we analyzed the influence of OXA and OXB on the production of estrogens and androgens in the porcine uterus during pregnancy and the estrous cycle. The present study and our recent investigations imply that orexins could have a regulatory role in the establishment and maintenance of pregnancy by influencing the production of steroids in the porcine uterus. However, further research is required to deepen our understanding of the role played by orexins in the uterus during pregnancy.

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