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EXPRESSION OF ADIPONECTIN RECEPTORS 1 AND 2 IN THE OVARY AND CONCENTRATION OF PLASMA ADIPONECTIN DURING THE OESTROUS CYCLE OF THE PIG

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The aim of this study was to compare the expression levels of adiponectin receptor 1 and adiponectin receptor 2 mRNAs and proteins in porcine ovaries during four stages (days 2 to 3, 10 to 12, 14 to 16, 17 to 19) of the oestrous cycle and to measure adiponectin plasma concentrations during the same phases of the cycle. Higher mRNA expression of adiponectin receptor 1 was detected in porcine granulosa cells than in corpora lutea and theca cells ($P < 0.01$). In contrast, higher gene expression of adiponectin receptor 2 occurred in newly developed and mature corpora lutea ($P < 0.01$). The adiponectin receptor 1 protein content was the highest in corpora lutea isolated on days 2 to 3 of the cycle and was the lowest in theca interna cells ($P < 0.01$). The profile of adiponectin receptor 2 protein was similar to that of adiponectin receptor 1. Adiponectin plasma concentrations were significantly higher throughout the luteal phase than in the follicular phase ($P < 0.01$). In conclusion, the presence of adiponectin receptor 1 and adiponectin receptor 2 mRNAs and proteins in the porcine ovary suggests that adiponectin may directly affect ovarian functions through its own specific receptors. The expression of both receptors and adiponectin plasma concentration were dependent on hormonal status related to the stage of the cycle.

Key words: Adiponectin receptors, adiponectin, pig, ovary, oestrous cycle

Adiponectin is an adipose-secreted 244-amino-acid protein with 30 kDa molecular weight, which circulates in the blood in the form of a trimer (low molecular weight, LMW), a hexamer (middle molecular weight, MMW) or a multimer (high molecular weight, HMW) (Maeda et al., 1996). Adiponectin exerts its effect by binding to two distinct but structurally related 7-transmembrane receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). AdipoR1 demonstrates higher affinity for adiponectin in the form of a trimer, whereas AdipoR2 shows greater affinity for the MMW and HMW forms. There are also differences in the expression of those receptors in tissues – AdipoR1 is

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found in higher concentration in skeletal muscles, whereas AdipoR2 in the liver (Yamauchi et al., 2003). Adiponectin regulates energy homeostasis by fatty acid oxidation, glucose uptake stimulation and inhibition of gluconeogenesis and, consequently, it leads to intensified thermogenesis and weight loss (Qi et al., 2004).

A considerable body of evidence has implicated the existence of a common endocrine system that controls metabolism and reproductive functions. The adipose tissue is regarded as an important organ whose hormonal products are involved in the regulation of both metabolism and reproduction. It is believed that the main adipokine secreted by adipose tissue – adiponectin – belongs to this hormone system (Palin et al., 2012). The presence of adiponectin receptor mRNAs and proteins was observed in human granulosa cells (Chabrolle et al., 2009), follicular cells and corpora lutea of rats (Chabrolle et al., 2007*a*) and cows (Lagaly et al., 2008; Tabandeh et al., 2010), in granulosa cells of prepubertal pigs (Ledoux et al., 2006) and in ovarian follicles of hens (mRNA only) (Chabrolle et al., 2007*b*). However, in animals there are no studies on the possible impact of hormonal status related to the oestrous cycle on adiponectin receptor expression in hormonally active structures of the ovary, such as corpora lutea, granulosa and theca interna cells. The level of adiponectin in porcine blood plasma during the oestrous cycle is also unknown. Thus, the present study was designed to determine whether the expression of ovarian adiponectin receptors and adiponectin plasma level are affected by the hormonal milieu in pigs.

