Steroid hormones, prostanoids, and angiogenic systems during rescue of the corpus luteum in pigs

E Przygrodzka, M M Kaczmarek, P Kaczynski and A J Ziecik

Department of Hormonal Action Mechanisms and Molecular Biology Laboratory, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (IARFR PAS), Tuwima 10, 10-748 Olsztyn, Poland

Correspondence should be addressed to A J Ziecik; Email: a.ziecik@pan.olsztyn.pl or to M M Kaczmarek; Email: m.kaczmarek@pan.olsztyn.pl

Abstract

In order to characterize the transition of the corpora lutea (CL) from acquisition of luteolytic sensitivity to rescue of luteal function: i) the expression of 38 factors associated with steroids, prostanoids, and angiogenic systems and ii) concentrations of the main hormones responsible for maintenance of CL function in cyclic and pregnant pigs were examined. Additionally, the effect of prostaglandin (PG) E \(_2\) and F \(_2\alpha\) on luteal function during the estrous cycle and pregnancy was evaluated in vitro. Significantly up-regulated gene expression was revealed in CL collected on day 14 of the estrous cycle (CYP19A1, ESR2, PTGS2, HIF1A, and EDN3) and on days 12–14 of pregnancy (SCARB1, PGRMC1, STAR, HSD3B1, NR5A1, PTGER, PTGER4, and VEGFA). Elevated concentrations of estradiol-17β and PGE\(_2\) occurred in CL on days 12 and 14 of pregnancy respectively, while an increased intraluteal PGF\(_2\alpha\) content was noted on day 14 of the estrous cycle. Both PGs increased the synthesis of progesterone by cultured luteal slices obtained on day 14 of pregnancy, in contrast to the action of PGF\(_2\alpha\) on the corresponding day of the estrous cycle. PGE\(_2\) stimulated cAMP production via PTGER2 and PTGER4, while PGF\(_2\alpha\) elevated the content of CREB in cultured luteal slices from CL of pregnant pigs. In silico analysis showed that infiltration of lymphocytes and apoptosis of microvascular endothelium were activated in CL on day 12 of the estrous cycle vs pregnancy. Summarizing, an abundance of E\(_2\) and PGE\(_2\) during pregnancy regulates specific pathways responsible for steroidogenesis, the prostanoid signaling system and angiogenesis during rescue from luteolysis in porcine CL.

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Introduction

The lifespan of the corpora lutea (CL) is tightly controlled by a balance between the supply of luteotropic and luteolytic as well as angiogenic and antiangiogenic factors. In the absence of live embryos in the uterus, regression of CL is initiated by prostaglandin F \(_2\alpha\) (PGF\(_2\alpha\)) originating from the endometrium. The luteolytic cascade involves, at first, disruption of progesterone (P\(_4\)) synthesis (functional luteolysis) followed by further degeneration of both luteal and endothelial cells (structural luteolysis).

The porcine CL are unique among domestic animals since a single injection of PGF\(_{2\alpha}\) does not induce luteolysis before day 12 of the estrous cycle (Guthrie & Polge 1976), although PGF\(_{2\alpha}\) receptors (PTGFR) are already abundant on the surface of porcine luteal cells during the early luteal phase (Gadsby et al. 1990, 1993). On the other hand, repeated administration of PGF\(_{2\alpha}\) on day 5 of the estrous cycle does promote luteolysis in pigs (Estill et al. 1993). Despite many studies, the molecular mechanism of luteolytic sensitivity (LS) acquisition in porcine CL still remains poorly understood.

To date, studies on LS acquisition in pigs were mostly concentrated on molecular changes occurring in CL during induced luteolysis (Diaz et al. 2000, 2011, 2013, Diaz & Wiltbank 2004, 2005, Luo et al. 2011) and revealed that PGF\(_{2\alpha}\) affects various molecular pathways in CL differently depending on whether LS is acquired. For example, increased expression of factors associated with synthesis and signaling pathways of PGF\(_{2\alpha}\) (Diaz et al. 2000) and estradiol-17β (E\(_2\); Diaz & Wiltbank 2004), decreased level of factors implicated in synthesis and signaling pathway of P\(_4\) (Diaz & Wiltbank 2005) and elevated expression of apoptotic genes (Luo et al. 2011, Diaz et al. 2013) were observed only in porcine CL with acquired LS and therefore were considered as markers of LS acquisition.

However, molecular changes involved in spontaneous luteolysis are not consistent with those occurring during induced luteolysis (Penny et al. 1999). Therefore, we decided to identify changes in gene expression related to the natural transition of porcine CL from PGF\(_{2\alpha}\)-resistant to PGF\(_{2\alpha}\)-sensitive status and compare them with changes taking place during prevention of luteolysis in early pregnant pigs. Based on our recent study (Przygrodzka et al. 2016).
structural luteolysis in pigs (Przygrodzka et al. 2015); we hypothesized that early pregnant CL do not possess full LS and can serve as a good model to illustrate differences between porcine CL with or without acquired LS and the rescue of CL during maternal recognition of pregnancy. Recently, we examined the expression of 12 genes associated with apoptosis in porcine CL of cyclic and pregnant pigs (Przygrodzka et al. 2015). These results revealed a simultaneous increase of TNFA and IFNG transcripts in porcine CL collected on day 12 of the estrous cycle. Thus, both cytokines seem likely to act synergistically in sensitizing porcine CL to further luteolytic action of PGF2α, whereas an up-regulated expression of FAS, FOS, and JUN mRNA noted in CL on day 14 of the estrous cycle suggests the involvement of these apoptotic factors in structural luteolysis in pigs (Przygrodzka et al. 2015).

