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Estradiol-17 β , prostaglandin E₂ (PGE₂) and the prostaglandin E₂ receptor are involved in PGE₂ positive feedback loop in the porcine endometrium

Agnieszka Waclawik^{1,*}, Henry N. Jabbour², Agnieszka Blitek¹, and Adam J. Ziecik¹

¹Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland ²Medical Research Council Human Reproductive Science Unit, The Queens Medical Research Institute, Edinburgh, EH16 4TJ, UK

Abstract

Before implantation, the porcine endometrium and trophoblast synthesize elevated amounts of luteoprotective prostaglandin E₂ (PGE₂). We hypothesized that embryo signal, estradiol-17 β (E₂) and PGE₂ modulate expression of key enzymes in PG synthesis: prostaglandin-endoperoxide synthase-2 (PTGS2), PGE synthase (mPGES-1), PGF synthase (PGFS), and prostaglandin 9-ketoreductase (CBR1); as well as PGE₂ receptor (PTGER2 and 4) expression and signaling within the endometrium. We determined the site of action of PGE₂ in endometrium during the estrous cycle and pregnancy. Endometrial tissue explants obtained from gilts (n=6) on days 11-12 of the estrous cycle were treated with vehicle (control), PGE₂ (100 nM), E₂ (1-100 nM) or phorbol 12-myristate 13-acetate (100 nM, positive control). E₂ increased PGE₂ secretion through elevating expression of mPGES-1 mRNA and PTGS2 and mPGES-1 protein in endometrial explants. By contrast, E₂ decreased PGFS and CBR1 protein expression. E₂ also stimulated PTGER2 but not PTGER4 protein content. PGE₂ enhanced mPGES-1 and PTGER2 mRNA as well as PTGS2, mPGES-1 and PTGER2 protein expression. PGE₂ had no effect on PGFS, CBR1 and PTGER4 expression and PGF_{2 α} release. Treatment of endometrial tissue with PGE₂ increased cAMP production. Co-treatment with PTGER2 antagonist (AH6809) but not PTGER4 antagonist (GW 627368X) inhibited significantly PGE₂-mediated cAMP production. PTGER2 protein was localized in luminal and glandular epithelium and blood vessels of endometrium, and was significantly up-regulated on days 11-12 of pregnancy. Our results suggest that E₂ prevents luteolysis through enzymatic modification of PG synthesis and that E₂, PGE₂ and endometrial PTGER2 are involved in PGE₂ positive feedback loop in porcine endometrium.

Keywords

endometrium; pig; prostaglandin synthesis; PGE₂ receptors; estradiol-17 β

*Corresponding author: Agnieszka Waclawik, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Tuwima 10, 10-747 Olsztyn, Poland, Telephone: +48-89-5357422, Fax: +48-89-5357421, e-mail: waclawik@pan.olsztyn.pl.

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Summary Sentence: Estradiol-17 β , prostaglandin E₂ (PGE₂) and the PTGER2 are involved in PGE₂ positive feedback loop in the porcine endometrium.

INTRODUCTION

Porcine embryos secrete enhanced levels of estrogens, mainly estradiol-17 β (E₂) between days 10 and 13 after fertilization, just before implantation in the process of the maternal recognition of pregnancy (1-4). It has been demonstrated that systemic estrogen administration between days 11 and 13 of the estrous cycle prevents corpus luteum (CL) regression and extends this gland life span in the pig (5). The action of estrogens may be luteoprotective and antiluteolytic (3, 4, 6).

However, estrogen alone, does not fully exert the effect of the embryo on growth and development of CL (7). It has been suggested that besides estrogens, prostaglandin E₂ (PGE₂) could be involved in the maternal recognition of pregnancy as a luteoprotective/antiluteolytic factor (8, 9). Endometrial PGE₂, and prostaglandin F_{2 α} (PGF_{2 α}) which has an opposite luteolytic effect, are involved in reproduction processes in many species (10, 11). It was demonstrated that inhibition of prostaglandin (PG) synthesis before implantation causes pregnancy failure in different species, including the pig (11, 12).

PGs are synthesized by prostaglandin-endoperoxide synthase (PTGS) and specific terminal prostaglandin synthases: PGE synthase and PGF synthase (PGFS) (13, 14). Moreover, PGE₂ can be converted into PGF_{2 α} by prostaglandin 9-ketoreductase/carbonyl reductase (CBR1) (15-17). It was found that highly inducible forms of prostaglandin-endoperoxide synthase and PGE synthase in the porcine endometrium are PTGS2, (known also as PGHS-2 or COX-2) (18, 19) and microsomal PGE synthase-1 (mPGES-1), respectively (14).

Before implantation, the endometrium and trophoblast synthesize elevated amounts of PGE₂ which results in high content of this prostanoid in the uterine lumen and/or utero-ovarian circulation in the pig (9, 14, 17, 20). Our recent study has demonstrated that expression of prostaglandin synthesis pathway enzymes is altered in the porcine conceptus and endometrium to favor luteoprotective PGE₂ synthesis between days 10 and 13 of pregnancy (14, 17).

PGE₂ mediates its effect via binding to either of four subtypes of G-protein-coupled receptors (PTGER1-4 also known as EP1-4), which are encoded by four separate genes (21). The effect of PGE₂ in endometrial angiogenesis, uterine receptivity, and decidualization is mediated by cAMP-dependent mechanisms in rats (22), rabbits (23) and human (24). Only two subtypes of PGE₂ receptors - PTGER2 and PTGER4 are coupled to adenylate cyclase and generate cAMP that activates the protein kinase A signaling pathway (21). Knockout studies in mice indicated that PTGER2, but not PTGER1, PTGER3, or PTGER4 is associated with reproductive dysfunction (25). PTGER2-deficient mice demonstrate impaired ovulation and dramatic reduction in litter size (25). In pigs, the PGE₂-binding sites were detected in both cycling and pregnant endometrium but their location within endometrium was not determined (26). Moreover, no mRNA or protein expression of any PGE₂ receptor subtype has been identified in the porcine endometrium yet. To-date, regulation of expression of PGE₂ receptors in porcine endometrium remains unknown. Therefore, the objective of the present study was to elucidate whether the well established porcine embryo signal, E₂ and the putative embryo signal, PGE₂ regulate expression of prostaglandin synthesis pathway enzymes: PTGS2, mPGES-1, PGFS and CBR1, as well as PGE₂ receptors (PTGER2 and PTGER4) in the porcine endometrium. Moreover, the aim of study was to determine the site of action of PGE₂ in the porcine endometrium during the estrous cycle and early pregnancy.