Materials and methods

Experimental animals

All studies were conducted in accordance with ethical standards of the institutional Animal Ethical Committee at the University of Warmia and Mazury in Olsztyn. Mature gilts (Large White & Polish Landrace) at 7 to 8 months age, weighing 130 to 140 kg and derived from private breeding were used. Twenty gilts were assigned to one of four experimental groups (n = 5 per group) as follows: days 2 to 3, 10 to 12, 14 to 16, and 17 to 19 of the oestrous cycle. Females were monitored daily for oestrous behaviour in the presence of an intact boar. The onset of the second oestrus was considered as day 0 of the oestrous cycle. Phases of the oestrous cycle were confirmed on the basis of ovarian morphology (Akins and Morrissette, 1968). Additionally, to fully confirm correctness of the evaluation of the oestrous cycle phase, the level of progesterone was determined (Nitkiewicz et al., 2010). Within a few minutes after slaughter the ovaries were removed and placed on ice for dissection. Ovarian tissue samples representing different stages of the oestrous cycle were collected from: (1) corpora haemorrhagica (days 2 to 3); (2) mature corpora lutea (days 10 to 12); and (3) regressing corpora lutea (days 14 to 16) and immediately frozen in liquid nitrogen and

stored at –80 °C until processing for RNA and protein analysis. Additionally, ovaries with preovulatory follicles (> 6 mm in diameter) from days 17 to 19 of the oestrous cycle were placed in cold PBS buffer and transported to the laboratory where they were subjected to the isolation of follicular granulosa and theca interna cells – precursor cells of large and small luteal cells, respectively. Tissue and cell material representing each phase of the cycle was subjected to RNA and protein isolation and came from the same pig. At slaughter, blood samples were collected into heparinised tubes, centrifuged (2500 × *g*, 15 min, 4 °C) and the obtained plasma was stored at –80 °C until the measurement.

Isolation of granulosa and theca interna cells

Granulosa and theca interna cells were isolated from large follicles (> 6 mm in diameter). The granulosa cells were aspirated with a syringe and additionally washed out with a strong stream of media directed to the internal wall of the follicle (Kaminski et al., 2004). Granulosa cells were scraped from theca interna layer and theca cells were enzymatically dispersed in 0.25% trypsin solution (Kaminski et al., 2003). Dispersed cells were centrifuged (800 × *g* for 10 min) and washed twice or three times for granulosa and theca interna cells, respectively. The cells were filtered through a nylon mesh (40 μm in diameter) and resuspended in Eagle's medium enriched with BSA (5%) and antibiotics. The cells were counted using a haemocytometer and their viability (~ 98%) was determined by 0.4% trypan blue dye exclusion. Granulosa and theca interna cells were immediately resuspended in TRIzol reagent (Invitrogen, USA) and stored at –80 °C until processing for RNA and protein analysis.

Determination of the expression of AdipoR1 and AdipoR2 genes

Total RNA was extracted from luteal tissues recovered on days 2 to 3, 10 to 12, and 14 to 16 of the oestrous cycle using the Absolutely RNA Miniprep Kit (Stratagene, USA). In the case of granulosa and theca cells, total RNA was extracted using the TRIzol reagent. Other methodological details were in accordance with those used in the study of Kiezun et al. (2013).

Quantitative real-time PCR analysis was performed using a PCR System 7300 (Applied Biosystems, USA). Sense and antisense primers (AdipoR1, AdipoR2, cyclophilin A) were chosen according to the study of Lord et al. (2005). Forward and reverse primers for GAPDH were used according to the paper of Nitkiewicz et al. (2010). The conditions of the reaction were the same as in our previous study (Kiezun et al., 2013). The relative expression levels of AdipoR1 and AdipoR2 were calculated based on the comparative cycle threshold method (ΔΔCt) (Livak and Schmittgen, 2001) and normalised using the geometrical mean of the expression level of reference genes GAPDH and cyclophilin A. To confirm that GAPDH and cyclophilin A were the suitable reference genes for

this study, we revealed that there were no statistically significant differences in Ct value between the examined ovarian structures throughout all investigated stages of the oestrous cycle.

