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The role of vaspin in porcine corpus luteum

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Abstract

Vaspin, visceral adipose tissue-derived serine protease inhibitor, plays important roles in inflammation, obesity, and glucose metabolism. Our recent research has shown the expression and role of vaspin in the function of ovarian follicles. However, whether vaspin regulates steroidogenesis and luteolysis in the corpus luteum (CL) is still unknown. The aim of this study was first to determine the expression of vaspin and its receptor GRP78 in porcine CL at the early, middle, and late stages of the luteal phase. Next, we investigated the hormonal regulation of vaspin levels in luteal cells in response to luteinizing hormone (LH), progesterone (P4), and prostaglandin PGE2 and PGF2 α . Finally, we determined vaspin's direct impact on luteal cells steroidogenesis, luteolysis and kinases phosphorylation. Our results are the first to show higher vaspin/GRP78 expression in middle and late vs early stages; immunohistochemistry showed cytoplasmic vaspin/GRP78 localization in small and large luteal cells. *In vitro*, we found that LH, P4, PGE2, and PGF2 α significantly decreased vaspin levels. Furthermore, vaspin stimulated steroidogenesis by the activation of the GRP78 receptor and protein kinase A (PKA). Also, vaspin increased the ratio of luteotropic PGE2 to luteolytic PGF2 α secretion via GRP78 and mitogen-activated kinase (MAP3/1). Moreover, vaspin, in a dose-dependent manner, decreased GRP78 expression, while it, in a time-dependent manner, increased kinases PKA and MAPK3/1 phosphorylation. Taken together, we found that vaspin/GRP78 expression depends on the luteal phase stage and vaspin affects luteal cells endocrinology, indicating that vaspin is a new regulator of luteal cells steroidogenesis and CL formation.

Key Words

- ▶ vaspin
- ▶ corpus luteum
- ▶ steroidogenesis
- ▶ luteolysis

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Introduction

Visceral adipose tissue-derived serpin – vaspin – is an adipocytokine identified in 2005 from the visceral adipose tissue of Otsuka Long-Evans Tokushima Fatty (OLETF) rat (Hida *et al.* 2005). Rat, mouse, and human vaspins are made up of 392, 394, and 395 amino acids, respectively; they exhibit approximately 40% homology with α 1-antitrypsin and are related to the serine protease inhibitor family. Previous studies found vaspin in human, mice, bovine, and porcine tissues including adipose tissue, hypothalamus, cerebrospinal fluid, liver, heart, kidney,

stomach, skeletal muscles and ovary (Li *et al.* 2008, Klötting *et al.* 2011, Lai *et al.* 2013, Kurowska *et al.* 2019a). The expression of vaspin in visceral and s.c. adipose tissue has been found to be positively correlated with BMI and body fat content, as well as with an increase in body fat and the deterioration of metabolic disorder (Heiker 2014). Studies have shown that vaspin has an insulin-sensitizing effect, improves glucose tolerance, and has actions in the body connected with obesity development and inflammation (Heiker 2014). Data in the literature show

that in endothelial cells, vaspin acts as a ligand for the cell-surface anion channel complex heat shock protein family A (Hsp70) member GRP78 receptor (Nakatsuka *et al.* 2013). Increasing evidence indicates the important role of vaspin in reproductive physiology. Significantly higher serum levels of vaspin have been observed in women than in men (González *et al.* 2009) and in women with ovarian pathologies, like polycystic ovarian syndrome (PCOS) (Tan *et al.* 2008). Vaspin expression was detected in the hypothalamus (Klötting *et al.* 2011) and ovarian follicles, including the granulosa, theca, and oocytes (Kurowska *et al.* 2019a). Furthermore, vaspin regulates the biology of ovarian follicular cells by activating various signaling pathways, stimulating steroidogenesis *via* GRP78 and protein kinase A (PKA) (Kurowska *et al.* 2020), and having anti-apoptotic properties *via* GRP78 and mitogen-activated kinase (MAP3/1), protein kinase B (AKT), and Janus kinase (STAT3) pathways (Kurowska *et al.* 2019b). However, vaspin expression and function in corpus luteum (CL) have never been studied in human or animal models.

The CL is a transient endocrine gland formed from ovulated follicles and is also a site of the rapid remodeling, growth, differentiation, and death of cells originating from granulosa, theca, capillaries, and fibroblasts (Arosh *et al.* 2004). The CL synthesizes progesterone (P4), a steroid hormone essential for pregnancy maintenance and ovarian periodicity, which plays a central role in the regulation of the estrous cycle (Mesen & Young 2015). Defects in CL function lead to infertility, miscarriages, and ovarian cycle disorders. If the oocyte is not fertilized, the CL regresses and a new cycle begins (Arosh *et al.* 2004). The lifespan of CL depends on interactions between luteotropic and luteolytic mediators like prostaglandin, which controls the maturation, maintenance, and regression of CL (Arosh *et al.* 2004). Prostaglandin E2 (PGE2) stimulates P4 secretion in porcine luteal cells, while the opposite effect has been observed for prostaglandin F2 α (PGF2 α) (Gregoraszczyk & Michas 1999). Decreasing P4 level is connected with functional luteolysis, leading to the degeneration of luteal and endothelial cells, which is defined as structural luteolysis (Diaz *et al.* 2002). Recent studies indicate that important factors connected with the development, lifespan, and regression of CL, include some novel factors such as adiponectin (Maleszka *et al.* 2014), and apelin (Różycka *et al.* 2018). Based on these observations, we hypothesized that vaspin, like other described adipokines, may be an important factor in the regulation of porcine luteal physiology: steroid synthesis or luteolysis. Our study is the first report on the expression

of the novel adipokine vaspin and its receptor GRP78 in porcine CL and characterization of its direct biological effects on steroidogenesis and luteolysis.

Materials and methods

Sample collection

Porcine ovaries were collected from sexually mature Polish Large White pigs (6–8 months old) at different stages of the luteal phase at a local abattoir; therefore, an agreement of ethical commission is not necessary. Sows were sacrificed during slaughter according to European Legislation (EFSA, AHAW/04-027). Ovaries were transported to the laboratory in PBS with an antibiotic–antimycotic solution within 30 min of collection, then CLs were isolated from the ovaries after morphological examination at the early (2–3 days after ovulation, the presence of corpora hemorrhagica), middle (10–12 days after ovulation, the phase when CL activity is the highest in the cycle), and late (14–16 days after ovulation, the phase of luteolysis) stages of the luteal phase (Rytelewska *et al.* 2020). To determine vaspin/GRP78 mRNA expression, CLs ($n = 10$ animal per stage, 1 CL from 1 animal, so the total number of animals was 30) were immediately frozen in liquid nitrogen and stored at -70°C . To determine vaspin/GRP78 protein expression, CLs ($n = 10$ /animal per stage) were homogenized twice in an ice-cold lysis buffer (50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl, 0.5% sodium deoxycholate (w/v), 0.5% NP-40 (v/v), 0.5% SDS (w/v), and protease inhibitor). Lysates were cleared by centrifugation (15,000 $\times g$ at 4°C for 30 min); subsequently, protein levels were determined using the Bradford method. To determine vaspin/GRP78 immunolocalization, CL at the middle luteal stage ($n = 4$, from four animals) were fixed in 4% (v/v) paraformaldehyde and subsequently dehydrated in an increasing gradient of ethanol and embedded in Paraplast.

Cell culture and experimental procedure

Based on results from vaspin/GPR78 expression in the *in vitro* experiments, we used only CL from the middle luteal stage of the estrous cycle. Luteal cell cultures were prepared according to Gregoraszczyk (1983). After isolation, luteal cells were resuspended in M199 with 10% FBS (v/v). The viability of cells was determined using a Trypan blue exclusion test (95%). Finally, the cells were subsequently plated in 96-well tissue culture plates at a

concentration of 6×10^4 cells/well for 24 h. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% O₂.