MATERIALS AND METHODS

Tissue Collection

Peripubertal crossbred gilts of similar age (approximately 5-5.5 months) were observed daily for onset of estrus. After exhibiting two natural estrous cycle gilts were assigned into two groups: pregnant and cycling. Gilts assigned to the pregnant groups were artificially inseminated at 12 h after onset of estrus (day 0) and 24 h later. Gilts were slaughtered at a local abattoir on either day 9, 11, 12 or 15 of pregnancy or the estrous cycle (n = 4-6 per every group). The stage of the estrous cycle was verified by utero-ovarian morphology (14) and pregnancy was confirmed by the presence of morphologically normal conceptuses. Uterine tissues from all groups were fixed in 4% neutral buffered formaldehyde overnight at 4 C, stored in 70% ethanol, and wax embedded (for immunohistochemical analyses). The endometrium was snap-frozen and stored until further use (for RNA and protein extraction). Additionally, uteri collected from gilts on days 11-12 of the estrous cycle were immediately placed in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory within 1 to 1.5 h for in vitro explant tissue and cell culture.

To determine if there are differences in expression of mPGES-1 protein between regions near the conceptus and without proximity to the conceptus, endometrial samples were collected from the implantation sites (n=4) and interimplantation sites (n=4) in day 25 pregnant gilts (n=4).

All procedures involving animals were approved by the Local Research Ethics Committee and were conducted in accordance with the national guidelines for agricultural animal care.

Endometrial Explant Culture

Uteri collected from gilts on days 11-12 of the estrous cycle (n=6) were washed three times in sterile PBS. The endometrial tissue from middle of uterine horn was dissected and cut into small pieces (2 × 3 mm) and then washed three times in medium M199 (Sigma-Aldrich Co., St Louis, MO, USA). A total of 100 mg of tissue was placed into each glass vial with 2 ml of M199 medium containing 0.1 % bovine serum albumin (ICN Biomedicals, Inc., Costa Mesa, CA, USA), 5% dextran/charcoal-stripped newborn calf serum (Sigma-Aldrich), penicillin (100 IU/ml) and streptomycin (100 µg/ml). The endometrial tissue explants were preincubated in a shaking water bath at 37 C in a humidified atmosphere of 5% CO₂ in air for 2 h.

After this period, explants were treated with vehicle only (control), or PGE₂ (100 nM, ICN), or E₂ (1, 10, 100 nM; Sigma-Aldrich Co.), or phorbol 12-myristate 13-acetate (PMA; 100 nM; Sigma-Aldrich Co.) and incubated for an additional 24 h period. The structure of PMA is analogous to diacylglycerol and serves as an agonist for activation of protein kinase C. Since it is known that PMA increases PG secretion, it was used as a positive control (27, 28).

All treatments were performed in duplicate in six independent experiments. After culture the medium were collected and stored at -20 C until PG concentrations were measured by EIA. Furthermore, endometrial tissue explants were snap-frozen in liquid nitrogen (for RNA and protein extraction) and stored at -80 C until further use.

Real-time PCR Quantitation

Total RNA was extracted from endometrial samples and tissue explants collected after in vitro experiment using the Total RNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland) and treated with DNase I (Invitrogen Life Technology Inc., Carlsbad, CA, USA) according

to the manufacturer's protocol. Real time PCR was performed with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, CA, USA) using QuantiTect SYBR Green PCR master mix (Qiagen GmbH, Hilden, Germany), as described previously (14, 17). Briefly, total RNA was reverse transcribed using oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR reaction (25 μ l) included 12.5 μ l QuantiTect SYBR Green PCR master mix, 0.5 μ M sense and antisense primers each and reverse transcribed cDNA (3.5 μ l of diluted RT product). The specific primers were used to evaluate the mRNA levels (Fig 1G). For quantification, standard curves consisting of serial dilutions of the appropriate purified cDNA were included. Before amplification, an initial denaturation (15 min at 95 C) step was used. The PCR programs for each gene were performed as follows: 36 cycles of denaturation (15 s at 95 C), annealing (30 s at 52.5 C for PGFS; or at 55 C for PTGS2, mPGES-1, CBR1 and β -actin) and elongation (60 s at 72 C). The PCR programs for PTGER2 and PTGER4 genes were performed as follows: 36 cycles of denaturation (15 s at 95 C), annealing (60 s at 60 C). After PCR, melting curves were acquired by stepwise increases in the temperature from 60-95 C to ensure that a single product was amplified in the reaction. Data obtained from the real-time PCR for PTGS2, mPGES-1, PGFS, CBR1, PTGER2 and PTGER4 were normalized against β -actin. Control reactions in absence of reverse transcriptase were performed to test for genomic DNA contamination.

Protein extraction

Endometrial samples and tissue explants were homogenized on ice in buffer containing 50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 1mM EDTA and supplemented with protease inhibitor cocktail (Sigma-Aldrich Co.). Homogenates were then centrifuged for 15 min at 800 x g at 4 C and stored at -70 C for further analysis. The protein concentration was determined by the Bradford (29) method.

