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EFFECT OF ADIPONECTIN ON THE STEROIDOGENIC ACUTE REGULATORY PROTEIN, P450 SIDE CHAIN CLEAVAGE ENZYME AND 3 β -HYDROXYSTEROID DEHYDROGENASE GENE EXPRESSION, PROGESTERONE AND ANDROSTENEDIONE PRODUCTION BY THE PORCINE UTERUS DURING EARLY PREGNANCY

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Adiponectin and its receptors are expressed in the human and porcine uterus and this endocrine system has important role in the regulation of reproductive processes. The expression of steroidogenic acute regulatory protein (*StAR*) and 3 β -hydroxysteroid dehydrogenase (*HSD3B1*) were observed in the human and porcine uterus during the oestrous cycle and pregnancy. The *de novo* synthesis of steroids in the uterus might be a crucial factor for effective implantation and maintenance of pregnancy. We hypothesized that adiponectin modulates the expression of key enzymes in the synthesis of the steroids: *StAR*, P450 side chain cleavage enzyme (*CYP11A1*) and *HSD3B1*, as well as progesterone (P_4) and androstenedione (A_4) secretion by the porcine uterus. Endometrial and myometrial explants harvested from gilts ($n = 5$) 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 oestrous cycle were cultured *in vitro* in the presence of adiponectin (1, 10 $\mu\text{g/ml}$), adiponectin with insulin (10 ng/ml) and insulin alone (10 ng/ml). Gene expression was examined by real-time PCR, and the secretion of the steroids was determined by radioimmunoassay. The content of *StAR*, *CYP11A1* and *HSD3B1* mRNAs and the secretion of P_4 and A_4 was modulated by adiponectin in endometrial and myometrial tissue explants during early pregnancy and the oestrous cycle. In this action adiponectin interacted with insulin. Insulin itself also regulated the steroidogenic activity of the porcine uterus. Here we reported, for the first time, the expression of *CYP11A1* genes in the porcine endometrium and myometrium. Our novel findings indicate that adiponectin affects basal and insulin-stimulated expression of key steroidogenic genes and production of steroid hormones by the porcine uterus during maternal recognition of pregnancy and implantation.

Key words: *adiponectin, uterus, steroidogenic enzymes, steroids, progesterone, pregnancy, porcine endometrium, oestrous cycle*

INTRODUCTION

Adiponectin is an adipocyte-derived hormone that acts as a major regulator of energy metabolism and insulin sensitivity (1, 2). In plasma, adiponectin occurs in different forms: the trimeric low molecular weight (LMW), the hexameric middle molecular weight (MMW) and the 12-18meric high molecular weight (HMW) complexes (3). Adiponectin can be found in a smaller globular form, which is produced through proteolytic cleavage (4). It circulates at high levels in the bloodstream, and its serum concentration is decreased in obesity and insulin resistance (5, 6). In an obese subject, reduction in body weight leads to an increase in plasma adiponectin levels (7). In diabetic mice, administration of recombinant adiponectin improves insulin resistance (1). Disturbed serum levels of adiponectin have also been demonstrated in patients with pancreatic cancer (8) and in rats with post-myocardial infarction heart failure (9). Adiponectin exerts its action by binding to specific seven-transmembrane adiponectin receptors 1 and 2 (AdipoR1, AdipoR2). AdipoR1 was identified as a high-affinity receptor for adiponectin in the form of a trimer and is predominantly expressed in skeletal

muscles, whereas AdipoR2 has a higher affinity for MMW and HMW adiponectin and is found mainly in the liver (10). The effects of adiponectin are mediated through the activation of the adenosine monophosphate activated protein kinase (AMPK), p38 mitogen-activated protein kinase (MAPK) and the peroxisome proliferators-activated receptor- α (PPAR α) (11).

It is well known that reproduction can be affected by nutritional status. Adiponectin may be a part of a common endocrine system that controls metabolism and the reproductive system. It is believed to play a key role regarding reproduction by regulating the hypothalamic-pituitary-gonadal (HPG) axis (12, 13). The adiponectin system (adiponectin, AdipoR1 and AdipoR2) was recently found in porcine, rat and human hypothalami (14, 15), pituitary glands (16, 17) and ovaries (18-21). The secretion of gonadotropin-releasing hormone (GnRH) from GT1-7 hypothalamic cells was inhibited by adiponectin (22). Adiponectin decreased basal and GnRH-stimulated luteinizing hormone (LH) secretion by the isolated pituitary cells of rats (16). At the level of the mammalian ovaries, *in vitro* studies have shown that adiponectin is able to affect steroidogenic gene expression and steroidogenesis. This effect depends on differences among

species, ovarian cell type and concentration of the hormone. Our group reported the inhibition of progesterone (P_4) release by adiponectin (1, 10 $\mu\text{g/ml}$) in the culture of porcine luteal cells on days 10 to 12 of the oestrous cycle. In the culture of porcine granulosa cells, adiponectin at a higher dose (10 $\mu\text{g/ml}$) increased basal secretion of oestradiol (E_2) (21). The addition of adiponectin to porcine granulosa cells increased the expression of steroidogenic acute regulatory protein (*StAR*) gene (23). However, no significant effect of both adiponectin doses was observed on basal production of P_4 by porcine granulosa cells (21). It was previously suggested that adiponectin interacts with insulin and insulin-like growth factor I (IGF-I). In our previous study, adiponectin at a higher dose (10 $\mu\text{g/ml}$) in combination with insulin enhanced P_4 secretion by porcine granulosa cells (21). In human granulosa cells, adiponectin also increased the IGF-I-induced secretion of P_4 and E_2 (19). The above mentioned data clearly demonstrates that adiponectin alters ovarian steroidogenic genes expression and the production of steroid hormones. This action may be modulated by insulin and IGF-I.

Little is known about the role of adiponectin in the uterus. The expression of adiponectin and its receptors has been found in human, mouse and porcine uteri (18, 24, 25). Our recent studies have demonstrated that the expression of adiponectin system in porcine endometrium and myometrium depends on the phase of the oestrous cycle and pregnancy (26, 27). It was reported earlier that the porcine uterus is a steroidogenic organ. *In vitro* studies have shown endometrial production of P_4 and oestrone (E_1) on days 30, 60 and 90 of gestation (28). *In vitro* secretion of P_4 , androstenedione (A_4), testosterone (T), E_1 and E_2 by the porcine endometrium and the myometrium during early pregnancy and the oestrous cycle was also demonstrated (29–31). The expression of *StAR*, which facilitates the entry of cytosolic cholesterol into the mitochondrion, mRNA content of P450 side chain cleavage enzyme (*CYP11A1*), which catalyses conversion of cholesterol to pregnenolone, and expression of 3β -hydroxysteroid dehydrogenase (*HSD3B1*) gene, which is responsible for P_4 and A_4 production, was identified in the human endometrium (32, 33). In the porcine endometrium, *StAR* gene expression was found (34). It was determined that the uterus of pigs also possesses active 3β -hydroxysteroid dehydrogenase (31). The authors of the above studies have suggested that uterine steroids may be an alternative signal for recognition of pregnancy and maintenance, and initiation of implantation. To our knowledge, there is an absence of published studies describing the expression of *CYP11A1* in the porcine uterus. We hypothesized that adiponectin is involved in the control of the steroidogenic activity of the porcine uterus and can interact with insulin in regulating this process. To accept or reject our hypothesis, we explored the influence of adiponectin on basal and insulin-induced: 1) expression of *StAR*, *CYP11A1* and *HSD3B1* genes; and 2) secretion of P_4 and A_4 by the endometrial and myometrial tissue explants harvested from pregnant pigs on days 10 to 11 of gestation (migration of the embryos to and within the uterus), days 12 to 13 (the maternal recognition of pregnancy), days 15 to 16 (implantation) and days 27 to 28 (the end of implantation) and cyclic pigs on days 10 to 11 of the cycle (the mid-luteal phase connected with period of fully active corpora lutea corresponding to the activity of corpora lutea during pregnancy).

MATERIALS AND METHODS

Experimental animals and tissue collection

The experiments were carried out in accordance with the ethical standards of the Animal Ethics Committee at the

University of Warmia and Mazury in Olsztyn (the number of ethical approval 91/2011/DTN).