To determine the effect of regulators on vaspin levels, following plating, media were removed and subsequently, luteal cells were incubated for 24 h in M199 with 1% FBS (v/v) as control or containing LH (50–150 ng/mL), P4 (10^{-8} – 10^{-6} M), and prostaglandin PGE2 and PGF2 α (10–250 ng/mL). Doses of hormones were based on Kurowska *et al.* (2019a). After incubation, the culture medium was stored at –20°C to measure vaspin concentrations, while cells were washed in PBS and then boiled in Laemmli buffer for 4 min and finally stored at –20°C to analyze vaspin protein expression. In this experiment we used five ovaries from five pigs and *in vitro* culture of luteal cells was repeated four times ($n = 4$) (each treatment in five wells); so the total number of animals was 20.

To investigate vaspin's effect on steroidogenesis, prostaglandin synthesis, and GRP78 expression, luteal cells were incubated for 24 h in M199 with 1% FBS (v/v) as a control or with vaspin at 0.01–100 ng/mL added alone or with LH (100 ng/mL). Doses of hormones were chosen based on Kurowska *et al.* (2020). After incubation, the culture medium was stored at –20°C for the quantification of P4, E2, PGE2, and PGF2 α concentrations, while cells were washed in PBS and then boiled in Laemmli buffer for 4 min and finally stored at –20°C to assess the protein expression of STAR, CYP11A1, HSD3B, CYP19A1, PTGER1, PTGFR, and GRP78. In this experiment we used five ovaries from five pigs and *in vitro* culture of luteal cells was repeated four times ($n = 4$) (each treatment in five wells); so the total number of animals was 20.

To study vaspin's effect on kinase phosphorylation, luteal cells were incubated in M199 with 1% FBS (v/v) as a control or with vaspin (1 ng/mL) for 1, 5, 15, 30, 45, and 60 min and then cells were washed in PBS and then boiled in Laemmli buffer for 4 min and finally stored at –20°C to evaluate pMAP3/1, MAP3/1, pPKA, PKA, pSTAT3, and STAT3 protein expression. In this experiment, we used four ovaries from four pigs and *in vitro* culture of luteal cells was repeated four times ($n = 4$) (each treatment in five wells); so the total number of animals was 16.

The GRP78 expression was decreased using a siRNA approach as previously described by Park *et al.* and validated in our laboratory using follicular cells (Kurowska *et al.* 2019b, 2020). To validate the GRP78 siRNA, luteal cells were incubated for 24 h in M199 without FBS, then the cells were cultured with GRP78 siRNA (1 or 2 nM) as follows (Park *et al.* 2017); the sequences of siRNA were

based on Kurowska *et al.* (2019b, 2020). After culturing, cells were stored at –20°C to analyze GRP78 protein expression. In this experiment, we used two ovaries from two pigs and *in vitro* culture of luteal cells was repeated four times ($n = 4$) (each treatment in five wells); so the total number of animals was 8.

To investigate whether P4 and prostaglandin secretion depend on GRP78 or kinase activation, luteal cells were incubated in M199 without FBS for 24 h. After starvation, they were pre-treated with GRP78 siRNA (2 nM) for 24 h or pre-treated for 1 h with MAP3/1 inhibitor PD09859 (50 μ M) or PKA inhibitor KT5720 (50 ng/mL). Finally, vaspin at 1 ng/mL was added for the following 24 h and then the culture medium was stored at –20°C to measure P4, and prostaglandin secretion. In this experiment, we used four ovaries from four pigs and *in vitro* culture of luteal cells was repeated four times ($n = 4$) (each treatment in five wells); so the total number of animals was 16. The total number of pigs in all experiments was 80.

RNA isolation and real-time PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Reverchon *et al.* 2014). RT and mRNA expression measured by real-time PCR were done as described previously (Kurowska *et al.* 2019a). The quantities of housekeeping genes, *PPIA* (cyclophilin A), and *GAPDH* were determined and normalized according to rules described by Vandesompele *et al.* (2002). The primers sequence was as follows: vaspin (forward 5'-GCTGTGAGTCGTGACCAAGT-3' and reverse 5'-CACAGAGATGCTCCAAGGG-3'), *GRP78* (forward 5'-ATCGAGTTGGCTTTCCGTGT-3' and reverse 5'-CCAGTCAGTCAGTCAGCAGG-3'), *PPIA* (forward 5'-GCATACAGGTCCTGGCATCT-3' and reverse 5'-TGTCCACATGCAGCAATGGT-3'), and *GAPDH* (forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-ATGGTGGTGAAGACGCCAGT-3'). RNA and cDNA quantities were evaluated by measuring absorbance at 260 and 280 nm using spectrophotometry with efficiency between 1.8 and 2.0.

Western blot

Tissue preparation, lysis, Western blotting, and quantification were performed as previously described (Rak *et al.* 2015). For each sample, 30–50 μ g of protein were subjected to Western blot. Primary and secondary antibodies are described in Table 1. An anti-actin antibody was used as the loading control. Signals were detected by

Table 1 Antibodies used to Western blot.

Antibody	Host species	Dilution	Vendor
Vaspin	Rabbit	1:1000	ThermoFisher Scientific product no. PA1-30989
GRP78	Rabbit	1:700	ThermoFisher Scientific product no. PA5-19503
STAR	Rabbit	1:500	Abcam product no. ab233427
CYP11A1	Goat	1:200	Santa Cruz Biotechnology product no. sc-18040
HSD3B	Mouse	1:1000	Abcam product no. ab75710
CYP19A1	Rabbit	1:200	ThermoFisher Scientific product no. PA1-21398
PTGER1	Rabbit	1:200	Abcam product no. 183073
PTGFR	Rabbit	1:200	ThermoFisher Scientific product no. PA5-70674
pMAP3/1	Rabbit	1:1000	Cell Signaling Technology product no. #9101S
MAP3/1	Rabbit	1:1000	Cell Signaling Technology product no. #9102S
pPKA	Rabbit	1:1000	Abcam product no. ab5815
PKA	Rabbit	1:500	Abcam product no. ab187515
pSTAT3	Rabbit	1:1000	Cell Signaling Technology product no. #9131S
STAT3	Rabbit	1:2000	Cell Signaling Technology product no. #4904S
Actin	Mouse	1:5000	Sigma-Aldrich product no. A5316
Anti-mouse	Horse	1:1000	Cell Signaling Technology product no. #7076
Anti-rabbit	Goat	1:1000	Cell Signaling Technology product no. #7074
Anti-goat	Mouse	1:1000	Santa Cruz Biotechnology product no. sc2354

chemiluminescence using WesternBright Quantum HRP substrate and visualized using the Chemidoc XRS+System (BioRad). All visible bands were quantified using a densitometer and ImageJ software (US NIH).

Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded CL sections (5 μ m) were processed by immunohistochemical analysis. Protein block was performed with 5% goat serum for 30 min in order to block nonspecific binding agents, then incubated with the primary antibodies (Table 1) anti-vaspin (1:100) or anti-GRP78 (1:50). Next, biotinylated goat anti-rabbit IgG (1:300), avidin-biotin-peroxidase complex and 3,3'-diaminobenzidine (Sigma-Aldrich) as chromogen antibodies (1:400) and avidin-biotinylated horseradish peroxidase complex (1:100) were applied.

Sections were then counterstained with hematoxylin, dehydrated, and mounted in DPX (Sigma-Aldrich). For negative control reaction, sections were incubated with non-immune rabbit IgG instead of primary antibodies and processed as above. Selected sections were photographed using a Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera (Nikon) with corresponding software. Objective Plan Apo λ (20 \times /0.75).

Enzyme-linked immunosorbent assay (ELISA)

Porcine vaspin ELISA kit (product no. MBS267502, MyBioSource, USA), P4 and E2 ELISA kits (product no. EIA-1561, EIA-2693, DRG Instruments GmbH, Germany), and PGE2 and PGF2 α ELISA kits (product no. ADI-900-001, ADI-900-069, Enzo Life Sciences, USA) were used according to the manufacturers' protocols. The sensitivity was 0.05 ng/mL for vaspin, 0.045 ng/mL for P4, 9.714 pg/mL for E2, 13.400 pg/mL for PGE2 and 6.710 pg/mL for PGF2 α . The inter- and intra-experimental coefficients of variation were <12 and <8%, respectively, for vaspin, <9.96 and <6.99% for P4, <9.39 and <6.81% for E2, <3.90 and <17.50% for PGE2 and <5.50 and <6.80% for PGF2 α . Absorbance was measured at the 405 nm wavelength using an ELx808 ELISA microplate reader and KC JUNIOR software (BioTek Instruments).