Western blot analysis

Western blot analysis was performed as we previously described (14, 17). Protein extracts (30 μ g) were dissolved in SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol and 2% β -mercaptoethanol), heated to 95 C for 4 min and separated on 15% (for mPGES-1), 12% (for PGFS and CBR1) and 10% (for PTGS2, PTGER2 and PTGER4) SDS-PAGE. Separated proteins were electroblotted onto 0.2 μ m nitrocellulose membrane in transfer buffer (20 mM Tris-HCl buffer, pH 8.2; 150 mM glycine, 20% methanol). After blocking in 5% non-fat dry milk in TBS-T buffer (Tris-buffered saline, containing 0.1% Tween-20) for 1.5 h at 25.6 C, the membranes were incubated overnight with 1:750 anti-COX-2 antibody (anti-PTGS2 antibody; Cayman Chemical, Ann Arbor, MI, USA), or 1:1000 polyclonal anti-mPGES-1 antibody (Cayman Chemical), or 1:2000 anti-lung-type PGFS antiserum (kindly donated from Prof. Kikuko Watanabe, University of East Asia, Yamaguchi, Japan), or 1:2000 polyclonal anti-human carbonyl reductase 1 antibody (Abcam, Cambridge, UK), or 1:200 rabbit polyclonal antibodies against human EP2 (anti-PTGER2 antibody; Cayman Chemical), or 1:50 rabbit polyclonal antibodies against human EP4 (anti-PTGER4 antibody; Cayman Chemical) at 4 C. Subsequently, the studied proteins were detected by incubating the membrane with 1:20000 dilution of secondary polyclonal anti-rabbit alkaline phosphatase-conjugated antibodies (for PTGS2, mPGES-1, PGFS, PTGER2 and PTGER4; Sigma-Aldrich Co.) and 1:2000 dilution of anti-goat alkaline phosphatase-conjugated antibodies (for CBR1, Abcam) for 1.5 h at 25.6 C. Immune complexes were visualized using alkaline phosphatase visualization procedure. Western blots were quantitated using Kodak 1D program (Eastman Kodak, Rochester, NY, USA). Sample loading was standardized to expression of β -actin using specific antibodies (1:3000; Abcam). Control experiments were performed for PTGER receptors by incubating the membranes with anti-PTGER2 or anti-PTGER4 antibodies preabsorbed with the

corresponding immunogenic blocking peptide (Cayman Chemical). Western blot analyses for PTGER receptors by incubating the membranes with the primary antibodies preabsorbed with the related blocking peptides did not give any signal. Specificity of other antibodies used was confirmed previously (14, 17, 30).

EIA of PGE₂ and PGF_{2α}

Concentrations of PGE₂ in medium were determined by an enzyme immunoassay (EIA) as described previously (31). Cross-reactivities of the anti-PGE₂ antiserum (donated by Dr. Seiji Ito, Kansai Medical University, Osaka, Japan) were as follows: PGE₁ 18%, PGA₁ 10%, PGA₂ 4.6%, PGB₂ 6.7%, PGD₂ 0.13%, PGF_{2α} 2.8%, PGJ₂ 14% and 15-keto-PGE₂ 0.05%. Assay sensitivity was 0.19 ng/ml and the intra- and interassay coefficients of variation were 4.9% and 8.5%, respectively. Concentrations of PGF_{2α} were determined by EIA test as described previously (31). Cross-reactivities of the anti-PGF_{2α} antiserum (Sigma-Aldrich Co.) were as follows: PGF_{1α} 60%, PGE₁ and PGE₂ <0.1%, and PGA₁, PGA₂, PGB₁ and PGB₂ <0.01%. Assay sensitivity was 0.23 ng/ml and the intra- and interassay coefficients of variation were 8.9% and 11.9%, respectively.

cAMP assay

Endometrial tissue (100 mg) collected from gilts on days 11-12 of the estrous cycle (n = 6) were minced finely into small pieces (2 × 3 mm). The tissue explants were incubated overnight at 37 C in a humidified 5% CO₂ incubator in 2 ml M199 medium (Sigma-Aldrich Co.) containing 100 IU penicillin and 100 µg streptomycin and 3 µg/ml indomethacin (Sigma-Aldrich Co.). Following overnight treatment, the tissue was incubated in M199 medium, containing 100 IU/ml penicillin and 100 µg/ml streptomycin, 3 µg/ml indomethacin and 1 mM 1-methyl-3-iso-butylxanthine (Sigma-Aldrich Co.) in the absence or presence of the appropriate receptor antagonist as shown in Figure 5C legend at 37 C for 30 min. It was then treated with vehicle, or 100 nM PGE₂ in presence/or absence of 10 µM AH6809 (PTGER2 antagonist; Sigma-Aldrich Co.) or 5 µM GW 627368X (PTGER4 antagonist, Cayman Chemical) (32) for 10 min at 37 C and washed in ice-cold PBS and snap-frozen. Tissue explants were homogenized in Lysis Buffer (100 mg of tissue per 1 ml of the buffer; R&D Systems, Abingdon, UK) and cAMP concentration was quantified in the homogenates by ELISA using a cAMP kit (R&D Systems) according to the manufacturer's protocol. The results were normalized to the protein concentration which was determined using protein assay kit (Bio-Rad, Hemel Hempstead, Herts, UK).

Immunohistochemistry

Immunohistochemical localization of PTGER2 in the endometrium on days 9, 11, 12, 15 of the estrous cycle and pregnancy (n=3-4 in every group) was performed using Bond-X automated immunostaining machine (Vision Biosystems, Newcastle, UK). The method on the Bond-X automated machine utilizes a specific polymer high contrast program. Briefly, cross-sections were made in the middle portion of the uterine horn. Five micron paraffin sections of uterine tissue were cut, dewaxed in xylene and dehydrated using decreasing grades of ethanol. Antigen retrieval was performed by pressure-cooking in 0.01 M sodium citrate (pH 6) before being placed on the Bond-X machine. Then, endogenous peroxidase activity was removed by fixing sections in 3% hydrogen peroxide for 5 min in methanol. Afterwards, slides were incubated with rabbit polyclonal anti-PTGER2 (1:1000; Cayman Chemical) antibody for 2 h, and treated with the polymer reagent (Vision Biosystems) for 15 min. Detection step was performed using 3,3'-diaminobenzidine tetrahydrochloride for 10 min and sections were counterstained in haematoxylin for 5 min. Slides were then removed from the machine and dehydrated and mounted using Pertex. To confirm antibody specificity, negative controls were performed by incubating sections with a control rabbit IgG at the matched concentrations to the primary antibodies and with the primary antibody

preabsorbed with the peptide it was raised (Cayman Chemical). Immunoreactivity was negligible with both preabsorbed antibody and with a control rabbit IgG.