Twenty five mature gilts (Large White x Polish Landrace; 7 to 8 months of age, body weight of 120 to 130 kg) descended from private breeding farm were used in the study. The gilts were assigned to one of five experimental groups ($n = 5$ per group) as follows: days 10 to 11, 12 to 13, 15 to 16 and 27 to 28 of pregnancy, days 10 to 11 of the oestrous cycle. Females were monitored daily for oestrus behavior in the presence of an intact boar. The day of onset of the second oestrus was designated as day 0 of the oestrous cycle. The stage of the oestrous cycle was also confirmed on the basis of the morphology of the ovaries (35). Insemination was performed on days 1 to 2 of the oestrous cycle. Uteri collected after slaughter from cyclic and early-pregnant gilts were immediately placed in ice-cold PBS supplemented with 100 IU/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin and transported to the laboratory on ice within 1 hour for *in vitro* explant tissue culture. Pregnancy was confirmed by the presence of conceptuses. On days 10 to 11 and 12 to 13 of pregnancy, the uterine horns were flushed with 20 ml of sterile phosphate-buffered saline (PBS) to recover conceptuses. On days 15 to 16 and 27 to 28 of pregnancy, conceptus/trophoblast was dissected from the endometrium. All slices of the uteri on days 15 to 16 and 27 to 28 of pregnancy were collected at the implantation sites.

Endometrial and myometrial explant culture

Uteri collected from gilts during the studied periods of pregnancy and on days 10 to 11 of the cycle were washed three times in sterile PBS. Endometrial and myometrial explants cultures were performed based on a modification of the technique of Franczak *et al.* (29, 30). Endometrial and myometrial tissues from the uterine horns were dissected and cut into small slices (100 mg) and then washed three times in medium M199 (Sigma-Aldrich Co., USA). Individual endometrial and myometrial slices were placed into culture glass vials with 2 ml medium M199 containing 0.1% BSA (MP Biomedicals, USA), 5% dextran/charcoal-stripped newborn calf serum (Sigma-Aldrich Co.), penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g/ml}$). The tissue cultures were preincubated in a shaking water bath for 2 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 . After preincubation, the slices were treated for 24 h with control medium and recombinant human adiponectin (1, 10 $\mu\text{g/ml}$, BioVendor, USA). To test interaction between adiponectin and insulin, explants were treated with combination of adiponectin and insulin (10 ng/ml; Sigma-Aldrich Co.) for 24 hours. The doses of adiponectin and insulin were established based on Ledoux *et al.* (23) and Maleszka *et al.* (20, 21). Control slices were incubated without any treatment. All cultures were performed in duplicates in five independent experiments. After culture, the media were collected and stored at -20°C until P_4 and A_4 concentration were measured by radioimmunoassay (RIA). Furthermore, endometrial and myometrial tissue explants were snap-frozen in liquid nitrogen (for RNA extraction) and stored at -80°C until further analysis. The viability of tissue explants was monitored by measuring lactate dehydrogenase (LDH) activity in medium at 2 hour of preincubation as well as at the end of the treatment period. The release of LDH was performed using a Liquick Cor-LDH kit (Cormay, Poland) following the manufacturer's instructions. The activity of LDH during the culture of tissue explants was compared to its activity in medium obtained after destruction of endometrial and myometrial cells by homogenization (positive control for causing cell death and the maximal release of LDH). Mean activity of LDH in 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 cells destruction).

Quantitative real-time PCR

Total RNA was extracted from all the tissue samples using peqGOLD TriFast isolation system (Peqlab, Germany). RNA concentration and quality were checked spectrophotometrically (Infinite M200 Pro, Tecan, Switzerland). One microgram of RNA was reversely transcribed into cDNA in a total volume of 20 μ l with 0.5 μ g oligo(dT)₁₅ primer (Roche, Germany) using the Omniscript RT Kit (Qiagen, USA) at 37°C for 1 hour. The process was terminated by incubation at 93°C for 5 min. Specific primer pairs used to amplify parts of *StAR*, *CYP11A1*, *HSD3B1*, cyclophilin A (*PPIA*) and β -actin (*ACTB*) genes are detailed in Table 1. Quantitative real-time PCR analysis was carried out using a PCR System 7300 (Applied Biosystems, USA), as described previously (26). The PCR reaction included 10 ng cDNA, the appropriate forward and reverse primer at various concentrations (Table 1), 12.5 μ l Power SYBR Green PCR Master Mix (Applied Biosystems, USA), and RNase free water in a final volume of 25 μ l. The constitutively expressed genes, *PPIA* and *ACTB*, were used as the internal control to verify the quantitative real-time PCR. During the preliminary experiments it was found that expression of *PPIA* and *ACTB* was very similar in the endometrium and myometrium and was stable during the oestrous cycle and pregnancy and with treatments. Real-time PCR cycling conditions for *StAR*, *CYP11A1*, *HSD3B1* and *ACTB* 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 conditions 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, annealing at 60°C for 1 min. Negative controls were performed in which cDNA was substituted by water, or reverse transcription was not performed before PCR. All samples were amplified in duplicates. The specificity of amplification was tested at the end of the PCR by melting-curve analysis. Product purity was confirmed by agarose gel electrophoresis. The real-time PCR amplification efficiencies of target and reference genes was 99 – 101%. Calculation of the relative expression level of *StAR*, *CYP11A1* and *HSD3B1* genes was conducted based on the comparative cycle threshold method ($\Delta\Delta C_T$) and normalized using the geometrical means of reference gene expression levels: *PPIA* and *ACTB*.

Radioimmunoassay of steroid hormones

Concentrations of P₄ were analysed by RIA according to the method described by Ciereszko *et al.* (36, 37) and concentrations of A₄ were determined according to the method of Dziadkowiec *et al.* (38). Cross-reactivities of the antiserum against P₄ and A₄ have been published previously (38, 39). The no-extraction assay was used to monitor P₄ levels. Efficiency of extraction for the A₄ assay was $85.3 \pm 0.3\%$. The sensitivities of the assays for P₄ and A₄ were 1 pg/ml and 2.2 pg/ml, respectively. The standard curve range was from 1 pg/ml to 1500 pg/ml for P₄, and from 1 pg/ml to 500 pg/ml for A₄. The intra- and interassay coefficients of variation were 1% and 7.36% for P₄, and 2.1% and 5.1% for A₄, respectively.

Statistical analysis

Statistical analysis was performed using the Statistica program (StatSoft Inc., USA). All data are reported as the mean \pm standard error of the mean from five independent observations. One-way analysis of variance and the Fisher's least significant difference *post hoc* test was used to compare the effect of treatments on gene expression and steroid secretion. The model included the effect of adiponectin in two different concentrations, the effect of adiponectin in two different concentrations with insulin and the effect of insulin alone. One-way analysis of variance and the Fisher's least significant difference *post hoc* test were also applied to analyse the effect of the oestrous cycle phase and pregnancy period on basal content of *CYP11A1* mRNA. Values for P < 0.05 were considered statistically significant.

RESULTS

The effect of adiponectin on steroidogenic acute regulatory protein (StAR) gene expression in the endometrial and myometrial tissue explants

StAR gene expression was found in the endometrium and myometrium during the early stage of pregnancy from day 10 to day 28 and on days 10 to 11 of the oestrous cycle (Fig. 1A and 1B).

In the endometrial tissue explants, the stimulatory effect of adiponectin at a dose of 1 μ g/ml and at both doses (1, 10 μ g/ml) on *StAR* mRNA content was observed on days 12 to 13 and 15 to 16 of pregnancy, respectively. The inhibitory effect of adiponectin at a dose of 10 μ g/ml on *StAR* mRNA expression

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'-GGAGAGCCGCGCAGGAGAATG-3' R: 5'-CTTCTGCAGGATCTTGATCTTCTTG-3'	U53020	416-435 575-599	300 300	184	(58)
<i>CYP11A1</i>	F: 5'-GTCCCATTTACAGGGAGAAGCTCG-3' R: 5'-GGCTCCTGACTTCTTCAGCAGG-3'	X13768	291-314 451-472	300 300	182	(58)
<i>HSD3B1</i>	F: 5'-AGGTTTCGCCGCTCATC-3' R: 5'-CTGGCACCAGAAATACTTG-3'	NM_001004 049.1	95-111 144-164	300 300	70	(31)
<i>PPIA</i>	F: 5'-GCACCTGGTGGCAAGTCCAT-3' R: 5'-AGGACCCGTATGCTTCAGGA-3'	AY266299	219-237 269-299	300 300	71	(18)
<i>ACTB</i>	F: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' R: 5'-GAGGGCGATGATCTTGATCTTCA-3'	U07786	266-289 608-631	500 500	366	(59)