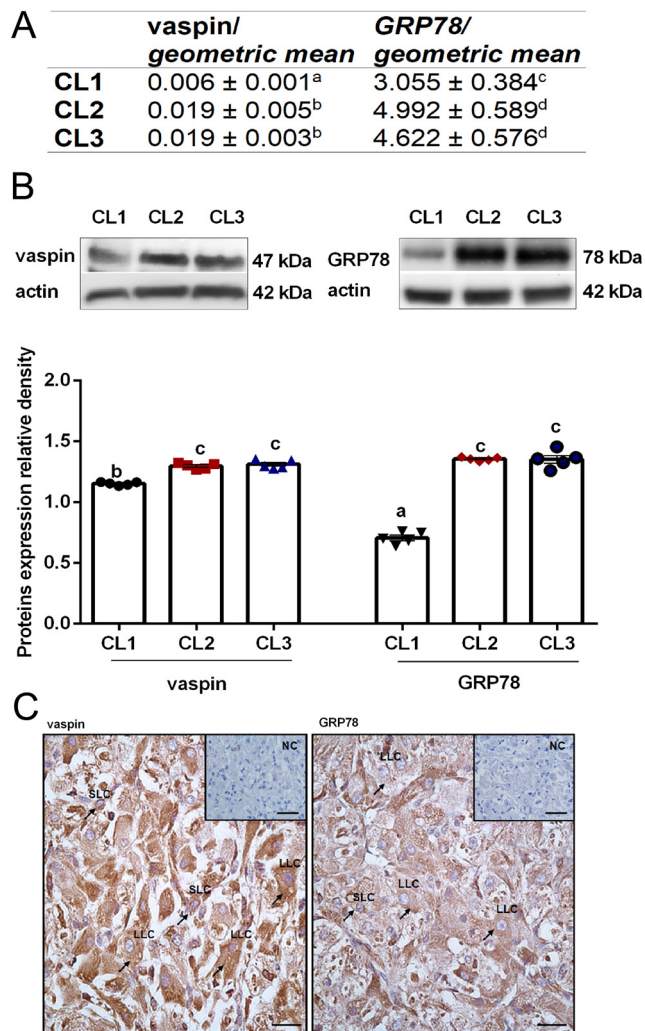
Statistical analysis

Statistical data are presented as the means \pm S.E.M. Distribution of normality was checked by the Shapiro-Wilk test. Statistical analysis was carried out using one-way or two-way ANOVA, followed by the Tukey test (PRISM software version 5; GraphPad). Statistical significance is indicated by different letters ($P < 0.05$). All outliers data have been included in statistical analysis and presented on graphs.

Results

Expression of vaspin and GRP78 in CLs at different stages of the luteal phase

Vaspin and *Grp78* mRNAs are expressed in porcine CL during the estrous cycle (Fig. 1A), suggesting that vaspin may play direct or indirect roles in the regulation of CL development. We observed that both the mRNA and protein expression of vaspin/GRP78 depend on the stage of the luteal phase: significantly higher levels were observed

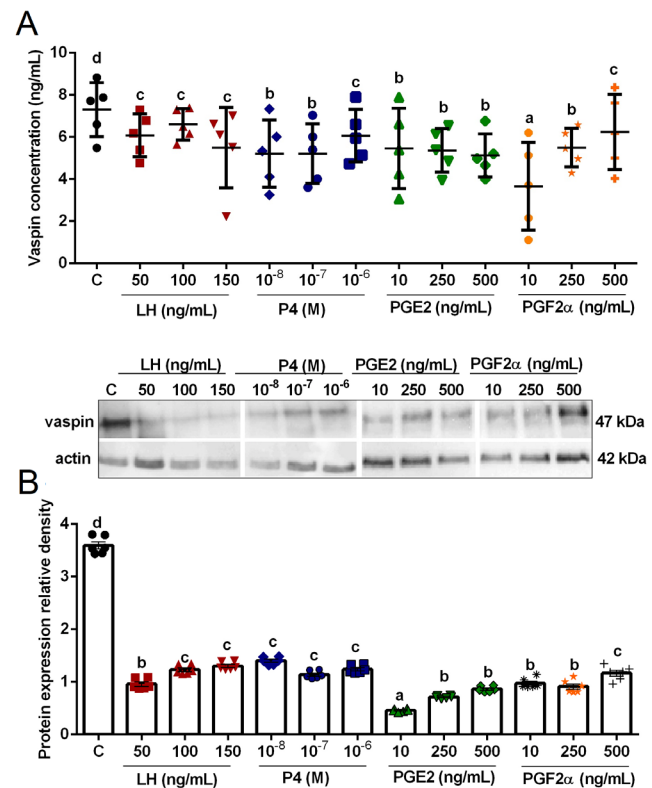
**Figure 1**

Vaspin and GRP78 mRNA (A) and protein (B) expression in porcine CLs at different stages of the luteal phase ($n = 10$). Positive vaspin/GRP78 staining were observed in the cytoplasm of large (arrows) and small (open arrows) luteal cells (C), bar = 50 μm . Genes expression levels were normalized to *PPIA* and *GAPDH* geometric mean. Data are plotted as the means \pm s.e.m. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). CL1, early; CL2, middle; CL3, late luteal phase. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

in the middle and late compared to the early stage (Fig. 1A and B, $n = 10$). Immunohistochemistry staining showed that vaspin and GRP78 were found in the cytoplasm of large (arrows) and small (open arrows) luteal cells (Fig. 1C, $n = 4$) indicating that the vaspin/GRP78 signaling may play a role in the functionality of the CL.

Effects of LH, P4, PGE2, and PGF2 α on vaspin levels

As studied before, some factors, including gonadotropin and steroid hormones, can regulate vaspin expression in

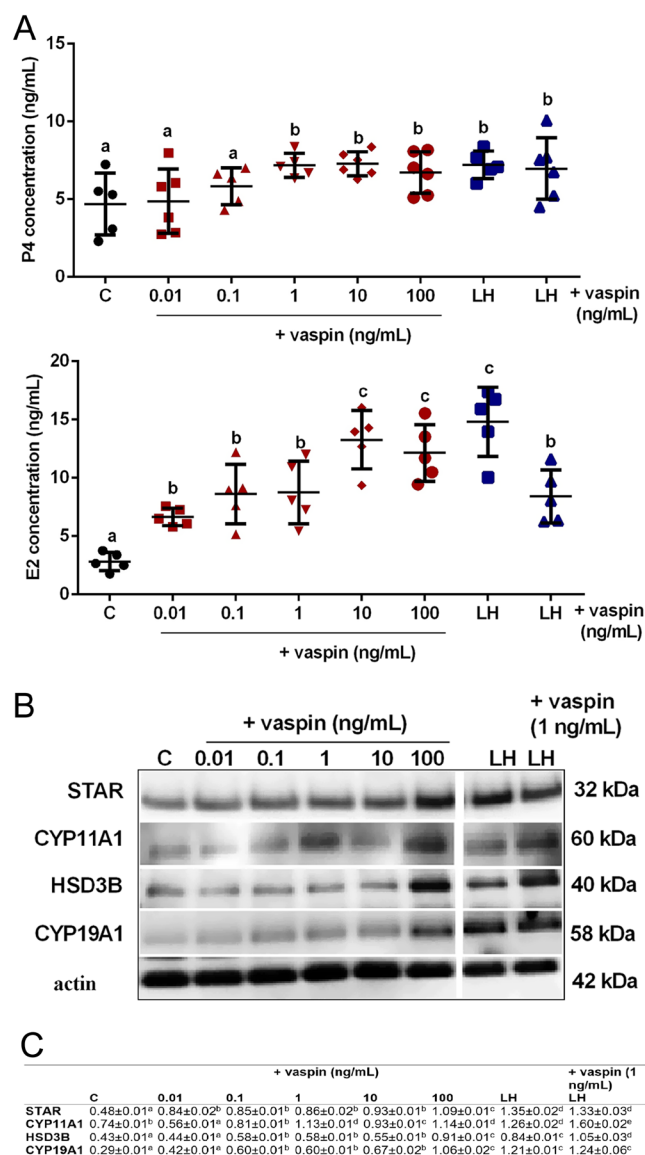
**Figure 2**

Vaspin secretion (A) and protein expression (B) regulation by LH, P4, PGE2, and PGF2 α in luteal cells ($n = 4$). Data are plotted as the means \pm s.e.m. from one representative culture. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

ovarian follicle cells (Kurowska *et al.* 2019a). In the present studies, we observed a reduction in vaspin secretion from luteal cells by LH, P4, and prostaglandins at all investigated doses (Fig. 2A, $n = 4$). Interestingly, the inhibitory effect of all investigated factors on vaspin expression was also confirmed for protein levels (Fig. 2B, $n = 4$).