Western blotting

Western blotting analysis was performed as described by Smolinska et al. (2007), using specific first and second antibodies which were presented in our previous study by Kiezun et al. (2013). The results of Western blotting were quantified by densitometric scanning of immunoblots with GelScan for Windows version 1.45 software (Kucharczyk, Poland). Data were expressed as a ratio of AdipoR1 or AdipoR2 protein relative to actin protein in arbitrary optical density units.

Measurement of plasma adiponectin concentration

Adiponectin plasma level was determined by immunoassay using a commercially available porcine-specific kit (CSB-E06839p; Cusabio, China) according to the manufacturer's protocol. The range of standard curve was 1.88–30 μg/ml. The minimum detectable dose of porcine adiponectin is less than 0.94 μg/ml. The sensitivity of this assay was defined as the lowest protein concentration that could be differentiated from zero samples. All samples were run in duplicate in the same assay. Absorbance values were measured at 450 nm using i-control software corresponding with Tecan Infinite m200 reader (Tecan, Switzerland).

Data analysis

The data obtained by real-time PCR and Western blot were analysed by one-way ANOVA followed by least significant difference (LSD) *post hoc* test, and are reported as the means ± SEM from five independent observations. Data concerning immunoassay analysis were analysed by ANOVA for repeated measurements and least significant difference (LSD) *post hoc* test, and are reported as the means ± SEM from five independent observations in each phase of the cycle. Statistical analysis was performed using the Statistica program (StatSoft Inc., Tulsa, USA). Differences at $P < 0.05$ were considered statistically significant.

Results

Gene and protein expression of AdipoR1

The highest AdipoR1 gene expression ($P < 0.01$) was found in granulosa cells, whereas the lowest transcript content ($P < 0.01$ compared to mature corpora lutea, granulosa and theca cells) was noted in regressing corpora lutea collected on days 14–16 of the cycle (Fig. 1A). The AdipoR1 protein content in the

ovary was the greatest on days 2–3 in comparison with the remaining stages of the oestrous cycle ($P < 0.01$). The protein concentration was higher in granulosa cells than in theca interna cells collected on days 17–19 ($P < 0.01$). Additionally, AdipoR1 protein concentration was the lowest in theca interna cells in comparison to all remaining stages of the cycle ($P < 0.01$) (Fig. 2A).

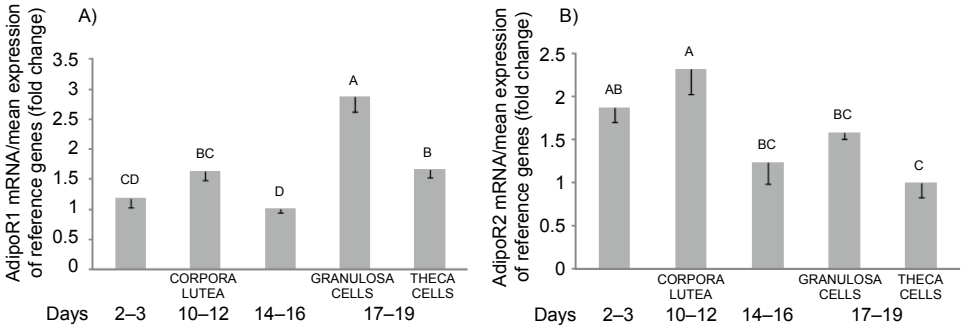

Fig. 1. (A) A comparison of adiponectin receptor 1 (AdipoR1) and (B) adiponectin receptor 2 (AdipoR2) mRNA expression determined by quantitative real-time PCR in the porcine corpora lutea, granulosa and theca interna cells on days 2–3, 10–12, 14–16 and 17–19 of the oestrous cycle. The results are expressed as means ± SEM (n = 5). Bars with different letters at the top are significantly different ($P < 0.01$)