Endometrial cell culture in vitro

Endometrial epithelial and stromal cells were isolated from uteri on days 11-12 of the estrous cycle (n=5) and pregnancy (n=3), as described previously (31), and seeded in 6-well plates. Cells cultures were incubated in M199 medium containing 2% bovine serum albumin (ICN Biomedicals), 10% newborn calf serum (Sigma-Aldrich Co.), penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 C in atmosphere of 95% air and 5% CO₂. At 80-90% cell confluence medium was replaced with serum-free M199. After 24 h cells were harvested and lysed in RIPA buffer (50 mM Tris; pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA; protease inhibitor cocktail) and the obtained protein extracts were used to study the expression of PTGER2 and PTGER4 by Western blot analyses.

Statistical analysis

Statistical analyses were performed using Repeated measures one-way ANOVA or Paired t-Test (GraphPad Prism 4.0, Graphpad Software, San Diego, CA, USA). Two-way ANOVA was used to evaluate changes in abundance of PTGER2 and PTGER4 mRNA and protein due to day of the estrous cycle and pregnancy as well as an effect of reproductive status. All numerical data are presented as the mean ± SEM and differences were considered as statistically significant when p<0.05.

RESULTS

Effect of E₂ on PTGS2, mPGES-1, PGFS, CBR1, PTGER2 and PTGER4 expression and PGE₂ and PGF_{2α} secretion in endometrial tissue explants

E₂ elevated expression of mPGES-1 mRNA (10 nM and 100 nM, p<0.05 and p<0.01, respectively) but had no effect on PTGS2, PGFS, CBR1, PTGER2 and PTGER4 mRNA content in endometrial tissue explants (Fig. 1). E₂ stimulated expression of PTGS2 (10 and 100 nM; p<0.05 and p<0.01, respectively), mPGES-1 (1 nM and 10 nM, p<0.05) and PTGER2 protein (10 nM and 100 nM) but decreased (p<0.05) expression of PGFS (10 nM and 100 nM) and CBR1 protein (100 nM) and had no effect on PTGER4 protein content (Fig. 2, A-F). E₂ treatment increased PGE₂ secretion in a dose dependent manner (Fig. 2G). However, there was no effect of E₂ treatment on PGF_{2α} secretion by endometrial tissue explants (Fig. 2H).

Effect of PGE₂ on PTGS2, mPGES-1, PGFS, CBR1, PTGER2 and PTGER4 expression and PGE₂ and PGF_{2α} secretion in endometrial tissue explants

PGE₂ stimulated mPGES-1 (p<0.01; Fig. 3C) and PTGER2 mRNA expression (p<0.05; Fig. 5A) and had no effect on PTGS2, PGFS and CBR1 mRNA content in endometrial explants (Fig. 3, A, B and D). PGE₂ enhanced PTGS2 (Fig. 4A), mPGES-1 (p<0.05, Fig. 4C) and PTGER2 protein expression (p<0.5, Fig. 5B) as well as PGE₂ secretion by endometrial tissue explants (p<0.05, Fig. 4E). By contrast, PGE₂ had no effect on PGFS and CBR1 expression and PGF_{2α} release (Fig 4, B, D and F) and PTGER4 mRNA and protein expression (Fig. 5, C and D). PMA used as a positive control stimulated both PTGS2 and mPGES-1 mRNA and protein expression (p<0.05, Fig. 3A, C and Fig. 4, A and C) and PGFS protein levels (p<0.05, Fig. 4B) but had no effect on CBR1 expression (Fig. 3D and Fig. 4D). Moreover, PMA increased secretion of PGE₂ (p<0.01, Fig. 4E) and PGF_{2α} (p<0.05, Fig. 4F).

PGE₂ and E₂ elevated about 2-fold the mPGES-1/PGFS protein ratios in endometrial tissue explants (Fig. 3F).

Endometrial expression of mPGES-1 protein in implantation and interimplantation sites

Expression of mPGES-1 protein was up-regulated in endometrium collected from the implantation sites when compared to interimplantation sites ($p < 0.01$; Fig 3G).

Effect of PGE₂ on cAMP accumulation in endometrial explants

Treatment with PGE₂ increased cAMP production in endometrial tissue explants in absence of any PTGER antagonists ($p < 0.001$; Fig. 5J). Co-treatment with PTGER2 antagonist (AH6809) inhibited significantly the PGE₂-mediated cAMP production. However, the response to PGE₂ was not suppressed by co-treatment with the PTGER4 antagonist (GW 627368X). No significant alteration in basal levels of cAMP was observed in endometrial tissue treated with the antagonists alone.

Expression of PTGER2 and PTGER4 in the endometrium during the estrous cycle and early pregnancy

No effect of day or reproductive status was detected for PTGER4 mRNA expression (Fig. 5F). PTGER4 protein and PTGER2 mRNA expression was affected by day but not reproductive status (Fig. 5H and E). PTGER2 mRNA content was significantly higher on day 12 and 15 in cyclic and pregnant gilts when compared to days of 9 and 11 the estrous cycle and pregnancy (Fig 5E). Expression of PTGER2 protein was affected by reproductive status but not day. PTGER2 protein expression was up-regulated on day 11 and 12 of pregnancy when compared to corresponding days of the estrous cycle ($p < 0.05$; Fig 5G).