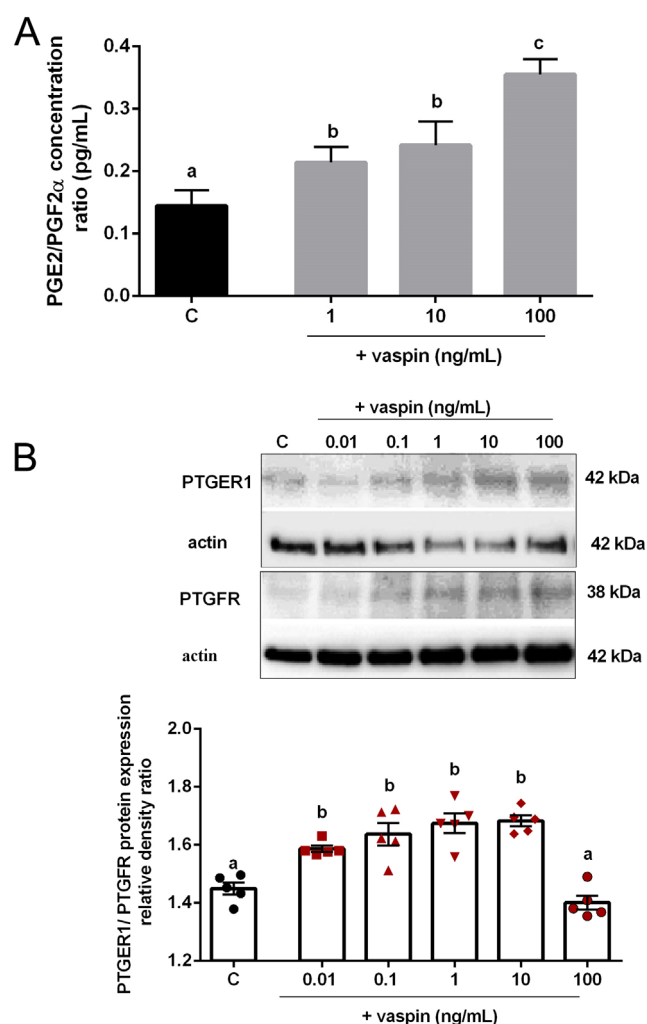
In vitro effect of vaspin on steroidogenesis

Vaspin is known to modulate steroidogenesis in ovarian follicular cells (Kurowska *et al.* 2020). Therefore, we demonstrated that vaspin increased steroidogenesis in luteal cells by stimulating steroid hormone secretion and the expression of steroid enzymes. Vaspin at doses of 1, 10, and 100 ng/mL increased P4 secretion, while added with LH did not change the stimulatory effect of LH on P4. Furthermore, vaspin, at all investigated doses, increased E2 secretion and decreased the stimulatory effect of LH, but not to the control level (Fig. 3A, $n = 4$). We observed the stimulatory effect of

**Figure 3**

Vaspin's effect on steroids P4 and E2 secretion (A), as well as steroidogenic enzymes protein expression (B) and densitometry analysis (C) in luteal cells ($n = 4$). Data are plotted as the means \pm S.E.M. from one representative culture. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

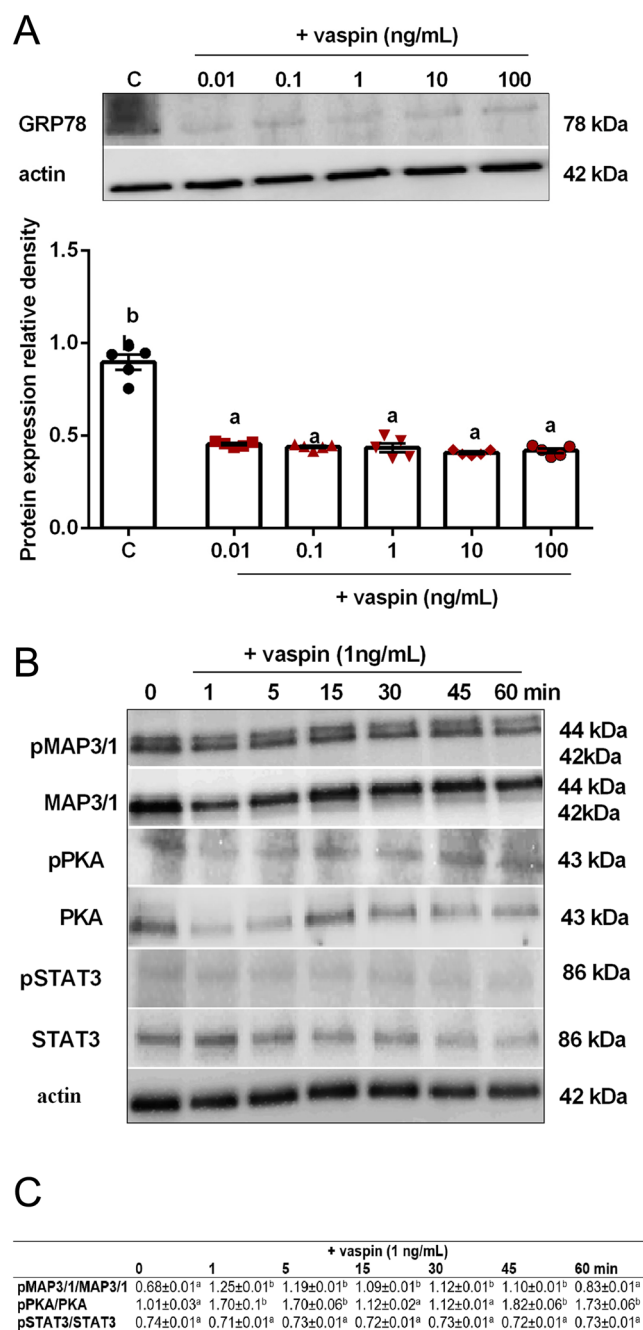
vaspin at all investigated doses on STAR, at 1–100 ng/mL on CYP11A1, and at 0.1–100 ng/mL on HSD3B and CYP19A1 protein expression. Interestingly, we noted that vaspin at 1 ng/mL increased LH-induced CYP11A1 and HSD3B expression (Fig. 3B and C, $n = 4$). These results indicate that vaspin is a regulator of luteal cells steroid synthesis.