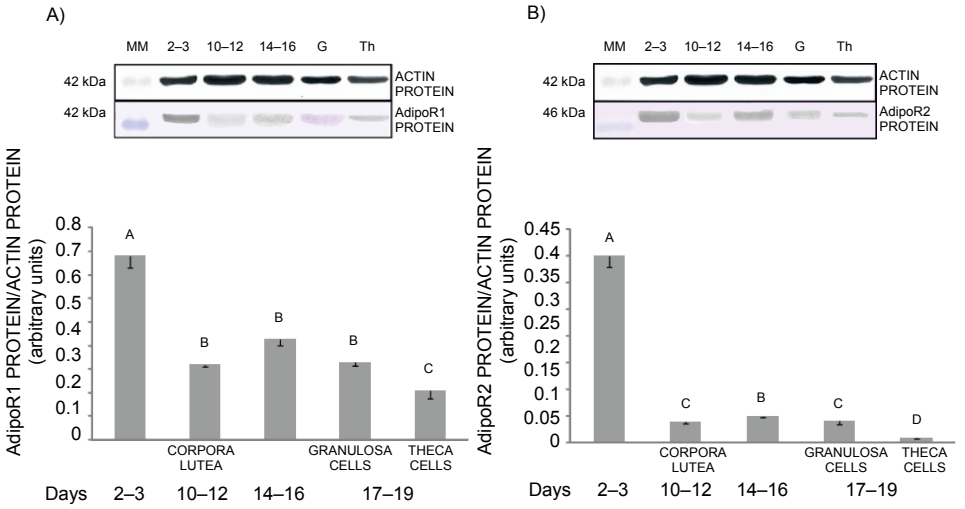

Fig. 2. (A) A comparison of adiponectin receptor 1 (AdipoR1) and (B) adiponectin receptor 2 (AdipoR2) protein expression determined by Western blotting analysis in the porcine corpora lutea, granulosa and theca interna cells on days 2–3, 10–12, 14–16 and 17–19 of the oestrous cycle. Upper panels: representative immunoblots (MM – molecular marker, G – granulosa cells, Th – theca interna cells); lower panels: densitometric analysis of adiponectin receptor proteins relative to actin protein. The noted values are expressed as means ± SEM in arbitrary optical density units (n = 5). Bars with different letters at the top are significantly different ($P < 0.01$)

Gene and protein expression of AdipoR2

The AdipoR2 gene expression pattern differed in comparison with adiponectin type 1 receptor mRNA. The highest transcript concentration ($P < 0.01$) was noted in mature corpora lutea (days 10–12). The lowest mRNA level of adiponectin type 2 receptor was observed in theca interna cells, isolated during the follicular phase of the cycle ($P < 0.01$ in relation to corpora lutea from days 2–3 and 10–12) (Fig. 1B). The protein concentration profile of AdipoR2 was similar to AdipoR1 and also was the highest in newly developed corpora lutea (days 2–3). The lowest protein concentration was indicated in theca interna cells ($P < 0.01$). The presence of AdipoR2 protein was more pronounced ($P < 0.01$) in regressing corpora lutea on days 14–16 compared to mature corpora lutea (days 10–12) and granulosa cells (days 17–19) (Fig. 2B).

Plasma adiponectin levels

The lowest plasma concentrations of adiponectin were observed during the follicular phase (days 17–19) ($P < 0.01$ in comparison with all remaining stages of the cycle representing the luteal phase). No differences were obtained in plasma adiponectin concentrations during the luteal phase (Fig. 3).