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

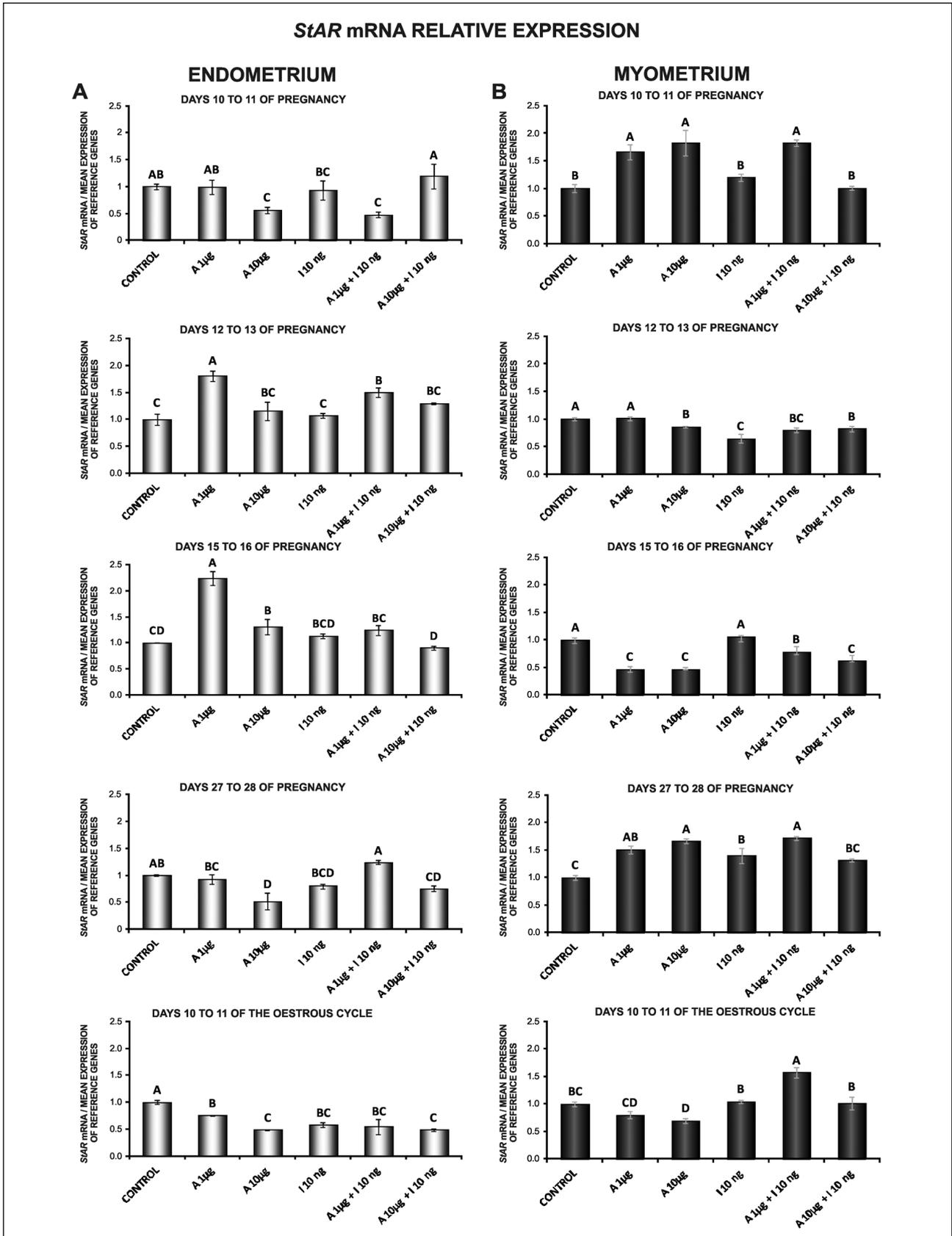


Fig. 1. The effect of adiponectin on basal and insulin-stimulated *StAR* gene expression. The influence of adiponectin (1, 10 µg/ml) and adiponectin (1, 10 µg/ml) in combination with insulin (10 ng/ml) on the relative expression of steroidogenic acute regulatory protein (*StAR*) gene in the endometrial (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 oestrous 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 ± standard error of the mean (n = 5). Bars with different superscript capital letters are significantly different at P < 0.05.

was determined on days 10 to 11 and 27 to 28 of pregnancy. On days 10 to 11 of the oestrous cycle, the expression of *StAR* gene was inhibited by adiponectin at both doses (1, 10 µg/ml) ($P < 0.05$; Fig. 1A).

Insulin alone in relation to the control reduced *StAR* gene expression in the endometrium on days 10 to 11 of the oestrous cycle ($P < 0.05$). During all studied days of pregnancy, insulin had no effect on *StAR* mRNA content (Fig. 1A).

In the endometrial tissue, adiponectin (10 µg/ml) in combination with insulin stimulated *StAR* mRNA expression compared to insulin alone on days 10 to 11 of pregnancy. Similarly, adiponectin at a dose of 1 µg/ml with addition of insulin enhanced the gene expression on days 12 to 13 and 27 to 28 of pregnancy when compared with the insulin treatment ($P < 0.05$; Fig. 1A).

In the myometrial tissue explants, on days 10 to 11 and 27 to 28 of pregnancy, *StAR* gene expression was increased in response to adiponectin (1, 10 µg/ml). On days 12 to 13 of pregnancy and on days 10 to 11 of the cycle, adiponectin (10 µg/ml) decreased expression of *StAR* gene. Similarly, on days 15 to 16 of pregnancy, adiponectin at both doses (1, 10 µg/ml) suppressed *StAR* mRNA expression ($P < 0.05$; Fig. 1B).

Insulin alone compared to the control diminished *StAR* mRNA content in the myometrium on days 12 to 13 of pregnancy ($P < 0.05$). In contrast, on days 27 to 28 of pregnancy, insulin enhanced the gene expression. On days 10 to 11 and 15 to 16 of pregnancy, and on days 10 to 11 of the cycle, insulin did not affect *StAR* gene expression (Fig. 1B).

In the myometrium, adiponectin at a lower dose (1 µg/ml) in combination with insulin provoked an increase in *StAR* mRNA expression in comparison with insulin alone on days 10 to 11 and 27 to 28 of pregnancy, and on days 10 to 11 of the cycle. Similarly, on days 12 to 13 of pregnancy, adiponectin at a higher dose (10 µg/ml) with insulin significantly increased *StAR* mRNA content. The expression of *StAR* gene was inhibited by adiponectin (1, 10 µg/ml) in combination with insulin compared to the insulin treatment on days 15 to 16 of pregnancy ($P < 0.05$; Fig. 1B).

The effect of adiponectin on CYP11A1 gene expression in the endometrial and myometrial tissue explants

CYP11A1 mRNA content was reported in the endometrium and myometrium during all studied days of pregnancy and on days 10 to 11 of the oestrous cycle (Fig. 2A and 2B). In endometrial tissue explants, adiponectin at both doses (1, 10 µg/ml) caused a decrease in *CYP11A1* mRNA content on days 10 to 11 and 15 to 16 of pregnancy, and on days 10 to 11 of the cycle. On days 12 to 13 of pregnancy, basal expression of *CYP11A1* gene was also reduced by adiponectin (1 µg/ml) ($P < 0.05$; Fig. 2A).

Insulin alone in relation to the control enhanced *CYP11A1* gene expression in the endometrium on days 12 to 13 and 27 to 28 of pregnancy. In contrast, on days 10 to 11 and 15 to 16 of pregnancy, and on days 10 to 11 of the cycle, insulin diminished the gene expression ($P < 0.05$; Fig. 2A).

In the endometrial tissue, adiponectin treatment at a higher dose (10 µg/ml) with insulin increased *CYP11A1* mRNA content in relation to insulin alone on days 15 to 16 of pregnancy. On days 12 to 13 and 27 to 28 of pregnancy, under the influence of 1 and 10 µg/ml of adiponectin a significant decrease in the gene expression was observed in insulin-induced cultures compared to insulin alone ($P < 0.05$; Fig. 2A).

Treatment of the myometrial explants on days 10 to 11 of pregnancy and on days 10 to 11 of the cycle with adiponectin (10 µg/ml) resulted in significant increase in *CYP11A1* mRNA content. On days 15 to 16 of pregnancy, adiponectin at a lower

dose (1 µg/ml) provoked an increase in *CYP11A1* gene expression. On days 12 to 13 of pregnancy, expression of *CYP11A1* mRNA content was attenuated by adiponectin (10 µg/ml) ($P < 0.05$; Fig. 2B).