Considering the obvious implication of a variety of signaling pathways in the process of LS acquisition, we aimed to examine the expression of 38 additional factors associated with i) steroids (P₄, E₂, and androgens), ii) prostanoids, and iii) angiogenic systems using CL tissue collected from the same animals that were recently studied (Przygrodzka et al. 2015), i.e., CL collected on days 8, 10, 12, and 14 of the estrous cycle/early pregnancy. Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, USA) Software was applied in order to better understand molecular changes noted in porcine CL with acquired LS and early pregnant CL (days 12–14 of pregnancy). Moreover, in order to test reproductive status-dependent effects of PGE₂ and PGF₂α on luteal function maintenance in vitro experiments were performed using luteal slices collected on day 14 of the estrous cycle and on the corresponding day of pregnancy.

Materials and methods

Animals and sample collection

Procedures performed on animals were conducted in accordance with the national guidelines for agricultural animal care and approved by the Local Animal Ethics Committee. In the present study, molecular and endocrine changes were examined using the same luteal tissues as in our recent study (Przygrodzka et al. 2015). Crossbred gilts (Polish Landrace; n = 42) 6–8 months of age exhibiting two estrus periods were randomly divided into two groups: cyclic (n = 21) and pregnant (n = 21). Gilts in each group were assigned into four subgroups (n = 4–6) representing days 8, 10, 12, and 14 of the estrous cycle and the corresponding days of pregnancy. Collection, preparation and storage of tissues, i.e., venous blood and CL were all described in detail (Przygrodzka et al. 2015). Moreover, in vitro experiments were performed using CL collected from gilts on days 12 and/or 14 of the estrous cycle and pregnancy (n = 5/reproductive status).

Luteal tissue homogenization

Homogenization of luteal tissue was performed as previously described (Diaz et al. 2000). Homogenates were centrifuged at 10 000 g for 5 min, and then supernatants were collected and stored at −20 °C.

Determination of E₂, P₄, and testosterone concentrations

The concentrations of E₂, P₄, and testosterone in blood plasma and homogenates of luteal tissue were assessed using the RIA method and commercially available kits (Orion Diagnostica (Oulu, Finland) and DIAsource ImmunoAssays S.A (Louvain-la-Neuve, Belgium) respectively) according to the manufacturers’ protocols. The sensitivity of the E₂, P₄, and testosterone assays was 3.12 pg/ml, 0.19 pg/ml, and 0.16 ng/ml respectively. The intra-assay coefficient of variation (CV) for the E₂, P₄, and testosterone assays was 6.5, 4.6, and 6.6% respectively.

Determination of PGE₂ and PGF₂α concentrations

PGE₂ and PGF₂α concentrations in homogenates of luteal tissue were determined using the EIA method (Wasielak et al. 2008, Blitek et al. 2010). Anti-PGE₂ and anti-PGF₂α antibodies were used at dilutions of 1:450 and 1:12 respectively. Assay sensitivity was 0.19 ng/ml for PGE₂ and 0.23 pg/ml for PGF₂α. The intra- and inter-assay CV were 8.5 and 8.1% for PGE₂, and 7.5 and 11.4% for PGF₂α respectively.

Total RNA isolation and real-time PCR

Isolation of RNA, RT, and real-time PCR analysis were performed as recently described (Przygrodzka et al. 2015). The integrity and quality of RNA were verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Genomic DNA contamination was removed according to the manufacturer’s instructions (DNAse I Kit, Invitrogen Life Technologies, Inc.). Then, RNA was reverse transcribed using a High Capacity cDNA RT Kit (Applied Biosystems) as previously reported (Blitek et al. 2010).

Analysis of genes was performed by real-time PCR using the Applied Biosystems 7900 HT Sequence Detection System (Applied Biosystems). In a final volume of 10 μl, 15 ng of cDNA was amplified using the TaqMan assays as shown in Supplementary Table S1, see section on supplementary data given at the end of this article. Each PCR was performed in duplicates on 384-well plates and consisted of the following steps: incubation for 10 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/elongation for 60 s at 60 °C. Real-time PCR Mainer Software was used to estimate the mean PCR amplification efficiency for each gene (Zhao & Fernald 2005). In order to select the most stable housekeeping gene among GAPDH, ACTB, and HPRT, the NormFinder algorithm was applied (Andersen et al. 2004).

Protein extraction and expression analysis

Protein extraction and expression analysis were performed as previously described (Przygrodzka et al. 2015). Briefly, fragments of porcine CL were homogenized on ice in homogenization buffer (50 mM Tris–HCl, pH 8.0; 150 mM...
NaCl, 1 mM EDTA) supplemented with 10 μg/ml of protease inhibitor cocktail (Sigma–Aldrich). Homogenates were then centrifuged for 15 min at 800 g at 4 °C and stored at −80 °C for further analysis. The protein concentration was determined by the Bradford method (Bradford 1976).

Protein samples from luteal tissue homogenates were dissolved in SDS gel-loading buffer (50 mmol/l Tris–HCl, pH 6.8; 4% SDS, 20% glycerol, and 2% β-mercaptoethanol), denatured at 95 °C for 4 min, and separated on 10% (CYP19A1, PTGSR2, and CREB) or 12% (HSD17B1, PTGFR, PTGER2, and PTGER4) SDS–PAGE. Separated proteins were electrotransferred onto 0.2 mm nitrocellulose or PVDF membranes in chilled transfer buffer (20 mmol/l Tris–HCl buffer, pH 8.2; 150 mmol/l glycine, and 20% methanol). After blocking in 5% non-fat dried milk in Tris-buffered saline buffer (TBS-T, containing 0.1% Tween-20) for 1.5 h at room temperature the membranes were incubated overnight at 4 °C with polyclonal rabbit or goat antibodies diluted in TBS-T buffer as follows: anti-CYP19A1 (1:100; sc-14244; Santa Cruz Biotechnology), anti-HSD17B1 (1:100; sc-26963; Santa Cruz Biotechnology), anti-PTGSR2 (1:100; ab15191; Abcam, Cambridge, UK), anti-PTGFR (1:100; sc-67029; Santa Cruz Biotechnology), anti-PTGER2 (1:100; 101775; Cayman Chemicals), and anti-CREB (1:50; sc-186, Santa Cruz Biotechnology). Subsequently, membranes were washed three times in fresh TBST-T and incubated with anti-rabbit or anti-goat secondary antibodies (Sigma–Aldrich) diluted 1:20 000 in TBS-T, at room temperature for 90 min. Afterwards, membranes were again washed three times in TBS-T. Immune complexes were visualized using the alkaline phosphatase visualization procedure. The intensity of bands was analyzed with the Kodak D Software v.3.5 visual quantitation system (Eastman Kodak). β-actin (1:3000; Abcam) or GAPDH (1:100; A4312; Sigma–Aldrich) were used as a loading control.