PTGER2 protein was highly expressed in luminal and glandular epithelium of endometrium and in blood vessels, and was present in myometrium (Fig. 5I). Protein expression of PTGER2 was detected on days 9 to 15 of the estrous cycle and pregnancy. Moreover, protein expression of PTGER2 and PTGER4 was confirmed in both luminal epithelial and stromal cells isolated from the uterus on days 11-12 of the estrous cycle and days 11-12 of pregnancy (Fig. 5K).

DISCUSSION

Present hypotheses about the antilutolytic mechanism and recognition of pregnancy in the pig are mainly focused on way of the PGF_{2 α} secretion (2, 4, 33). Although PGE₂ has been reported to have luteoprotective effects (8, 9), currently there are no studies describing the molecular mechanisms regulating PGE₂ synthesis in the endometrium during early pregnancy. To our knowledge, this is the first study to address the effect of PGE₂ and embryo signal, E₂ on the prostaglandin biosynthesis pathway and PGE₂ signaling in the porcine endometrium. PTGS2 is an enzyme involved in nonselective production of PGs, whereas PG synthases (mPGES-1 and PGFS) and CBR1 control the selective production and associated specific functions of PGE₂ and PGF_{2 α} . We have found that PGE₂ biosynthetic pathways are selectively activated in the porcine endometrium (14). Our hypothesis was that selective changes in expression of enzymes involved in PG synthesis and in the porcine endometrium are a result of embryo signals.

Even spherical day 10-11 blastocysts sized 5-7 mm in diameter are capable for enhanced estrogen synthesis (34, 35). Synthesis and secretion of estrogens by conceptuses dramatically increase during trophoblast elongation on days 11-12 of pregnancy, decline rapidly on day 13, and then initiate a second more sustained increase on days 16-25 of pregnancy (4, 36). In the present studies, we demonstrated that E₂ stimulated PGE₂

synthesis through increase of PTGS2 protein and mPGES-1 mRNA and protein expression and decreased the protein content of enzymes involved in PGF_{2α} production – PGFS and CBR1 in the porcine endometrium on days 11-12 after estrus. Stimulation of PTGS2 protein expression in endometrial explants by estradiol-17β in vitro observed by us is in agreement with the observation that administration of estrogen in vivo affects expression pattern of PTGS2 (18). The data presented herein are also consistent with our previous findings that mPGES-1 expression is higher and PGFS and CBR1 - lower in the porcine endometrium between days 10 and 13 of pregnancy when compared to following days of gestation (14, 17). The observations in the explant cultures after treatment with conceptus factors corresponds also with comparison of CBR1 and PGFS protein expression in pregnant versus cyclic animals on days 10-11 and 10-13, respectively (14, 17). In contrast, our previous data showed no differences in mRNA and protein expression of mPGES-1 in the endometrium on days 11-12 of pregnancy and days 11-12 of the estrous cycle (14). This discrepancy between stimulation of mPGES-1 expression in explants by conceptus factors (E₂ and PGE₂) and lack of effect of pregnancy on mPGES-1 mRNA and protein can be a result of local changes induced in the endometrium by the conceptus. It is not possible to distinguish which endometrial samples collected on day 11-12 of pregnancy had direct contact with the embryo since implantation begins on day 14. It is tempting to speculate that effect of conceptus factors on endometrial explants can mimic local changes in the endometrium which is near the conceptus. This hypothesis, although needs further study, is supported by results concerning later stage of pregnancy and indicating two-fold up-regulation of mPGES-1 protein expression in the endometrium in the implantation sites when compared with interimplantation sites (Fig. 3G). It is likely that effects of estrogen on the endometrium are restricted to regions in close proximity to the conceptus due to metabolic activity of trophoblast. During pregnancy, pig endometrium rapidly converts estradiol-17β to the biologically inactive estrone sulfate which is present in high concentrations within the uterine lumen of pregnant pigs (37). The trophoblast has sulfatase enzyme activity that restores the biological activity of estrogen, allowing for a localized effect of estrogen to up-regulate genes such as SPP1 in luminal epithelium (38).

Profile of estrogen secretion by conceptus (36) highly corresponds with selective and dramatic changes in PG synthesis enzymes during early pregnancy, reported previously by us (14, 17). Endometrial mPGES-1 expression exhibits a biphasic profile, similar to estrogen synthesized and secreted by conceptuses. By contrast, rapid increase of PGFS and CBR1 after initiation of implantation corresponds with the decline in conceptus estrogen synthesis. Our findings are in agreement with in vivo observations which demonstrate that estrogens increase PGE₂ content in the porcine uterus (5) and therefore may be an important factor in increase of the PGE₂:PGF_{2α} ratio during early pregnancy.

CBR1 is the enzyme which can modulate both PG concentrations since it converts PGE₂ into PGF_{2α} (15, 16). In the present study, E₂ inhibited CBR1 protein expression. This is consistent with reports demonstrating inhibition of prostaglandin 9-ketoreductase activity in the sheep endometrium during the maternal recognition of pregnancy (39). Similarly, interferon-τ, the conceptus signal in ruminants, down-regulates expression of prostaglandin 9-ketoreductase gene and increases PGE₂ secretion in epithelial cells of bovine endometrium (40). Moreover, E₂ inhibits prostaglandin 9-ketoreductase activity in human placenta (41).

E₂ had a significant effect mainly on protein levels but did not influence the mRNA content of most factors investigated (except of mPGES-1). This can be explained by the fact that steroids affect stability of many mRNAs that are important in reproductive processes and in this way may influence gene expression levels and function (42).

In vivo studies demonstrated that timing of endometrial exposure to estrogen is critical to establishment of pregnancy (18). Early administration of estrogen on days 9 and 10 of pregnancy probably desynchronizes the uterine environment for conceptus implantation resulting in later embryonic loss as well as alters the pattern of many gene expression, including endometrial PTGS2 mRNA and protein expression (18, 43). Therefore, in our experiment we used gilts on days 11-12 of the estrous cycle that corresponds to the normal period of conceptus E₂ secretion. We did not choose the 11-12 day pregnant gilts because their endometrium would have been exposed already to embryo signal and hence resulted in altered expression of target genes of interest.