**Figure 4**

Vaspin's effect on prostaglandin secretion (A) and receptors PTGER1/PTGFR protein expression (B) in luteal cells ($n = 4$). Data are plotted as the means \pm S.E.M. from one representative culture. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

In vitro effect of vaspin on prostaglandin secretion and PTGER1/PTGFR receptors expression

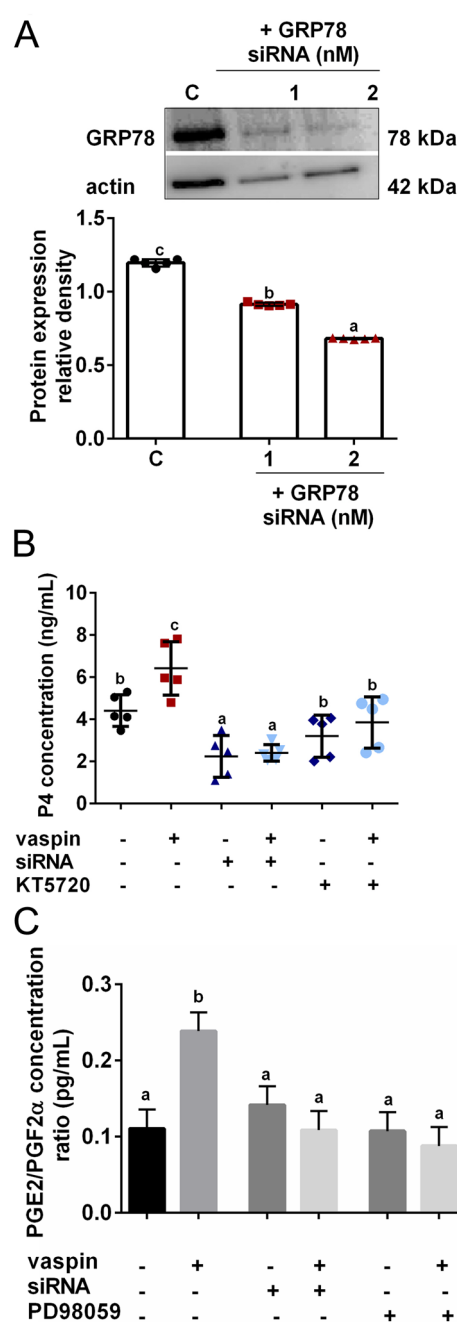
Prostaglandins balance luteotropic and luteolytic properties in the porcine luteal cells (Waclawik 2011). Hence, we documented that vaspin at doses of 1, 10, and 100 ng/mL significantly increased the ratio of luteotropic PGE2 to luteolytic PGF2 α secretion (Fig. 4A, $n = 4$). Likewise, we noted that vaspin at doses of 0.01–10 ng/mL increased the ratio of protein expression of receptor PTGER1 to PTGFR (Fig. 4B, $n = 4$), suggesting that vaspin plays an important role in CL luteolysis.

**Figure 5**

Vaspin's effect on GRP78 expression (A), kinases phosphorylation (B) and densitometry analysis (C). Data are plotted as the means \pm S.E.M. from one representative culture. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

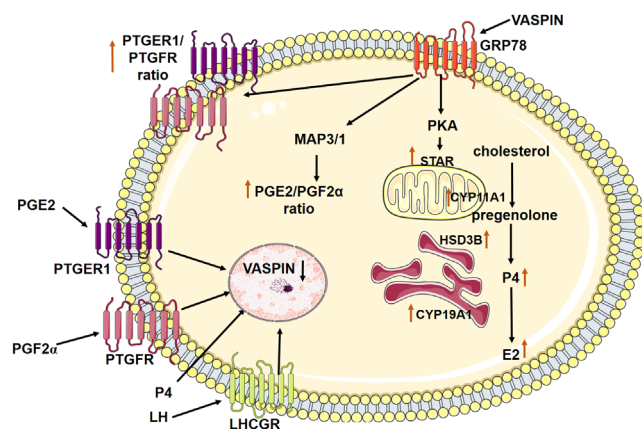
Involvement of GRP78 and kinase pathways in vaspin's action on P4 and prostaglandin secretion

We found that vaspin, at all investigated doses, significantly decreased GRP78 protein expression (Fig. 5A, $n = 4$).

**Figure 6**

The molecular mechanism of vaspin effect on P4 (B) and prostaglandin secretion (C) by luteal cells ($n = 4$). GRP78 siRNA (A). Data are plotted as the means \pm S.E.M. from one representative culture. Statistical analysis was carried out using one-way ANOVA, followed by Tukey's test. Different letters indicate differences between groups ($P < 0.05$). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

However, vaspin at a dose of 1 ng/mL, depending on the time of the incubation, increased phosphorylation of MAP3/1 kinase after 1, 5, 15, 30, and 45 min and PKA kinase after 5, 15, 45, and 60 min, while no effect on STAT3 kinase phosphorylation was observed (Fig. 5B and C, $n = 4$).

**Figure 7**

Summary of vaspin's expression, regulation and direct action on biological effects on steroidogenesis and luteolysis in the luteal cells. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0332>.

To validate the siRNA silencing method, we noted that 1 and 2 nM of GRP78 siRNA decreased GRP78 protein expression (Fig. 6A, $n=4$). Consequently, to investigate the molecular mechanism of observed changes in luteal cells, we examined the roles of the GRP78 receptor and the kinases MAP3/1 and PKA in vaspin-mediated P4 and prostaglandin secretion. We observed that simultaneous treatment with GRP78 siRNA (2 nM) or KT5720 (50 ng/mL) added with vaspin (1 ng/mL) reversed the stimulatory effect of vaspin on P4 secretion (Fig. 6B, $n=4$). Next, treatment with GRP78 siRNA or PD098059 (50 μ M) added with vaspin reversed the stimulatory effect on the ratio of PGE2/PGF2 α secretion (Fig. 6C, $n=4$). Furthermore, we noted that GRP78 siRNA and each inhibitor added alone had no effect on P4 or prostaglandin secretion.

Discussion

In the present study, we found, for the first time, that the expression of vaspin and those of GRP78 were significantly different in porcine CL at different stages of the luteal phase. We observed that the expression of vaspin in luteal cells is regulated by several factors like LH, P4, and prostaglandin PGE2 and PGF2 α (Fig. 7). Furthermore, vaspin was shown to affect luteal cells steroid and prostaglandin secretion through GRP78 and PKA and MAPK3/1 activation, respectively, indicating that vaspin is a new regulator of luteal cells function.

We noted that vaspin and its receptor GRP78 are more highly expressed in the CL of the middle and late stages of

the luteal phase compared to CL of the early luteal phase. Differences in vaspin levels between individual CL are probably hormonally controlled by LH or steroids secreted by luteal cells, the concentrations of which change during the luteal phases. Lower vaspin levels in the early luteal phase may be connected with ovulation LH peak at the beginning of the luteal phase. We suggest that this factor can negatively regulate vaspin levels at an early stage and can indicate an autocrine interaction; interestingly, when levels of LH start to decrease during the middle and late luteal phases, we noted that vaspin/GRP78 expression in CL of middle and late stages significantly increased. Furthermore, we observed immunolocalization of vaspin/GRP78 in the cytoplasm of small and large luteal cells. Large luteal cells form from granulosa cells after ovulation, while small luteal cells form from theca; our previous paper documented vaspin abundance in the granulosa and theca cells (Kurowska *et al.* 2019a). Studies have demonstrated the luteal-phase-dependent expression of other adipokines like apelin (Różycka *et al.* 2018), adiponectin (Maleszka *et al.* 2014) or chemerin (Rytelewska *et al.* 2020) in CL.

Our findings demonstrated differences in vaspin/GRP78 expression in CL at different stages of the luteal phase. In the following experiments, we determined the factors that regulate vaspin expression. We found that P4, LH and prostaglandins are negative regulators of vaspin levels in luteal cells. Hormonal interactions in the hypothalamic–pituitary–ovary axis are essential for proper ovarian physiology, including CL. For example, LH produced by pituitary cells is the parent luteotropic factor in mammals and its pulsative release is necessary for CL development in pigs, cows, and sheep (Stouffer & Hennebold 2015). P4 is the main hormone released from CL in pregnancy maintenance, while prostaglandins regulate CL formation/regression. PGF2 α is a luteolytic factor, while PGE2 is involved in maintaining the hormonal function of CL (Stouffer & Hennebold 2015). Our previous findings demonstrated that gonadotropins and steroids stimulated vaspin expression in porcine ovarian follicles by the activation of several kinase pathways (Kurowska *et al.* 2019a). The different actions of LH and P4 on vaspin levels in luteal and follicular cells can be explained by the type of structure and the phase of the estrus cycle. Likewise, von Loeffelholz *et al.* (2010) showed that vaspin expression increased in women using contraceptives, indicating the role of steroids in vaspin regulation. Data from Siawrys and Smolińska (2013) document that LH and P4 stimulated leptin expression in

porcine luteal cells, while in bovine, apelin expression was regulated by PGF2 α (Shirasuna *et al.* 2008). Interestingly, we observed a stronger inhibitory effect in the vaspin protein expression in luteal cells than vaspin secretion to the culture medium; this effect is probably that some other factors can have an inhibitory effect into luteal cells. For example, leptin mRNA can be regulated negatively by tumor necrosis factor- α or interleukin-1 β in human adipocytes (Bruun *et al.* 2002). In agreement with our observation, Bruun *et al.* (2002) observed a stronger inhibitory effect on the leptin mRNA level in adipose cells compared to its secretion to the culture medium.