In vitro experiments

The effect of testosterone on E2 production

In order to determine activity of aromatase we performed in vitro experiment using precision-cut luteal slices obtained on days 12 and 14 of the estrous cycle/pregnancy. The slices were obtained as previously described (Przygrodzka et al. 2015) and incubated (one slice per well) in culture medium M-199 medium (Sigma–Aldrich) supplemented with: 0.1% BSA (ICN Biomedicals, Inc., Costa Mesa, CA, USA), antibiotics and anti-fungal additive (amphotericin B; Sigma–Aldrich) for 24 h at 37 °C in a humified atmosphere containing 95% air and 5% CO₂. Slices were pre-incubated for 1.5 h in culture medium only and then incubated for 24 h with testosterone (10 and 1 μM). Afterwards, incubation media were collected and stored for later analysis of E₂ concentration using the RIA method (see section ‘Determination of E₂, P₄, and testosterone concentrations’).

The effect of PGs on P₄ production and CREB expression

In order to determine reproductive status-dependent effects of both PGs on P₄ synthesis by porcine CL, precision-cut luteal slices obtained on day 14 of the estrous cycle/pregnancy were incubated with PGE₂ (10 and 1 μM) or PGF₂α (1 and 0.1 μM) in the presence or absence of the appropriate antagonist PTGER2 (AH6809, 10 μM), PTGER4 (GW627368x, 5 μM), or PTGFR (AL8810, 50 μM). After 24 h of incubation at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂, media were collected and stored for later analysis of P₄ concentration using the RIA method as recently described (Przygrodzka et al. 2015). Luteal slices treated with PGF₂α were washed in ice-cold PBS and snap–frozen in order to analyze CREB expression using Western blots (see section ‘Protein extraction and expression analysis’).

The effect of PGE₂ on cAMP production

To elucidate whether two PGE₂ receptors are involved in cAMP production, an in vitro experiment was performed as previously described (Waclawik et al. 2009). Luteal slices collected from CL of pregnant pigs on day 14 of pregnancy were incubated in culture medium containing 3 μg/ml indomethacin. After overnight incubation at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂, the medium was replaced with fresh culture medium supplemented with 3 μg/ml indomethacin and 1-methy-3-iso-butylxanthine as well as an appropriate receptor antagonist (AH6809 or GW627368x) at 37 °C for 30 min. Subsequently, luteal slices were treated with PGE₂ (1 μM) in the presence or absence of the aforementioned antagonist for 10 min at 37 °C. Afterwards, slices were washed in ice-cold PBS and snap–frozen. Luteal slices were homogenized and cAMP concentration was quantified in the homogenates using a cAMP kit (R&D System, Minneapolis, MN, USA) according to the manufacturer’s protocol. Concentrations of cAMP were normalized to the protein content, which was determined using the Bradford method (Bradford 1976).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 5.02 Software (GraphPad Software, Inc., San Diego, CA, USA). Two-way ANOVA followed by the Bonferroni’s post hoc test was used to determine: i) changes of mRNA/protein expression in luteal tissue; ii) the luteal and blood plasma concentrations of hormones in samples collected on days 8, 10, 12, and 14 of the estrous cycle and pregnancy; iii) concentration of E₂ and P₄ in post-incubation medium; and iv) content of CREB protein in cultured luteal slices. The initial model included day, reproductive status and day of estrous cycle/pregnancy × reproductive status interaction. The production of cAMP by cultured luteal slices treated with PGE₂ was assessed using one-way ANOVA. Numerical data are expressed as means ± s.e.m. and results were considered statistically significant at P<0.05.

IPA and Venn diagrams

IPA Software was used to investigate possible interactions between genes with altered expression and to obtain insight into processes occurring in porcine CL during the luteal phase and early pregnancy. To achieve this, we performed the
following comparisons: i) day 14 of the estrous cycle (CL with acquired LS) vs day 8 (CL without LS) and ii) day 12 of the estrous cycle (LS acquisition) vs day 12 of pregnancy (rescue from luteolysis). Datasets representing 50 genes, i.e., 38 genes presented in Supplementary Table S1 and 12 genes described in our recent study (Przygrodzka et al. 2015) expressed as an fold change (FC) were uploaded to the IPA and analyzed using the Ingenuity Knowledge Base. The right-tailed Fisher’s exact test using a threshold $P$ value < 0.05 after application of the Benjamini–Hochberg method for multiple testing correction and z-score were used as two statistical measures for identification of significant canonical pathways, biofunctions, and upstream regulators. The z-score value showed the state of inhibition ($z$-score $< -2$) or activation ($z$-score $> 2$) for each of the above mentioned analyses. In order to increase clarity of the results, all canonical pathways and biofunctions associated with cancer and disease were removed. Furthermore, in order to determine markers of LS acquisition and rescue of CL we compared transcriptomic data obtained on day 14 of the estrous cycle vs day 8 of the estrous cycle as well as vs days 12 and 14 of pregnancy using Venn diagrams (Oliveros 2007). Up-regulated (FC > 1.5) genes for three of the above mentioned comparisons were used in each comparison.