Insulin alone compared to the control stimulated *CYP11A1* mRNA content in the myometrium on days 15 to 16 and 27 to 28 of pregnancy. On days 12 to 13 of pregnancy, insulin diminished the expression of *CYP11A1* gene ($P < 0.05$). On days 10 to 11 of pregnancy and on days 10 to 11 of the cycle, the administration of insulin did not affect *CYP11A1* mRNA expression (Fig. 2B).

In the myometrial explants, adiponectin (1, 10 µg/ml) in combination with insulin increased *CYP11A1* gene expression in comparison with insulin alone on days 10 to 11 of pregnancy and on days 10 to 11 of the cycle. In contrast, on days 27 to 28 of pregnancy, the expression of *CYP11A1* mRNA content was suppressed by adiponectin (1, 10 µg/ml) with insulin compared to insulin alone. A similar effect was caused by adiponectin (1 µg/ml) in combination with insulin on days 15 to 16 of pregnancy ($P < 0.05$; Fig. 2B).

Basal expression of CYP11A1 gene in the endometrial and myometrial tissue explants on days 10 to 11 of the cycle and during pregnancy

In the endometrium, the expression of *CYP11A1* gene was lower during all studied stages of pregnancy relative to days 10 to 11 of the porcine oestrous cycle. During pregnancy, *CYP11A1* mRNA content was higher on days 10 to 11 of gestation compared to days 12 to 13, 15 to 16 and 27 to 28 of gestation ($P < 0.05$; Fig. 3A).

In the myometrium, the expression of *CYP11A1* gene was lower on days 10 to 11 of oestrous cycle than during all studied stages of pregnancy (except for days 10 to 11). During pregnancy, the highest *CYP11A1* mRNA expression was noted on days 27 to 28 of gestation, and the lowest on days 10 to 11 of gestation ($P < 0.05$; Fig. 3B).

The effect of adiponectin on 3β-hydroxysteroid dehydrogenase (HSD3B1) gene expression in the endometrial and myometrial tissue explants

HSD3B1 mRNA expression was observed in the endometrial and myometrial tissue explants during the early stage of pregnancy from day 10 to day 28 and on days 10 to 11 of the oestrous cycle (Fig. 4A and 4B).

In the endometrium, on days 15 to 16 of pregnancy, *HSD3B1* gene expression was enhanced by adiponectin at the concentration of 1 µg/ml. A similar effect was caused by adiponectin (10 µg/ml) on days 10 to 11 of the cycle. On days 10 to 11 of pregnancy, adiponectin at doses of 1 and 10 µg/ml reduced *HSD3B1* mRNA expression. Adiponectin at a higher dose (10 µg/ml) decreased *HSD3B1* mRNA content on days 12 to 13 of pregnancy ($P < 0.05$; Fig. 4A).

Insulin alone compared to the control increased *HSD3B1* mRNA content in the endometrium on days 10 to 11 of the cycle. On days 27 to 28 of pregnancy, insulin decreased the gene expression ($P < 0.05$). There was no effect of insulin on the content of *HSD3B1* mRNA in these explants on days 10 to 11, 12 to 13 and 15 to 16 of pregnancy (Fig. 4A).

In the endometrium, on days 10 to 11 of pregnancy, adiponectin at a higher dose (10 µg/ml) caused an increase in insulin-stimulated *HSD3B1* gene expression in relation to insulin alone, while administration of adiponectin at a lower dose (1 µg/ml) with insulin resulted in significant decrease in the expression. On days 10 to 11 of the cycle, adiponectin (1 µg/ml) with insulin diminished *HSD3B1* mRNA expression ($P < 0.05$; Fig. 4A).

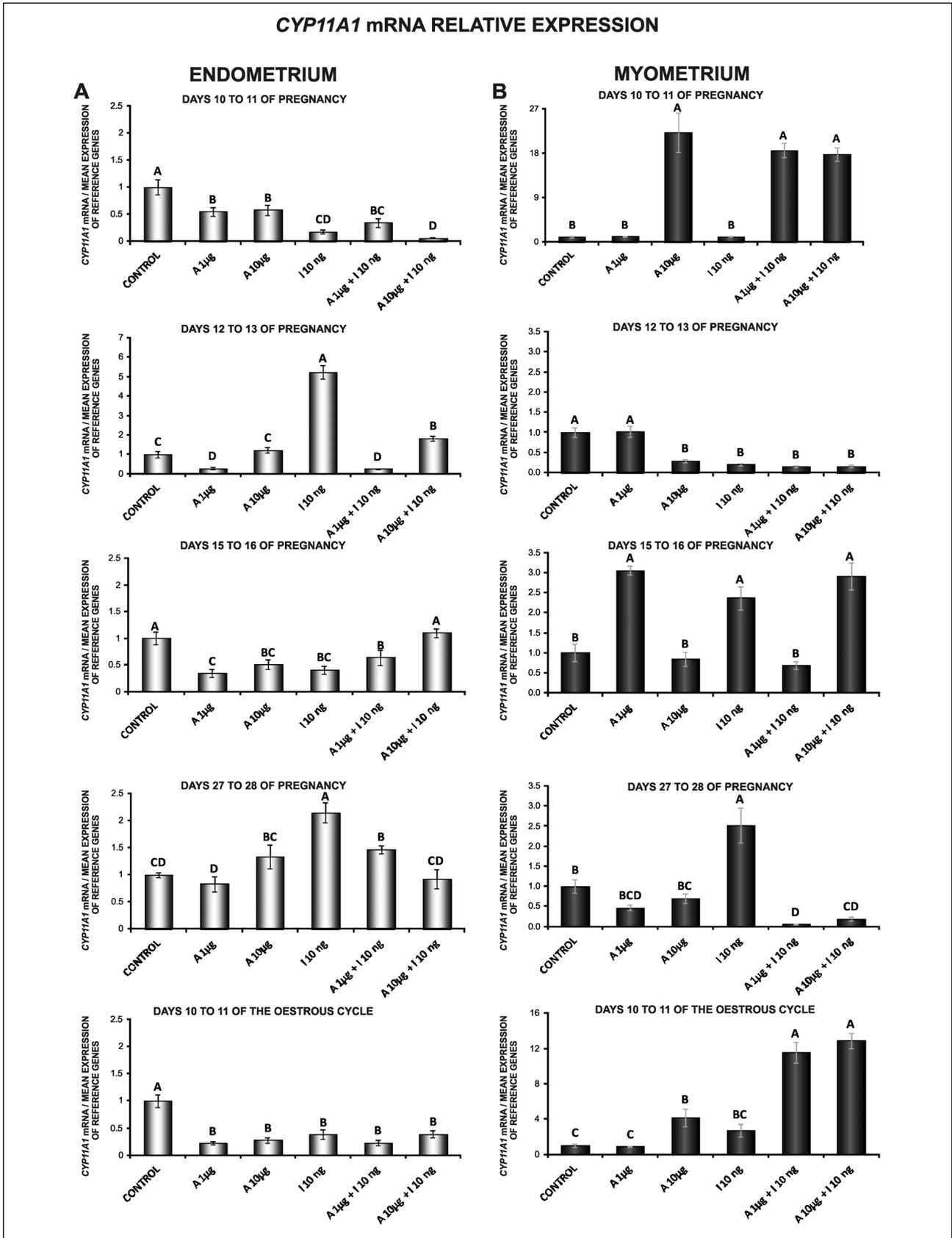


Fig. 2. The effect of adiponectin on basal and insulin-stimulated *CYP11A1* gene expression. The influence of adiponectin (1, 10 µg/ml) and adiponectin (1, 10 µg/ml) in combination with insulin (10 ng/ml) on the relative expression of P450 side chain cleavage enzyme (*CYP11A1*) gene in the endometrial (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 oestrous cycle preincubated for 2 hours and subsequently incubated for 24 hours. The expression levels were normalized with the geometric mean expression of reference genes: cyclophilin and β-actin. Data are expressed as the mean ± standard error of the mean (n = 5). Bars with different superscript capital letters are significantly different at P < 0.05.