These studies indicate for the first time, the existence of PGE₂ autoamplification loop in the porcine endometrium that can be involved in the increase of PGE₂:PGF_{2α} ratio during the maternal recognition of pregnancy. Porcine endometrial tissue explants produced cAMP in response to PGE₂ treatment. Increase of PGE₂ mediated-cAMP production occurred via PTGER2 since cAMP production in response to PGE₂ was inhibited following co-treatment with the PTGER2 specific antagonist. Our study suggests that PGE₂ acts through endometrial PTGER2 receptor, activates cAMP signaling pathway and elevates the mRNA and protein expression of enzymes involved in PGE₂ synthesis (namely PTGS2 and mPGES-1) and secretion of this prostanoid by the endometrium (Fig. 6). It is consistent with the reports that PGE₂ itself may induce its own secretion in other cell types (44-46). Both PGE₂ and estradiol-17β increased the mPGES-1:PGFS protein ratios which are indicative of the relative PGE₂ to PGF_{2α} production in the endometrium. Moreover, E₂ and PGE₂ stimulated endometrial PTGER2 expression that corresponds to up-regulation of PTGER2 protein content in the endometrium on days 11-12 of pregnancy when compared to corresponding days of the estrous cycle. These data provide a novel mechanism of positive feedback loop that contributes to increased PGE₂ synthesis in the porcine uterus during the peri-implantation window (Fig. 6). Similar observations have been reported for the bovine embryo recognition signal, interferon-τ (47).

It is likely that PGE₂ produced in the uterus acts in autocrine/paracrine manner via PGE₂ receptors, resulting in the local increase of endometrial vascular permeability and preparation for angiogenesis and implantation (11, 48). We have identified the mRNA and protein expression of two subtypes of PGE₂ receptors coupled with cAMP signaling pathway, in the porcine endometrium, for the first time. PTGER2 protein was highly expressed in the luminal and glandular epithelium of endometrium and in uterine blood vessels, and moderately in myometrium in both pregnant and cycling gilts. Both epithelial and stromal cells isolated from the uterus of cycling and pregnant gilts expressed PTGER2 and PTGER4 protein. On basis of our present and previous results (14), it may be concluded that the site of PGE₂ action in the porcine endometrium corresponds highly to localization of PGE₂ synthesis. The present results are also consistent with localization of PTGER2 in bovine and human endometrium (49, 50). It is suggested that in the rat and mouse PTGER2 is involved in implantation, whereas PTGER4 is important in the process of decidualization (11). Role of PTGER4 could vary in different species. This hypothesis is supported by fact that in contrast to rodents and human, in the bovine endometrium PTGER4 expression is very low (50). The specific roles of these receptors in the porcine endometrium remains to be elucidated. However, the present results indicate that expression of PTGER4 is not affected by reproductive status or by factors secreted by conceptus during the maternal recognition of pregnancy. On the other hand, our studies suggest an important role for PTGER2 in the porcine maternal-embryo communication.

In contrast to PTGER2 and PGE synthase, expression of the enzymes involved in PGF_{2α} production (PGFS and CBR1) was not affected by PGE₂ treatment. As predicted, expression of PTGS2, mPGES-1 and PGFS as well as PGE₂ and PGF_{2α} secretion from endometrial

explants was stimulated by PMA, used in the present study as a positive control. PMA mimics diacylglycerol activation of protein kinase C and it was demonstrated that this phorbol ester stimulates both PG secretion and expression of PG synthesis enzymes (27, 28).

In summary, the present study provides the first direct evidence that primary conceptus signal, E_2 and another conceptus factor - PGE_2 regulate the protein expression of endometrial PG synthesis pathway enzymes to favor production of luteoprotective PGE_2 in pigs. Our novel findings indicate that one potential mechanism of inhibition of luteolysis in the pig is the up-regulation of PTGS2 and PGE_2 synthase protein expression and the down-regulation of endometrial PGFS and CBR1 protein concentrations by E_2 . Our results suggest that E_2 produced by the porcine trophoblast prevents luteolysis through enzymatic modification of PG synthesis in endometrial cells.

PGE_2 could exert a luteoprotective effect as well as it may act locally through endometrial PGE_2 receptors, especially PTGER2. Moreover, endometrial PTGER2 may be involved in a positive feedback loop during increased PGE_2 synthesis in the porcine uterus in peri-implantation window.

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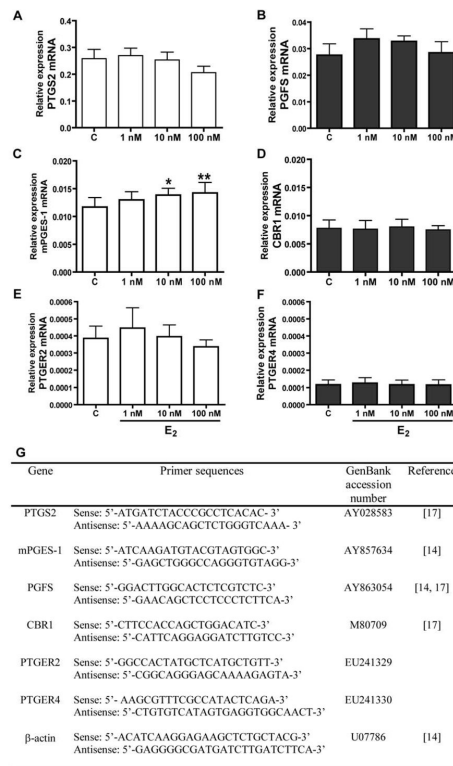


Figure 1. Effect of E_2 (1, 10 or 100 nM) on mRNA expression of prostaglandin-endoperoxide 2 (A), PGF synthase (B), PGE synthase (C), prostaglandin 9-ketoreductase (D) and PGE₂ receptors: PTGER2 (E) and PTGER4 (F) in endometrial tissue explants *in vitro*. Asterisks indicate significant differences between control (C) and experimental groups (* $p < 0.05$; ** $p < 0.01$). (G) Primers used for Real-time PCR.