Results

Molecular and endocrine changes in porcine CL during the estrous cycle and early pregnancy

Factors related to $P_4$ production and action

The expression of LDLR, STAR, HSD3B1, and NR5A1 decreased on day 14 vs day 12 of the estrous cycle ($P<0.05$; Fig. 1A). During pregnancy, levels of SCARB1, STAR, HSD3B1, and PGRMC1 increased on day 14 vs day 12 ($P<0.05$) and the corresponding day of the

**Figure 1** The mRNA (A and B) and protein (C) expression of factors related to steroids synthesis as well as testosterone and E2 (ng/g and pg/g of luteal tissue, respectively; D) concentrations in the luteal tissue throughout the luteal phase of the estrous cycle and corresponding days of early pregnancy. Data of genes expression were normalized to GAPDH as the best reference gene, while for protein expression $\alpha$-actin or GAPDH was chosen. Data are expressed in arbitrary units as means ± S.E.M. and were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. Information about effects of day (D), reproductive status (S), and day × reproductive status (D×S) interaction on content of hormones examined in the luteal tissue are placed above each graph. Asterisks denote significant differences between parallel days of cyclic and pregnant animals (*$P<0.05$, **$P<0.01$, and ***$P<0.001$), whereas different letters (capital letters for cyclic and small for pregnant) above bars present differences among groups of cyclic and pregnant animals. Samples of western blots are presented at the top of C.
estrous cycle (P<0.05). The level of NR5A1 transcript increased as early as on day 12 of pregnancy vs days 8 and 10 (P<0.05; Fig. 1A), and its expression was greater (P<0.01) on day 14 of pregnancy vs parallel day of the estrous cycle. The highest level of PGR mRNA was noted on day 8 vs the other days of the estrous cycle (P<0.001) as well as the corresponding day of pregnancy (P<0.01; Fig. 1A). The abundance of PGRMC2 mRNA was observed on day 12 of the estrous cycle vs other days (P<0.01). The decreased levels of LDLR and PGRMC2 mRNA (P<0.05) were noted on day 12 of pregnancy when compared with parallel day of the estrous cycle. The levels of CYP11A1 and YY1 remained unchanged in CL of cyclic and pregnant pigs (Supplementary Figure S1, see section on supplementary data given at the end of this article).

Factors related to production and action of androgens and estrogens

An increased level of CYP17A1, an enzyme involved in androstenedione (A4) synthesis, was observed on days 10 and 12 of the estrous cycle vs day 8 (P<0.05; Fig. 1B), while during pregnancy its level was augmented only on day 10 vs day 8 (P<0.05). An enzyme converting A4 to testosterone, HSD17B1, was maintained at a constant level in CL of cyclic pigs (Fig. 1B and C). In contrast, during pregnancy elevated mRNA (Fig. 1B) and protein (Fig. 1C) expression of HSD17B1 were noted on day 10 vs the other days (P<0.05), as well as the corresponding day of the estrous cycle (P<0.001). The amounts of intraluteal testosterone were greater on day 14 vs day 8 of the estrous cycle (P<0.05), while during pregnancy its content was already abundant on days 12 and 14 vs day 8 (P<0.05; Fig. 1D). The level of AR remained unchanged in cyclic and pregnant animals (Supplementary Figure S1).

Intraluteal testosterone can be converted to E2 by CYP19A1, whose mRNA markedly increased on day 14 vs other days of the estrous cycle (P<0.01) and day 14 of pregnancy (P<0.05; Fig. 1B). The protein content of CYP19A1 increased on day 12 of the estrous cycle vs days 8 and 10 (P<0.05; Fig. 1C). In pregnant animals, the amount of CYP19A1 was augmented on day 12 vs days 8 and 10 (P<0.05). The elevated concentration of luteal E2 was noted on day 14 of the estrous cycle vs day 10 (P<0.05), while during pregnancy the increase started earlier, reaching its highest level on day 12 vs days 8 (P<0.01) and 14 (P<0.05), as well as vs the corresponding day of the estrous cycle (P<0.05; Fig. 1D).

The expression of ESR1 remained unchanged in cyclic pigs, whereas during pregnancy its highest level was determined on day 12 vs days 8 and 10 (P<0.01 and P<0.05 respectively). In contrast, the expression of ESR2 was raised on day 14 of the estrous cycle vs days 10 and 12 (P<0.01), but remained constant in pregnant animals.

Factors related to production and action of PGs

The ratio between luteotropic PGE2 and luteolytic PGF2α determines the lifespan of CL, thus factors related to their synthesis, signaling and metabolism were investigated. The level of PTGS2 was raised on day 14 of the estrous cycle vs day 10 (protein) and day 12 (mRNA and protein; P<0.05; Fig. 2A and B). During pregnancy, the highest expression of PTGS2 was noted on day 8 vs day 10 (protein) and day 12 (mRNA and protein; P<0.05). An effect of reproductive status on PTGS2 expression was observed on day 14 (P<0.01). The expression of PTGFS was constant during the course of the estrous cycle and pregnancy (Supplementary Figure S1). The mRNA level of HPGD, an enzyme converting PGF2α to its inactive metabolite, 3,14-dihydro-15-keto PGF2α (PGFM), remained unchanged in cyclic animals but was increased on day 10 vs other days of pregnancy (P<0.01) and day 10 of the estrous cycle (P<0.01). The greatest mRNA expression of CBR1, an enzyme showing among other actions the ability to convert PGE2 to PGF2α, occurred on day 10 vs the other days of the estrous cycle (P<0.01 and P<0.001 respectively) and the corresponding day of pregnancy (P<0.05; Fig. 2A). During pregnancy, the highest expression of CBR1 transcript was found on day 8 vs day 12 (P<0.05). An abundant level of PTGFR mRNA was noted on day 14 of the estrous cycle vs days 8 (P<0.01) and 10 (P<0.001; Fig. 2A). During pregnancy, the greatest expression of PTGFR was found on days 12 and 14 vs other days (P<0.001). In contrast, the expression of PTGFR protein dropped on day 14 of the estrous cycle (P<0.05) vs days 8 and 12 (P<0.05; Fig. 2B), while during pregnancy its levels were constant. However, the increased level of PTGFR expression was found on days 12 (mRNA; P<0.01) and 14 (protein, P<0.05) of pregnancy vs the corresponding days of the estrous cycle. The intraluteal content of PGF2α was higher on day 8 of the estrous cycle vs day 10, while it remained unchanged during pregnancy. Moreover, an abundant concentration of PGF2α was found on day 14 of the estrous cycle vs corresponding day of pregnancy (P<0.01; Fig. 2C). The intraluteal content of PGE2 was elevated on days 8 and 10 of the estrous cycle vs day 12 (P<0.001 and P<0.05 respectively) and on day 8 vs day 14 (P<0.01). In contrast, during pregnancy the highest amount of PGE2 was found on day 14 vs other days of pregnancy (P<0.001) and the corresponding day of the estrous cycle (P<0.001; Fig. 2C).