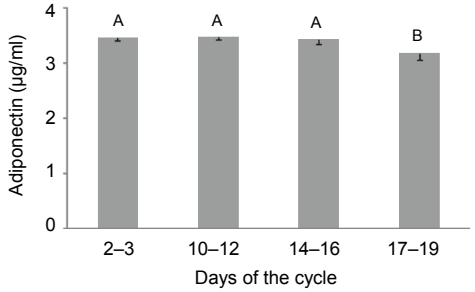

Fig. 3. Adiponectin concentration in porcine blood plasma during the oestrous cycle. The results are expressed as means ± SEM (n = 5). Bars with different letters at the top are significantly different ($P < 0.01$)

Discussion

The results of this study provide evidence for the expression of AdipoR1 and AdipoR2 mRNAs and proteins in the porcine ovary at different stages of the oestrous cycle and confirm the possible involvement of adiponectin in the control of the porcine reproductive system. To our knowledge, this is the first report demonstrating the presence of adiponectin in porcine blood plasma in relation to the oestrous cycle although no causal relationship between individual hormones

and plasma adiponectin levels were examined at this time. Interestingly, while protein concentration of both receptors mirrored the tendency of mRNAs expression during the follicular phase, some discrepancies in gene expression and protein concentration in corpora lutea were observed. As indicated in the study, a lack of correlation between the protein concentration and gene transcript in corpora lutea may result from transcriptional regulation, post-transcriptional regulation (RNA processing and stability), translational regulation or protein stability, as well as functioning feedbacks, i.e. high protein concentration may suppress mRNA expression, and a high level of gene expression may diminish the post-transcriptional processes (Gry et al., 2009; Vogel and Marcotte, 2012). The low concentration of protein with the simultaneous high gene expression level can also be caused by the action of interference RNA (RNAi). Additionally, the luteal tissue is more heterogeneous than granulosa and theca interna cells which might be a reason for discrepancies in gene expression and protein concentrations in corpora lutea.

To study the involvement of adiponectin in the reproductive functions of animals, it is crucial to localise the components of the adiponectin system in gonads. The presence of receptor mRNAs and proteins has already been demonstrated in the ovarian structures of different species. Chabrolle et al. (2009) determined the expression of both receptors (at mRNA and protein levels) in human granulosa and theca interna cells. Of these two types of follicular cells, a higher expression was observed in theca cells. Theca interna cells, corpora lutea, oocytes and granulosa cells of rats were also confirmed to express AdipoR1 and AdipoR2 genes and proteins, with a relatively less abundant presence of these transcripts and proteins in granulosa cells (Chabrolle et al., 2007*a*). Adiponectin receptors are also located in the ovarian structures of some domestic animals. Research on changes in the gene expression pattern of AdipoR1 and AdipoR2 in dairy cows was conducted by Tabandeh et al. (2010). According to this study, mRNAs of both receptors are weakly expressed in granulosa and cumulus cells in comparison with theca interna cells. In the bovine ovary, the transcript level of AdipoR1 mRNA was higher than that of AdipoR2 in granulosa and theca cells from large follicles, whereas the opposite expression pattern was observed in oocytes from large follicles. Finally, the presence of AdipoR1 and AdipoR2 mRNAs in the whole porcine ovary, without distinction between structures, was determined by Lord et al. (2005). These studies were advanced by Ledoux et al. (2006), who indicated gene expression of AdipoR1 and AdipoR2 in granulosa cells of prepubertal gilts.

The differentiated expression of adiponectin receptor genes and proteins during the oestrous cycle with the peak of receptor protein occurrence in the corpora haemorrhagica implies the impact of ovarian steroid hormones and/or pituitary gonadotropins on this expression. These data are in accordance with previously published observations showing that treatment with hCG, after PMSG pre-

treatment, resulted in an AdipoR1 and adiponectin transcript increase in rat ovaries (Chabrolle et al., 2007*a*). There is also evidence that AdipoR2 mRNA content in bovine theca, but not granulosa, cells was stimulated by LH (Lagaly et al., 2008). Further evidence for the dependence of AdipoR1 and AdipoR2 expression on the hormonal milieu comes from the studies by Tabandeh et al. (2010) on cows, indicating that the transcript concentration of both receptors increased in bovine granulosa and theca cells during follicular growth. Additionally, a higher expression was revealed for AdipoR1 and AdipoR2 in theca cells isolated from large follicles compared with the cells harvested from medium and small follicles. In the case of corpora lutea, the expression of both receptors increased during the progress of the luteal phase of the cycle.