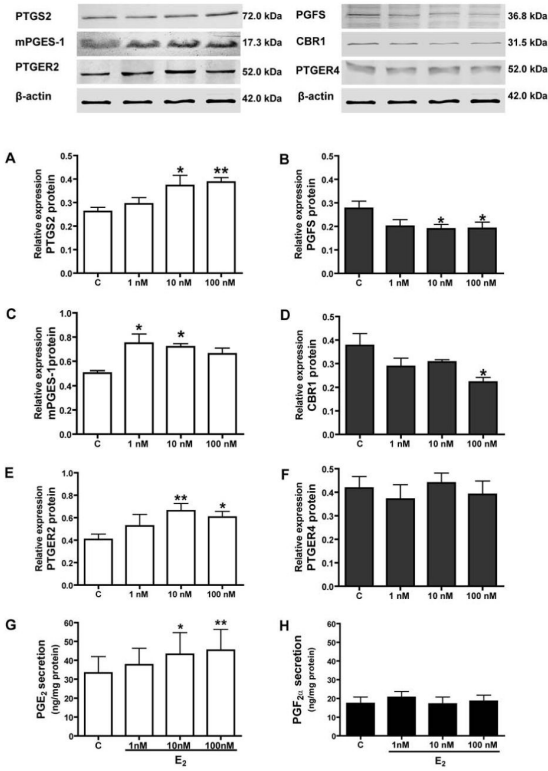


Figure 2. Effect of E₂ (1, 10 or 100 nM) on protein expression of prostaglandin-endoperoxide 2 (A), PGF synthase (B), PGE synthase (C), prostaglandin 9-ketoreductase (D) and PGE₂ receptors: PTGER2 (E) and PTGER4 (F) in endometrial tissue explants *in vitro*. The representative samples of Western blots are shown in upper panels. Effect of E₂ (1, 10 or 100 nM) on PGE₂ (G) and PGF_{2α} secretion (H) in endometrial tissue explants *in vitro*. Asterisks indicate significant differences between control (C) and experimental groups (* p<0.05; ** p<0.01).

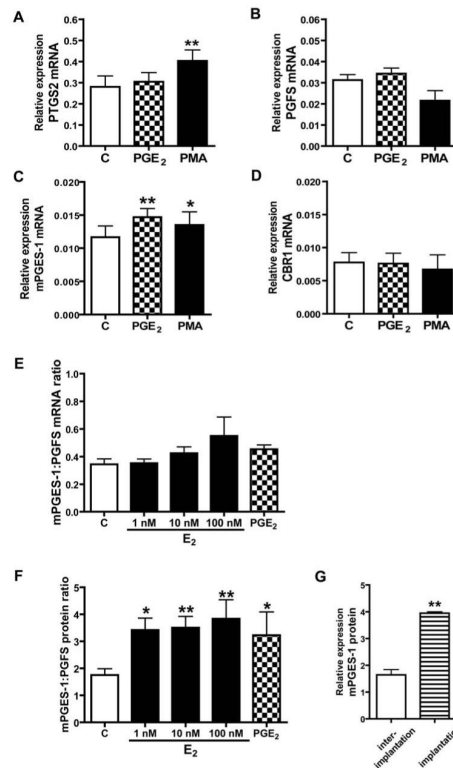


Figure 3.

Effect of PGE₂ (100 nM) and phorbol-12-myristate-13-acetate (100 nM) on mRNA expression of prostaglandin-endoperoxide 2 (A), PGF synthase (B), PGE synthase (C) and prostaglandin 9-ketoreductase (D) in endometrial tissue explants *in vitro*. Effect of PGE₂ and E₂ on the ratios of mPGES-1:PGFS mRNA (E) and protein (F). Expression of mPGES-1 protein in implantation and interimplantation sites. Asterisks indicate significant differences between control (C) and experimental groups (* p<0.05; ** p<0.01).

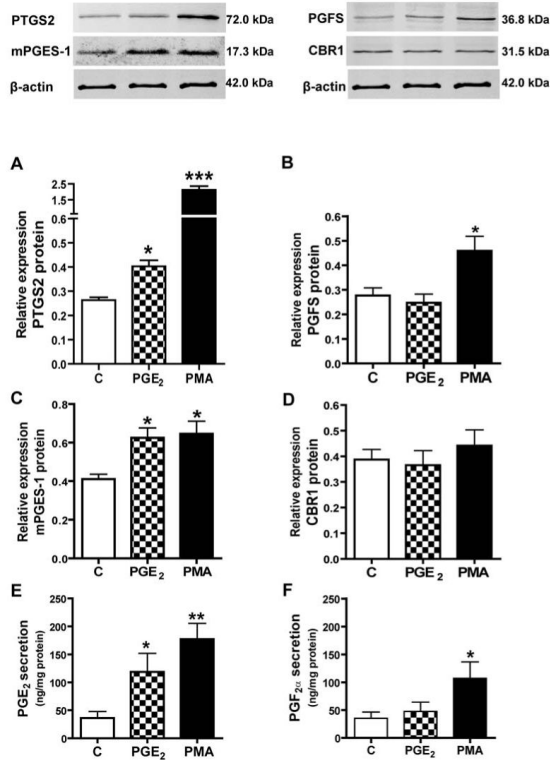


Figure 4. Effect of PGE₂ (100 nM) and phorbol-12-myristate-13-acetate (100 nM) on protein expression of prostaglandin-endoperoxide 2 (A), PGF synthase (B), PGE synthase (C) and prostaglandin 9-ketoreductase (D) in endometrial tissue explants *in vitro*. The representative samples of Western blots are shown in upper panels. Effect of PGE₂ (100 nM) and phorbol-12-myristate-13-acetate (100 nM) on PGE₂ (E) and PGF_{2 α} secretion (F) in endometrial tissue explants *in vitro*. Asterisks indicate significant differences between control (C) and experimental groups (* p<0.05; ** p<0.01; *** p<0.001).