The level of PTGES1 decreased on day 14 of the estrous cycle vs days 8 (P<0.01), 10 (P<0.001), and 12 (P<0.01; Fig. 2D). During pregnancy, a drop in PTGES1 transcript was seen as early as on day 10 vs day 8 (P<0.05). Moreover, higher PTGES1 mRNA expression
was observed on day 12 of the estrous cycle vs the corresponding day of pregnancy (P<0.01). The level of PTGER2 mRNA increased on day 14 of the estrous cycle vs days 8 and 10 (P<0.05), while during pregnancy the expression of PTGER2 was higher only on day 12 (vs day 8, P<0.05; Fig. 2D). The PTGER2 protein expression was similar in cyclic and pregnant animals, and its level increased on day 12 vs day 8 (cycle) and day 10 (pregnancy) (P<0.05) respectively. A significant increase in PTGER4 mRNA was found on day 14 vs other days (P<0.05) in cyclic pigs, while during pregnancy its level had already increased on day 12 vs days 8 and 10 (P<0.05; Fig. 2D). In cyclic animals, a higher content of PTGER4 protein was noted on days 12 and 14 vs day 8 (P<0.05), whereas in pregnant pigs it was already elevated on day 10 vs day 8 (P<0.05; Fig. 2E). The elevated PTGER4 protein expression was found on days 12 (P<0.001) and 14 (P<0.05) of pregnancy vs the corresponding days of the estrous cycle.

An increased level of NFKB1 mRNA was observed on day 14 of the estrous cycle vs days 8 (P<0.001), 10 (P<0.01), and 12 (P<0.05; Fig. 2F), while during pregnancy it remained constant. Significant changes in NFKB1A transcript expression were noted only during the estrous cycle, with the greatest level on day 14 vs day 8 (P<0.05).

Factors related to blood vessels/angiogenesis

Although the expression of VEGFA mRNA was constant during the estrous cycle, during pregnancy it increased on day 14 vs the other days (P<0.01) as well as on the corresponding day of the estrous cycle (P<0.05; Fig. 3). FLT mRNA expression was elevated on days 12–14 of the estrous cycle vs days 8 and/or 10 (P<0.05). During pregnancy, a significant increase (P<0.01) in FLT1 level was detected on day 14 vs day 8. The level of KDR was constant in both cyclic and pregnant animals (Supplementary Figure S1). The highest level of HIF1A occurred...
on day 14 of the estrous cycle vs day 12 and the corresponding day of pregnancy (P < 0.05). In contrast, it was unchanged during early pregnancy.

The level of FGF2 was elevated on day 14 of the estrous cycle vs day 10 (P < 0.05), in contrast to pregnancy when it was maintained at almost constant levels. Expression of FGFR2 increased on day 14 of the estrous cycle vs days 8 and 10 (P < 0.001) as well as day 12 (P < 0.01). During pregnancy, augmented expression of FGFR2 was noted on day 12 vs days 8 (P < 0.001) and 10 (P < 0.05). The level of PTX3, a known antagonist of FGF2, decreased along with the development of CL in cyclic and pregnant pigs and its highest expression was observed on day 8 of the estrous cycle and early pregnancy (vs day 14, P < 0.05).

The expression of ANGPT1 increased on day 14 of the estrous cycle vs days 8 and 10 (P < 0.01). During pregnancy, the level of ANGPT1 mRNA was elevated on days 12–14 vs day 8 (P < 0.01; Fig. 3). The expression of ANGPT2 mRNA was unchanged in cyclic animals, while a higher level was noted on day 14 of pregnancy vs day 8 (P < 0.05). Although expression of TIE2 and EDN1 mRNA was maintained at constant levels during pregnancy, it was increased on day 14 of the estrous cycle vs the other days (P < 0.01). Additionally, the level of EDN1 was augmented on day 14 of the estrous cycle in comparison to the corresponding day of pregnancy (P < 0.05).

**IPA analysis and Venn diagram**

Since it is well known that porcine CL on day 14 of the estrous cycle have acquired LS, while CL on day 8 of the estrous cycle have not, we compared molecular changes on those days to identify events characteristic for CL sensitivity to PGF₂α. The second comparison, i.e., day 12 of the estrous cycle (CL during LS acquisition) vs day 12 of pregnancy (CL without acquired LS) was selected to define events taking place during acquisition of LS, while being inhibited in CL during maternal recognition of pregnancy.