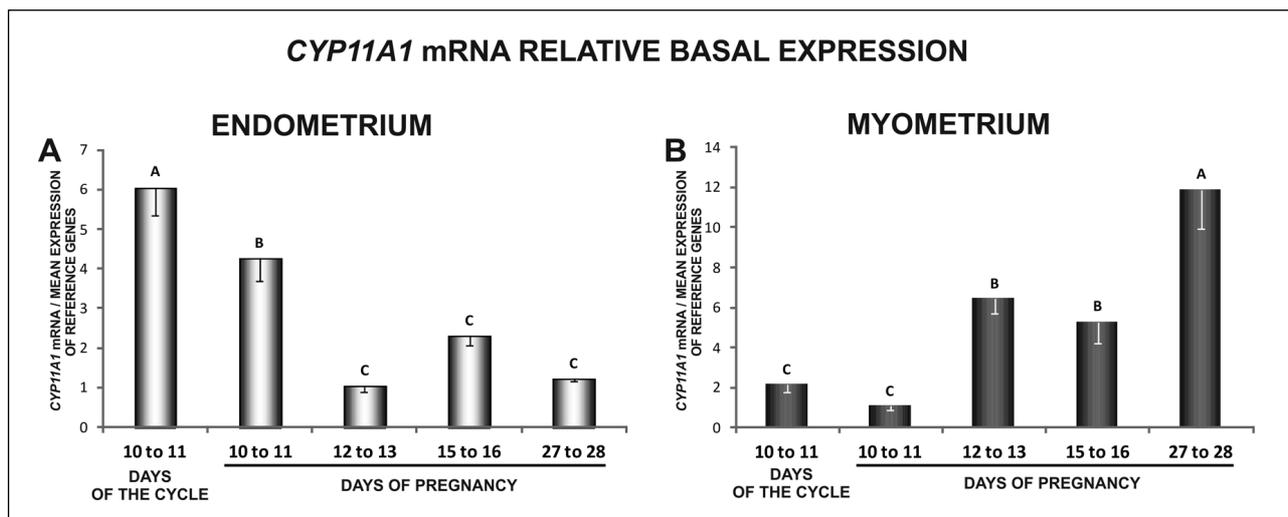


Fig. 3. Basal expression of *CYP11A1* gene in endometrial and myometrial tissue explants. A comparison of P450 side chain cleavage enzyme (*CYP11A1*) mRNA expression determined by quantitative real-time PCR in the endometrium (A) and myometrium (B) between days 10 to 11 of the oestrous cycle and days 10 to 11, 12 to 13, 15 to 16 and 27 to 28 of pregnancy. Data are expressed as the mean \pm standard error of the mean ($n = 5$). Bars with different superscript capital letters are significantly different at $P < 0.05$.

In the myometrial explants, adiponectin at both doses (1, 10 $\mu\text{g/ml}$) increased *HSD3B1* mRNA content on days 12 to 13 of pregnancy. On days 15 to 16 of pregnancy, under the influence of 1 and 10 $\mu\text{g/ml}$ of adiponectin a significant decrease in basal *HSD3B1* mRNA expression was observed. Adiponectin at a higher dose (10 $\mu\text{g/ml}$) on days 27 to 28 of pregnancy, and adiponectin at a lower dose (1 $\mu\text{g/ml}$) on days 10 to 11 of the cycle decreased *HSD3B1* gene expression ($P < 0.05$; Fig. 4B).

Insulin alone in relation to the control increased *HSD3B1* mRNA expression in the myometrium on days 10 to 11 of the cycle ($P < 0.05$). During all studied days of pregnancy, insulin had no effect on the gene expression (Fig. 4B).

In the myometrial tissue explants, on days 15 to 16 of pregnancy and on days 10 to 11 of the cycle, adiponectin at a dose of 1 $\mu\text{g/ml}$ with insulin attenuated *HSD3B1* mRNA expression compared to insulin alone ($P < 0.05$; Fig. 4B).

The effect of adiponectin on P₄ secretion by the endometrial and myometrial tissues explants

In endometrial explants, on days 10 to 11 and 27 to 28 of pregnancy, the secretion of P₄ was enhanced in response to 10 $\mu\text{g/ml}$ of adiponectin. On days 12 to 13 of pregnancy, the addition of this treatment at a dose of 1 $\mu\text{g/ml}$ caused an increase in P₄ production. On days 10 to 11 of the cycle, adiponectin at a higher dose (10 $\mu\text{g/ml}$) decreased P₄ release ($P < 0.05$; Fig. 5A).

Insulin alone compared to the control decreased P₄ release in the endometrium on days 10 to 11 of the cycle ($P < 0.05$). During all studied days of pregnancy, insulin did not affect P₄ output (Fig. 5A).

In the endometrial tissue explants, on days 10 to 11 of the cycle, the secretion of P₄ was stimulated by adiponectin (10 $\mu\text{g/ml}$) in combination with insulin compared to insulin alone ($P < 0.05$). During all studied days of pregnancy, the effect of adiponectin at both doses (1, 10 $\mu\text{g/ml}$) on insulin-stimulated P₄ production was non-significant (Fig. 5A).

In the myometrium, adiponectin at a higher dose (10 $\mu\text{g/ml}$) on days 10 to 11 of pregnancy and at both doses (1, 10 $\mu\text{g/ml}$) on days 12 to 13 of pregnancy provoked an increase in P₄ secretion. On days 10 to 11 of the cycle, adiponectin at a lower dose (1 $\mu\text{g/ml}$) reduced P₄ production ($P < 0.05$; Fig. 5B).

Insulin alone in relation to the control decreased P₄ production in the myometrium on days 10 to 11 of the cycle ($P < 0.05$). During all studied days of pregnancy, insulin did not affect the release (Fig. 5B).

In the myometrial tissue explants, on days 10 to 11 of the cycle, adiponectin at both doses (1, 10 $\mu\text{g/ml}$) with insulin enhanced the secretion of steroid compared to insulin alone ($P < 0.05$). During pregnancy, the release of P₄ was not changed in response to adiponectin (1, 10 $\mu\text{g/ml}$) with insulin (Fig. 5B).

The effect of adiponectin on A₄ secretion by the endometrial and myometrial tissue explants

In the endometrium, adiponectin at both concentrations (1, 10 $\mu\text{g/ml}$) decreased basal secretion of A₄ on days 10 to 11 of pregnancy and on days 10 to 11 of the cycle ($P < 0.05$; Fig. 6A).

Insulin alone compared to the control diminished A₄ production in the endometrium on days 10 to 11 and 12 to 13 of pregnancy, and on days 10 to 11 of the cycle ($P < 0.05$). On days 15 to 16 and 27 to 28 of pregnancy, A₄ release was not changed under insulin influence (Fig. 6A).

Treatment of the endometrial explants on days 10 to 11 and 27 to 28 of pregnancy with adiponectin (1, 10 $\mu\text{g/ml}$) and insulin in relation to insulin alone caused an increase in A₄ secretion. Adiponectin at a lower dose (1 $\mu\text{g/ml}$) in combination with insulin increased A₄ secretion on days 12 to 13 of pregnancy, and decreased the steroid production on days 15 to 16 of pregnancy ($P < 0.05$; Fig. 6A).

In the myometrial tissue explants, on days 15 to 16 and 27 to 28 of pregnancy, basal secretion of A₄ was enhanced by a lower dose of adiponectin (1 $\mu\text{g/ml}$). On days 10 to 11 of the cycle, adiponectin at a higher dose (10 $\mu\text{g/ml}$) decreased A₄ production ($P < 0.05$; Fig. 6B).

Insulin alone compared to the control increased A₄ secretion on days 12 to 13 of pregnancy and decreased the production of A₄ on days 10 to 11 of the cycle ($P < 0.05$). On days 10 to 11, 15 to 16 and 27 to 28 of pregnancy, insulin had no effect on A₄ release (Fig. 6B).