Based on this interesting finding, we have hypothesized that vaspin may be involved in steroid and prostaglandin synthesis. Overall, the expression of both components (ligand and receptor) of the vaspin signaling system allows for analysis of the direct role of vaspin in luteal cells function. The formation, maintenance, regression, and steroidogenesis of CL are among the most significant and closely regulated events in mammalian reproduction. Any dysfunction in luteal steroidogenesis leads to LPD, which results in the failure of embryo implantation and abortion. In general, we observed stimulatory effects of vaspin on steroids P4, E2 secretion, and steroid enzymes protein expression in a dose-dependent manner. We noted a similar observation in follicular cells (Kurowska *et al.* 2020). Moreover, E2 is a potent tropic hormone that stimulates P4 biosynthesis (Stocco *et al.* 2007). The middle luteal phase is the most stable phase of mature CL, with full secreting capacity (Stouffer & Hennebold 2015). Stimulating E2 secretion and decreasing P4 secretion starts at CL from the late stage, where the expression of STAR and HSD3B decrease and CYP19A1 increases; in contrast, CYP11A1 expression is constant (Przygodzka *et al.* 2016). Moreover, E2 is essential for CL function, as described in rats, as it participates in the hypertrophy of luteal cells (Stocco *et al.* 2007). Similarly, in the porcine reproductive tract E2 is a part of embryo signaling systems for CL rescue during pregnancy (Ziecik *et al.* 2018). Furthermore, in our study, we observed that vaspin combined with LH, decreased E2 secretion, and caused additional stimulation of CYP11A1 and HSD3B expression; no effects on other study parameters were observed. Similarly, in ovarian follicular cells, vaspin decreased E2 production in culture induced LH (Kurowska *et al.* 2020). No additional effect of vaspin on P4 and an inhibitory effect on E2 secretion can be a consequence of the negative effect of LH on vaspin expression in the luteal cells. Further investigations are required to understand the molecular mechanisms

involved in vaspin interaction with different hormones. However, the differences observed in the present study after LH stimulation between P4 secretion/HSD3B expression or E2/CYP19A1 can be explained by enzyme activity; future studies are needed to discover the activity of the 3BHSD and CYP19A1 enzymes. In rat testes, HSB3B activity can be decreased without affecting enzyme expression (Hierlihy *et al.* 2006). To confirm our results, previous papers have documented that other adipokines affect luteal steroidogenesis, for example, leptin increased P4 secretion in equine (Galvão *et al.* 2014) and water buffalo (Kumar *et al.* 2012). A similar effect of apelin was observed in porcine luteal cells (Różycka *et al.* 2018), whereas adiponectin decreased P4 secretion (Maleszka *et al.* 2014).

A balance between luteotropic and luteolytic prostaglandin, which exert their effects throughout membrane receptors PTGER1/2/3 and PTGFR, regulates the lifespan of CL. PGE2 stimulates P4 synthesis and is involved in maintaining CL function; conversely, PGF2 inhibits P4 synthesis and leads to the apoptosis of luteolytic cells (Arosh *et al.* 2004). PGF2 α causes CL regression by inhibiting cholesterol transport, its conversion to steroid hormones and the stimulation of P4 synthesis by gonadotropin leading to the reduced secretion of P4 and lowered concentrations in the CL and serum. Besides, increases expression of factors associated with PGF2 α synthesis decrease P4 production pathways (Przygodzka *et al.* 2016) and PGE2 synthesis in pigs. Data in the literature show that leptin has a luteotropic effect: in equine stimulated PGE2 secretion (Galavao *et al.* 2014), while in rabbits, it decreased PGF2 α (Sirotkin *et al.* 2009). Vaspin's effects on prostaglandin synthesis have never been shown in ovaries, but in rat chondrocytes, vaspin combined with interleukin 1 β increased PGE2 secretion (Bao *et al.* 2014). In the present study, we documented vaspin's effect on luteal prostaglandin secretion. In our experiments, we chose luteal cells from the middle stage of the luteal phase, because the enzymatic apparatus that mediates luteolysis is responsive to luteolytic stimulus from day 12 of the porcine estrus cycle and allows the possibility of studying CL regression. Interestingly, levels of both prostaglandins in the middle stage are almost constant (Waclawik 2011), which allows directly studying vaspin's effects. We noted that vaspin increased the ratio of PGE2/PGF2 α secretion and PTGER1/PTGFR expression. The importance of the prostaglandin system ratio has been shown by many authors, for example, in cow ovaries: the prostaglandin synthesis ratio PGES to

PGFS starts to decrease in the late luteal phase leading to luteolysis (Arosh *et al.* 2004). In porcine CL at the middle stage, changing the PGE2 to PGF2 α ratio from 1:1 to 2:1 protects luteal cells against the inhibitory effect of PGF2 α on P4 secretion (Gregoraszczyk & Michas 1999).

Finally, to investigate the molecular mechanism of vaspin's role in luteal cells function, we studied the involvement of the GRP78 and kinase phosphorylation. Our study showed that vaspin decreased GRP78 protein expression, which is in good agreement with data we obtained on ovarian follicles (Kurowska *et al.* 2020). Increasing doses of vaspin lead to the downregulation of the GRP78 receptor in the cell membrane and finally lower protein expression of GRP78 in luteal cells. A similar phenomenon has been observed for leptin and its receptor in mouse liver (Hegyi *et al.* 2004) and E2 and ER- α in mice mammary glands (Hatsumi & Yamamuro 2006), indicating the ligand-induced desensitization or internalization of those receptors occurs when receptors have been chronically exposed to an excessive amount of a ligand. To our knowledge, the phosphorylation of kinases is necessary for proper ovarian physiology, including CL. As we described previously MAP3/1, PKA, and STAT3 participate in the regulation of multiple processes, including steroidogenesis, proliferation, and apoptosis (Kurowska *et al.* 2019b, 2020). Furthermore, differentiation of a luteal phenotype implies numerous changes in the intracellular signaling network; steroid production in bovine luteal cells is mediated by PKA (Jiang *et al.* 2011), while prostaglandin secretion is regulated by MAP3/1 (Arosh *et al.* 2004). In this study, we observed vaspin's effects on MAP3/1 and PKA phosphorylation, but not on STAT3. Interestingly, in porcine ovarian follicles, we observed the phosphorylation of all investigated kinases by vaspin (Kurowska *et al.* 2020), which suggests that vaspin's effect depends on the ovarian structure. Using pharmacological inhibitors of kinases and GRP78 silencing, we noted that vaspin stimulated P4 secretion through GRP78 and the activation of PKA, while prostaglandin secretion *via* GRP78 and MAP3/1. Our results are in agreement with previous work, where the stimulatory effect of vaspin on steroidogenesis in ovarian follicular cells was activated by GRP78 and PKA (Kurowska *et al.* 2020) or in human placenta cells, apelin affected steroid production by PKA activation (Dawid *et al.* 2019). Further, in endometrial cancer cells (Gao *et al.* 2009) and human placenta (Lappas *et al.* 2005), leptin stimulated PGE2 secretion by MAP3/1 pathways.

In conclusion, vaspin/GRP78 signaling was found in CL at different stages of the estrous cycle. We indicated vaspin increased steroid and prostaglandin synthesis, as well as the phosphorylation of kinases, suggesting that this adipokine can be a new regulator in CL function maintenance. The effect of vaspin/GRP78 on luteal cells steroidogenesis and luteolysis could potentially lead to therapeutic interventions for infertility caused by LPD. However, to confirm the role of vaspin in female fertility, experiments on animal models like vaspin knockout mice should be developed in future studies. Interestingly, data from Cheng *et al.* (2016) described that female adiponectin null mice displayed impaired folliculogenesis, reduced retrieval of oocytes, disrupted estrous cycle, an elevated number of atretic follicles, which suggested that adipokines are essential for female fertility. Importantly, recent published studies clearly documented the role of vaspin in reproductive pathologies; for example, vaspin could be a potential novel biomarker for the prediction and early diagnosis of PCOS (Tan *et al.* 2008) or pregnancy pathologies (Mierzyński *et al.* 2019). Moreover, lower plasma vaspin level is a predictor of successful ovulation in PCOS patients (Dogan *et al.* 2017).