For days 12 and 14 of the estrous cycle, the following canonical pathways were found to be activated: TNFR1 signaling (ratio 4/59 and 5/59 respectively), apoptosis signaling (ratio 4/89 and 6/89 respectively), production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages (ratio 4/180 and 7/180 respectively). In contrast, peroxisome proliferator-activated receptors (PPAR) signaling (ratio 4/94 and 7/94 respectively) and liver X receptor/retinoic acid receptor activation (ratio 4/121 for both comparisons) were inhibited. Among the top ten biofunctions identified by IPA, the following were found for day 12 of the estrous cycle (vs day 12 of pregnancy; Supplementary Table S2A, see section on supplementary data given at the end of this article and Fig. 4A): movement and infiltration of lymphocytes (P = 1.40 × 10⁻¹² and P = 1.80 × 10⁻¹⁴ respectively), cleavage and synthesis of lipid (P = 3.68 × 10⁻¹¹ and 9.29 × 10⁻¹² respectively), release of eicosanoid (P = 2.70 × 10⁻¹⁰), as well as apoptosis of, e.g., microvascular endothelial cells (P = 1.36 × 10⁻¹¹). However, synthesis of ROS (P = 1.10 × 10⁻¹⁰), synthesis of lipid and steroid (P = 8.00 × 10⁻²² and P = 1.61 × 10⁻¹⁵ respectively), activation of leukocytes (P = 1.09 × 10⁻¹²) and antigen-presenting cells (P = 2.10 × 10⁻¹¹), as well as recruitment of cells, e.g., leukocytes (P = 2.14 × 10⁻⁸) were all found for day 14 of the estrous cycle (vs day 8 of the estrous cycle; Supplementary Table S2B). Several upstream regulators of genes showing changed expression on day 14 of the estrous cycle (Fig. 4B) were found, including EDN1 (P = 4.07 × 10⁻¹¹), HIF1A

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**Figure 3** The mRNA expression of factors related to angiogenesis in the luteal tissue throughout the luteal phase of the estrous cycle and corresponding days of early pregnancy. Data were normalized to GAPDH as the best reference gene and are presented in arbitrary units as mean ± S.E.M. Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. Information about effects of day (D), reproductive status (S), and day × reproductive status (D × S) interaction on genes expression in the luteal tissue are placed above each graph. Asterisks denote significant differences between parallel days of cyclic and pregnant animals (*P < 0.05), whereas different letters (capital letters for cyclic and small for pregnant) above bars present differences among groups of cyclic and pregnant animals.

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In vitro experiments

The effect of testosterone on E2 production

Testosterone (10 µM) increased the concentration of E2 in culture medium after incubation of luteal slices obtained from days 12 and 14 of the estrous cycle and pregnancy (P<0.05; Fig. 5A). The higher concentration of E2 was noted in the culture medium after treatment with testosterone of slices from day 12 of pregnancy vs the estrus cycle and from day 14 of the estrous cycle vs pregnancy (P<0.05 and P<0.01 respectively).

The effect of PGs on P4 production

Both PGE2 and PGF2α increased the concentration of P4 in culture medium after incubation of luteal slices obtained from pregnant pigs (P<0.05; Fig. 5B and C). Treatment of luteal slices with an antagonist of PTGFR (AL8810) in the presence of PGF2α did not affect P4 secretion. P4 secretion by luteal slices pre-treated with an antagonist of PTGER2 (AH6809) or PTGER4 (Gw627368x; P<0.05; Fig. 5C) was enhanced by PGE2 but not when these antagonists were added without PGE2. Incubation of luteal slices obtained from CL on day 14 of the estrous cycle with PGF2α (1 µM) decreased the concentration of P4 in post-incubation medium (P<0.05), while PGE2 had no effect (Fig. 5B and C).

The effect of PGF2α on CREB expression

Considering the important role of CREB in the control of luteal steroidogenesis, as well as the effect of PGF2α on cAMP accumulation stimulated by LH or PGE2 (Mamluk et al. 1999), we examined the effect of PGF2α on CREB content in luteal slices collected from CL on day 14 of the estrous cycle and the corresponding day of pregnancy. Treatment with PGF2α increased the content of CREB in luteal slices from early pregnant pigs (P<0.05), while no effect was observed in luteal slices from cyclic animals (Fig. 5D).

The effect of PGE2 on cAMP production

Treatment with PGE2 in the presence or absence of PTGER2 or PTGER4 antagonists (AH6809 and Gw627368x respectively) increased production of cAMP by luteal slices from CL on day 14 of the estrous cycle and pregnancy (P<0.05; Fig. 5E). Treatment with antagonists of PTGER2 or PTGER4 only did not affect cAMP production.

Discussion

In the present report we examined molecular and endocrine changes occurring in porcine CL before and after spontaneous acquisition of LS in cyclic gilts as well as during natural rescue of luteal function in early pregnant pigs. Our investigations focused on steroid and prostanoid synthesis, action and metabolism, as well as angiogenic signalling pathways crucial for CL function. Furthermore, we showed the effect of both PGs and blocking of their receptors (PTGFR, PTGER2, and PTGER4) in vitro P4 production by porcine luteal slices on day 14 of the estrous cycle and pregnancy. Additionally, new elements of PGE2 and PGF2α signalling pathways in porcine CL during pregnancy were identified.
concentration (Przygrodzka et al. 2015) in CL on the late luteal phase. Concomitantly, in the present report we noted lower levels of genes associated with steroidogenesis, i.e., LDLR, STAR, and HSD3B1 in CL on above mentioned stage of the luteal phase. Interestingly, the expression of CYP11A1, an enzyme responsible for conversion of cholesterol to pregnenolone, remained unchanged during the course of the estrous cycle. Similar molecular changes associated with steroidogenesis were determined in porcine CL with acquired LS during induced luteolysis (Diaz & Wiltbank 2005). We also observed a decrease of NR5A1, a transcription activator of steroidogenic genes, in CL at the late luteal phase. It is possible that its expression is inhibited by PGF2α in porcine CL with acquired LS, as demonstrated in cows (Atli et al. 2012).