In the myometrium, insulin-induced secretion of A₄ was enhanced by both doses of adiponectin (1, 10 $\mu\text{g/ml}$) in relation

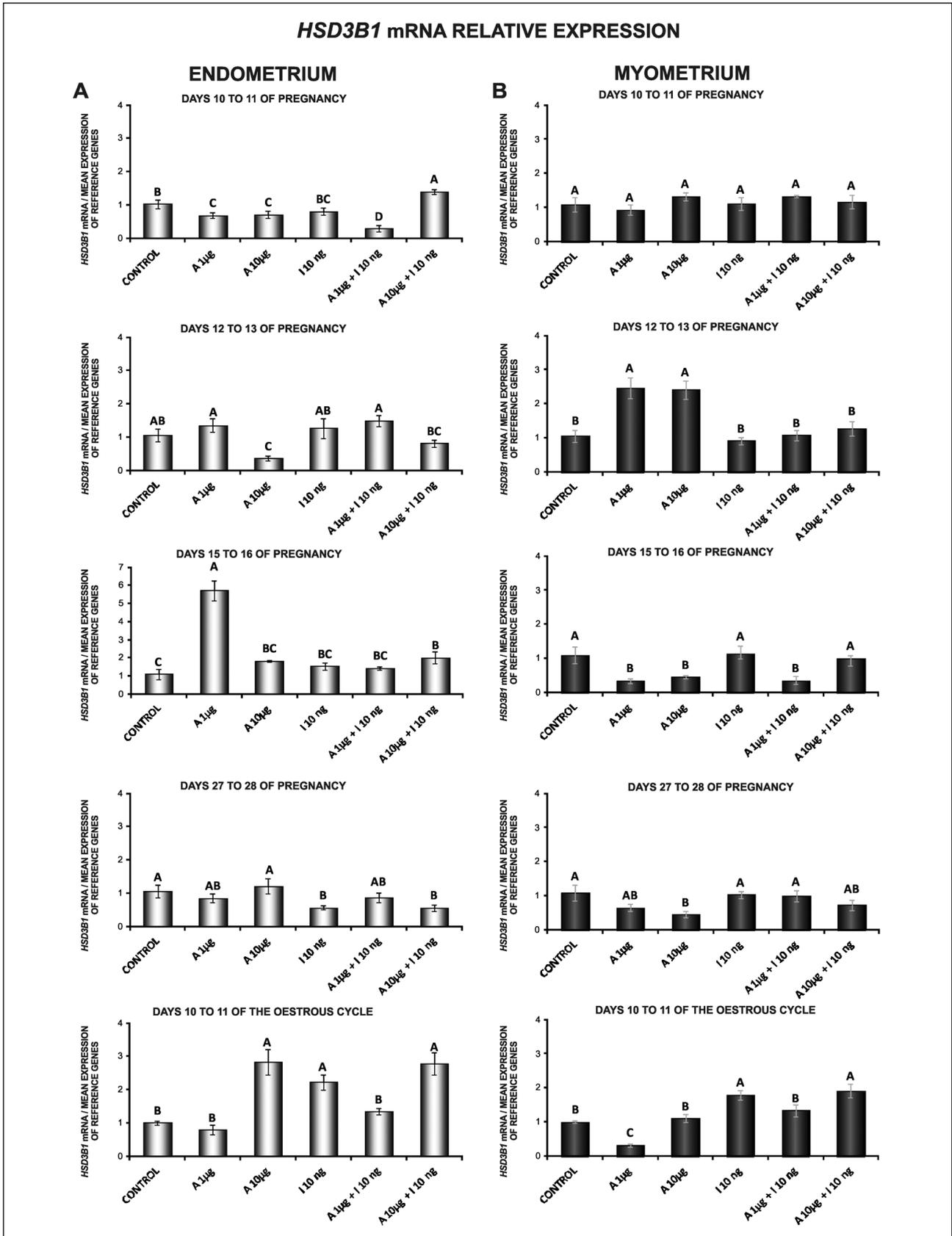


Fig. 4. The effect of adiponectin on basal and insulin-stimulated *HSD3B1* gene expression. The influence of adiponectin (1, 10 µg/ml) and adiponectin (1, 10 µg/ml) in combination with insulin (10 ng/ml) on the relative expression of 3β-hydroxysteroid dehydrogenase (*HSD3B1*) gene in the endometrial (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 oestrous cycle preincubated for 2 hours and subsequently incubated for 24 hours. The expression levels were normalized with the geometric mean expression of reference genes: cyclophilin and β-actin. Data are expressed as the mean ± standard error of the mean (n = 5). Bars with different superscript capital letters are significantly different at P < 0.05.

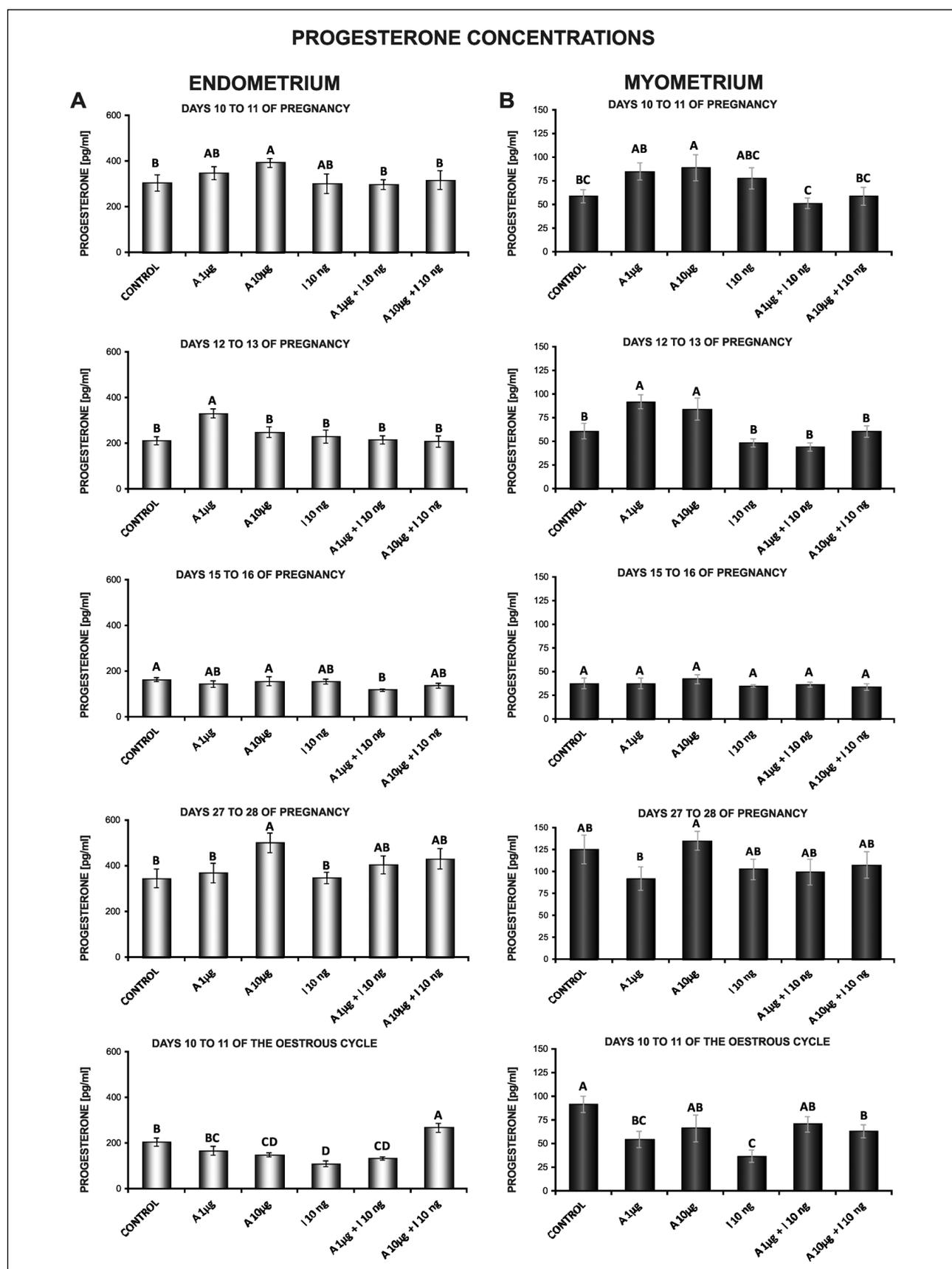


Fig. 5. The effect of adiponectin on basal and insulin-stimulated progesterone secretion. The influence of adiponectin (1, 10 $\mu\text{g}/\text{ml}$) and adiponectin (1, 10 $\mu\text{g}/\text{ml}$) in combination with insulin (10 ng/ml) on *in vitro* progesterone secretion by the endometrial (A) and 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 oestrous cycle preincubated for 2 h and subsequently incubated for 24 hours. Data are expressed as the mean \pm standard error of the mean ($n = 5$). Bars with different superscript capital letters are significantly different at $P < 0.05$.