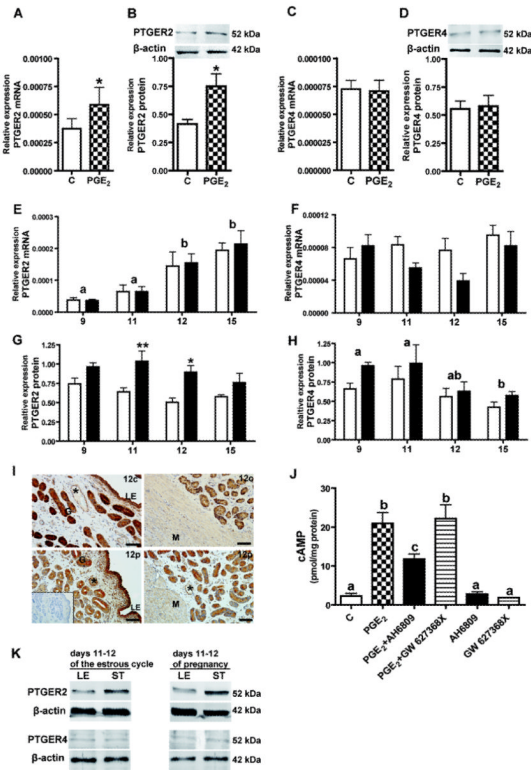


Figure 5. Regulation of PTGER2 and PTGER4 expression, cAMP signaling and localization of PTGER2 expression in the porcine endometrium. Effect of PGE₂ (100 nM) on mRNA and protein expression of PGE₂ receptors: PTGER2 (**A**, **B**) and PTGER4 (**C**, **D**) in endometrial tissue explants *in vitro*. Asterisks indicate significant differences between control (C) and experimental group (* p<0.05). Expression of PTGER2 (**E**) and PTGER4 (**F**) mRNA during the estrous cycle (white bar) and early pregnancy (black bar). Expression of PTGER2 (**G**) and PTGER4 (**H**) protein during days 9-15 of the estrous cycle (white bar) and early pregnancy (black bar). Days with different letters for expression of PTGER2 mRNA and PTGER4 protein are statistically different. PTGER4 mRNA is not affected by day or reproductive status (p>0.05). The asterisk represents differences (* p<0.05; ** p<0.01) for PTGER2 protein abundance between cyclic and pregnant gilts on days 11 and 12 of the estrous cycle and pregnancy. **I**, Immunolocalization of PTGER2 protein in luminal (LE) and glandular epithelium (G) endometrium, blood vessels (*) and myometrium (M) in the uterus of pregnant and cycling gilts. Representative immunohistochemical analyses are presented: 12c – day 12 of the estrous cycle; 12p – day 12 of pregnancy. Insets: negative control with preabsorbed antibodies. Scale bar, 50 μm. **J**, cAMP accumulation in endometrial tissue explants in response to treatment with 100 nM PGE₂ in the presence/absence of PTGER2 antagonist (AH6809), or PTGER4 antagonist (GW 627368X). Additionally, endometrial explants were treated with PTGER2 or PTGER4 antagonists alone. Means with different letters indicate significant differences (p<0.05). **K**, Protein expression of PTGER2 and PTGER4 in luminal epithelial (LE) and stromal cells (ST) isolated from uterus on days 11-12 of the estrous cycle and pregnancy.

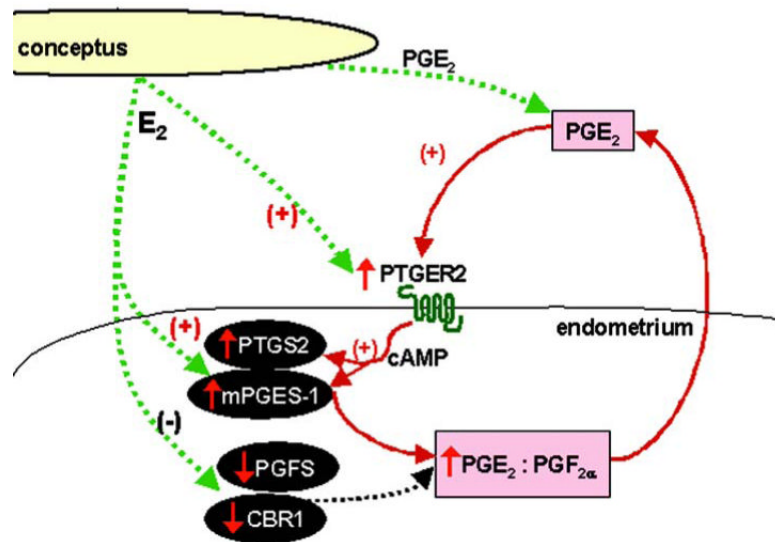


Figure 6. Potential mechanism involved in increasing luteoprotective PGE₂ level and the PGE₂:PGF_{2α} ratio during the maternal recognition of pregnancy in the pig - involvement of estradiol-17β, PGE₂ and endometrial PGE₂ receptors (PTGER2) in PGE₂ positive feedback loop in the porcine endometrium.

The primary conceptus signal, E₂ and another conceptus factor - PGE₂ regulate the protein expression of endometrial PG synthesis pathway enzymes to favor production of luteoprotective PGE₂. E₂ up-regulates PTGS2 and PGE synthase protein expression and down-regulates endometrial PGFS and CBR1 protein concentrations increasing PGE₂ secretion and the PGE₂:PGF_{2α} ratio. PGE₂ acts through endometrial PTGER2 receptor, activates cAMP signaling pathway and elevates the mRNA and protein expression of enzymes involved in PGE₂ synthesis (PTGS2 and mPGES-1) and its own secretion in the endometrium.