PGF2α mediates its actions via nuclear and membrane receptors (PGR and PGRMC1, PGRMC2 respectively). As shown in the present study, the expression of PGR dropped along with the development of porcine CL, similar to bovine CL (Misao et al. 1998). Thus, it is likely that a negative regulatory loop exists between the intraluteal concentration of P4 and expression of PGR in CL of cyclic pigs, as demonstrated in bovine CL (Misao et al. 1998).

As shown in the current report, a marked increase of both CYP19A1 and ESR2 transcripts, as well as elevated concentrations of E2 occurred in porcine CL collected in the late luteal phase. Similar results were observed in porcine CL with acquired LS during induced luteolysis (Diaz & Wiltbank 2005). Interestingly, we did not observe any changes in the content of CYP19A1 protein during the late luteal phase of the natural estrous cycle. However, treatment of luteal slices obtained from cyclic and pregnant animals with testosterone increased concentrations of E2 in post-incubation culture medium. This confirms the activity of CYP19A1 in CL at the mid and late luteal phase of cyclic and pregnant animals. Therefore, we cannot exclude the possibility that E2 can mediate luteolytic actions in porcine CL, as suggested previously (Diaz & Wiltbank 2005). It is also possible that post-transcriptional modifications occur, leading to such a difference between CYP19A1 mRNA and protein content.

The role of EDN1 in functional and structural luteolysis was described in detail in bovine CL (Girsh et al. 1996, Meidan & Levy 2002), while further studies suggest its participation in luteolysis of porcine CL (Diaz et al. 2000, Zorrilla et al. 2010). As observed in the current study, an increase of EDN1 in porcine CL at the late luteal phase in comparison to pregnancy gives additional support for the involvement and crucial role of EDN1 in the process of luteolysis in pigs.

As shown in the current study, the level of HIF1A, a transcription factor regulating expression of VEGFA, was higher in CL collected at the late luteal phase. Since hypoxia and HIF1A were found to be involved in...
luteolysis of the bovine CL (Nishimura et al. 2008, Nishimura & Okuda 2010), it is possible that HIF1A also participates in the regression of porcine CL, while increased HIF1A expression may be characteristic of CL with acquired LS. However, further studies on HIF1A expression at the protein level should be performed to confirm this supposition.

The balance between ‘luteotropic’ and ‘luteolytic’ PGs regulates the lifespan of CL. In the present report, reduced levels of PTGES1 and intraluteal content of PGE2 were noted in CL from the late luteal phase. These results are in agreement with previous studies (Waclawik et al. 2008) and suggest that PGF2α can affect accumulation/production of luteotropic PGE2 in porcine CL with acquired LS. We did not detect any changes in the expression of PTGFS in CL of cyclic pigs, while the expression of PTGS2 at mRNA and protein levels, as well as the content of PGF2α in porcine CL, were elevated at the time of luteolysis. These results indicate an extraluteal (uterine) source of PGF2α synthesis rather than its intraluteal synthesis in porcine CL with acquired LS as proposed by Diaz et al. (2000). On the other hand, it is possible that other isoforms of PTGFS can mediate intraluteal synthesis of PGF2α in pigs as demonstrated in ovine CL (Lee et al. 2012). A decreased level of PTGFR observed at the late luteal phase could be a result of the existence of a negative feedback between increasing concentrations of intraluteal PGF2α and PTGFR expression, as previously suggested by Diaz et al. (2000).

Although Zorrilla et al. (2013) did not observe any changes in NFκB1A expression during the course of the estrous cycle in pigs, as described here the highest expression of NFκB1A was in CL at the late luteal phase, confirming previous studies in pigs (Luo et al. 2011) showing the involvement of NFκB signaling pathway in acquisition of LS.

The IPA tools identified TNFR1 signaling, apoptosis signaling and production of NO and ROS among the canonical pathways activated in CL collected on days 12–14 of the estrous cycle. Among the most important biofunctions identified by IPA in CL collected as early as on day 12 of the estrous cycle were movement and infiltration of lymphocytes, cleavage and synthesis of lipid, and release of eicosanoid, as well as apoptosis of microvascular endothelial cells, while activation of leukocytes and antigen-presenting cells as well as recruitment of leukocytes were identified on day 14 of the estrous cycle. Therefore, it seems likely that pulses of PGF2α originating from the endometrium (Ziecik & Kotwica 2001) induce synthesis of NO and initiate production of chemoattractant proteins leading to infiltration of immune cells into porcine CL, as demonstrated in bovine CL (Bauer et al. 2001). Additionally, EDN1, HIF1A, AP1, ESR2, PTGS2, and PGF2α were identified as up-stream regulators of dataset genes in CL on day 14 of the estrous cycle. The majority of the aforementioned genes were common for all comparisons included in Venn diagram and up-regulated on day 14 of the estrous cycle, thus indicating the possible paramount role of these factors in luteolysis in pigs.

**Rescue of luteal function during early pregnancy**

Fertilization and the presence of live embryos in the uterus lead to prolongation of luteal function in pigs due to the luteotropic and/or antiluteolytic action of signals from conceptuses, i.e., E2 and PGE2. Interestingly, we observed an increased intraluteal concentration of P4 (Przygrodzka et al. 2015) together with altered expression of crucial genes involved in steroidogenesis (i.e. SCARB1, STAR, HSD3B1, and LHCGF) in porcine CL collected on day 14 of pregnancy. Concomitantly, an elevated level of NR5A1, a transcription activator of steroidogenic genes, was observed in CL on day 14 of pregnancy. Thus, it is possible that the presence of NR5A1 can be important for sustaining P4 production in pigs through stimulation of transcription of the main steroidogenic genes, as suggested for bovine CL (Taniguchi et al. 2009). The abundance of PGRMC1 observed in porcine CL on day 14 of pregnancy could be essential for enhancement of steroidogenesis (Hughes et al. 2007) and sustain anti-apoptotic mechanisms mediated by P4 in luteal cells (Engmann et al. 2006). Kowalik & Kotwica (2008) observed a correlation between PGRMC1 level and P4 concentration in the luteal tissue of cyclic cows.