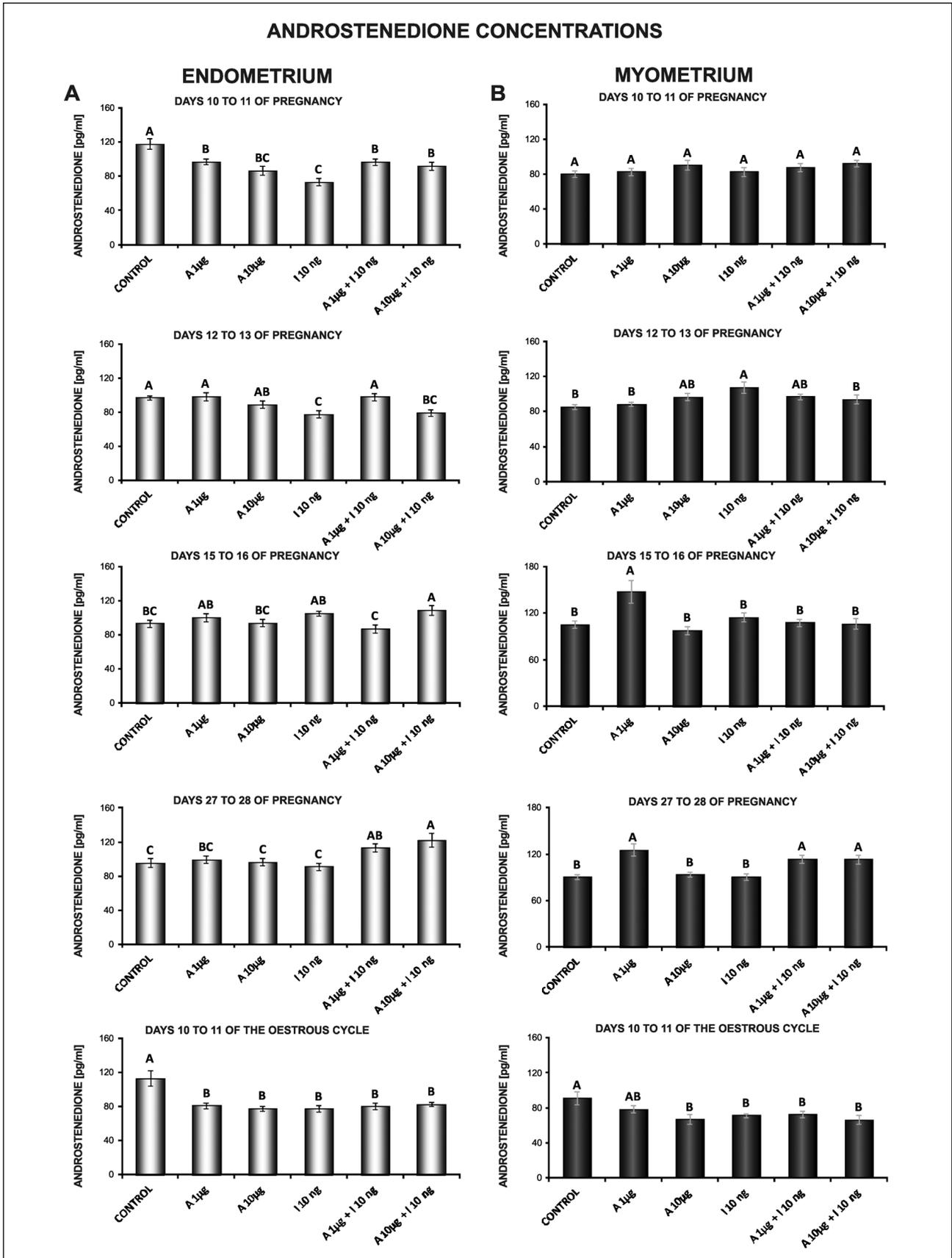


Fig. 6. The effect of adiponectin on basal and insulin-stimulated androstenedione secretion. The influence of adiponectin (1, 10 µg/ml) and adiponectin (1, 10 µg/ml) in combination with insulin (10 ng/ml) on *in vitro* androstenedione secretion by the endometrial (A) and myometrial (B) tissue explants 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 oestrous cycle preincubated for 2 hours and subsequently incubated for 24 hours. Data are expressed as the mean ± standard error of the mean (n = 5). Bars with different superscript capital letters are significantly different at P < 0.05.

to insulin alone on days 27 to 28 of pregnancy. On days 12 to 13 of pregnancy, adiponectin (10 µg/ml) with insulin diminished A₄ production ($P < 0.05$; Fig. 6B).

DISCUSSION

To our knowledge, this is the first report of the influence of adiponectin and insulin on uterine steroidogenesis. Our results demonstrate that adiponectin modulates basal and insulin-induced expression of *StAR*, *CYP11A1* and *HSD3B1* genes and secretion of P₄ and A₄ by endometrial and myometrial tissue explants harvested from pregnant pigs on days 10 to 11, 12 to 13, 15 to 16 and 27 to 28 of gestation, and from cyclic pigs on days 10 to 11 of the cycle. In the present study, we have shown, for the first time, the expression of *CYP11A1* mRNA in the porcine uterus during the early stage of pregnancy and the mid-luteal phase of the oestrous cycle. In the endometrium, the expression of *CYP11A1* gene was higher on days 10 to 11 of the cycle compared to all tested days of pregnancy. In contrast, in the myometrium, *CYP11A1* mRNA content was higher during pregnancy than on days 10 to 11 of the cycle. The expression of *HSD3B1* gene and P₄ secretion in endometrial tissue explants was more pronounced on days 10 to 11 of pregnancy than on days 10 to 11 of the oestrous cycle. During pregnancy, the highest *StAR*, *CYP11A1* and *HSD3B1* mRNA expression and P₄ and A₄ release was detected on days 10 to 11 of gestation. In the myometrium, the highest *StAR* gene expression was found on days 15 to 16 of pregnancy, *CYP11A1* mRNA content on days 27 to 28 and *HSD3B1* gene expression on days 15 to 16 and 27 to 28 of pregnancy. The production of P₄ was the highest on days 27 to 28 of gestation, whereas the release of A₄ on days 15 to 16 of pregnancy (*StAR*, *HSD3B1*, P₄ and A₄ data not shown). Our results disagree with the studies of *HSD3B1* gene expression, P₄ and A₄ production levels obtained previously by Wojciechowicz *et al.* (31). Disagreement can be result of methodological differences. Their results were obtained on endometrial and myometrial slices weighing 200 – 210 mg and the incubation time was 12 hours.

The expression of key steroidogenic enzymes was found earlier in the uterus. Indeed, the presence of *StAR*, *CYP11A1* and *HSD3B1* transcripts was reported in the human endometrium (32, 33). In mice, Ben-Zimra *et al.* (40) reported the gene expression of *CYP11A1* and *HSD3B1* in the endometrium during pregnancy. In pigs, the expression of *StAR* gene was found in the endometrium on days 12 and 16 of the oestrous cycle and pregnancy (34). It has been documented that in pigs, the expression of *HSD3B1* gene and the activity of 3β-hydroxysteroid dehydrogenase were different in the endometrium and myometrium during early pregnancy and the oestrous cycle (31). Here we demonstrated, the expression of *StAR* gene, which facilitates the entry of cytosolic cholesterol into the mitochondrion, mRNA content of *CYP11A1*, which catalyses conversion of cholesterol to pregnenolone, and *HSD3B1* gene expression, converting dehydroepiandrosterone into A₄ and pregnenolone into P₄, in the porcine endometrium and myometrium during early pregnancy and the mid-luteal phase of the cycle. In the present study, we observed that the influence of adiponectin on expression of studied steroidogenic genes depends on the type of tissue, stage of pregnancy and presence of insulin.

Steroid hormones are essential for the establishment of normal endometrial receptivity, the recognition of pregnancy and the successful establishment of implantation. Adiponectin is considered to be important modulator of these processes. As indicated in our previous study, the higher porcine plasma concentrations of adiponectin were observed on days 2 to 3, 10

to 12 and 14 to 16 of the oestrous cycle relative to the follicular phase (20). Our unpublished studies have shown the highest level of adiponectin in porcine plasma on days 15 to 16 and 27 to 28 of pregnancy compared to days 10 to 11 of the cycle and days 10 to 11, 12 to 13 and 30 to 32 of pregnancy. Our recent studies have shown that the adiponectin system (genes and proteins) is present in the porcine endometrium and myometrium during the oestrous cycle and early pregnancy (26, 27). In the endometrium, adiponectin protein concentration was higher on days 12 to 13 of gestation (which is when recognition of pregnancy occurs in pigs) than on days 10 to 11, 15 to 16 and 27 to 28 of gestation. The highest AdipoR2 protein content was found during implantation (days 15 to 16). The high expression of AdipoR1 and AdipoR2 mRNAs in the porcine endometrium was also reported by Lord *et al.* (18).