The role of adiponectin in the control of ovarian functions has been explored to a limited degree. However, it is worth noting that the identified polymorphism in the porcine adiponectin, AdipoR1 and AdipoR2 genes is possibly associated with reproductive traits such as litter size and weaning-to-oestrus interval (Houde et al., 2008). Moreover, Lagaly et al. (2008) revealed that adiponectin decreased mRNA abundance of LH receptor in granulosa cells. Simultaneously, inhibition of insulin- and LH-stimulated release of progesterone and androstenedione from bovine theca cells was observed. The IGF-I-induced LH receptor and CYP11A1 and CYP17A1 gene expression was attenuated as well. Different to cows, granulosa cells of rats and women, cultured in the presence of IGF-I, responded to adiponectin by an increase in progesterone and oestradiol secretion (Chabrolle et al., 2007*a*; Chabrolle et al., 2009). There is only one study in which porcine granulosa cells from medium follicles of prepubertal gilts were treated with adiponectin. In response to adiponectin, higher levels of cyclooxygenase-2, prostaglandin E synthase and VEGF gene expression were observed in cells treated with the hormone. Increased expression of the StAR and a lower expression of P450aromatase gene was also noted (Ledoux et al., 2006). New information on the role of adiponectin in ovaries was obtained by an experiment where RNAi was used to block AdipoR1 and AdipoR2 expression in the KGN cell line derived from human granulosa cells (Pierre et al., 2009). The absence of AdipoR1 in the cells intensified apoptosis, whereas the elimination of AdipoR2 inhibited FSH- and IGF-I-induced secretion of progesterone and oestradiol and decreased mitogen-activated kinase activity (relative to a control) in response to adiponectin or FSH treatment. These results suggest that AdipoR1 controls the survival of granulosa cells, whereas AdipoR2 regulates steroid production through mitogen-activated protein kinases (MAPK) activation.

Our findings suggest the influence of gonadal steroid hormones, the concentrations of which change throughout the oestrous cycle, on plasma adiponectin concentrations in swine. Our results have shown similar levels of adiponectin in all investigated stages of the luteal phase and a statistically significant decrease of adiponectin plasma level during the follicular phase, which may

suggest an inhibitory effect of oestradiol on plasma adiponectin concentrations. The noted decrease in adiponectin concentrations during the follicular phase may be supported by the observations of Liu et al. (2006) which indicated an inhibitory effect of oestradiol on adiponectin content in women. However, the role of oestrogens is not fully explained. Another study (Chalvatzas et al., 2009) did not show any effect of oestradiol on human adiponectin plasma level. Additionally, no changes were observed in circulating adiponectin plasma concentrations during the menstrual cycle in healthy women (Dafopoulos et al., 2009). Adiponectin levels seem to be also determined by other gonadal steroid hormones. It was demonstrated in men that serum adiponectin concentrations vary between childhood and adulthood in a negative correlation with serum androgen levels (Bottner et al., 2004). Lafranco et al. (2004) demonstrated that increased testosterone levels in humans are inversely correlated with circulating adiponectin levels, and a similar inverse relationship has been observed in mice (Nishizawa et al., 2002). An increase in the levels of circulating adiponectin in women receiving hCG and a decrease in the hormone levels in patients treated with FSH were also observed (Liu et al., 2006).

Our present study demonstrates the expression of AdipoR1 and AdipoR2 genes and proteins in the porcine ovary and shows an impact of the stage of the oestrous cycle on the level of receptor transcripts and proteins. Similarly, adiponectin plasma concentrations are dependent on the phase of the oestrous cycle. Our results suggest that adiponectin may directly affect porcine ovarian functions through binding to the specific receptors. The adiponectin effect may be related to regulations of its receptors' expression fluctuating during the cycle. However, further studies are needed to fully explain the role of adiponectin in the control of the reproductive system.

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