Declaration of interest

The authors declare that there is no conflict of the interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J & Fortier MA 2004 Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. *Endocrinology* **145** 2551–2560. (<https://doi.org/10.1210/en.2003-1607>)
- Bao JP, Jiang LF, Li J, Chen WP, Hu PF & Wu LD 2014 Visceral adipose tissue-derived serine protease inhibitor inhibits interleukin-1 β -induced catabolic and inflammatory responses in murine chondrocytes. *Molecular Medicine Reports* **10** 2191–2197. (<https://doi.org/10.3892/mmr.2014.2478>)
- Bruun JM, Pedersen SB, Kristensen K & Richelsen B 2002 Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. *Molecular and Cellular Endocrinology* **190** 91–99. ([https://doi.org/10.1016/s3033-7207\(02\)00007-2](https://doi.org/10.1016/s3033-7207(02)00007-2))
- Cheng L, Shi H, Jin Y, Li X, Pan J, Lai Y, Lin Y, Jin Y, Roy G, Zhao A, *et al.* 2016 Adiponectin deficiency leads to female subfertility and ovarian

- dysfunctions in mice. *Endocrinology* **157** 4875–4887. (<https://doi.org/10.1210/en.2015-2080>)
- Dawid M, Mlyczyńska E, Kurowska P, Sierpowski M & Rak A 2019 Apelin decreased placental hormone secretion by human trophoblast BeWo cells via apelin receptor, protein kinase A and extracellular signal-regulated kinases 1/2 activation. *Journal of Physiology and Pharmacology* **70** 95–907. (<https://doi.org/10.26402/jpp.2019.6.08>)
- Diaz FJ, Anderson LE, Wu YL, Rabot A, Tsai SJ & Wiltbank MC 2002 Regulation of progesterone and prostaglandin F2alpha production in the CL. *Molecular and Cellular Endocrinology* **191** 65–80. ([https://doi.org/10.1016/S0303-7207\(02\)00056-4](https://doi.org/10.1016/S0303-7207(02)00056-4))
- Dogan K, Ekin M, Helvacioğlu Ç & Yaşar L 2017 Can serum vaspin levels predict clomiphene resistance in infertile women with PCOS? *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **217** 6–11. (<https://doi.org/10.1016/j.ejogrb.2017.08.006>)
- Galvão A, Tramontano A, Rebordão MR, Amaral A, Bravo PP, Szóstek A, Skarzynski D, Mollo A & Ferreira-Dias G 2014 Opposing roles of leptin and ghrelin in the equine corpus luteum regulation: an in vitro study. *Mediators of Inflammation* **2014** 682193. (<https://doi.org/10.1155/2014/682193>)
- Gao J, Tian J, Lv Y, Shi F, Kong F, Shi H & Zhao L 2009 Leptin induces functional activation of cyclooxygenase-2 through JAK2/STAT3, MAPK/ERK, and PI3K/AKT pathways in human endometrial cancer cells. *Cancer Science* **100** 389–395. (<https://doi.org/10.1111/j.1349-7006.2008.01053.x>)
- González CR, Caminos JE, Vázquez MJ, Garcés MF, Cepeda LA, Angel A, González AC, García-Rendueles ME, Sangiao-Alvarellos S, López M, *et al.* 2009 Regulation of visceral adipose tissue-derived serine protease inhibitor by nutritional status, metformin, gender and pituitary factors in rat white adipose tissue. *Journal of Physiology* **587** 3741–3750. (<https://doi.org/10.1113/jphysiol.2009.172510>)
- Gregoraszczuk E 1983 Steroid hormone release in cultures of pig corpus luteum and granulosa cells: effect of LH, hCG, PRL and estradiol. *Endocrinologia Experimentalis* **17** 59–68.
- Gregoraszczuk EL & Michas N 1999 Progesterone and estradiol secretion by porcine luteal cells is influenced by individual and combined treatment with prostaglandins E2 and F2alpha throughout the estrus cycle. *Prostaglandins and Other Lipid Mediators* **57** 231–241. ([https://doi.org/10.1016/S0090-6980\(99\)00009-X](https://doi.org/10.1016/S0090-6980(99)00009-X))
- Hatsumi T & Yamamuro Y 2006 Downregulation of estrogen receptor gene expression by exogenous 17beta-estradiol in the mammary glands of lactating mice. *Experimental Biology and Medicine* **231** 311–316. (<https://doi.org/10.1177/153537020623100311>)
- Hegyí K, Fülöp KA, Kovács KJ, Falus A & Tóth S 2004 High leptin level is accompanied with decreased long leptin receptor transcript in histamine deficient transgenic mice. *Immunology Letters* **92** 193–197. (<https://doi.org/10.1016/j.imlet.2003.11.029>)
- Heiker JT 2014 Vaspin (serpinA12) in obesity, insulin resistance, and inflammation. *Journal of Peptide Science* **20** 299–306. (<https://doi.org/10.1002/psc.2621>)
- Hida K, Wada J, Eguchi J, Zhang H, Baba M, Seida A, Hashimoto I, Okada T, Yasuhara A, Nakatsuka A, *et al.* 2005 Visceral adipose tissue-derived serine protease inhibitor: a unique insulin-sensitizing adipocytokine in obesity. *PNAS* **102** 10610–10615. (<https://doi.org/10.1073/pnas.0504703102>)
- Hierlihy AM, Cooke GM, Curran IH, Mehta R, Karamanos L & Price CA 2006 Effects of ciprofibrate on testicular and adrenal steroidogenic enzymes in the rat. *Reproductive Toxicology* **22** 37–43. (<https://doi.org/10.1016/j.reprotox.2005.11.001>)
- Jiang YF, Tsui KH, Wang PH, Lin CW, Wang JY, Hsu MC, Chen YC & Chiu CH 2011 Hypoxia regulates cell proliferation and steroidogenesis through protein kinase A signaling in bovine corpus luteum. *Animal Reproduction Science* **129** 152–161. (<https://doi.org/10.1016/j.anireprosci.2011.12.004>)
- Klötting N, Kovacs P, Kern M, Heiker JT, Fasshauer M, Schön MR, Stumvoll M, Beck-Sickinger AG & Blüher M 2011 Central vaspin administration acutely reduces food intake and has sustained blood glucose-lowering effects. *Diabetologia* **54** 1819–1823. (<https://doi.org/10.1007/s00125-011-2137-1>)
- Kumar L, Panda RP, Hyder I, Yadav VP, Sastry KV, Sharma GT, Mahapatra RK, Bag S, Bhure SK, Das GK, *et al.* 2012 Expression of leptin and its receptor in corpus luteum during estrous cycle in buffalo (*Bubalus bubalis*). *Animal Reproduction Science* **135** 8–17. (<https://doi.org/10.1016/j.anireprosci.2012.08.030>)
- Kurowska P, Mlyczyńska E, Barbe A, Staub C, Gregoraszczuk E, Dupont J & Rak A 2019a Vaspin in the pig ovarian follicles: expression and regulation by different hormones. *Reproduction* **158** 135–146. (<https://doi.org/10.1530/REP-19-0034>)
- Kurowska P, Mlyczyńska E, Dawid M, Opydo-Chanek M, Dupont J & Rak A 2019b In vitro effects of vaspin on porcine granulosa cell proliferation, cell cycle progression, and apoptosis by activation of GRP78 receptor and several kinase signaling pathways including MAP3/1, AKT, and STAT3. *International Journal of Molecular Sciences* **20** 5816. (<https://doi.org/10.3390/ijms20225816>)
- Kurowska P, Mlyczyńska E, Dawid M, Dupont J & Rak A 2020 Role of vaspin in porcine ovary: effect on signaling pathways and steroid synthesis via GRP78 receptor and protein kinase A. *Biology of Reproduction* **102** 1290–1305. (<https://doi.org/10.1093/biolre/iaaa027>)
- Kurzynska A, Chojnowska K, Bogacki M & Bogacka I 2016 PPAR ligand association with prostaglandin F2α and E2 synthesis in the pig corpus luteum-an in vitro study. *Animal Reproduction Science* **172** 157–163. (<https://doi.org/10.1016/j.anireprosci.2016.07.014>)
- Lai X, Zhang C, Wang J, Wang C, Lan X, Zhang C, Lei C & Chen H 2013 mRNA expression pattern and association study with growth traits of bovine vaspin gene. *Molecular Biology Reports* **40** 4499–4505. (<https://doi.org/10.1007/s11033-013-2542-2>)
- Lappas M, Permezel M & Rice GE 2005 Leptin and adiponectin stimulate the release of proinflammatory cytokines and prostaglandins from human placenta and maternal adipose tissue via nuclear factor-kappaB, peroxisomal proliferator-activated receptor-gamma and extracellularly regulated kinase 1/2. *Endocrinology* **146** 3334–3342. (<https://doi.org/10.1210/en.2005-0406>)
- Li Q, Chen R, Moriya J, Yamakawa J, Sumino H, Kanda T & Takahashi T 2008 A novel adipocytokine, visceral adipose tissue-derived serine protease inhibitor (vaspin), and obesity. *Journal of International Medical Research* **36** 625–629. (<https://doi.org/10.1177/147323000803600402>)
- Maleszka A, Smolinska N, Nitkiewicz A, Kiezun M, Chojnowska K, Dobrzyn K, Szwaczek H & Kaminski T 2014 Adiponectin expression in the porcine ovary during the oestrous cycle and its effect on ovarian steroidogenesis. *International Journal of Endocrinology* **2014** 957076. (<https://doi.org/10.1155/2014/957076>)
- Mesen TB & Young SL 2015 Progesterone and the luteal phase: a requisite to reproduction. *Obstetrics and Gynecology Clinics of North America* **42** 135–151. (<https://doi.org/10.1016/j.ogc.2014.10.003>)
- Mierzyński R, Poniedziałek-Czajkowska E, Dłuski D, Patro-Małyza J, Kimber-Trojnar Ż, Majsterek M & Leszczyńska-Gorzelak B 2019 Nesfatin-1 and vaspin as potential novel biomarkers for the prediction and early diagnosis of gestational diabetes mellitus. *International Journal of Molecular Sciences* **20** 159. (<https://doi.org/10.3390/ijms20010159>)
- Nakatsuka A, Wada J, Iseda I, Teshigawara S, Higashio K, Murakami K, Kanzaki M, Inoue K, Terami T, Katayama A, *et al.* 2013 Visceral adipose tissue-derived serine proteinase inhibitor inhibits apoptosis of endothelial cells as a ligand for the cell-surface GRP78/voltage-dependent anion channel complex. *Circulation Research* **112** 771–780. (<https://doi.org/10.1161/CIRCRESAHA.111.300049>)
- Park HJ, Park JY, Kim JW, Yang SG, Jung JM, Kim MJ, Park JJ & Koo DB 2017 Regulation of the endoplasmic reticulum stress by BIP/GRP78 is involved in meiotic maturation of porcine oocytes *in vitro*. *Development and Reproduction* **21** 407–415. (<https://doi.org/10.12717/DR.2017.21.4.407>)