Adiponectin and both receptors genes and proteins were detected in the uterus of pregnant rabbits and mice during the peri-implantation period. Before implantation, adiponectin protein was observed mainly in glandular and luminal epithelial cells, as well as in the stromal cells of rabbit endometrium and in the epithelial cells of mouse uterus. During implantation, the expression of adiponectin and its receptor in the endometrium of rabbits and adiponectin protein within the endometrium of mice was significantly higher in implantation sites (localized mainly in decidual cells) than in interimplantation sites (localized mainly in the epithelial cells) (25, 41). The termination of delayed implantation elevated the expression of adiponectin and both receptors in mice luminal epithelia, which suggests that adiponectin system expression is important for blastocyst activation and uterine receptivity. The stimulatory effect of decidualization on the expression of the adiponectin system in mice uterine tissue was confirmed by using artificial decidualization and an *in vitro* decidualization system (25). An increase of both adiponectin receptors in human endometrial cells (both stromal and epithelial) during the decidualization process was also demonstrated (42). The previously mentioned results suggest that adiponectin signalling by an auto/paracrine mechanism may play a significant role in embryo development and decidualization. During the implantation window, the expression of AdipoR1 and AdipoR2 was decreased in the endometrium of infertile women compared to the fertile control group. These results suggest that adiponectin plays a role in human uterine receptivity, and this observation may constitute a novel factor for prediction of implantation failure (43). The aforementioned results provided further evidence that adiponectin may play an essential role in pregnancy recognition and implantation.

The *de novo* synthesis of steroids in the uterus might be a crucial factor for effective implantation and maintenance of pregnancy. Available data indicate that uterine receptivity for conceptus implantation requires actions of P₄ and/or oestrogens on the uterus to modulate locally produced growth factors, cytokines and prostaglandins through auto- and paracrine pathways (44). P₄ concentration remained fairly unchanged throughout pregnancy; however, not statistically significant decrease was found in the mean P₄ level in the porcine peripheral blood plasma from days 14 to days 28 of pregnancy (45, 46). A downward trend, although no significant, was also observed in the P₄ concentration in peripheral blood measured in the jugular vein, in uterine arteries close to the ovary as well as close to the cervix in pigs from days 12 to 25 of pregnancy. Moreover, the level of P₄ in the branch of uterine artery close to the ovary and close to the cervix was significantly greater than in the jugular vein on days 12 of pregnancy (47). It was reported that endometrial secretion of P₄ (31) and the concentration of P₄ in uterine washings (48) were higher in pregnant pigs than in cyclic ones on days 12 to 13, when maternal recognition of pregnancy

in pigs occurs, which suggests that P_4 found in uterine arteries may be derived not only from the ovaries, but also from uterine tissues. In our study, the expression of *StAR* gene and secretion of endometrial P_4 were stimulated by adiponectin at a lower dose on days 12 to 13 of gestation. In the myometrium, during this stage of pregnancy, adiponectin at both doses increased *HSD3B1* mRNA content and P_4 release. On days 10 to 11 of pregnancy, adiponectin enhanced P_4 production in the endometrium, and *StAR*, *CYP11A1* gene expression and P_4 release in the myometrium. In our previous study, in the myometrium, the highest concentration of AdipoR1 protein was found on days 12 to 13 of pregnancy, and the highest expression of AdipoR2 protein was detected on days 10 to 11 and 12 to 13 of gestation (27). Thus, our findings suggest that adiponectin may contribute to a higher level of P_4 in the uterus during maternal recognition of pregnancy and to an elevated concentration of P_4 in porcine peripheral blood plasma before day 14 of pregnancy.

A_4 is converted to E_1 by $P450_{\text{arom}}$ and is the major circulating androgen in pigs (49). It has been proposed that in pigs, uterine-derived A_4 may serve as a substrate for oestrogens production mainly around days 12 to 13 of both pregnancy and the oestrous cycle and that porcine gravid uterus on days 15 to 16 is a higher source of A_4 than the cyclic uterus (31). Concentration of A_4 in porcine uterine fluid was also greater than respective plasma values of pregnant gilts between days 9 and 15 (48). Two phases of oestrogens secretion are essential for establishment of pregnancy. Pig conceptuses secrete oestrogens between days 10 to 12 of gestation providing the initial signal for maternal recognition of pregnancy in pig (50), and between days 15 and 25 to 30 of gestation (51). We observed that *StAR* mRNA expression and A_4 production were enhanced by adiponectin with insulin compared to insulin alone in the endometrium on days 10 to 11 of pregnancy (10 $\mu\text{g/ml}$) and on days 12 to 13 (1 $\mu\text{g/ml}$) of pregnancy. During implantation, adiponectin (1 $\mu\text{g/ml}$) stimulated *StAR* and *HSD3B1* genes expression in the endometrium, which correlates with the highest level of adiponectin in porcine plasma (our not published results) and the concentration of AdipoR2 protein in the endometrium presented in our previous studies (27). In the myometrium, adiponectin increased *CYP11A1* mRNA content and A_4 secretion. On days 27 to 28 of pregnancy, adiponectin and/or adiponectin with insulin enhanced *StAR* gene expression and A_4 release in the uterus. We propose that adiponectin and/or adiponectin with insulin have a positive effect on the level of A_4 , which could be a substrate for oestrogens in the uterus during early pregnancy. Based on the current study, we suggest that adiponectin modulates the local expression of steroidogenic enzymes and secretion of P_4 and A_4 , which may be important for maternal recognition of pregnancy and implantation.

The above results are in agreement with previously published studies on other steroidogenic tissues such as adrenal cells and Leydig cells. Adiponectin has been shown to increase steroid production through increase in *StAR* expression (52, 53). However, in human Leydig cells, it has been also shown that a repressive action by adiponectin at a low dose on cAMP-dependent *CYP11A1* promoter activity did not translate into decreased P_4 levels (53). Here we also reported that steroidogenic genes expression was not correlated each other and to steroid hormones production as well. Such discrepancy may be a result of the absence of correlations between the protein concentration and gene transcript. This phenomenon could be attributed to transcriptional and post-transcriptional regulation (RNA processing and stability), differences in mRNA and protein stability and functioning feedbacks that suppress mRNA expression through high protein concentrations and attenuate post-transcriptional processes through high levels of gene expression. The absence of correlations between the

protein concentration and gene expression of *StAR* was found by Blomberg and Zuelke (54) in developing porcine conceptuses. The authors suggested that *StAR* is regulated post-transcriptionally, either through mRNA stability, translation, or protein degradation. It cannot be ruled out that a similar phenomenon occurs in porcine uterus. Moreover, these inconsistencies may also derive from the activity of steroidogenic enzymes that can be modified post-translationally.

Specific binding sites for insulin have been identified in human endometrium (55). To our knowledge, there are very few studies investigating insulin's effect on the uterus. It has been previously shown that insulin stimulates proliferation of mouse endometrial epithelial cell (56). In porcine endometrial epithelial cells, insulin has been demonstrated to stimulate transepithelial sodium transport by activation of a protein phosphatase that increases Na-K ATPase activity (57). Besides the fact that insulin modulated the effect of adiponectin on the expression of steroidogenic enzymes and secretion of hormones, our results revealed for the first time the influence of insulin itself on the steroidogenic activity of the uterus in mammals.

In summary, we have shown, for the first time to our knowledge, the expression of *CYP11A1* gene in the porcine endometrium and myometrium during early pregnancy and the oestrous cycle. Our present observations are the first to disclose the role of adiponectin and insulin in the steroidogenic activity of the uterus. We found that adiponectin and insulin alone regulates the expression of *StAR*, *CYP11A1* and *HSD3B1* genes and secretion of P_4 and A_4 by the porcine endometrial and myometrial tissue explants during early pregnancy and the oestrous cycle. In this action adiponectin interacts with insulin. We documented that the influence of adiponectin on uterine steroidogenesis depends on the reproductive status, type of tissue and dose of adipokine. The present work provides new elements towards understanding the role of adiponectin within the porcine uterus. Most authors agree that 30 to 40% of porcine embryos are lost between days 12 to 18 of pregnancy. This critical period for embryos has not been fully understood. Periodic expression of genes for many factors controlling effective maternal recognition of pregnancy and implantation takes place during that time in various uterine structures. We proposed that adiponectin belongs to the group of factors that affect these processes by controlling the synthesis of steroid hormones.

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