In the current study, we observed an increase of HSD17B1 at mRNA and protein levels in porcine CL collected on day 10 of pregnancy. Moreover, the content of testosterone was already higher on day 12 of pregnancy. The abundance of CYP19A1 protein, increased intraluteal E2 and enhanced ability of luteal tissue to convert testosterone to E2 were also observed in CL on days 12 and 14 of pregnancy, which suggests that CL of early pregnant pigs are able to synthesize E2 in substantial amounts. Taking into consideration the augmented expression of ESR1 in CL on day 12 of pregnancy, it seems likely that expression of this receptor in porcine luteal tissue might be regulated by E2, while the increase of ESR1 may be necessary for E2 to exert its luteotropic action.

The luteal content of PGE2 was fourfold higher in CL on day 14 of pregnancy in comparison to the corresponding estrous cycle day, whereas the concentration of PGF2α at this time was fourfold lower. However, the level of PTGES1 in CL did not change between days 10–14 of pregnancy and did not differ compared to day 14 of the estrous cycle. Collectively, our and earlier studies (Wasielak et al. 2008) indicate that the high concentration of PGE2 in CL is not necessarily due to its intraluteal synthesis, in contrast to bovine CL (Arosh et al. 2004). Thus, we propose that the increased PGE2 content in CL of pregnant pigs

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originates mainly from the endometrium and/or conceptuses rather than from the CL itself. A local transfer of PGE$_2$ from the uterus to the ovary has already been shown in the pig (Stefanczyk-Krzymowska et al. 2006) and sheep (Lee et al. 2012). Moreover, results presented here confirm the high abundance of PTGER2 and PTGER4 in luteal tissue of early pregnant gilts (Waclawik et al. 2010) and showed a higher content of PTGER4 compared to CL of cyclic pigs. Additionally, elevated expression of HPGD in CL of early pregnant pigs suggests activation of an intraluteal mechanism protecting CL against the lutolytic action of PGE$_2$ during pregnancy.

Interestingly, the present study revealed that expression of PTGFR mRNA and protein were more abundant in pregnant than cyclic CL. These results are not in agreement with previous studies (Gadsby et al. 1990, 1993), in which a decreased amount of PGE$_2$ binding sites was noted in luteal cells of pregnant and pseudopregnant pigs. It is worthwhile to mention that Gadsby et al. (1990, 1993) measured the capacity of [3H]PGE$_2$ binding to its membrane receptors in isolated porcine luteal cells. Thus, the observed discrepancy could result from the different animal models and methods used.

Considering the increased level of PTGFR and PTGER2/4 in porcine luteal tissue during early pregnancy, we examined in vitro effects of both PGs on luteal function maintenance. Our results showed that PGE$_2$ as well as PGE$_2$ increased secretion of P$_4$ by luteal slices collected from early pregnant pigs. Similar results were observed in previous studies on porcine luteal cells (Wiesak et al. 1992). Moreover, PGE$_2$ increased P$_4$ synthesis and cAMP production via both PTGER2 and PTGER4. Thus, binding of PGE$_2$ to PTGER2 and PTGER4 can activate the cAMP signaling pathway leading to increased P$_4$ synthesis in CL of early pregnant pigs. Since PGE$_2$ also augmented the content of CREB in CL of early pregnant pigs, it seems likely that PGE$_2$ is responsible for enhancement of cAMP action, and can also increase availability of CREB which is activated further by lutetropic hormones acting via cAMP, e.g., PGE$_2$ and LH. While expression of PTGFR was prominent in endothelial cells (Zannoni et al. 2007) of cyclic CL, we postulate that PGE$_2$ might also be engaged in luteal function maintenance during early pregnancy in pigs by stimulation of angiogenesis. A positive effect of PGE$_2$ on angiogenesis was previously demonstrated in bovine CL (Shirasuna et al. 2012, Zalman et al. 2012).

Taking into account the results of our in vivo, in vitro and in silico studies, it can be proposed that an abundance of lutetropic E$_2$ and PGE$_2$ in CL of early pregnant pigs prevents infiltration of immune cells into porcine CL and consequently inhibits events leading to acquisition of LS, despite the high content of PTGFR in CL of early pregnant pigs. It is also possible that intraluteal PGE$_2$ acting via the cAMP/PKA pathway inhibits activation of signaling pathways induced by PGE$_2$ in porcine CL with acquired LS. Our data, together with the earlier report of Zorrilla et al. (2009), suggest that for acquisition of LS or CL rescue the activation of different post-PTGFR signaling pathways is more important than maintenance of a specific amount of PTGFR in porcine CL.

In summary, the results presented here: i) confirm that PGFS2, EDN1, CYP19A1, ESR2, FOS, and JUN can serve as markers of porcine CL with acquired LS; ii) suggest a potential role of HIF1A in LS acquisition; iii) suggest that a high intraluteal content of E$_2$ and the presence of PGE$_2$ originating from the endometrium or conceptuses are required to extend luteal function in pigs; iv) propose lutetropic and lutetropic actions of PGE$_2$ depending on the reproductive status of pigs; v) propose an alternative mechanism of PGE$_2$ action in CL of early pregnant pigs based on elevated content of CREB; and vi) demonstrate that the lutetropic action of PGE$_2$ is mediated by increased production of cAMP via both PTGER2/4 receptors. It needs to be emphasized that application of IPA analysis strengthened our statement about the importance of immune cells in the process of LS acquisition in pigs, however, further in vitro studies are required to confirm the participation of these signaling pathways in cyclic regression of porcine CL.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0332.

**Declaration of interest**

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

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