- Przygodzka E, Kaczmarek MM, Kaczynski P & Ziecik AJ 2016 Steroid hormones, prostanoids, and angiogenic systems during rescue of the corpus luteum in pigs. *Reproduction* **151** 135–147. (<https://doi.org/10.1530/REP-15-0332>)
- Rak A, Drwal E, Wróbel A & Gregoraszczuk EL 2015 Resistin is a survival factor for porcine ovarian follicular cells. *Reproduction* **150** 343–355. (<https://doi.org/10.1530/REP-15-0255>)
- Rak A, Drwal E, Rame C, Knapczyk-Stwora K, Słomczyńska M, Dupont J & Gregoraszczuk EL 2017 Expression of apelin and apelin receptor (APJ) in porcine ovarian follicles and in vitro effect of apelin on steroidogenesis and proliferation through APJ activation and different signaling pathways. *Theriogenology* **96** 126–135. (<https://doi.org/10.1016/j.theriogenology.2017.04.014>)
- Reverchon M, Bertoldo MJ, Ramé C, Froment P & Dupont J 2014 Chemerin (RARRES2) decreases in vitro granulosa cell steroidogenesis and blocks oocyte meiotic progression in bovine species. *Biology of Reproduction* **90** 102. (<https://doi.org/10.1095/biolreprod.113.117044>)
- Różycka M, Kurowska P, Grzesiak M, Kotula-Balak M, Tworzydło W, Rame C, Gregoraszczuk E, Dupont J & Rak A 2018 Apelin and apelin receptor at different stages of corpus luteum development and effect of apelin on progesterone secretion and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in pigs. *Animal Reproduction Science* **192** 251–260. (<https://doi.org/10.1016/j.anireprosci.2018.03.021>)
- Rytelewska E, Kisiełowska K, Kieżun M, Dobrzyn K, Gudelska M, Rak A, Dupont J, Kaminska B, Kaminski T & Smolinska N 2020 Expression of chemerin and its receptors in the ovaries of prepubertal and mature gilts. *Molecular Reproduction and Development* **87** 739–762. (<https://doi.org/10.1002/mrd.23391>)
- Shirasuna K, Shimizu T, Sayama K, Asahi T, Sasaki M, Berisha B, Schams D & Miyamoto A 2008 Expression and localization of apelin and its receptor APJ in the bovine corpus luteum during the estrous cycle and prostaglandin F2 α -induced luteolysis. *Reproduction* **135** 519–525. (<https://doi.org/10.1530/REP-07-0409>)
- Siawrys G & Smolinska N 2013 In vitro effects of luteinizing hormone, progesterone and oestradiol-17 β on leptin gene expression and leptin secretion by porcine luteal cells obtained in early pregnancy. *Journal of Physiology and Pharmacology* **64** 513–520.
- Sirotkin AV, Rafay J & Kotwica J 2009 Leptin controls rabbit ovarian function in vivo and in vitro: possible interrelationships with ghrelin. *Theriogenology* **72** 765–772. (<https://doi.org/10.1016/j.theriogenology.2009.05.011>)
- Stocco C, Telleria C & Gibori G 2007 The molecular control of corpus luteum formation, function, and regression. *Endocrine Reviews* **28** 117–149. (<https://doi.org/10.1210/er.2006-0022>)
- Stouffer R & Hennebold J 2015 Structure, function, and regulation of the corpus luteum. In *Knobil and Neill's Physiology of Reproduction*, 4th ed., pp. 1023–1076. Eds T Plant & A Zeleznik. Amsterdam, Netherlands: Elsevier Inc.
- Tan BK, Heutling D, Chen J, Farhatullah S, Adya R, Keay SD, Kennedy CR, Lehnert H & Randeve HS 2008 Metformin decreases the adipokine vaspin in overweight women with polycystic ovary syndrome concomitant with improvement in insulin sensitivity and a decrease in insulin resistance. *Diabetes* **57** 1501–1507. (<https://doi.org/10.2337/db08-0127>)
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (<https://doi.org/10.1186/gb-2002-3-7-research0034>)
- von Loeffelholz C, Möhlig M, Arafat AM, Isken F, Spranger J, Mai K, Randeve HS, Pfeiffer AF & Weickert MO 2010 Circulating vaspin is unrelated to insulin sensitivity in a cohort of nondiabetic humans. *European Journal of Endocrinology* **162** S07–S13. (<https://doi.org/10.1530/EJE-09-0737>)
- Waclawik A 2011 Novel insights into the mechanisms of pregnancy establishment: regulation of prostaglandin synthesis and signaling in the pig. *Reproduction* **142** 389–399. (<https://doi.org/10.1530/REP-11-0033>)
- Ziecik AJ, Przygodzka E, Jalali BM & Kaczmarek MM 2018 Regulation of the porcine corpus luteum during pregnancy. *Reproduction* **156** R57–R67. (<https://doi.org/10.1530/REP-17-0662>)

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